Genetic disorders of HDL metabolism: from model to mechanism
Holleboom, A.G.

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Adriaan G. Holleboom

Leuvensehoofdstad, 1981, perhaps better known as Onno, graduated from Jeroen Bosch College gymnasium in 1999. As a youngster already eager to reach the sky, he almost succeeded to become Dutch basketball league champion. After crossing the Rhine northwards, he attended medical school at the Academic Medical Center, University of Amsterdam, where he graduated with honours in 2007. During his study he was vice-president of the Medical Student Association, a member of the Student Council, the restless reporter of Emphasis studying human-bird interrelations in their natural habitat, whilst working on HDL research at the Department of Vascular Medicine. In 2004 he took off for Columbia University, New York to study TNF and ABCA1 in the lab of prof. Alan R. Tall. In 2006 he was awarded the dr. I. Snapper Award for his extracurricular activities in internal medicine. In 2008, he was awarded a TopTalent grant from the Netherlands Organisation of Scientific Research NWO to conduct his PhD research under guidance of dr. J.A. Kuivenhoven and prof. dr. E.S.G. Stroes. In 2011, he started his residency in Internal Medicine under supervision of prof. J.B.L. Hoekstra.

Onno lives together with Miriam van Lieshout and their son Imme.

The promise of HDL

According to the World Health Organization, cardiovascular disease is the number one cause of mortality in the entire world. Ever since Barr and co-workers described a strong inverse relationship between the plasma concentration of cholesterol in high-density lipoproteins (HDL) and the risk of cardiovascular disease in 1951, many scientific efforts have been undertaken to study the role of HDL in atherosclerosis, in order to develop therapies to prevent cardiovascular diseases. However, the question whether HDL causally protects from atherosclerosis remains to be answered to date. The studies presented in this thesis focus on patients with genetic disorders of HDL metabolism, and on how these disorders impact HDL metabolism and the development of atherosclerosis. They range from an infusion protocol based on the tracer dilution principle and kinetic modelling, to validation of a gene locus found in genome-wide association studies (GWAS) through the identification of a linking molecular mechanism: From Model to Mechanism.
Genetic Disorders of HDL Metabolism: from Model to Mechanism

Adriaan G. Holleboom
Genetic disorders of HDL metabolism: from model to mechanism

Academic thesis

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Cover: by studying genetics, the blueprint of life, and by using scientific models (sketches of cog wheels), we try to decipher how HDL metabolism really works (cog wheels): from model to mechanism.

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Genetic Disorders of HDL Metabolism: from Model to Mechanism

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Faculteit der Geneeskunde
Voor Miriam en Imme
Voor mijn ouders
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Chapter 1

Journal of Lipid Research 2010, 51(8)

The HDL hypothesis: does high-density lipoprotein protect from atherosclerosis?

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Chapter 1

There is unequivocal evidence of an inverse association between plasma high-density lipoprotein (HDL) cholesterol concentrations and the risk of cardiovascular disease, a finding that has led to the hypothesis that HDL protects from atherosclerosis. This review details the experimental evidence for this “HDL hypothesis”. In vitro studies suggest that HDL has a wide range of anti-atherogenic properties but validation of these functions in humans is absent to date. A significant number of animal studies and clinical trials support an atheroprotective role for HDL, however, most of these findings were obtained in the context of marked changes in other plasma lipids. Finally, genetic studies in humans have not provided convincing evidence that HDL genes modulate cardiovascular risk. Thus, despite a wealth of information on this intriguing lipoprotein, future research remains essential to prove the HDL hypothesis correct.
In 1951, Barr and coworkers\(^1\) reported that plasma levels of high-density lipoprotein (HDL) cholesterol were reduced in patients with coronary artery disease. In 1977, Gordon et al subsequently showed that low HDL cholesterol is a risk factor for coronary heart disease in the Framingham study.\(^2\) These important findings have given rise to a large number of diverse HDL studies over the last few decades. The numerous different and apparently unrelated beneficial effects that have since been ascribed to HDL appeal to the imagination. Because of a general consensus that HDL protects against atherosclerosis – what we shall term the HDL hypothesis – strategies have been developed to raise plasma HDL levels, or to improve HDL function. However, it is increasingly questioned whether such interventions will indeed reduce the risk of atherosclerosis. This review summarizes the reported evidence that supports the HDL hypothesis.

Epidemiology

A low plasma HDL-C concentration is among the strongest, statistically independent risk factors for cardiovascular disease (CVD).\(^2\) In a widely cited meta-analysis of four large studies (total number of individuals studied: 15,252), a 1 mg/dL increase of HDL-C levels was reported to be associated with a 2-3% decreased CVD risk.\(^3\) This result provides an epidemiological argument in favor of therapeutically raising HDL-C levels. One may draw parallels with the detrimental consequences of elevated low-density lipoprotein cholesterol (LDL-C) levels and blood pressure, which have been successfully controlled through therapy resulting in significant reductions of cardiovascular mortality and morbidity.\(^4,\)\(^5\) It should be noted, however, that the associations between elevated LDL-C and blood pressure and increased CVD risk reflect causal relationships while such a relation between low HDL-C levels and increased CVD is not undisputed.\(^6,\)\(^7\) This is related to the fact that HDL-C levels are influenced by many different variables that also affect CVD risk:

- Men have on average lower HDL-C levels than women.\(^8\)
- Smokers have 14% lower HDL-C levels than non-smokers, and this relationship appears to be dose-dependent,\(^9\) whereas individuals who quit smoking show a subsequent increase in HDL-C levels.\(^10\) Even in individuals who acutely smoke 2 cigarettes, HDL-C levels drop 6 mg/dL.\(^11\)
- A recent meta-analysis of 25 studies (total number of individuals studied: 2,027) shows that programs of regular aerobic exercise increased HDL-C levels by 2.5 mg/dL on average.\(^12\)
- Obesity, especially abdominal obesity, is also associated with lower HDL-C levels\(^13\) while weight loss results in an elevation of HDL-C levels.\(^14\)
- Patients with type 2 diabetes mellitus display several lipid abnormalities of which is a low HDL-C level is a prominent feature.\(^15\)
Metabolic syndrome, a cluster of pathologies comprising abdominal obesity, hypertension, impaired glucose tolerance, high triglycerides and low HDL-C levels, is regarded by investigators as a single disease entity resulting from insulin resistance.\textsuperscript{16} In parallel, (postprandial) hypertriglyceridemia results in low HDL-C levels, associated with an increased cholesteryl ester transfer protein (CETP) driven exchange of cholesteryl esters and triglycerides between pro-atherogenic apolipoprotein (apo) B containing lipoproteins (very low-density lipoprotein [VLDL] and LDL) and HDL.\textsuperscript{17} In fact, over 50\% of the patients with low HDL-C levels also present with increased fasting triglycerides.\textsuperscript{18} With this in mind, HDL-C levels may be seen as a stable reflection of disturbances in triglyceride metabolism, whereas plasma triglyceride levels themselves are subject to large inter-individual and intra-individual variability.

Systemic inflammation (as observed in rheumatoid arthritis and systemic lupus erythematosus) - a recognized risk factor for CVD\textsuperscript{19} - is associated with a secondary dyslipidemia characterized by low HDL-C levels.\textsuperscript{20} Finally, a low socioeconomic status is an independent predictor of low HDL-C.\textsuperscript{21} Interestingly, even in baboons, socially subordinate males show 31\% lower HDL-C levels than dominant males, possibly related to chronic social stress.\textsuperscript{22}

Thus, many different factors affect both CVD risk as well as HDL-C levels. A recent very large meta-analysis comprising 302,430 individuals has once again made clear that HDL-C is a strong predictor of cardiovascular events, even after statistical adjustment for these variables.\textsuperscript{23} Such adjustments, however, do not guarantee the absence of residual confounding (Box 1)\textsuperscript{24}, and one may ask what the meaning of statistical outcomes is when the adjustments concern parameters that are intrinsically related to HDL metabolism. In addition to plain HDL-C measurements, many investigators have focused on HDL-related parameters, such as levels of apolipoprotein A-I (apoA-I; HDL's most important structural protein), or HDL subclasses (as assessed by e.g. nuclear magnetic resonance or 1- and 2-dimensional gel electrophoresis) and have shown that some of these parameters are superior to HDL-C in cardiovascular-risk assessment.\textsuperscript{25} Although these studies are of interest, these epidemiological relationships are subject to the same confounding variables as those concerning HDL-C itself and are therefore not fit to prove that HDL is responsible for atheroprotection.

In conclusion, the epidemiological association between HDL and CVD is strong, and is largely responsible for the formulation of the HDL hypothesis, but in itself does not prove a causal relationship.

HDL function

In line with the HDL hypothesis, HDL has been reported to exhibit many anti-atherogenic properties (for an extensive review see \textsuperscript{26}) that are addressed in this section.
Reverse cholesterol transport

In 1982, Fielding and Fielding demonstrated that HDL can act as an acceptor of cellular cholesterol, which is proposed to constitute the first step in a hypothetical pathway that is known as reverse cholesterol transport (RCT). In its broadest sense, RCT is defined as the uptake of cholesterol from peripheral cells by lipid-poor apoA-I and HDL that is mediated by lipid transporter molecules such as ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1) and scavenger receptor B-I (SR-BI), and the subsequent delivery to the liver for ultimate excretion into the feces as neutral sterols or bile acids. HDL can deliver cholesterol to the liver through hepatic SR-BI, or, alternatively, CETP shuttles cholesterol from HDL to (V)LDL which can be taken up via the LDL receptor (LDLr) pathway. The overall RCT hypothesis, especially with respect to the relationship between plasma HDL cholesterol and fecal cholesterol excretion, has been challenged by findings in both mice and humans:

For mice, it has been reported that (total body) ABCA1 deficiency, causing near complete HDL deficiency, does not affect hepatobiliary flux of cholesterol. Similarly, modulation of apo A-I, lecithin-cholesterol acyl transferase (LCAT; an enzyme critically involved in HDL maturation), and SR-BI, resulting in large changes in plasma HDL-C concentrations, had no effect on sterol excretion. In addition, the infusion of apoA-I-phospholipid complexes in apoA-I knock out mice did not increase fecal sterol excretion. Thus, marked manipulation of HDL metabolism in mice apparently does not result in changes in net fecal cholesterol excretion. Another method to study whole body RCT in mice has been developed by Rader and coworkers. In short, macrophages loaded with radiolabeled cholesterol are injected into the peritoneal cavity of mice, after which the distribution of this cholesterol in plasma, liver and feces is quantified. These studies have provided insight in which factors control the RCT pathway in mice but have not provided data on net fecal cholesterol output.

In humans, the data on HDL dependent fecal cholesterol output are equivocal. Both a negative as well as the absence of a relationship between plasma HDL-C concentrations and CVD. Statistical adjustment for these potential confounders relies on three assumptions: that the relationships between confounders and HDL-C and CVD are linear, independent of each other and assessed with great precision. It is, however, safe to state that, for many confounders, none of these assumptions hold. It is well-established that associations between parameters in epidemiological studies can only aid the formulation of hypotheses, which need to be tested by experimental research. In this respect, the results of vitamin E supplementation, hormonal replacement therapy, or homocysteine lowering by folic acid and vitamin B supplementation are unsettling examples of promising epidemiological relationships which turned sour in a clinical trial setting. The HDL hypothesis too should be subjected to rigorous experimental testing.
and fecal sterol excretion have been reported\textsuperscript{31-33} and a recent study in 7 individuals with genetically determined low HDL-C indicated reduced fecal cholesterol output.\textsuperscript{34} Intervention studies that increase plasma HDL-C levels also provided mixed results: the infusion of pro-apolipoprotein A-I or reconstituted HDL (rHDL; apoA-I purified from human blood, reconstituted with phospholipids) was reported to result in an increase of sterol excretion\textsuperscript{35, 36}, but CETP inhibition (which blocks the exchange of cholesterol from HDL to LDL, resulting in a marked HDL-C increase as well as a LDL-C decrease) had no effect.\textsuperscript{37} Thus, human studies have only provided little evidence that HDL-C levels correlate with fecal cholesterol output. In fact, according to cholesterol flux modelling studies by Schwartz et al, the role of HDL in total body cholesterol homeostasis is suggested to be minimal.\textsuperscript{38} Studying cholesterol exchange processes from another angle, Hellerstein and coworkers have recently developed a technique which estimates tissue cholesterol efflux by means of the steady-state isotope dilution principle.\textsuperscript{39} Although its value remains to be determined, a potential strength of this technique is that it may be applied in both case-control settings as well as to assess the impact of novel drugs.

HDL may have its most relevant role regarding vascular protection in the initial steps of the RCT pathway. Macrophages in the vessel wall take up (oxidized) LDL, turn into foam cells and add to a pro-inflammatory environment that promotes atherosclerotic plaque formation and ultimately plaque instability. In this context, apoA-I and HDL-mediated cholesterol efflux from lipid-laden macrophages is a conceptually attractive atheroprotective mechanism. Accordingly, the cholesterol acceptor capacity of (apoB depleted) serum, or isolated HDL has been studied as a biomarker for studies that test HDL modulating therapies.\textsuperscript{30} Some of these studies showed promising data. For example, HDL isolated from patients after treatment with the CETP inhibitor torcetrapib was shown to be able to elicit more cholesterol efflux from cultured cholesterol-loaded cells than HDL taken from patients at baseline.\textsuperscript{40} However, the only study in humans to report on the predictive value of serum cholesterol acceptor capacity for CVD showed that those patients with a recurrent cardiovascular event were, paradoxically, characterized by a high serum cholesterol acceptor capacity.\textsuperscript{41} The authors speculated that a high serum acceptor capacity reflects an abundance of small lipid-poor HDL particles, as a consequence of an inherent inability of these patients to saturate their serum HDL with cholesterol.\textsuperscript{41} While this may or may not be the correct interpretation, this finding clearly illustrates the difficulties that arise in the translation of in vitro findings to the in vivo situation.

Another method to study cholesterol efflux is to assess the efflux capacity of donor cells. It is known that cellular cholesterol homeostasis is controlled by ABCA1\textsuperscript{42}, ABCG1\textsuperscript{43} and SR-BI\textsuperscript{44-47}, although a large percentage of cholesterol efflux remains unexplained.\textsuperscript{48} To date, only 2 studies addressed the relationship between cellular cholesterol donor capacity and atherosclerosis progression in humans. The first study showed a significant association between cholesterol efflux from human skin fibroblasts and increased carotid intima media thickness (cIMT; a surrogate endpoint for cardiovascular endpoints)
in 9 individuals. The second study, in 142 subjects undergoing coronary angiography, showed that cholesterol efflux from cultured primary macrophages to HDL and apoA-I was not different between subjects with and without significant stenosis. After adjustment for age and sex - the group with stenosis was on average 6 years older and consisted of significantly more men - cholesterol efflux to HDL became significantly lower in the stenosis group.

Altogether, there is no evidence for a role of HDL in the net removal of cholesterol from the body (or vascular wall) and subsequent excretion into feces in mice, while evidence in humans is very scarce. To date, there are no assays to measure cholesterol efflux that have proven value in predicting cardiovascular events in humans. This reflects the inherent difficulty of finding a measure for the complex dynamics of cellular cholesterol exchange in atherosclerotic lesions.

Other potentially atheroprotective properties

In in vitro experiments, HDL has been shown to inhibit the expression of endothelial adhesion molecules and to inhibit LDL-induced monocyte transmigration. By blocking lipopolysaccharide activity and decreasing CD11b/CD18 upregulation, rHDL has also been shown to decrease lipopolysaccharide-induced adhesion of leukocytes to human endothelial cells. In addition, the anti-oxidative activity of HDL is typically characterized by its ability to inhibit LDL oxidation but it has also been shown to inhibit the formation of reactive oxygen species. In rabbits, the infusion of rHDL or low dose apoA-I can moreover prevent or even reverse an inflammatory response when provoked by placement of a non-occlusive, silastic collar around a carotid artery. Several years ago, two assays assessing the anti-oxidative and anti-inflammatory capacity of HDL were reported to distinguish between CVD patients and healthy controls with similar HDL-C levels. These results held promise with regard to the need of valuable biomarkers to study HDL modulating therapies. Unfortunately, these findings have as of yet not been reproduced.

Apoptotic cell death following injury of vascular endothelium is assumed to play an important role in the pathogenesis of atherosclerosis. HDL has been shown to protect endothelial cells from apoptosis induced by mildly oxidized LDL. Similarly, preincubation of human umbilical venous endothelial cells with HDL can prevent apoptosis induced by tumor necrosis factor-α. HDL also potently protects endothelial cells against growth factor deprivation-induced apoptosis via suppression of the mitochondrial apoptotic pathway. Both apoA-I, and sphingosine-1-phosphate, a lipid component of HDL, have been proposed to contribute significantly to HDL-mediated cytoprotection.

HDL can affect platelet function through the promotion of nitric oxide production and coagulation by the inhibition of several coagulation factors, such as tissue factor, factor Va, VIIIa and Xa. Purified HDL, but not LDL, significantly enhanced inactivation of factor Va by activated protein C (APC) and protein S. HDL has also been shown to scavenge...
anionic phospholipids, thereby abolishing their pro-coagulant properties. Whether these anti-thrombotic effects apply to humans is unknown. There are reports of an epidemiological relationship between HDL-C and thrombosis risk, but this relationship is subject to confounding by important health parameters (e.g. obesity or the metabolic syndrome) along the lines discussed above.

One study in 2001 has shown that HDL can induce vasodilation by stimulating the release of nitric oxide by endothelial cells in mice. In line, infusion of rHDL into hypercholesterolemic, type 2 diabetic patients and subjects with low HDL-C was reported to partially restore endothelium-dependent vasodilation. These studies point towards a direct role for HDL in atheroprotection taken that endothelial dysfunction precedes atherosclerosis. To date, however, there are no assays that can be used to assess this type of HDL functionality ex vivo in a clinical setting.

In conclusion, there is considerable evidence for a direct protective role of HDL in inflammatory, oxidative, apoptotic and thrombotic processes but these studies are primarily performed in in vitro settings. With respect to protection from endothelial dysfunction, some evidence suggests that in vitro findings may apply to in vivo situations as well, but studies are small. In each case, a direct link between ex vivo measurements (biomarkers) and cardiovascular risk is yet to be established. Since such evidence could strengthen the concept that HDL is an actor rather than a bystander in atherogenesis, an important challenge for the coming years will be the translation of HDL function assays from bench to bed-side. These assays are particularly needed in view of the ongoing testing of novel HDL drugs in clinical trials, where early indications of success or failure are absolutely vital.

Animal models

Many animal models have been developed that mimic human atherosclerosis (Box 2). This section focuses on evidence that HDL provides direct atheroprotection in animal models in which key HDL modulators were studied through overexpression, knock-out and cross-breeding experiments.

ApoA-I

ApoA-I is the most important structural protein of HDL. Consequently, apoA-I knock-out mice have abnormal (apoE-enriched) HDL and 75% lower HDL-C levels. However, on an atherogenic diet these mice do not exhibit increased atherosclerosis. Knocking out apoA-I in human (h)apoB transgenic mice did result in increased atherosclerosis, but only in female mice after feeding a Western-type diet for 6 months and in the context of profound hypertriglyceridemia. Similarly, knocking out apoA-I in LDLr-/ mice increased atherosclerosis, but these mice also displayed a robust increase of VLDL cholesterol and abnormal and potentially pro-atherogenic apoA-I negative HDL,
with a density overlapping that of LDL.\textsuperscript{85} In another study, a lack of apoA-I was studied in LDLr-/-/apobec-/- (LA) mice\textsuperscript{86} which express full-length apoB100 and have 3-fold higher plasma levels of apoB100 than LDLr−/− mice.\textsuperscript{87} In this case, there was a gene-dose-dependent increase in atherosclerosis with each missing apoA-I allele in both male and female mice. Interestingly, the lipid profiles of these mice barely differed with respect to LDL and VLDL cholesterol, suggesting that the apoA-I HDL fraction was indeed responsible for the observed differences in atherosclerosis formation. Together, these data suggest more atherosclerosis as a result of a lack of apoA-I. Also, the data may imply a direct protective role for apoA-I since some observations were made in the absence of concurrent deleterious changes in other (apo)lipoprotein fractions.

Extending these findings, overexpression of hApoA-I in mice has provided very uniform results. Expression of hApoA-I inhibited pre-atherosclerotic fatty streak lesion formation in C57Bl/6 mice on an atherogenic diet and increased HDL-C levels in the absence of significant changes in non-HDL-C.\textsuperscript{88} In addition, the expression of hApoA-I in apoE deficient mice and in hApo(a)–transgenic mice yielded similar results.\textsuperscript{89–91} A reduction of atherosclerosis has been reproduced in various apoA-I gene transfer protocols in different animal models.\textsuperscript{92, 93, 94, 95} Finally, weekly infusions of purified rabbit apoA-I\textsuperscript{96} and transgenic overexpression of hApoA-I\textsuperscript{97} in cholesterol-fed rabbits also reduced progression of atherosclerosis.

\textbf{Box 2. Strengths and limitations of animal models.}

The most studied animal, the mouse, almost exclusively uses HDL to transport cholesterol in the circulation, whereas in humans most cholesterol is carried by LDL. This major difference is closely related to the natural absence and presence of CETP in mice and humans, respectively. Since mice are not normally prone to atherosclerosis, this urges investigators to use genetic and dietary manipulation to induce this phenotype. Another point that deserves attention — especially when interpreting earlier mouse studies (1990-2000) — is the use of a mixed genetic background, which in view of strong differences in atherosclerosis susceptibility amongst congenic lines makes correct interpretation difficult.\textsuperscript{206} The wide-spread use of cholate-containing atherogenic diets in especially that period, causing liver toxicity and gall stone formation, further complicates matters.\textsuperscript{207} Furthermore, most data have been generated in supraphysiological settings using high copy-number transgensics and adenovirus to mediate strong hepatic expression. Taken together, it is not surprising that many mouse studies have provided equivocal data and interpretations with regard to effects of HDL-related factors on atherosclerosis. In more recent years, more dedicated mouse models have been developed that closely mimic human atherosclerosis, e.g. the CETP\textit{tg}xLDLrKO208 and CETP\textit{tg}apoE3Leiden\textsuperscript{121} mice. In addition, the use of (conditional) tissue-specific knock-out models rather than whole body knock-out models, the use of endogenous promoters, and knock-in models have provided better tools to study lipid biology and the natural course of atherogenesis. Bone marrow transplantation finally represents another promising tool to study vascular macrophages.
Taken together, the currently published animal data provide good evidence in favour of a direct atheroprotective effect of apoA-I.

**ABCA1 and ABCG1**

ATP-binding cassette (ABC) transporter A1 is instrumental for the *de novo* synthesis of HDL in the liver and small intestine by mediating the efflux of phospholipids and cholesterol to apoA-I that is produced in these organs.\(^9^8\) ABCG1 constitutes another important player in HDL metabolism with a special role in the delivery of cholesterol to larger HDL.

Total body knock out studies have provided the following data on these transporters: ABCA1-/- mice have virtually no HDL but show no increased atherosclerosis, not even when fed an atherogenic diet on a LDLr-/- or on an apoE-/- background.\(^9^9\) ABCG1-/- mice specifically accumulate cholesterol esters in lung macrophages, but show unaltered plasma lipid levels and atherosclerosis development on a Western-type diet.\(^1^0^0\) Interestingly, knocking out both genes in the same mouse again induced marked HDL-C reductions but did not increase lipid accumulation in the vascular wall (after one year).\(^1^0^1\) Thus, a complete loss of ABCA1, ABCG1 or both does not result in increased atherosclerosis in mice.

Five studies have addressed whether increasing ABCA1 or ABCG1 function affects atherosclerosis. Transgenic ABCA1 overexpression in the liver of LDLr-/- mice was shown to result in high apoB levels and a 10-fold accelerated atherosclerosis development\(^1^0^2\) while it was associated with minimal changes in the lipid profile and a 2 to 3-fold increase of atherosclerosis in apoE-/- mice.\(^1^0^3\) In contrast, overexpression of hABCA1 under control of its endogenous promoter was shown to protect against atherosclerosis in apoE-/- mice.\(^1^0^4\) Two studies on ABCG1 overexpression showed increased or unchanged atherosclerosis in LDLr-/- mice\(^1^0^5\) and apoE-/- mice, respectively.\(^1^0^6\) Thus, only one out of these five studies showed a beneficial effect on atherosclerosis of the overexpression of either ABCA1 or ABCG1.

Bone marrow transplantation studies have offered a different view on the role of both transporters in the vascular wall. In the absence of effects on plasma lipids, repopulating apoE-/- mice with ABCA1-/- macrophages resulted in accelerated atherosclerosis.\(^9^9\) In the context of unexplained hypertriglyceridemia, an ABCA1-/- bone marrow transplantation in LDLr-/- mice also resulted in larger and more advanced atherosclerotic lesions.\(^1^0^7\) In line with an atheroprotective role of ABCA1, transplantation of macrophages of ABCA1tg mice into LDLr-/- mice resulted in decreased atherosclerotic lesion progression again without effects on lipid profiles.\(^1^0^8\) On the other hand, data on ABCG1-/- bone marrow transplantation studies provide a less clear picture: two studies showed reduced and one study increased atherosclerosis.\(^1^0^9\)

In conclusion, total body knock-out and transgenic overexpression experiments have indicated that ABCA1 and ABCG1 do not have the anticipated effect on
atherosclerosis (with the exception of 1 out of 7 studies). With bone marrow transplantation experiments, however, ABCA1 was clearly shown to provide atheroprotection, albeit in the absence of effects on plasma lipids, while similar experiments with a focus on ABCG1 have given contrasting results. Despite a clear indication that upregulation of ABCA1 may be beneficial, none of these studies support a direct protective effect of plasma HDL against atherosclerosis.

**LCAT**

Lecithin-cholesteryl acyltransferase (LCAT) is an enzyme that plays a key role in regulating HDL-C levels through the esterification of free cholesterol on HDL.\(^{110}\) In contrast to what one may expect, a marked reduction of HDL-C as a result of LCAT deficiency causes reduced atherosclerosis in C57/Bl6, LDLr-/-, apoE-/- and CETP transgenic mice.\(^{111}\) These results, however, sharply contrast with those reported by Furbee et al who showed that LCAT deficiency led to increased atherosclerosis in apoE-/- and LDLr-/- mice.\(^{112}\) The latter authors hypothesized that differences in diet and plasma apoB lipoprotein levels might explain this discrepancy.\(^{112}\) A further study by Mertens et al supported a beneficial role for LCAT by showing that LCAT gene transfer reduces atherosclerosis by reducing oxidative stress in LDLr-/-;ob/ob mice, but these effects were seen in the absence of changes in HDL-C levels.\(^{113}\)

In line with this series of diverse results when studying loss of LCAT, transgenic studies have not provided an answer whether LCAT is pro- or antiatherogenic either. A 100-fold overexpression of human LCAT in C57Bl/6N mice resulting in a 2.5-fold rise of HDL-C and a 3.5-fold rise of apoA-I levels in absence of a change in apoB levels was associated with a dose-dependent increase of diet-induced atherosclerosis.\(^{114}\) Interestingly, overexpressing hCETP in these mice normalized HDL-C levels which reduced the atherogenic phenotype.\(^{115}\) Finally, other investigators showed that moderate overexpression of human LCAT had no effect on atherogenesis.\(^{116-118}\)

In addition to mouse studies, there are a number of interesting studies on LCAT in rabbits. A 15-fold LCAT overexpression in transgenic rabbits (with endogenous CETP) has been shown to protect against diet-induced atherosclerosis\(^ {119}\) in the context of both marked increases in apoA-I and decreases in apoB containing lipoproteins, as further underlined in another study by these investigators.\(^ {120}\) However, in a later study, overexpression of LCAT on a LDLr-/- background did not affect aortic lipid concentrations nor the extent of aortic atherosclerosis in rabbits.\(^ {121}\)

**Taken together, LCAT studies in mice and rabbits have not provided clear answers when it comes to the role of this enzyme in atherogenesis, which in rabbits may be due to effects on apoB lipoproteins. These studies illustrate the impossibility to predict a shift in atherosclerosis vulnerability on the basis of changes in plasma HDL-C concentration.**
**CETP**

CETP mediates the transfer of cholesteryl esters and triglycerides between apoA-I and apoB lipoproteins. Wild-type mice are naturally CETP deficient, carry most of their cholesterol in HDL and are not prone to atherosclerosis. The introduction of human CETP in mice has greatly improved our insight into lipoprotein metabolism. In cholesterol-fed C57BL/6 mice it led to a marked shift of cholesterol from apoA-I to apoB containing lipoproteins, resulting in reduced HDL-C and increased LDL-C levels. These changes were associated with increased atherosclerosis. In line, the expression of hCETP in apoE-/- or apoE*3-Leiden mice decreased HDL-C levels, elevated VLDL, LDL and IDL cholesterol, and also increased atherosclerosis. Interestingly, inhibition of CETP with torcetrapib in the latter mice did not offer improvement when used on top of atorvastatin mimicking the torcetrapib studies in humans (addressed below). In Dahl salt-sensitive hypertensive rats (also naturally CETP-deficient) the introduction of hCETP led to a dose-dependent increase of atherosclerosis. However, these animals also displayed very strong dose-dependent increases in plasma cholesterol (up to 6-fold) and triglycerides (up to 35-fold). In contrast, in hApoCIII transgenic mice (severely hypertriglyceridemic with an increased tendency for atherosclerosis), CETP expression led to lower plasma HDL-C concentrations, lower total cholesterol levels and decreased atherosclerosis. Similarly, CETP expression has been shown to rescue the atherogenic phenotypes of LCATtg mice and SR-B1-/- mice, in conjunction with a reduction of the elevated HDL-C levels.

Rabbits naturally express CETP at high levels. When fed cholesterol, CETP inhibition using various methods generally resulted in a lower atherosclerosis burden. However, in most of these studies, the drop in non-HDL-C was stronger than the increase in HDL-C. Of note, one study failed to show an effect of CETP inhibition on atherosclerosis in the context of a non-significant decrease of non-HDL-C levels. On the other hand, a more recent study demonstrated a linear association between HDL-C increase and atherosclerosis reduction after using torcetrapib in rabbits.

In conclusion, studies in rabbits show that CETP inhibition can offer atheroprotection, while many mouse studies indicate that CETP may be pro- or anti-atherogenic depending on the metabolic context. In line with the role of CETP in lipoprotein metabolism, any modulation with an impact on CETP has marked effects on all lipoprotein parameters. As for LCAT, the CETP mouse studies show that it is not possible to predict how changes in plasma HDL-C concentrations affect atherosclerosis.

**SR-B1**

Scavenger receptor class B type 1 (SR-B1) is a hepatic HDL receptor and plays a role in cellular cholesterol homeostasis. Paradoxically, SR-B1-/- mice have 2.5 fold increased plasma HDL-C but show accelerated atherosclerosis. On an apoE-/- background,
these mice even develop spontaneous myocardial infarctions because of coronary atherosclerosis on a chow diet.\textsuperscript{136} As mentioned above, the introduction of human CETP in SR-B1/-/ mice normalized HDL-C levels and attenuated atherosclerosis.\textsuperscript{127} The anti-atherogenic role of SR-B1 was furthermore underlined by Kozarsky et al who showed that gene transfer and hepatic overexpression of SR-B1 can protect from atherosclerosis.\textsuperscript{137} SR-B1 is also expressed in macrophages, where it mediates cellular cholesterol exchange.\textsuperscript{44-47} In bone-marrow transplantation studies, SR-B1 has been shown to modulate atherosclerosis development without effects on circulating lipids.\textsuperscript{138, 139}

These SR-B1 studies reiterate that modulation of HDL-C levels may have a paradoxical impact on atherosclerosis.

**HL**

Hepatic lipase (HL) hydrolyzes both triglycerides and phospholipids and exerts this catalytic function on all classes of lipoproteins.\textsuperscript{140, 141} Overexpression of human HL in mice resulted in decreased total and HDL cholesterol levels, and decreased aortic cholesterol content.\textsuperscript{142} This effect on lipids and atherosclerosis was also seen in a murine HL/LDLR double -/- context.\textsuperscript{143, 144} In HL/apoE double -/- mice, the introduction of liver-specific human HL also lowered total and HDL cholesterol. However, in this study an increase in plaque size was observed.\textsuperscript{145} HL/-/ mice display a mildly altered lipid phenotype, including a 21\% increase in HDL-C and a 35\% increase in total cholesterol, but showed no change in atherosclerosis. Female HL/apoE double -/- mice presented with reduced aortic atherosclerosis, in the context of increases in all lipoproteins including HDL.\textsuperscript{146} In contrast, in HL/LDLR double -/- mice more atherosclerosis was observed.\textsuperscript{147}

Thus, mouse studies into the role of HL in atherosclerosis have provided equivocal data, illustrating the impact of the type of mouse model applied. Here too, levels of HDL-C are a poor predictor of the atherogenicity of a particular model.

**EL**

Endothelial lipase (EL) exhibits a high phospholipase activity and low triglyceride lipase activity.\textsuperscript{143} It primarily hydrolyzes HDL lipids. Mouse studies into the effect of EL overexpression on atherosclerosis have not yet been reported. EL/apoE double -/- mice had reduced atherosclerotic aortic lesion area in one study,\textsuperscript{148} but no change in plaque area compared to apoE -/- mice in another study.\textsuperscript{149} Both studies reported an increase in HDL-C and phospholipids and no change in triglyceride levels.

More data are needed to evaluate the link between EL, HDL and atherosclerosis.
Liver X receptors (LXR) α and β are nuclear receptors that control cellular cholesterol homeostasis through transcriptional regulation of target genes such as *ABCA1*, *APOE* and *ABCG1*. The importance of these receptors was shown by knocking out LXRα and β which caused a 26% reduction of HDL-C, a 83% increase of LDL-C, and a 27-fold accumulation of cholesterol in arterial wall macrophages in mice on a normal chow diet (although aortic lesions did not progress to advanced atheroma). It has been hypothesized that upregulation of LXR target genes can provide atheroprotection. Indeed, systemic administration of synthetic LXR agonists has been shown to reduce plaque formation in mice. Bone marrow transplantation studies have moreover shown that this atheroprotection can be attributed to macrophage LXRs. In addition, others have shown that LDLr-/- mice transplanted with LXRαβ-/- bone marrow demonstrate a 3-fold increase in atherosclerosis in absence of changes in plasma lipids. Unfortunately, systemic administration of first generation LXR agonists results in elevated plasma triglycerides, fatty liver, and disturbance of growth hormone and thyroid axes.

These studies show that cholesterol mobilization from the vascular macrophage constitutes an important step in atheroprotection. However, these studies do not support a direct role for HDL as an atheroprotective entity.

Reviewing the data of major animal studies that have focused on primary modulators of HDL metabolism, it is clear that the vast majority of animal studies have thus far not provided evidence that HDL protects from atherosclerosis. Although many studies may have indicated that modulation of HDL metabolism is associated with atheroprotection, a careful analysis shows that this is often seen in the context of a simultaneous modulation of apoB-containing lipoproteins which obscures the reported effects of HDL on atherosclerosis. The most positive and convincing data have been generated by studies of overexpression of human apoA-I in mice and apoA-I infusion in rabbits. Paradoxically, high HDL-C levels have also been associated with accelerated atherosclerosis in SR-BI-/- or LCAT transgenic animals. The capacity to reverse atherosclerosis in these mice through CETP expression underlines the interdependency of various HDL modulators to achieve atheroprotection. Finally, bone marrow transplantation experiments and studies with LXR agonists have provided good evidence that promotion of cellular cholesterol efflux from the vascular macrophage confers atheroprotection by promoting the initial steps of the RCT pathway. However, it should be noted that these processes bear no apparent relationship to plasma lipid levels, including those of HDL-C.
Genetic studies in humans

Studying families with genetic disorders of HDL metabolism and thus a life-long exposure to high or low levels of HDL-C may help to unravel whether HDL provides direct atheroprotection or not. A life-long exposure to 30-60% reductions of HDL-C levels as a consequence of mutations in ABCA1, APOAI or LCAT has been reported to be associated with increased cIMT and CVD risk in several family studies but other investigators have not found this. The APOAI defect, leading to very low levels of HDL-C, has been associated with a normal cIMT in carriers. This has led some to believe that apoA-I Milano is more atheroprotective than normal apoA-I, but one may also interpret this as evidence that HDL does not protect. Although this specific apoA-I mutation has been reported to induce enhanced cellular cholesterol efflux compared to wild-type apoA-I, others have disputed this.

Human studies of functional mutations in genes that are associated with higher HDL-C plasma levels have not given a clear picture thus far. In line with the HDL hypothesis, carriers of CETP mutations were initially reported to have vascular benefit but this concept was not supported by later studies. Several reports have indicated that HL deficiency is associated with high HDL-C but the number of subjects studied is too small to draw conclusions on the effect on atherosclerosis. Individuals with functional mutations in EL have been reported to exhibit increased HDL-C levels but to date there is no information on the risk of atherosclerosis in these carriers. Thus far, only one family study by our group describes a mutation in SR-B1 affecting HDL metabolism (manuscript in preparation).

Under the assumptions of Mendelian randomization, the existence of a causal relationship between HDL-C levels and CVD would imply that associations between a gene variant and HDL-C levels will translate into an altered risk of disease. This concept was recently used by Danish investigators who studied the impact of mutations in ABCA1 and HL on future CVD in the general population. In two population cohorts and a large case-control study, Frikke-Schmidt et al have studied ABCA1 mutations and found that a ~30% lower HDL-C associated with functional mutations in ABCA1 did not predict cardiovascular risk in the general population and in CVD patients. For HL, three functional variants have been shown to be associated with a modest rise in HDL-C levels, without changing the risk of CVD.

Single nucleotide polymorphisms (SNP) in CETP presumably have the highest impact on HDL-C of all common genetic variants that have hitherto been studied throughout the genome. A recent meta-analysis on CETP genotypes and CAD risk in 196,367 subjects (46 studies combined) showed 5% risk reductions for three CETP polymorphisms, all of which are associated with lower CETP concentration and activity. Because these variants conferred both ~5% higher HDL-C levels as well as ~1% lower LDL-C and ~2% lower triglyceride levels, it cannot be excluded that the association of these variants with apoB lipoproteins was responsible for the observed impact on CVD risk. In fact, a 5% risk...
reduction for a lifelong 1% reduction in LDL-C is proportional to that found for a common genetic variant in the LDL receptor gene, where a 5% increase in LDL-C was associated with an odds ratio for CVD of 1.29.\textsuperscript{174} Genome wide-association studies (GWAS) have recently provided new data on loci that modulate lipids and (apo) lipoproteins as well as CVD risk. In two thousand CAD cases and more than twelve thousand controls, it was shown that 11 SNPs associated with elevated LDL-C levels increased CVD risk.\textsuperscript{174} In contrast, 16 SNPs associated with changes in HDL-C levels (in or near CETP, LIPC, LPL, GALNT2, LIPG, ABCA1, MVK/MMAB, LCAT and GRIN3A) were not associated with CVD risk.\textsuperscript{174} In addition, Kathiresan and coworkers reported that a genotype score, crafted from a combination of 4 HDL SNPs and 5 LDL SNPs was an independent risk factor for CVD.\textsuperscript{175} Genotype scores for HDL-C or LDL-C also predicted CV events separately, but the effects of the individual SNPs on risk were not reported.\textsuperscript{175}

In conclusion, the published cross-sectional family-based candidate gene studies suffer from small numbers, ascertainment bias and lack of prospective follow-up. This means that the results may be skewed because samples are taken from families who have come to a physician’s attention, rather than from the general population.\textsuperscript{171} Bypassing referral bias and cross-sectional designs, large prospective population studies have indicated that non-synonymous mutations in ABCA1 and HL have no effect on CVD risk, notwithstanding profound effects on HDL metabolism. Recent evidence obtained from large epidemiological and GWAS analyses do not support the HDL hypothesis either. In fact, a direct head-to-head comparison between genetic variants affecting either plasma LDL-C or HDL-C in a very large study\textsuperscript{174} indicated that only the former predict CVD.

Clinical trials

This section focuses on therapeutic modalities that affect HDL and have been studied for anti-atherosclerotic efficacy in humans or show particular promise for the future.

Fibric acid

Fibrates have primarily been developed on the basis of their cholesterol- and triglyceride-lowering activity in rodents. This class of drugs comprises agonists of the peroxisome proliferator-activator receptor-alpha (PPAR-\(\alpha\)) mostly expressed in muscle and liver. They induce triglyceride catabolism and hepatic fatty acid uptake, reduce hepatic triglyceride production, increase the removal of LDL from plasma, reduce CETP activity, and increase the production of apoA-I and apoA-II.\textsuperscript{176} In humans, fibrates have been shown to reduce triglyceride levels by 18-48% and LDL-C levels by 8-13% while increasing HDL-C up to 10%.\textsuperscript{177, 178} In a post-hoc analysis of the VA-HIT trial, which studied the atheroprotective effect of gemfibrozil in 2,531 men with a history of coronary heart disease,
cardiovascular event rate was found to be inversely associated with on-trial HDL-C levels. This has been interpreted as evidence in favor of a causal relationship between HDL-C and CVD. However, in light of the much stronger triglyceride-lowering effect, the strong inverse biological relationship between HDL-C and triglycerides, and the much higher variability of triglyceride measurements, this interpretation is not conclusive. On the other hand, a recent meta-analysis suggests that fibrates are efficacious: they were shown to reduce the risk of non-fatal myocardial infarction by 22% without influencing mortality, cancer or stroke.

Nicotinic acid
Nicotinic acid, also known as niacin or vitamin B3, lowers triglycerides and LDL-C by 20% and 12%, respectively, and can increase HDL-C levels up to 23%. Niacin inhibits hepatic triglyceride synthesis, thereby decreasing plasma apoB-containing lipoproteins, and raises HDL-C levels by decreasing hepatic holoparticle HDL uptake through inhibition of the expression of hepatic ATP synthase β chain. A third target for niacin is the G-protein-coupled receptor GPR109A (PUMA-G in mice). Although this receptor has been shown to be critical to niacin’s lipid-altering effects in mice, the relevance of this receptor in humans is controversial. An argument against a role for GPR109A is the absence of its expression in the human liver, which is thought to be a primary target organ of niacin. Finally, experiments with APOE*3-Leiden/CETPtg mice showed that the HDL-C increasing effect of niacin is at least partly mediated by inhibition of hepatic CETP expression. Niacin was reported to improve subclinical measures of atherosclerosis as well as cardiovascular event rates in a few small trials. In 160 patients, the HATS study showed that a niacin simvastatin combination therapy led to a lower cardiovascular event rate compared with placebo. The subsequent ARBITER 2 study examined the impact of the addition of extended-release niacin to statin therapy on cIMT progression in 167 patients with established coronary heart disease (and low HDL-C). In this study, no significant difference in cIMT progression was found between the treatment arms, but a significant cIMT progression occurred in the statin-only arm versus baseline, in absence of a significant progression in the niacin arm. In the ARBITER 3 study, 104 participants were treated with extended-release niacin and these subjects showed a net cIMT regression. However, in these cases an open-label design and the lack of a control arm constitute serious limitations. Very recently, two carotid imaging studies provided additional encouraging evidence of atheroprotection conferred by extended-release niacin on top of statins. Although these reports strongly emphasized the impact of niacin on HDL-C levels, the studies show similar profound changes in triglyceride levels. The development of fibrates and nicotinic acid derivatives was not founded on the HDL hypothesis; rather, their potential to increase HDL-C has been welcomed as a side-effect. Although these drugs unmistakably have atheroprotective potential, given their broad spectrum of effects on lipid metabolism it is difficult to confidently ascribe this beneficial effect to their impact on HDL-C.
**HDL-like particles**

Several investigators have studied the effects of infusion of HDL-like particles. In a study performed by Eriksson et al, the intravenous infusion of human proapoA-I liposome complexes in 4 subjects with heterozygous familial hypercholesterolemia was shown to increase fecal cholesterol excretion, lending support to the classical RCT concept.\(^{35}\) In 2003, a widely-cited study reported regression of coronary plaque atheroma volume compared to baseline after infusion of recombinant apoA-I\(_{\text{Milano}}\)/phospholipid complexes.\(^{187}\) This study was of great conceptual importance but it only comprised 45 patients in the active arm and 12 patients in the placebo arm, leaving it underpowered for a formal direct comparison between treatment arms. In addition, the investigators did not observe a dose-effect relation.\(^{188}\) Other investigators used a similar compound, rHDL, in a larger study. In this placebo-controlled trial with 145 patients, four weekly infusions of rHDL proved ineffective in reducing coronary plaque atheroma volume versus placebo, although plaque regression relative to baseline in the active arm of the study was observed.\(^{189}\) Larger studies with HDL infusion protocols showing reduced plaque burden and a reduction in hard clinical endpoints could provide a good basis for the HDL hypothesis. At this point however, the data on the effect of relatively short-term HDL infusion studies are scarce and in our opinion disappointing.

**CETP inhibition**

By inhibiting CETP, the normal exchange of neutral lipids between apoA-I and apoB-containing lipoproteins is blocked, which results in higher HDL-C and lower LDL-C levels.\(^{190}\) Administration of CETP inhibitors dalcetrapib (previously denoted as JTT-705), torcetrapib and anacetrapib has been shown to potently increase plasma HDL-C concentrations in humans.\(^{191-193}\) Although phase III trials with dalcetrapib and anacetrapib were recently initiated, similar studies with torcetrapib were prematurely halted in 2006: the large phase III mortality and morbidity trial ILLUMINATE was terminated because of significantly higher cardiovascular and non-cardiovascular mortality rates in the atorvastatin/torcetrapib arm.\(^{194}\) Concurrent imaging trials did not show positive effects of torcetrapib treatment on cIMT\(^{195, 196}\) nor on coronary percent atheroma volume when applied on top of atorvastatin therapy.\(^{197}\) The question is whether the failure of torcetrapib can be attributed to CETP inhibition in general or to off-target toxicity. Focusing on the latter, torcetrapib use has consistently been associated with increased blood pressure, elevated plasma aldosterone levels and electrolyte changes.\(^{194-198}\) These effects appear to be specific to torcetrapib since other CETP inhibitors have no effect on blood pressure.\(^{199}\) These effects were moreover independent of the inhibition of CETP since torcetrapib also evoked an acute increase in blood pressure and plasma adrenal steroids in naturally CETP deficient mice while anacetrapib had no such effects.\(^{200}\) Although these findings indicate off-target toxicity for torcetrapib, they leave open the question whether CETP
inhibition constitutes a viable strategy for atheroprotection. Results from ongoing CETP inhibition trials may provide the answer in the next few years.

*ApoA-I mimetic peptides and stimulation of apoA-I synthesis*

These peptides, seldom affecting HDL cholesterol levels, shift the focus to functional attributes of HDL. Some apoA-I mimetic peptides have been reported to result in the formation of pre-beta like HDL particles, to increase anti-oxidative paraoxonase activity and to decrease HDL lipid hydroperoxides. One of these peptides, orally administered D-4F, has been show to be safe and well-tolerated in a first trial in humans but data in humans on the efficacy of these compounds is unavailable at this point. In contrast to the scant data in humans, mouse studies with apoA-I mimetic peptides have shown promise to reduce atherosclerosis and endothelial dysfunction.

Based on the positive data on apoA-I overexpression in animal models, strategies to increase apoA-I synthesis are vigorously pursued. RVX-208 is a compound that was specifically developed to increase endogenous apoA-I production. In African green monkeys, oral administration resulted in increased levels of plasma apoA-I and HDL-C. Serum taken from these animals was shown to mediate enhanced cholesterol efflux from J774 macrophages via the ABCA1 pathway, probably related to higher concentrations of small HDL particles. At this point there are no published data available regarding effects on atherosclerosis. A phase III clinical trial is currently planned.

In summary, there is evidence for fibrates and niacin as anti-atherosclerotic agents, but since both drugs have major effects on other blood lipids, this does not prove that HDL is causal to the observed atheroprotection. A large ongoing outcome trial with niacin (HPS2-THRIVE) may provide the statistical power to indirectly assess the effect of an increase in HDL-C in 2013. The use of apoA-I mimetic peptides has yet to be proven to have clinical relevance, whereas infusions of HDL-like particles have shown promise in preclinical studies and small-scale clinical trials, but have thus far not improved cardiovascular outcome in larger studies. To date, the most powerful tools to raise plasma HDL-C concentrations are CETP inhibitors. Whether these drugs decrease CVD risk may be learned from two large outcome trials of which the results are expected in 2012. Drugs that specifically upregulate apoA-I may be the ultimate means to prove that HDL protects from CVD in humans, but these compounds are in early clinical development.

**Conclusions**

A considerable and diverse body of evidence has been generated to support the hypothesis that HDL protects against atherosclerosis. However, caution is needed as the strong urge for novel drugs to decrease CVD risk may have biased this field of research. The most convincing evidence for the HDL hypothesis has been generated through tissue
culture and animal experiments, with suggestive evidence from epidemiological studies. However, direct evidence from human studies is almost absent.

Adjusting for confounders in the relationship between HDL and CVD

Many risk factors for CVD also influence HDL-C levels, thereby blurring the relationship between HDL and CVD. Statistical adjustment for these potential confounders relies on

Although epidemiological studies have established a strong negative relationship between HDL cholesterol and cardiovascular disease, there are many variables which affect HDL cholesterol levels as well as cardiovascular disease risk simultaneously; the role of these potential confounders is unclear. On the basis of in vitro experiments, HDL has been credited with many putative atheroprotective qualities, but clinical relevance has not been convincingly proven for any of these properties. Animal models yielded conflicting results for most of the key players in HDL metabolism with respect to their effect on plasma HDL levels and atherosclerosis propensity. Only apoA-I, the primary structural protein of HDL, showed a strong and consistent atheroprotective tendency in overexpression and infusion models. In humans, both high-quality genetic association studies and clinical trials have thus far not provided clear-cut evidence in favour of the hypothesis that HDL protects from atherosclerosis.
three assumptions: that the relationships between confounders and HDL-C and CVD are linear, independent of each other and assessed with great precision. It is, however, safe to state that, for many confounders, none of these assumptions hold. It is well-established that associations between parameters in epidemiological studies can only aid the formulation of hypotheses, which need to be tested by experimental research. In this respect, the results of vitamin E supplementation, hormonal replacement therapy, or homocysteine lowering by folic acid and vitamin B supplementation are unsettling examples of promising epidemiological relationships which turned sour in a clinical trial setting. The HDL hypothesis too should be subjected to rigorous experimental testing.

In our view, the HDL hypothesis remains a hypothesis to date (summarized in Figure 1). This relates first of all to the challenge to study HDL independent from effects on other plasma lipids and lipoproteins which is true for epidemiology, animal studies as well as for clinical trials. The heterogeneity of HDL represents a second major hurdle. Most studies carried out thus far have focused on plasma HDL-C concentrations as a marker of the protection that is associated with HDL. However, it has become increasingly clear that this may be an oversimplification. The studies with the CETP inhibitor torcetrapib are a hallmark in this respect, with increased levels of HDL-C obviously not being predictive of a better outcome. Earlier studies have moreover shown that interventions in HDL metabolism may affect atherosclerosis in the absence of effects on plasma HDL-C concentrations. Although it is conceivable that HDL subspecies, or individual HDL components will better predict atherosclerosis, studies into these matters have thus far only provided many hypotheses and the translation of such data to clinical practice is in its infancy. Strategies aimed at improving HDL function may prove useful, but HDL function, a poorly defined concept to date, is not easily assessed. What is needed first is convincing evidence that HDL function parameters can both predict the occurrence of CVD, as well as a beneficial therapeutic intervention in humans.

Genetic studies offer another interesting means to study the HDL hypothesis. Data from very large genetic association studies will soon provide the statistical power needed to test whether HDL gene variation affects atherosclerosis or not. However, many of the genetic variants that affect HDL-C levels also affect other lipid parameters. With respect to clinical trials, only those compounds causing an isolated HDL increase can be expected to offer a direct answer to the primary question of this review. Atherosclerosis imaging and clinical endpoint trials with specific apoA-I agonists are best suited to this goal, since these compounds target the de novo HDL production at its natural production site and have little or no effect on other lipid parameters. The infusion of HDL-like macromolecules constitutes another means to study whether HDL provides atheroprotection but, if successful, the intravenous route of administration is bound to limit its application in daily clinical practice.

Irrespective of the outcome of ongoing trials, the identification of putative drug targets related to HDL pathways remains of great importance. Research into macrophage cholesterol homeostasis and, more specifically, cholesterol efflux has already provided
interesting targets for therapy. With macrophages at the culprit of atherosclerotic plaque
development, the right intervention directed at these cells can be expected to provide
atheroprotection. However, here too, stronger evidence to link macrophage cholesterol
efflux to CVD in humans would be welcome.

Acknowledgements
We are indebted to Haydn Pritchard (University of British Columbia, Vancouver) for
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021.001.035).
Reference list


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Chapter 1


The HDL hypothesis


Chapter 1


Chapter 1


Chapter 1


Chapter 2

Outline
Adriaan G. Holleboom

Image in background: figure from paper entitled “Protein-lipid Relationships in Human Plasma, II. In Atherosclerosis and Related Conditions” by David P. Barr et al, American Journal of Medicine 1951, in effect depicting the “birth” of the HDL hypothesis. Figure used with permission. See Chapter 1 for review of evidence for this hypothesis.
According to Fact Sheet No. 317 of the World Health Organisation of January 2011, cardiovascular diseases (CVD) are now the primary cause of mortality in the world.\(^1\) 60 years ago, Barr and co-workers reported a strong inverse relationship of plasma levels of cholesterol contained in high density lipoproteins (HDL-c) and the risk of CVD.\(^2\) In several large population-based studies, this association proved to be strong, reproducible and independent of other factors that influence the risk of CVD, such as age, gender, smoking behaviour and plasma levels of low density lipoprotein cholesterol (LDL-c).\(^3, 4\)

Ever since, these studies have driven doctors, biologists, statisticians and other scientists to investigate HDL, which popularly also became known as ‘the good cholesterol’. This scholarship has greatly advanced our knowledge of HDL metabolism, but it also clearly demonstrates that HDL metabolism is complex. Many molecules are involved and interrelated, which makes it difficult to answer the two fundamental questions in this field of research:

- does HDL itself actually and causally protect from atherosclerosis?
- which process in HDL metabolism should be targeted to achieve the desired therapeutic potential?

To advance our understanding of these matters, in this thesis we have studied HDL metabolism in patients with monogenic disorders that affect receptors, enzymes and other proteins that are essential to this macromolecule. This approach takes advantage of a reduction in the complexity of HDL metabolism, because only one factor at time is altered, in a stable way, whilst at the same time studying human pathophysiology, thus not turning to \textit{in vitro} reflections of HDL biology.

With these disorders as a cornerstone, we have addressed the questions in the HDL field by conducting an range of studies, from infusion protocols based on the tracer dilution principle and kinetic modelling, to validation of a gene locus found in genome wide association studies (GWAS) through the identification of a linking molecular mechanism by the use of high throughput sequencing, mass spectrometry and molecular studies of protein glycosylation: from \textit{Model to Mechanism}

This thesis consists of three parts. In the first part, we study how mutations in genes encoding HDL-related proteins affect its metabolism in humans. In the second part, we investigate lecithin:cholesterol acyl transferase (LCAT), an enzyme crucial to the maturation of HDL particles, and the consequences of mutations in the gene that encodes for this enzyme. In the final part, we look beyond cholesterol concentrations of HDL by investigating a number of its functions in human homeostasis.
Part 1 - HDL candidate and GWAS genes

Chapter 3 provides a summary of the current literature on the genetics of HDL. We discuss a number of HDL genes that have been identified in humans and mice, we review genetic association studies and the use of SNP panels in CVD risk prediction, and we provide an overview of newly proposed HDL genes.

Earlier research in vitro and in animals suggested a role for scavenger receptor class B, type I (SR-BI) as an HDL receptor, in platelet function, in atherosclerosis susceptibility and in adrenal steroidogenesis. Chapter 4 examines the function of SR-BI in humans. To this end, we identified a family in which we were able to compare patients with an inborn error in SR-BI with their unaffected relatives.

Genome-wide association studies have linked variation in GALNT2, the gene encoding ppGalNac-T2, to variation in HDL-c and triglycerides in the general population. ppGalNAC-T2 is an enzyme responsible for the initiation of mucin-type O-linked glycosylation of proteins. In chapter 5 we investigate the linking molecular mechanism in carriers of a mutation in GALNT2.

To better understand the molecular pathology of low plasma HDL-c levels, we sequenced the gene encoding the ATP-binding cassette protein A1 (ABCA1), crucial to the lipidation of HDL particles with cholesterol effluxed from macrophages, in patients with very low HDL-c levels in chapter 6. We describe the identification of ABCA1 gene variants and perform in vitro experiments to study the consequences of these genetic variants for the function of this transmembrane cholesterol transporter.

Part 2 – LCAT and its clinical consequences

Lecithin:cholesterol acyl transferase is a crucial enzyme to HDL metabolism in that it catalyses the esterification of cholesterol, leading to the maturation of HDL particles from nascent discs to spherical lipoproteins. Mutations in the LCAT gene cause defective maturation of HDL particles and low HDL-c levels in mutation carriers. In a similar effort to study the ABCA1-related molecular pathology of low HDL-c (chapter 6), chapter 7 describes the frequency of LCAT gene mutations in patients with a low HDL-c referred to our hospital. The effects of these mutations on the enzymatic function of lecithin:cholesterol acyl transferase are studied in vitro.

In chapter 8 we investigated the relationship between plasma levels of LCAT protein, lipid metabolism, and risk of coronary artery disease (CAD) in a prospective study of 933 individuals who developed CAD during follow-up and 1,852 age- and gender-matched controls who did not develop CAD. On the basis animal studies and studies of patients with LCAT deficiency, we hypothesized that low plasma levels of LCAT would be associated with low HDL-c levels.

In chapter 9 we study the molecular defect in a patient who presented with HDL deficiency, early signs of corneal opacification, severe proteinuria and glomerular
pathology, fitting the clinical phenotype of familial LCAT deficiency. Early stages of this
disease are studied, as well as optimal symptomatic treatment of the nephropathy.
In the subsequent three chapters, we study arterial wall thickness (chapter 10, 11) and
stiffness (chapter 12) in carriers of LCAT mutations to address the question whether
patients who suffer from this specific form of genetically low HDL-c levels have
increased atherosclerosis. Chapter 10 describes a case control study of heterozygous
LCAT mutation carriers. With the use of B-mode ultrasound, we measured carotid intima
media thickness (cIMT) as a marker of subclinical atherosclerosis in these patients. In
chapters 11 and 12, we applied 3.0 T MRI technology and a dynamic assessment of
arterial stiffness to further investigate the arterial condition in these patients.
Through its ability to function as a phospholipase A₂, LCAT is proposed to have anti-
oxidant properties. It is not known whether this function is relevant to lipid oxidation
in humans. In chapter 13, we therefore studied lipid oxidation in carriers of LCAT
mutations. We measured activities of the three anti-oxidative enzymes associated with
HDL, i.e. LCAT, paraoxonase 1 and platelet-activating factor acetyl hydrolase, as well as
arachidonic and linoleic acids and their oxidative derivatives, (auto-)antibodies against
oxidized phospholipids and apolipoproteins and finally, the anti-oxidant capacity of HDL,
and compared these to family controls.

Part 3 – HDL function: beyond its cholesterol concentration
Atheroprotection by HDL is generally considered to be mediated by reverse cholesterol
transport (RCT) from peripheral tissues to cholesterol excretion via the liver. In humans,
however, little is known about in vivo cholesterol fluxes through this pathway. In chapter
14, we therefore investigated whether tissue cholesterol efflux as well as fecal sterol
excretion was altered in patients with genetically low HDL-c due to mutations in APOA1
or ABCA1 by the use of an infusion protocol based on the tracer-dilution principle and
kinetic modelling.
Lipoprotein-independent secretion of 27-hydroxycholesterol (27OHC) from macrophages
has been proposed to offer an alternative to HDL-mediated RCT. In chapter 15, we
investigated 27OHC concentrations in plasma of humans and mice with monogenic
disorders of HDL metabolism to study whether 27-OHC is indeed independent of HDL
metabolism.
In plasma, HDL is the major carrier of both sphingosine-1-phosphate (S1P), a bioactive
sphingolipid, and of apolipoprotein M (apo M), which is involved in the formation of
nascent HDL particles. Apo M has been proposed to bind S1P within HDL. In chapter 16,
we tested the hypothesis that plasma HDL-c or apo M determine plasma S1P levels by
measuring S1P and apo M in patients with both mildly and severely lowered HDL-c or
apo A-I due to mutations in APOA1, ABCA1 or LCAT and in patients with high levels of
HDL-c and apo A-I due to mutations in CETP, SCARB1, LIPC or LIPG.
Cardiovascular death is often preceded by ventricular tachyarrhythmias. Prolongation of the cardiac repolarization time, which has been shown to predict sudden cardiac death, often underlies these rhythm disturbances. HDL might protect from cardiovascular death including sudden cardiac death.\textsuperscript{4} \textbf{Chapter 17} tests the hypothesis that increasing HDL shortens cardiac repolarization. We treated isolated rabbit cardiomyocytes with reconstituted HDL (rHDL) as well as purified human apo A-I and studied repolarization. In addition, the effect of rHDL infusion on heart rate-corrected QT interval on surface electrocardiograms of patients with genetically low HDL-c as well as controls was studied. \textbf{In chapter 18} we study a potentially antiatherogenic consequence of tumor necrosis factor $\alpha$ (TNF$\alpha$) signalling in vascular cells in relation to cellular cholesterol efflux from macrophages via ABCA1. TNF$\alpha$ is released by free cholesterol-loaded apoptotic macrophages, and the clearance of these cells by phagocytic macrophages may help to limit plaque development. Macrophage cholesterol uptake induces ATP-binding cassette transporter A1 (ABCA1) to promote cholesterol efflux to apolipoprotein A-I, potentially reducing atherosclerosis. We study the effect of TNF$\alpha$ on ABCA1 expression and function in macrophages and the linking intracellular signalling pathway.

Reference List


Part 1

Mutations in HDL candidate and GWAS genes
Image on previous page: Immunoblot of 2D gel electrophoresis of apolipoprotein C-III isoforms of a carrier of the D314A-mutation in GALNT2, showing an increase in the nonsialylated apo C-III0. See Chapter 5 for more detail.
Chapter 3


The value of HDL genetics

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Abstract

Purpose of review. Hereditary disorders of high-density lipoprotein (HDL) metabolism and studies in mice have provided valuable insight into the pathways of this intriguing lipoprotein and moreover revealed targets to raise HDLc to reduce atherosclerosis.

Recent findings. To date, as many as 11 genes are considered key players in the synthesis, maturation, conversion and/or catabolism of HDL. Five of these genes have been identified in humans: APOA1, LCAT, ABCA1, HL, and CETP, while the other six genes have been identified in mice: SCARB1, ABCG1, ATPB5, PLTP, LIPG and APOM. Genetic association studies are as of yet the best line of evidence of the roles of the ‘murine genes’ in human HDL pathways. In addition to recent genetic association studies, a third section describes exciting news on six newly proposed HDL genes: VNN1, GALNT2, MMAB/MVK, CTα, BMP-1 and SIRT1.

Summary. This review provides a summary of the current literature on the genetics of HDL. New information from this research area may assist us in obtaining a better understanding of HDL biology and identifying novel pharmacological targets.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABCA1</td>
<td>ATP binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP binding cassette protein G1</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Angiopoietin protein like 4</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>Apolipoprotein A-I</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>Apolipoprotein A-II</td>
</tr>
<tr>
<td>ApoC-III</td>
<td>Apolipoprotein C-III</td>
</tr>
<tr>
<td>ApoM</td>
<td>Apolipoprotein M</td>
</tr>
<tr>
<td>ATP5B</td>
<td>ATP synthase H+ transporting mitochondrial F1 complex β-polypeptide</td>
</tr>
<tr>
<td>BMP-1</td>
<td>Bone-morphogenetic protein 1</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CTα</td>
<td>CTP:phosphocholine cytidylyltransferase-alpha</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>EL (LIPG)</td>
<td>Endothelial lipase</td>
</tr>
<tr>
<td>eQTL</td>
<td>Expression quantitative trait locus</td>
</tr>
<tr>
<td>GALNT2</td>
<td>N-acetylgalactosaminyltransferase 2</td>
</tr>
<tr>
<td>GWA</td>
<td>Genome wide association</td>
</tr>
<tr>
<td>HDLc</td>
<td>High-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HIDS</td>
<td>Hyperimmunoglobulinemia D syndrome</td>
</tr>
<tr>
<td>HL (LIPC)</td>
<td>Hepatic lipase</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin:cholesterol acyl transferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>LDL receptor</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>MMAB</td>
<td>Cob(I)alamin adenosyltransferase</td>
</tr>
<tr>
<td>MVK</td>
<td>Mevalonate kinase</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid transfer protein</td>
</tr>
<tr>
<td>PON1</td>
<td>Paraoxonase 1</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low density lipoprotein</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SR-B1 (SCARB1)</td>
<td>Scavenger receptor B1</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>VLDLR</td>
<td>Very low density lipoprotein receptor</td>
</tr>
<tr>
<td>VNN1</td>
<td>Vanin-1 (pantetheinase)</td>
</tr>
</tbody>
</table>
Introduction

HDL represents a highly heterogeneous pool of the smallest of lipoproteins in the circulation. These dynamic macromolecules, rich in protein, carry neutral lipids (cholesteryl ester and triglycerides) packaged in a monolayer of phospholipids and (apolipo)proteins. HDL is generally considered to protect our body from lipid accumulation, especially in macrophages in the vascular wall, but other functions have also been suggested, including protective roles in inflammation, apoptosis, and platelet aggregation (reviewed by Rader[1]). Ever since epidemiological studies have provided unequivocal evidence of an inverse relationship between HDLc levels and cardiovascular disease (CVD)[2], it has been hypothesized that raising HDL is anti-atherogenic.

Agents known to raise HDLc levels, such as fibrates and niacin, have indeed been shown to reduce atherosclerosis-related morbidity and mortality (reviewed by Joy and Hegele[3]) but convincing evidence is yet to be provided[4] (results of 2 clinical endpoint trials are expected in 2010[5] and 2013[6]). Another means to increase HDL is the infusion of HDL-like particles. In 2003, a widely-cited study reported regression of coronary plaque atheroma volume compared to baseline after infusion of recombinant apoA-I Milano/phospholipid complexes.[7] However, in a larger placebo-controlled trial published in 2007, a similar compound proved ineffective although plaque regression relative to baseline in the active arm of the study was observed.[8] In the same year, the promise of CETP inhibition, a novel class of HDL drugs [9], suffered a major blow after a dramatic failure of one of its class.[10] These unfortunate results have challenged the idea that increasing HDLc is guaranteed to provide atheroprotection. As a result, this issue is now more vigorously and openly discussed (reviewed by Joy and Hegele[3], and by Carlquist[11]).

Numerous reviews have been published on HDL as a promising target for pharmacological intervention [3;12-14] but predicting the effects of intervention in HDL metabolism has proven difficult and only large cardiovascular endpoint trials can currently provide the ultimate answer. At the same time, the basic scientific community has attempted to improve our understanding of HDL biology. A major contribution to this understanding comes from studying HDL genetics, the topic of this review. In an attempt to not duplicate other reviews[15-18], we have limited ourselves to the following topics:

The first section discusses a set of more or less established HDL genes that have long been identified in humans and mice. The second section summarizes the latest news on genetic association studies and the use of SNP panels in CVD risk prediction. The third section summarizes exciting information on newly proposed HDL genes.
Established HDL genes

Twin studies have indicated that 50% of the variation of HDLc levels is genetically determined.[19] An extensive literature search indicates that, in fact, 50 different genes are reported to be associated with HDL. However, in many cases it is unclear how the respective gene product affects HDL metabolism (see following sections). On the basis of the present literature, we arbitrarily denoted 11 of these genes as ‘established HDL genes’. This was performed on the basis of either long-established literature or reports published in high-profile journals. These genes encode proteins directly affect HDL synthesis, maturation, conversion and/or catabolism. Table 1 also shows how these genes were identified and to what extent their role in human metabolism has been established to date. While classical linkage analysis in families with hereditary HDL disorders has traditionally led to major breakthroughs in our understanding of HDL pathways[20;21], multiple other major HDL genes have been identified through studies in mice. In view of excellent reviews and books, this section only provides a short summary.

Human studies. The genes encoding apoAI, LCAT and ABCA1 are essential for the de novo synthesis of HDL. A complete lack of either of these factors confers severe HDL deficiency.[22;23] On the other hand, HL and CETP deficiency induce the accumulation of HDL in the circulation.[23;24] A high HDL phenotype has also been linked to apoCIII deficiency [25] while apoAII deficiency is reported to have no effect on HDLc levels.[26]

Mouse studies. SCARB1[27], ABCG1[28-30], ATP5B[31], PLTP[32] LIPG[33] and APOM[34] represent an important set of HDL genes that have been identified in mice. With regard to the impact of these factors on human HDL biology, many investigators have found associations of genetic variation at the respective loci with HDLc levels in cohort studies (see table 1).

To date, it is not known to what extent it would be possible to explain a low or high HDL phenotype in a person by studying his or her genetic make-up. Only very few studies report on systematic screening for mutations in subjects at both ends of the normal distribution of HDLc level and these studies are moreover primarily restricted to the genes identified in humans. Kiss et al.[35] found that sequencing of APOAI, LCAT, ABCA1 and PLTP only explained 12% of the phenotype of a cohort of 124 subjects with low HDLc levels (<10th percentile). On the other hand, our group reported that mutations in CETP (2 out of 95) and SCARB1 (1 out of 162) are very rare in subjects with high HDLc (>90th percentile; Van der Steeg & Vergeer et al, abstract AHA 2006). The current literature thus suggests that other major HDL genes are yet to be identified but only routine sequencing of the known candidate genes will learn whether this is true. It is very well possible that the frequency of mutations in the current HDL candidate genes is underestimated in the general population. This has been elegantly illustrated by a large resequencing effort by
Cohen and Hobbs.[36] In this study rare ABCA1, APOA1 and LCAT alleles were shown to significantly contribute to low plasma HDLc levels in the general population.

**Genetic association studies**

Genetic variability that causes or is associated with functional changes at mRNA or protein level confers a lifelong exposure to a certain condition and can thus provide information when e.g. stratifying for CVD risk. The ease of performing SNP studies constitutes the basis of many reports which may have to be interpreted with caution as recently pointed out.[76] The requirements for publishing SNP studies nowadays include the use of tagSNPs and replication in different, preferentially large cohorts.[76-78] Several reviews focused on the results of HDL SNPs studies [15-18;79] and this section therefore only summarizes the results of insightful newly published data.

**Single Nucleotide Polymorphisms**

**ABCA1.** The discovery of ABCA1 has prompted direct therapeutic strategies to increase ABCA1 expression to raise HDLc (reviewed by Beaven and Tontonoz).[80] The concept that ABCA1 affects CVD through changes in HDLc levels is, however, challenged [50]: in 9,259 subjects, the minor alleles of 6 nonsynonymous SNPs were either associated with an increase, decrease or no change of HDLc levels. Despite this heterogeneity, 5 out of these 6 SNPs were associated with an increased CVD risk. These data agree with an earlier study [81] and several other studies indeed point at functions of ABCA1 that affect CVD risk but have little to do with plasma HDLc levels.[82-85].

**CETP.** The relation between CETP, HDL, and CVD risk is the topic of many reviews (for a recent review see [3]) primarily because CETP inhibition represents an potent tool to raise HDL. Yet, there is no decisive evidence on whether CETP is a friend or a foe. In a recent meta-analysis of 13,677 subjects, a firm association between a CETP SNP, HDLc levels, and CVD risk was reported.[60] The original studies included in this meta-analysis were, however, mostly published before the requirements for publishing SNP studies became stringent and publication bias of the original studies cannot be excluded.

**PON1** is reported to be physically associated with HDL without affecting HDLc levels[86]. In 1,399 CVD patients, it was demonstrated that a SNP encoding for Q192R is associated with higher PON1 activity and reduced fatty acid oxidation. During a 4-year follow-up, significantly more major cardiac events and deaths occurred in subjects with the QQ192 genotype (lowest PON1 activity, n=584), compared to the other genotypes (higher PON1 activity, n=681). When combining cases and controls and adjustment for classical risk factors, more events and deaths were observed in those with low compared to those with high PON1 activity. This study then provides evidence of a PON1-associated atheroprotective property of HDL [87] but these data may need confirmation in larger studies.
TG/HDL genes. Many genes control lipolysis of plasma triglycerides (TG), a process that also affects HDLc levels through the delivery of apolipoproteins and phospholipids to HDL. This paragraph discusses a few studies that provide novel insight and those based on large-scale population studies.

**ANGPTL4** inhibits lipoprotein lipase but its exact role in lipid metabolism is unclear. In 3,551 subjects, rare ANGPTL4 gene variants were found to be associated with lower TG levels. The same SNPs were typed in 2 other large cohorts (15,792 subjects and 10,135 subjects, respectively) and the E40K variant was associated with a lower TG/high HDLc phenotype.

**CD36** is a liver scavenger receptor that affects fatty acid metabolism. Five out of 36 tagSNPs in 2020 subjects were associated with increased odds for metabolic syndrome while 15 SNPs were associated with HDLc levels.

**Uncoupling proteins (UCPs)** are mitochondrial anion carrier proteins expressed in skeletal muscle. Reduced function or expression decreases energy expenditure and

### Table 1. Established HDL candidate genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Human genetic deficiency</th>
<th>Human genetic association studies</th>
</tr>
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<tbody>
<tr>
<td><strong>APOA1</strong></td>
<td>HDL deficiency, variable effect on atherosclerosis[37]</td>
<td>SNPs are associated with increased or decreased HDLc levels[38]</td>
</tr>
<tr>
<td><strong>LCAT</strong></td>
<td>Fish-eye disease/familial LCAT deficiency, HDL deficiency, increased atherosclerosis [37;42]</td>
<td>Not performed due to lack of genetic variation</td>
</tr>
<tr>
<td><strong>ABCA1</strong></td>
<td>Tangier disease, HDL deficiency, increased atherosclerosis[47;48]</td>
<td>SNPs are variably associated with HDLc levels and CAD risk[49;50]</td>
</tr>
<tr>
<td><strong>LIPC</strong></td>
<td>High levels of HDLc, apoAI, TG and VLDL Rare allele of -514 C&gt;T promoter variant associated with increased HDLc, and increased CAD risk in men[57;58]</td>
<td>Increased HDLc; decreased atherosclerosis on apoE-/- background[23]</td>
</tr>
<tr>
<td><strong>CETP</strong></td>
<td>High HDLc levels; effect on atherosclerosis unclear [23]</td>
<td>SNPs are associated with HDLc and CAD risk [59;60]</td>
</tr>
</tbody>
</table>

**Murine studies**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Human genetic deficiency</th>
<th>Human genetic association studies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SCARB1</strong></td>
<td>-</td>
<td>Gender-dependent association of SNPs with HDLc, and LDLc[61;62]</td>
</tr>
<tr>
<td><strong>ABCG1</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>ATP5B</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>PLTP</strong></td>
<td>-</td>
<td>rs3843763 C/T: effect on HDLc[69]; association with CAD not assessed</td>
</tr>
<tr>
<td><strong>LIPG</strong></td>
<td>-</td>
<td>SNPs are associated with HDLc[72]; association with CAD not assessed</td>
</tr>
<tr>
<td><strong>APOM</strong></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
increases fat storage. Several recent studies identified significant associations between SNPs in genes encoding UCPs and HDLc levels. [91-93]. None of the abovementioned studies assessed the association with CAD risk.

**SNP panels and CVD risk**
Prevalent SNPs normally only explain a minor portion of the variance of plasma lipid levels and several investigators have attempted to analyze the combined effects of multiple SNPs. Boekholdt et al. genotyped 546 men with coronary atherosclerosis for SNPs in *ABCA1*, *APOA1*, *APOE*, *CETP*, *LIPC*, *LCAT* and *SCARB1*. These variants together explained 12.4% (95% CI: 6.9–17.9%) of variation in HDLc levels multivariate linear regression analyses.[94] Using different statistical methods, Spirin et al. documented that common SNPs in *APOA1*, *ABCA1*, *CETP*, *LIPC* and *LIPG* only explained 2.2% of the variance in HDLc levels in 3,306 subjects the Dallas Heart Study.[69] In 5,287 subjects, Kathiresan et al. studied genetic variation in 5 LDL genes (*APOB*, *PCSK9*, *APOE*, *HMGCR*, *LDLR*) and 4 HDL/TG genes (*ABCA1*, *CETP*, *LIPC*, *LPL*). It was tested whether
a panel of 9 SNPs could predict a first cardiovascular event during a 10-year follow-up. The number of alleles with a deleterious effect on the lipid profile was assessed with a genotype score. After adjustment for covariates, this score was strongly associated with future cardiovascular events. The association was reported to be mainly driven by SNPs in \textit{LDLR}, \textit{PCSK9}, \textit{APOE}, \textit{LIPC} and \textit{LPL}. ATP-III risk classification improved significantly when the genotype score was included in the risk model but an improvement in clinical risk prediction as assessed by the C statistic in ROC analysis was not found.\cite{95}
The small numbers of studies of this kind, which heavily depend on the statistical models that are used, may indicate the need of replication of these findings and further evaluation of statistical methods used.

**Novel loci that are proposed to have a role in HDL metabolism**

This section discusses newly proposed HDL genes. They are summarized in table 2 and depicted schematically in figure 1.

\textbf{VNN1.} Inter-individual differences in baseline gene transcription represent an interesting target for genetic studies that can be realized to date. Göring et al.\cite{96} generated a transcription map derived from lymphocytes of 1,240 subjects. 85 percent of 19,648 transcripts were considered to be significantly heritable. Transcripts that were linked

| Table 2. Novel genes that are proposed to have a role in HDL metabolism. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Gene | Genetic variance | Study cohort (ethnicity) | Influence on metabolism | Influence on CAD risk |
| VNN1 | eQTL; SNPs in promoter region | 1,240 subjects of Mexican descent (SAFHS#) | Strong association with HDL-c levels; vanin-1 produces factor that prevent lipid peroxidation | Not assessed \cite{96} |
| GALNT2 | GWA: rs4846914 | ~ 27,000 Caucasians | O-linked glycosylation | Unknown \cite{97;100} |
| MMAB | GWA: rs2338104 | ~ 18,000 Caucasians | Role in production of succinyl-coA | Unknown \cite{100} |
| MVK | GWA: rs2338104 | ~ 19,000 Caucasians | Synthesis of steroloprenoids such as cholesterol | Unknown \cite{100} |
| CTα | - | - | KO mice: hepatic PC biosynthesis regulates plasma HDL-c levels | Unknown \cite{114} |
| BMP-1 | - | - | HepG2 and CHO cells: converts pro-apoAI to mature apoAI | Unknown \cite{115} |
| SIRT1 | - | - | Activation of LXRa and LXRβ. KO mice: decreased ABCA1 expression and HDL-c levels | Unknown \cite{117} |

† Dallas Heart Study
§ Atherosclerosis Risk in Communities
* Copenhagen Heart Study
# San Antonio Family Heart Study
‡ Hypertension Genetic Epidemiology Network
The value of HDL genetics

Figure 1. Novel genes that are proposed to affect HDL: schematic model of possible mechanisms of involvement

Clockwise, starting in the upper left corner: SIRT1 activates LXRα and β, thereby upregulating ABCA transporter expression and cholesterol efflux to HDL. MVK codes for mevalonate kinase, which catalyzes an early step in the biosynthesis of isoprenoids. This pathway leads amongst others to sterolisoprenoids production, including cholesterol. Protein function can be regulated by O-linked glycosylation, a process that involves activity of N-acetylgalactosaminyltransferase 2, encoded by GALNT2; in this graph, LCAT represents an example of enzyme in lipid metabolism that is O-glycosylated with N-acetylgalactosamine residues. VNN1 codes for vanin-1, which leads to the production of factors that prevent lipid peroxidation, possibly associated with the anti-oxidative properties of HDL. CTP:phosphocholine cytidylyltransferase-alpha (CTα) encodes for a key enzyme in the biosynthesis of phosphatidylcholine (PC) which affects HDLc plasma levels in mice. BMP-1 codes for C-terminal procollagen endopeptidase/bone morphogenetic protein-1 bone-morphogenetic protein, which converts the proprotein of apoAI into the mature apoAI that is able to bind phospholipids.

with microsatellite polymorphisms near the particular gene (eQTLs) were subsequently identified. For these “cis-regulated” genes, sequence variants apparently substantially influence the abundance of their own gene product. 67 cis-regulated transcripts that correlated with HDLc levels were identified, with VNN1 (or vanin 1) representing the most promising factor. VNN1 codes for pantetheinase, which produces cysteamine, an aminothiol that potently prevents lipid peroxidation. A further sequencing effort yielded 4 SNPs in the VNN1 promoter region that showed a high correlation with transcript abundance and HDLc levels. The biological link between VNN1 and HDL remains to be established, but this method may represent an important addition to the available gene-finding instrumentation.
The scientific community has recently witnessed a surge of genome-wide association studies (GWAs) in search of novel genetic causes of human disease. High-density SNP maps in combination with high-throughput chip-analyses of tens of thousands of individuals have provided a tremendous amount of new information. Several of these studies focused on genetic loci associated with plasma lipid parameters and CVD.[97-100] Although this type of study may have its drawbacks[101], the strength of this approach was validated by the identification of nearly all established candidate genes in lipid metabolism. For HDLc levels, these were: *CETP, LIPC, LPL, LIPG, ABCA1, LCAT* and the *APOA1-C3-A4-A5* gene cluster.[97;98;100] Given this impressive validation, we wish to give special attention to the novel HDL gene loci.

**GALNT2**. This gene was linked to HDLc levels in 2 different GWA studies.[97;100] Not surprisingly, *GALNT2* was also associated with TG levels. *GALNT2* is located on chromosome 1 and codes for N-acetylgalactosaminyltransferase 2, which is involved in O-linked glycosylation of proteins, i.e. the transfer of N-acetylgalactosamine to serine or threonine residues. Since O-linked glycosylation can regulate protein function, it is hypothesized that GALNT2 affects HDLc/TG levels indirectly through the glycosylation of the proteins involved.[97] In this regard, LCAT, apoCIII, the VLDL receptor and the LDL receptor are all O-glycosylated with N-acetylgalactosamine residues.[102-105]

**MVK and MMAB**. Also SNPs in these genes on chromosome 12 were associated with HDLc levels[100]. Both are regulated by SREBP2.[106] *MVK* encodes for mevalonate kinase, which catalyzes an early step in the biosynthesis of isoprenoids. This pathway leads to amongst others sterolisoprenoids production, including cholesterol. In man, homozygosity for severe mutations in *MVK* is known to cause mevalonic aciduria, a rare metabolic disorder characterized by psychomotor retardation, failure to thrive, progressive cerebellar ataxia, dysmorphic features, progressive visual impairment and recurrent febrile crises. On the other hand, homozygosity for milder *MVK* mutations causes hyperimmunoglobulinemia D syndrome (HIDS), a predominantly Northern-European disease, characterized by recurrent fever episodes and increased levels of immunoglobulin D and A.[107;108] In line with the GWA findings, patients suffering from HIDS present with low HDLc levels (~0.75 mmol/L), even when in remission, but effects on CVD risk have not been reported. The same holds true for patients suffering from mevalonic aciduria.[109]

**MMAB**, the gene in close proximity to *MVK* - and under control of the same promoter - encodes for cob(l)alamin adenosyltransferase, an enzyme involved in the synthesis of 5'-deoxyadenosylcobalamine.[110] This is a cofactor for the production of an intermediate metabolite in the Krebs cycle (succinyl-coA).[111] In humans, deficiency of cob(l)alamin adenosyltransferase results in methylmalonic aciduria, a disease that afflicts 1 in 50,000 newborns in Quebec[110;112], but the role of MMAB in cholesterol metabolism is not understood. Only one report shows a negative correlation between urinary methylmalonic acid and red blood cell membrane cholesterol content in schizophrenic patients.[113]
The exact function of GALNT2 and MVK/MMAB in lipid metabolism and atherogenesis need to be clarified in future studies which may reveal their potential as targets for intervention. It is interesting in this regard that most HDL genes identified in GWAs were not associated with future CVD.[95;100] However, an association with CVD risk is not a condition sine qua non in the identification of a good target as clearly illustrated by the identification of a SNP near the HMGCR locus[97] - the targets of statins - while this SNP (conferring only a ~4 mg LDLc difference) was not found to be linked to CVD risk.[95]

Three other recent studies in mice and in tissue culture showed key roles of entirely different factors in HDL metabolism. These are:

**CTα.** CTP:phosphocholine cytidylyltransferase-alpha is a key enzyme in the CDP-choline pathway for the biosynthesis of phosphatidylcholine (PC). CTα KO mice as well as liver-specific CTα KO mice show marked decreases of plasma HDLc and VLDLc levels. In knockout hepatocytes, PC content as well as ABCA1 expression were reduced but this could be restored by adenoviral-mediated CTα expression. In vivo, plasma lipid levels of CTα KO mice could also be restored with adenoviral delivery of CTα. This study demonstrates for the first time that hepatic PC biosynthesis is an important regulator of plasma HDL, underlining the importance of hepatic HDL formation.[114]

**BMP1.** C-terminal procollagen endoproteinase/bone morphogenetic protein-1 was recently identified as an enzyme that converts the proprotein of apoAI into the mature apoAI, which is able to bind phospholipids (in HepG2 and CHO cells stably expressing apoAI). It is speculated that the decrease of plasma apoAI levels seen during a systemic inflammatory response may be mediated by BMP-1, as this factor is inhibited by alpha2-macroglobulin, a protease inhibitor secreted during the innate immune response.[115]

**SIRTI.** Sirtuin 1 is a NAD+-dependent deacetylase that is involved in apoptosis, fat metabolism, glucose homeostasis, and neurodegenerative processes by regulation of nuclear activities such as transcription, DNA replication, and DNA repair.[116] Li. et al demonstrated that SIRT1 activates liver X receptor (LXR) proteins α and β. In SIRT KO mice, expression of ABCA1, a well-known LXR target, is reduced which resulted in defective cholesterol efflux, lower HDLc levels, fatty livers and a blunted response to LXR agonists.[117]

**Genetics to study HDL**

A continuous exchange of neutral lipids, phospholipids, apolipoproteins and non-structural proteins makes HDL a challenging array of molecules to study, and more so when considering its many proven and proposed functions. Can HDL genetics help us here?
HDL genes

The complexity of HDL appears to be reflected by the large number of genes that are discussed in this review while even more candidates are likely to be discovered in the years to come. Although the biological (and clinical) significance in humans of many genes has yet to be proven, it is unlikely that they will not play a role in man at all. Studying carriers of mutations - using rapidly advancing sequencing technology - will likely provide insight in this matter. Another important aspect with special regard to CVD risk prediction is the study of gene-gene and gene-environment interactions. This research area has only been given little attention and needs to be explored with novel bioinformatic tools in large genetic databases.

HDL genes and atherosclerosis

HDL enjoys most of its current attention due to the promise that HDL raising drugs protect from atherosclerosis. While experimental proof in human studies still has to be provided, there is indeed convincing evidence from studies in animals that this can be achieved. When focusing on candidate HDL genes and atherosclerosis in man, however, only few studies have unequivocally demonstrated a relation. In fact, the first GWAs show no impact of SNPs in HDL genes on CVD risk. Whether this latter is the result of a lack of statistical power needs to be resolved in the future. Along the same line, however, genetically determined low or high HDL in man (and mice) has not provided clear positive answers either. Subjects with complete HDL deficiency in the absence of overt atherosclerosis underline our incomplete understanding and caution when extrapolating epidemiological HDL/CVD data to individual HDL changes and risk of atherosclerosis. can be rightfully argued that many of these studies suffer from a lack of power. For CETP deficiency, however, many subjects have been studied but it has remained difficult to draw conclusions on their risk of atherosclerosis.

In conclusion

Studying novel HDL genes is bound to help unravelling novel pathways and their relations with vascular biology and beyond. Improved insight in HDL biology may help identifying biomarkers that are needed in HDL intervention studies in man. Finally, studies of HDL genetics have already proven to be of value to find pharmacological targets and this holds promise for the future.

Acknowledgements

The work on HDL genetics in the research group of dr. Jan Albert Kuivenhoven is supported by a grant of the European Community (FP6-2005-LIFESCIHEALTH-6; STREP contract number 037631). A.G. Holleboom is supported by a grant of the Netherlands Organisation for Scientific Research (NWO).
The value of HDL genetics

Reference List


   Excellent review on HDL HDL as a pharmacological target


   Excellent systematic review outlining the clinical evidence for raising HDL as an anti-atherosclerotic therapy


   Ref Type: Internet Communication


   Ref Type: Internet Communication


Association study presenting an apparent paradox: ABCA1 SNP have diverse effects on plasma HDL cholesterol levels, but the minor alleles are almost uniformly associated with an increased risk of CAD


54. Joyce C, Freeman L, Brewer HB, Jr., Santamarina-Fojo S: Study of ABCA1 function in transgenic
Chapter 3


The value of HDL genetics


Large resequencing study pointing to ANGPTL4 as a new HDL locus
Chapter 3


Very elegant study, in which the development and application of a whole genome transcription map shows its worth by identifying a new HDL locus, VNN1


First genome-wide association study to show decisively that known lipid (and CAD risk) modifying genes can be picked up using this approach


The value of HDL genetics


CTα is demonstrated to play a crucial role in lipoprotein metabolism as a key protein in phospholipid synthesis


BMP-1 is demonstrated to convert pro-ApoAI into the actual apolipoprotein. Interestingly, BMP-1 is inhibited in the inflammatory state, which might explain the low HDL-c levels seen in the acute phase


SIRT1 is demonstrated to regulate LXR activation and subsequent ABCA1 expression and function. SIRT1 KO mice display low HDL-c plasma levels.
Chapter 4

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Genetic Variant of the Scavenger Receptor BI in Humans


1Department of Vascular Medicine, Academic Medical Centre, University of Amsterdam, The Netherlands. 2Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden, The Netherlands.
Abstract

Background. In mice, scavenger receptor class B type I (SR-BI) is essential for the delivery of high-density lipoprotein (HDL) cholesterol to the liver and steroidogenic organs. Paradoxically, elevated HDL cholesterol levels are associated with increased atherosclerosis in SR-BI knockout mice. To date, it is unclear what role SR-BI plays in human metabolism.

Methods. We sequenced the gene encoding SR-BI in individuals with elevated HDL cholesterol levels and identified a family with a new missense mutation (P297S). The functional effects of the P297S mutation on HDL binding, cellular cholesterol uptake and efflux, atherosclerosis, platelet function and adrenal function were studied.

Results. Cholesterol uptake from HDL by primary murine hepatocytes that expressed mutant SR-BI was reduced by half as compared to wild-type SR-BI. Carriers of the P297S mutation in SR-BI (n=18) had increased HDL cholesterol levels (1.82 versus 1.38 mmol/L, p<0.001), a reduced capacity to efflux cholesterol from macrophages but carotid intima media thickness was similar in carriers and family controls. Platelets from carriers displayed increased unesterified cholesterol content and impaired function. Finally, carriers displayed attenuated adrenal steroidogenesis as evidenced by decreased urinary excretion of sterol metabolites, a decreased response to adrenocorticotrope stimulation and symptoms of diminished adrenal function.

Conclusions. We identified a family with a functional mutation in the scavenger receptor SR-BI. Carriers of the mutation had increased HDL cholesterol levels and a reduction in macrophage cholesterol efflux but this was not associated with increased atherosclerosis in this small group of individuals. Reduced SR-BI function was furthermore associated with altered platelet function and decreased adrenal steroidogenesis.
Introduction

A low plasma concentration of high-density lipoprotein (HDL) cholesterol is a strong risk factor for cardiovascular disease. This observation explains the interest in the development of HDL cholesterol increasing drugs to reduce atherosclerosis. To develop such drugs, in-depth knowledge of human HDL metabolism is necessary. Our understanding of several potential targets for drug development has greatly benefited from the identification of families with specific HDL gene mutations. A prominent example is the interest in inhibiting cholesterol ester transfer protein (CETP). Scavenger receptor class B type I (SR-BI), a major HDL receptor, is also regarded as a potential target for pharmacological modulation. In mice, the SR-BI gene (*SCARB1*) is expressed in the liver and steroidogenic tissues, where it mediates the selective uptake of HDL cholesteryl esters. An interesting feature of SR-BI knockout mice is that they suffer from more atherosclerosis despite 2-fold elevated HDL cholesterol levels. The atheroprotective effects of SR-BI are primarily attributed to its role in cellular cholesterol efflux from lipid-laden macrophages to HDL and the delivery of HDL cholesteryl esters to the liver. SR-BI knockout mice furthermore display adrenal glucocorticoid insufficiency under stress due to the depletion of lipid stores in the adrenal cortex. Finally, these mice have an altered cholesterol distribution in platelets, which interferes with platelet aggregation.

In humans, it is known that genetic SR-BI variants are associated with HDL cholesterol levels and SR-BI protein levels in peripheral cells, but thus far there exists no evidence that impaired SR-BI function affects human physiology. We here report on a family with a functional SR-BI mutation and show that this HDL receptor plays an important role in cholesterol homeostasis in humans.

Methods

The studies reported were designed by the authors and supported by grants from the European Community, the Netherlands Organization for Scientific Research, and the Netherlands Heart Foundation. Human studies were approved by the Institutional Review Board of the Academic Medical Center, Amsterdam, The Netherlands. All participants signed written informed consent. Animal experiments were approved by the Ethics committee for animal experiments of Leiden University, Leiden, The Netherlands.

**Study subject identification and mutation analysis**

A group of 162 unrelated Caucasian subjects with HDL cholesterol above the 95th percentile for age and gender were identified from the lipid clinic at the Academic Medical Center, The Netherlands (for details see the Supplementary Appendix). The *SCARB1* gene (NM_005505.4; encoding SR-BI) was sequenced, and a novel SR-BI mutation (nucleotide mutation, c.889C>T; resultant amino acid substitution, P297S) was identified in one of these individuals. Her family was invited for further studies.
Plasma measurements

Fasting plasma total cholesterol and triglyceride levels were measured using standard assays (see the Supplementary Appendix). HDL cholesterol was measured as cholesterol remaining after precipitation of apolipoprotein (apo) B-containing lipoproteins. Low-density lipoprotein (LDL) cholesterol was calculated. In a random subset of carriers and family controls, lipoproteins were characterized through fast protein liquid chromatography (FPLC).

Functional analyses of the P297S variant of SR-BI

SR-BI knockout mice were provided by Dr. M. Krieger (Department of Biology, MIT, Cambridge, MA, USA). Adenoviruses expressing human wild-type SR-BI (Ad.SR-BIWT), SR-BI containing the P297S mutation (Ad.SR-BIP297S) or without insert (Ad.mock) were prepared as described (see the Supplementary Appendix). In two independent experiments, mice were injected with Ad.mock, followed by injection of Ad.SR-BIP297S or Ad.SR-BIWT. Blood samples were collected after an overnight fast before and four days after virus administration. The data of both experiments were combined.

Primary hepatocytes were isolated from the livers of SR-BI knockout mice injected with Ad.SR-BIP297S or Ad.SR-BIWT. These cells were tested for their potential to interact with human HDL (see the Supplementary Appendix).

Carotid artery intima-media thickness

Carotid artery intima-media thickness was assessed as described (see the Supplementary Appendix). The mean combined intima-media thickness of both common carotid arteries was used for calculations.

Macrophage and platelet studies

Peripheral monocytes, isolated from carriers of the P297S mutation and from non-carriers, were cultured for 10 days and incubated with [3H]cholesterol for 24 hours. After medium aspiration, the cells were washed and incubated with 0.5 ml of efflux medium (D-MEM:F-12(1:1), Glutamax, 0.1% P/S, 0.2% BSA and 5 µg (protein)/ml HDL). Efflux medium without HDL was used as a control. Fractional cholesterol efflux was calculated by measuring the release of radiolabeled cholesterol into the medium after 4 or 24 hours (see the Supplementary Appendix).

Platelets were isolated from freshly drawn venous blood, collected into 0.1 volume 130 mmol/L trisodium citrate and used for platelet activation studies, including platelet aggregation and analysis of platelet spreading on fibrinogen (see the Supplementary Appendix).

Adrenal function tests

Urinary steroid excretion was analysed in 24-hr urine samples as described. Tetracosactin (a synthetic adrenocorticotropic hormone analog) tests were performed
at 9 am after an overnight fast. Following two baseline blood samples (t=-15, t=0 min), participants were given a 1 µg tetracosactin bolus, followed by another blood sample after 30 minutes. Total cortisol was measured by an enzyme immunoassay and cortisol binding globulin (CBG) was measured using a commercial radioimmunoassay. Free cortisol was calculated (see the Supplementary Appendix).25

Statistical analyses
Quantitative trait locus analyses were carried out for HDL cholesterol levels using Mendel software, version 4.0.26 In univariate analyses, SR-BI genotype was used as the only independent variable. Multivariate quantitative trait locus analyses incorporated gender, age, alcohol use, body-mass index and smoking behaviour as covariates. The likelihood ratio test was used to compute P values. Other statistical analyses were performed in SPSS, version 12. To compare data between groups, Students t-tests or Mann-Whitney U tests were used where appropriate. All P values were two-sided and P values <0.05 were considered to be statistically significant.

Results
A genetic variant of SR-BI and HDL cholesterol levels
Among 162 unrelated individuals with high HDL cholesterol, one was found to be heterozygous for a novel point mutation in SCARB1 (c.889C>T) which resulted in a proline to serine substitution at amino acid position 297 (P297S). The mutation was not found in 150 normolipidemic controls. The proline at this position is highly conserved across species (Supplementary Appendix Figure 1), and in silico analysis predicts the P297S mutation to be functionally important.27

Whole-genome linkage analysis showed the SCARB1 locus at 12q24.31 to be the only region in the genome significantly co-segregating with the high HDL cholesterol phenotype in this family (maximum multipoint parametric LOD score 3.31 between rs1345015 and rs7297801; see Supplementary Appendix Figure 2). The likelihood of another gene in linkage disequilibrium with SCARB1 being responsible for the high HDL cholesterol phenotype was reduced by the identification of two different recombination events upstream of and close to the SCARB1 gene in two carriers. These upstream recombination sites together with a downstream recombination site delimit a ~30MB genomic region co-segregating with the high HDL cholesterol phenotype (Figure 1).

In a further evaluation of 124 family members, 19 were carriers of the P297S mutation in SR-BI. One carrier was not included in additional studies since she suffered from multiple sclerosis and used multiple types of medication. The remaining 18 carriers were matched for body-mass index, gender and age on an aggregate basis to 36 family controls (baseline characteristics in Table 1). Carriers had significantly increased HDL cholesterol levels (1.82 mmol/L vs. 1.38 mmol/L, p<0.001) but no significant differences in other plasma lipids.
Figure 1. Pedigree used for linkage analysis and the obtained haplotype information. The red haplotype, including rs3782287 which is located in intron 7 of the SCARB1 gene, is present in all individuals with the high HDL cholesterol phenotype. Two recombination events upstream of, and very close to, the SCARB1 gene (observed in IV:1 and III:5) and one recombination event downstream of SCARB1 (observed in V:2) determined the borders of a ~30MB linkage interval. (For color figure, see page 416)

Figure 2. P297S carriers exhibit an isolated high HDL phenotype. QTL analyses for the allelic effect of P297S as explanatory variable for HDL cholesterol levels in the entire pedigree (n=124). The upper bar shows the results for univariate analysis. The bars below depict results for multivariate analysis, with corrections for gender, body-mass index, smoking behaviour, alcohol use and age. (a) SR-BI genotype was not associated with total cholesterol, LDL cholesterol or triglyceride levels (see Table 1). Fast performance liquid chromatography was performed for 15 P297S carriers and 15 family controls. P297S carriers have more cholesterol in the HDL fraction, concentrated in particles of larger size (shift of the HDL peak to the left, b). Data are shown as means ± standard error. * p<0.001.
Quantitative trait locus analysis for the entire family revealed that the P297S mutation was associated with higher HDL cholesterol levels before and after adjustment for gender, age, body-mass index, smoking habits and alcohol use (Figure 2a).

In a random subset of 15 carriers and 15 controls (matched for age, gender and body-mass index), FPLC profiles revealed larger HDL particles in heterozygotes (Figure 2b; see Supplementary Appendix Table 1 for further details). Apo A-I, apo A-II and apo E levels were all markedly higher in carriers than controls, whereas apo B levels were slightly lower. Differences in plasma levels of cholesteryl ester transfer protein (CETP) or phospholipid transfer protein were excluded as explanations for the high HDL phenotype (Supplementary Appendix Table 2).

**Table 1.** Demographic characteristics of P297S carriers and matched family controls.

<table>
<thead>
<tr>
<th></th>
<th>P297S carriers</th>
<th>Non-carriers</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>18</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Age (range)</td>
<td>45 ± 20 (15-81)</td>
<td>45 ± 21 (13-84)</td>
<td>0.96</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>7-nov</td>
<td>15/21</td>
<td>0.85</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>24.1 ± 4</td>
<td>24.7 ± 4</td>
<td>0.58</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.5 ± 1.4</td>
<td>5.5 ± 1.5</td>
<td>0.9</td>
</tr>
<tr>
<td>LDL</td>
<td>3.2 ± 1.4</td>
<td>3.5 ± 1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>HDL</td>
<td>1.82 ± 0.49</td>
<td>1.38 ± 0.33</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides (median, IQR; mmol/L)</td>
<td>1.1 (0.8-1.4)</td>
<td>1.1 (0.6-1.7)</td>
<td>0.71</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>0 (0%)</td>
<td>4 (11%)</td>
<td>0.29</td>
</tr>
<tr>
<td>Daily alcohol users (%)</td>
<td>9 (50%)</td>
<td>21 (58%)</td>
<td>0.58</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>0</td>
<td>1</td>
<td>0.48</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1</td>
<td>1</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Values are means ± SD unless otherwise indicated. IQR = interquartile range. HDL = high-density lipoprotein, LDL = low-density lipoprotein.

**Functional assessment of the P297S variant**

We compared the effects of adenoviral expression of wild-type SR-BI and the P297S variant on high cholesterol levels in SR-BI knockout mice. Mean total cholesterol levels were reduced by 60% and 46% in mice injected with Ad.SR-BIWT and Ad.SR-BIP297S, respectively (p<0.001, Figure 3a). Mice injected with Ad.SR-BI P297S also exhibited reduced normalization of HDL particle size compared to those injected with Ad.SR-BIWT (Supplementary Appendix Figure 3a). Hepatic SR-BI mRNA expression and protein levels were similar in both groups (Figures 3b and 3c and Supplementary Appendix Figure 3b).

The functionality of the P297S variant was also tested in primary hepatocytes isolated from the livers of mice transduced with either Ad.SR-BIWT or Ad.SR-BIP297S. Hepatocytes were incubated with increasing concentrations of human [3H]cholesteryl ester-HDL or [125I]-HDL. The average ratio of cell-associated [3H]cholesteryl ester to cell-associated...
with increasing HDL concentration was 6.7 and 3.4 for hepatocytes transduced with wild-type SR-BI and the P297S variant, respectively (p<0.001). Figure 3d shows that hepatocytes expressing the P297S variant show a similar cellular association of $^{125}$I HDL in the presence of a markedly reduced efficiency in the selective uptake of $^3$H cholesteryl ester from HDL compared to cells expressing wild-type SR-BI. The actual cholesteryl ester uptake was 56% lower in cells expressing the P297S variant compared to cells expressing wild-type SR-BI (p<0.001).

The P297S variant and carotid intima-media thickness
Carriers of the P297S variant did not differ from family controls regarding the prevalence of cardiovascular disease and carotid intima-media thickness (averaging 585 µm in 15 carriers versus 595 µm in 15 controls).
The P297S variant and macrophage cholesterol efflux

Cholesterol efflux from monocyte-derived macrophages of P297S carriers and non-carriers to HDL was significantly lower in carriers after 4 and 24 hours of incubation with HDL (9.6% vs. 13.2%, p=0.04 and 22.4% vs. 27.6%, p=0.01 respectively; Supplementary Appendix Figure 4a). No statistically significant effects of the mutation on monocyte mRNA levels of the ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1, two proteins involved in cholesterol efflux; Supplementary Appendix Figure 4b) were found. A non-significant (p=0.10) increase in SR-BI mRNA expression levels accompanied significantly increased SR-BI protein levels in carriers of the mutation (Supplementary Appendix Figure 4c and 4d).

The P297S variant and platelet function

Platelet counts did not differ between P297S carriers and controls (3.0 ± 0.8 x10¹¹ vs. 3.0 ± 0.6 x10¹¹ platelets/L), but platelets from carriers contained more unesterified cholesterol (Figure 4a). Platelet aggregation response to six different agonists was significantly diminished in carriers (Figure 4b). P-selectin expression, a measure of platelet activation, was higher in circulating platelets from carriers (Figure 4c). Platelets from carriers also displayed increased adhesion and spreading when exposed to immobilized fibrinogen (Supplementary Appendix Figure 5 and Supplementary Video 1 and 2, available online at www.nejm.org).

The P297S variant and adrenal steroidogenesis

Since murine SR-BI deficiency affects adrenal steroidogenesis¹⁸,¹⁹, we investigated urinary excretion of adrenal hormones. Most urinary steroids were diminished in P297S carriers versus controls (Figure 5). SR-BI knockout mice have higher hepatic expression of CBG¹⁸, as well as 2-fold higher plasma levels of CBG (median 20 AU [IQR 18-22] vs. 9 AU [IQR 6-12], p=0.03). Consistent with these findings, human P297S carriers also displayed higher CBG levels (median 77 mg/dL vs. 51 mg/dL, p=0.04; Supplementary Appendix Table 3).

Tetracosactin stimulation tests revealed that baseline plasma levels of free cortisol were similar in carriers and non-carriers (11.2 nmol/L vs. 11.2 nmol/L, p=0.94), but the percentage increase in free cortisol following tetracosactin stimulation was significantly lower in carriers (127% vs. 223% in controls, p=0.04; Supplementary Appendix Table 3). Several P297S carriers had consulted their physicians for complaints of fatigue, dizziness and/or fainting, and these complaints were more prevalent in those with lower indices of adrenal function (Supplementary Appendix Figure 6). In addition, one member of this family died in 1959 from the consequences of adrenal insufficiency at 53 years of age (personal communication treating physician). Both he and his father’s brother had offspring carrying the P297S mutation, indicating that this patient was also a carrier.
Figure 4. Platelet characteristics. To detect membrane cholesterol, coverslips with platelets were incubated with filipin. Filipin staining indicates unesterified cholesterol content of platelets of a non-carrier and a P297S carrier (representative fluorescence microscope images, a). For aggregation studies, platelet-rich plasma was stimulated with 2 μg/mL ADP, 0.25 mg/ml arachidonic acid, 1 μg/ml collagen, 1.2 mg/ml ristocetin, 100 nM phorbol 12-myristate 13-acetate (PMA), and 20 μM thrombin-receptor activating peptide SFLRN (TRAP). Platelet aggregation response to different stimuli is lower in P297S carriers than in controls (n=4-7, b). To investigate the activation state of circulating platelets, surface expression of P-selectin on non-stimulated platelets was determined by flow cytometry. Resting platelets of a P297S carrier express more P-selectin (n=6-9; p=0.02), indicative of a higher baseline state of activation. (c) Data are shown as means ± standard error.

Figure 5. Urinary steroid excretion in carriers of the P297S mutation (n=15) and non-carriers (n=15) over 24 hours. Carriers show a reduction in the excretion of most steroids. Data are shown as means ± standard error. # p=0.06, * p<0.05, ** p<0.01.
Discussion

In a large family, we identified a functional mutation (P297S) in the scavenger receptor SR-BI. In mice, SR-BI mediates the hepatic uptake of HDL cholesterol, which explains the high HDL cholesterol levels in SR-BI knockout mice. In our family, HDL cholesterol levels were elevated in carriers of the P297S mutation. Adenoviral expression of the P297S variant in SR-BI knockout mice was less efficient in normalizing plasma cholesterol levels than wild-type SR-BI. Moreover, the P297S variant was found to have a 56% reduced capacity to mediate cholesteryl ester uptake from HDL in primary hepatocytes. Mice carry the bulk of their plasma cholesterol in HDL, whereas plasma cholesterol in humans is mostly carried in LDL. This difference is due to CETP, which mediates the exchange of cholesterol and triglycerides between HDL and LDL. CETP is present in humans but absent in mice. Thus, humans have an effective alternative route for HDL cholesterol to reach the liver, by transferring cholesterol to LDL followed by hepatic uptake through the LDL receptor. Although CETP levels are normal in P297S carriers, they have on average a 32% increase in plasma HDL cholesterol levels, suggesting that, as in mice, SR-BI also plays a role in hepatic HDL cholesterol uptake in humans.

SR-BI mediates the bidirectional transfer of cholesterol between cells and HDL. Although a role for SR-BI in cholesterol efflux from macrophages is disputed, it has been shown that a SR-BI blocking antibody can reduce cholesterol efflux to HDL by half. Using cultured macrophages of P297S carriers, we found that efflux of cholesterol to HDL was significantly reduced compared to controls.

SR-BI knockout mice are more susceptible to atherosclerosis than wild-type mice despite a more than 2-fold increase of HDL cholesterol. This phenomenon may be a consequence of reduced cholesterol efflux from macrophages to HDL and an impaired delivery of HDL cholesteryl esters to the liver. Studying the number of cardiovascular events and carotid intima-media thickness in this family, however, did not reveal differences between carriers and non-carriers but the statistical power to detect a difference was low, given the small numbers of carriers and their relatively young age. Since it is questioned that HDL cholesterol levels are per definition atheroprotective, this topic may need further investigation.

In SR-BI knockout mice, high plasma levels of unesterified cholesterol are associated with increased platelet cholesterol and blunted platelet aggregation. P297S carriers do not show increased plasma levels of unesterified cholesterol, but increased platelet unesterified cholesterol content was observed in conjunction with altered platelet characteristics, most notably increased basal activity and reduced platelet aggregation. These findings are in line with those of Imachi et al., who showed that human platelet aggregation negatively correlated with platelet SR-BI expression.

In mice, SR-BI is the only receptor that mediates the adrenal uptake of cholesteryl esters from HDL. This explains why SR-BI knockout mice show depletion of adrenal lipid stores and a lack of inducible glucocorticoid synthesis. In humans, the adrenal LDL receptor...
Chapter 4

is generally believed to control cholesterol uptake, based on the observation of mildly reduced adrenal cortical function in patients with complete LDL receptor deficiency. In heterozygous carriers of LDL receptor mutations, however, no impact on adrenal function could be demonstrated. In the current study, P297S carriers exhibited a marked reduction in urinary steroid secretion, a reduced response to adrenocorticotrope stimulation, and complaints consistent with diminished adrenal function (with one historical fatal case consistent with adrenal insufficiency). These observations support a role for SR-BI in adrenal steroidogenesis and suggest that HDL fulfils a thus far unanticipated role in human adrenal steroid synthesis. This may have consequences for the development of strategies to increase HDL cholesterol. For example, torcetrapib, an agent specifically developed to increase HDL cholesterol, was found to cause an increase in total mortality associated with profound increases in circulating aldosterone levels.

In conclusion, we identified a functional mutation in humans in SR-BI, a scavenger receptor that in mice mediates uptake of HDL cholesterol by the liver and the adrenal glands. We demonstrated that this mutation was associated with increased HDL cholesterol levels and a reduction in macrophage cholesterol efflux but we were unable to confirm an association with increased severity of atherosclerosis in the small group of carriers. Reduced SR-BI function was furthermore associated with altered platelet function, and decreased adrenal steroidogenesis.

Disclosure
The authors report no potential conflicts of interest.

Acknowledgments
This work was supported by grants of the European Community (FP6-2005-LIFESCIHEALTH-6; STREP contract number 037631), the Netherlands Organisation for Scientific Research (project numbers 021.001.035 and 917.66.301) and the Netherlands Heart Foundation (2006BI107, 2007T056, 2008T070 and 2009B027). We would like to thank A.H. Zwinderman for his help with the statistical analyses. We furthermore gratefully acknowledge the family members studied for their willingness to cooperate with our investigations. In addition, we would like to thank Reeni B. Hildebrand, J. Kar Kruijt, Ilze Bot, Ronald J. van der Sluis, Mirjam van Alderen, Joram van Miert, Alinda Schimmel, Han Levels, and Jorge Peter for excellent technical support and Kobie Los and Claartje Koch for genetic fieldwork.

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References


### Supplementary Appendix

**Supplementary Table 1.** Lipid profiles as measured by fast performance liquid chromatography in a randomly chosen subgroup of P297S carriers and family controls

<table>
<thead>
<tr>
<th></th>
<th>P297S carriers</th>
<th>Non-carriers</th>
<th>p</th>
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<tbody>
<tr>
<td><strong>N</strong></td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td><strong>Total cholesterol</strong></td>
<td>5.71 ± 1.08</td>
<td>5.30 ± 0.89</td>
<td>0.22</td>
</tr>
<tr>
<td>HDL fraction</td>
<td>2.05 ± 0.54</td>
<td>1.25 ± 0.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL fraction</td>
<td>3.51 ± 0.97</td>
<td>3.79 ± 0.80</td>
<td>0.44</td>
</tr>
<tr>
<td>VLDL fraction</td>
<td>0.15 ± 0.09</td>
<td>0.27 ± 0.18</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Free cholesterol</strong></td>
<td>1.29 ± 0.33</td>
<td>1.34 ± 0.30</td>
<td>0.54</td>
</tr>
<tr>
<td>HDL fraction</td>
<td>0.40 ± 0.12</td>
<td>0.29 ± 0.08</td>
<td>0.003</td>
</tr>
<tr>
<td>LDL fraction</td>
<td>0.75 ± 0.22</td>
<td>0.84 ± 0.24</td>
<td>0.20</td>
</tr>
<tr>
<td>VLDL fraction</td>
<td>0.15 ± 0.10</td>
<td>0.21 ± 0.10</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Cholesteryl ester</strong></td>
<td>4.41 ± 0.91</td>
<td>3.96 ± 0.69</td>
<td>0.08</td>
</tr>
<tr>
<td>HDL fraction</td>
<td>1.65 ± 0.46</td>
<td>0.96 ± 0.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL fraction</td>
<td>2.77 ± 0.80</td>
<td>2.95 ± 0.61</td>
<td>0.57</td>
</tr>
<tr>
<td>VLDL fraction</td>
<td>0.00 ± 0.09</td>
<td>0.05 ± 0.13</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>0.88 ± 0.38</td>
<td>1.29 ± 0.62</td>
<td>0.10</td>
</tr>
<tr>
<td>HDL fraction</td>
<td>0.12 ± 0.08</td>
<td>0.12 ± 0.07</td>
<td>0.90</td>
</tr>
<tr>
<td>LDL fraction</td>
<td>0.39 ± 0.13</td>
<td>0.51 ± 0.20</td>
<td>0.06</td>
</tr>
<tr>
<td>VLDL fraction</td>
<td>0.38 ± 0.23</td>
<td>0.65 ± 0.52</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Values are means ± SD, in mmol/L. Cholesteryl ester values were calculated by subtracting free cholesterol values from total cholesterol values.
### Supplementary Table 2. Additional characteristics of a randomly chosen subgroup of P297S carriers and family controls

<table>
<thead>
<tr>
<th></th>
<th>P297S carriers</th>
<th>Unaffected family controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Age (range)</td>
<td>42 ± 19 (15-70)</td>
<td>43 ± 19 (14-73)</td>
<td>0.84</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>5-10</td>
<td>5-10</td>
<td>1.0</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>24.6 ± 4</td>
<td>26.4 ± 4</td>
<td>0.24</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>139 ± 25</td>
<td>136 ± 21</td>
<td>0.76</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>86 ± 14</td>
<td>82 ± 8.6</td>
<td>0.39</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>0 (0%)</td>
<td>2 (13%)</td>
<td>0.48</td>
</tr>
<tr>
<td>Daily alcohol users (%)</td>
<td>7 (47%)</td>
<td>8 (53%)</td>
<td>0.72</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.9 ± 1.0</td>
<td>4.9 ± 1.0</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>LDL</td>
<td>2.7 ± 0.9</td>
<td>3.1 ± 0.9</td>
<td>0.14</td>
</tr>
<tr>
<td>HDL</td>
<td>1.82 ± 0.42</td>
<td>1.17 ± 0.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides (median, IQR; mmol/L)</td>
<td>1.0 (0.5-1.3)</td>
<td>0.9 (0.7-1.9)</td>
<td>0.14</td>
</tr>
<tr>
<td>Apo B (mg/dL)</td>
<td>84 ± 22</td>
<td>102 ± 24</td>
<td>0.04</td>
</tr>
<tr>
<td>Apo A-I (mg/dL)</td>
<td>176 ± 42</td>
<td>138 ± 26</td>
<td>0.005</td>
</tr>
<tr>
<td>Apo A-II (mg/dL)</td>
<td>36 ± 6</td>
<td>31 ± 3</td>
<td>0.007</td>
</tr>
<tr>
<td>Apo E (mg/dL)</td>
<td>4.8 ± 1.2</td>
<td>3.5 ± 0.7</td>
<td>0.001</td>
</tr>
<tr>
<td>CETP concentration (mg/dL)</td>
<td>2.0 ± 0.4</td>
<td>2.0 ± 0.4</td>
<td>0.91</td>
</tr>
<tr>
<td>CET (nmol/ml/hr)</td>
<td>12.1 ± 5.8</td>
<td>12.1 ± 3.2</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>PLTP activity (%)</td>
<td>98 ± 11</td>
<td>99 ± 11</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Values are means ± SD unless otherwise indicated. HDL = high-density lipoprotein, LDL = low-density lipoprotein, Apo = apolipoprotein. CETP = cholesteryl ester transfer protein. CET = cholesteryl ester transfer rate. PLTP = phospholipid transfer protein. PLTP activity is expressed as a percentage of normal pool plasma.

### Supplementary Table 3. Response of P297S carriers and non-carriers to tetracosactin (a synthetic adrenocorticotropic hormone) stimulation

<table>
<thead>
<tr>
<th></th>
<th>P297S carriers</th>
<th>Non-carriers</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>11</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>42 ± 19</td>
<td>42 ± 18</td>
<td>0.98</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>3-8</td>
<td>3-7</td>
<td>0.89</td>
</tr>
<tr>
<td>Body mass index</td>
<td>25.2 ± 4</td>
<td>25.6 ± 4</td>
<td>0.86</td>
</tr>
<tr>
<td>Cortisol binding globulin</td>
<td>77 (56-121)</td>
<td>51 (46-64)</td>
<td>0.04</td>
</tr>
<tr>
<td>Baseline total cortisol (nmol/L)</td>
<td>364 (298-608)</td>
<td>294 (261-498)</td>
<td>0.41</td>
</tr>
<tr>
<td>Stimulated total cortisol (nmol/L)</td>
<td>715 (549-847)</td>
<td>673 (599-875)</td>
<td>0.97</td>
</tr>
<tr>
<td>Relative change in total cortisol (%)</td>
<td>80 (39-105)</td>
<td>124 (37-158)</td>
<td>0.16</td>
</tr>
<tr>
<td>Baseline free cortisol (nmol/L)</td>
<td>11.2 (9.7-13.5)</td>
<td>11.2 (7.9-13.8)</td>
<td>0.94</td>
</tr>
<tr>
<td>Stimulated free cortisol (nmol/L)</td>
<td>23.3 (15.0-32.9)</td>
<td>36.5 (22.1-39.2)</td>
<td>0.12</td>
</tr>
<tr>
<td>Relative change in free cortisol (%)</td>
<td>127 (33-173)</td>
<td>223 (81-296)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Baseline values are the average (free) cortisol levels at t=-15 and t=0. Stimulated (free) cortisol values represent cortisol levels 30 minutes after a 1 µg bolus of tetracosactin. Free cortisol levels are a more accurate reflection of adrenal status than total cortisol levels in the context of altered levels of cortisol binding globulin (see Extended Methods: Adrenal function tests). Values are means ± SD or medians (interquartile range).
### Supplementary Table 4. Primer sequences to sequence SCARB1 (NM_005505.4)

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence (5' → 3')</th>
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<tr>
<td>1</td>
<td>GGCATAAAACCACTGGCCACC</td>
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<td></td>
<td>TGGCCTCCCTGTTGCTCTGC</td>
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<td>2</td>
<td>CGACAGCCTCATATGGCGAAGG</td>
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<td></td>
<td>GGGGCTAGTACAAGCAGCTTC</td>
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<td>3</td>
<td>TGTTCCCAGGGCAGGCAATG</td>
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<td></td>
<td>GTCCCACGCTCGACCACC</td>
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<tr>
<td>4</td>
<td>TGTTGTCAGTAGCTGGCCAC</td>
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<tr>
<td>5</td>
<td>GGAAGAGGGCTCAAGCCAGAATG</td>
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<td></td>
<td>AAGGAGCTCTGGTCTCCCTGC</td>
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<tr>
<td>6</td>
<td>TGTCTACATAGCCCGCTCTTC</td>
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<td>7</td>
<td>GCCATAGCTACGCCAGAAAC</td>
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<td>8</td>
<td>ATCTGGGCAAGCTTGGCAG</td>
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<td>9</td>
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<tr>
<td>10</td>
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<td>ACCACCCCCAGCCACAGCAG</td>
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<td>11</td>
<td>AAGCAAGCTCACTGGCCAGC</td>
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<td></td>
<td>CCCATGGGCTGTTTCTGG</td>
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<td>12</td>
<td>GCTGTGATGATAAACAGCCCTG</td>
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<td>ACCCTGGCTCAGGCTTCTCC</td>
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<td></td>
<td>ACCACCCCCAGCCACAGCAG</td>
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<td>14</td>
<td>AAGCAAGCTCACTGGCCAGC</td>
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<td>CCCATGGGCTGTTTCTGG</td>
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**Promoter region**

<table>
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<tr>
<td>AGGACCTCTGGCTCTGATGA</td>
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<td>GCCATGCCGGCGCAAGACAAG</td>
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<td>AAAATGGGACGATTGGTCAG</td>
</tr>
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<td>ATCTCCGCACTCCTCTCC</td>
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<td>GAAATGGGACGATTGGTCAG</td>
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<tr>
<td>CAGGGAGGTACAGGGCTGTG</td>
</tr>
<tr>
<td>CTGGGAGGCTCAGGGCTGTG</td>
</tr>
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</table>

**Supplementary Figure 1.** Evolutionary conservation of the proline residue at position 297 in the SR-BI protein.
Supplementary Figure 2. Whole-genome linkage analysis results in the family. The SCARB1 locus is the only locus on the entire genome which is significantly linked to the high HDL cholesterol phenotype in this family (multipoint parametric LOD score 3.31 between SNP markers rs1345015 and rs12581579).

Supplementary Figure 3. Adenoviral expression experiments in mice. SR-BI KO mice FPLC profiles at baseline and after adenoviral overexpression of SR-BIWT or SR-BIP297S. Overexpression of SR-BIWT results in a marked reduction in size and volume of the HDL cholesterol peak, compared to baseline, whereas overexpression of SR-BIP297S results in an intermediate phenotype (n=3 per group, a). Western blot showing similar hepatic SR-BI protein levels after administration of Ad.SR-BIWT or Ad.SR-BIP297S. (b)

Supplementary Figure 4. Macrophages from P297S carriers show reduced fractional cholesterol efflux to HDL despite increased SR-BI expression. Cholesterol efflux from primary macrophages induced by HDL (5 µg/ml apoA-I) is lower in P297S carriers than in non-carriers, both in 4hr efflux experiments (n=12 vs. 6) as well as in 24hr efflux experiments (n=14 vs. 14, a). Cholesterol efflux to medium without added HDL did not differ between groups (p=0.59; data not shown). Monocytes from P297S carriers show a trend towards an increase in SR-BI mRNA expression (n=11 vs. 11; p=0.10), while ABCG1 and ABCA1 expression were not affected. (b) Macrophages from P297S carriers display increased SR-BI protein levels (n=8 vs. 8, c). Representative SR-BI Western blot. (d) * p<0.05, ** p<0.02
Supplementary Figure 5. Platelets from P297S carriers display increased spreading under static conditions. For static adhesion studies, platelet suspensions were seeded on fibrinogen-coated coverslips and allowed to spread for 45 min. Platelet spreading was analyzed by differential interface contrast microscopy. Arrows indicate fully spread platelets. See also Supplementary Video 1 and 2, available online at www.nejm.org.

Supplementary Figure 6. Representation of urinary corticosteroid excretion and the response to a 1 µg bolus of tetracosactin in P297S carriers and non-carriers. Carriers appear to cluster in the left lower corner, indicative of decreased adrenal corticosteroid production. a) F, age 25. Often fatigued and listless, with regular complaints of dizziness after rising. Reports that fatigue has a profound negative impact on her everyday life. Has consulted a physician for these complaints, but no cause was found. b) F, age 23. Regular complaints of fatigue (for which she has consulted a physician) and dizziness upon rising. Very occasional fainting. c) F, age 51. Often fatigued, regularly listless. Occasional complaints of dizziness but has never consulted a physician for these complaints. d) F, age 27. Regular complaints of fainting and near-fainting, for which she has consulted a physician (no cause was found). Occasionally fatigued and listless. e) F, age 66. Occasional dizyness, for which her GP prescribed chronic treatment with cinnarizine. f) F, age 33. Has consulted her physician for complaints of fatigue and listlessness; these were diagnosed as being secondary to alpha-thalassemia.
Extended methods

Study population and sequence analysis

Over the past decade, we have used our lipid clinic network and contacts with general practitioners in The Netherlands to collect plasma and DNA of individuals with hyperalphalipoproteinemia, with the intent to identify novel genes that control HDL cholesterol levels. For the present study, we selected 162 unrelated Caucasian subjects with hyperalphalipoproteinemia, as defined by HDL cholesterol above the 95th percentile for age and gender. Most of these index subjects were selected from families in which a high HDL cholesterol trait was established. The family of the index patient in which a novel SR-BI mutation (P297S) was found was expanded for this study. Informed consent was obtained for blood sampling and storage, and genetic analysis. The study was approved by the Institutional Review Board of the Academic Medical Center in Amsterdam, the Netherlands.

Genomic DNA was isolated from peripheral blood leukocytes using salt-chloroform extraction methodology, as described. To select primers for polymerase chain reactions (PCR), the SR-BI mRNA sequence was retrieved via the NCBI Nucleotide online database (NM_005505.4) and blasted against the human genome (UCSC Genome Bioinformatics; http://genome.ucsc.edu) to identify the intron/exon boundaries and the amplicons (exonic regions or promoter sequences). All amplicons were inserted into Oligos version 9.9 (a software tool to design PCR primers by Ruslan Kalender, Institute of Biotechnology, University of Helsinki) to select optimal PCR primer combinations. Primers were selected in intronic sequences at least 100 base pairs upstream or downstream of the intron/exon boundaries and were obtained from Eurogentec (Seraing, Belgium). Supplementary Table 4 shows the primer sequences that were used. Genomic DNA of the selected subjects was subjected to PCR in a Thermocycler PCR Apparatus (Biometra/Westburg, The Netherlands) and the products were sequenced using fluorescently labelled dideoxy chain terminations with a Big Dye Terminator ABI Prism kit (Applied Biosystems, Foster City, CA, USA) according manufactury’s protocol and analysed on an Applied Biosystems automated DNA sequencer (model 3730).

Whole genome SNP typing was performed on DNAs of the index patient (IV:2), nine of her close family members as well as 2 more distant relatives (IV:1 and III:5) using the GeneChip® Human Mapping 250K array (Affymetrix). SNP data were checked for Mendelian inheritance errors using software packages GRR3 and PedCheck. Multipoint parametric linkage analysis and haplotyping was performed using Allegro software5 with 20369 informative SNP markers over the entire genome.

Blood was collected after overnight fasting. Total plasma cholesterol (TC) and triglycerides (TG) were determined by an enzymatic colorimetric procedure (CHOD-PAP, Roche Diagnostics, Basel, Switzerland), HDL-C was measured as cholesterol remaining after precipitation of apolipoprotein (apo) B-containing lipoproteins by MnCl₂.
density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. Plasma apolipoprotein (apo) A-I, A-II, and B concentrations were determined by immunonephelometry. Apo E concentration was determined by electroimmunoassay using a Hydragel LpE kit supplied by Sebia (Issy-les-Moulineaux, France). Distribution of lipids over lipoproteins was assessed by FPLC. The system contained a PU-980 ternary pump with an LG-980-02 linear degasser, a FP-920 fluorescence and UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra reagent pump (Besta, Uppsala, Germany) was used for in-line enzymatic lipid reagent addition at 0.1 ml/min. Plasma lipoprotein separations were performed with a Superose 6 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) with TBS, pH 7.4, as eluent at a flow rate of 0.31 ml/min. In-line total cholesterol and triglyceride measurements were performed using PAP 250 cholesterol and PAP 250 triglyceride enzymatic methods (Biomerieux, Le Fontanille, France) and in-line free cholesterol was determined by COD-PAP reagent (WAKO Chemicals, Neuss, Germany). Cholesteryl ester concentrations for each lipid fraction were calculated by subtraction of the free cholesterol level from total cholesterol. Commercially available lipid plasma standards (low, medium and high) were used for quantitative analysis (SKZL, Nijmegen, the Netherlands) of TC, FC and TG in the main lipoprotein classes. Computer analysis of the chromatograms was carried out using the Chrompass Chromatography Data System, Rev 1 (Jasco Corporation, Tokyo, Japan).

Plasma CETP concentration was analyzed using a double-antibody sandwich ELISA as described. Plasma cholesteryl ester transfer (CET), was assayed essentially as described. In brief, [3H]cholesterol was equilibrated for 24 h with plasma cholesterol at 4 °C followed by incubation at 37 °C for 3 h. Subsequently, apoB-containing lipoproteins were precipitated by addition of phosphotungstate/MgCl2. Lipids were extracted from the precipitate and the labelled cholesteryl esters were separated from labelled unesterified cholesterol on silica columns and assayed by liquid scintillation counting. Plasma PLTP activity was measured in a liposome vesicles-HDL system as described. In short, plasma samples were incubated for 45 min, 37°C, with [14C]dipalmitoyl-phosphatidylcholine-labeled liposomes and an excess of HDL, followed by precipitation of the liposomes with a mixture of NaCl, MgCl2, and heparin (final concentrations 230 mmol/l, 92 mmol/l, and 200 units/ml, respectively). The measured PLTP activities are linearly related to the amount of plasma used in the incubations (0.5–1.0 µl). The method is not influenced by the phospholipid transfer-promoting capacities of CETP. Plasma PLTP activity was related to the activity measured in a reference plasma pool, based on plasma collected from 200 randomly selected healthy individuals, and is expressed as the percentage of the activity measured in this reference plasma, which was included in every assay. PLTP activity in reference plasma was 21.2 µmol · ml⁻¹ · h⁻¹. The between- and within-assay coefficients of variation (CV) were 4.8 and 3.5%, respectively.
Functional analyses of the P297S variant

Mutagenesis of human SR-BI cDNA (ATCC, Teddington, Middlesex, UK) was performed using the Quickchange Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, California). Adenoviruses expressing human SR-BI<sup>wt</sup> (Ad.SR-BI<sup>wt</sup>) or SR-BI<sup>P297S</sup> (Ad.SR-BI<sup>P297S</sup>) were essentially prepared as described. In brief, the SR-BI open reading frame was cloned into a pAdTrack-CMV vector. pAdTrack-CMV-encoding SR-BI was digested with PmeI and inserted into pAdeasy using recombination in E.coli BJ5183. Similarly, the empty pAdTrack-CMV vector was inserted into the pAdeasy construct (Ad. mock).

In vivo studies. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research, Leiden, the Netherlands in accordance with national laws and conducted in conformity with the Public Health Service Policy. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University, Leiden, The Netherlands. SR-BI KO mice were kindly provided by Dr. M. Krieger (Department of Biology, MIT, Cambridge, MA, USA). Mice were housed in sterilized filter-top cages and given unlimited access to food and water. Mice were maintained on sterilized regular chow, containing 4.3% (w/w) fat and no added cholesterol (RM3; Special Diet Services, Witham, UK). Mice were injected via the tail vein with 2x10<sup>8</sup> pfu Ad.mock to saturate the uptake of viral particles by Kupffer cells, followed by a 1x10<sup>8</sup> pfu injection of Ad.SR-BI<sup>P297S</sup> (n=3), Ad.SR-BI<sup>wt</sup> (n=3) or Ad.Mock (n=2). In a second, independent experiment, and using new virus batches, mice were injected with 4x10<sup>8</sup> pfu Ad/mock, followed by a 2x10<sup>8</sup> pfu injection of Ad.SR-BI<sup>P297S</sup> (n=3), Ad.SR-BI<sup>wt</sup> (n=3) or Ad.Mock (n=3). Blood samples were collected after an overnight fast before and five days post injection. The distribution of lipids over the different lipoproteins in serum was determined by fractionation of 30 µl of serum using a Superose 6 column (3.2 x 300 mm, Smart-System; Pharmacia, Uppsala, Sweden). Plasma TC was determined using an enzymatic colorimetric assay (Roche Diagnostics).

Hepatic mRNA was isolated using Trizol (Invitrogen) and cDNA was prepared using Superscript II reverse transcriptase (Invitrogen). QPCR was performed on a MyiQ (Biorad) using primers for SR-BI (sequences CTGTGGTGAGATCATGTGG and CTTGTCCTTGAGGGGAACA) and for GAPDH, cyclophiline and 36B4 as controls. Hepatic protein concentrations were determined using BCA Protein Assay Kits (PIERCE) according to the manufacturer’s protocol. For quantitative Western blot analysis (SR-BI protein and β-Actin as control), 40 µg of liver lysate was subjected to 10% SDS-polyacrylamide gelelectrophoresis. Protein was transferred onto PVDF Transfer Membrane (PerkinElmerTM Life Sciences, Inc) at 15V overnight and pre-incubated with 5% milk in phosphate-buffered saline-0.1% Tween20 (PBS-T). Polyclonal rabbit anti-SR-BI (ab396, abcam) was diluted 1:5,000 in 0.5% milk in PBS-T buffer and incubated overnight at 4°C. After washing with PBS-T 4 times for 5 minutes, 1:10,000 diluted polyclonal swine anti-rabbit immunoglobulins/HRP (P0399, Dakocytomation) in 0.5% milk in PBS-T buffer
were added and incubated for 1 hour at room temperature. Membranes were rinsed with PBS-T 4 times for 15 minutes and the signal was detected by chemiluminescence ECL blot detection system (34080, PIERCE). Anti-β-Actin (ab8227, abcam) was used to normalize for quantification of SR-BI protein expression.

In vitro studies. Parenchymal liver cells were isolated from SR-BI KO mice injected with Ad.SR-BIP297S or Ad.SR-BIWT by collagenase perfusion (0.06% (w/v) collagenase) of the liver for 12 min as described.12 Liver cells were collected by mincing the liver in ice-cold Hank’s buffer containing 0.3% (w/v) BSA followed by filtration through nylon gauze to remove large debris. Three subsequent washing steps (10 min, 50 g, 4°C) with ice-cold Hank’s buffer were performed to separate the parenchymal cells (pellet) from the endothelial and Kupffer cells (supernatant). The pellet consisted of pure parenchymal cells as judged by light microscopy. Cell viability was examined by trypan blue exclusion and was >95%. After resuspension in oxygenated DMEM supplemented with 2% (w/v) BSA (pH 7.4), 0.75 mg of parenchymal cell protein was incubated with the indicated amounts of HDL at 37°C for 3 h in a final volume of 0.5 ml. HDL was isolated from blood of healthy subjects by differential ultracentrifugation as described 13 and dialyzed against PBS with 1 mM EDTA in PBS. HDL (1.063 < d < 1.21) was labelled with [3H]CE via exchange from donor particles as reported previously.14 In short, donor particles were formed by sonication of egg yolk phosphatidylcholine supplemented with 50 µCi of [3H]CE. Sonication was carried out with a MSE soniprep 150 for 40 min (amplitude, 12 µm) at 52°C under a constant stream of argon in a 0.1 M KCl, 10 mM Tris, 1 mM EDTA. 0.025% NaN3 buffer, pH 8.0. Donor particles with a density of 1.03 g/ml were isolated by density gradient centrifugation. Native HDL was labelled by incubating with donor particles (mass ratio of HDL protein/particle phospholipid = 8:1) in the presence of human lipoprotein-deficient serum as the CE transfer protein source (1:1, v/v) for 8 h at 37°C in a shaking-water bath under argon. Ethylmercurithiosalicylate (thimerosal; 20 mM) was added to stimulate CE transfer and to inhibit PLTP-mediated transfer and lecithin:cholesterol acyltransferase activity. 3H-CE radiolabeled HDL was then isolated by density gradient ultracentrifugation, dialysed against PBS/EDTA and labelled with 125I according to McFarlane.15 During the incubation, the cells were shaken at 150 rpm and briefly oxygenated every hour. The viability of the cells remained greater than 90% during these incubations. Subsequently, the cells were washed twice with wash buffer (0.15 M NaCl, 2.5 mM CaCl2, and 50 mM Tris-HCl, pH 7.4) containing 0.2% (w/v) BSA and once with the same buffer without BSA, after which the cells were lysed in 0.1 N NaOH and protein content and radioactivity were measured. Values for [3H]CE and [125I] association are expressed per mg cellular protein and are corrected for SR-BI independent association of HDL with hepatocytes by subtracting the [3H]CE and [125I] association values obtained in control (untransfected) SR-BI knockout hepatocytes. Western blot for SR-BI protein quantification was carried out as described above.
**Carotid intima-media thickness**

The intima-media thickness (cIMT) measurements were performed as described. In short, high-resolution B-mode ultrasound images were acquired using an Acuson 128XP/10v (Acuson Corp., Mountainview, California), with a 7.0-MHz linear array transducer. Segments of 10 mm of the common carotid artery were scanned. The acquired images were saved as JPEG image files. One image reader, blinded to the genetic status of the subjects, measured the cIMT of the far wall of those segments. The mean combined cIMT of both common carotid arteries was used for calculations.

**Macrophages studies**

Per subject, 30 ml of heparinized blood was collected and diluted 1:1 with phosphate-buffered saline/0.1% bovine serum albumin + heparin (LeoPharma). The diluted blood was layered on Lymphoprep 1.077 g/ml (Lucron Bioproducts N1114544) and centrifuged for 15 minutes at 1000g. Mononuclear cells were collected with a sterile plastic Pasteur pipet and washed twice. Platelets were depleted by centrifugation for 10 minutes at 250g. The mononuclear cells were subsequently layered on a percoll (Pharmacia 17-0891-01) density gradient and centrifuged for 45 minutes at 1750g. The upper layer of platelets and aggregates was removed and the monocytes were harvested from the upper interface and washed twice. Cells were seeded in RPMI 1640/glutamax/hepes (Gibco 72400) with 10% human serum, in a 24-wells cell culture plate, 1 ml and 0.4 x 10^6 cells per well. Cells were cultured for 10 days and subsequently loaded with [3H]cholesterol, by adding 0.5 ml of labeling medium (D-MEM:F-12(1:1),Glutamax, 0.1% Pen/Strep, 0.2% BSA, 30 µg/ml cholesterol, 0.5 µCi/ml [3H]cholesterol in ethanol) and incubated for 24 hours (at 37ºC, 5% CO₂). Labeling medium was removed and the cells were washed 4 times, after which 0.5 ml of efflux medium, consisting of D-MEM:F-12(1:1), Glutamax, 0.1% P/S, 0.2% BSA and 5 µg apoA-I protein/ml HDL (Calbiochem 437641) was added to each well. Efflux medium without HDL was used as a control. Fractional cholesterol efflux was obtained by measuring the release of radiolabeled cholesterol into the medium over 4 or 24 hours.

**mRNA analysis.** SR-BI, ABCA1 and ABCG1 mRNA levels in primary monocytes were measured as detailed above, with primers for human SR-BI (CTGTGGGTGAGATCTGTGG and CTTGTCCTTGAAGGGGAACA), ABCG1 (ACTCCGGCTTCCTCTTCTTC and CATGGTCCTTGCCAGGTAGT), and ABCA1 (ATGAGGACAACAATACAAAGCC and GGGAAAGAGGAAGTAGACTCCAAA), respectively.

**SR-BI protein.** Macrophages were washed twice with warm PBS, and lysed in ice-cold buffer (pH 8.2) containing 1mg/ml BSA, 5mM EDTA, 10mg/ml Tx-100, 1mg/ml SDS, 10mM KCl, 1.5mM MgCl₂ and 1mg/ml protease inhibitor (Roche) for 20 minutes, and sonicated for 5 seconds. After centrifuging at 10,000g for 2 minutes, the supernatants were transferred to new tubes. Protein concentrations were determined using BCA
Protein Assay Kits (PIERCE) according to the manufacturer’s protocol. Western blot analyses were performed as described above. THP1 cell lysate was used as a positive control.

Platelet studies
Freshly drawn venous blood from P297S carriers and non-carriers was collected into 0.1 volume 130 mmol/L trisodium citrate. For aggregation studies, platelet-rich plasma was prepared by centrifugation (150 x g, 15 min, 20°C) and adjusted to a final concentration of 2.0 × 10^{11} platelets/L with platelet-poor plasma prepared from the remaining blood (1100 g, 10 min, 20°C). Washed platelets were prepared by adding 0.1 volume of ACD (2.5 g trisodium citrate, 1.5 g citric acid, and 2 g D-glucose in 100 mL distilled water) to lower the pH of the PRP to 6.5, followed by two cycles of centrifugation (330 g, 15 min, 20°C) and resuspension in Hapes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na2HPO4, 1 mmol/L MgSO4, 10 mmol/L Hapes, 5 mmol/L D-glucose, pH 6.5). Prostacyclin (10 ng/mL, f.c.) was present during the final wash step, and the final resuspension was in Hapes-Tyrode buffer (pH 7.2) to a final concentration of 2.0 × 10^{11} platelets/L. For static adhesion studies, platelet suspensions were diluted to a concentration of 0.3 × 10^{11} platelets/L.

Activation state. To investigate the activation state of circulating platelets, surface expression of P-selectin on non-stimulated platelets was determined by flow cytometry. Whole blood was incubated with RPE-conjugated anti-human P-selectin (20 min, 20°C), fixed with 0.5 ml 0.1% formaldehyde in 150 mmol/L NaCl for 10 min (20°C), diluted in 0.5 ml fixative (1:10, v/v) and analyzed by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, CA, USA). P-selectin expression was corrected for platelet count.

Aggregation. Human platelet-rich plasma was stimulated with 2 μg/mL ADP, 0.25 mg/ml arachidonic acid, 1 μg/ml collagen, 1.2 mg/ml ristocetin, 100 nM phorbol 12-myristate 13-acetate (PMA), and 20 μM thrombin-receptor activating peptide SFLLRN (TRAP) under constant stirring at 1000 rpm (37 °C). Optical aggregation was monitored in a Chrono-Log lumiaggregometer (Chrono-Log Corporation, Havertown, PA).

Spreading of platelets under static conditions. Coverslips were coated with 100 μg/ml human fibrinogen (1 hr, 20°C) and then blocked with 1% human serum albumin in PBS. Washed platelet suspensions were diluted to a concentration of 0.3 × 1011 platelets/L in Hapes-Tyrode buffer. After seeding on the fibrinogen-coated coverslips, platelets were allowed to spread for 45 min. Platelet spreading was analyzed by differential interface contrast microscopy (Carl Zeiss BV, Göttingen, Germany). Images were taken every 30 sec and analyzed using Axiovision 4.6.3. software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).
**Cholesterol content.** Coverslips were washed with PBS, incubated in 50 mmol/L NH₄Cl/ PBS (10 min, 20°C), washed again and blocked with PBS containing 3% BSA (10 min, 37°C). To detect membrane cholesterol, coverslips were incubated with 50 mg/mL filipin III in PBS/1% BSA (1 h, 20°C). Finally, coverslips were washed with PBS and mounted in Aqua/ Poly Mount. Cells were visualized with a Nikon Eclipse E600 fluorescence microscope (60x Nikon objective) equipped with a CoolSNAP-Pro camera (Media Cybernetics, Inc. Silver Spring, MD), using Image Pro Plus software (Media Cybernetics, Inc.) for analysis.

**Adrenal function tests**
Urinary steroid excretion was analysed in 24hr urine samples by quantitative gas chromatography and mass spectrometry selected-ion-monitoring, as previously described.¹⁷,¹⁸ In brief, steroids were released from conjugation enzymatically and, after extraction, subjected to selected-ion-monitoring analysis. Selected-ion-monitoring is a form of quantitative mass spectrometry: compounds fragment and ionise in a mass spectrometer in selective fashion, which is dependent on molecular weight and structure. The machine is programmed only to monitor ions of particular mass as compounds elute from the chromatography system, e.g. gas chromatography. Ions chosen for selected-ion-monitoring must be distinctive for the compounds of interest present in the sample.¹⁹ Tetracosactin (a synthetic adrenocorticotropic hormone [ACTH] analog) tests were performed at 9am after an overnight fast. Following two baseline blood samples (t=-15, t=0 min) participants were given a 1 µg tetracosactin bolus, followed by another blood sample after 30 minutes. Total cortisol was measured by an enzyme immunoassay and cortisol binding globulin (CBG) was measured using a commercial RIA. Plasma levels of CBG in SR-BI knockout (n=3) as well as wildtype (n=4) mice (after overnight fasting) were determined by Western blot using rabbit anti-mouse CBG antiserum (gift from Dr. G. L. Hammond, University of Western Ontario).

We show that P297S carriers as well as SR-BI knockout mice present with higher plasma levels of cortisol binding globulin (CBG). Hoekstra et al. already reported previously that SR-BI knockout mice have increased hepatic expression of CBG²⁰, related to their adrenal insufficiency and the notion that CBG expression is under negative control of corticosteroids via the hepatic glucocorticoid receptor²¹,²² In human plasma more than 90% of cortisol is bound to CBG, which acts as a reservoir. Therefore, at the same rate of cortisol synthesis, subjects with higher plasma levels of CBG will show higher plasma total cortisol levels. However, because CBG-bound cortisol has no hormonal activity, total cortisol measurements do not adequately reflect the adrenal status of a subject in the presence of altered CBG levels. To deal with this, we have calculated free cortisol levels using a widely cited method by Coolens et al.¹ Briefly, two simultaneous binding equilibria determine the binding of cortisol in human plasma: the saturable binding of cortisol to CBG and the non-saturable binding to albumin. The relationship between unbound and bound cortisol is described by the following formula, with U, T and G representing the molar concentrations of unbound cortisol, total cortisol and CBG.
respectively, $K$ representing the affinity of CBG for cortisol at 37°C and $N$ the ratio of albumin-bound to unbound cortisol:

$$U^2 \cdot K (1 + N) + U [1 + N + K(G - T)] - T = 0$$

Using a value of $3.10^7 \text{M}^{-1}$ for $K$ and a constant value of 1.74 for $N$, the following equations apply (all concentrations expressed as µM):

$$Z = 0.00167 + 0.182 (G - T)$$

$$U = \sqrt{Z^2 + 0.00122T} - Z$$

For a more extensive description of this method, we refer to the paper by Coolens et al.$^{25}$
Heterozygosity for a loss-of-function mutation in \textit{GALNT2} improves plasma triglyceride lipolysis in man

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Summary

Genome-wide association studies have identified GALNT2 as a novel candidate gene in lipid metabolism but it is not known how the encoded enzyme ppGalNAc-T2, which contributes to the initiation of mucin-type O-linked glycosylation, mediates this effect. In two families, we identified a loss-of-function mutation in GALNT2 (D314A) which segregates with elevated plasma high-density lipoprotein cholesterol (HDL-c) and reduced triglycerides. The favourable effect of this mutation was evidenced by improved postprandial triglyceride catabolism in carriers which is likely attributable to attenuated glycosylation of apolipoprotein (apo) C-III that was also found in these carriers. Apo C-III inhibits lipoprotein lipase, an enzyme that hydrolyses plasma triglycerides thereby also affecting HDL-c levels. We show that a synthetic apo C-III based peptide is a substrate for wild-type ppGalNAc-T2 while its glycosylation by mutant ppGalNAc-T2 is impaired. It is furthermore shown that in the post-prandial phase, apo C-III distribution over lipoproteins was different between carriers and controls, and endogenous VLDL of the carriers had reduced potential to inhibit LPL. Neuraminidase treatment of apo C-III was also shown to decrease its potential to inhibit LPL. Combined, these data suggest that ppGalNAc-T2 affects lipid metabolism through apo C-III glycosylation. In conclusion, we have found a loss-of-function mutation that establishes GALNT2 as a lipid-modifying gene, thereby linking the cardiovascular and glycosylation research fields.
Introduction

Genome-wide association studies (GWAS) have identified many novel loci to affect plasma lipids and cardiovascular disease (CVD). However, validation of these candidate genes by unravelling of pathways through which they affect human metabolism is awaited. (Ku et al., 2010) This also holds true for GALNT2 (locus NM_004481). In recent GWAS, SNPs in intron 1 of the GALNT2 gene were found to be associated with high-density lipoprotein cholesterol (HDL-c) and triglyceride levels (Kathiresan et al., 2008). Teslovich et al. subsequently showed that hepatic overexpression and silencing of GALNT2 in wild-type mice resulted in a reduction and increase of HDL-c levels, respectively. (Teslovich et al., 2010) To date, however, it is not known through which mechanism the encoded enzyme UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-2 (ppGalNAc-T2) mediates these effects in mice and man. The enzyme belongs to a family of ppGalNAc transferases comprising 20 members in humans (Ten Hagen et al., 2003), all catalyzing the transfer of GalNAc residues onto proteins, thereby initiating mucin-type O-glycan chain synthesis on threonine and/or serine residues. The size of the enzyme family, its level of evolutionary conservation and the spatio-temporal expression patterns during embryonic development point to important and isoform specific functions for ppGalNAc-transferases in mammalian physiology (Ten Hagen et al., 2003; Gerken et al., 2011), but to date these are largely unknown. In the current study, we identify a loss-of-function mutation in subjects with high HDL-c, and provide the first evidence of a molecular pathway through which ppGalNAc-T2 affects plasma lipid levels.

Results

A rare GALNT2 variant

GALNT2 was sequenced in 243 subjects who were referred to our lipid clinic for high HDL-c levels (>95th percentile for age and gender). We identified 2 unrelated heterozygotes for a same point mutation (c.941A>C). This mutation causes the exchange of an aspartic acid for an alanine residue at an evolutionary conserved position (D314A; Supplementary Table 1) and was not found in 1472 normolipidemic controls, nor in 68 individuals with HDL-c levels <5th percentile for age and gender. In one family, 7 carriers and 14 unaffected family members were identified. In the second family, 1 carrier and 3 unaffected family members were recruited. In both families, the mutation segregated with a phenotype characterized by elevated HDL-c and decreased triglyceride levels (Supplementary Figure 1).

Clinical examination

Carriers of the mutation were subjected to a routine physical examination and blood tests, including protein spectrum, platelet aggregation and plasma coagulation assays. Since von Willebrand Factor levels are affected by mucin-type O-linked glycosylation (van
Chapter 5

In addition, carotid intima media thickness was measured as a surrogate marker for atherosclerosis. None of the above analyses revealed abnormalities in the carriers compared to non-carriers.

**Lipids and (apo)lipoproteins**

Fasting plasma lipids of 8 carriers revealed 30% higher HDL-c levels (p=0.017), 20% lower triglyceride levels (p=0.05), and 29% increased total cholesterol (p=0.004; Table 1) compared to 17 family controls. Low-density lipoprotein cholesterol (LDL-c) tended to be higher in carriers (p=0.07).

Several proteins and enzymes with established roles in lipid metabolism are known to be decorated with mucin-type O-glycans including apo E (Zanni et al., 1989), apo A-II (Remaley et al., 1993; Vaith et al., 1978), apo C-III (Vaith et al., 1978), and

Table 1: Demographic, lifestyle and lipid characteristics of carriers of the GALNT2D314A mutation and controls.

<table>
<thead>
<tr>
<th>Demographic and lifestyle characteristics</th>
<th>Carriers (n = 8)</th>
<th>Non-carriers (n = 17)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>47.8 (24)</td>
<td>44.9 (13)</td>
<td>0.69</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>25</td>
<td>53</td>
<td>0.28*</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.4 (4.5)</td>
<td>24.4 (2.4)</td>
<td>0.95</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>1 (13)</td>
<td>5 (29)</td>
<td>0.36*</td>
</tr>
<tr>
<td>Alcohol use (U/wk) †</td>
<td>1 (0-9)</td>
<td>4 (0-15)</td>
<td>0.48</td>
</tr>
<tr>
<td>Diabetes mellitus type 2 (n)</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Cardiovascular events (n)</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

| Lipids and lipoproteins                  |                |                      |    |
| Total cholesterol (mg/dl)                | 251 (46)       | 195 (38)             | 0.004|
| LDL cholesterol (mg/dl)                  | 157 (32)       | 128 (37)             | 0.070|
| HDL cholesterol (mg/dl)                  | 79 (23)        | 55 (13)              | 0.017‡|
| Triglycerides (mg/dl) †                  | 73 (57-89)     | 91 (68-114)          | 0.05‡|

| Apolipoproteins and LCAT activity        |                |                      |    |
| Apo B (mg/dl)                            | 117 (25)       | 110 (31)             | 0.58 |
| Apo A-I (mg/dl)                          | 165 (39)       | 135 (36)             | 0.07 |
| Apo A-II (mg/dl)                         | 28.4 (5.2)     | 29.4 (4.4)           | 0.66 |
| Apo E (mg/dl)                            | 2.31 (0.61)    | 3.19 (1.1)           | 0.06 |
| Apo C-II (mg/dl)                         | 4.17 (3.8)     | 4.05 (1.8)           | 0.92 |
| Apo C-III (mg/dl)                        | 10.0 (2.3)     | 10.3 (2.5)           | 0.83 |
| LCAT activity (cholesteryl ester/ml/hr)  | 17.6 (3.6)     | 16.9 (1.3)           | 0.72 |

Data are presented as mean (SD) unless otherwise specified. P for T-test of independent samples. *P for chi-square test. †Alcohol use and triglycerides are presented as median (interquartile range) and were log-transformed prior to comparison of means by T-test. ‡ retained statistical significance (P<0.05 for both) upon adjustment for age, gender and body mass index in a linear regression model.

Schooten et al., 2007), von Willebrand Factor antigen and activity were also studied. In addition, carotid intima media thickness was measured as a surrogate marker for atherosclerosis. None of the above analyses revealed abnormalities in the carriers compared to non-carriers.

**Lipids and (apo)lipoproteins**

Fasting plasma lipids of 8 carriers revealed 30% higher HDL-c levels (p=0.017), 20% lower triglyceride levels (p=0.05), and 29% increased total cholesterol (p=0.004; Table 1) compared to 17 family controls. Low-density lipoprotein cholesterol (LDL-c) tended to be higher in carriers (p=0.07).

Several proteins and enzymes with established roles in lipid metabolism are known to be decorated with mucin-type O-glycans including apo E (Zanni et al., 1989), apo A-II (Remaley et al., 1993; Vaith et al., 1978), apo C-III (Vaith et al., 1978), and
ppGalNAc-T2 glycosylates apolipoprotein C-III

lecithin:cholesterol acyltransferase (LCAT; Schindler et al., 1995)). Plasma levels of the respective apolipoproteins and LCAT activity were, however, not significantly different in cases and controls (Table 1).

Identification of ppGalNAc-T2 substrate

Since glycan chains affect the size and charge of proteins, we ran 2-dimensional gel electrophoresis (2-DE) on plasma proteins to identify possible ppGalNAc-T2 substrates. This was carried out by direct head-to-head comparisons of pairs of carriers and age-and-gender matched family controls. No changes were found in apo A-II and apo E isoforms (Supplementary Figure 2). However, we identified significant differences in the

![Graph showing differences in apo C-III isoforms](image)

**Figure 1.** 2-DE analysis of apo C-III isoforms in carriers of the GALNT2<sup>D314A</sup> mutation compared to non-carriers. Panel (a) shows significantly decreased levels of monosialylated apo C-III (apo C-III<sub>1</sub>) and increased levels of non-sialylated apo C-III<sub>0</sub> isoforms (comprising apo C-III that is not sialylated and apo C-III that is not glycosylated (Bruneel et al., 2007)) Data are expressed as means (SE). P values for Mann-Whitney U tests. (b) A carrier with increased levels of non-sialylated apo C-III<sub>0</sub> isoforms, while all apo C-III of a family control is either mono- (apo C-III<sub>1</sub>) or disialylated (apo C-III<sub>2</sub>).
relative distribution of apo C-III isoforms which result from differences in sialylation of a single O-glycan at the Thr74 position of this apolipoprotein (Vaith et al., 1978)(Figure 1). Carriers showed a pronounced 6.6-fold increased concentration of non-sialylated apo C-III0 (p<0.01), and a 32% decrease of monosialylated apo C-III1 (p<0.05) while disialylated apo C-III2 concentrations was not different between the groups (Figure 1). MALDI-TOF mass spectrometry confirmed that the experimentally detected aberrant proteins were the denoted apo C-III isoforms (Supplementary Table 2).

Functional characterization of ppGalNAc-T2\textsuperscript{D314A}

GALNT2 mRNA and protein levels in lysates of cultured skin fibroblasts were identical in carriers and controls (Supplementary Figure 3). To study the effects of the D314A mutation on enzyme function, mutant and wild-type ppGalNAc-T2 were expressed in COS7 cells. A study of enzyme kinetics showed that the maximum number of enzymatic reactions catalyzed per second (K\textsubscript{cat}) by mutant ppGalNAc-T2 was more than 2-fold lower compared to wild-type ppGalNAc-T2 (p<0.05, Figure 2a) when using a standard ppGalNAc-T substrate (EA2)(Ten Hagen et al., 2003)), and an 11-mer apo C-III peptide harbouring the Thr74 residue as a substrate (p<0.01, Figure 2b). These reductions in K\textsubscript{cat} were observed in the absence of significant reductions in K\textsubscript{m}, ppGalNAc-T1, the only other ppGalNAc-T reported to be highly expressed in human liver (Ten Hagen et al., 2003; Gene Sorter, 2011) and an enzyme with broad substrate specificity, was unable to use the apo C-III peptide as substrate (Supplementary Figure 4).

Molecular modelling studies suggest that the observed loss of catalytic activity may be due to a loss of enzyme stability: residue D314 is at the “N-cap” position(Richardson and Richardson, 1988) of a catalytic domain alpha helix (Figure 3a) and its side chain forms a hydrogen bond to the amide nitrogen of Y317 within the helix. The negative charge of D314 is expected to neutralize the partial positive charge of the helix dipole. Thus, the exchange of D for A at position 314 would remove both the hydrogen bond and negative charge and would be expected to destabilize the enzyme (see movie in Figure 3b and accompanying legends for more detailed information).(Nicholson et al., 1988)

Maximal oral fat challenge

4 carriers and 4 family controls were willing to participate in a fat load study in which they ingested cream corresponding with 40 g of fat per square meter of body surface area. We observed a significantly improved postprandial plasma triglyceride clearance in carriers (p=0.014; Figure 4). In addition, triglyceride levels peaked at 3 hours in carriers instead of 4 hours in non-carriers, while plasma LPL levels were similar at any time point in both groups (Supplementary Figure 5). Under fasting conditions, the distribution of apo C-III over lipoproteins was similar in carriers and non-carriers. Four hours postprandial, apo C-III was significantly more abundant in the HDL fraction in carriers compared to controls (area under the curve for HDL fractions, and apo C-III differences between carriers and controls for peak HDL fraction (#15); for both p=0.029; see Supplementary Figure 6).
ppGalNAc-T2 glycosylates apolipoprotein C-III

Figure 2. Enzyme kinetics of wild-type and mutant ppGalNAc-T2 during O-linked glycosylation of EA2 and an 11-mer apo C-III peptide.

2a Kinetic parameters for EA2 peptide glycosylation.

<table>
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<td>sec⁻¹</td>
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<tr>
<td>Wildtype ppGalNAc-T2</td>
<td>457.3 ± 53.6 (n = 5)</td>
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<td>ppGalNAc-T2D314A</td>
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2b Kinetic parameters for apo C-III peptide glycosylation.

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<td>sec⁻¹</td>
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<td>Wildtype ppGalNAc-T2</td>
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<tr>
<td>ppGalNAc-T2D314A</td>
<td>880.6 ± 243.3 (n = 4)</td>
<td>2.4 ± 0.4 (n = 3) b</td>
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</table>

Vertical axis indicates rate of transfer as dpm/h/densitometric unit of enzyme. The different GalNAc transferases were overexpressed in COS7 cells. Representative kinetic plots and averages of replicate peptide glycosylation experiments catalyzed by human wild-type ppGalNAc-T2 or mutant ppGalNAc-T2D314A. Purified recombinant human ppGalNAc-T2 (hT2) or ppGalNAc-T2D314A (D314A) mixed with various concentrations of peptide substrates, EA2 (2a) or Apo C-III (2b), was used for enzymatic kinetic studies. Kinetic plots were fit to Michaelis-Menten equation. Kinetic parameters for peptide glycosylation by wild-type ppGalNAc-T2 or ppGalNAc-T2D314A are given in tables as averages ± SD. N indicates number of replicate experiments.
Chapter 5

Inhibition of lipoprotein lipase (LPL)

Having established that apo C-III is a specific substrate for ppGalNAc-T2 and the knowledge that apo C-III is an established inhibitor of LPL (Jong et al., 1999), we studied whether sialylation of the sole O-linked glycan of apo C-III affects this property. To this end, apo C-III was treated with neuraminidase. Figure 5a shows that the loss of sialic acids results in a shift from apo C-III1 and apo C-III2 (with one and two sialyl groups, respectively) to apo C-III0 (with no sialic acids). Native untreated apo C-III inhibited LPL by 54%, while neuraminidase-treated apo C-III inhibited LPL by 26% (p<0.001; Figure 5b). In a substrate competition assay, very-low density lipoprotein (VLDL) isolated from plasma of GALNT2\textsuperscript{D314A} carriers (containing endogenous apo C-III) showed reduced ability to inhibit recombinant human LPL to hydrolyse a standard [\textsuperscript{3}H]triolein substrate compared

Figure 3. Molecular modeling of D314A mutation in ppGalNAc-T2 (a) Ribbon diagram of the human T2 structure (PDB ID 2flu). Alpha helices (except as noted) are shown in orange, beta strands in blue and loop regions in gray. Residue D314 (green carbons) is at the N-terminus of an alpha helix (yellow ribbon) which lies on the side of the catalytic domain opposite to that of the substrate binding face. The enzyme is shown with bound EA2 peptide (white carbons), UDP (green carbons) and a manganese ion (yellow sphere). A portion of the lectin domain is shown in the upper right of the figure. The figure was created using CCP4mg software. (Potterton et al., 2004) (for color figure, see page 417) (b) Movie showing the location of D314 and the hydrogen bond it forms with the amide nitrogen of Y317. ppGalNAc-T2 (PDB ID 2flu) is shown as a ribbon diagram with orange alpha helices, blue beta strands and gray loops. The enzyme is bound to EA2 peptide (white carbons), UDP (green carbons) and a manganese ion (yellow sphere). The active site initially faces forward and the view of the enzyme then rotates 180 degrees to the side containing D314 (green carbons) and the alpha helix (yellow ribbon) to which D314 forms a hydrogen bond. A close up view shows the hydrogen bond (dashed blue line) between the D314 side chain and the amide nitrogen of Y317. The movie was created using CCP4mg software. (Potterton et al., 2004)

(b) See movie file
ppGalNAc-T2 glycosylates apolipoprotein C-III to VLDL from plasma of non-carriers (p=0.017; Figure 5c). In this experiment, we normalized for apo B concentrations. Figure 5c also shows that increasing concentrations of VLDL isolated from carriers did not further decrease LPL activity whereas control VLDL led to a 50% reduction of LPL activity in this assay (at 80mg/dl VLDL apoB).

Discussion

Genome-wide association studies have identified an association between variation at the GALNT2 gene locus and plasma lipids. (Cho et al., 2008; Willer et al., 2008) Manipulation of GALNT2 expression in mice has subsequently been shown to affect plasma lipids, but the molecular mechanism was not addressed. (Teslovich et al., 2010) The current study identifies a missense mutation in GALNT2 causing a reduction of GalNAc-T2 catalytic activity which is associated with increased HDL-c and decreased triglycerides in carriers. The favourable effect of this mutation on lipid metabolism was evidenced by improved post-prandial triglyceride removal in carriers of the mutation. Our molecular studies lead to propose that ppGalNac-T2 mediates these effects through glycosylation of apo C-III, an established inhibitor of LPL.

We observed segregation of the mutation with high HDL-c and low triglyceride levels in the families of 2 probands. Such an inverse relationship between these lipid parameters is commonly encountered. In fact, low HDL-c and increased triglycerides are...
Figure 5. Inhibition of lipoprotein lipase-mediated triglyceride hydrolysis. (a) 2-DE analysis shows that treatment of apo C-III isolated from human VLDL (obtained from Academy Bio-Medical Company) with neuraminidase results in desialylation of the protein, as evidenced by an increase in the non-sialylated isoform apo C-III0. (b) Desialylation of apo C-III with neuraminidase abolishes its potential to inhibit LPL. rhLPL activity is inhibited by 54% after incubation with untreated apo C-III. Desialylation with neuraminidase reduces the inhibitory capacity of apo C-III (p<0.001). Vertically shaded bar: catalytic activity of rhLPL is not affected by neuraminidase treatment. Experiments were conducted in triplicate. Data are expressed as means (SE). (c) VLDL of carriers (closed symbols) has reduced ability to inhibit LPL-mediated triglyceride hydrolysis compared to VLDL of non-carriers (open symbols). Given are activities of recombinant human LPL (rhLPL) on a [3H] triolein substrate co-incubated with increasing amounts of VLDL. VLDL was isolated using gradient ultracentrifugation from pooled plasma of 4 carriers or 4 non-carriers, and normalized for apo B concentration. Measurements were carried out in triplicate. Data are expressed as means (SE). In linear regression, ß and intercept were -0.46 ± 0.39 and 238.9 ± 18.8 mU/ml in carriers, and -1.79 ± 0.12 and 236 ± 5.8 mU/ml in non-carriers, p=0.017.
often seen in patients at increased risk of atherosclerosis. (Malloy and Kane, 2001) In this case, however, the GALNT2 mutation is associated with a favourable lipid profile which reflects improved metabolic capacity to clear plasma triglycerides. Because homozygosity for a mutation in GALNT3, encoding the related ppGalNAc-T3 enzyme, has been shown to cause tumoral calcinosis, a severe metabolic disorder (Topaz et al., 2004), the GALNT2^{D314A} carriers underwent a general clinical screening in our institute. Clinically, we could not discriminate between heterozygotes and family controls, which is not unexpected when considering that congenital disorders of glycosylation (CDG) are autosomal recessive diseases.

It is shown that the mutant ppGalNAc-T2 enzyme has reduced catalytic activity which may be related to decreased enzyme stability. Thus a decrease of ppGalNAc-T2 activity is associated with an increase in HDL-c which is in line with the finding that GALNT2 silencing increases HDL-c in mice (Teslovich et al., 2010). Plasma concentrations or activities of potential ppGalNAc-T2 substrates (identified through a literature search) did not reveal natural substrates for ppGalNAc-T2 and we refrained from using in silico studies to identify potential substrates in view of the poorly conserved consensus sequence for mucin type O-linked glycosylation. (Ten Hagen et al., 2003) After coupling of GalNAc to Thr or Ser residues of protein substrates, the O-glycan chain may be elongated with a β1,3-linked galactose and negatively charged sialic acids to form mucin-core 1 structures. (Ten Hagen et al., 2003) Failure of GalNAc addition can thus alter charge and/or mass of target proteins. Using 2-DE and subsequent immunoblotting of plasma samples led to the discovery of marked changes in apo C-III isoforms in the carriers. This protein could indeed be a target for ppGalNAc-T2 since it has a single O-glycan chain (Thr74) (Vaith et al., 1978) and no N-linked glycans. (Wopereis et al., 2003) Under normal conditions, 3 different apo C-III isoforms are discriminated, i.e. apo C-III_{10}, apo C-III_{11}, and apo C-III_{12}, with zero, one or two sialic acid residues, respectively. (Wopereis et al., 2003) Comparing carriers and controls, we identified a marked 6.6-fold increased concentration of non-sialylated apo C-III_{10}, and a 32% decrease of monosialylated apo C-III_{1}. Apo C-III inhibits LPL-mediated hydrolysis of plasma triglycerides (Jong et al., 1999) in VLDL and chylomicrons, thereby affecting both HDL-c and triglyceride levels. Interestingly, apo C-III is also used as a model molecule to study O-linked glycosylation CDG disorders. (Wopereis et al., 2003) Using an 11-mer apo C-III peptide harboring the Thr74 residue, we show that ppGalNAc-T2 but not ppGalNac-T1 can use this peptide as a substrate. Since ppGalNAc-T1 is the only other ppGalNAc-T expressed in the human liver (Ten Hagen et al., 2003; Gene Sorter, 2010; Gene Sorter, 2011), this result suggests that apo C-III is preferentially glycosylated by ppGalNAc-T2, an intriguing finding given the generally broad substrate specificity of ppGalNAc-T1. Functionality of the D314 mutation was illustrated by a reduction of glycosylation activity (K_{cat} but with retained K_{m}) when using either a general ppGalNAc-T substrate or the apo C-III peptide.
Combined, these findings suggest that altered glycosylation of apo C-III due to defective ppGalNAc-T2 causes an increased capacity to hydrolyze plasma triglycerides in carriers of the mutation. To test this hypothesis, we challenged carriers and controls with an oral fat load and observed a significantly improved postprandial plasma triglyceride clearance. Although this observation supports the involvement of increased LPL-mediated triglyceride lipolysis, the molecular details of this process are difficult to unravel due to the complexity of in vivo plasma triglyceride lipolysis. From what is currently known, this process involves the simultaneous interactions of apo C-II as cofactor of LPL, apo A-V as modulator of LPL function, and angiopoietin-like factors 3 and 4 as established LPL inhibitors. While most of these factors are known to be present on HDL, VLDL and chylomicrons, the apolipoproteins rapidly exchange between these lipoproteins. In addition, the cocktail of activation and inhibitory processes at specific and differentially regulated sites of action (adipose tissue, skeletal muscle, heart muscle) occur with simultaneous effects on VLDL and chylomicron remnant uptake by the liver which also reduce plasma triglyceride levels. Finally, the molecular background of hypertriglyceridemia and hypotriglyceridemia is only unravelled in a minority of the patients seen in the clinic, illustrating that many other unknown factors may play important roles as well. With this complexity in mind, we have used several approaches to unravel the molecular mechanism that may explain our findings. Having identified changes in apo C-III isoform distribution in carriers of the GALNT2 mutation and having provided evidence that establishes that glycosylation of apo C-III by mutant ppGalNAc-T2 is impaired, we subsequently addressed the question whether the O-linked glycan chain of apo C-III affects LPL activity. Using neuraminidase, we showed that desialylation of apo C-III affects its potential to inhibit LPL in vitro. In another experiment, we show that the distribution of apo C-III over lipoproteins is similar in carriers and controls under fasting conditions. After an oral fat load, however, apo C-III was profoundly increased in the HDL fraction in only carriers. Taken that lipolysis occurs at the surface of VLDL and chylomicrons, a change of apo C-III concentration over lipoproteins can be expected to affect plasma triglyceride hydrolysis. In a third experiment, we show that VLDL (as an endogenous source of apo C-III) isolated from plasma of carriers had a 50% reduced capacity to inhibit LPL compared to control VLDL. Taken together, these results suggest that attenuated ppGalNac-T2-mediated glycosylation of apo C-III is involved in mediating the effects on lipids that are observed in carriers of the GALNT2 mutation. This is a new insight when considering the existing literature on apo C-III, a topic which has thus far not been studied in the context of reduced ppGalNAc-T2 activity.

There are, however, several important aspects that merit further discussion. First, others have shown that the amino terminus of apo C-III is responsible for inhibiting LPL while...
the glycan chain at Thr74 is located in the C-terminal domain, and that synthetic apo C-III lacking the carbohydrate moiety, can still inhibit LPL. (McConathy et al., 1992) These and other investigators (Roghani and Zannis, 1988) have, however, not addressed the potential of the different apo C-III isoforms to inhibit LPL activity. Also, the effect of a loss of the carbohydrate moiety as a result of loss of ppGalNAc-T2 activity has not been studied in vivo. In addition, we also show that carriers show an altered distribution of apo C-III over lipoproteins in the postprandial phase. The notion that negative charge of apo C-III has effects on the distribution of apo C-III over lipoproteins was previously demonstrated by Luttman et al. (Luttmann et al., 1994) and it is likely that this effect also mediates part of the lipid changes that we observe. Such changes have even been implicated in the prediction of the progression of coronary heart disease. (Blankenhorn et al., 1990) Second, Maeda et al. described a family with high apo C-III, (Maeda et al., 1981) due to heterozygosity for an APOCIII gene mutation that changes the Thr74 to an Ala residue. (Maeda et al., 1987) Thus theoretically, half of their apo C-III cannot be glycosylated. Although Maeda and coworkers did not find marked effects on lipid levels, 2 out of a total of 3 carriers had reduced triglyceride levels. It was also shown that the mutation did not affect expression and/or secretion of the mutant apo C-III which agrees with a study by Roghani et al. (Roghani and Zannis, 1988) showing that recombinant apo C-III with the same mutation was normally secreted. In 8 carriers of the GALNT2 mutation, we found a significant difference in triglyceride and HDL-c levels compared to 17 non-carriers. Maeda et al. did not investigate the effect of fat loading but their study and our study combined suggest that defects in apo C-III and ppGalNAc-T2 can translate into a similar fasting plasma lipid profiles. Pollin et al (Pollin et al., 2008) indeed show that an APOC3 non-sense mutation, has a similar effect on both fasting and postprandial lipid levels.

Third, apo C-III isoforms have been extensively studied in man. In support of our hypothesis, Mauger et al. have demonstrated that of the three apo C-III isoforms, the non-sialylated apo C-III0 is least associated with plasma triglyceride levels. Stocks and Galton furthermore show that neuraminidase treatment of hypersialylated apo C-III from VLDL of hypertriglyceridemic patients reduces its capacity to inhibit LPL. In addition, Kashyap et al. showed decreased levels of the apo C-III0 isoform in 10 subjects with severely increased triglyceride levels. (Kashyap et al., 1981) By contrast, others showed an increase of apo C-III0 in 12 patients with severe familial hypertriglyceridemia. (van Barlingen et al., 1996) The latter contrasting data show that apo C-III0 levels are not necessarily predictive of triglyceride levels, especially not in patients with hypertriglyceridemia of different molecular aetiology. Many other parameters may affect apo C-III sialylation levels in plasma such as the levels of sialyltransferase, sialidase activity, the number of asialoglycoprotein receptors (Maeda et al., 1981) which may differ under pathophysiological conditions in patients and beneficial conditions observed in our study subjects. Van Barlingen e.g. postulated that their findings were related to a longer residence time of VLDL in the circulation.
Fourth, in the current study, we did not address the possible effects of the GALNT2 defect on the hepatic uptake of lipoproteins (Shelburne et al., 1980; Windler et al., 1980). However, Windler and Havel (Windler and Havel, 1985) showed that the apo C-III isoforms are not different in this regard.

Finally, it is also possible that ppGalNac-T2 affects lipid metabolism through additional target proteins. Ter-Borch Gram Schjoldager et al. e.g. recently proposed that ppGalNac-T2 affects proteolytic processing of angptl3 which is required to inhibit LPL. Our data do not support this hypothesis: we find identical plasma concentrations of processed/unprocessed angptl3 in plasma of carriers and non-carriers (Supplementary Figure 7). According to these investigators, loss of ppGalNac-T2 activity would result in increased LPL inhibition, increased plasma triglyceride and decreased HDL-c levels while both our family analyses and the knock down of GALNT2 in mice show the exact opposite. (Teslovich et al., 2010) We can, however, not exclude that other substrates of ppGalNac-T2 affect lipid metabolism.

In conclusion, the current study shows that a rare loss-of-function mutation in GALNT2 causes a beneficial lipid phenotype in humans. The data identify a mechanism through which ppGalNac-T2 may affect plasma lipids in man, i.e. through glycosylation of apo C-III, an inhibitor of LPL-mediated hydrolysis of plasma triglycerides. This family-based study connects the research fields of protein glycosylation and lipoprotein biology by showing how a mucin-type O-linked glycosylation defect can cause changes in lipid metabolism. As of yet, lipid metabolism in heterozygotes for congenital disorders of glycosylation has to our knowledge not been studied which offers new opportunities for future research, likely resulting in further insight in the relevance of glycosylation in lipid metabolism. Finally, this study underlines the relevance of genome-wide association studies to identify and unravel new pathways in complex disorders.
**ppGalNAC-T2 glycosylates apolipoprotein C-III**

**Experimental procedures**

**Participants, DNA analysis, lipids.** All participants gave written informed consent. Oral fat challenges were carried out as described. (Nierman et al., 2005) Unaffected individuals of a screening program for familial hypercholesterolemia and donors of the Dutch blood bank were used to screen for DNA variants. The mutation in GALNT2 was identified through massive parallel sequencing (Herman et al., 2009), and was confirmed by Sanger sequencing. Allelic discrimination with pre-designed primers (Applied Biosystems) on a Lightcycler 480 (Roche) was used to check controls for this variant. Total cholesterol, LDL-c, HDL-c, triglycerides, and apo A-I, E, C-II, and C-III were measured using commercially available assays (Randox, Wako) on a COBAS MIRA analyser. Fast protein liquid chromatography to separate lipoproteins and determine their cholesterol content was carried out as previously described (Levels et al., 2003). Fractions were collected for subsequent apo C-III immunoblotting (see Supplemental Methods for more detail).

**Two dimensional gel electrophoresis and mass spectrometry.** 2-DE of plasma proteins was performed using IPGphor and Multiphor (Amersham Biosciences). Aliquots (200µg) for Western blots were applied on pH 4-7 IPGs and plasma (300µg) for preparative gels on pH 3-10 IPGs (Karlsson et al., 2005). Separated proteins for quantification and identification were detected by silver staining. (Shevchenko and Shevchenko, 2001) For protein identification, Tryptic digests of proteins excised from the gels were analyzed by mass spectrometry (see Supplemental Methods for more detail).

In Western blots, proteins were transferred to a PVDF membrane. After blocking and incubation with primary antibodies against candidate proteins, the membranes were incubated with HRP-conjugated secondary antibodies. Proteins were visualized using an ECL plus Western blotting detection system, exposed to X-ray film and developed. Isoform intensities were determined as optical density per mm² and expressed as % of the total protein level for each protein.

**In vitro analyses.** Secretion constructs for wild-type human (h) T1, wild-type hT2, and mutant hT2 are described in the supplement. COS7 cells were grown to 90% confluence and transfected with pIMKF4 vector, pIMKF4-wild-type hT1, pIMKF4-wild-type hT2, or pIMKF4-mutant hT2 as described. (Hagen et al., 1997) The activities of enzymes were measured against EA2 (PTTDSTTPPTTK) and an apo C-III peptide (PEVRPTSAVAA) using radioactively labeled UDP-GalNAc. (Hagen et al., 1997).

Wild-type and mutant ppGalNAC-T2 activities were measured by quantifying the transferred radilabeled GalNAC from UDP-[14C]GalNAC to peptide substrates (O'Connell and Tabak, 1993). Reactions (25 µl final volume) were initiated by adding purified enzyme (4.6-27.4 fmol) to reaction mixtures containing 40 mM sodium cacodylate, 10 mM MnCl2, 40 mM b-mercaptoethanol, 20 mM UDP-GalNAC, 7.3 mM UDP-[14C]GalNAC (54.70 mCi/mmol), and 0.1% Triton X-100 at pH 6.6. Reactions were incubated at 37 °C for 1 hour. Concentrations of EA2 and apo C-III peptides were 6-200 mM and 100-500
mM, respectively. Reactions were stopped by adding 75 ml of 30 mM EDTA and then acidified by adding 100 ml of 0.1% TFA before Sep-Pak peptide purification (O’Connell and Tabak, 1993). Kinetic parameters were calculated using Hanes plot. To calculate $k_{cat}$, enzyme concentration were measured by western blots according to a standard curve made with known concentrations of FLAG-BAP (Sigma-Aldrich).

VLDL was isolated from plasma by gradient ultracentrifugation and added to recombinant human LPL. A $[^3H]$triolein substrate (Nilsson-Ehle and Schotz, 1976) was used to test LPL activity and the potential of apo C-III to inhibit the triolein hydrolysis. Apo C-III isolated from human VLDL (Academy Bio-Medical Company), was treated with neuraminidase (NorthStar). The potential of treated and untreated apo C-III to inhibit triolein hydrolysis was assessed in the above mentioned LPL assay (see Supplemental Methods).

Statistical analyses. Parameters were compared between carriers and non-carriers using Students t-tests or Mann-Whitney U tests, where appropriate, for continuous variables. Chi-square tests were used for categorical variables. Data on LPL inhibition by increasing amounts of VLDL were analyzed by linear regression, fat challenge data were analyzed in a mixed linear model. Statistical analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago, Illinois). Probability values of <0.05 were considered statistically significant.

Acknowledgements

We are indebted to the study participants and thank Jonathan Seidman, Claartje Koch, Alinda Schimmel, Joanna Coelho Amado de Azevedo, Jorge Peter, Jaap Legemate, Anne van der Made, and Bas van den Bogaard for their assistance to facilitate this family study. We thank the Dutch blood bank Sanquin for providing control DNA samples; Max Nieuwdorp for his help designing the desialylation experiments and Xenon Genetics for their help financing the collection of DNA samples. This study was supported by the European Union (FP6-2005-LIFESCIHEALTH-6; STREP contract number 037631), the Le Ducq Foundation and in part by the Intramural Research Program of NIDCR, NIH, USA. A.G. Holleboom is supported by the Netherlands Organisation for Scientific Research (project number 021.001.035). Dr. Kastelein is a recipient of the Lifetime Achievement Award (2010) of The Dutch Heart Foundation (2010 T082).
**Supplementary Table 1.** Evolutionary conservation of an aspartic acid residue at position 314 of ppGalNAc-T2

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**Supplementary Table 2.** Characterization of apo C-III isoforms in HDL of a carrier of ppGalNAc-T2-D314 with peptide mass fingerprinting using 2-DE and MALDI-TOF mass spectrometry. The apo C-III isoforms (accession no. P02656) correspond to the di-sialylated (C-III2), mono-sialylated (C-III1) and non-sialylated (C-III0, 8.8 and 8.9 kDa) variant as shown in figure 1. (Bruneel et al., 2007) Bold T indicates residue threonine 74 which carries the O-glycan chain.

<table>
<thead>
<tr>
<th>Apo C-III isoforms</th>
<th>pI/Mass (Da)</th>
<th>Peptide Masses (Da)</th>
<th>Amino acid positions and corresponding sequences</th>
<th>Sequence coverage (%)</th>
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<td>52-58, DYWSTVK</td>
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<td></td>
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<td></td>
<td>2380.16</td>
<td>59-79, DKFSEFWDLPEVRPTSSAVAA</td>
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</tr>
</tbody>
</table>
Supplementary methods

**GALNT2 qPCR and ppGalNAc-T2 western blot of fibroblast lysates**

mRNA was isolated from cultured fibroblasts (obtained by skin biopsies from the forearm) using Trizol (Invitrogen) and cDNA was prepared using Superscript II reverse transcriptase (Invitrogen). QPCR was performed on a MyiQ (Biorad) using primers for GALNT2 (sequences available upon request) and for 36B4 as control. ppGalNAc-T2 protein was assessed in fibroblast lysates by standard western blotting techniques using anti-human ppGalNAc-T2 (Sigma no. HPA-011222) as primary antibody. Peptides were visualized using Licor Odyssey InfraRed detection system.

**Two dimensional gel electrophoresis and Western blotting**

Aliquots (5ul) of EDTA plasma from carriers (n=7) and controls (n=7) were used for 2-DE/Western blot analysis. Prior to 2-DE the protein concentrations were determined (Bradford, 1976) and the samples were dissolved in 0.25 ml sample solution according to Görg. (Gorg et al., 2000) 2-DE was performed using IPGphor and Multiphor (Amersham Biosciences) as described previously (Karlsson et al., 2005). Shortly, proteins (300ug) for identification were separated on 3-10 IPGs and prior to Western blot samples containing 200ug of plasma protein were applied to pH 4-7 IPGs by in-gel rehydration for 12 h using low voltage (30 V). The proteins were then focused at 53 000 Vh at maximum voltage of 8000 V. The second dimension (SDS-PAGE) was performed by transferring the proteins to a homogenous (T=14%, C=1.5%) home-cast gel on gel bond running at 40–800 V, at 10 °C, 20–40 mA overnight. Proteins for identification were detected by silver staining (Shevchenko and Shevchenko, 2001) and images were evaluated by spot detection and intensities using a CCD camera and a Fluor-S Multi-imager in combination with a computerised imaging 12-bit system, PDQuest V8.0.1. (Bio-Rad).

In Western blots, proteins were transferred to a PVDF membrane in a trans-blot cell system (Bio-Rad) running for 2 h, 60V, 400 mA, in transfer buffer at pH 8.3. The membranes were blocked for 1 h in blocking buffer (5% non-fat dry milk/TBS pH 7.5), then washed in TTBS pH 7.5 and incubated in 2% non-fat milk in TTBS with primary antibodies overnight. Primary antibodies used were rabbit polyclonal anti–human apo C-III (Abcam, No. 21032; 1:5000), rabbit polyclonal anti-human apo A-II (Abcam, No. 24241; 1:5000) or goat polyclonal anti-human apoE (GenWay, No. 18272197662; 1:5000). After another wash in TTBS the membrane was incubated in 2% non-fat milk in TTBS with HRP conjugated secondary antibodies against goat, rabbit and mouse IgG ( Bio Rad;1:40 000), respectively, for one hour. Proteins were visualized using the ECL plus western blotting detection system, exposed to X-ray film and developed. Isoform intensities were determined as optical density*mm2 (ODU) and expressed as % of the total protein level for each protein.
Mass spectrometry
Proteins from silver stained gels prepared as described above were excised from the gel with a syringe and transferred to small Eppendorf tubes (0.5 mL). The gel pieces were washed twice with 50% acetonitrile/25 mM ammonium bicarbonate, with 100% acetonitrile once and dried in a SpeedVac vacuum concentration system (Savant, Farmingdale, NY, USA). About 0.25 mL of trypsin, (20 mg/mL in 25 mM ammonium bicarbonate) was added to the gel piece and the sample was incubated overnight at 37°C. The supernatant was transferred to a separate tube and the peptides were further extracted from the gel piece by incubation in 50% acetonitrile/5% TFA for 5 h in room temperature. The supernatants from the two steps were then pooled, dried in SpeedVac and dissolved in 5 ul 0.1% TFA. Peptides obtained after tryptic digestion were mixed 1:1 with a matrix (DHB, 0.02 mg/mL) in 70% acetonitrile/0.3% TFA, and then spotted on a stainless steel target plate. Analyses of peptide masses were performed using MALDI-TOF MS (Voyager DE PRO; Applied Biosystems) equipped with a 337 nm N2 laser operated in reflector mode with delayed extraction. Instrument settings optimized for DHB as described previously(Ghafouri et al., 2007) and positive ionization, delay time of 170 ns and accelerating voltage of 20 kV were used to collect spectra in the mass range 700–3600 Da. Data processing of spectra was performed in Data Explorer V4.0 (Applied Biosystems). External mass calibration with a standard peptide mixture and internal calibration using known trypsin autolysis peaks (m/z: 842.5100, 2211.1046) were also performed prior to the database search. Peptide masses (plus H+) with the greatest intensity (major peaks), in the spectra were submitted to database search. NCBI and Swiss-Prot were used with AIdente or MS-Fit as search engines. Restrictions were human species, mass tolerance 50 ppm, maximum one missed cleavage by trypsin and cysteine modification by carbamido-methylation.

FPLC and immunoblot for apo C-III in lipid fractions
Plasma lipids were fractionated by fast performance liquid chromatography.(Levels et al., 2003) Apo C-III in the fractions was quantified by spot blot with the anti-human apo C-III described under ‘Two dimensional gel electrophoresis and Western blotting’.

Desialylation of apo C-III
63 ug of apo C-III isolated from human VLDL (Academy Bio-Medical Company) was incubated with 60 mU neuraminidase (NorthStar) for 3 hours at 37°C in 50 mM sodium phosphate pH 5.0. As a control reaction the same amount of apo C-III was incubated without neuraminidase. 15 ug Apo C-III was incubated with human recombinant LPL for 2 hrs on ice prior to LPL activity measurement. LPL activity was measured as previously described.(Nilsson-Ehle and Schotz, 1976)

Generation of secretion constructs
DNA constructs of wild-type human (h) T1, wild-type hT2, and mutant hT2 were generated using pKN55 (containing stem, catalytic, and lectin domains of human T1(Fritz et al., 2004) and T2(Fritz et al., 2006)). Subcloning into pIMKF4 was performed
using MluI and AgeI. The mutation changing the aspartic acid to an alanine at position 314 was created to replicate the naturally occurring mutation in hT2 using the Quikchange II XL Site Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol using pIMKF4-hT2 as a template and using the following primers: 5′- GTGGGCTGTTTGTGATGGCTAAGTTCTATTTTGAAGAAC-3′ and 5′- GTTCTTCAAATAGAACTTAGCCATCACAAACAGCCCAC -3′.

**ANGPTL3 Western blotting**

Full length and processed ANGPTL3 were assessed in plasma of carriers and non-carriers by standard western blotting techniques using anti-human ANGPTL3 (R&D no. AF3829) as primary antibody. Peptides were visualized using Licor Odyssey InfraRed detection system (Westburg).
Reference List


Ref Type: Online Source
Ref Type: Online Source


Ref Type: Online Source

Ref Type: Online Source


Chapter 5


Pollin, T.I., Damcott, C.M., Shen, H., Ott, S.H., Shelton, J., Horenstein, R.B., Post, W., McLenithan, J.C.,


Willer, C.J., Sanna, S., Jackson, A.U., Scuteri, A., Bonnycastle, L.L., Clarke, R., Heath, S.C., Timpson, N.J.,
Chapter 5


Supplementary data

Supplementary Figure 1. Pedigrees of two families with carriers of the GALNT2<sup>D314A</sup> mutation. Symbols: clear is non-carrier; half-filled is heterozygous for the D314A mutation; strike-through is deceased. Arrow and red square indicate probands of the families. Values for HDL-c and triglycerides (TG) are given in mg/dl.

First family:

Second family:

One non-carrier is not indicated in pedigree. This is a son of the deceased grandnephew of the proband. His HDL-c is 64 mg/dl and triglycerides were 55 mg/dl.

Supplementary figure 2. 2-DE analysis of apo A-II and apo E isoforms in a carrier of the D314A mutation compared to a non-carrier. No detectable variation in isoform distribution of apo A-II (Expasy 2DE page ApoA-II, 2011) and apo E (Expasy 2DE page ApoE, 2010) was found after Western Blotting.
Supplementary Figure 3. GALNT2 mRNA and ppGalNAc-T2 protein levels in cultured fibroblasts of 3 D314A carriers and one control subject. (a) GALNT2 expression in cultured fibroblasts. GALNT2 mRNA levels as assessed by qPCR in fibroblast lysates of three GALNT2 D314A carriers are comparable to those of an unrelated control. GALNT2 expression normalized for 36B4. (b) Western blot of ppGalNAc-T2 protein in lysates of cultured fibroblasts. Protein levels are similar in the carriers and the control.

Supplementary figure 4. O-linked glycosylation of EA2 and an 11-mer apo C-III peptide with human ppGalNAc-T1 and ppGalNAc-T2. Vertical axis indicates rate of transfer as dpm/h/densitometric unit of enzyme. The different GalNAc transferases were overexpressed in COS7 cells. Panel (a) shows that the EA2 peptide, a standard ppGalNAc-T substrate, is a substrate for ppGalNAc-T1 (T1, grey bar) and for ppGalNAc-T2 (T2, white bar). (b) Remarkably, ppGalNAc-T1 exhibited no catalytic activity towards the apo C-III compared to wild-type ppGalNAc-T2. Reactions lacking peptide yielded background values that were averaged for each isoform and subtracted from each value obtained using either substrate. Adjusted values were averaged to give values shown. Error bars indicate standard deviations. The data are obtained through 3 independent experiments that were carried out in triplicate.
Supplementary Figure 5. LPL concentration levels in plasma during an oral fat challenge. In both carriers (n=4, open symbols) and non-carriers (n=4, closed symbols), the oral fat challenge did not change plasma LPL levels. LPL levels did not differ significantly between carriers and controls at any time point. Data are depicted as means (SD). Plasma LPL levels were measured in plasma using a commercially available ELISA (Dainippon).

Supplementary figure 6. Distribution of apolipoprotein C-III over lipid fractions during oral fat challenge. Prior to ingestion of the cream (top panel), apo C-III was similarly distributed over the lipoprotein fractions in 4 carriers of the D314A-mutation (closed symbols) and the 4 non-carriers (open symbols). 4 hours after cream ingestion, however, the % of total apolipoprotein C-III in the HDL fraction was significantly increased in carriers compared to controls (bottom panel), average area under curve HDL fraction for carriers 23.4 (SD 6.0), for controls 8.2 (SD 2.6), P for MW-U = 0.029). Symbols represent means, whiskers represent SD.
Supplementary Figure 7. Plasma concentrations of (unprocessed) full length ANGPTL3 and the processed N-terminal fragment in carriers and non-carriers. Western blotting of plasma proteins indicates that ANGPTL3 is similarly processed in carriers and non-carriers. The left panel shows full length ANGPTL3 (upper bands) and the processed N-terminal fragment of ANGPTL3 (lower bands) in a carrier and a non-carrier. Quantification in 7 carriers and 11 non-carriers revealed no differences between these groups. Whiskers: SD.
Chapter 6

Atherosclerosis 2010, 213(2)

Identification and characterization of novel loss of function mutations in ATP-binding cassette transporter A1 in patients with low plasma high density lipoprotein-cholesterol

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1- Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy.
2- Department of Experimental Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands.
3- Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands.
4- Department of Endocrinology, University Medical Center Groningen, Groningen, University of Groningen, Groningen, The Netherlands.
5- Department of Cardiac, Thoracic and Vascular Surgery Pantai Medical Centre, Kuala Lumpur, Malaysia.
Abstract

Objectives. The current literature provides little information on the frequency of mutations in the ATP binding cassette transporter A1 (ABCA1) gene in patients with low high-density lipoprotein cholesterol (HDL) levels that are referred to the clinic. In 78 patients with low plasma levels of HDL cholesterol that were referred to our clinic, we routinely screened for ABCA1 gene mutations and studied the functionality of newly identified ABCA1 missense mutations.

Methods. The coding regions and exon-intron boundaries of the ABCA1 gene were sequenced in 78 subjects with HDL cholesterol levels below the 10th percentile for age and gender. Novel mutations were studied by assessing cholesterol efflux capacity (using apolipoprotein A-I as acceptor) after transient expression of ABCA1 variants in BHK cells.

Results. Sixteen out of 78 patients (21%) were found to carry 19 different ABCA1 gene variants (1 frameshift, 2 splice-site, 4 nonsense and 12 missense variation) of which 14 variations were novel. Of three patients with homozygous mutations and three patients having compound heterozygous mutations only one patient presented with the clinical characteristics of Tangier Disease (TD) in the presence of nearly complete HDL deficiency. Seven out of 8 newly identified ABCA1 missense mutations were found to exhibit a statistically significant loss of cholesterol efflux capacity.

Conclusion. This study shows that 1 out of 5 patients who are referred to our hospital because of low HDL cholesterol levels have a functional ABCA1 gene mutation. It is furthermore demonstrated that in vitro studies are needed to assess functionality of ABCA1 missense mutations.
Introduction

twin studies have indicated that the variation in plasma high-density lipoprotein (HDL) cholesterol levels is largely determined by genetic factors [1]. Many genes have been implicated in HDL metabolism [2] and this number is still expanding [3,4]. One of the major HDL candidate genes is the ATP-binding cassette transporter A1 (ABCA1) which is a cell membrane double transporter protein that plays an important role in cholesterol homeostasis. It is generally accepted that ABCA1 controls the rate-limiting step in the transport of cellular free cholesterol and phospholipids to apolipoprotein (apo) A-I which leads to the formation of pre-β high-density lipoprotein (pre-β HDL). Through the action of lecithin:cholesterol acyltransferase (LCAT), this pre-β HDL can mature into larger HDL subspecies [5-7]. Defects in the ABCA1 gene cause Tangier Disease (TD) [8], an autosomal recessive disorder characterized by HDL deficiency and accumulation of cholesterol in peripheral tissues. TD patients suffer from many symptoms including peripheral neuropathy, hepatosplenomegaly, and corneal opacification. While carriers of mutations in the ABCA1 gene are reported to exhibit an increased risk of atherosclerosis [9], it has also been reported that not all TD patients suffer from overt atherosclerosis [10]. Recent epidemiological data suggest that loss of ABCA1 function is not necessarily associated with increased risk of ischemic heart disease or cerebrovascular disease [11,12]. Heterozygous carriers for detrimental ABCA1 mutations do not present with specific clinical symptoms but present with markedly lower HDL cholesterol levels compared to age- and gender-matched controls [13]. ABCA1 is mainly expressed in the small intestine, liver, brain and cells of reticuloendothelial system. The 220 kDa protein is synthesized in the endoplasmic reticulum and transported to the plasma membrane via vesicles, but it is also found in intracellular compartments such as late endosomes/lysosomes, the trans-Golgi network and endoplasmic reticulum [6,14]. More than 50% of the over 90 identified ABCA1 mutations in the current literature [15] are missense mutations. Most of these mutations appear to be localized in extracellular loops, nuclear binding domains and carboxy terminal region [12,16,17]. Functionally defective ABCA1 variants fail to mediate lipid efflux to apo A-I and as a consequence the non-lipidated apo A-I fails to undergo maturation into larger HDL subspecies and will undergo rapid renal clearance [18]. Previous studies have demonstrated that missense mutations in ABCA1, identified in patients with Tangier disease or individuals with Familial hypoalphalipoproteinemia, can cause different degrees of impairment in lipid transfer activity [13]. Thus far, only few investigators have routinely sequenced the ABCA1 gene. There is only one report on patients with isolated low HDL cholesterol that are referred to the clinic [19]. Two other groups have reported ABCA1 gene variation at the lower end of the HDL cholesterol distribution curve of prospective epidemiological studies [11,17].
Chapter 6

the current study, we sequenced the ABCA1 gene in 78 patients that were referred to our clinic who presented with HDL cholesterol levels below the 10th percentile for age and gender. We identified an unexpected high number of ABCA gene variants (n=19), of which 14 had not been described earlier. In vitro as well as confocal imaging experiments were carried out to evaluate whether the newly identified mutations were functional and could therefore explain the low HDL cholesterol phenotype of the respective patients.

Materials and methods

Study population
The current study is part of a research effort aiming at the characterization of mutations in established and newly proposed HDL genes, and the identification of novel genes that regulate HDL cholesterol levels. In a first step, we have selected 78 individuals with extremely low HDL cholesterol (<10th percentile for age and gender). In a second step, we have sequenced the coding regions of established HDL genes, i.e. ATP binding cassette transporter Al (ABCA1), apolipoproteins A-I (apo A-I), and lecithin:cholesterol acyltransferase (LCAT). Patients were either seen in our outpatient clinic or they were referred to our hospital. With the exception of 5 patients from south-east Asia, 1 patient from Belgium and one patient from Spain, all other patients were of Dutch ancestry. Of note, patients #2 and #14 (see table 1) were referred with a suspicion of Tangier Disease. In this screening effort, we identified one mutation in APOAI in two subjects, 13 mutations in LCAT in 20 subjects and 19 mutations in ABCA1 in 16 individuals.

Biochemical measurements
Blood was obtained after an overnight fast in EDTA-coated tubes and directly placed on ice. Plasma was isolated by centrifugation at 4°C, 3000g for 15 minutes and stored at -80°C for further analyses. Plasma cholesterol, LDL cholesterol, HDL cholesterol and triglyceride levels were analyzed using a commercially available enzymatic method (Randox, Westburg, USA) on the Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

Mutation screening in ABCA1
Genomic DNA was extracted from 10 ml whole blood on an AutopureLS apparatus according to manufacturer’s protocol (Gentra Systems, Minneapolis, USA). Primers were designed to amplify coding sequence and exon-intron boundaries of the ABCA1 gene using web-based Primer3 software [20]. PCR amplification was carried out with 50ng of genomic DNA in a 25μl reaction volume containing 1x Taq DNA polymerase buffer (Qiagen, Hilden, Germany), 50μmol/l of each dNTP, 0.4μmol/l of each primer, and 1U Taq DNA polymerase. A Touchdown PCR program (96°C for 5 minutes, then 20 cycles of 30 seconds at 96°C, 30 seconds at 65°C to 55°C with 0.5°C decrement/cycle and 30 seconds at 72°C, followed by 30 cycles of 30 seconds at 96°C, 30 seconds at 55°C and 30 seconds at 72°C) on a T3 biocycler PCR apparatus (Biometra, Germany) was used for
DNA amplification. The sequence reactions were performed using fluorescently labelled
dideoxy chain terminations with a BigDye terminator ABI prism kit (Applied Biosystems,
Foster City, CA, USA) according to manufacturer’s protocol and analyzed on an Applied
Biosystems automated DNA sequencer (model 370). Sequences were analyzed with the
Sequencher Package (Gene Codes Co, Ann Arbor, Mi, USA).

Generation of ABCA1 gene expression vectors
Wild-type ABCA1-GFP pcDNA3.1 vector was provided by Prof. S. Calandra (University
of Modena, Italy). This vector carries the human cDNA of ABCA1 fused in frame with
a Green Fluorescence Protein (GFP) cDNA. 8 novel missense variations [c.299C>G(p.
S100C), c.1724A>G (p.D575G), c.1779C>G (p.F593L), c.3167T>C (p.L1056P), c.3757G>A
(p.E1253K), c.4535C>T (p.T1512M), c.5573T>C (p.V1858A), c.5821T>C (p.C1941R)] were
introduced into this chimeric construct by site-directed mutagenesis using Stratagene
QuikChange XL site-directed mutagenesis kit according to manufacturer’s instructions
(La Jolla, CA, USA).

Functional assessment of ABCA1 gene mutations
Baby hamster kidney (BHK) cells were obtained from ATCC (Manassas, VA). Cells were
cultured in DMEM F-12 GlutaMax (GIBCO) containing 10% fetal bovine serum (FBS) and
Penicillin (100U/ml)-Streptomycin (100ug/ml) at 37°C in a humidified 5% CO2 incubator.
Transient transfections were carried out when the cells were at 90% confluency using
Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s
instructions. Two days after transfection, transfection efficiency was evaluated by FACS
analysis. The cells were harvested, centrifuged, extensively washed with phosphate
buffered saline, resuspended in the same buffer and analyzed in a FacsCalibur cell sorter
(BD Biosciences, Bedford, MA) using CellQuestPro software. Non-transfected cells were
used as negative control. To assess cholesterol efflux potential, the transfected cells were
incubated with 2μCi/ml [3H]cholesterol for 24 hours. Cholesterol efflux was measured
after 4 h incubation with or without apoA-I (20μg/ml; Calbiochem). Radioactivity in the
medium and cells was determined by scintillation counting and the fractional cholesterol
efflux was calculated as the percentage of cpmmedium/(cpmmedium+cpmcell). For each
construct, efflux to apo A-I was measured in triplicate in 3 independent experiments.

Confocal microscopy
BHK cells were plated on coverslips in 12-well plates at 30% confluency and transiently
transfected with wild-type and mutant vectors after 24 hours. Cells were fixed with
Methanol-Acetone 48 hours after transfection and mounted on slide using VECTASHIELD®
Mounting Medium (VECTOR laboratories). Images were prepared using a Leica TCS-SP2
Confocal Microscope at 40x magnification and 488 nm wavelength.
Statistical analysis

Statistical analyses were performed in SPSS version 16. Efflux data were analyzed using unpaired Student’s T-test statistics. Efflux data are presented as mean ± SD and p-values <0.05 were considered statistically significant.

Results

ABCA1 gene defects in low HDL-c individuals

In 78 patients with HDL cholesterol levels below the 10th percentile for age and gender, who were referred to our clinic, the coding sequence and exon-intron boundaries of the APOA1, LCAT and ABCA1 genes were sequenced. We identified 2 carriers of the APOA1p.L202P mutation, and 20 carriers of 13 different mutations in LCAT (data not shown). In ABCA1, we identified 14 novel and 5 known genetic variations in 16 subjects including one frameshift (p.C978fsX988), 2 splice site (IVS11-1G>C and IVS48+2T>C), 4 nonsense (p.R282X, p.W424X, p.Q1038X, p.W1747X) and 12 missense variations (p.S100C, p.D575G, p.F593L, p.L1056P, p.E1172D, p.S1181F, p.E1253K, p.C1477R, p.T1512M, p.N1800H, p.V1858A, p.Y1941R). None of the 78 patients carried mutations in more than one of the 3 genes that were sequenced. The identified ABCA1 variations as well as the lipid profile and demographic data of the carriers are listed in table 1. Seven ABCA1 variants were

Table 1: Identification of the ABCA1 mutation and baseline characteristics of the carriers (cDNA NM_005502).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Amino acid (‘Nucleotide’*)</th>
<th>TC (mmol/l)</th>
<th>TG (mmol/l)</th>
<th>LDL-c (mmol/l)</th>
<th>HDL-c (mmol/l)</th>
<th>Clinical manifestations of CVD</th>
<th>Other relevant clinical data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1 (female 42)</td>
<td>p.L506P (c.1518T&gt;C)</td>
<td>2.4</td>
<td>0.6</td>
<td>1.99</td>
<td>-0.13</td>
<td>Absent</td>
<td>OAD</td>
</tr>
<tr>
<td>Patient 2 (male 40)</td>
<td>p.W424X (c.1272C&gt;A)</td>
<td>1.75</td>
<td>1.29</td>
<td>0.52</td>
<td>0.1 (0.3)</td>
<td>Neuropathy, Splenomegaly, Thrombocytopenia</td>
<td>Moderate (20-30)% of coronary artery disease</td>
</tr>
<tr>
<td>Patient 3 (male 55)</td>
<td>p.S100C (c.300C&gt;T)</td>
<td>2.4</td>
<td>1.4</td>
<td>2.6</td>
<td>-0.15</td>
<td>Absent</td>
<td>OAD</td>
</tr>
<tr>
<td>Patient 4 (male 53)</td>
<td>p.L1056P (c.3168C&gt;T)</td>
<td>2.4</td>
<td>1.4</td>
<td>2.6</td>
<td>-0.15</td>
<td>Absent</td>
<td>OAD</td>
</tr>
</tbody>
</table>

* Nomenclature based on guidelines of Human Genome Variation Society. Plasma Lipid values are presented as mmol/l. TC: Total cholesterol, TG: Triglyceride, CVD: Cardiovascular disease, TD: Tangier Disease, N.A.: Not Applicable, DM1: Diabetes Mellitus type 1, DM2: Diabetes Mellitus type 2
ABCA1 mutations in patients with low HDL-cholesterol

considered to be functional due to obvious destructive effects of frameshift, splice site and nonsense variations on protein structure and/or function. In addition, 5 missense variations have previously been reported to be functional (references are given in Table 1) and were not analyzed in the current study.

From 8 novel missense variations identified in our cohort, one is localized in the first transmembrane domain (p.S100C), two in the first large extracellular loop (p.D575G and p.F593L), two in the first Nuclear Binding Domain (p.L1056P and p.E1253K), one in the second large extracellular loop (p.T1512M), one in the extracellular region, close to the plasma membrane (p.V1858A) and one is localized in the C-terminal domain (p.C1941R). The estimated positions of these variants are given in Figure 1.

**In silico prediction of the effect of ABCA1 variations**

*In silico* analysis was performed using PolyPhen (=Polyorphism Phenotyping) [21] and SIFT [22] software to predict functional significance of the 8 novel missense variations (see supplementary table 1). Four out of 8 mutations were predicted to be probably damaging (p.S100C, p.D575G, p.T1512M, p.C1941R), two as possibly damaging (p.F593L and p.L1056P) and two were described as benign (p.E1253K and p.V1858A) by PolyPhen. Using SIFT, 7 novel variations were predicted to affect protein function, while one (p.V1858A) was predicted to be tolerated.

**Figure 1:** Position of the newly identified ABCA1 variations in ABCA1 protein structure.
Chapter 6

In vitro characterization of novel missense ABCA1 variations in BHK cell system

All newly identified missense variations were studied in vitro by assessing the ABCA1-mediated cholesterol efflux to apo A-I in BHK cells that were transiently transfected with wild-type or mutant ABCA1 constructs. FACS analysis of transfected cells (using GFP-tagged ABCA1 protein) indicated that all ABCA1 variants were expressed at comparable levels to that of the wild-type protein (data not shown). Efflux data of three independent triplicate experiments were normalized to the values from the cells transfected with the wild-type ABCA1 construct. Figure 2 shows that the ABCA1- p.S100C, p.D575G, p.F593L, p.L1056P, p.E1253K, p.T1512M, p.C1941R mutant proteins all had a significantly reduced capacity to efflux cholesterol to apo A-I compared to wild-type ABCA1 which is in line with the low HDL cholesterol levels of the individuals in whom the mutations were identified. The ABCA1- p.V1858A, however, had a normal potential to efflux cholesterol to apo A-I which is in line with the prediction that the mutation is benign (both PolyPhen and SIFT) suggesting that the low HDL cholesterol in this individual is likely the result of other, yet unknown molecular defect.

Figure 2: Normalized cholesterol efflux to ApoA-I in BHK cells transfected with ABCA1 variants relative to cells transfected with wild-type ABCA1 (WT).

Cellular localization of mutant ABCA1 proteins

GFP-tagged wild-type and novel mutant proteins were expressed in BHK cells and their localization was evaluated using confocal microscopy. While WT-ABCA1 and ABCA1-V1858A variant show normal localization on plasma membrane, ABCA1-L1056 protein shows a complete intracellular retention. All other mutant ABCA1 proteins show partial intracellular retention (Supplementary figure 1).

Discussion

our group has a long-standing interest in the characterization of the genetic background of low HDL cholesterol levels of patients that are referred to our hospital. In the current
paper, we focus on the identification and function of newly identified ABCA1 gene mutations.

**Frequency of ABCA1 gene mutations**

Routine screening for ABCA1 mutations in 78 patients with HDL cholesterol levels below the 10th percentile revealed 5 known and 14 novel gene variations. An approximate 21% mutation rate suggests a high prevalence of ABCA1 mutations in patients with low HDL-c that are referred to our institute. A similar sequencing effort in patients that were referred to the hospital was carried out by Kiss et al [19] who identified a much lower prevalence of ABCA1 mutations of 6%. We have no clear explanation for the discrepancy other than that Kiss et al. have used less strict inclusion criteria (including diabetes, short bowel syndrome, nephrotic syndrome, malignancies including multiple myeloma, or treatment with stanozolol or danazol). Instead, we have solely selected patients on the basis of low HDL cholesterol levels. We can moreover not exclude a referral bias for inherited HDL traits, since our group has a long history in the characterization of genetic disorders of HDL metabolism. In fact, 2 of 78 patients were referred due to TD symptoms. The ABCA1 gene has also been sequenced in individuals at the lower end of the HDL cholesterol distribution curve of prospective epidemiological studies [11,17]. The observed frequencies were also lower, i.e. 10.9 % in the Dallas Heart Study (HDL cholesterol < 5th percentile; HDL cholesterol <0.76 mmol/l for men and <0.81 mmol/l for women) and 10% in the Copenhagen City Heart Study (bottom 1st percentile; HDL cholesterol <0.6 mmol/l for men and <0.8 mmol/l for women). Several investigators have identified ABCA1 promoter polymorphisms that are associated with HDL cholesterol levels [11,23] although only one study shows functionality of a mutation in the promoter region [11]. In the present study, we did not sequence the regulatory sequences of the ABCA1 gene, indicating that we may underestimate the frequency of ABCA1 mutations in our cohort.

**Impact of novel missense mutations on ABCA1 function**

The newly identified missense mutations were further studied in *in silico* and *in vitro* experiments which allowed for head-to-head comparisons between the outcomes. Nowadays, *in silico* prediction of the functionality of mutations has become very popular [17] mainly due to its simplicity and ease of use in the context of the magnitude of genetic data that are generated by second generation sequencing technology. It has previously been shown that these methods have their limitations [24]. In line, the current study shows that predictions made by PolyPhen were consistent with *in vitro* characterization studies in only 50% of the cases (Supplementary table 1). The most striking discrepancy was found for the new ABCA1-p.L1056P variant which was only defined as possibly damaging while our data show that this variant is amongst those with the most profound loss of ABCA1 mediated efflux and confocal microscopy revealed complete intracellular retention (Supplementary figure 1). In line, cholesterol efflux of fibroblasts grown from
skin biopsies of the index patient who was homozygous for this mutation (a classical test for diagnosis of TD) indicated complete abrogation of cholesterol efflux to apo A-I as an acceptor (Supplementary figure 2).

In contrast to PolyPhen, SIFT predictions for the assessment of the novel variations studied here were more compatible to the outcome of our in vitro experiments, i.e. all variants with significant reduction in cholesterol efflux to apo A-I in our in vitro assay were proposed to be deleterious while ABCA1-p.V1858A was predicted to be tolerated.

Combined, the current data indicate the importance of assessing the effect of missense mutations through functional studies and not to solely rely on in silico predictions. Many investigators have shown that ABCA1 mutations can have an impact on the 3-dimensional folding of the protein. The position of the mutation can affect maturation, localization or interaction of ABCA1 with early/late endosomes, lysosomes, phospholipids of the plasma membrane. More importantly it may interfere with its capacity to interact with apo A-I [25-27] and thus lead to a decrease in the synthesis of pre-β HDL. The missense mutations ABCA1-p.L1056P and ABCA1-p.E1253K, identified in patients with near HDL deficiency, are located in the intracellular region, inside the nuclear binding domain 1, close to the Walker A motif and after the Walker B motif respectively (Figure 1). This region is known to be involved in the ATP hydrolysis function of ABCA1 [28,29]. Mutations located in this region may have an impact on the ability of ABCA1 to hydrolyze ATP which is necessary to transport its substrates [30]. Thus, lack of proper localization to the plasma membrane for ABCA1-p.L1056P and partial intracellular retention for ABCA1-p.E1253K (Supplementary figure 1) results in low cholesterol efflux potential of both ABCA1 mutants and confirms the vital role of this ABCA1 domain (Figure 2).

Also, mutations in extracellular loops have been shown to affect the protein’s folding, localization, lipid transport ability [29,31] or apo A-I binding [6]. The ABCA1- p.F593L and ABCA1-p.D575G mutations are located in the first large extracellular loop, while ABCA1-p.T1512M is located in the second extracellular loop. All 3 mutant proteins are indeed hampered in their ability to efflux cholesterol to apo A-I (Figure 2). In line, the mutant proteins are retained into the intracellular compartments and have a limited expression at the cell membrane (Supplementary figure 1). All mutations were identified in patients with almost complete HDL deficiency.

The cholesterol efflux assay performed for ABCA1-p.S100C revealed a significant reduction in efflux potential to apo A-I. The mutation is located in the first transmembrane domain and may impair the protein’s ability to properly interact with the plasma membrane. The ABCA1-p.C1941R mutation showed a marked reduction in cholesterol efflux.

This mutation is localized in the C-terminal domain which may lead to defective localization or oligomerization [32], especially due to proximity to the PDZ binding domain. Confocal microscopy also revealed considerable intracellular retention of this mutant (Supplementary figure 1).
The ABCA1-p.V1858A variant was the only missense variation that was found to have no significant effect on cholesterol efflux and cellular localization. It is possible that the extracellular region, where the variant is located, is not fundamental for ABCA1 folding and function.

In conclusion, the current study shows that ABCA1 gene defects explain up to 20% of the low HDL cholesterol phenotype of patients that are referred to our clinic. In view of the scarcity of routine ABCA1 gene sequencing efforts in referred patients, further studies are needed to verify partial ABCA1 deficiency as a frequent cause of low HDL cholesterol levels. Through functional studies we furthermore showed that the majority of the newly identified missense mutations were functional.

Acknowledgements

This work was supported by the European Union (EU FP6-2005-LIFESCIHEALTH-6; STREP contract number 037631) and by the Netherlands Organisation for Scientific Research (NWO; project number 021.001.035). We thank R. Franssen (supported by NHS2008B070) for his help with the efflux experiments. We would like to thank Xenon Genetics Inc. (Burnaby, BC, Canada) for their help in obtaining part of the genomic DNA samples of the patients investigated.
Chapter 6

References


ABCA1 mutations in patients with low HDL-cholesterol


Chapter 6


Supplementary Figure 1: Localization of GFP-tagged ABCA1 WT and GFP-tagged ABCA1 mutant proteins in BHK cells. (for color figure, see page 417)

Supplementary table 1: Relative percentage of apo A-I-mediated cholesterol efflux using BHK cells transfected with ABCA1 variants compared to the wild-type protein.

<table>
<thead>
<tr>
<th>ABCA1 Variant</th>
<th>% efflux relative to WT (Mean±SD)</th>
<th>p-value</th>
<th>SIFT prediction</th>
<th>PolyPhen prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>10.4 ± 1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p.L1056P</td>
<td>36.4 ± 2.7</td>
<td>&lt;0.0001</td>
<td>Affects protein function</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>p.F593L</td>
<td>70.9 ± 5.6</td>
<td>0.0006</td>
<td>Affects protein function</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>p.E1253K</td>
<td>81.6 ± 6.3</td>
<td>0.0085</td>
<td>Affects protein function</td>
<td>Benign</td>
</tr>
<tr>
<td>p.T1512M</td>
<td>87.8 ± 4.8</td>
<td>0.0244</td>
<td>Affects protein function</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>p.D575G</td>
<td>60.9 ± 9.3</td>
<td>0.0022</td>
<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.C1941R</td>
<td>49.8 ± 6.4</td>
<td>0.0002</td>
<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.S100C</td>
<td>75.6 ± 3.4</td>
<td>0.0004</td>
<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.V1858A</td>
<td>89.1 ± 5.2</td>
<td>0.0831</td>
<td>Tolerated</td>
<td>Benign</td>
</tr>
</tbody>
</table>

NT: Non-transfected cells. Analysis is performed using unpaired Student’s T-test statistics.
Absence of cholesterol efflux to apo A-I could clearly be seen in monocytes isolated from a patient with homozygote p.L1056P mutation (L1056P-/-). Monocytes from a parent (L1056P+/-) showed reduced efflux to apoA-I (<50% of efflux values in control monocytes). **Experimental setup:** 30 ml of heparinised blood was collected and diluted 1:1 with PBS/0.1% bovine serum albumin (BSA) + heparin (LeoPharma). To isolate the mononuclear cells, this mixture was layered on Lymphoprep 1.077g/ml (Lucron Bioproducts N1114544) and centrifuged for 15 minutes at 1000g. The interface was collected and washed twice. Thrombocytes were subsequently depleted by centrifugation for 10 minutes at 250g without brake. In a next step, the mononuclear cells were layered on a Percoll (Pharmacia 17-0891-01) density gradient and centrifuged for 45 minutes at 1750g. The monocytes were harvested from the upper interface and washed twice. 0.4 x 10⁶ cells were seeded in 1 ml of RPMI 1640/glutamax/hepes (Gibco 72400) with 10% human serum, in a 24-wells cell culture plate. Cells were cultured for 10 days. Cells were then loaded overnight at 37°C with free [3H]cholesterol, by adding 0.5ml of labeling medium (RPMI, Glutamax, 0.1% Pen/Strep, 0.2% BSA, 30μg/ml cholesterol, 0.5μCi/ml [3H]cholesterol in ethanol) in the presence of 3mM LXR agonist TO-901317 (#71810, Cayman Chemical Company). Labeling medium was removed and the cells were washed four times, after which 0.5ml of efflux medium, consisting of RPMI, Glutamax, 0.1% P/S, 0.2% BSA and 10μg/ml human apoA-I (Calbiochem) or native human HDL 12μg/ml (Calbiochem) was added to each well. Efflux medium without apoA-I or HDL was used as control. Efflux was measured over a 4-hour time span.
Part 2

LCAT and its clinical consequences
Image on previous page: Immunoelectrophoretogram of plasma from the first patient in whom familial LCAT deficiency is described, by K.R. Norum and E. Gjone in the Scandinavian Journal of Clinical and Laboratory Investigation, 1967. HDL (α1-lipoprotein) is present in the immunoelectrophoretogram of a normal control (lower part) but absent in the patient (upper part). Figure used with permission. See Chapter 9 for a description of the pathology and treatment of the youngest patients with this disorder to date.
Chapter 7

Submitted for publication

High prevalence of mutations in \textit{LCAT} in patients with low high-density lipoprotein cholesterol levels in The Netherlands

Identification and characterization of 8 new mutations in \textit{LCAT}

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Abstract

Objective. Lecithin: cholesterol acyltransferase (LCAT) is crucial to the maturation of high-density lipoprotein (HDL). Homozygosity for LCAT mutations underlies rare disorders characterized by HDL-cholesterol (HDL-c) deficiency while heterozygotes have half normal HDL-c levels. We studied the prevalence of LCAT mutations in referred patients with low HDL-c to better understand the molecular basis of low HDL-c in our patients.

Methods and Results. LCAT was sequenced in 98 patients referred for HDL-c <5th percentile and in 4 patients referred for low HDL-c and corneal opacities. LCAT mutations were highly prevalent: in 28 of the 98 participants (29%), heterozygosity for missense mutations was identified while 18 patients carried the same mutation (p.T147I). The 4 patients with corneal opacities were compound heterozygotes. All previously identified mutations are documented to cause loss of catalytic activity. Nine novel mutations – p.E134D/p.Y135N, p.R322C, p.W99S, p.V246F, p.R268C, p.W315X, p.L338F and p.R347C - were shown to be functional through in vitro characterization. The effect of several mutations on the core protein structure was studied by a 3D model.

Conclusion. Unlike previous reports, functional mutations in LCAT were found in 29% of patients with low HDL-c, thus constituting a common cause of low HDL-c in referred patients in The Netherlands.
LCAT mutations in patients with low HDL-cholesterol

Introduction

Twin studies have shown that high-density lipoprotein cholesterol (HDL-c) levels are to a large extent determined by genetic variation (heritability of 50%). (Goode et al., 2007) Deleterious mutations in three specific genes have thus far been shown to cause Mendelian forms of HDL deficiency. (Miller et al., 2003; Holleboom et al., 2008) These are APOA1, encoding apolipoprotein (apo) A-I, ABCA1 encoding ATP-binding cassette transporter, and LCAT, encoding lecithin:cholesterol acyltransferase. Homozygosity for deleterious mutations in APOA1 causing a loss of apo A-I, the major constitutive apolipoprotein of HDL, results in complete HDL deficiency. (Yokota et al., 2002; Hovingh et al., 2004a; Miller et al., 1998). Homozygosity for functional mutations in ABCA1 also cause HDL deficiency, severely decreased cellular cholesterol efflux and cholesteryl ester accumulation in macrophages. Affected patients often present with hepatosplenomegaly, peripheral neuropathy, enlarged yellow tonsils and fatty deposits in the rectal mucosa, collectively termed Tangier Disease. (Hovingh et al., 2004b) Also, patients suffering from a complete loss of LCAT have no HDL (Norum and Gjone, 1967).

LCAT is a plasma enzyme that is synthesized and secreted by the liver and small intestine and is a member of the lipase superfamily which includes lipoprotein lipase, endothelial lipase and hepatic lipase. In plasma, LCAT primarily associates with HDL (Jonas, 2000) where it esterifies free cholesterol molecules with acyl groups derived from phosphatidylcholine (lecithin). This leads to the maturation of HDL from a nascent discoidal particle, consisting of apolipoprotein (apo) A-I, phospholipids and free cholesterol, to mature spherical HDL with a core of cholesterol esters. (Glomset, 1968)

LCAT deficiency syndromes are considered rare metabolic disorders with an autosomal recessive mode of inheritance. (Santamarina-Fojo et al., 2001) Individuals with deleterious mutations on both alleles present with HDL deficiency while heterozygotes typically have half normal HDL-c levels, usually below the 5th percentile for age and gender. (Santamarina-Fojo et al., 2001; Hovingh et al., 2005) Homozygosity or compound heterozygosity for mutations in LCAT underlie 2 distinct clinical phenotypes, i.e. fish-eye disease (FED) and familial LCAT deficiency (FLD). FED is only characterized by progressive corneal opacification which eventually leads to loss of vision. FLD is a much more severe condition, in which apart from corneal opacification, mild anemia and progressive loss of renal function often occur. With no causal therapy available to date, many of these patients develop renal insufficiency in the fourth or fifth decade of life (Santamarina-Fojo et al., 2000). The difference in clinical presentation between FED and FLD is a result of the effect of mutations on LCAT activity towards different lipoprotein substrates in plasma. In FED, LCAT does not esterify cholesterol on HDL (alpha-activity) but remains active on low-density lipoprotein (LDL; beta-activity). FLD on the other hand is characterized by a complete loss of LCAT activity on both substrates which leads to the accumulation of free cholesterol.
cholesterol and phospholipids in various tissues such as the erythrocyte membrane and the glomeruli.(Santamarina-Fojo et al., 2001)

To date, it is unclear whether low HDL-c in patients with partial or complete LCAT deficiency leads to increased atherosclerosis.(Hovingh et al., 2005; Calabresi et al., 2009) Studying atherosclerosis in LCAT deficiency syndromes is obstructed by the low prevalence of these disorders. Currently, 78 causative mutations in LCAT have been described(Human Gene Mutation Database, 2010) while the prevalence of LCAT mutations in subjects with low HDL-c has been estimated at 2-9%. (Cohen et al., 2004; Recalde et al., 2002; Kiss et al., 2007; Miettinen et al., 1998). In the present study, we set out to determine the prevalence of LCAT mutations in referred patients with low HDL-c to better understand the molecular basis of low plasma HDL-c in our patients.

Materials and Methods

Study population

The current study is part of an ongoing effort that aims at the characterization of mutations in established and newly proposed HDL genes, and the identification of novel genes that affect HDL-c levels. To this purpose, we sequenced the coding regions and exon-intron boundaries of established HDL genes, i.e. ABCA1 (Candini et al., 2010), APOA1, and LCAT in patients with HDL-c <5th percentile for age and gender. Patients were either seen in our outpatient clinic or they were referred to our hospital. With the exception of 5 patients from south-east Asia, 1 patient from Belgium, 1 from Spain, 1 from Morocco and 1 from Turkey, all patients were of Dutch ancestry. Of note, 4 patients were referred under the specific clinical suspicion of FED, i.e. low HDL-c combined with corneal opacities (see table 2). The study protocol was approved by the institutional review board of the AMC, Amsterdam. All participants provided written informed consent.

Plasma measurements

Blood was obtained after an overnight fast in EDTA-coated tubes and directly placed on ice. Plasma was isolated by centrifugation at 3000g for 15 minutes at 4°C, and stored at -80°C for further analyses. Total cholesterol, LDL-c, HDL-c, triglycerides, apo A-I and apo B were measured using commercially available assays (Randox, Crumlin, UK; Wako San Diego, CA, USA) on a COBAS MIRA analyser. Plasma LCAT activity was measured with a proteoliposome assay as described earlier.(Frohlich et al., 1988)

Mutation screening in LCAT

Genomic DNA was extracted from 10 ml whole blood on an AutopureLS apparatus according to manufacturer’s protocol (Gentra Systems, Minneapolis, MN, USA). Primers were designed to amplify coding sequence and exon-intron boundaries of the LCAT using web-based Primer3 software.(Rozen and Skaletsky, 2000) PCR and sequencing reactions
were carried out as described. (Candini et al., 2010) Nomenclature of the mutations is based on guidelines of Human Genome Variation Society.

**Generation of LCAT expression vectors**

Wild-type LCAT-p.ENTR221 vector was obtained from Invitrogen (Carlsbad, CA, USA). The following novel LCAT mutations were introduced into this construct using the Stratagene QuikChange XL site-directed mutagenesis kit according to manufacturer’s instructions (La Jolla, CA, USA): c.296G>C (p.W99S), c.402G>T (p.E134D), c.403T>A (p.Y135N), c.402G>T-c.403T>A (p.E134D-Y135N), c.736G>T (p.V246F), c.802C>T (p.R268C), c.945G>A (p.W315X), c.964C>T (p.R322C), c.1012C>T (p.L338F) and c.1039C>T (p.R347C). To determine the effect of both mutations separately and combined, the double mutation c.402G>T (p.E134D)/ c.403T>A (p.Y135N) was introduced into one construct and also into two separate constructs. The c.440C>T (p.T147I) mutation, known to cause FED in homozygotes (Funke et al., 1991), was also introduced into the LCAT vector and the mutant protein was used as a control for the specific loss of LCAT alpha-activity. All mutant LCAT cDNAs were subcloned into pcDNA™DEST40 expression vector (Invitrogen systems) by a LR Cloning reaction (Gateway® Invitrogen systems, Carlsbad, CA, US).

**In vitro characterization of LCAT mutations**

COS7 cells were cultured in DMEM media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, and 100 IU/ml penicillin/100μg/ml streptomycin in 24-well plates with a density of 35,000 cells/well. Cells were transfected with pcDNA™DEST40_LCAT wild-type or pcDNA™DEST40_LCAT mutant vectors at 60-90% confluency (24 hours after seeding) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Twenty-four hours after transfection, the medium was substituted with Optimem (GIBCO Invitrogen). Seventy-two hours after transfection, the medium was collected and centrifuged at 4°C for 10 minutes at 2000g to remove cellular debris. Aliquots of the media were kept at -80°C.

Recombinant LCAT in media was quantified by Western blot analyses (Invitrogen systems). Mutant and wild-type media containing 20 µg of total protein were run on a 4-12% gradient polyacrylamide gel and subsequently blotted onto a nitrocellulose membrane. After washing, membranes were incubated with rabbit anti-LCAT monoclonal antibody (Novus Biologicals, EPR1384Y, Littleton, CO, USA). After a second wash, membranes were incubated with polyclonal donkey anti-rabbit Infrared Dye800CW in Odyssey block/0.1% Tween 20. Blotted LCAT protein was visualized on LI-COR® Odyssey (LI-COR, Lincoln, NE, USA). Alpha- and beta-activity of recombinant LCAT were measured as described previously. (Frohlich et al., 1988; O K et al., 1993)

**Construction of a partial 3D model for LCAT to model effect of mutations**

A family of LCAT-like sequences was assembled using extensive PSI-BLAST searches. The family members were aligned using MAFFT, allowing identification of highly conserved
regions in the LCAT family. Models for LCAT were predicted using I-tasser, HHpred, pro-
sp3 tasser and LOMETS software. (Roy et al., 2010; Hildebrand et al., 2009; Zhou et al.,
2009; Wu and Zhang, 2007) Most models are based on bacterial lipases as templates.
Only models were retained that allow disulfide bridge formation between C337 and
C380, having C55 as oxyanion hole residue and having the proper catalytic triad. All
models were superposed in Molecular Operating Environment (chemical computing
group), allowing the identification of regions that are similar in all models. Only residues
with <5 Å root mean square deviation in all models were retained in the final model,
which is based on HHpred using the Pseudomonas aeruginosa lipase (pdb code 1EX9) as
template.

Statistical analysis
Differences in concentration or activity of mutant LCAT compared to wild-type LCAT were
assessed by Student’s T test, after log-transformation in case of a skewed distribution. All
analyses were carried out by the use of SPSS software, version 16.0.

Results

LCAT variation in patients with a low HDL-c

Of the 98 participants who were referred to our clinic for low HDL-c, 28 (29%) were
identified as heterozygotes for non-synonymous missense mutations in LCAT (Table 1).
The p.T147I mutation, originally characterized by Funke et al. (Funke et al., 1991) in
patients with FED, was identified in 18 patients of only Dutch descent. Of the 98 patients
referred with only a low HDL-c, 7 were of non-Caucasian descent (2 of these subjects were
heterozygous for an LCAT mutation; patient #10 and #15). In the 91 patients of Dutch
descent only, the prevalence of LCAT mutations remained 29%. The four patients referred
for clinical suspicion of FED (i.e. low HDLc and corneal opacities) were all compound
heterozygotes for non-synonymous missense or nonsense mutations (Table 2).
Nine LCAT mutations have been described earlier (see Table 1 for references) but the

Figure 1: Position of new mutations identified in LCAT

Black boxes: LCAT exon 1 – 6
p.R268C, p.W315X, p.L338F and p.R347C (see Figure 1 for the position of the mutations in the LCAT gene). All amino acid residues at the respective positions are evolutionary conserved (Peelman et al., 1999) and accordingly, all these mutations were associated with reduced plasma LCAT activity in their carriers (Table 3). For p.E134D/p.Y135N, p.W99S, p.V246F and p.R268H mutations, additional family members participated in this study. Figure 2 shows clear segregation of the respective LCAT defects with a low HDL-c phenotype in these families. Through studying segregation of genotype in one of these families, it could be established that the nucleotide substitutions responsible for the p.E134D and p.Y135N changes resided on the same allele.

**Figure 2:** Pedigrees of four Dutch families with new mutations in LCAT

Right filled symbols indicate heterozygosity, clear symbols unaffected family members, all with HDL-c levels within normal range. HDL-c <5th: HDL-c levels below 5th percentile for age and gender. Squares are males, circles are females. N/A: not available for analysis.
Characteristics of LCAT mutation carriers

Compared to pooled control plasma of 100 healthy volunteers, average HDL-c was reduced by 59% and triglycerides were increased by 37% in heterozygotes (Table 1). On average, plasma LCAT activity in heterozygous carriers of new mutations was reduced...
by 33% compared to pooled control plasma (Table 3). Eleven heterozygotes (34%) had suffered from some clinical manifestation of atherosclerotic vascular disease, 7 (22%) carriers of mutations in LCAT had been diagnosed with hypertension, 1 with diabetes mellitus type 1 and 2 with diabetes mellitus type 2. Clinically manifest atherosclerotic cardiovascular disease (CVD) was present in 9 (28%) mutation carriers (all heterozygotes) and in two mutation carriers (patient 28 and 29, both heterozygotes), subclinical atherosclerosis was detected (Tables 1 and 2).

In the 4 compound heterozygotes with corneal opacification, HDL-c was on average reduced by 79% and triglycerides were increased by 45% (Table 2). The patients did not
present with loss of visual acuity, probably related to their young age. In none of the compound heterozygotes, proteinuria was reported. Of note, patient #29 and patient #32 had relatively high plasma levels of HDL-c and of LCAT activity compared to patients #30 and #31 (Tables 2 and 3).

In silico prediction of the effect of LCAT mutations

In silico analysis was performed using PolyPhen (Ramensky et al., 2002) and SIFT (Ng and Henikoff, 2003) software to predict functional significance of the 8 novel missense variations (see Supplementary Table 1). 5 mutations were predicted to be probably damaging (p.W99S, p.E134D, p.Y135N, p.V246F and p.R268C), one as possibly damaging (p.L338F) and two were described as benign (p.R322C and p.R347C) by PolyPhen. Using

<p>| Table 2: Characteristics of Fish-Eye-Disease patients (compound heterozygotes for LCAT gene mutations) |
|-------------------------------------------------|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Patients (gender, age(y))</th>
<th>Amino acid* (Nucleotide*) Change</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference values controls</td>
<td></td>
<td>195</td>
<td>122</td>
</tr>
<tr>
<td>Compound heterozygotes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 30 (female, 58)</td>
<td>p.T147I(Funke et al., 1991b) (c.440C&gt;T) / p.W315X (c.945G&gt;A)</td>
<td>195</td>
<td>196</td>
</tr>
<tr>
<td>Patient 31 (male, 34)</td>
<td>p.T147I(Funke et al., 1991b) (c.440C&gt;T) / p.W99S (c.296G&gt;C)</td>
<td>144</td>
<td>205</td>
</tr>
<tr>
<td>Patient 32 (male, 70)</td>
<td>p.N29I(Okubo et al., 1996) (c.86A&gt;T) / p.R347C (c.1039C&gt;T)</td>
<td>144</td>
<td>223</td>
</tr>
</tbody>
</table>

| Table 3: Plasma LCAT activities of patients with new LCAT gene mutations |
|--------------------------|---------------------------------|-----------------|-----------------|
| Patients (gender, age (y)) | Amino acid (Nucleotide) Change | LCAT activity* (% of pooled control plasma: 13 nmol CE/h*ml) |
|--------------------------|---------------------------------|-----------------|-----------------|
| Heterozygotes            |                                 |                 |                 |
| Patient 1 (female, 76)   | p.R268C (c.802C>T)              | 58              |                 |
| Patient 2 (male, 52)     | p.V246F (c.736G>T)              | 46              |                 |
| Patient 3 (male, 49)     | p.R322C (c.964C>T)              | 73              |                 |
| Patient 4 (male, 52)     | p.E134D (c.402G>T)/ p.Y135N (c.403T>A)† | 71              |                 |
| Patient 5 (male, 66)     | p.V246F (c.736G>T)              | †               |                 |
| Compound heterozygotes   |                                 |                 |                 |
| Patient 30 (female, 58)  | p.T147I(Funke et al., 1991a) / p.W315X (c.440C>T, c.945 G>A) | 7.6             |                 |
| Patient 31 (male, 34)    | p.T147I / p.W99S (c.440C>T, c.296G>C) | 7.1             |                 |

Plasma LCAT activity was measured with a proteoliposome assay as described.(Frohlich et al., 1988) † patient had deceased before moment of plasma collection for LCAT activity assay.

In silico prediction of the effect of LCAT mutations

In silico analysis was performed using PolyPhen (Ramensky et al., 2002) and SIFT (Ng and Henikoff, 2003) software to predict functional significance of the 8 novel missense variations (see Supplementary Table 1). 5 mutations were predicted to be probably damaging (p.W99S, p.E134D, p.Y135N, p.V246F and p.R268C), one as possibly damaging (p.L338F) and two were described as benign (p.R322C and p.R347C) by PolyPhen. Using
**LCAT** mutations in patients with low HDL-cholesterol

SIFT, 6 novel variations were predicted to affect protein function, while two (p.E134D and P.L338F) were predicted to be tolerated.

**In vitro characterization of new mutations**

Panels a, b and c of figure 3 give plasma LCAT protein levels, and LCAT alpha- and beta-activity, respectively, as assessed in the medium of COS7 cells transfected with the LCAT constructs harbouring the respective mutations. The concentrations and activities of the LCAT mutants are expressed as a percentage of wild-type LCAT (taken along in each of the experiments). The frequently present and previously characterized p.T147I mutation (Funke et al., 1991) was used as control in these analyses, since this mutant LCAT is known to be secreted upon expression in vitro, and it has been reported to result in loss of alpha-activity but retained beta-activity of the enzyme (O K et al., 1993). In the current study, the p.T147I mutant was secreted at 20% of wild-type LCAT and had as expected lost alpha activity but retained 50% of beta activity. The p.E134D and p.Y135N mutants were secreted at 30% and 72% of wild-type LCAT, respectively, but both mutants had lost catalytic activity. The double p.E134D/p.Y135N mutant was not secreted at all. The p.R322C mutant was secreted at 18% of wild-type LCAT and showed residual alpha activity (17%) and beta activity (17%). The p.W99S mutant was secreted at 106% normal levels but was catalytically inactive. The p.V246F and p.R268C mutants were hardly secreted (9% and 4% of wild-type LCAT, respectively) and were also catalytically inactive. The p.W315X mutant was not secreted at all. The p.L338F mutant was secreted at only 5% of wild-type LCAT but had 26% of normal alpha activity and 48% of normal beta activity. Finally, the p.R347C mutant was secreted at 32% of wild-type LCAT and had 43% of alpha-activity and 26% of beta-activity. In summary, these analyses show that the newly identified LCAT mutants were secreted at lower levels or showed a marked loss of catalytic activity or both.

<table>
<thead>
<tr>
<th></th>
<th>LDL-c (mg/dl)</th>
<th>HDL-c (mg/dl)</th>
<th>Clinical manifestations of LCAT deficiency</th>
<th>CVD (age (y) of first manifestation)</th>
<th>Other relevant clinical data</th>
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<tbody>
<tr>
<td>Patients</td>
<td></td>
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<td>Nucleotide*</td>
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<tr>
<td>TC</td>
<td>75</td>
<td>22</td>
<td>Corneal opacification</td>
<td>DM2, hypertension</td>
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</tr>
<tr>
<td>TG</td>
<td>146</td>
<td>4</td>
<td>Corneal opacification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-c</td>
<td>116</td>
<td>4</td>
<td>Corneal opacification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL-c</td>
<td>89</td>
<td>16</td>
<td>Corneal opacification</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** Characteristics of Fish-Eye-Disease patients (compound heterozygotes for LCAT gene mutations)

<table>
<thead>
<tr>
<th></th>
<th>LDL-c (mg/dl)</th>
<th>HDL-c (mg/dl)</th>
<th>Clinical manifestations of LCAT deficiency</th>
<th>CVD (age (y) of first manifestation)</th>
<th>Other relevant clinical data</th>
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</thead>
<tbody>
<tr>
<td>Patients</td>
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<td>Amino acid*</td>
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<td>Nucleotide*</td>
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<td>TC</td>
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</tr>
<tr>
<td>TG</td>
<td>124</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-c</td>
<td>144</td>
<td>205</td>
<td>Corneal opacification</td>
<td></td>
<td></td>
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<tr>
<td>HDL-c</td>
<td>89</td>
<td>16</td>
<td>Corneal opacification</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 7

Figure 3: In vitro characterization of new mutations in LCAT.
3a: Recombinant LCAT protein levels

UT denotes untransfected COS7 cells, WT is wild-type LCAT protein. *Previously described mutation, used as control in all analyses.

3b: LCAT alpha activity
UT denotes untransfected COS cells, WT is wild-type LCAT protein. Alpha activity of WT LCAT was 1.2 nmol CE/hr*ml. *Previously described mutation, used as control

3c: LCAT beta activity
UT denotes untransfected COS7 cells, WT is wild type LCAT protein. Beta-activity of WT LCAT was 52 nmol CE/h/µg LCAT. *Previously described mutation, used as control
**Molecular modelling of mutations**

Peelman et al. previously proposed a 3D homology model for a part of the LCAT protein which predicts a lipase-like alpha/beta hydrolase structure with the S205, D369 and H401 residues as catalytic triad. (Peelman et al., 1998) We here updated this model using a combination of several new and improved fold-recognition methods. Only 192 out of 416 residues of LCAT are similar in the different models and were retained in the final model. Five mutants that were identified in the current study could be mapped to this model, i.e. p.T147I, p.R159Q, p.R182C, p.I202T and the novel variant p.L338F (see Figure 4).

**Figure 4:** Molecular modelling of mutated residues in LCAT (for color figure, see page 418)

Residues S205, D369 and H401 form the catalytic triad of LCAT. T147, R159 and R182: mutated residues – p.T147I, p.R159Q and p.R182C - identified in patients of the present study and characterized in a previous model. (Peelman et al., 1998) I202 and L338: residues that are mutated – p.I202T and p.L338F - in patients identified in the present study that have not been modelled before.
Chapter 7

The p.I202T and p.L338F mutations probably affect the hydrophobic core of the protein, as they are part of the central beta strands 5 and 7 of the alpha/beta hydrolase fold of LCAT. It is therefore quite likely that these mutations affect the proper folding of the protein. The other three mutations, at the surface of the protein were previously modeled (Peelman et al., 1999), and are again found at the same position in the current model: p.T147 is found in the alpha 3-4 helix, where it is very close to the position of 2 mutations that are also associated with FED (p.N155D and p.N415S; not shown in Figure 4). The p.R182C mutation introduces a surface exposed cysteine which may make the enzyme susceptible to oxidation. The p.R159Q mutation is found in the connection between the α3-4 helix and the β4 strand. The p.R159 residue is strongly conserved in LCAT of different species and is totally surface exposed in this model and it is not obvious how a conservative mutation at this position affects the structure or activity of the enzyme. However, residues 143 -165 are involved in LCAT activation by apo A-I (Jonas, 2000)

Discussion

This study shows that LCAT mutations cause low HDL-c in almost a third (29%) of patients referred for analysis of low HDL-c levels. This is partly explained by a high prevalence of a founder mutation (present in 50% of the patients with LCAT mutations). The relevance of this finding is underlined by establishing that all mutations studied are functional.

We identified a striking 5-10 fold higher prevalence of LCAT gene mutations compared to 4 previous reports on this field. This is remarkable when considering that the mutations identified in these previous studies were not functionally characterized (except for p.G254R (LCAT[Finn]; (Miettinen et al., 1998). This suggests that the prevalence of mutations really causing low HDL-c may be even lower in the other studies. The high prevalence of one specific mutation (p.T147I) in the current study can to a large extent explain the apparent discrepancy with the previous studies. Fifty percent of the carriers of LCAT mutations (n=18) in our cohort carried this defect, thereby suggesting that T147I is a founder mutation in The Netherlands (is in fact found in all provinces). Without this mutation, however, the prevalence of LCAT mutations in our cohort would still be 10% which is close to the prevalence reported by Recalde et al. i.e. 9% (6 carriers in 66 subjects of Spanish descent with an HDL-c < 10th percentile). It is of interest to note that the respective study subjects were not referred by a physician because of low HDL-c but by contrast recruited during health checks at work. It is thus possible to find such high prevalences of LCAT mutations in very different groups of individuals. On the other hand, Miettinen et al.(Miettinen et al., 1998) reported a lower prevalence, i.e. 5.6%, in participants of a population study (9 carriers in 156 Finnish patients with an HDL-c between 7.8 and 27 mg/dl, i.e. <5th percentile). However, these participants were only smokers, which reduced the chance to find genetic causes for low HDL-c. More
importantly, this cohort was only genotyped for 2 specific LCAT defects (p.G254R and p.R423C) and therefore other mutations in LCAT could have been missed and could thus have led to underestimation of the prevalence. Other investigators reported an even lower prevalence of 3.9% (6 carriers in a study of 155 Caucasian Canadians with an HDL-c<5th percentile for age and gender). (Cohen et al., 2004) This was also a study in non-referred subjects who were enrolled in general population study. The difference in prevalence with the study by Recalde (Recalde et al., 2002) et al is likely related to this difference in study design.

Our study best compares with that of Kiss et al. who investigated 124 patients referred for clinical evaluation of HDL-c below the 5th percentile for age and gender. (Kiss et al., 2007) They found a prevalence of only 3% (4 carriers in 124 Canadian patients of Caucasian descent). A marked difference with our study is that these investigators excluded patients with diabetes mellitus and other conditions that are associated with low HDL-c, such as malignancies, short bowel syndrome and the nephrotic syndrome. This could theoretically result in enrichment for monogenetic disorders that cause a low HDL-c phenotype which makes the low prevalence compared to that of the current study even more remarkable. On the other hand, Kiss et al. also excluded patients with plasma triglycerides levels > 95th percentile for age and gender while carriers of LCAT mutations have elevated triglyceride levels. (Hovingh et al., 2005) This could have led to the exclusion of carriers of LCAT gene defects. Illustrative in this respect is that when excluding hypertriglyceridemic patients, we would have missed three heterozygotes (#6, 21 and 22). Taken together, we do not have a satisfying explanation for the difference in prevalence of LCAT gene mutation in referred low HDL-c patients between their and our study other than referral bias. It is likely that in the advent of next generation sequencing and its application in patient diagnostics (Almomani et al., 2011) that future studies will unequivocally reveal to what extent LCAT mutations cause low HDL-c in patient and general population studies.

In this study, we characterized 9 novel missense mutations to study whether the respective amino acid changes had an effect on LCAT secretion and/or LCAT activity. In vitro, all mutants were shown to affect LCAT either by reduced or absent secretion, by loss of catalytic activity, or both. In silico prediction, on the other hand, predicted 2 mutations to be benign and 2 others to be tolerated, underlining that in silico predictions should be used with care when analyzing the effect of mutations in LCAT.

Of note, the in vitro characterization can fully explain the discrepancy between the relatively high plasma HDL-c and LCAT activity of compound heterozygous patients #29 and #32 and the very low HDL-c and LCAT activity of the two other compound heterozygotes. Patient #29, HDL-c 39% and LCAT activity 56% of normal, carries two mutations that both lead to mild loss of LCAT activity upon expression in vitro: p.L338F had 26% and 48% of normal alpha- and beta-activity, and p.R347C 43% and 26% of normal alpha- and beta-activity. Patient #32, plasma HDL-c and LCAT activity of 29% and
35% of normal, respectively – both also relatively high for a FED patient – also carries the mild p.R347C mutation, and the severe p.N29I that causes FLD in homozygous patients. (Okubo et al., 1996) In contrast, the other compound heterozygotes both carry two highly detrimental mutations in LCAT. Both carry the p.T147I mutation, with known loss of alpha activity, patient #30 also carries the p.W315X nonsense mutation, and patient #31 the p.W99S mutation with no catalytic activity upon expression in vitro.

Even between the two mildly affected compound heterozygotes #29 and #32, the differences in LCAT activities correspond to the differences in plasma HDL-c and plasma LCAT activity: of the two, patient #29 had two mild mutations instead of one, and also had the highest HDL-c and plasma LCAT activity of the two, underlining the accuracy of this molecular characterization.

In the absence of an X-ray structure of the LCAT protein, interpretation of the effect of mutations on enzyme structure is made possible through 3D molecular modelling. The current analysis is restricted to the structurally conserved core of LCAT, as the loops are highly variable elements in the lipase family. (Peelman et al., 1998) The p.I202T and the novel p.L338F mutations could be modelled in this way, and both residues were found to be part of the central beta strands of the alpha/beta hydrolase fold of LCAT. Substitution of these amino acids is likely to affect normal folding and therefore enzyme secretion and/or function.

Wild-type LCAT has 2 disulfide bonds (p.C74-p.C98 and p.C337-p.C380). The first bridge allows for the formation of the lid region, which covers the catalytic center of LCAT and is opened upon contact to a lipid surface. (Jonas, 1998) Substitution C for Y at position 337 causes FLD in three siblings in our clinic illustrating the importance of the second disulfide bond. (Holleboom et al, manuscript accepted for publication in Atherosclerosis, DO10.1016/j.atherosclerosis.2011.01.025). The novel p.W99S and p.L338F mutations, identified in the current study, affect the residues directly adjacent to the cysteines that form these bridges and might affect the formation of these bridges thereby affecting protein folding. Interestingly, p.W99S is normally secreted but catalytically inactive. In contrast p.L338F affected secretion but this mutant had retained partial enzymatic function. The other six novel missense mutations – p.E134D/p.Y135N, p.R322C, p.V246F, p.R268C and p.R347C lie in regions of LCAT with unknown specific function (Jonas, 1998; Jonas, 2000), but clearly these residues are important for normal secretion and function of the enzyme given the severe reduction of these upon expression in vitro.

In a previous study we already showed that mutations in ABCA1 often explain a low HDL-c in our patients (21%). (Candini et al., 2010) The current study indicates that this also holds true for LCAT. Combined, routine sequencing of these genes in referred low HDL-c patients in The Netherlands is warranted since it improves insight in the pathophysiology of low HDL-c. The identification of so many carriers of LCAT mutations enables further studies into the unresolved relation between LCAT and atherosclerosis.
(LCAT is seen as a target for pharmaceutical drugs to reduce atherosclerosis) through adequately powered imaging studies of subclinical atherosclerosis. In addition, patients could enroll in future clinical trials aimed at increasing LCAT by enzyme or gene therapy (in preclinical development (Rousset et al., 2009) (Amar et al., 2009)). In conclusion, we report an unexpectedly high prevalence of LCAT mutations in a cohort of patients who were investigated for low HDL-c levels.

Acknowledgments
We thank all participants and the referring physicians to make this study possible. We are also indebted to Ms. M. van Aalderen and Mr. J.N. van Miert BSc for technical assistance, and to Mrs. J.F. Los, Mrs. C. Koch and Mrs. J.G.A. Olsman-Bruins for their work to recruit participants. We would like to thank Xenon Genetics Inc. (Burnaby, BC, Canada) for their help in obtaining some of the genomic DNA samples of the patients investigated.

Funding sources
This work has been made possible through a grant of the European Community (FP6-2005-LIFESCIHEALTH-6; STREP contract number 037631) and by a grant from the Fondation Leducq. A.G. Holleboom is supported by a grant of the Netherlands Organisation for Scientific Research (NWO; project number 021.001.035). This study was furthermore supported by a grant from the Dutch Heart Foundation (2008B070). Dr. Kastelein is a recipient of the Lifetime Achievement Award (2010) of The Dutch Heart Foundation.
Reference List


**Supplementary Table 1:** *In silico* prediction of the effect of LCAT mutations

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<thead>
<tr>
<th>Missense variant</th>
<th>SIFT</th>
<th>PolyPhen</th>
</tr>
</thead>
<tbody>
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<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.E134D</td>
<td>Tolerated</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.Y135N</td>
<td>Affects protein function</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>p.V246F</td>
<td>Affects protein function</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>p.R268C</td>
<td>Affects protein function</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>p.R322C</td>
<td>Affects protein function</td>
<td>Benign</td>
</tr>
<tr>
<td>p.L338F</td>
<td>Tolerated</td>
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<tr>
<td>p.R347C</td>
<td>Affects protein function</td>
<td>Benign</td>
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</tbody>
</table>

In *silico* analysis performed using PolyPhen (Ramensky et al., 2002) and SIFT (Ng and Henikoff, 2003) software to predict functional significance of the 8 novel missense variations.

Reference List to tables, figures and supplementary table 1


Chapter 7


Plasma levels of lecithin:cholesterol acyltransferase and risk of future coronary artery disease in apparently healthy men and women
A prospective case-control analysis nested in the EPIC-Norfolk population study

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& Both authors equally contributed to this study
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Abstract

Lecithin:cholesterol acyltransferase (LCAT) plays a key role in the maturation of high-density lipoprotein (HDL) as evidenced by low HDL-cholesterol levels in carriers of deleterious mutations in \textit{LCAT}. These carriers present with increased carotid intima media thickness, but in the general population, the role of LCAT in atherosclerosis is unclear. We set out to study this in a prospective case-control study. Plasma LCAT levels, which strongly correlate with LCAT activity, were measured in baseline non-fasting samples of 933 apparently healthy men and women who developed coronary artery disease (CAD) and 1,852 matched controls who remained free of CAD during 6-year follow-up. LCAT levels did not differ between cases and controls, but were higher in women than in men. Stratification into LCAT quartiles revealed a positive association with plasma LDL-cholesterol and triglyceride levels, in the unexpected absence of an association with HDL-cholesterol. In mixed-gender analysis, the odds ratio (OR) for future CAD in the highest LCAT quartile versus the lowest was 1.00 (confidence interval (CI): 0.76-1.29, \( P \) for linearity = 0.902), although opposite trends were observed in men and women. In fact, high LCAT levels were associated with an increased CAD risk in women (unadjusted OR 1.45, CI: 0.94-2.22, \( P \) for linearity: 0.036). In contrast to our studies in carriers of \textit{LCAT} mutations, the current data show that low LCAT plasma levels are not associated with increased atherosclerosis in the general population.
Introduction

LCAT hydrolyzes the sn-2 acyl group of phosphatidylcholine and subsequently transfers and esterifies the fatty acid to free cholesterol, thereby using apolipoprotein (apo) A-I as co-factor. The reaction products are thus cholesteryl ester (CE) and lysophosphatidylcholine. The vast majority of CE in the blood circulation is generated by this enzymatic reaction. LCAT is primarily active on HDL and as such drives the maturation of small nascent HDL discs to larger spherical HDL. In catalyzing the esterification of free cholesterol, LCAT has been proposed to maintain a concentration gradient of free cholesterol from cells to HDL, thereby facilitating reverse cholesterol transport. LCAT deficient patients present with almost complete HDL deficiency because they are unable to form mature HDL, which in turn leads to a rapid clearance of nascent HDL from the circulation.

Based on our current understanding of HDL metabolism, it is not clear whether LCAT is pro- or anti-atherogenic: the generation of CE on HDL by LCAT can be regarded as anti-atherogenic since this action increases HDL cholesterol (HDL-c) levels but in the presence of cholesteryl ester transfer protein (CETP) and triglyceride-rich lipoproteins, the CE will be transferred to apolipoprotein (apo) B-containing lipoproteins which are atherogenic. Animal studies have, unfortunately, not provided clear answers: both LCAT knockout mice and LCAT overexpression models yielded mixed results with respect to atherogenesis, as recently reviewed by Ng. Very recently, Amar et al studied adenoviral expression of human LCAT in squirrel monkeys, which express CETP at a level comparable to humans and develop diet-induced atherosclerosis. In this study, overexpression of LCAT led to an antiatherogenic lipoprotein phenotype by increasing HDL-c and lowering LDL-c.

Contrary to the numerous animal studies on LCAT and atherosclerosis, only very few studies were carried out in humans but these have also provided conflicting results. Genetic association studies have, historically, not been performed due to the lack of frequent LCAT gene variation in the general population. Recently, however, genome wide association studies reported 2 SNPs near the LCAT gene locus that were associated HDL-c levels. Willer et al. showed that the rs255052 SNP, located 49 kb downstream of LCAT had an effect size on HDL-c of + 0.019 mmol/l, while this SNP was not found associated with coronary artery disease (CAD). Another recent paper described a SNP 7.7 kb upstream of LCAT to be correlated with HDL-c but this effect was not reproduced in another cohort. To date, it remains to be shown whether these SNPs are indeed associated with transcriptional changes at the LCAT gene locus.

Large LCAT studies using biochemical means have been difficult to conduct because LCAT activity measurements are cumbersome and time-consuming. In this light, only 2
reports on small cross-sectional studies have shown that LCAT activity is low in patients with either angiographically documented CAD\textsuperscript{12} or acute myocardial infarction\textsuperscript{13}. Finally, the use of LCAT plasma levels as a tool was until recently very limited due to the absence of commercially available LCAT antibodies. Families with LCAT deficiency syndromes have thus far been one of the few sources to study LCAT and atherosclerosis in man. We have previously shown that loss of LCAT function in carriers of LCAT gene mutations is associated with an increased carotid intima media thickness (cIMT), a surrogate marker for cardiovascular endpoints.\textsuperscript{15} To shed more light on the role of LCAT in human atherosclerosis, the objective of the present study was to assess the association between plasma LCAT levels and risk of future CAD events in a large cohort representing the general population.

Methods

Study design and participants

A prospective nested case-control study was performed among participants of the EPIC (European Prospective Investigation into Cancer and Nutrition)-Norfolk study. The EPIC-Norfolk cohort has been described in detail\textsuperscript{20}. For the present analysis, only individuals who did not report a history of heart attack or stroke at the baseline clinic visit were enrolled while participants treated with lipid-lowering medication at baseline were excluded. Cases were 993 individuals who had a hospital admission and/or died from CAD as underlying cause during follow-up (average of 6 years). CAD was defined as code 410 to 414 according to International Classification of Diseases-9\textsuperscript{th} revision. Controls (n=1,852) were individuals who remained free of CAD during follow-up. Two controls were matched to each case for sex, age (within 5 years), general practice, and time of enrolment (within 3 months). The study was approved by the Norwich District Health Authority Ethics Committee, and all participants gave signed informed consent.

Biochemical analysis

Non-fasting blood samples were drawn into plain and citrate bottles. Blood samples were processed directly at the Department of Clinical Biochemistry, University of Cambridge, or stored at -80°C. Serum levels of total cholesterol, HDL-c, and triglycerides were measured in fresh samples with the RA 1000 (Bayer Diagnostics, Basingstoke, United Kingdom). LDL cholesterol (LDL-c) levels were calculated using the Friedewald formula. Serum levels of apoA-I and apoB were measured by rate immunonephelometry (Behring Nephelometer BNII, Marburg, Germany) with calibration traceable to the International Federation of Clinical Chemistry primary standards.\textsuperscript{21} Serum concentrations of apolipoprotein (apo) A-II were measured with a commercially available immunoturbidimetric assay (Wako Pure Chemicals Industries, Ltd, Osaka, Japan) on a Cobas-Mira autoanalyzer (Roche, Basel, Switzerland). Lipoprotein subclass concentrations and average size of LDL and HDL were measured by nuclear magnetic resonance (NMR) spectroscopic assay (LipoScience,
Prospective analysis of LCAT and coronary artery disease

Inc., North Carolina).\textsuperscript{22} Gradient gel electrophoresis was performed as described.\textsuperscript{23, 24} CETP concentrations were measured with a validated 2-antibody sandwich-type ELISA.\textsuperscript{25} Lipoprotein lipase (LPL) levels were measured in serum using a commercially available ELISA (Dainippon). LCAT plasma levels were measured using a commercially available sandwich-type ELISA (Daiichi, Japan), which has been described in detail.\textsuperscript{26} Depending on the absolute LCAT concentration, these investigators reported intra-assay variations of 2.7 to 5.2% with inter-assay coefficients of variation varying from 4.5 to 6.1%. All EPIC-Norfolk assays were carried out in random order to avoid systemic bias and were analyzed in a blinded fashion.

**Statistical analysis**

Baseline characteristics were compared between cases and controls using analysis of variance for continuous variables and the chi-square test for categorical variables. Plasma LCAT levels were analyzed as categorical variables after division into quartiles based on the distribution of LCAT levels in controls. Risk factor levels per quartile of LCAT plasma level were calculated. Associations between LCAT level quartiles and traditional risk factors were calculated using analysis of variance for continuous variables and traditional risk factors were calculated using analysis of variance for continuous variables and traditional risk factors were calculated using analysis of variance for continuous variables and traditional risk factors were calculated using analysis of variance for continuous variables and traditional risk factors were calculated using analysis of variance for continuous variables and traditional risk factors were calculated using analysis of variance for continuous variables and traditional risk factors were calculated using analysis of variance for continuous variables and traditional risk factors were calculated using analysis of variance for continuous variables.

<table>
<thead>
<tr>
<th>Table 1: Baseline characteristics of cases and matched controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study population, n</strong></td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>Women, % (n)</td>
</tr>
<tr>
<td>Body mass index (kg/m(^2))</td>
</tr>
<tr>
<td>Diabetes, % (n)</td>
</tr>
<tr>
<td>Smokers, % (n)</td>
</tr>
<tr>
<td>Alcohol use, units per week*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
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</table>

**Lipids and lipoproteins**

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.45 (1.2)</td>
<td>6.26 (1.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)*</td>
<td>1.9 (1.40-2.80)</td>
<td>1.6 (1.20-2.30)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>4.27 (1.1)</td>
<td>4.08 (1.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.26 (0.37)</td>
<td>1.37 (0.40)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apolipoprotein A-I (mg/dl)</td>
<td>155.1 (29.5)</td>
<td>162.2 (28.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dl)</td>
<td>136.6 (31.7)</td>
<td>128.4 (29.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LCAT (μg/ml)</td>
<td>8.89 (2.10)</td>
<td>8.91 (2.25)</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Controls were matched to cases for age, sex and enrolment time. Data are presented as mean values with standard deviation in brackets, or as a percentage of the total group. P values are for one way ANOVA on continuous variables, or for Chi-square analysis on categorical variables. *Mean and interquartile range. Because of skewed distribution, data on alcohol and triglycerides were log-transformed prior to performing a one way ANOVA test.
the chi-square test for trend for categorical variables. In addition, Pearson correlation coefficients were calculated to assess the correlation between LCAT levels and other continuous risk factors. Conditional logistic regression analysis was used to calculate odds ratios (ORs) and corresponding 95% confidence intervals (CI) as an estimate of the relative risk of incident CAD, taking into account the matching for sex, age, and enrolment time. The lowest quartile was used as reference category. The ORs were adjusted for the cardiovascular risk factors included in the Framingham Risk Score: systolic blood pressure, LDL-c, HDL-c, smoking, and diabetes mellitus, and for CETP. Statistical analyses were performed using SPSS software (version 12.0.2, SPSS Inc., Chicago, Illinois). A P value of <0.05 was considered to be statistically significant.

Results

Baseline characteristics of study participants

268 out of 933 cases (28.7%) died of CAD, whereas the remaining cases suffered from nonfatal CAD events. Table 1 gives demographic and lifestyle parameters, lipids, (apo) lipoproteins and LCAT levels of cases and controls (for sex-specific tables: see data supplement). Cases had a higher BMI, were more likely to smoke, to have diabetes, and consumed less alcohol compared to controls. As expected, levels of total cholesterol, LDL-c, triglycerides, apoB, systolic and diastolic blood pressure were significantly higher in cases than in controls while HDL-c and apoA-I levels were significantly lower in cases.

Table 2: Plasma LCAT levels, demographic and lifestyle characteristics and lipid parameters

<table>
<thead>
<tr>
<th>LCAT Quartile</th>
<th>1 (n=700)</th>
<th>2 (n=684)</th>
<th>3 (n=691)</th>
<th>4 (n=710)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66.3 (7.4)</td>
<td>65.5 (7.7)</td>
<td>65.0 (7.9)</td>
<td>64.7 (7.7)</td>
</tr>
<tr>
<td>Current smoker, % (n)</td>
<td>16 (110)</td>
<td>10 (69)</td>
<td>10 (69)</td>
<td>6.9 (49)</td>
</tr>
<tr>
<td>Former smoker, % (n)</td>
<td>46 (321)</td>
<td>52 (355)</td>
<td>53 (366)</td>
<td>55 (389)</td>
</tr>
<tr>
<td>Never smoked, % (n)</td>
<td>38 (269)</td>
<td>38 (260)</td>
<td>37 (256)</td>
<td>38 (272)</td>
</tr>
<tr>
<td>Alcohol use, units/week</td>
<td>2.5 (1.0-8.0)</td>
<td>3.0 (1.0-9.5)</td>
<td>4.0 (1.0-11)</td>
<td>4.0 (1.0-12)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.8 (3.7)</td>
<td>26.5 (3.4)</td>
<td>26.8 (3.6)</td>
<td>27.3 (3.6)</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>90.0 (12.2)</td>
<td>92.1 (11.3)</td>
<td>92.7 (11.0)</td>
<td>93.9 (11.2)</td>
</tr>
<tr>
<td>Waist hip ratio</td>
<td>0.88 (0.09)</td>
<td>0.89 (0.08)</td>
<td>0.89 (0.08)</td>
<td>0.90 (0.08)</td>
</tr>
<tr>
<td>Diabetes, % (n)</td>
<td>3.1 (22)</td>
<td>3.7 (25)</td>
<td>3.5 (24)</td>
<td>3.5 (25)</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>138 (18.5)</td>
<td>140 (18.9)</td>
<td>141 (17.4)</td>
<td>143 (18.1)</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>82 (11.6)</td>
<td>84 (11.8)</td>
<td>85 (11.2)</td>
<td>86 (11.2)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.9 (1.0)</td>
<td>6.2 (1.0)</td>
<td>6.4 (1.1)</td>
<td>6.8 (1.2)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>3.8 (0.9)</td>
<td>4.1 (0.9)</td>
<td>4.2 (1.0)</td>
<td>4.5 (1.1)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.3 (0.4)</td>
<td>1.3 (0.4)</td>
<td>1.3 (0.4)</td>
<td>1.3 (0.4)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.4 (1.0-1.9)</td>
<td>1.6 (1.2-2.2)</td>
<td>1.7 (1.3-2.4)</td>
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</tr>
<tr>
<td>Apolipoprotein A-I, mg/dl</td>
<td>155.8 (33.2)</td>
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</tr>
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</table>
Validation of the LCAT concentration assay

In our hands, intra-assay and inter-assay coefficients of variations for this ELISA were 1.9%, and 7.4%, respectively. These data were obtained after running 176 measurements of LCAT concentration in the same pooled plasma (harvested from 160 healthy volunteers employed in our department in 2006; this pooled plasma was stored in aliquots for single use purposes).

Kobori et al. previously reported strong correlations between LCAT concentration measurements with LCAT activity as assessed with a liposome substrate (r = 0.871, P<0.001) or endogenous substrates (r=0.864, P<0.001). We verified this by LCAT concentration (using the same ELISA) and LCAT activity (using proteoliposomes) measurements in plasma of heterozygotes for a mutation in the LCAT gene that causes premature truncation of the mature LCAT protein, a 28kD protein that was intracellularly retained. LCAT levels were 3.44, 3.82, and 4.08 ug/ml compared to 7.85 ug/ml in pool plasma. Thus, LCAT levels were 44%, 49% and 52% of normal in these individuals. These findings are in line with our previous measurements using a radio-immuno assay: LCAT concentration averaged 2.5±0.4 ug/ml compared to 4.4±0.5 ug/ml in apparently healthy family controls. Importantly, this approximate 50% reduction of LCAT concentration is proportional to the markedly reduced LCAT activities in these heterozygotes compared to controls (19.7±2.6 vs. 28.8±3.2 nmol.h⁻¹.ml⁻¹ in the controls, respectively).

<table>
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<td>6.4 (1.1)</td>
<td>6.8 (1.2)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>3.8 (0.9)</td>
<td>4.1 (0.9)</td>
<td>4.2 (1.0)</td>
<td>4.5 (1.1)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.3 (0.4)</td>
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</tr>
<tr>
<td>Triglycerides, mmol/l§</td>
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<tr>
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<td>162.7 (27.2)</td>
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<td>128.9 (29.9)</td>
<td>132.7 (30.5)</td>
<td>141.1 (30.6)</td>
</tr>
</tbody>
</table>

Values are mean ± SD or percentage and number of participants
*P for ANOVA of risk factor levels between LCAT quartiles.
†R indicates Pearson’s correlation coefficient between LCAT levels and risk factors and the corresponding P value (P†).
‡P value for smoking by Chi-square test for trend.
§Median (interquartile range).
|| Spearman’s correlation coefficient between LCAT levels and risk factors and the corresponding P value (P||).
Chapter 8

**Plasma LCAT levels, demographic and lifestyle parameters, and lipid parameters**

Plasma LCAT levels were normally distributed and were close to identical in cases and controls (8.89 μg/ml (standard deviation [SD] 2.10) vs. 8.91 μg/ml (SD 2.25), respectively, \( P = 0.78 \)). Considering only the males, again no differences were observed (8.69 μg/ml (SD 2.0) vs. 8.72 μg/ml (SD 2.0) amongst cases and controls, respectively \( P = 0.3 \)). Among women, however, LCAT levels tended to be higher in cases (9.44 μg/ml (SD 2.3) vs. 9.16 μg/ml (SD 2.3) in controls; \( P = 0.06 \)). When analyzing cases and controls together, average LCAT levels were significantly higher in women compared to men (9.25 μg/ml versus 8.69 μg/ml, respectively; \( P < 0.001 \)).

Table 2 summarizes the associations of quartiles of LCAT levels with established cardiovascular risk factors and lipid profiles. LCAT levels were inversely associated with age. On the other hand, LCAT levels were positively associated with alcohol use, smoking, body mass index (BMI), waist circumference, waist-to-hip ratio, and both systolic and diastolic blood pressure. The prevalence of diabetes did not differ among the quartiles. LCAT levels were positively associated with total cholesterol, LDL-c and triglycerides. In the entire cohort, apoA-I, apoA-II and apoB levels were all positively associated with LCAT levels \( (P<0.001, P<0.0001, P<0.0001, \text{ respectively}) \). See data supplement for gender-specific data. The inverse association of LCAT with age could be attributed to associations observed in only the male participants.

We did not observe a correlation between LCAT levels and HDL-c when analyzing men and women separately or when both sexes were combined. Table 3 gives Pearson’s correlations between LCAT levels and additional lipid parameters. LCAT was strongly negatively correlated with HDL size and LDL size as determined by gradient gel electrophoresis and NMR measurements. Furthermore, LCAT levels were positively associated with apoA-II, CETP and LPL.

**Plasma LCAT levels and risk of future CAD for men and women**

In a mixed-gender analysis, LCAT quartiles were not associated with the risk of CAD (OR = 1.00; 95%CI 0.76-1.29 comparing the top versus bottom quartile; \( P \) for linearity over the quartiles 0.902; see table 4). Adjustment for the Framingham Risk Score did not change this result (OR = 0.90; 95%CI 0.69-1.17 for a comparison of top versus bottom quartile; \( P = 0.519 \)).

For men, the risk of CAD decreased with increasing LCAT levels. This linearity proved statistically significant after adjustment for the Framingham Risk Score (OR = 0.71; 95%CI 0.51-0.97 for a comparison of top versus bottom quartile; \( P \) for linearity = 0.03). In contrast, the risk of CAD in women increased with increasing LCAT levels (OR = 1.35; 95%CI 0.87-2.09 for a comparison of top versus bottom quartile; \( P \) for linearity = 0.036). However after adjustment for the Framingham Risk Score, this statistical significance was lost (\( p=0.08 \); see table 4). Adjustment for plasma CETP levels did not affect the results of the mixed-gender nor the gender-specific analyses.
Discussion

this prospective analysis shows for the first time that low plasma levels of LCAT are not associated with an increased risk of future CAD in the general population. Although a gender-specific effect was identified which needs further investigation, this finding does not support our previous finding of increased atherosclerosis in patients with a marked loss of LCAT function.\textsuperscript{15} The current analyses furthermore show that LCAT levels were
positively associated with alcohol use, smoking, BMI, waist circumference, waist-to-hip ratio, blood pressure, total cholesterol, triglycerides and LDL-c levels, in the absence of an association with HDL-c.

**Plasma LCAT levels and lipid metabolism**

The absence of an association between plasma LCAT levels and HDL-c was previously described by Albers et al. in both normolipidemic and hyperlipidemic volunteers, but not in two other small cross-sectional studies in which a positive correlation between LCAT levels, LCAT activity and HDL-c was found. Taken the results of the current larger and prospective analysis, it could be argued that LCAT concentration does not reflect LCAT activity but there is ample evidence from other studies that it does. We confirmed this by measuring 50% LCAT activity and 50% LCAT plasma concentration in heterozygotes for a mutation in LCAT which causes premature truncation of the mature LCAT protein. It can also be argued that the LCAT reaction is not a rate-limiting step in HDL genesis but this does not agree with the notion that loss of LCAT function causes marked reduction of plasma HDL-c levels in individual with LCAT gene mutations. On the other hand, it was recently shown that SNPs near the LCAT gene locus are associated with HDL-c levels but it remains to be shown whether these SNP are indeed associated with e.g. transcriprional regulation of the LCAT gene.

The positive association of LCAT levels with LDL-c levels in the current study also corroborates the findings of Albers et al. In line, patients with familial LCAT deficiency present with reduced LDL-c levels while kinetic analyses in humans have shown that 30% of plasma CE are formed on apoB-containing particles.

We here also show that plasma triglycerides and waist circumference/waist-to-hip ratio are positively associated with plasma LCAT levels which confirms an independent positive association that was recently reported between LCAT activity and presence of the metabolic syndrome. The respective authors postulated that increased LCAT synthesis by the liver might represent a salvage pathway against the dyslipidemia in metabolic syndrome. Whether this is true cannot be concluded from the current study.

**Plasma LCAT levels and risk of future CAD**

This study shows that plasma LCAT levels do not predict risk of future CAD. This result is not what we expected on the basis of our previous finding of increased cIMT in carriers of functional LCAT gene mutations compared to unaffected family members. This discrepancy may be explained by relatively large reductions of LCAT in our families whilst the variation of LCAT concentration in the studied population is by comparison limited.

On the other hand, a recent study showed a positive association of LCAT levels with carotid atherosclerosis in patients with the metabolic syndrome as well as in control subjects. Other investigators previously showed that LCAT activity was reduced in 90 patients with CAD and in 60 patients with acute myocardial infarction. Although these small studies lacked a prospective design, it can be reasoned that LCAT activity
might be reduced in the acute phase of a myocardial infarction – probably in parallel
with the reduced HDL-c levels – but may normalize over time.\textsuperscript{33} Gender specific analyses furthermore showed that higher LCAT levels in women were
associated with an increased risk of CAD. In the males, an opposite non-significant trend
was observed. It is possible that the opposite trends in men and women underlie the
absence of a relation between LCAT levels and CAD in the mixed-gender analysis. At this
point, we have no explanation for this effect (most women were post-menopausal at
enrolment) and it should be confirmed in other sex-specific prospective studies.

\textit{Considerations}

Certain aspects of the present study merit further consideration. Plasma levels of
LCAT were determined in a non-fasting sample, however most studies report no large
effect of diet or feeding condition on LCAT.\textsuperscript{19} Furthermore, a single measurement of
LCAT plasma level may fail to decipher the role of this enzyme in the physiology of pro-
and antiatherogenic lipid changes and associated atherosclerosis. Also, CAD events
were scored through death certification and hospital admission data, which may have
resulted in under-ascertainment or misclassification. Previous validation studies in this
study cohort, however, indicate high specificity of such case ascertainment.\textsuperscript{34} Finally, we
adjusted for confounding factors, but residual confounding by imperfectly measured or
unmeasured confounders cannot be excluded. However, this is a common limitation of
a non-randomized study. In this respect, treatment with rosuvastatin has recently been
found to decrease LCAT activity.\textsuperscript{35} Although subjects treated with lipid-lowering therapy
were excluded from the present analysis, the potential initiation of such therapy during
follow-up might be a confounder of unknown importance.

\textit{Conclusions}

This study shows that low plasma LCAT levels (reflecting low LCAT activity) are not
associated with an increased risk of future CAD in the general population. Although this
may be related to opposite trends in both genders which merits further investigation,
this finding does not match our finding of increased in our studies in families with LCAT
genre mutations.\textsuperscript{15}

\textit{Acknowledgments}

We thank the participants, general practitioners, and staff in EPIC Norfolk.

\textit{Funding Sources}

HDL research by the group of Dr. Jan Albert Kuivenhoven is supported by a grant of the
European Community (FP6-2005-LIFESCIHEALTH-6; STREP contract number 037631).
A.G. Holleboom is supported by a grant of the Netherlands Organisation for Scientific
Research (NWO; project number 021.001.035).
Chapter 8

Reference List


Prospective analysis of LCAT and coronary artery disease


Data supplements

Baseline characteristics of male cases and matched controls

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td>Men, n</td>
<td>598</td>
<td>1161</td>
<td>...</td>
</tr>
<tr>
<td><strong>Demographic and lifestyle parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>64.7</td>
<td>64.6</td>
<td>Matched</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.2 (3.5)</td>
<td>26.6 (3.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes, %(n)</td>
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<td>&lt;0.0001</td>
</tr>
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<td>Smokers, %(n)</td>
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<td>8.6 (100)</td>
<td>&lt;0.0001</td>
</tr>
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<td>Alcohol use, units/week*</td>
<td>4 (1-12)</td>
<td>6 (2-13.5)</td>
<td>0.041</td>
</tr>
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<td>Systolic RR (mmHg)</td>
<td>145 (18.6)</td>
<td>139 (17.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic RR (mmHg)</td>
<td>87 (12.2)</td>
<td>85 (11.1)</td>
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</tr>
<tr>
<td><strong>Lipids and lipoproteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>6.2 (1.0)</td>
<td>6.1 (1.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>LDLc (mmol/l)</td>
<td>4.1 (1.0)</td>
<td>4.0 (0.9)</td>
<td>0.001</td>
</tr>
<tr>
<td>HDLc (mmol/l)</td>
<td>1.2 (0.3)</td>
<td>1.3 (0.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoAI (mg/dl)</td>
<td>147 (26.0)</td>
<td>153 (24.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoB (mg/dl)</td>
<td>135 (30.4)</td>
<td>127 (28.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG (mmol/l)*</td>
<td>1.85 (1.3-2.7)</td>
<td>1.7 (1.2-2.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LCAT (μg/ml)</td>
<td>8.69 (2.0)</td>
<td>8.72 (2.0)</td>
<td>0.277</td>
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</table>

Data are presented as mean values with standard deviation in brackets, or as a percentage of the total group. P values are for one way ANOVA on continuous variables, or for Chi-square analysis on categorical variables. *Mean and interquartile range. Because of skewed distribution, data on alcohol and triglycerides were log-transformed before performing a one way ANOVA test.
Baseline characteristics of female cases and matched controls

<table>
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<th>Cases</th>
<th>Controls</th>
<th>P</th>
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<td>Women, n</td>
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<td>690</td>
<td>...</td>
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<tr>
<td><strong>Demographic and lifestyle parameters</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>66.8</td>
<td>66.5</td>
<td>Matched</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.4 (4.5)</td>
<td>26.2 (3.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes, % (n)</td>
<td>5.4 (18)</td>
<td>1.0 (7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers, % (n)</td>
<td>14.9 (50)</td>
<td>8.1 (56)</td>
<td>0.001</td>
</tr>
<tr>
<td>Alcohol use, units/week*</td>
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<td>2 (0.5-6)</td>
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</tr>
<tr>
<td>Systolic RR (mmHg)</td>
<td>142 (19)</td>
<td>138 (18)</td>
<td>0.003</td>
</tr>
<tr>
<td>Diastolic RR (mmHg)</td>
<td>85 (12)</td>
<td>82 (11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Lipids and lipoproteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>6.8 (1.3)</td>
<td>6.6 (1.1)</td>
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<tr>
<td>LDLc (mmol/l)</td>
<td>4.5 (1.1)</td>
<td>4.3 (1.1)</td>
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<tr>
<td>HDLc (mmol/l)</td>
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<td>1.6 (0.4)</td>
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</tr>
<tr>
<td>ApoAI (mg/dl)</td>
<td>168 (30.4)</td>
<td>177 (29.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoB (mg/dl)</td>
<td>139 (33.7)</td>
<td>131 (31.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG (mmol/l)*</td>
<td>1.8 (1.3-2.4)</td>
<td>1.5 (1.1-2.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LCAT (μg/ml)</td>
<td>9.16 (2.3)</td>
<td>9.44 (2.3)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Data are presented as mean values with standard deviation in brackets, or as a percentage of the total group. P values are for one way ANOVA on continuous variables, or for Chi-square analysis on categorical variables. *Mean and interquartile range. Because of skewed distribution, data on alcohol and triglycerides were log-transformed before performing a one way ANOVA test.
Chapter 8

Plasma LCAT levels, demographic and lifestyle characteristics and lipid parameters in men

<table>
<thead>
<tr>
<th>LCAT Quartiles (men)</th>
<th>1 (n=453), (&lt;7.32 µg/ml)</th>
<th>2 (n=441), (7.32-8.52 µg/ml)</th>
<th>3 (n=434), (8.52-8.86 µg/ml)</th>
<th>4 (n=431), (&gt;8.86 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66.3 (7.6)</td>
<td>65.1 (7.9)</td>
<td>63.8 (8.1)</td>
<td>63.3 (8.0)</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>17 (77)</td>
<td>9.8 (43)</td>
<td>9.0 (39)</td>
<td>7.4 (32)</td>
</tr>
<tr>
<td>Former smoker, %</td>
<td>54 (244)</td>
<td>60 (264)</td>
<td>60 (262)</td>
<td>64 (276)</td>
</tr>
<tr>
<td>Never smoked, %</td>
<td>29 (132)</td>
<td>30 (134)</td>
<td>31 (133)</td>
<td>28.5 (123)</td>
</tr>
<tr>
<td>Alcohol use, units/week§</td>
<td>3.0 (1.0-9.5)</td>
<td>5.0 (1.5-11.5)</td>
<td>6.0 (2.0-14.1)</td>
<td>7.0 (2.5-16.5)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.0 (3.4)</td>
<td>26.6 (3.2)</td>
<td>26.8 (3.2)</td>
<td>27.2 (3.2)</td>
</tr>
<tr>
<td>Participant’s waist circumference, cm</td>
<td>95 (10.3)</td>
<td>97 (9.4)</td>
<td>97 (9.0)</td>
<td>98 (9.3)</td>
</tr>
<tr>
<td>Waist hip ratio</td>
<td>0.92 (0.06)</td>
<td>0.94 (0.05)</td>
<td>0.94 (0.06)</td>
<td>0.95 (0.06)</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>3.8 (17)</td>
<td>5.0 (22)</td>
<td>3.2 (14)</td>
<td>4.2 (18)</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>139 (18.1)</td>
<td>141 (18.8)</td>
<td>141 (17.4)</td>
<td>144 (17.7)</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>83 (12)</td>
<td>85 (12)</td>
<td>86 (11)</td>
<td>87 (11)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.7 (1.0)</td>
<td>6.0 (1.0)</td>
<td>6.3 (1.1)</td>
<td>6.5 (1.1)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l§</td>
<td>1.4 (1.1-2.0)</td>
<td>1.7 (1.2-2.25)</td>
<td>1.8 (1.3-2.6)</td>
<td>2.0 (1.6-2.7)</td>
</tr>
<tr>
<td>HDLc, mmol/l</td>
<td>3.7 (0.9)</td>
<td>4.0 (0.9)</td>
<td>4.1 (0.9)</td>
<td>4.3 (0.9)</td>
</tr>
<tr>
<td>Apolipoprotein AI, mg/dl</td>
<td>146 (28.3)</td>
<td>152 (22.3)</td>
<td>152 (24.5)</td>
<td>155 (23.8)</td>
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<tr>
<td>Apolipoprotein B, mg/dl</td>
<td>119 (28.7)</td>
<td>127 (27.5)</td>
<td>133 (29.7)</td>
<td>139 (29.0)</td>
</tr>
</tbody>
</table>

Plasma LCAT levels, demographic and lifestyle characteristics and lipid parameters in women

<table>
<thead>
<tr>
<th>LCAT Quartiles (women)</th>
<th>1 (n=247), (&lt;7.70 µg/ml)</th>
<th>2 (n=243), (7.70-8.92 µg/ml)</th>
<th>3 (n=257), (8.92-10.28 µg/ml)</th>
<th>4 (n=279), (&gt;10.28 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66.4 (7.2)</td>
<td>66.3 (7.4)</td>
<td>67.1 (7.0)</td>
<td>66.9 (6.8)</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>13 (33)</td>
<td>11 (26)</td>
<td>12 (30)</td>
<td>6.1 (17)</td>
</tr>
<tr>
<td>Former smoker, %</td>
<td>31 (77)</td>
<td>37 (91)</td>
<td>40 (104)</td>
<td>41 (113)</td>
</tr>
<tr>
<td>Never smoked, %</td>
<td>55 (137)</td>
<td>52 (126)</td>
<td>48 (123)</td>
<td>53 (149)</td>
</tr>
<tr>
<td>Alcohol use, units/week§</td>
<td>2.0 (0.5-5.5)</td>
<td>1.5 (0.5-5.5)</td>
<td>2.0 (0.5-5.75)</td>
<td>1.0 (0.0-4.5)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.5 (4.2)</td>
<td>26.3 (3.8)</td>
<td>26.8 (4.2)</td>
<td>27.6 (4.2)</td>
</tr>
<tr>
<td>Participant’s waist circumference, cm</td>
<td>80.9 (10.0)</td>
<td>83.8 (9.5)</td>
<td>85.6 (10.3)</td>
<td>87.3 (10.7)</td>
</tr>
<tr>
<td>Waist hip ratio</td>
<td>0.79 (0.06)</td>
<td>0.81 (0.06)</td>
<td>0.82 (0.06)</td>
<td>0.83 (0.06)</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>2.0 (5)</td>
<td>1.2 (3)</td>
<td>3.9 (10)</td>
<td>2.5 (7)</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>136 (19)</td>
<td>138 (19)</td>
<td>141 (17)</td>
<td>142 (19)</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>81 (11)</td>
<td>82 (11)</td>
<td>83 (11)</td>
<td>85 (11)</td>
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<tr>
<td>Total cholesterol, mmol/l</td>
<td>6.2 (1.1)</td>
<td>6.6 (1.1)</td>
<td>6.7 (1.1)</td>
<td>7.2 (1.3)</td>
</tr>
<tr>
<td>HDLc, mmol/l</td>
<td>4.0 (1.0)</td>
<td>4.3 (1.0)</td>
<td>4.3 (1.0)</td>
<td>4.7 (1.2)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l§</td>
<td>1.3 (0.9-1.8)</td>
<td>1.6 (1.1-2.0)</td>
<td>1.7 (1.2-2.3)</td>
<td>1.9 (1.3-2.5)</td>
</tr>
<tr>
<td>Apolipoprotein AI, mg/dl</td>
<td>172 (34.5)</td>
<td>174 (28.9)</td>
<td>177 (28.2)</td>
<td>174 (28.3)</td>
</tr>
<tr>
<td>Apolipoprotein B, mg/dl</td>
<td>124 (30.0)</td>
<td>132 (31.2)</td>
<td>132 (32.0)</td>
<td>144 (32.8)</td>
</tr>
</tbody>
</table>
Prospective analysis of LCAT and coronary artery disease

Values are mean ± SD or percentage and number of participants. *P for ANOVA of risk factor levels between LCAT quartiles. †R indicates Pearson’s correlation coefficient between LCAT levels and risk factors and the corresponding P value (P†). ‡P value for smoking by Chi-square test for trend. § Median (interquartile range) || Spearman’s correlation coefficient

<table>
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<th>P*</th>
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<td>&lt;0.0001‡</td>
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<td>0.216</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are mean ± SD or percentage and number of participants. *P for ANOVA of risk factor levels between LCAT quartiles. †R indicates Pearson’s correlation coefficient between LCAT levels and risk factors and the corresponding P value (P†). ‡P value for smoking by Chi-square test for trend. § Median (interquartile range) || Spearman’s correlation coefficient
Chapter 9

Proteinuria in early childhood due to familial LCAT deficiency caused by loss of a disulfide bond in lecithin:cholesterol acyl transferase

Atherosclerosis 2011, in press

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3) Department of Nephrology, Haga Hospital, The Hague, The Netherlands
4) Department of Pediatrics, Haaglanden Medical Center, The Hague, The Netherlands
5) Department of Endocrinology, Academic Medical Center, Amsterdam, The Netherlands
Abstract

Introduction. Familial lecithin:cholesterol acyltransferase (LCAT) deficiency (FLD) is a rare recessive disorder of cholesterol metabolism characterized by the absence of high density lipoprotein (HDL) and the triad of corneal opacification, hemolytic anemia and glomerulopathy.

Patients. We here report on FLD in three siblings of a kindred of Moroccan descent with HDL deficiency. In all cases (17, 12 and 3 years of age) corneal opacification and proteinuria were observed. In the 17 year-old female proband, anemia with target cells was observed.

Results. Homozygosity for a mutation in LCAT resulted in the exchange of cysteine to tyrosine at position 337, disrupting the second disulfide bond in LCAT. LCAT protein and activity were undetectable in the patients’ plasma and in media of COS7 cells transfected with an expression vector with mutant LCAT cDNA. Upon treatment with an ACE inhibitor and a thiazide diuretic, proteinuria in the proband decreased from 6g to 2g/24 hours.

Conclusion. This is the first report that FLD can cause nephropathy at a very early age.
Introduction

Lecithin:cholesterol acyl transferase (LCAT) is a key enzyme in HDL metabolism. It catalyses the maturation of HDL particles by esterifying free cholesterol molecules.(1) LCAT has two disulphide bridges. The first bridge (Cys74-Cys98) allows for the formation of the lid region, which covers the catalytic center and is opened upon contact to a lipid surface.(1) The function of the second disulfide bond (Cys337-Cys380) is less clear.(5) Homozygosity or compound heterozygosity for LCAT mutations that result in complete loss of function cause familial LCAT deficiency (FLD), characterized by corneal opacification, mild hemolytic anemia, and progressive renal disease.(6) Proteinuria is on average detected between the age of thirty and forty (see Supplementary Table 2 for an inventory of mutations in LCAT described to result in FLD with reported proteinuria and age of first documentation of proteinuria).(6) Therapeutic options for FLD are poorly defined, associated with the rareness of the disease (<100 patients described worldwide). Plasma transfusions transiently normalized plasma lipids and lipoproteins, as well as the abnormal erythrocyte membranes.(8;9) Recently, combined treatment with nicotinic acid and fenofibrate was associated with a reduction in plasma lipoprotein X levels and a concomitant reduction in proteinuria in one FLD patient.(10) Intensive renoprotective therapy with blockade of the RAS, as well as adequate treatment of hypertension, may delay renal deterioration.(11) Enzyme replacement (12) and gene therapy (13) are eagerly awaited. Here, we describe FLD in three children of Moroccan descent due to a mutation in LCAT that causes loss of the second disulfide bond in LCAT. This is the first description of renal pathology at a very early age. Effects of ACE inhibition in the proband are described.

Methods

Genetic analysis
PCR amplification and sequence reactions and analysis were performed as described.(14)

Biochemical analysis
Plasma lipids and lipoproteins were analyzed as described.(14) Plasma LCAT levels were measured by ELISA.(15) Plasma LCAT α-activity was measured with a proteoliposome assay as described in Supplemental Methods.(16)

In vitro characterization of p.C337Y mutation
Wild-type LCAT and LCAT^{C337Y} were expressed in COS7 cells as described in Supplemental Methods. LCAT protein and α- and β-activity of media were measured as described in Supplemental Methods.(16;17)
Chapter 9

Carotid Ultrasound Imaging

Ultrasound scans of the carotid arterial wall were assessed according to a standardized protocol. (18)

Results

Patients

A 17 year old girl was referred to our clinic for renal pathology compatible with a metabolic disorder, including FLD. At age 15, she presented with abdominal pain for which no cause could be identified. Routine urinalysis revealed excess urinary protein, subsequently quantified at 6g/24 hours, indicating severe proteinuria. Blood pressure was 115/70 mmHg, mild pitting edema was noted on both lower extremities and plasma EM shows vacuoles in the glomerular basement membrane containing electron dense material. Examples of lesions indicated by arrows.

Figure 1. Ocular pathology in the proband with familial LCAT deficiency (for color figure, see page 418)

Peripheral corneal opacification

Slit lamp examination showing opaque dots located centrally in the cornea

EM shows vacuoles in the glomerular basement membrane containing electron dense material. Examples of lesions indicated by arrows.
albumin was low at 25 g/l. Complement factors were normal and anti-nuclear antibodies or anti-dsDNA could not be detected. Creatinine clearance calculated from a 24 hour urine collection was elevated at 164 ml/min, indicative of hyperfiltration due to early renal damage. Kidneys were of normal size and morphology on ultrasound examination. Peripheral corneal opacification was apparent (Figure 1a), and on slit lamp examination, central opaque dots were observed, with a clear zone separating the limbus and the lipid arc (Figure 1b). Her plasma HDL cholesterol level was 0.14 mmol/l, and hemoglobin was mildly decreased at 11.4 g/dl, with normal mean corpuscular volume and number of reticulocytes. Target cells were seen in the blood smear. Haptoglobin was not decreased, nor was LDH increased. Light microscopy of a renal biopsy revealed non-amyloid mesangial and pericapillary depositions in the majority of the glomeruli. Upon electron microscopic analysis, vacuoles in the glomerular basement membrane were identified, containing electron dense material (Figure 1c). To exclude Fabry disease, alpha-galactosidase activity was measured in leucocytes of the patient, and found to be normal.

Parents of the proband were first cousins of Morroccan descent. The proband had a 12 year old sister, and two younger brothers, aged 6 and 3 years (See pedigree in Table 1). The sister and the youngest brother were asymptomatic, but had proteinuria of 0.45 g/l with a urinary protein to creatinine ratio of 34 mg/mmol and glomerular filtration rate of 196 ml/min/1.73 m^2 (eGFR, estimated with Schwartz equation), and of 0.16 g/l with a urinary protein to creatinine ratio of 15 mg/mmol and eGFR rate of 127 ml/min/1.73 m^2, respectively. No edema was noted in these two siblings, and plasma albumin levels and creatinine clearance were normal. In both, slight limbal opacification of the cornea was observed.

**Genetic analysis**

In the proband, a homozygous mutation at position c.1010 G>A was identified (TGT>TAT), leading to substitution of cysteine by tyrosine at position 337 (p.C337Y). Her sister and youngest brother were also homozygous for this mutation while the parents and oldest brother were heterozygous (for pedigree, see Table 1).

**Plasma lipids and LCAT measurements**

The 3 homozygous patients presented with very low HDL cholesterol levels ranging from 0.14 – 0.38 mmol/l averaging 0.33 mmol/l. The heterozygotes had on average 50% lower levels of HDL cholesterol and apoA-I compared to reference values. In the homozygotes, levels of apoA-I were also reduced, but in the heterozygotes these were remarkably normal. LCAT protein and α-activity were undetectable in plasma of the homozygotes and reduced by 52% and 27% compared to reference values, respectively, in the heterozygotes. (20) (see Table 1)
In vitro characterization of p.C337Y mutation in LCAT

In the media of COS7 cells transfected with p.C337Y-LCAT, no immunoreactive LCAT protein could be detected, compared to clearly detectable LCAT media of cells transfected with wild-type LCAT. Compared to media of cells transfected with wild-type LCATT, both α- and β-LCAT activity were severely reduced in the media of cells transfected with p.C337Y-LCAT. (See Supplemental Table 1)

Carotid ultrasound imaging

Carotid intima media thickness (IMT), a surrogate endpoint for atherosclerosis, was within normal range in the four oldest family members (I:01, I:02, II:01 and II:02). (21) The two other siblings (II:03 and II:04) were considered too young to be assessed.

Symptomatic therapy

After one year of treatment with an ACE inhibitor, lisinopril 20 mg once daily, and hydrochlorothiazide 25 mg once daily, the proteinuria in the proband was 3-fold decreased from 6 g/24 hours to 2 g/24 hours and the pitting edema had resolved. Kidney function calculated from 24 hour urine was initially elevated and remained elevated.

Table 1. Pedigree, and individual lipid and LCAT parameters, and carotid intima media thickness

<table>
<thead>
<tr>
<th>Pedigree ID</th>
<th>Genotype</th>
<th>TC mmol/l</th>
<th>LDL-c mmol/l</th>
<th>Apo B g/l</th>
<th>HDL-c mmol/l</th>
<th>Apo A-I g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>I:01</td>
<td>+/-</td>
<td>3.0</td>
<td>2.1</td>
<td>0.67</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>I:02</td>
<td>+/-</td>
<td>3.8</td>
<td>3.0</td>
<td>1.1</td>
<td>0.74</td>
<td>1.1</td>
</tr>
<tr>
<td>II:01 (proband)</td>
<td>+/-</td>
<td>2.8</td>
<td>1.8</td>
<td>0.36</td>
<td>0.15</td>
<td>0.42</td>
</tr>
<tr>
<td>II:02</td>
<td>+/-</td>
<td>1.2</td>
<td>0.8</td>
<td>0.21</td>
<td>0.26</td>
<td>0.30</td>
</tr>
<tr>
<td>II:03</td>
<td>+/-</td>
<td>1.4</td>
<td>1.0</td>
<td>0.81</td>
<td>0.38</td>
<td>0.82</td>
</tr>
<tr>
<td>II:04</td>
<td>+/-</td>
<td>3.6</td>
<td>2.9</td>
<td>0.21</td>
<td>0.62</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Arrow in pedigree indicates proband. TC denotes total cholesterol; LDL-c, low density lipoprotein-cholesterol; apoB, apolipoprotein B; HDL-c, high density lipoprotein-cholesterol; apo A-I, apolipoprotein A-I; TG, triglycerides; IMT, intima media thickness. LCAT α-activity is expressed as nmol CE/h*mL. Reference values in parentheses (not available for IMT). +/- denotes heterozygous, -/- homozygous. LCAT α-activity and IMT were measured in duplicate and expressed as means ± SD. ND denotes not determined.

In vitro characterization of p.C337Y mutation in LCAT

In the media of COS7 cells transfected with p.C337Y-LCAT, no immunoreactive LCAT protein could be detected, compared to clearly detectable LCAT media of cells transfected with wild-type LCAT. Compared to media of cells transfected with wild-type LCATT, both α- and β-LCAT activity were severely reduced in the media of cells transfected with p.C337Y-LCAT. (See Supplemental Table 1)

Carotid ultrasound imaging

Carotid intima media thickness (IMT), a surrogate endpoint for atherosclerosis, was within normal range in the four oldest family members (I:01, I:02, II:01 and II:02). (21) The two other siblings (II:03 and II:04) were considered too young to be assessed.

Symptomatic therapy

After one year of treatment with an ACE inhibitor, lisinopril 20 mg once daily, and hydrochlorothiazide 25 mg once daily, the proteinuria in the proband was 3-fold decreased from 6 g/24 hours to 2 g/24 hours and the pitting edema had resolved. Kidney function calculated from 24 hour urine was initially elevated and remained elevated.
proteinuria and the urinary protein to creatinine ratio in the 12-year old homozygous sister of the proband (II:02) had decreased from 0.45 g/l to 0.20 g/l and from 34 mg/mmol to 25 mg/mmol, respectively, after a year of treatment with another ACE inhibitor enalapril at 5 mg twice daily. The 3 year old brother was followed but has so far remained untreated. His proteinuria level and urinary protein to creatinine ratio remained stable around 0.20 g/l and 15 mg/mmol, respectively. eGFRs remained unchanged in the two younger patients.

**Discussion**

Here we describe FLD in three siblings of Moroccan descent and report that this metabolic disorder can cause renal damage in early childhood. A novel causal mutation in LCAT in this first family from Northwest Africa in which FLD is described, results in loss of a disulfide bond in the enzyme, associated with a complete loss of immunoreactive protein in plasma and a complete loss of LCAT activity.

To date 78 mutations in LCAT have been described.(22) The current study identifies the first naturally occurring mutation leading to disruption of a disulfide bond in LCAT, due to the substitution from cysteine to tyrosine at position 337 (p.C337Y). By definition, this causes a change in the secondary structure of the enzyme. Based on the 3D model of LCAT(2), the substitution possibly induces a shift in the stereotypic position of His377, a component of the catalytic triad (2;3), thus disrupting the structure of this triad causing the enzyme to lose activity. Alternatively, since the mutant protein was not identified in the patients’ plasma or in the media of transfected cells, it is likely that misfolding of
the protein results in defective secretion as proposed by Qu et al. (4) and probably rapid degredation.

To our knowledge, this is the first description of renal damage due to FLD at such an early age. In our proband, routine urinalysis during the work-up for abdominal pain had led to the surprising finding of severe proteinuria at age 17. Additional family screening showed that proteinuria due to FLD can already be present at the age of three. Of note, only 26 mutations in LCAT are reported to result in FLD with documented proteinuria and age of first detection (see Supplementary Table 2 for an inventory of several databases). The youngest FLD patient documented with a trace of proteinuria thus far was a 13 year old girl.(23)

As in other chronic glomerulopathies, proteinuria and hyperfiltration can be present before any symptoms are apparent. Since renal insufficiency is the main cause of morbidity and mortality in FLD(24), it may be important to start intensive renoprotective therapy by optimal control of blood pressure and reduction of proteinuria.(25). Indeed, antihypertensive therapy, inhibition of the RAS and lipid-lowering therapy resulted in decreased proteinuria and stabilization of pathological sequelae of FLD during 5 years of follow-up.(11) Thus, close clinical monitoring from an early age, and genetic screening of younger siblings is recommended, especially since in our patients, a stable 2- to 3- fold reduction in proteinuria could be achieved with ACE-inhibition.

In conclusion, a naturally occurring variant disrupting a disulfide bond (Cys337-Cys380) causes FLD and renal damage at a very early age. Clearly, a medical need exists for these patients which may be met by enzyme replacement and gene substitution therapy (12;13). Until then, the current study supports intensive renoprotective therapy, and early molecular diagnosis and monitoring of affected family members.

Acknowledgements

We would like to thank the patients and family members for their participation, Mr. J. van Miert, Ms. M. van Aalderen, Dr. E. de Groot and Mr. J. Gort for technical support, and prof. F. Wijburg (Dept. of Pediatrics, AMC), prof. S. Florquin (Dept. of Pathology, AMC), The Department of Ophthalmology (AMC) and Ms. K. Los for clinical assistance.

Funding

HDL research by the group of Dr. Jan Albert Kuivenhoven is supported by a grant of the European Community (FP6-2005-LIFESCIHEALTH-6; STREP contract number 037631). A.G. Holleboom is supported by a grant of the Netherlands Organisation for Scientific Research (NWO; project number 021.001.035)
Reference list


22. Human Genome Mutation Database. 2010. Internet Communication


Supplemental methods

Genetic analysis
Genomic DNA was extracted from 10 ml whole blood on an AutopureLS apparatus according to the manufacturer’s protocol (Gentra Systems, Minneapolis, USA). Design of PCR primers, PCR amplification and sequence reactions and analysis were performed as described.(1)

Plasma LCAT α-activity
Plasma LCAT α-activity was measured with a proteoliposome assay. This LCAT activity assay was performed using radioactively labeled vesicles composed of egg yolk phosphatidylcholine (EYPC) (Sigma-Aldrich, Oakville), unesterified cholesterol (UC) (Sigma-Aldrich, Oakville), [\(^3\)H]cholesterol (Perkin Elmer, Waltham, MA) (in a molar ratio of 4:1 5 EYPC/UC) and human apoA-I (0.0167 mg/ml) purified from plasma as previously described.(2) Plasma was incubated with proteoliposomes for 30 minutes, and the reaction was terminated by the addition of 1 ml of absolute ethanol. After centrifugation at 3,750 rpm for 15 min, the unesterified cholesterol and cholesterol ester in the supernatant were separated by TLC on Merck silica gel 60 glass plates (Sigma-Aldrich, Oakville) with the solvent system petroleum ether-diethyl etheracetic acid 70:12:1 (v/v). The radioactivity associated with cholesterol and cholesterol ester was determined by liquid scintillation counting after TLC. The results are expressed as molar esterification rate (MER) of cholesterol (% esterified cholesterol x nmoles of free cholesterol at the start of the reaction per ml of media per h).(3)

In vitro characterization of p.C337Y mutation
The c.1010 G>A (p.C337Y) was introduced into wild-type LCAT-p.ENTR221 vector from Invitrogen (Carlsbad, CA, USA) using Stratagene QuikChange XL site-directed mutagenesis kit (La Jolla, CA, USA). The mutant LCAT cDNA was cloned into expression vector pcDNA™DEST40 (Invitrogen systems) by LR Cloning reaction (Gateway® Invitrogen systems) and expressed in COS7 cells. Cells were grown in DMEM media (Invitrogen) supplemented with 10% FBS and 100 IU/ml penicillin/100μg/ml streptomycin, seeded into wells of 24-well plates at a density of 35,000 cells/well and transfected with either pcDNA™DEST40_LCAT wild type or pcDNA™DEST40_C337Y vectors at 60-90% of confluence (24 hours after seeding) with Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection the medium was substituted with OptiMEM cell free medium (GIBCO Invitrogen). Seventy-two hours after transfection, medium was harvested and centrifuged for 10 minutes at 2000 g at 4°C. Aliquots were kept at -80°C.
LCAT in media was quantified by western blot: culture media containing 20 µg of protein were run on a 4-12% gradient polyacrylamide gel and blotted onto a nitrocellulose membrane. After washing, the membrane was incubated with rabbit anti-LCAT monoclonal antibody (Novus EPR 1384Y, Littleton, CO, USA). After a second wash, the
membrane was incubated with polyclonal swine anti-rabbit immunoglobulins/HRP (DAKO, Glostrup, Denmark). LCAT protein was visualized by enhanced chemiluminescence (SuperSignal® West Pico, Thermo Scientific, Logan, Utah, USA).

LCAT α-activity of media was measured as described for plasma, but 100 ul of media was incubated with proteoliposomes instead of 15 ul of plasma, and for 120 instead of 30 minutes. LCAT β-activity of media was measured as described by O et al.(2) Radiolabeled lipoprotein was prepared by equilibration with [3H]cholesterol at 4°C as described by Dobiasova and Schutzova.(4) An aliquot of heatinactivated isolated LDL was added to a precooled test tube containing [3H]cholesterol-labeled filter paper discs and the mixture was incubated at 4°C for 20 h. The labeled fraction was incubated with recombinant LCAT and the rate of [3H]cholesterol was determined.(2)

Supplemental tables

**Supplemental Table 1. In vitro characterization of p.C337Y mutation in LCAT**

<table>
<thead>
<tr>
<th>Protein concentration (% of wild type)*</th>
<th>Transfected with wild type LCAT</th>
<th>Transfected with C337Y LCAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>0.0</td>
</tr>
<tr>
<td>LCAT α- activity (nmol CE/h*mL, mean ± SD)†</td>
<td>1.5 ± 0.6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>LCAT β-activity (nmol CE/h/µg LCAT, mean ± SD)‡</td>
<td>52 ± 3.3</td>
<td>2.6 ± 0.47</td>
</tr>
</tbody>
</table>

Protein level and α- and β-activity were determined in media of untransfected COS7 cells, cells transfected with vector containing wild type LCAT and with vector containing LCAT with C337Y. All values given are adjusted for values in media of untransfected cells. *media from three separate transfections blotted in duplicate. †media from three separate transfections measured in duplicate. ‡single activity experiment measured in duplicate.
Supplemental Table 2. Reported mutations in LCAT that result in clinical and molecular FLD with reported proteinuria and age at first documentation of proteinuria

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Codon</th>
<th>Reference</th>
<th>Age of first documentation proteinuria (years)</th>
<th>Identified in the same FLD patient</th>
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<td><strong>Missense/nonsense mutations</strong></td>
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<td>AAT-ATT</td>
<td>Asn-Ile</td>
<td>29</td>
<td>-5</td>
<td>64</td>
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<tr>
<td>GGC-AGC</td>
<td>Gly-Ser</td>
<td>54</td>
<td>-6</td>
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<td>GGG-CGG</td>
<td>Gly-Arg</td>
<td>95</td>
<td>-7</td>
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<td>TGG-CGG</td>
<td>Trp-Arg</td>
<td>99</td>
<td>-8</td>
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<td>TAC-TAA</td>
<td>Tyr-Term</td>
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<td>-9</td>
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<td>GCC-ACC</td>
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<td>117</td>
<td>-9</td>
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<td>CGG-TGG</td>
<td>Arg-Trp</td>
<td>159</td>
<td>-9</td>
<td>18</td>
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<tr>
<td>TAC-AAC</td>
<td>Tyr-Asn</td>
<td>180</td>
<td>-10</td>
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Sources of inventory: Human Gene Mutation Database(22), Scriver et al, The Metabolic and Molecular Bases of Inherited Diseases(23), uniprot.org, pubmed.com, Criteria for inclusion in list: clinical and molecular diagnosis of FLD, including report of proteinuria/renal damage with age of first documentation of proteinuria. Symbols in final column: Pairs of symbols represent mutations identified in the same compound heterozygous FLD patient.
Chapter 9

Reference list to supplemental tables


22. Human Gene Mutation Database. Human Gene Mutation Database.  2010. Internet Communication

Chapter 10

Compromised LCAT Function Is Associated With Increased Atherosclerosis

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Chapter 10

Abstract

**Background.** Prospective epidemiological studies have shown that low plasma levels of HDL cholesterol (HDL-C) are associated with an increased risk for cardiovascular disease (CVD). Despite nearly 40 years of research, however, it is unclear whether this also holds true for individuals with severely reduced levels of HDL-C due to mutations in the lecithin:cholesterol acyltransferase (LCAT) gene. Better insight into CVD risk in these individuals may provide clues toward the potential of LCAT as a pharmaceutical target to raise HDL-C levels.

**Methods and Results.** Lipids, lipoproteins, high-sensitivity C-reactive protein (CRP), and carotid artery intima-media thickness (IMT) were assessed in 47 heterozygotes for LCAT gene mutations and 58 family controls. Compared with controls, heterozygotes presented with a mean 36% decrease in HDL-C levels ($P<0.0001$), a 23% increase in triglyceride levels ($P<0.0001$), and a 2.1-fold increase in CRP levels ($P<0.0001$). Mean carotid IMT was significantly increased in heterozygotes compared with family controls (0.623±0.13 versus 0.591±0.08 mm). After adjustment for age, gender, and alcohol use, this difference proved statistically significant ($P<0.0015$).

**Conclusions.** The data show that heterozygosity for LCAT gene defects is associated with low HDL-C levels and elevated concentration of triglycerides and CRP in plasma. This phenotype underlies increased IMT in carriers versus controls, which suggests that LCAT protects against atherosclerosis. This in turn indicates that targeting LCAT to raise HDL-C may reduce CVD risk.
Increased carotid IMT in LCAT mutation carriers

Introduction

Despite the widespread use of pharmacological agents that can effectively decrease LDL cholesterol (LDL-C), the vast majority of patients at increased risk for atherosclerosis continue to have cardiovascular disease (CVD). This underscores the need for additional therapeutic strategies. In this respect, drugs that increase HDL cholesterol (HDL-C) hold promise. In addition to its pivotal role in reverse cholesterol transport, HDL also exerts antithrombotic, antioxidant, and antiinflammatory properties, which illustrates that increasing HDL-C levels may result in various beneficial effects.

Hereditary disorders of human HDL metabolism, such as deficiencies of apolipoprotein (apo) A-I, ATP binding cassette A1 (ABCA1), lecithin:cholesterol acyltransferase (LCAT), and cholesteryl ester transfer protein (CETP) have been crucial to our understanding of the reverse cholesterol transport pathway. Consequently, they have proven important in the identification of therapeutic targets to increase HDL-C. This is exemplified by the recent materialization of inhibitors of CETP that exhibit strong HDL-C-raising potential in clinical trials. Also, increasing the function of ABCA1, a transmembrane protein that promotes the efflux of cholesterol and phospholipids, is thought to reduce CVD risk. This assumption is among others that are based on the finding that mutations in the ABCA1 gene are associated with increased risk for atherosclerosis in humans. This has led to the search for molecules that selectively enhance ABCA1 function. HDL deficiency and increased risk of coronary artery disease have also been described in families with apoA-I mutations. Supported by convincing epidemiological evidence of the protective role of apoA-I, this has further established this protein as a promising target to reduce coronary artery disease risk. This knowledge has recently prompted the use of infusion of apoA-I-containing phospholipid complexes in patients with acute coronary syndrome, which was shown to induce regression of coronary atheroma.

LCAT deficiency represents another rare, recessive genetic disorder that underlies HDL deficiency. LCAT is a plasma enzyme that esterifies free cholesterol, primarily at the surface of the HDL particle, after which the cholesteryl ester molecules migrate to the inner core of this lipoprotein. Through this action, LCAT plays a key role in the maturation of HDL particles. Norum and Gjone first described that LCAT gene mutations underlie familial LCAT deficiency. Later, it was recognized that a less severe clinical phenotype, fish-eye disease, results from mutations in this very gene. Although LCAT deficiency and fish-eye disease are both characterized by HDL deficiency (5% to 10% of normal HDL-C levels). (For a review of the effect of LCAT mutations on lipid metabolism, see Pritchard and Hill.) Although LCAT has thus long been known to play an important role in HDL metabolism, its association with atherosclerosis has remained elusive. This is mainly because of the very limited numbers of carriers of LCAT gene mutations. In addition, animal data have provided conflicting results. We therefore set out to study the relationships between LCAT and atherosclerosis using B-mode ultrasound intima-media

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thickness (IMT) measurements of the carotid arteries as a validated marker for CVD risk and atherosclerosis in large cohort of LCAT mutation carriers.

Methods

Study Groups
Over the past 14 years, our laboratory has, in collaboration with the laboratory of Dr. P.H. Pritchard, characterized 5 families of Dutch Caucasian descent with LCAT deficiency disorders. Families 1, 2, 17, 18 and 319 have been described previously. The probands of these 3 families experienced corneal clouding and consulted ophthalmologists before being referred to our Lipid Clinic. More recently, we identified 2 additional probands with LCAT deficiency disorders (families 4 and 5, respectively), but these data were not published.

Briefly, the proband of family 4, a male, aged 68 years, was referred to our Lipid Clinic for low HDL-C levels. Upon physical examination, corneal opacities were noted, whereas laboratory tests showed an HDL-C level of 0.15 mmol/L. LCAT activity was assessed by using a proteoliposome substrate20 and was severely reduced, which was related to compound heterozygosity for 2 missense mutations (underlying P10Q and V309M, respectively). The proband of family 5, a 20-year-old woman, was referred to our Lipid Clinic for corneal clouding. She had HDL deficiency (HDL-C 0.09 mmol/L), and her plasma LCAT activity was severely reduced due to compound heterozygosity for point mutations that caused amino acid substitutions at positions 123 (T123I) and 309 (V309M) of the mature LCAT protein.

For the present cross-sectional analysis, the probands and their family members of the aforementioned 5 families were invited to participate in an IMT study irrespective of cardiovascular or genetic status; no inclusion or exclusion criteria were used. The recruitment of study individuals was performed as follows: we contacted the index patients of the families who were previously found to be homozygous or compound heterozygous for LCAT gene mutations. Using these individuals as central spokespersons, family members were invited to participate by our physicians and genetic field workers (sometimes through the organization of family reunions).

This was done through a letter and/or by telephone. Family members included both first- and second-degree relatives and those who had married into the family.

All LCAT gene defects under study have previously been shown to underlie marked loss of LCAT activity (30% to 42% compared with family controls), which has as its direct consequence a 30% to 38% reduction of HDL-C levels.16,19,21 We therefore only genotyped the participants (using previously described methods16,18,19,21) to distinguish between carriers and noncarriers. Heterozygotes for LCAT gene mutations do not have clinical signs or complaints despite having these marked reductions of HDL-C levels. Thus, importantly, there existed no clinical recruitment bias for the heterozygotes. From families 1, 2, 3, 4, and 5, we recruited 3, 2, 30, 6, and 6 heterozygotes and 6,
Increased carotid IMT in LCAT mutation carriers

0, 45, 3, and 4 controls, respectively. Nine homozygotes or compound heterozygotes, characterized by near complete HDL-C deficiency, also underwent IMT measurements (average IMT was 0.73±02 mm). However, the low number and the completely different age distribution (60.9±15.6 versus 41.9±16.1 and 42.2±17.4 years in controls and heterozygotes, respectively) unfortunately could not provide the required statistical foundation for solid conclusions with regard to the impact of LCAT on atherosclerosis. The data from this group were therefore not used for further analysis. Past medical history, presence of cardiovascular risk factors, and use of medication were assessed by questionnaire. The data on clinical events were verified by studying the clinical records. Informed consent was obtained for plasma sampling, storage, genetic analysis, and IMT measurements. The study was approved by the Ethics Committee of the Academic Medical Center in Amsterdam.

Blood Analyses

Blood was collected in EDTA-coated tubes after overnight fasting. Total cholesterol, triglycerides, and HDL-C were measured by established methods, and LDL-C was calculated by the equation of Friedewald.

Carotid Artery IMT and CVD

IMT measurements were performed in a standardized fashion for both the carriers of LCAT gene mutations and the controls, as described previously. Briefly, an Acuson 128XP/10v (Acuson Corporation) equipped with a 7.0-MHz linear-array transducer was used to obtain B-mode ultrasound images. The following wall segments were bilaterally scanned over a length of 10 mm: the common carotid artery, the carotid bulb, and the internal carotid artery. Images were saved as JPEG image files, and a reader, blinded for the genetic status of the patient, measured the IMT of the far wall of the respective segments. The mean combined outcome of the 6 segments was used for analysis. A vascular event was defined by the presence of at least 1 of the following: acute myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting, and angina pectoris, as well as by the presence of peripheral or cerebrovascular disease. Cardiovascular events were ascertained by use of questionnaires and verified by studying the clinical records.

Statistical Analysis

Results are expressed as means (SD), except for triglycerides, lipoprotein(a), and hs-CRP levels, which are expressed as medians (interquartile range) because of a skewed distribution. These variables were log-transformed before statistical analysis. Differences
in terms of demographic and lifestyle characteristics between heterozygotes for \textit{LCAT} gene mutations and family controls were evaluated with linear or logistic regression analyses with generalized estimating equations in the SAS procedure GENMOD to account for correlations within families. For differences in blood pressure, smoking, and alcohol use, we made adjustments in a multivariate model for age and gender. To evaluate differences between the 2 groups in biochemical characteristics and mean carotid IMT, we used the same SAS procedure, allowing for clustering within families (due to clustering of genetic and/or environmental factors). For the biochemical characteristics, we adjusted for age, gender and smoking using multivariate models. For mean carotid IMT, the main outcome of this study, a more elaborate procedure was used. We first explored univariately the relation between mean carotid IMT and baseline variables. Hereafter, using multivariate models, we identified independent predictors after stepwise backward selection. For all generalized estimating equation models, the exchangeable correlation structure was used. The difference between controls and \textit{LCAT} heterozygotes was tested by assessing their interaction (age and group). Probability values < 0.05 were considered significant. For statistical analyses the SAS package (release 8.02; SAS Institute Inc) was used.

\textbf{Results}

\textit{Genetic and Demographic Characteristics of the Study Groups}

A total of 47 heterozygotes for \textit{LCAT} gene mutations and 58 controls (ascertained by genotyping) were recruited from 5 families of Dutch descent originating from different parts of The Netherlands. Mean average age was nearly identical among the heterozygotes and controls (42.2 versus 41.9 years of age, respectively; Table 1). Males were slightly more prevalent among the heterozygotes (62\%) than among controls (47\%), but this did not reach statistical significance ($P = 0.062$). Systolic and diastolic blood pressure and

\begin{table}[h]
\centering
\caption{Demographic and Lifestyle Characteristics and \textit{LCAT} Activity Levels of Heterozygotes for \textit{LCAT} Gene Mutations and Family Controls}
\begin{tabular}{lccccc}
\hline
 & Controls (n=58) & Heterozygotes (n=47) & P(unadjusted) & P(Adjusted)* \\
\hline
Age, y & 41.9±16.1 & 42.2±17.4 & 0.848 & ... \\
Male gender, n (%) & 27 (47) & 29 (62) & 0.062 & ... \\
Diastolic blood pressure, mmHg & 81.8±21.9 & 88.8±25.9 & 0.183 & 0.303 \\
Systolic blood pressure, mmHg & 131.6±24.0 & 126.5±34.5 & 0.951 & 0.909 \\
Smoking, n (%) & 8(14) & 10(21) & 0.007 & 0.015 \\
Alcohol users, n (%) & 41 (71) & 33 (72) & 0.805 & 0.580 \\
LCAT activity, nmol*ml⁻¹*h⁻¹ & >25 & 14-18 & ... & ... \\
\hline
\end{tabular}
\end{table}

*P values for the variables blood pressure (diastolic and systolic), smoking, and alcohol use were adjusted for age and sex. †\textit{LCAT} levels as measured against an apoA-I– containing liposome substrate. Data were derived from previous publications.16,18,19,21
alcohol use did not differ among the groups. The percentage of smokers, however, was significantly higher in the heterozygotes than in the controls (21% versus 14%; \( P = 0.015 \)). One heterozygous carrier and 2 subjects in the control group had been prescribed a statin. No patient had diabetes mellitus, defined as use of oral antidiabetic drugs or insulin. The data on LCAT activity levels represent data from previous publications.\(^{16,18,19,21}\) In these reports, the LCAT mutations in the respective families have been shown to result in 30% to 42% reductions in LCAT activity, with concomitant 30% to 38% reductions in HDL-C levels in the heterozygous carriers compared with family controls.

**Lipids, (Apo)lipoproteins, and C-Reactive Protein Levels**

Table 2 gives the raw data accompanied by probability values adjusted for age, gender, smoking, and family. Compared with family controls, heterozygotes exhibited a mean 36% decrease in HDL-C levels and a mean 22% decrease in apoA-I levels (\( P < 0.0001 \) for both). Mean triglyceride levels were, in contrast, increased by 23% (\( P = 0.026 \)). In the presence of unchanged total cholesterol levels, the heterozygotes also displayed a significant 7.1% increase in LDL-C levels (\( P < 0.0001 \)), with a concomitant nonsignificant 7.5% increase in apoB levels. Finally, heterozygotes presented with a marked 2.2-fold increase in C-reactive protein (CRP) plasma levels (\( P < 0.0001 \)).

**IMT and Cardiovascular Events**

Mean IMT values were 0.591 \pm 0.08 mm for controls and 0.623 \pm 0.13 mm for heterozygotes (Table 3). After adjustment for age, gender, use of alcohol, and family, this difference in average IMT proved to be highly statistically significant (\( P = 0.0015 \)). This probability value was obtained with multivariate backward stepwise regression analysis, whereby age, gender, and use of alcohol remained in the model but smoking and blood pressure did not. Although the family controls did not experience cardiovascular events, 1 male
heterozygote had angina pectoris at age 50 years and a myocardial infarction at age 54 years, and a second heterozygote had a myocardial infarction at age 58 years.

Discussion

It has long been difficult to assess the risk of atherosclerosis in individuals with genetically determined low HDL-C levels. This knowledge, however, can provide important insight when it comes to targeting HDL metabolism to reduce CVD risk. After almost 40 years of LCAT research, the present study provides evidence that heterozygotes for LCAT gene defects, who present with an average 36% decrease in HDL-C levels, exhibit an increased risk for atherosclerosis as assessed by IMT measurements. These data suggest that intact LCAT function is important in the protection against atherosclerotic vascular disease.

**LCAT, Lipids, and Lipoproteins**

The present analysis confirms the strong impact of LCAT gene mutations on HDL-C levels. All LCAT gene defects under study have previously been shown to underlie marked loss of LCAT activity (30% to 42% compared with family controls), and as a direct consequence, to result in 30% to 38% reductions in HDL-C levels\(^\text{16,19,21}\) (for a review, see Pritchard and HILL\(^\text{13}\)). LCAT is primarily active at the phospholipid monolayer of nascent HDL particles, where it converts cholesterol into cholesteryl ester on activation by its cofactor, apoA-I. Even a single change in the amino acid sequence of LCAT has been shown to result in a decreased interaction with apoA-I, with a consequential decrease in LCAT activity.\(^\text{26}\) This will, in turn, cause defective maturation of small HDL into larger, spherical, cholesteryl ester–enriched HDL and hence lower HDL-C levels. The data show that this effect is not counteracted by upregulation of the unaffected LCAT allele in heterozygotes or by other key players in HDL metabolism. Additionally, the present study shows the effect of LCAT mutations on plasma triglycerides levels, as has been reported previously.\(^\text{20,27}\)

Although heterozygotes presented with a moderate 22% increase in triglyceride levels, homozygotes presented with a marked 337% increase in triglyceride levels (data not shown). Mild hypertriglyceridemia is also observed in other genetic HDL deficiencies.\(^\text{28,29}\)

For LCAT, hepatic triglyceride overproduction and reduced lipoprotein lipase activity, as found in LCAT-deficient mice, may explain this phenotype.\(^\text{30}\) Finally, we identified a small but significant increase in LDL-C levels compared with controls, for which we have no explanation.

<table>
<thead>
<tr>
<th>Table 3. Carotid IMT and CVD Events for Heterozygotes for LCAT Gene Mutations and Family Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Mean carotid IMT, mm</td>
</tr>
<tr>
<td>Cardiovascular events, n (%)</td>
</tr>
</tbody>
</table>

*Adjusted for all demographic and lifestyle characteristics that stayed in the model with backward stepwise regression analysis (age, gender, and alcohol use).
**LCAT and Atherosclerosis**

Despite the fact that LCAT has long been recognized as a key regulator of HDL metabolism, the role of this enzyme in human atherogenesis has remained controversial. Prospective studies that have assessed LCAT activity or LCAT concentration at baseline are nonexistent, whereas cross-sectional observational studies have reported increased and decreased LCAT activity in subjects with CVD.\(^1\)\(^2\) Initially, the paradoxical finding of complete HDL deficiency and the reported absence of CVD in LCAT-deficient patients has been used to reject the hypothesis that HDL is important in the protection against atherosclerosis. Potential mechanisms to explain these findings were subsequently postulated, such as preferential clearance of HDL fractions that have less atherogenic potential.\(^3\)\(^3\) In addition, decreased LDL-C and apoB levels have also been put forward as an explanation for the lack of marked CVD. In the large group of LCAT mutation carriers in the present study, however, LDL-C was clearly not decreased, which refutes this argument. Studies focusing on the role of LCAT in human atherosclerosis have thus far been hampered by the paucity of clinical events in small numbers of individuals with LCAT mutations. The advent of validated surrogate markers for atherosclerosis has proved very useful in this context. IMT measurements have been used to show that carriers of ABCA1 and apoA-I defects are at increased risk for coronary artery disease,\(^8\)\(^3\)\(^4\)\(^3\) which illustrates the power of this tool to study small, interesting groups of patients. The present study now shows that heterozygotes for LCAT mutations also have increased atherosclerosis compared with family controls. This finding was not based on a few heterozygous outliers with a very thick carotid intima-media complex but on solid IMT data. This is illustrated by the fact that the 30 heterozygotes of the largest family (family 3) presented with a thicker IMT than their 45 family control members (0.614 versus 0.589 mm). This effect, however, did not reach statistical significance, which illustrates the need to recruit and study more families with the same rare genetic disorder to answer our primary research question. During the preparation of this report, Ayyobi et al\(^3\) also reported vascular abnormalities in a small cohort of 9 heterozygotes for 1 specific LCAT mutation. The investigators could not, however, study family controls, and because of the small number of study subjects, these investigators could not draw conclusions regarding the risk for atherosclerosis.\(^3\) A potential but dangerous drawback of studying atherosclerosis in relatively small cohorts, such as investigated here, is the referral basis of the index patients. In this respect, we would like to emphasize that the probands of the currently studied families came to our attention because they experienced corneal opacifications, or they were referred because of previously identified low HDL-C levels. This excludes a bias for selecting those families with LCAT mutations with an increased or established risk for CVD. Exactly how reduced LCAT function affects IMT, however, cannot be deduced from the present study. LCAT mutations may directly compromise reverse cholesterol transport by reducing the flux of cholesterol from cholesterol-loaded peripheral macrophages to the HDL fraction. The markedly reduced HDL-C levels in carriers also may affect endothelial function directly.\(^3\)\(^7\) Alternatively, loss of LCAT activity may cause enhanced oxidation of LDL.\(^3\)\(^8\)
Chapter 10

**LCAT and Inflammation**

Elevated levels of CRP are an established predictor for coronary artery disease, but there is much debate about whether this plasma factor plays an active role in atherosclerosis or whether it merely represents an innocent bystander. We were surprised to find that hs-CRP levels were 2.2-fold increased in the heterozygotes. From the present study, however, it can not be appreciated whether increased levels of CRP are a direct consequence of reduced LCAT activity or of the consequentially reduced HDL-C levels. Pirro and colleagues also reported significantly higher CRP levels in other subjects with hypoalphalipoproteinemia, which suggests a proinflammatory status in subjects with low HDL-C levels per se.

**Concluding Remarks**

Upregulation of LCAT function has been proposed as an HDL-C increasing therapy, but its atheroprotective effects have been questioned. The present study suggests that increasing LCAT activity may reduce atherosclerosis progression, at least in subjects with subnormal levels of LCAT. Efforts to develop LCAT protein therapy have failed, but LCAT gene therapy protocols are more promising, as recently illustrated by decreased atherosclerosis in dyslipidemic obese mice after adenovirus-mediated transfer of the LCAT gene.

Also, Zhang et al recently showed that a similar strategy increased reverse cholesterol transport in hamsters but, interestingly, not in mice. The present report indicates that increasing LCAT activity in plasma might be speculated to result not only in increased HDL-C levels but also in decreased concentrations of triglycerides and hs-CRP. Moreover, increased LCAT activity might also result in an augmented capacity of HDL to exhibit antioxidative, antiinflammatory, and antithrombotic effects.

**Acknowledgements**

We thank all participants of this study for their cooperation. Moreover, we thank Joris Butzelaar for his work as a reader of the IMT files, and Kobie Los, who drew blood samples from the participants.
Increased carotid IMT in LCAT mutation carriers

References


Carriers of Lecithin: Cholesterol Acyltransferase Gene Mutations have Accelerated Atherogenesis as Assessed by Carotid 3T-MRI

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Abstract

Objectives. Aim of this study was to investigate the role of reduced Lecithin:cholesterol acyltransferase (LCAT) function on atherogenesis using 3 Tesla carotid magnetic resonance imaging (MRI) and B-mode ultrasound.

Background. The role of low high-density lipoprotein cholesterol (HDL-c) as a causal factor in atherogenesis has recently been questioned. LCAT plays a key role in HDL-c metabolism.

Methods. Carotid 3.0 Tesla MRI and B-mode ultrasound measurements were performed in 40 carriers of LCAT gene mutations and 40 controls, matched for age. Patients with cardiovascular disease were excluded.

Results. Carriers had 31% lower LCAT activity levels and 38% decreased HDL-c levels (both p<0.001 vs. controls). Carriers presented with a 10% higher normalized wall index (0.34±0.07 vs 0.31±0.04, p=0.002), a 22% higher mean wall area (17.3±8.5mm² vs 14.2±4.1mm², p=0.01), and a 22% higher total wall volume (1039±508mm³ vs 851±247mm³, p=0.01 vs. controls) as measured by MRI. The prevalence (20 vs 5, p=0.002) and the total volume (102mm³ vs 3mm³) of atherosclerotic plaque components on MRI relating to lipid-rich tissue or calcification were also higher in carriers than controls. All differences retained significance after adjustment for age, gender, blood pressure, LDL-c, BMI, smoking and family history of cardiovascular disease. Common carotid intima-media thickness measured with ultrasound was increased in carriers by 12.5% (0.72±0.33mm vs 0.64±0.15mm, p=0.14).

Conclusion. Carriers of LCAT gene mutations exhibit increased carotid atherosclerosis, indicating an increased risk for cardiovascular disease. The present findings imply that raising LCAT activity may be an attractive target in cardiovascular prevention strategies.
Introduction

A low plasma high-density lipoprotein cholesterol (HDL) level is among the strongest risk factors for cardiovascular disease (CVD)\(^1\). One of the mechanisms by which HDL is considered to convey atheroprotection is the removal of excess cholesterol from lipid-laden foam cells in the artery wall and transport it to the liver for fecal excretion, a process referred to as “reverse cholesterol transport”\(^2\). A crucial enzyme in HDL metabolism is lecithin:cholesterol acyl transferase (LCAT)\(^3,4\). This plasma enzyme, produced in the liver and small intestine, is predominantly associated with HDL and esterifies free cholesterol using apolipoprotein A-I (apoA-I) as a cofactor\(^3\). Homozygotes for deleterious mutations in the LCAT gene are characterized by near complete HDL-c deficiency (~90% reduction), while heterozygotes have profoundly reduced HDL-c levels (~40% reduction) compared to normal\(^5-8\).

To date, the relation between LCAT and atherosclerosis has been a matter of debate. Animal studies have not been able to provide a clear answer, as both LCAT knockout models as well as LCAT overexpression models yielded mixed results with respect to atherogenesis as was recently reviewed\(^9\). Human studies have also been conflicting. Hovingh et al. reported that carriers of LCAT gene mutations have increased carotid intima-media thickness (IMT as quantified by B-mode ultrasound imaging) compared to family controls\(^10\). In contrast, Calabresi et al. recently reported that carotid IMT was decreased in carriers while using the same ultrasound methodology\(^11\). These contradictory outcomes are difficult to interpret and may result from population differences. Furthermore, a limitation of previous studies is that carotid ultrasound lacks statistical power to reliably measure arterial wall thickness in small population studies, since ultrasound provides two dimensional longitudinal images, where atherosclerosis is a three dimensional eccentric developing disease. Magnetic resonance imaging (MRI) might overcome these imaging limitations, as it enables transverse three dimensional imaging of atherosclerosis at high resolution with excellent interscan reproducibility\(^12\).

In the present study, we set out to assess the relationship between LCAT and carotid atherosclerosis using carotid 3.0 Tesla MRI, in parallel to carotid B-mode ultrasound imaging, comparing carriers of LCAT mutations and controls. We hypothesized that carriers of LCAT gene mutations had increased atherosclerosis compared to controls.

Methods

Study Design

In this study the extent of carotid atherosclerosis in subjects with LCAT gene mutations and age-matched controls was compared. The study was conducted at the Academic Medical Center in Amsterdam, The Netherlands from October 2008 to October 2009.
The study protocol was reviewed and approved by the institutional review board and all subjects gave written informed consent. The 3 probands of the families in which we identified LCAT mutations, had presented to the ophthalmologists with corneal clouding, after which they were referred to our lipid clinic. Subsequently, we performed genetic testing in their family members to identify subjects with LCAT mutations. Carriers of molecularly diagnosed LCAT mutations (DNA and LCAT activity) were enrolled in this study, irrespective of their age and gender. Probands with cardiovascular disease and their family members were excluded. For the control group, family members of the included carriers were asked to participate in the study, comprising first, second or third degree family members or spouses. They were included if they could be matched for age with a carrier. As insufficient numbers of unaffected family controls (N=19) volunteered, we complemented the control group with unrelated controls (N=21) recruited by advertisement. Exclusion criteria for both carriers and controls were history of CVD, prior carotid surgery or any contraindication for MRI.

Questionnaire, Biometric and Biochemical Measurements
Presence of cardiovascular risk factors, use of medication and family history of CVD, were assessed by a questionnaire. Brachial artery blood pressures were measured using an oscillometric blood pressure device (Omron 705IT). Presence of hypertension was defined as a systolic blood pressure (SBP) >140 mmHg, a diastolic blood pressure (DBP) >90 mmHg or use of antihypertensive medication. Weight and length were measured to calculate body mass index (BMI).
EDTA plasma obtained through venous blood samples were obtained after overnight fasting and stored using standardized protocols. Plasma total cholesterol, HDL-c and triglyceride levels were analyzed using a commercially available enzymatic methods (Westburg, USA) on a Cobas Mira autoanalyzer (Roche, Switzerland). Low-density lipoprotein cholesterol (LDL-c) levels were calculated using the Friedewald equation. LCAT activity was measured using a proteoliposome substrate as previously described10.

Carotid Magnetic Resonance Imaging
Scans were obtained in a 3.0 Tesla Philips whole-body scanner, using a single-element microcoil with a diameter of 5cm. Cardiac gated axial T1-weighted Turbo Spin Echo image stacks were acquired at end-diastole using double inversion recovery preparation and active fat suppression. Sequence parameters were: slice thickness 3 mm, imaging matrix size 240, FOV of 60 x 60 mm, non-interpolated pixel size 0.25 x 0.25 mm, TE 9 ms, TR according to the subjects’ heart rate (approximately 900 ms), echo train length 7, echo train duration 63 ms. To localize the left and right common carotid artery and carotid bifurcation, axial Magnetic Resonance Angiography (MRA) images were acquired using a Time of Flight (TOF) sequence. These images together with projection images were used for positioning the scan planes perpendicular to the vessel at a predefined distance.
LCAT mutation carriers have increased atherosclerosis assessed by MRI

distal to the flow divider. Ten slices were scanned of the distal 3.0 cm of the left and right common carotid artery. The slices were located from 9 mm to 39 mm proximal to the carotid flow divider. Each carotid was scanned individually. A total of 20 images were obtained per scan. All images were saved in DICOM format. Standardized equipment and protocols were used for image storage and data management. The imaging protocol and image analysis have been described previously.\textsuperscript{12,13} Quantitative image analysis was performed using semi-automated measurement software (VesselMass, Leiden University Medical Center, The Netherlands).\textsuperscript{14} One reader analyzed all the images, blinded for group and any other data of the participants. Mean wall area (MWA), lumen area (LA), outer wall area (OWA), and total wall volume (TWV) were measured. Normalized wall index (NWI) was calculated as:

$$\text{NWI} = \frac{\text{MWA}}{\text{OWA}}.$$  

Also prevalence of plaque components (PC) and total PC volume (mm$^3$) was assessed. PC was defined as a T1-weighted image on which an area of decreased signal intensity within the artery wall was identified. Previous studies have shown that areas in the artery wall with decreased signal intensity on T1-weighted images represent either lipid-rich tissue or calcification.\textsuperscript{15} Prevalence of PC was reported as the total number of images per group that showed PC. Also the volume of PC's was quantified, and reported as the sum of all PC volumes of all subjects per group.

**Carotid Ultrasound Imaging**

Carotid B-mode ultrasound scans of the left and right common, bulb and internal carotid arterial far walls were assessed in a single-angle imaging protocol, with the transducer axis parallel to a virtual ear-to-ear line, according to our standardized protocol as previously described.\textsuperscript{16} One experienced and certified sonographer performed all scans, and one reader analyzed all the images, blinded for group and any other data of the participants. Images were analyzed quantitatively off-line by one certified image analyzer using validated software (eTrack, Academic Medical Center, The Netherlands). The primary ultrasound parameter was defined as mean common carotid intima-media thickness (CCIMT, defined as the average far wall IMT of the left and right distal 1 cm of the common carotid artery). A secondary ultrasound endpoint entailed the mean carotid IMT (IMT), defined as the average far wall IMT of the left and right common, bulb and internal carotid arterial wall segments.

**Outcome Parameters**

Normalized wall index (NWI) was the primary outcome parameter of the study. A priori, based on previous study data and assuming a two-sided $\alpha$ of 0.05 and a $\beta$ of 0.2 (power of 80%), we calculated a sample of at least 38 subjects per group was required to detect a 0.02 difference in NWI between groups. Secondary MRI outcome parameters were MWA (mm$^2$), and TWV (mm$^3$). Secondary ultrasound outcome parameters were CCIMT.
(mm), and IMT (mm). Exploratory endpoints were PC prevalence (n) and total PC volume (mm³) assessed by MRI.

Statistical Analysis

Continuous variables are expressed as means ± standard deviations (SD), unless otherwise specified. Differences in demographic, biometrical and biochemical parameters between carriers of LCAT gene mutations and controls were assessed using unpaired Student’s t-tests or Chi square tests, where appropriate. Differences in carotid imaging parameters between carriers of LCAT gene mutations and controls were assessed using unpaired Student’s t-tests, unless otherwise specified. In addition, a multivariate model was used with generalized estimating equations in the SAS procedure GENMOD to account for age, gender, hypertension (SBP >140 mmHg, DBP >90 mmHg or use of antihypertensive medication), LDL-c, BMI, smoking, family history of CVD and correlations within families due to clustering of genetic and/or environmental factors. To compare the agreement between MRI and US scans within patients we assessed the intraclass correlation coefficients (r) and mean paired difference between MWT (MRI) and CCIMT (ultrasound). Statistical analyses were done using SPSS (Statistical Package for the Social Sciences) version 16.0 and SAS package (SAS Institute Inc.). The authors had full access to the raw data and take responsibility for its integrity.

Results

Population Characteristics

We studied 40 carriers of LCAT gene mutations (from 14 families of Dutch descent) and 40 age-matched controls of which 19 were family members and 21 were unrelated individuals. Mutations in the LCAT gene can either cause loss of enzymatic activity on only HDL (α-activity), or loss of activity on both HDL and LDL (α- and β-activity respectively)⁸. Clinically, this translates into two different autosomal recessive disorders: fish eye disease (FED, only loss of α-activity) and familial LCAT deficiency (FLD, loss of both α- and β-activity)⁵⁻⁷. Whereas both FED and FLD patients present with low HDL-c, only FLD patients also exhibit lower LDL-c levels⁵⁻⁷. Of the carriers, 38 had one mutant LCAT allele, while 2 were homozygotes for a defect which underlied clinically manifest FED (corneal opacification). Thirty-three out of the 40 carriers had a mutation of which it is known that it causes FED when present on both alleles. Four individuals were heterozygotes for a mutation which is known to cause FLD when present on both alleles. Finally, 3 subjects carried LCAT gene point mutations of which it is unknown whether they cause FED or FLD when present on both alleles (no homozygous patients described). Table 1 summarizes the demographic, lifestyle, and clinical characteristics of carriers and controls. Age, gender, smoking, alcohol use, blood pressure, diabetes, fasting glucose level, fasting insulin level, Homeostatic Model Assessment index (HOMA) index, hypertension and the Framingham risk score were similar. BMI tended to be higher in the carriers, but this
LCAT mutation carriers have increased atherosclerosis assessed by MRI

Table 1. Characteristics in Carriers of LCAT Gene Mutations and Controls.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Carriers of LCAT gene mutations (n=40)</th>
<th>Controls (n=40)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42.4 (13.0)</td>
<td>42.3 (14.1)</td>
<td>0.97</td>
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<td>Male sex, n (%)</td>
<td>27 (68)</td>
<td>23 (58)</td>
<td>0.36</td>
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<td>Body Mass Index (kg/m²)</td>
<td>25.7 (4.0)</td>
<td>24.5 (3.5)</td>
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<td>Smokers, n (%)</td>
<td>6 (15)</td>
<td>6 (15)</td>
<td>1.0</td>
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<td>Alcohol use (units per week)</td>
<td>5.8 (5.5)</td>
<td>7.8 (7.9)</td>
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<td>Medication use, n (%)</td>
<td></td>
<td></td>
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<tr>
<td>Statin</td>
<td>11 (28)</td>
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<tr>
<td>Ezetimibe</td>
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<td>0.08</td>
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<td>Fibrate</td>
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<td>Blood pressure</td>
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<tr>
<td>Systolic (mmHg)</td>
<td>131 (13)</td>
<td>128 (13)</td>
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<tr>
<td>Diastolic (mmHg)</td>
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<td>76 (9)</td>
<td>0.26</td>
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<td>Hypertension, n (%)</td>
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<td>6 (15)</td>
<td>0.26</td>
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<td>Glucose metabolism</td>
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<tr>
<td>Fasting glucose (mmol/L)</td>
<td>5.2 (0.8)</td>
<td>5.3 (0.9)</td>
<td>0.49</td>
</tr>
<tr>
<td>Fasting insulin (mU/L)</td>
<td>5.1 (8.9)</td>
<td>5.2 (7.3)</td>
<td>0.94</td>
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<td>HOMA index</td>
<td>1.2 (2.1)</td>
<td>1.5 (2.4)</td>
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<td>Diabetes, n (%)</td>
<td>0 (0)</td>
<td>1 (3)</td>
<td>0.31</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>175.4 (44.1)</td>
<td>190.8 (41.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dL)</td>
<td>126.5 (33.4)</td>
<td>123.6 (31.4)</td>
<td>0.69</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>34.1 (13.9)</td>
<td>54.4 (16.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>104.4 (80.8 – 146.2)</td>
<td>82.3 (55.3 – 126.1)</td>
<td>0.06</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dL)</td>
<td>103.1 (25.6)</td>
<td>100.7 (23.8)</td>
<td>0.68</td>
</tr>
<tr>
<td>Apolipoprotein A-I (mg/dL)</td>
<td>119.9 (31.9)</td>
<td>150.6 (25.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LCAT activity (nmol· mL⁻¹ · h⁻¹)</td>
<td>9.24 (2.95)</td>
<td>12.85 (2.92)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Framingham Risk Score</td>
<td>3.9 (4.6)</td>
<td>3.1 (4.6)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Values are indicated as mean ± SD unless otherwise indicated. Male sex, smokers, medication use, hypertension, diabetes: p for X² test; for other parameters: p for Students’ t-test. LCAT is lecithin:cholesterol acyltransferase, HOMA index is Homeostatic Model Assessment index, hypertension was defined as systolic blood pressure > 140 mmHg, diastolic blood pressure > 90 mmHg or use of antihypertensive medication. For triglycerides we report median and interquartile range; P for T-test after log-transformation. Framingham Risk Score shows the 10-year risk for coronary heart disease.

was not statistically significant. Also more lipid lowering medication, especially statins, and ascals were prescribed in the carriers.

Table 1 also gives the results of lipid, (apolipoproteins and LCAT activity measurements. Carriers had 8% lower total cholesterol (p<0.04) which could be attributed to a 38% reduction of HDL-c levels (p<0.001) with similar LDL-c levels in both groups. Plasma
triglyceride levels were 27% higher in carriers compared to controls (p<0.06). Apo B levels were identical while carriers had 20% lower apo A-I levels (p<0.001). LCAT activity levels were 31% lower in carriers compared to controls (p<0.001).

**Carotid MRI and Ultrasound**

Mean values (±SD) of MRI and ultrasound parameters are shown in Table 2. NWI, the primary endpoint of this study, was significantly increased in carriers compared to controls (p=0.02), shown in Figure 1. Statistical corrections for differences in age, gender, hypertension, LDL-c, BMI, smoking, family history of cardiovascular disease (CVD) and clustering of genetic and/or environmental factors in families rendered stronger

<table>
<thead>
<tr>
<th>3.0 Tesla MRI</th>
<th>Carriers of LCAT gene mutations (n=40)</th>
<th>Controls (n=40)</th>
<th>P1</th>
<th>Adj. P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NWI</td>
<td>0.34 (0.07)</td>
<td>0.31 (0.04)</td>
<td>0.02</td>
<td>0.002</td>
</tr>
<tr>
<td>MWA (mm²)</td>
<td>17.3 (8.5)</td>
<td>14.2 (4.1)</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>TWV (mm³)</td>
<td>1039 (508)</td>
<td>851 (247)</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>LA (mm²)</td>
<td>32.5 (6.7)</td>
<td>31.3 (5.1)</td>
<td>0.32</td>
<td>0.72</td>
</tr>
</tbody>
</table>

**Plaque Composition Analysis**

| Total PC volume (mm³) | 102 | 3 |

**B-Mode ultrasound**

| CCIMT (mm) | 0.72 (0.33) | 0.64 (0.15) | 0.19 | 0.14 |
| IMT (mm) | 0.75 (0.36) | 0.69 (0.23) | 0.39 | 0.53 |

P1 for the unadjusted model, P2 for multivariate model adjusting for age, gender, BMI, hypertension, LDL-cholesterol, smoking status, family history of cardiovascular disease (CVD) and accounting for clustering of genetic and/or environmental factors in families, *P for X² test. LCAT is lecithin:cholesterol acyltransferase. NWI is normalized wall index, MWA is mean wall area, TWV is total wall volume, LA is lumen area, PC is plaque component. CCIMT is the mean common carotid intima media thickness. IMT is the average mean intima media thickness of the common, bulb and internal carotid arteries.

**Figure 1.** Comparison of Carotid Atherosclerosis between Carriers of LCAT Gene Mutations and Controls. The mean and 95% confidence intervals for normalized wall index (NWI) measured by 3.0 Tesla MRI are shown for each group. P value is adjusted for age, gender, BMI, hypertension, LDL-cholesterol, smoking status, family history of cardiovascular disease (CVD) and clustering of genetic and/or environmental factors in families.
LCAT mutation carriers have increased atherosclerosis assessed by MRI

statistical significance (p=0.002). NWI in unaffected family controls was similar to that of unrelated controls (0.30 ± 0.04 versus 0.31 ± 0.05, p=0.57). We also assessed differences in plaque composition. The prevalence of plaque component related to lipid-rich tissue or calcification (PC prevalence) was 25% higher (20 versus 6) and total PC volume was 34 times larger in carriers than in controls. Ultrasound CCIMT, and IMT were increased in carriers, but these differences did not reach statistical significance. There was excellent agreement between MWT (MRI) and CCIMT (ultrasound), with an intraclass correlation coefficient of 0.91 (95%CI 0.86 – 0.94, p<0.001), and a mean paired difference of 0.01 (SD 0.11) mm.

Discussion

the present study shows that carriers of LCAT gene mutations exhibit increased carotid artery wall thickening as assessed by 3.0 Tesla MRI compared to age-matched controls. This finding bear clinical relevance since carotid artery wall thickening is associated with an increased risk of cardiovascular events17,18. Whereas previous carotid ultrasound studies were unable to bring consensus on the impact of LCAT gene mutations on carotid atherosclerosis10,11, the current MRI data lend support to the concept that decreased LCAT function, as a result of LCAT gene mutations, is associated with accelerated atherogenesis.

The aim of our study was to test the hypothesis that decreased LCAT function is associated with accelerated atherogenesis. To this purpose, we investigated two parameters of atherosclerosis with MRI: arterial wall thickening, and presence of plaque components. First, the data show that the carriers of LCAT gene mutations have thickened carotid artery walls compared to controls with significant increases of NWI, MWA and TWV. These differences remained statistically significant after adjustments for age, gender, hypertension, LDL-c, BMI, smoking, family history of cardiovascular disease (CVD) and clustering of genetic and/or environmental factors in families. Second, carriers presented with a 32% increased prevalence of PC and 16.5 times larger total PC volume compared to controls. These are features of carotid artery plaques which have been associated with increased CV event rates17,18. Combined, these findings point towards accelerated atherogenesis in individuals with reduced LCAT function.

We also assessed atherogenesis by means of carotid ultrasound IMT measurements. Whereas IMT parameters tended towards an increase in carriers, the differences did not reach statistical significance. The latter most likely pertains to lack of power, as attested to by the significant MRI findings. In fact, we previously showed that the measurement variability of carotid 3.0 Tesla MRI is less compared to that of ultrasound IMT12.

Various studies have attempted to unravel the relation between LCAT function and CVD in humans. Recent genome wide-association studies (GWAS) revealed that LCAT
correlates to HDL-c levels, but not to CVD risk\cite{19,20}. The prevalence of single nucleotide polymorphisms (SNPs) in the population, however, is low and it is unknown if these SNPs actually affect LCAT function. Moreover, total variation in HDL-c explained by LCAT SNPs was very small. Therefore, GWAS may not be the most sensitive technique to detect a relation between LCAT and CVD. In cross-sectional studies in patients with either angiographically documented CAD or acute myocardial infarction, both decreased and increased LCAT activity have been observed\cite{21-23}. A recent prospective nested case-control study studied LCAT concentration in 2785 healthy subjects with a follow-up of 6 years\cite{24}. In this study, LCAT levels did not differ between cases and controls. However, since the variation of LCAT concentration in this population was small, a potential contribution of LCAT to CVD risk may have been overcome by other risk factors, such as diabetes mellitus, smoking, blood pressure, BMI and LDL-c.

Two prior imaging studies have addressed the relation between LCAT and atherosclerosis in carriers of LCAT gene mutations using carotid IMT. Hovingh \textit{et al.} showed that carotid IMT was significantly increased in the carriers\cite{10}, while Calabresi \textit{et al.} showed the opposite with carotid IMT being significantly decreased in carriers\cite{11}. This apparent discrepancy may be explained by differences in the populations. The carriers in the current study and in Hovingh’s study predominantly had a different type of LCAT mutation than those in the study of Calabresi. Both in Hovingh’s as well as the present study, the vast majority of carriers of LCAT gene mutations exhibited loss of α-activity (LCAT activity on HDL), whereas Calabresi \textit{et al.} predominantly investigated individuals with loss of function mutations of α- and β-activity (LCAT activity on HDL and LDL). Accordingly, LDL-c levels in the current study were 23% higher compared to those in Calabresi’s study (127 mg/dl vs 103 mg/dl respectively). In fact, the average LDL-c of the patients studied by Calabresi was on target of the National Cholesterol Education Program (NCEP) ATPIII guidelines. In fact, the absence of the primary trigger of atherosclerosis, that is increased LDL-c, in Calabresi’s study may be an important explanation why they did not observe increased atherogenesis in their familial LCAT deficiency patients.

To date, it has been unclear how to monitor and treat carriers of LCAT gene mutations. Whereas ideally a prospective randomized controlled trial is required to settle this issue, this type of evidence is unlikely to become available given the rareness of the disease. Considering the present data combined with the fact that decreased levels of HDL-c are strongly associated with CVD risk, we propose to closely monitor as well as treat CVD risk factors in both heterozygous as well as homozygous patients with LCAT gene mutations. The current study results support a distinct role of LCAT in atherogenesis. Whether this effect relates to the effects on HDL-c or to e.g. the anti-inflammatory properties\cite{25,26} that are attributed to LCAT, cannot be determined by the current study.
LCAT mutation carriers have increased atherosclerosis assessed by MRI

Limitations
A limitation inherent to this type of small cohort studies is referral bias of the examined individuals. Carriers and family controls were recruited with the same method, while unrelated controls were recruited by advertisement. Nonetheless, related and unrelated controls were similar in terms of NWI, so it is unlikely that differences in recruitment methods introduced bias. Furthermore, we have attempted to minimize this effect by excluding patients with pre-existent CVD and included only carriers identified in families of which the probands were asymptomatic for CVD. These probands presented either with marked corneal clouding, or low HDL-c levels identified through (random) screening for CVD risk factors.

Conclusions
The present study shows that carriers of LCAT gene mutations have increased carotid atherosclerosis compared to controls. Our data have two clinical implications. First, as carriers of LCAT gene mutations have experienced lifelong exposure to marked dyslipidemia and the current data suggest that they are at increased risk of developing atherosclerosis, close monitoring and treatment of CVD risk factors is advocated. Second, based on these data it is tempting to speculate that increasing LCAT activity is an interesting target to reduce cardiovascular risk27,28.

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Chapter 11

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LCAT mutation carriers have increased atherosclerosis assessed by MRI


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Chapter 12

Submitted for publication

Patients with low HDL-cholesterol caused by mutations in \textit{LCAT} have increased arterial stiffness

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* these authors contributed equally
Abstract

A low high-density lipoprotein cholesterol level and increased arterial stiffness are both strong predictors of cardiovascular disease. Carriers of mutations in LCAT, encoding lecithin:cholesterol acyl transferase, are exposed to lifelong low high-density lipoprotein cholesterol levels. Because structural imaging studies of atherosclerosis were inconclusive, we investigated functional alterations in large arteries by measuring arterial stiffness by carotid-femoral pulse wave velocity in 45 carriers of LCAT mutations (mean age±SD 46±13 yrs) and 45 age-matched controls. Probands referred with established cardiovascular disease were excluded.

In carriers, high-density lipoprotein cholesterol was lower (32±12 vs. 59±16 mg/dl; p<0.0001) and triglycerides higher (median 116 [IQR 80-170] vs. 71 [IQR 53-89] mg/dl; p<0.001) vs. controls. Pulse wave velocity was higher in carriers vs. controls (7.9±2.0 m/s vs. 7.1±1.6 m/s; p<0.01). This difference retained significance in multivariate analysis after adjustment for age, sex, systolic blood pressure and body mass index, and also after exclusion of matched pairs of which carrier or control had cardiovascular disease. Also, pulse wave velocity was correlated with wall thickening of the carotid arteries assessed by 3.0 Tesla magnetic resonance imaging and ultrasound in carriers (Pearson’s R 0.54; p<0.001 and 0.50; p<0.001, respectively), in controls (R 0.58, p<0.001 and 0.36, p<0.04, respectively), and in the groups combined (R 0.56, p<0.001 and 0.47, p<0.001, respectively).

In conclusion, pulse wave velocity is increased in patients with low high-density lipoprotein cholesterol levels due to LCAT mutations and is also associated with carotid wall thickening. It may be a useful adjunct to assess cardiovascular risk in these patients.
Arterial stiffness is increased in LCAT deficiency

Introduction
A low plasma level of high-density lipoprotein cholesterol (HDL-c) is a strong and independent predictor of cardiovascular disease (CVD)\(^1\)\(^2\). Carriers of mutations in LCAT, encoding lecithin:cholesterol acyl transferase, are exposed to lifelong low HDL-c levels. LCAT is a crucial enzyme in HDL metabolism produced in the liver and small intestine.\(^3\)\(^4\) Upon secretion into the circulation, it associates predominantly with HDL where it esterifies free cholesterol using apolipoprotein A-I (apoA-I) as a cofactor\(^4\). Carriers of LCAT mutations have been reported to suffer from increased atherosclerosis but this is not undisputed. In this respect, two ultrasound studies reached opposite conclusions, one study reporting increased intima media thickness (IMT) in the carotid arteries of carriers as a surrogate outcome for atherosclerosis\(^5\) and the other reporting decreased IMT in carotid arteries of carriers\(^6\). Using 3.0 T magnetic resonance imaging of the carotid arteries, we recently found support for the conclusion of the first study (see Chapter 11 of this thesis). However, whether the observed atherosclerotic structural changes in LCAT mutation carriers are associated with functional alterations of large arteries is undetermined. In carriers of ABCA1 mutations, who also suffer from low HDL-c levels, endothelial function of the brachial artery as assessed by flow mediated dilation was reduced and infusion of reconstituted HDL (rHDL) restored endothelial function in these patients\(^7\). Also in patients with type 2 diabetes and diabetic dyslipidemia, rHDL infusion restored endothelial function\(^8\). This direct temporary improvement in vascular tone by HDL infusion is probably mediated by induction of endothelial nitric oxide synthase via the HDL-receptor scavenger receptor B-1\(^9\).

In the present study, we examined the effect of reduced LCAT function on long-term functional alterations of large arteries by assessment of arterial stiffness, another strong and independent predictor of cardiovascular disease (CVD)\(^10\)\(^-\)\(^13\). We studied carotid-femoral PWV, the gold standard of non-invasive measurement of arterial stiffness\(^14\), in patients with LCAT gene mutations and age-matched controls. In addition to our primary study objective, we also examined the association between arterial stiffness and structural changes in large arteries by assessment of wall thickening of the carotid arteries using B-mode ultrasound (intima media thickness [IMT]) and 3.0 Tesla magnetic resonance imaging (MRI).

Methods

Study design and participants
The design of this study has been described in detail in Chapter 11. In brief, the study was conducted at the Academic Medical Center in Amsterdam, The Netherlands from October 2008 to October 2009. The study protocol was approved by the local institutional review board and all subjects provided written informed consent. Patients molecularly diagnosed with LCAT mutations were enrolled in this study, irrespective of their age and...
sex. In order to limit referral bias, we excluded family probands who were referred to our outpatient clinic with clinically manifest CVD. For the control group, unaffected family members of the included carriers were asked to participate in the study, comprising first, second or third degree family members or spouses. These controls were included if they could be individually matched for age to carriers. As insufficient numbers of family controls volunteered, the control group was complemented with unrelated controls recruited by advertisement. Family history of CVD, presence of cardiovascular risk factors, use of medication and alcohol were assessed. Presence of hypertension was defined as a systolic blood pressure (SBP) >140 mmHg, a diastolic blood pressure (DBP) >90 mmHg or use of antihypertensive medication.

**Blood pressure and arterial stiffness**

Participants visited the hospital after an overnight fast and were asked to refrain from smoking (if applicable) at least three hours before the visit. All measurements were carried out in supine position after 15 minutes rest in a quiet, temperature-controlled room. All hemodynamic measurements were performed by a single investigator (BvdB) who was blinded for the genetic status of the participants. Brachial blood pressure was measured 3 times at 1-minute intervals in supine position at the right arm after 15 minutes rest using a validated oscillometric device (Omron 705IT) in a quiet and temperature controlled room. The mean of the last 2 measurements was used for analysis. Measurements of carotid-femoral pulse wave velocity (PWV) were performed with the SphygmoCor system (Atcor Medical Pty Ltd, West Ryde, Australia). Pulse waveforms were recorded at the right carotid and femoral artery sequentially. Wave travel distance was calculated by subtracting carotid artery – suprasternal notch distance from suprasternal notch – femoral artery distance. Measurements were conducted in duplicate and means were used for analysis.

**Carotid ultrasound and magnetic resonance imaging**

Ultrasound scans of the carotid arterial wall were assessed as surrogate outcome for atherosclerosis according to a standardized protocol. Values given are means of left and right common carotid artery, carotid bulb and internal carotid. Bilateral 3.0 T MRI scans of the carotid arteries were obtained as previously in Chapter 11. Normalized wall index (NWI) represents mean vessel wall area normalized for the transverse size of the vessel, measured as the outer wall area. Carotid IMT and NWI were assessed during the same hospital visit as PWV.

**Plasma lipids**

Blood was obtained after overnight fasting and stored using standardized protocols. Plasma total cholesterol, HDL-c and triglyceride levels were analyzed using a commercially available enzymatic method (Westburg, USA) on a Cobas Mira autoanalyzer (Roche,
Arterial stiffness is increased in LCAT deficiency

Switzerland). Low density lipoprotein cholesterol (LDL-c) levels were calculated using the Friedewald equation.

**Statistical analysis**

Data are expressed as means±standard deviations (SD), median (interquartile range [IQR]) or numbers and percentages where appropriate. Differences between carriers and controls were assessed by comparison of continuous data: independent t-tests for parametric data and Mann-Whitney for non-parametric data; chi-square test was applied to compare categorical data. Missing data were imputed with the mean values of the group. Correlations of PWV, cIMT and NWI are expressed as Pearson’s correlation coefficient. A multivariate model was used with generalized estimating equations in the SAS procedure GENMOD to account for potential confounders, i.e. age, gender, SBP and body mass index (BMI) and correlations within families due to clustering of genetic and/or environmental factors, using stepwise backward elimination. Statistical analyses were done using SPSS (Statistical Package for the Social Sciences) version 16.0 and SAS package version 9.1 (SAS Institute Inc., Cary, NC USA). The authors had full access to the raw data and take responsibility for its integrity.

**Results**

**Population characteristics**

We studied 45 carriers of LCAT gene mutations (from 15 families of Dutch descent) and 45 age-matched controls of which 19 were family members and 26 were unrelated individuals. Of the carriers, 43 had one mutant LCAT allele, while 1 was homozygous for T147I and one was compound heterozygous for T147I and V333M. Both the homozygote and the compound heterozygote had previously presented with corneal opicification and HDL deficiency, without the presence of proteinuria, and had thus been diagnosed with fish eye disease (FED).

Table 1 summarizes the demographic, lifestyle, and clinical characteristics of carriers and controls. Proper matching for age was achieved for carriers of an LCAT mutation and controls. The percentage of males did not differ between the two groups. On average, carriers had a 1.9 kg/m2 higher BMI (p<0.02). More carriers had experienced cardiovascular events and received statin treatment than controls, and carriers tended to receive antihypertensive treatment more frequently than controls. Systolic/diastolic blood pressures were 135±15 / 79±9 mmHg for LCAT mutation carriers and 131±13 / 77±9 mmHg for controls (p=0.12 / p=0.19). HDL-c levels in carriers of LCAT mutations were lower compared to controls (32±12 vs. 59±16 mmol/l, p<0.0001), while LDL-c levels were identical (125±35 vs. 125±31 mmol/l, p=0.65). Triglycerides were higher in carriers of LCAT mutations (116 [IQR 80-170] vs. 71 [IQR 53-89] mmol/l, p<0.001).
Pulse wave velocity in carriers of LCAT mutations and age-matched controls

PWV was higher in carriers of a mutation in LCAT compared to controls, 7.9±2.0 vs. 7.1±1.6 m/s (p<0.01), see Figure 1. In a multivariate regression model that adjusted for age, sex, SBP, BMI and family clustering, this difference retained statistical significance (p<0.01). After exclusion of matched pairs of which the carrier (n=6) and/or control (n=1) had suffered from CVD, the PWV of the 38 remaining carriers remained significantly higher compared to the respective matched controls (7.7±2.0 vs. 6.9±1.6 m/s, p<0.05). Again, this difference retained significance after adjustment for age, sex, SBP and family clustering in the multivariate regression model (p<0.01).

PWV and carotid wall thickening assessed by ultrasound and 3.0 T MRI

In a random set of 36 carriers and 36 controls individually matched for age, carotid wall thickening had also been assessed by ultrasound (cIMT) and by using 3.0 T MRI scanning. (see Chapter 11), cIMT tended to be higher in carriers (0.85±0.08 mm) compared to controls (0.70±0.04 mm, p = 0.07). NWI was significantly higher in carriers (0.34±0.08) compared to controls (0.31±0.04, p = 0.04). Table 2 and Figure 2 show that PWV correlated well with both cIMT and carotid NWI obtained by MRI. We observed strong correlations in carriers: Pearson’s R for PWV to cIMT: 0.50, p<0.001, Pearson’s R for PWV to NWI: 0.54, p<0.001. In controls PWV was also correlated to cIMT and NWI: Pearson’s R for PWV to cIMT: 0.36, p<0.04, and for PWV to NWI: Pearson’s R 0.58, p<0.001.

Table 1. Clinical characteristics of LCAT mutation carriers compared to unaffected controls

<table>
<thead>
<tr>
<th></th>
<th>LCAT mutation carriers</th>
<th>Matched controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>46 ± 13</td>
<td>45 ± 14</td>
<td>0.83</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>35 (78%)</td>
<td>29 (64%)</td>
<td>0.16</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.3±4.1</td>
<td>24.6±3.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>7 (16%)</td>
<td>5 (11%)</td>
<td>0.54</td>
</tr>
<tr>
<td>History of CVD, n (%)</td>
<td>6 (13%)</td>
<td>1 (2%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>17 (38%)</td>
<td>13 (29%)</td>
<td>0.37</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>135 ± 15</td>
<td>131 ± 13</td>
<td>0.12</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>79 ± 9</td>
<td>77 ± 9</td>
<td>0.19</td>
</tr>
<tr>
<td>Statin users, n (%)</td>
<td>16 (36%)</td>
<td>1 (2%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total Cholesterol, mg/dl</td>
<td>172 ± 47</td>
<td>191 ± 35</td>
<td>0.05</td>
</tr>
<tr>
<td>LDL-c, mg/dl</td>
<td>125 ± 35</td>
<td>125 ± 35</td>
<td>0.75</td>
</tr>
<tr>
<td>HDL-c, mg/dl</td>
<td>32 ± 12</td>
<td>59 ± 16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>116 [IQR 80-170]</td>
<td>71 [IQR 53-89]</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are indicated as means ± SD or median [IQR] unless otherwise indicated.
Arterial stiffness is increased in LCAT deficiency.

**Figure 1.** Carotid-femoral pulse wave velocity in carriers of an LCAT mutation and matched controls.

Boxed values are means ± SD for PWV.

*Difference retained significance in multivariate analysis, independent of age, sex, systolic blood pressure and BMI and correlations within families due to clustering of genetic and/or environmental factors, and also after exclusion of 6 matched pairs with members with a history of CVD.

**Figure 2.** Scatter plot of PWV to IMT (carotid ultrasound) and NWI (carotid MRI)

2a: Carotid-femoral PWV plotted to carotid IMT. Continuous line indicates correlation between PWV and cIMT in controls (open symbols; n=36), Pearson’s R = 0.36 (p < 0.04); dashed line indicates correlation between PWV and cIMT in LCAT mutation carriers (closed symbols; n = 36), Pearson’s R = 0.50 (p < 0.001) (see Table 2 for combined analysis).

2b: Carotid-femoral PWV plotted to carotid NWI, assessed by 3.0 T MRI. Continuous line indicates correlation between PWV and NWI in controls (open symbols; n=36), Pearson’s R = 0.58 (p<0.001); dashed line indicates correlation between PWV and NWI in LCAT mutation carriers (closed symbols; n=36), Pearson’s R = 0.54 (P < 0.001) (see Table 2 for combined analysis).
Discussion

In this well matched case-control study, we demonstrate that aortic pulse wave velocity is increased in LCAT mutation carriers compared to controls, indicative of increased arterial stiffness in these patients. This difference retained significance in multivariate analysis and after exclusion of patients with CVD. In addition to our primary study objective, this study also reveals a strong correlation between arterial stiffness and thickness of the carotid arterial wall as assessed by ultrasound (IMT) and 3.0 T MRI.

Relevant to the question whether LCAT is a feasible target for HDL-enhancing strategies is the matter of atherogenesis in carriers of LCAT mutations. Two ultrasound studies have reached opposite conclusions.

In order to more dynamically investigate the condition of large arteries in carriers of LCAT mutations in our center, we assessed carotid-femoral pulse wave velocity, considered the gold standard of non-invasive arterial stiffness measurements. The observed increase in arterial stiffness in LCAT mutation carriers might result from accelerated atherosclerosis in these patients, which in turn might be caused by decreased reverse cholesterol transport from the vascular wall due to the impaired maturation of HDL. We did, however, not observe a correlation of PWV with HDL-c (not in carriers, nor in controls nor in the groups combined; data not shown), although this might be explained by large standard deviations in both parameters. Previously, PWV was found to be inversely related to HDL-c levels. In a population-based study, 122 middle-aged subjects with low HDL-c levels had significantly higher PWV independent of age, sex, physical activity and smoking status, compared to 795 subjects with normal HDL-c levels. In a cross-sectional study among postmenopausal women, HDL-c was also reported to be inversely and independently related to PWV. However, neither of these two studies adjusted for SBP, an important determinant of PWV.

Our study shows increased levels of plasma triglycerides in carriers compared to controls, a finding that has also been reported by others and which is possibly caused...

<table>
<thead>
<tr>
<th>Normalized Wall Index (MRI)*,†</th>
<th>Carotid IMT (ultrasound)*,‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWV LCAT mutation carriers (n = 36)§</td>
<td>0.54 (&lt;0.001)</td>
</tr>
<tr>
<td>PWV matched controls (n=36)§</td>
<td>0.58 (&lt;0.001)</td>
</tr>
<tr>
<td>PWV carriers and controls combined§</td>
<td>0.56 (&lt;0.001)</td>
</tr>
</tbody>
</table>

Values are Pearson's correlation coefficients R, with respective p-values in brackets. § For 9 of 45 matched controls in which PWV was assessed, no MRI or ultrasound data were available. Respective matched carriers were excluded from correlation analysis, rendering 36 matched pairs. † Average NWI in 36 carriers: 0.34 (SD 0.08), in 36 controls: 0.31 (SD 0.04), p for T-test = 0.04 (see Chapter 11). ‡ Average carotid IMT in 36 carriers: 0.85 mm (SD 0.08), in 36 controls: 0.70 mm (SD 0.04), p for T-test = 0.07 (see Chapter 11). * NWI was very strongly correlated to IMT: in combined analysis, R was 0.84, P< 0.0001. This was previously demonstrated (see Chapter 11).
Arterial stiffness is increased in LCAT deficiency by increased de novo lipogenesis in the liver. However, in a recent population-based study, PWV was not associated with triglycerides, making it unlikely that the increase in triglycerides contributes to the increased PWV in carriers. Lastly, we identified a slight increase in BMI in carriers. However in multivariate analysis, BMI did not affect the relation between LCAT genotype and PWV. This is in accordance with a systematic review, indicating that risk factors other than age and blood pressure contribute only modestly to arterial stiffness.

Arterial stiffness predicts CVD independently of traditional cardiovascular (CV) risk factors. In a Danish population-based study of 1968 participants, PWV significantly improved CV risk prediction, especially in subjects with low estimated CVD risk. In a recent analysis of the Framingham Heart Study, CVD risk was increased by 48% per standard deviation increase in PWV.

In a recent editorial, Wilkinson et al postulate three hypotheses concerning the nature of the relation between arterial stiffness and CVD. First, the relationship might be causal: arterial stiffness might promote CVD independent of other CV risk factors, by causing: 1) increased wave reflection, leading to elevated central aortic systolic pressure and pulse pressure which in turn increases left ventricular work load and (2) altered hemodynamics and shear stress, stimulating the formation of atherosclerotic plaques. Secondly, PWV could reflect the integrated consequences of established risk factors over time. Thirdly, PWV might simply reflect the burden of atherosclerotic plaque in the arterial wall. In this respect, we observed strong relationships between arterial stiffness and carotid wall thickness assessed by ultrasound and MRI, in line with previous observations in the general population and in patients with type 2 diabetes mellitus. To study the basis of the relationship between PWV and CVD, large prospective studies are called for.

Two aspects of our study merit closer consideration. First, to minimize potential referral bias in our case control study, we excluded LCAT gene carriers who were referred to our outpatient clinic with a history of CVD. We only included carriers identified in families of which the probands were asymptomatic for cardiovascular disease. The use of statins and antihypertensive medication is considerably higher in LCAT mutation carriers, and since both statins and antihypertensive drugs have shown to decrease PWV, the actual difference in PWV might even be larger between carriers and controls.

Perspectives

In addition to statins and antihypertensive medication, increasing HDL-c through lifestyle modifications or pharmacological treatment might also reduce arterial stiffness by improvement of cholesterol efflux, reduction of inflammation or improvement of endothelial function. In future studies, it would be of interest to examine whether treatment specifically aimed at increasing HDL-c will affect arterial stiffness.
Chapter 12

This first report of a strong relation between PWV and arterial wall thickness assessed by MRI underscores the interrelation between arterial stiffness and arterial wall thickening in atherogenesis and provides a solid basis for large prospective studies designed to characterize the role of arterial stiffness in atherogenesis.

In conclusion, carriers of LCAT mutations characterized by low HDL-c levels have increased arterial stiffness compared to unaffected controls. This study supports close clinical monitoring of cardiovascular risk factors in carriers of LCAT mutations. Because PWV is a reproducible, non-invasive and readily applicable functional measure of arterial stiffness, it may be an useful method to assess and monitor the increased CVD risk in these patients. Lastly, our data bolsters the notion that LCAT might be an interesting target to reduce cardiovascular risk and supports LCAT enhancing strategies currently evaluated in preclinical studies\textsuperscript{31-33}.

\textit{Funding sources}

Part of the study was sponsored with an educational research grant by Merck Sharp and Dohme (MSD, USA) and by a grant from the Dutch Heart Foundation (2008B070). A.G. Holleboom is supported by a grant of the Netherlands Organisation for Scientific Research (NWO; project number 021.001.035). Dr. Kastelein is a recipient of the Lifetime Achievement Award (2010) of The Dutch Heart Foundation (2010 T082).
Arterial stiffness is increased in LCAT deficiency

References


Arterial stiffness is increased in LCAT deficiency


Chapter 13

Submitted for publication

Lipid Oxidation in Carriers of LCAT Gene Mutations

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Chapter 13

Abstract

**Background.** Lecithin:cholesterol acyltransferase (LCAT), a key enzyme in high-density lipoprotein (HDL) metabolism, hydrolyzes oxidized acyl groups from phospholipids. We investigated whether this affects lipid oxidation in man by studying carriers of functional LCAT mutations.

**Methods.** In 4 carriers of two mutant LCAT alleles, 63 heterozygotes and 63 family controls, we measured activities of LCAT, paraoxonase 1 (PON1) and platelet-activating factor-acetylhydrolase (PAF-AH); levels of arachidonic (AA) and linoleic acid (LA) and their oxidized derivatives; oxidized phospholipids on apolipoprotein (apo) B-, A-I- and (a)- containing lipoproteins (oxPL/apoB, oxPL/apoA-I and oxPL/apo(a)); IgM and IgG autoantibodies to malondialdehyde (MDA) and IgG and IgM apoB-100-immune complexes (IC/apoB); and the potential of HDL to neutralize oxidized low-density lipoprotein.

**Results.** In patients with LCAT mutations, HDL-cholesterol, apo A-I, arachidonic acid and its oxidized derivatives, oxPL/apo(a), activities of LCAT, PON1 and PAF-AH on HDL, and the anti-oxidative capacity of HDL were gene-dose-dependently decreased, the latter effect being independent of PON1- and PAF-AH-activities. OxPL/apoB were 17% increased in heterozygotes (p<0.001) but not in carriers of two defective LCAT alleles.

**Conclusion.** Carriers of LCAT mutations present with markedly reduced plasma activities of LCAT and PAF-AH, and a reduced anti-oxidative potential of HDL, but this was not accompanied by severe lipid oxidation in these patients, suggesting that alternative mechanisms, such as the clearance of oxidized LDL from plasma by scavenger receptors, may more strongly determine the level of plasma lipid oxidation than HDL-associated anti-oxidative enzymes.
Introduction

Decreased levels of high-density lipoprotein cholesterol (HDL-c) are strongly associated with the risk of coronary artery disease (CAD). HDL is thought to exhibit anti-atherogenic properties through its role in reverse cholesterol transport. However, HDL also exerts anti-oxidant properties through the actions of its associated proteins and enzymes, which may provide additional atheroprotection. These include paraoxonase-1 (PON1), platelet-activating factor acetyl hydrolase (PAF-AH) and lecithin:cholesterol acyltransferase (LCAT). In the current study, we have focused on the role of LCAT in lipid oxidation and in determining the antioxidant capacity of HDL.

Oxidized phospholipids (OxPL) are key mediators of further lipid oxidation and inflammation. Importantly, the detrimental biological effects of OxPL are attenuated upon removal of the sn-2 oxidized fatty acids, a reaction that can be catalyzed by LCAT. This enzyme is secreted by the small intestine and the liver and is primarily associated with HDL in the circulation. It catalyzes the hydrolysis of the acyl group at the sn-2 position of phosphatidylcholine (PC or lecithin) and the subsequent transfer and esterification of this acyl group to the hydroxyl group of free cholesterol, thereby maturing the HDL pool and generating most of the cholesteryl esters present in human plasma. Via its phospholipase-A_2 (PLA_2) activity, LCAT can also hydrolyze oxidized acyl chains from PC-based oxidized phospholipids (OxPL), generating less bioactive lysophosphatidylcholine and an oxidized free fatty acid which it subsequently used by LCAT to transesterify diacylglycerol to generate triglyceride. Several in vitro and animal studies have shown that through its PLA_2 activity, LCAT is capable of diminishing oxidative stress by hydrolyzing oxidized sn-2 fatty acids from OxPL.

In humans, rare naturally occurring mutations in LCAT result in loss of enzymatic LCAT activity and consequentially low HDL-c levels. It is, however, not known whether a loss of LCAT activity and reduced HDL-c levels increase lipid oxidation in carriers of LCAT mutations. We hypothesize that the combined loss of LCAT activity and HDL would result in an increase in lipid oxidation. To test this, a) we assessed the activity of anti-oxidative enzymes, b) measured oxidized lipoproteins and oxidized phospholipids per apolipoprotein fraction, and c) measured free radical oxidation products of arachidonic acid and linoleic acid. In addition, we studied the anti-oxidant capacity of HDL of subjects with no, one or two defective LCAT alleles.

Methods

Study Design

Homoyzgous, compound heterozygous and heterozygous carriers of molecularly diagnosed LCAT mutations (DNA and LCAT activity) were enrolled in this study, irrespective
of their age and gender. For the control group, family members of the included patients were asked to participate in the study, comprising first, second or third degree family members or spouses. Informed consent was obtained for blood sampling, storage, genetic and biochemical analysis. The study was approved by the Institutional Review Board of the Academic Medical Center in Amsterdam.

**Plasma lipids and LCAT activity**

Serum and EDTA plasma were obtained through venous blood samples after an overnight fast; tubes were directly placed on ice. Plasma was isolated by centrifugation at 4°C, 3000g for 15 minutes and stored at -80°C for further analyses. Blood samples were collected between August 2008 and October 2009. All plasma analyses were conducted between November 2009 and November 2010.

Total cholesterol, low-density lipoprotein (LDL) cholesterol, HDL-c, triglycerides, and apolipoproteins A-I and B were measured using a COBAS MIRA analyser. LCAT activity was measured using a proteoliposome substrate as described. Levels of lipoprotein(a) (Lp(a)) were measured by quantitative immunoprecipitation analysis with commercially available antiserum (Diasorin, Stillwater, MN, USA).

**HDL preparation**

The HDL preparation from subjects’ serum was performed by precipitation as described.

**Dichlorofluorescein (DCF) assay**

The antioxidant/anti-inflammatory properties of HDL were tested in the presence or absence of LDL as described previously with some modifications. LDL was isolated from normal plasma by ultracentrifugation as described. Dichlorofluorescin diacetate (DCFH-DA, Molecular Probes/Invitrogen, Carlsbad, CA, USA) was dissolved in fresh methanol at 2.0 mg/ml and incubated at room temperature for 20 min in the dark, resulting in the release of DCFH. On interaction with lipid peroxidation products DCFH forms DCF, producing intense fluorescence. Normal HDL inactivates oxidized LDL and therefore prevents the oxidation of DCFH and release of DCF. To determine the functional properties of HDL, patient and control HDL was used in this assay in the presence or absence of air-oxidized LDL. HDL (final concentration 50 μg cholesterol/ml) in the presence or absence of oxidized LDL (final concentration 100 μg cholesterol/ml) was added into a black 96-well plate in a final volume of 100μl. The plate was incubated at 37°C on a rotator for 1 h in the dark. At the end of this incubation period, 10μl of DCFH solution (0.2 mg/ml) was added to each well, mixed, and incubated for an additional 2 hours at 37°C with rotation in the dark. Fluorescence was measured with a plate reader (Fluo-Star Galaxy, bMG) at an excitation wavelength of 465 nm and an emission wavelength of 535 nm.
Paraoxonase-1 activity
PON1 activity in HDL, prepared by the dextran-Mg\(^{2+}\) method, was determined using paraoxon as substrate. Briefly, the assays were performed in a final volume of 250 µL containing 5µL of HDL, 5.5 mmol/L paraoxon, 2 mmol/L CaCl\(_2\) and 100 mmol/L Tris-HCl, pH 8.0. The rate of p-nitrophenol formed by the hydrolysis of paraoxon was measured by monitoring the increase in absorbance at 405nm for 25min at room temperature in a microplate spectrophotometer. PON1 activity was expressed as U per L of HDL. 1U is the activity that catalyzes the formation of 1µmol p-nitrophenol per min.

PAF-acetylhydrolase activity
1-O-hexadecyl-2-[\(^{3}\)H-acetyl]-sn-glycero-3-phosphocholine (Hexadecyl PAF, [acetyl-\(^{3}\)H], 0.1 mCi/ml, specific activity range 10-30 Ci/mmol) was purchased from Perkin Elmer (Waltham, MA, USA). Unlabeled PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) was from Sigma Aldrich. PAF-AH activity in HDL, prepared by the dextran-Mg\(^{2+}\) method, was measured by the trichloroacetic acid (TCA) precipitation procedure using \(^{3}\)H-PAF (100 mM final concentration, specific activity 15000 cpm/nmol) as a substrate. Fifty microlitres of HDL diluted 1:3 (v/v) with HEPES buffer (pH 7.4) were mixed with HEPES in a final volume of 90 µl and used as the source of the enzyme. Incubations were performed for 10 min and PAF-AH activity was expressed as nmole PAF degraded per min per ml of HDL.

Oxidized phospholipids per apolipoprotein, apoB-immune complexes and autoantibodies to oxidized LDL
OxLDL biomarkers were measured by ELISA, as previously described. All samples for a given analysis were obtained in a single assay. Each sample was assayed in triplicate, and data are expressed as relative light units (RLU) in 100 milliseconds. The levels of circulating oxPL/apoB, oxPL/apoA-I and oxPL/apo(a) were measured as described, using a sandwich ELISA of anti-apolipoprotein antibodies and the murine monoclonal antibody E06, which binds oxidized but not native phospholipids. Titers of IgG and IgM autoantibodies binding to malondialdehyde (MDA)-LDL were determined at 1:200 dilutions of plasma as previously described. Apolipoprotein B-100 (apoB-100)-immune complexes (IC/apoB) were measured as previously described.

Arachidonic and linoleic acids and their oxidized derivatives
High performance liquid chromatography (HPLC) with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) was employed to quantify total levels - i.e. free fatty acids, plus esterified fatty acids to cholesterol, phospholipids and triglycerides - of multiple distinct oxidation products of arachidonic acid (AA) and linoleic acid (LA), including individual hydroxy-eicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs), using established methods. Briefly, 10 ng each of two deuterated internal standards, 15(S)-hydroxy-5,8,11,13-eicosatetraenoic-5,6,8,9,11,12,14,15-d8...
acid (15-HETE-d8) and prostaglandin F2α (PGF2α-d4) (Cayman Chemical Company, Ann Arbor, MI) were added to plasma samples, fatty acids were released by base hydrolysis with 1 N NaOH at 60°C for 120 min under argon atmosphere, samples acidified to pH 3.0 with 2 M HCl, and then fatty acids were extracted twice with 4 ml hexane. The combined hexane extracts were dried under N2 flow, resuspended in 200 µl 50% methanol/water (v/v). Samples were injected onto a C-18 column (2 × 250 mm, 5 m ODS, 100A; Phenomenex, Rancho Palos Verdes, CA) at a flow rate of 0.2ml/min. Separations were performed using a gradient starting from 85% methanol over 15 min, then to 100% methanol over 1 min, following by 100% methanol for 15 min. HPLC column effluent was split so that only 50% was introduced into a Quattro II triple quadrupole mass spectrometer (Micromass, Inc., Manchester, UK). Analyses were performed using electrospray ionization in negative-ion mode with multiple reaction monitoring of parent and characteristic daughter ions specific for each isomer monitored. The transitions monitored were mass-to-charge ratio (m/z): m/z 295 → 171 for 9-HODE; m/z 295 → 195 for 13-HODE; m/z 279 → 261 for LA; m/z 319 → 115 for 5-HETE; m/z 319 → 155 for 8-HETE; m/z 319 → 151 for 9-HETE; m/z 319 → 167 for 11-HETE; m/z 319 → 179 for 12-HETE; m/z 319 → 175 for 15-HETE; m/z 303 → 259 for AA; m/z 327 → 182 for 15-HETE-d8. Collision-induced dissociation was obtained using argon gas. The internal standard 15-HETE-d8 was used to for quantification of HETEs, as well as to calculate extraction efficiencies of HODEs and HETEs (which control studies revealed were > 85%). The internal standard PGF2α-d4 was used to for quantification of F2-isoprostanes.

Statistical analyses

All data presented are means with standard deviations (SD), unless otherwise indicated. Parameters were compared between two groups using Student’s T-test for continuous variables and X² test for categorical variables. Analysis of variance was applied to study differences over three groups in case of stepwise differences. In case of a significant difference in univariate analysis, multivariate analysis by linear regression was carried out to assess independence of observed effects. Statistical analyses were performed using SPSS software (version 12.0.2, SPSS Inc., Chicago, Illinois). A P value of <0.05 was considered statistically significant.

Results

Study group characteristics

Two patients with familial LCAT deficiency (FLD) and two patients with fish eye disease (FED) participated. One FED patient was homozygous for the T147I mutation in LCAT, the other compound heterozygous for T147I and V309M. The FLD patients were homozygous for either C337Y or T321M. In addition, 63 heterozygous carriers of LCAT gene mutations and 63 unaffected family controls participated in the study. Mutations in the LCAT gene can either cause loss
of enzymatic activity on only HDL (α-activity), or loss of activity on both HDL and LDL (α- and β-activity respectively). A complete loss of only α-activity underlies FED while a complete loss of both α- and β-activity causes FLD. Thirty-nine out of the 63 heterozygotes had a mutation of which is known that it causes FED when present on both alleles. Twenty individuals were heterozygotes for a mutation which is known to cause FLD when present on both alleles. Four subjects carried LCAT gene point mutations of which it is unknown whether they cause FED or FLD when present on both alleles (no patients homozygous for these mutations described).

Table 1 summarizes the demographic, lifestyle, and clinical characteristics of cases and controls. Age, body mass index (BMI), alcohol use and the percentage smokers were similar heterozygotes and family controls. Among the heterozygotes were more men but this did not reach statistical significance. Heterozygotes experienced more CVD (p<0.01) concordant with a more frequent use of HMG-CoA-reductase inhibitors (statins) compared to controls (p<0.001). HDL-c and apo A-I were gene-dose dependently decreased in carriers of LCAT mutations, while levels of LDL-c, apo B and triglycerides did not differ between the groups. The gene-dose dependent decrease in TC could therefore be attributed to the decrease in HDL-c. Carriers of two defective LCAT alleles presented with severe HDL-c deficiency, and severely reduced lipoprotein(a).

Table 1. Clinical characteristics, lipids and lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Homozygous/compound heterozygous (n=4)</th>
<th>Heterozygotes (n=63)</th>
<th>Family controls (n=63)</th>
<th>P HZ/cht vs. controls</th>
<th>P heterozygotes vs. controls</th>
<th>P for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>34.0 ± 19</td>
<td>44.8 ± 1.7</td>
<td>44.2 ± 2.1</td>
<td>0.69</td>
<td>0.82</td>
<td>N/A</td>
</tr>
<tr>
<td>Males, n (%)</td>
<td>2 (50)</td>
<td>38 (60)</td>
<td>28 (44)</td>
<td>0.92†</td>
<td>0.07†</td>
<td>N/A</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4 ± 4.8</td>
<td>25.6 ± 0.64</td>
<td>24.5 ± 0.55</td>
<td>0.44</td>
<td>0.19</td>
<td>N/A</td>
</tr>
<tr>
<td>Alcohol (units per wk)*</td>
<td>3 (6)</td>
<td>4 (5)</td>
<td>3 (6)</td>
<td>0.59</td>
<td>0.64</td>
<td>N/A</td>
</tr>
<tr>
<td>Current smokers (n, %)</td>
<td>0 (0)</td>
<td>6 (10)</td>
<td>5 (8)</td>
<td>0.72†</td>
<td>0.76†</td>
<td>N/A</td>
</tr>
<tr>
<td>CVD cases, n (%)</td>
<td>0 (0)</td>
<td>7 (11)</td>
<td>1 (2)</td>
<td>0.79†</td>
<td>&lt;0.01†</td>
<td>0.08†</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1‡ (2)</td>
<td>0.15†</td>
<td>0.15†</td>
<td>0.43</td>
</tr>
<tr>
<td>Statin use, n (%)</td>
<td>2 (50)</td>
<td>15 (24)</td>
<td>2 (3)</td>
<td>&lt;0.001†</td>
<td>&lt;0.001†</td>
<td>N/A</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>115 ± 56</td>
<td>178 ± 45</td>
<td>195 ± 41</td>
<td>&lt;0.001</td>
<td>0.034</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>87 ± 40</td>
<td>126 ± 35</td>
<td>130 ± 30</td>
<td>&lt;0.01</td>
<td>0.55</td>
<td>0.041</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>6.4 ± 2.5</td>
<td>33 ± 12</td>
<td>55 ± 20</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)*</td>
<td>89 (63)</td>
<td>118 (114)</td>
<td>92 (80)</td>
<td>0.49</td>
<td>0.11</td>
<td>N/A</td>
</tr>
<tr>
<td>Apo A-I (mg/dl)</td>
<td>31 ± 0.2</td>
<td>126 ± 26</td>
<td>159 ± 33</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apo B (mg/dl)</td>
<td>74 ± 8.6</td>
<td>103 ± 26</td>
<td>100 ± 23</td>
<td>0.12</td>
<td>0.56</td>
<td>N/A</td>
</tr>
<tr>
<td>Lp(a) (mg/dl)*</td>
<td>0.35 (0.1)</td>
<td>1.9 (38)</td>
<td>2.0 (25)</td>
<td>&lt;0.001</td>
<td>0.86</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*median (interquartile range). † P for X2 test. Data for alcohol use, triglycerides and Lp(a) were log-transformed before T-test/ANOVA because of a skewed distribution. N/A not applicable. ‡ diabetes mellitus type 2. P HZ/ cHT vs. controls: P for unpaired two-tailed T-test homozygotes/compound heterozygotes vs. controls.
Chapter 13

**LCAT, PON1 and PAF-AH activities**

Table 2 shows a highly significant gene-dose dependent decrease of LCAT and PAF-AH activities in carriers of *LCAT* mutations (for both, P for ANOVA < 0.001). PON1 activity levels were, however, similar in heterozygotes and controls while carriers of 2 defective *LCAT* alleles showed a 85% reduction in PON1 activity (p<0.001).

**Arachidonic and linoleic acids and their oxidized derivatives**

Loss of LCAT activity was associated with decreased total levels of essential fatty acids (free plus esterified) (Table 3): total plasma levels of arachidonic acid (AA) were gene-

<table>
<thead>
<tr>
<th>Table 2. LCAT, PAF-AH and PON1 activities</th>
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<tbody>
<tr>
<td><strong>Homozygous/compound heterozygous patients</strong></td>
</tr>
<tr>
<td><strong>Heterozygotes</strong></td>
</tr>
<tr>
<td>(n=63)</td>
</tr>
<tr>
<td>LCAT activity (nmol/ mL/ h)</td>
</tr>
<tr>
<td>PAF-AH activity (nmol/min/ml)*</td>
</tr>
<tr>
<td>PON1 activity (U/l)*</td>
</tr>
</tbody>
</table>

* median and interquartile range. Data were log-transformed before statistical analysis because of a skewed distribution. P HZ/cHT vs. controls: P for unpaired two–tailed T test homozygotes/compound heterozygotes vs. controls, P (unadj.): P for unpaired two–tailed T test, P (adj.): P for linear regression model including age and gender as potential confounders, P for ANOVA to assess differences over the three groups.

<table>
<thead>
<tr>
<th>Table 3. Arachidonic and linoleic acids and their oxidized derivatives</th>
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</thead>
<tbody>
<tr>
<td><strong>Homozygous/compound heterozygous patients</strong> (n=4)</td>
</tr>
<tr>
<td><strong>Non-oxidized fatty acids</strong></td>
</tr>
<tr>
<td>Arachidonic acid (nmol/l)</td>
</tr>
<tr>
<td>Linoleic acid (nmol/l)</td>
</tr>
<tr>
<td><strong>Oxidized fatty acids</strong></td>
</tr>
<tr>
<td>5-HETE (nmol/l)</td>
</tr>
<tr>
<td>8-HETE (nmol/l)</td>
</tr>
<tr>
<td>9-HETE (nmol/l)</td>
</tr>
<tr>
<td>11-HETE ((nmol/l)</td>
</tr>
<tr>
<td>12-HETE (nmol/l)</td>
</tr>
<tr>
<td>15-HETE ((nmol/l)</td>
</tr>
<tr>
<td>9-HODE (nmol/l)</td>
</tr>
<tr>
<td>13-HODE (nmol/l)</td>
</tr>
</tbody>
</table>

P HZ/cHT vs. controls: P for unpaired two–tailed T test homozygotes/compound heterozygotes vs. controls (unadj.): P for unpaired two–tailed T test, P (adj.): P for linear regression model including age and gender as potential confounders, N/A not applicable, P for ANOVA to assess differences over the three groups.

* Retained significance after exclusion of 8 participants (7 heterozygotes, 1 control) who had experienced atherosclerotic CVD, † Due to lack of plasma, arachidonic and linoleic acids and their oxidized derivatives could not be measured in three heterozygotes and two family controls.
Anti-oxidation by HDL and lipid oxidation in LCAT deficiency

dependently decreased in carriers of *LCAT* mutations (P for ANOVA p<0.049). Total plasma levels of linoleic acid (LA) were also significantly decreased in heterozygotes compared to controls (P=0.007; P=0.022 after adjustment for age and gender). Carriers of two defective alleles however showed similar levels of LA as the heterozygotes.

Measuring peroxidized AA, we found that all HETEs, i.e. 5-, 8-, 9-, 11-, 12-, 15-HETEs, were significantly reduced in carriers of two defective LCAT alleles compared to controls. For 8-, 9-, 11- and 12-HETEs, levels were also significantly reduced when comparing heterozygotes and controls and for these parameters we observed a gene-dose effect (ANOVA: p=0.008, p=0.024, p=0.006, p=0.005, respectively). For peroxidized LA, we found no significant differences in 9- and 13-HODEs when comparing the carriers of LCAT mutations and controls.

Supplementary table 1 shows that ratios of HETEs/AA and HODEs/LA are not different in heterozygotes and controls. In carriers of two defective LCAT alleles, 9-, 11- and 12-HETEs/AA ratios were significantly lower compared to controls.

### (Auto-)antibodies against oxidized phospholipids and apolipoproteins

Oxidized phospholipids on apo-B-containing lipoproteins were significantly increased in heterozygotes compared to family controls (p = 0.01; Table 4) and remained significantly increased (p=0.017) after correction for age and gender. This difference also retained significance after exclusion of participants who had experienced atherosclerotic CVD (7 heterozygotes and 1 control) (p = 0.030). In a linear regression model with only PAF-AH activity as a potential confounder, the difference also retained significance (p = 0.049). However, significance was lost in a linear regression model including age, gender and PAF-AH activity (p = 0.125). In carriers of 2 defective *LCAT* alleles, however, oxidized phospholipids on apoB-

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>P heterozygotes vs. controls (unadj.)</th>
<th>P heterozygotes vs. controls (adj.)</th>
<th>P for ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCAT activity (nmol/mL/h)</td>
<td>0.45 ± 0.2</td>
<td>9.6 ± 2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PAF-AH activity (nmol/min/ml)*</td>
<td>1.4 (1.8)</td>
<td>3.2 (2.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PON1 activity (U/l)*</td>
<td>7.5 (38)</td>
<td>46 (79)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* median and interquartile range. Data were log-transformed before statistical analysis because of a skewed distribution, P HZ/cHT vs. controls: P for unpaired two–tailed T test homozygotes/compound heterozygotes vs. controls, P (unadj.): P for unpaired two–tailed T test, P (adj.): P for linear regression model including age and gender as potential confounders, N/A not applicable, P for ANOVA to assess differences over the three groups.
containing lipoproteins were similar to controls. Only in carriers of two defective LCAT alleles, oxidized phospholipids on apolipoprotein (a) were markedly decreased (p<0.001; 87% reduction). Oxidized phospholipids on apoA-I containing lipoproteins did not differ between the groups.

We also measured IgG and IgM immune complexes on apoB-containing lipoproteins and IgG and IgM against MDA-LDL (Table 4). Of these 4 parameters, only one parameter was significantly different amongst the groups: IgM immune complexes on apoB-containing lipoproteins were significantly decreased in heterozygotes compared to controls (p=0.03).

### Table 4. (Auto-) antibodies to oxidized phospholipids and apolipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Homozygous/compound heterozygous patients (n=4)</th>
<th>Heterozygotes (63)</th>
<th>Family controls (n=63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized phospholipids/apoB (RLU)*</td>
<td>2.2<em>10^4 (3.1</em>10^3)</td>
<td>2.8<em>10^4 (8.6</em>10^3)</td>
<td>2.4<em>10^4 (6.6</em>10^3)</td>
</tr>
<tr>
<td>Oxidized phospholipids/apoAI (RLU)*</td>
<td>693 (902)</td>
<td>910 (851)</td>
<td>1.0*10^4 (864)</td>
</tr>
<tr>
<td>Oxidized phospholipids/apo(a) (RLU)*</td>
<td>726 (1.1*10^3)</td>
<td>4.4<em>10^3 (3.5</em>10^3)</td>
<td>5.8<em>10^3 (2.4</em>10^3)</td>
</tr>
<tr>
<td>IgG immune complexes/apoB (RLU)*</td>
<td>1.9<em>10^3 (1.1</em>10^3)</td>
<td>1.8<em>10^3 (1.1</em>10^3)</td>
<td>1.9*10^3 (817)</td>
</tr>
<tr>
<td>IgM immune complexes/apoB (RLU)*</td>
<td>1.9<em>10^3 (1.3</em>10^3)</td>
<td>931 (840)</td>
<td>1.2*10^3 (833)</td>
</tr>
<tr>
<td>IgG MDA-LDL (RLU)*</td>
<td>1.9<em>10^3 (1.6</em>10^3)</td>
<td>1.8<em>10^3 (1.2</em>10^3)</td>
<td>1.8<em>10^3 (1.6</em>10^3)</td>
</tr>
<tr>
<td>IgM MDA-LDL (RLU)*</td>
<td>9.3<em>10^3 (7.0</em>10^3)</td>
<td>9.5<em>10^3 (6.4</em>10^3)</td>
<td>1.1<em>10^4 (6.4</em>10^3)</td>
</tr>
</tbody>
</table>

* median and interquartile range. Data were log-transformed before statistical analysis because of a skewed distribution. P HZ/CHT vs. controls: P for unpaired two–tailed T test homozygotes/compound heterozygotes vs. controls. P (unadj.): P for unpaired two–tailed T test. P (adj.): P for linear regression model including age and gender as potential confounders, N/A not applicable, P for ANOVA to assess differences over the three groups † Retained significance after exclusion of 8 participants (7 heterozygotes, 1 control) who had experienced atherosclerotic CVD. ** Retained significance in linear regression model with PAF-AH activity as only potential confounder; significance lost upon inclusion of PAF-AH activity in the model already including age and gender

### Table 5. Anti-oxidative capacity of HDL

<table>
<thead>
<tr>
<th></th>
<th>Homozygous/compound heterozygous patients (n=4)</th>
<th>Heterozygotes (n=63)</th>
<th>Family controls (n=63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-oxidative potential of HDL to LDL (arbitrary units)*</td>
<td>1.3<em>10^2 (7.5</em>10^4)</td>
<td>3.0<em>10^2 (1.1</em>10^4)</td>
<td>2.3<em>10^2 (5.5</em>10^3)</td>
</tr>
</tbody>
</table>

* median and interquartile range. Data were log-transformed before statistical analysis because of a skewed distribution. † degradation of the substrate PAF. P HZ/CHT vs. controls: P for unpaired two–tailed T test homozygotes/compound heterozygotes vs. controls. P (unadj.): P for unpaired two–tailed T test. P (adj.): P for linear regression model including age, gender, activities of PON1 and PAF-AH, and of HDL-c, BMI and statin use as potential confounders. P for ANOVA: to assess differences over the three groups. ** independent of age, gender, activities of PON1 and PAF-AH, and of HDL-c, BMI and statin use. † Retained significance after exclusion of 8 participants (7 heterozygotes, 1 control) who had experienced atherosclerotic CVD
Anti-oxidative capacity of HDL

The anti-oxidative capacity of HDL (‘cell-free assay’,23) was gene-dose-dependently decreased: fluorescence intensity reflecting oxidized LDL was 1.3*10⁵ arbitrary units in carriers of two mutant alleles, 3.0*10⁴ arbitrary units in heterozygotes, and 2.3*10⁴ arbitrary units in controls (Table 5, p<0.001 for all comparisons including ANOVA). Multivariate regression analysis shows that this difference retained significance after adjustments for age, gender, BMI, HDL-c, and statin use (p<0.001). Interestingly, additional corrections for PON1 and PAF-AH activities in plasma did not change this result either (p<0.001). After exclusion of participants who had experienced atherosclerotic CVD, the
anti-oxidative capacity of HDL also remained significantly lower in heterozygous carriers of LCAT gene mutations compared to controls.

Discussion
First proposed by Klimov et al. in 1989\textsuperscript{18}, LCAT can hydrolyze oxidized fatty acids from the sn-2 position of OxPL\textsuperscript{7, 17}, in particular long chain oxidized fatty acids, whereas PAF-AH has been reported to hydrolyze short chain oxidized fatty acids.\textsuperscript{16} In mice deficient for both the LDL-receptor and leptin, LCAT overexpression decreased auto-antibodies to oxidized LDL\textsuperscript{38}, but the role of LCAT in plasma lipid oxidation in humans is unknown. In this case-control study, we therefore investigated lipid oxidation in patients with mutations in the LCAT gene. Compared to family controls, the anti-oxidative capacity of HDL against oxidized LDL was gene-dose dependently reduced in carriers of \textit{LCAT} mutations, and independent of potential confounders. Also, carriers of \textit{LCAT} mutations had decreased HDL associated PAF-AH activity, and heterozygotes had increased levels of circulating OxPL on apoB-containing lipoproteins (OxPL/apo B). However, other parameters of lipid oxidation assessed in this study did not differ between carriers of \textit{LCAT} mutations and controls.

Activities of \textit{PON1} and PAF-AH
In addition to LCAT, two other anti-oxidative enzymes are associated with HDL particles, i.e. PON1\textsuperscript{5} and PAF-AH.\textsuperscript{6} We measured the activities of these two enzymes to study their potential contribution to the anti-oxidative capacity of HDL in carriers of \textit{LCAT} mutations with 30\% reduced LCAT activity and 40\% reduced HDL-c.
In vitro studies have demonstrated that PON1 can protect LDL from undergoing oxidative modification,\textsuperscript{39} but the exact substrate(s) are not known. Some have suggested that PON1, like LCAT and PAF-AH, also hydrolyzes OxPL\textsuperscript{39, 40}, but Marathe et al. reported that PON1 has no phospholipase activity towards OxPL and that previous findings may have been due to contamination with PAF-AH.\textsuperscript{41} Although its natural substrates are not known, PON1 activity has been suggested to influence the development of atherosclerosis and CVD risk.\textsuperscript{5} In a recent prospective general population study of 3,375 subjects, the association of PON1 activity with CVD risk was shown to strongly depend on levels of HDL-c\textsuperscript{42} which may be related to the fact that PON1 in the circulation is almost exclusively associated with HDL.\textsuperscript{5} In line, carriers of two defective LCAT alleles in the present study demonstrate that complete HDL deficiency results in very low PON1 activity. On the other hand, we show that a 40\% reduction in HDL cholesterol in heterozygotes for \textit{LCAT} mutations has no effect on HDL-associated PON1 activity suggesting that HDL may become rate-limiting for PON1-activity only at very low levels of HDL-c. These findings also suggest that PON1 is unlikely to contribute to altered lipid oxidation in heterozygotes for \textit{LCAT} mutations.
PAF-AH – also named lipoprotein-associated PLA$_2$ (Lp-PLA$_2$) - is known to hydrolyze OxPL. There is abundant evidence that HDL-associated PAF-AH has anti-atherogenic and anti-inflammatory properties through its ability to abrogate the biological activity of oxidized LDL, and its capacity to attenuate phospholipid oxidation (for review, see Tselepis et al). The clear gene-dose dependent reduction in the HDL-associated PAF-AH activity in our carriers of LCAT mutations suggests that PAF-AH activity is more sensitive than PON1 activity to disturbances of HDL homeostasis. The reduced PAF-AH activity may contribute to oxidative stress in our patients. Of note, LCAT has been reported to hydrolyze PAF, but at a 200 times slower rate compared to PAF-AH. It is therefore unlikely that the loss of LCAT in the carriers contributed to the decreased hydrolysis of PAF in the PAF-AH activity assay.

The reductions in activities of PAF-AH and PON1 in carriers of two defective LCAT alleles are in notable contrast to observations in patients with genetic PAF-AH deficiency, who have normal HDL-c levels. In these patients, the loss of PAF-AH activity is not accompanied by a decrease in activities of LCAT or PON1 indicating that the disturbed HDL maturation due to LCAT deficiency in our patients underlies the observed decreased activity of PON1 and PAF-AH.

(Auto-)antibodies against oxidized phospholipids and apolipoproteins

To study the relevance of the anti-oxidative PLA$_2$ activity of LCAT in humans, we assessed OxPL on apolipoproteins in sandwich-ELISAs with the specific E06 antibody. OxPL on apoB-containing lipoproteins are important products of free radical oxidation, and major contributors to the proinflammatory properties of oxidized LDL. In the general population, increased levels of OxPL/apoB measured with the E06 antibody were recently found to be associated with increased risk of future coronary artery disease. In our study, OxPL/apoB were higher in the heterozygotes for mutations in LCAT, also after statistical correction for age and gender as possible confounders. Thus, PLA$_2$ activity of LCAT may be relevant for OxPL in humans but the reductions in HDL-associated PAF-AH and 40% decrease of HDL cholesterol could also play a role here. In this respect, a separate linear regression analysis with only PAF-AH activity as potential confounder, showed that the difference on oxPL/apoB between heterozygotes and controls was still statistically significant, but this significance was lost upon inclusion of PAF-AH activity in the previous model already including age and gender.

In our 4 patients with two defective LCAT alleles we did not find a further increase in OxPL/apoB. However, Iitabe et al. previously showed that 5 patients with familial LCAT deficiency had increased plasma levels of LDL-associated OxPL compared to family controls, measured by monoclonal antibody DLH3 which binds a very similar epitope to antibody E06. We have no explanation for this discrepancy.

In contrast to apoB-containing lipoproteins, OxPL on apo(a) containing lipoproteins, significant predictors of atherosclerotic lesion size and development, and of coronary artery disease, were not increased in carriers of one LCAT mutation and were even
decreased in carriers of two defective LCAT alleles. We have no explanation for the first observation, but the latter observation might be explained by severely reduced levels of Lp(a) itself in carriers of two defective LCAT alleles, compared to no significant reduction in heterozygotes. Severe reductions in Lp(a) in patients with LCAT deficiency have been previously reported. For apo(a) to bind to apoB-containing particles and thus form Lp(a), these particles need to contain normal amounts of cholesterol esters. In LCAT deficiency, characterized by absent esterification of free cholesterol in plasma, this binding has been reported to be disturbed resulting in very low Lp(a) levels in these patients. In heterozygotes for LCAT mutations, Lp(a) levels were comparable to those in family controls, implying that modest reduction in LCAT activity does not affect the CE-content of apoB-containing lipoproteins to the extent that apo(a) can no longer normally bind to these lipoproteins.

We also assessed OxPL on apoA-I containing lipoproteins using the same methodology as used for apoB and Lp(a) containing lipoproteins. These did not differ between the groups, which might indicate that LCAT does not contribute significantly to the hydrolysis of OxPL associated with apo A-I containing lipoproteins. Unfortunately, literature on oxidation of phospholipids on HDL is absent to date, making it difficult to interpret these particular results.

In more advanced stages of LDL oxidation, IgG and IgM immune complexes target oxidized LDL epitopes. In our patients with LCAT mutations, we observed no differences in IgG serotypes on LDL compared to family controls. This indicates that the oxidation of LDL in these patients is not increased to advanced stages compared to their family controls. On the other hand, IgM immune complexes were significantly decreased in the heterozygotes which may indicate reduced protection to oxidative stress; these IgM serotype immune complexes have been proposed to protect instead of contribute to oxidation-induced inflammation.(for review, see Lopes-Virella et al). This effect was, however, absent in carriers of two defective alleles.

We also measured antibodies against MDA-LDL, i.e. LDL with MDA bound to apoB of these particles (with MDA in turn derived from oxidized phospholipids on these lipoproteins). MDA-LDL are known to induce a humoral immune response. Both IgM and IgG auto-antibodies against MDA-LDL were not significantly different in subjects with one or two LCAT gene mutations compared to controls, which may indicate that LCAT does not contribute to protection against this form of LDL oxidation. These data need to be interpreted with care however, because we observed large interindividual variations in these parameters.

In summary, heterozygotes for LCAT gene mutations have increased phospholipid oxidation on LDL but not on HDL and Lp(a). A strong reduction of OxPL on Lp(a) in carriers of 2 LCAT gene mutations was related to severe reductions of Lp(a) levels. Analyses of IgG and IgM immune complexes and of MDA-LDL were not indicative of pronounced oxidative stress.
Arachidonic and linoleic acids and their oxidized derivatives

To study whether the reduced activities of the HDL-associated enzymes LCAT and PAF-AH, and the lower HDL-c in carriers of LCAT mutations would be associated with further oxidative stress, we measured plasma levels of multiple specific fatty acid oxidation products. Compared to controls, none of the oxidized derivatives of AA or LA were increased in carriers of one or two defective alleles. On the contrary, we found four of the oxidized derivatives of AA to be reduced in a gene-dose dependent manner. The lower oxidized fatty acids found in our carriers are most probably secondary to the decrease in plasma levels of total AA and LA, from which they are derived. In turn, the lower total plasma levels of AA and LA are most probably due to the reduction in cholesterol esters (CE) caused by the loss of LCAT activity in our patients. The total plasma levels of AA and LA represent the sum of these fatty acids, present as free fatty acids, but also as acyl groups esterified to cholesterol (CE), triglycerides (TG) and phospholipids (PL). In a recent systematic review, Hodson et al estimated from data of a typical lipoprotein composition of 1989 healthy individuals that 19% of total fatty acid in plasma is esterified to cholesterol (CE). Furthermore, they meta-analyzed plasma CE fatty acid composition in 3739 men and 2163 women from 11 studies and found that LA on average account for 52% of all fatty acids esterified to cholesterol. Thus, the reduction in CE can very well explain our observations of modestly lower total LA in our patients. The 30% reduction in AA in our patients (both in carriers of one and two defective alleles) is more difficult to explain, since Hodson et al report that AA accounts for only 5% of all fatty acids esterified to cholesterol, but might be due to altered proportions of fatty acids on cholesterol esters, which has been suggested by earlier studies. After normalisation for total levels of LA or AA, none of the oxidized fatty acids were elevated in LCAT mutation carriers. In conclusion, this analysis does not support the hypothesis of increased oxidative stress in these patients.

Anti-oxidant capacity of HDL

On the basis of the original publication of Navab et al, we have set up an assay that assessed the ability of HDL to inactivate oxidized phospholipids on LDL. Application of this assay, in our cohort of patients with one or two defective LCAT alleles revealed that the HDL of these patients have a strongly decreased ability to inhibit exogenously isolated and oxidized LDL from releasing the fluorescent DCF. In this assay we normalized for HDL cholesterol. The observed decrease might be directly due to a loss of LCAT on HDL. Oxidative modification of phospholipids on HDL itself is unlikely to contribute to this loss in our patients, since levels of oxidized phospholipids on apo A-I containing lipoproteins were unaltered. Also, these effects may be related to gene-dose dependent loss of PAF-AH in our study. However, statistical adjustments for PAF-AH, but also differences in PON1 and other possible confounders, as well as exclusion of subjects treated with statins did not affect the finding that loss of LCAT activity is strongly related to reduced anti-oxidant capacity of HDL in our study. It has been shown that HDL of
Chapter 13

LCAT mutations carriers consists of altered HDL subpopulations with altered composition of apolipoproteins compared to controls. Thus, one can speculate that changes in composition of HDL may affect the antioxidant properties of HDL.

Conclusions and future perspectives

Studying multiple parameters to assess lipid oxidation in carriers of LCAT gene mutations, we only observed a mild increase of OxPL on apo B-containing lipoproteins and a (detrimental) decrease of IgM immune complexes with no indications of further lipid oxidation. These results could be interpreted to parallel the finding that despite a life-long and marked reduction in HDL-c, these patients have only mildly increased atherosclerosis. On the other hand, they are surprising when considering that these patients not only present with significant decreases of LCAT activity which is thought to provide direct anti-oxidative potential (through hydrolysis of OxPL at the sn-2 position of PC), but also with marked reductions of HDL-c, and of apo A-I, which has been implicated to confer anti-oxidative properties itself. On top, a significant and dose-dependent decrease of both PAF-AH and anti-oxidative potential of HDL in carriers of LCAT gene mutations would also be expected to translate in a more severe oxidative phenotype. The apparent mild oxidative status could be related to unchanged PON1 activity levels in carriers of one defective LCAT allele. However, a severe reduction of PON1 in carriers of 2 defective LCAT alleles did not result in severe lipid oxidation in these four patients. Although the reductions in activities of LCAT and PAF-AH observed in our patients coincided with a strong decrease in the potential of their HDL to protect LDL from undergoing oxidative modification ex vivo, our findings in their plasma suggest that alternative mechanisms, such as clearance of oxLDL by CD36 and other scavenger receptors, may be stronger determinants of circulating oxidized LDL and lipid oxidation products than these HDL-associated enzymes.

Acknowledgements

We would like to thank the patients and family members for their participation. HDL research by the groups of Dr. Angeliki Chroni and Dr. Jan Albert Kuivenhoven is supported by a grant of the European Community (FP6-2005-LIFESCIHEALTH-6; STREP contract number 037631) and by Fondation Leducq. Adriaan G. Holleboom is supported by a grant of the Netherlands Organisation for Scientific Research (NWO; project number 021.001.035). George Daniel is supported by the graduate student fellowship program of National Center for Scientific Research “Demokritos”. Dr. Kastelein is a recipient of the Lifetime Achievement Award (2010) of The Dutch Heart Foundation.
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Chapter 13


**Supplementary Table 1.** Oxidized fatty acids normalized for total levels of arachidonic/linoleic acid

<table>
<thead>
<tr>
<th>Compound</th>
<th>Homozygotes/compound (n=4)</th>
<th>Heterozygotes (61)†</th>
<th>Family controls (n=60)†</th>
<th>P heterozygotes vs. controls (unadj.)</th>
<th>P homozygotes vs. controls (unadj.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HETE/AA (mmol/mol)</td>
<td>2.0 ± 0.5</td>
<td>3.3 ± 1.6</td>
<td>2.9 ± 1.3</td>
<td>0.10</td>
<td>0.18</td>
</tr>
<tr>
<td>8-HETE/AA (mmol/mol)</td>
<td>0.9 ± 0.15</td>
<td>1.4 ± 0.52</td>
<td>1.5 ± 0.64</td>
<td>0.39</td>
<td>0.09</td>
</tr>
<tr>
<td>9-HETE/AA (mmol/mol)</td>
<td>1.1 ± 0.18</td>
<td>1.9 ± 0.74</td>
<td>1.9 ± 0.73</td>
<td>1.0</td>
<td>0.047</td>
</tr>
<tr>
<td>11-HETE/AA (mmol/mol)</td>
<td>0.84 ± 0.21</td>
<td>1.6 ± 0.60</td>
<td>1.6 ± 0.58</td>
<td>0.88</td>
<td>0.012</td>
</tr>
<tr>
<td>12-HETE/AA (mmol/mol)</td>
<td>0.87 ± 0.34</td>
<td>1.5 ± 0.60</td>
<td>1.6 ± 0.59</td>
<td>0.83</td>
<td>0.025</td>
</tr>
<tr>
<td>15-HETE/AA (mmol/mol)</td>
<td>1.9 ± 0.53</td>
<td>2.8 ± 1.2</td>
<td>2.7 ± 1.2</td>
<td>0.47</td>
<td>0.15</td>
</tr>
<tr>
<td>9-HODE/LA (mmol/mol)</td>
<td>0.83 ± 0.34</td>
<td>1.1 ± 1.6</td>
<td>0.99 ± 0.55</td>
<td>0.36</td>
<td>0.56</td>
</tr>
<tr>
<td>13-HODE/LA (mmol/mol)</td>
<td>1.3 ± 0.23</td>
<td>2.0 ± 0.8</td>
<td>1.9 ± 0.79</td>
<td>0.44</td>
<td>0.12</td>
</tr>
</tbody>
</table>

P (unadj.): P for unpaired two-tailed T test, P (adj.) † Due to lack of plasma, arachidonic and linoleic acids and their oxidized derivatives could not be measured in three heterozygotes and two family controls.
Part 3

HDL function: beyond plasma cholesterol concentration
Image on previous page: John A. Glomset postulated this mechanism for the transport of cholesterol from membranes of peripheral cells by HDL to the liver in a review published in the *Journal of Lipid Research* in 1968 (figure used with permission of the American Society for Biochemistry and Molecular Biology). In Chapter 14, we describe evidence for the presence of this mechanism.
Chapter 14

Submitted for publication

*In vivo* tissue cholesterol efflux is reduced in carriers of mutations in *APOA1* and *ABCA1*

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Abstract

Objective. Atheroprotection by high density lipoprotein (HDL) is considered to be mediated by reverse cholesterol transport (RCT) from peripheral tissues. In humans, however, little is known about in vivo cholesterol fluxes through this entire pathway. We therefore investigated whether RCT cholesterol fluxes were altered in patients with genetic HDL deficiencies.

Methods and results. Five carriers of an APOA1 mutation, two patients homozygous for an ABCA1 mutation (mean HDL-c of all 7: 13±13 mg/dl) and seven unaffected controls (mean HDL-c: 54±11 mg/dl, p<0.0001) received a 20-hour infusion of $^{13}$C$_2$-cholesterol ($^{13}$C-C). Enrichment profiles of plasma and erythrocyte free cholesterol and plasma cholesterol esters were measured repetitively. A 4-compartment SAAM-II model was applied to calculate tissue cholesterol efflux (TCE). Fecal $^{13}$C recovery and total sterol excretion were measured during 7 days post-infusion. TCE was reduced by 19% in carriers (3.5±0.63 mg/kg/hr versus 4.2±0.38 mg/kg/hr in controls, p = 0.03) and associated with plasma HDL-c ($\beta$ for linear regression: 0.66, p<0.05). Fecal $^{13}$C recovery and sterol excretion did not differ significantly between carriers and controls: 9.9±6.2% versus 13.3±6.3% (p=0.33), and 1098±705 mg/day versus 1456±404 mg/day (p=0.47), respectively.

Conclusions. TCE is reduced in carriers of mutations in APOA1 or ABCA1, substantiating that HDL contributes to efflux of tissue cholesterol in humans. The residual TCE and unaffected fecal sterol excretion in severely affected subjects indicate, however, that non-HDL pathways contribute significantly.
Introduction

Epidemiological studies have demonstrated a strong inverse relation between plasma high density lipoprotein cholesterol (HDL-c) concentrations and the risk of cardiovascular disease.\textsuperscript{1-4} The anti-atherogenic properties of HDL could not be substantiated however in a recent meta-regression analysis, in which pharmacological increases in HDL-c did not translate into a decreased CVD risk.\textsuperscript{5} In fact, inhibition of cholesterol ester transfer protein (CETP) by torcetrapib was even associated with an increased mortality in spite of a 70\% increase in HDL-c levels.\textsuperscript{6} Although the latter has been largely attributed to off-target toxicities of the compound\textsuperscript{7}, these findings have emphasized the need for other measures than plasma HDL-c concentrations to assess the atheroprotective properties of the HDL particle. Ideally, such measures should relate directly to the mechanistic pathways that form the basis of the anti-atherogenic effects of HDL in humans.\textsuperscript{3, 8}

The most frequently studied function of HDL is its role in the reverse transport of cholesterol (RCT) from peripheral tissues. RCT has been characterized as the uptake of cholesterol from peripheral cells by lipid-poor apolipoprotein A-I (Apo A-I) and HDL, mediated by lipid transporter molecules such as ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1) and scavenger receptor type B-I (SR-BI), to the delivery of cholesterol to the liver for excretion into the faeces as neutral sterols or bile acids.\textsuperscript{9} Indirect proof for a role of HDL in tissue RCT in humans has come from patients with HDL deficiency syndromes, in whom accumulation of cholesterol in peripheral tissues such as corneas\textsuperscript{10}, tonsils\textsuperscript{11} and glomeruli\textsuperscript{10} is a central feature. The contribution of individual components in RCT has been addressed predominantly by \textit{in vitro} quantification of the cholesterol efflux capacity of macrophages and the capacity of plasma to induce cellular cholesterol efflux, as well as by human studies of cholesterol excretion as bile acids and faecal sterols (for review, see \textsuperscript{8,9}). However, very few studies have addressed \textit{in vivo} measurements of the RCT pathway in humans. Such studies are highly relevant, considering the current conflicting results regarding the role of HDL in RCT in murine studies\textsuperscript{12-16}, as well as in human studies of fecal sterol excretion.\textsuperscript{17-19} A major hurdle to resolve this ongoing debate pertains to the methodological complexity of quantifying \textit{in vivo} cholesterol fluxes in humans.

In the present study, \textit{in vivo} tissue cholesterol efflux (TCE) was quantified in carriers of mutations in \textit{APOA1} or \textit{ABCA1} as compared to healthy controls. We used a recently developed stable isotope infusion method combined with a 4-compartment SAAM-II model (Turner et al, co-submission). To assess the entire RCT pathway, fecal recovery of the cholesterol tracer and total fecal sterol excretion were measured as well. We demonstrate that tissue cholesterol efflux is significantly reduced in carriers of mutations in \textit{APOA1} or \textit{ABCA1} as compared to controls, indicating that Apo A-I and ABCA1 indeed contribute to tissue cholesterol efflux in humans.
Methods

Subjects

Subjects were considered a case if they were between 18 and 70 years of age and were molecularly diagnosed with mutations in *APOA1* or *ABCA1* in our tertiary University Lipid Clinic. Control subjects were unaffected and unrelated normolipidemic individuals recruited via advertisements. Subjects were excluded from participation if they had any of the following conditions: a body mass index (BMI) > 35 kg/m², alcohol or drug abuse, uncontrolled hypertension defined as systolic pressure > 160 mmHg and/or diastolic pressure > 100 mmHg, diabetes mellitus or cardiovascular disease within six months prior to inclusion. Lipid lowering drugs (statins, fibrates, nicotinic acid derivatives, ezetimibe) had to be discontinued at least 6 weeks before the screening visit. The study protocol was approved by the Institutional Review Board of the Academic Medical Center, University of Amsterdam, The Netherlands. Each participant provided written informed consent.

Experimental procedures

To ensure stable cholesterol intake, participants maintained a standardized diet from 2 days prior to the infusion until the end of study. Daily cholesterol intake was monitored using a dietary record. In order to normalize the fecal isotope recovery measurements for variations in fecal flow, participants used capsules containing 3 mg [2H₄] sitostanol (Medical Isotopes Inc., Pelham, AL, USA) 3 times daily with their meals from 2 days prior until 7 days following infusion. One day post-infusion, participants started collecting daily stool samples for 7 days, using a FSC specimen collection system (Fisher Scientific, Hampton, NH, USA).

On the day of infusion, participants were admitted to the hospital at noon after a light breakfast in the morning. Two intravenous catheters were placed, one used for blood sampling and the other for a 20h-constant infusion of ¹³C-cholesterol (¹³C-C). Infusates were prepared by dissolving 200 mg of [2,3-¹³C₂] cholesterol (99%, Isotec, Miamisburg, OH, USA) into 13 ml of warm USP ethanol. This solution was mixed slowly into 120 ml of 10% Liposyn III (Hospira Inc, Lake Forest, IL, USA) to a final concentration of 1.5 mg/ml ¹³C-C. The infusate, piggybacked into normal saline (100 ml/hr) was administered over 20 hours at a rate of 5.5 ml/hr. Blood samples were collected directly before the start of infusion and at subsequent hourly intervals. Immediately after drawing, blood was placed on ice. After centrifugation, plasma was aliquoted and stored at −80°C. Every 3 hours residual red blood cells (RBCs) were washed with saline twice and stored at −80°C. Two and seventeen hours after start of the infusion, two standardized light meals were served. For clinical applicability, the infusion protocol originally developed by Turner et al (co-submission), was slightly modified regarding the duration of ¹³C-C infusion. Since SAAM-II fitting gave comparable output for either a 32h- or a 20h-infusion period (data not shown), a 20-hour infusion period was used in the present study.
**Analytical procedures**

**Plasma analyses**

Total and free cholesterol, HDL-c and triglycerides were determined with commercially available enzymatic methods (Wako Diagnostics, Richmond, VA, USA, and Roche Diagnostics GmbH, Mannheim, Germany). LDL-cholesterol (LDL-c) was calculated using the Friedewald formula. Apo A-I and apo B were determined by nephelometric immunochemistry (Behring GmbH, Marburg, Germany).

**Analysis of cholesterol and its metabolites**

Plasma free cholesterol was extracted with ethanol-acetone, acetylated with toluene/pyridine/acetyl chloride, and dissolved in toluene for analysis of isotopic enrichment of $^{13}$C-C by mass spectrometry (MS). Free cholesterol from RBCs was analyzed after homogenization with silicate beads and extraction in chloroform-methanol. For measurement of cholesterol ester (CE) enrichment by MS, extracted plasma free cholesterol and CE were first separated on an amino-propyl SPE cartridge, the fatty acid moiety of the cholesterol–ester cleaved by methanolic HCl, and the resultant free cholesterol was subsequently acetylated.

Stool samples were first homogenized with an equal mass of water after which neutral sterols (NS) and bile acids (BA) were extracted separately under basic and acidic conditions, respectively, in the presence of the internal standards 5-α-cholestane and 5-β-cholanic acids. The BA extract was split into two portions. The first, used for compositional analysis by FID, was directly subjected to a two-step derivatization: butylation with butanolic HCl followed by silylation by BSTFA-pyridine. The second portion was further purified for mass spectrometric analysis with an octadecyl SPE cartridge, selectively eluting primarily deoxycholic acid with a 20% aqueous-methanol solution prior to butylation and silylation. The NS fraction was silylated directly for both compositional and isotope analysis. Isotopic enrichments of $^{13}$C-C were measured using GC-C-IRMS (Thermo Finnegan MAT 253 IRMS, Bremen, Germany) and determined as atom percent excess (APE) by comparison of the unknown samples to a standard curve, generated with gravitametrically prepared working lab standards with known enrichments. Molar percent excess (MPE) was calculated as 14.5 or 15 × APE for the acetyl or silyl derivative of cholesterol, respectively and by 17 × APE for the butyl-silyl derivative of deoxycholic acid.

Compositional analysis and excretion measurement of BA and NS was performed by GC/FID by comparison to the internal standards and sitostanol. GC peak areas of cholesterol, coprostanol, epicoprostanol, coprostan-3-one, and cholestanol were used to calculate NS mass. GC peak areas of isolithocholic, isodeoxycholic, lithocholic, deoxycholic, cholic, chenodeoxycholic, ursodeoxycholic, 7-ketolithocholic were used to calculate acidic sterol mass.

**Calculation of cholesterol fluxes**

Tissue cholesterol efflux and additional plasma cholesterol fluxes were calculated by use of a 4-compartmental kinetic model (SAAM-II software, University of Washington, Seattle, WA, USA) and changes in plasma cholesterol over time were calculated using the relationship:

$$\text{rate} = \frac{\text{total}}{t} \times \frac{\text{change}}{\text{change}}$$

where rate is the rate of change of total cholesterol, total is the total cholesterol, change is the change in total cholesterol, and $t$ is time.

The kinetic model was calibrated to experimental data using the software package SAAM-II (SAAM II software, University of Washington, Seattle, WA, USA). The model was then used to estimate tissue cholesterol efflux and additional plasma cholesterol fluxes.
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Seattle, WA, USA, Version 1.2.1). This model’s compartments, assumptions and equations are summarized in Figure 1. Its biological background, development and validation are described in detail in co-submission by Turner et al. In short, the model is based upon several assumptions: 1) subjects are at metabolic steady state (i.e. constant weight and total cholesterol concentrations); 2) each pool is at steady state (i.e. flux in = flux out); 3) esterification of plasma FC is irreversible and 4) there is no direct removal of RBC FC. Inputs into the model are:

**Figure 1: Four-compartment SAAM-II model**

![Four-compartment SAAM-II model diagram](image)

Parameters:
- \( R^* \) infusion rate (mg/kg/hr)
- \( V_1^* \) plasma FC pool size (mg/kg body weight)
- \( V_2^* \) RBC FC pool size (mg/kg body weight)
- \( V_3^* \) plasma CE pool size (mg/kg body weight)
- \( V_4 \) size of pool in rapid exchange with plasma FC pool (mg/kg body weight)
- \( k_{(0,1)} \) rate constant for transfer of tracer from plasma FC pool to environment (hr⁻¹)
- \( k_{(0,3)} \) rate constant for transfer of tracer from plasma CE pool to environment (hr⁻¹)
- \( k_{(3,1)} \) rate constant for transfer of tracer from plasma FC pool to plasma CE pool (hr⁻¹)
- \( k_{(1,2)} \) rate constant for transfer of tracer from RBC FC pool to plasma FC pool (hr⁻¹)
- \( k_{(2,1)} \) rate constant for transfer of tracer from plasma FC pool to RBC pool (hr⁻¹)
- \( k_{(1,4)} \) rate constant for transfer of tracer from rapidly exchanging pool 4 to plasma FC pool (hr⁻¹)
- \( k_{(4,1)} \) rate constant for transfer of tracer from plasma FC pool to rapidly exchanging pool 4 (hr⁻¹)
- \( s_1, s_2, s_3 \) sampling sites, corresponding with V1, V2, V3

* entered as fixed parameters into the model, others are calculated by SAAM II

Steady-state equations:
- Flux 1 = \( k_{(0,1)}V_1 = \) flux of plasma FC to the environment (mg/kg/hr)
- Flux 2 = \( k_{(2,1)}V_1 + k_{(1,2)}V_2 = \) exchange flux between plasma FC and RBC FC (mg/kg/hr)
- Flux 3 = \( k_{(0,3)}V_3 + k_{(3,1)}V_1 = \) flux of plasma FC to plasma CE pool (mg/kg/hr)
- Flux 4 = \( k_{(4,1)}V_1 + k_{(1,4)}V_4 = \) exchange flux between plasma FC and FC in rapidly exchanging pool 4 (mg/kg/hr)

Flux 1 + Flux 3 equals tissue cholesterol efflux (mg/kg/hr).
the plasma FC pool: \( V_1 = FC \text{ (mg/L)} \times \text{plasma volume (L/kg)} \) estimated as 7% of body weight, adjusted for hematocrit,
the RBC pool: \( V_2 = \text{mg FC/g RBC} \times \text{hematocrit} \times \text{blood volume estimated as 7% of body weight} \),
the CE pool: \( V_3 = \text{mg CE/kg} \times \text{plasma volume estimated as 7% of body weight, adjusted for hematocrit} \),
the infusion rate of \(^{13}\text{C}-\text{C} (R)\)
the \(^{13}\text{C}-\text{C} \) enrichments of FC sampled in V1 and V2, and \(^{13}\text{C}-\text{CE} \) enrichments in V3.

The model was applied to fit the \(^{13}\text{C}-\text{C} \) enrichment curves of plasma FC, RBC FC and plasma CE, separately for each individual. An example of such \(^{13}\text{C} \)-incorporation curves is shown in Figure 2. The calculations of the other parameters are summarized in Figure 1.

**Tissue cholesterol efflux (TCE)**
In Figure 1, cholesterol can leave the system, either to the environment – represented by Flux 1 = \( k(0,1) \times V_1 \), the rate constant for transfer of tracer from plasma FC to the environment (hr\(^{-1}\)), multiplied by the plasma FC pool size (mg/kg body weight) - or in the form of CE production, represented by \( k(3,1) \times V_1 \), the rate constant for transfer of tracer from plasma FC to the plasma CE pool (hr\(^{-1}\)), multiplied by the plasma FC pool size (mg/kg body weight). At steady state, CE production equals CE loss, therefore, the FC

**Figure 2. Rise-toward-plateau curves for the \(^{13}\text{C} \)-enrichment of plasma free cholesterol, cholesterol esters and red blood cells during \(^{13}\text{C} \)-cholesterol infusion.**

13C enrichment profiles of plasma free cholesterol, cholesterol esters and red blood cells of a representative carrier (closed symbols, i.e. a patient with compound heterozygosity for ABCA1 mutations (Q1038X/N1800H) and a representative control participant (open symbols). Data are presented as molar percent excess.
estherification flux (Flux 3) equals k(0.3)*V3, the rate constant for transfer of tracer from the plasma CE pool to the environment (hr⁻¹) multiplied by plasma CE pool size (mg/kg). Under the assumption of steady state, both these losses, i.e. Flux 1 and Flux 3, have to be balanced by an equally large flux from a non-rapidly miscible pool. Hence, the thus derived efflux of free cholesterol from peripheral tissues into the plasma compartment, i.e. tissue cholesterol efflux (TCE) equals the sum of Flux 1 and Flux 3.

Additional cholesterol fluxes
The plasma FC esterification flux (Flux 3 in mg/kg/hr) represents the mass of plasma FC per kg body weight that is converted into plasma CE every hour, and thus the LCAT-mediated esterification step of RCT. The model calculates two additional exchange fluxes in equilibrium with plasma FC (V1). These include the FC exchange flux with the RBC FC pool (Flux 2) and Flux 4, the exchange flux with a fourth pool of FC (V4), which is in rapid exchange with plasma FC (V1). Although this rapidly miscible pool of FC has multiple anatomical correlates, the liver is an important component (Turner et al, co-submission).²⁰ Flux 2 is calculated as k(2,1)*V1 = k(1,2)*V2, the rate constant for transfer of tracer from plasma FC pool to RBC pool (hr⁻¹), multiplied by plasma FC pool size (mg/kg body weight) and equals the rate constant for transfer of tracer from RBC FC pool to plasma FC pool (hr⁻¹) multiplied by RBC FC pool size (mg/kg body weight, Figure 1).

Fecal sterol excretion
Percentage fecal ¹³C recovery was calculated as: % ¹³C enrichment * mg of sterol excreted. Average daily mass excretion of neutral and acidic sterols (NS and bile acids (BA)) in mg/day was measured as the sterol concentration ([μg/sample] / [²H₄] sitostanol (μg/sample)) * fecal sitostanol (μg/day).

Statistical analyses
All data presented are means with standard deviations (SD), unless otherwise indicated. Parameters were compared between cases and controls using Student’s T-test for continuous variables and the chi-square test for categorical variables. In addition, linear regression was applied to assess the association between variables. Statistical analyses were performed using SPSS software (version 12.0.2, SPSS Inc., Chicago, Illinois). A P value of <0.05 was considered statistically significant.

Results
Baseline characteristics
Five carriers of a previously described mutation in APOA1 (c.C643T, p.L202P)²¹, one patient homozygous for a mutation in ABCA1 (c.3167T>C, p.L1056P), one patient with compound heterozygosity for a mutation in ABCA1 (c.3112C>T, p.Q1038X and
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A total of five carriers of mutations in \textit{APOA1} (c.5398A>C, p.N1800H) and seven unaffected controls were included in this study. All carriers of a mutation in \textit{APOA1} and all control participants were males; both patients with mutations in \textit{ABCA1} were females. Baseline demographic and lifestyle parameters, lipids and lipoproteins of cases and controls are listed in Table 1. Plasma HDL-c and Apo A-I concentrations of carriers were 76\% and 62\% lower than in controls (p < 0.0001 and p < 0.01, respectively). Carriers tended to have higher concentrations of triglycerides and apo B, and to use statins more often than controls. Several months after the molecular diagnosis of ABCA1 deficiency, the patient homozygous for the p.L1056P mutation in \textit{ABCA1} was diagnosed with coronary artery disease. No clinically manifest atherosclerotic cardiovascular disease was present in the other participants. Plasma FC and CE concentrations were comparable in cases and controls (Table 1).

\textit{Tissue cholesterol efflux}

Figure 2 displays the $^{13}$C-incorporation curves of plasma FC, CE and RBC FC for a representative carrier and control participant during the 20h infusions of $^{13}$C-C. There were no significant changes in plasma concentrations of free cholesterol or cholesterol esters during the $^{13}$C-C infusions. The SAAM-II kinetic modeling results are shown in Table 2. Tissue cholesterol efflux was on average 19\% lower in carriers compared to controls: (3.5 ± 0.63 mg/kg/hr versus 4.2 ± 0.38 mg/kg/hr, p = 0.03 (Table 2 and Figure 3a). This difference retained statistical significance upon adjustment for age and BMI in linear regression analysis (p = 0.02). In the carrier group, TCE was comparably low in the ABCA1-deficient patients (3.4 ± 0.33 mg/kg/hr) and the carriers of the \textit{APOA1} mutation.
3.5 ± 0.75 mg/kg/hr). Tissue cholesterol efflux was significantly correlated with plasma concentrations of HDL-c and Apo A-I in the entire study group (β for HDL-c 0.66, p = 0.01, β for ApoA-I 0.71, p < 0.01, see Figure 3b). Both associations also retained statistical significance upon adjustment for age and BMI: p for HDL-c: 0.045; p for Apo A-I: 0.017. Carriers and controls did not differ in any of the other kinetic parameters (Table 2).

Cholesterol flux from plasma towards the environment (flux 1) tended to be lower in carriers compared to controls (2.6 ± 0.82 vs. 3.4 ± 0.44 mg/kg/hr, p = 0.10). The plasma cholesterol esterification flux (Flux 3) did not differ between carriers and controls. RBC FC concentrations of carriers were comparable to controls, as was the exchange flux of
In vivo cholesterol efflux is reduced in genetic HDL deficiency

Figure 3. Tissue cholesterol efflux (TCE) in carriers of mutations in APOA1 or ABCA1 and unaffected controls

a) Tissue cholesterol efflux (mg/kg/hr) was calculated by SAAM II as the sum of Flux 1 and Flux 3 (Figure 1). *Patients homozygous/compound heterozygous for mutations in ABCA1. **P value for univariate analysis (unpaired Student’s T test). The observed difference was statistically independent of age and BMI (p after adjustment for age and BMI: 0.02).

b) Scatter plot of tissue cholesterol efflux and HDL-c (top panel) and ApoA-I (lower panel) in carriers (closed symbols) and family controls (open symbols). Line fit with linear regression, for carriers and controls combined. β's and p values for linear regression also for carriers and controls combined.

*Patients homozygous/compound heterozygous for mutations in ABCA1. **Univariate linear regression analysis. Both associations retained statistical significance upon adjustment for age and BMI: p for HDL-c: 0.045; p for Apo A-I: 0.017.
plasma with red blood cells (Flux 2) and the exchange flux with the rapidly miscible pool (Flux 4).

Fecal sterol excretion

Although the excretion of neutral sterols and bile acids in feces as well as the percentage $^{13}$C recovery in fecal sterols and bile acids appeared to be numerically lower compared to controls (table 3), no statistical difference was observed due to the considerable intra-day as well as intersubject variability. In addition, none of the fecal parameters was significantly correlated with plasma HDL-c levels (Supplemental Figure).

Table 3. Fecal sterol excretion and $^{13}$C-recovery

<table>
<thead>
<tr>
<th>Fecal excretion</th>
<th>Carriers of mutations in APOA1 (n = 5) or ABCA1 (n = 2)</th>
<th>Controls (n = 7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral sterols (mg/day)</td>
<td>1098 (705)</td>
<td>1456 (404)</td>
<td>0.47</td>
</tr>
<tr>
<td>$^{13}$C Recovery in neutral sterols (%)</td>
<td>8.2 (4.9)</td>
<td>10.9 (5.8)</td>
<td>0.35</td>
</tr>
<tr>
<td>Fecal bile acids (mg/day)</td>
<td>331 (194)</td>
<td>484 (218)</td>
<td>0.19</td>
</tr>
<tr>
<td>$^{13}$C Recovery in fecal bile acids (%)</td>
<td>1.7 (1.6)</td>
<td>2.3 (1.6)</td>
<td>0.54</td>
</tr>
<tr>
<td>Total fecal $^{13}$C recovery (%)</td>
<td>9.9 (6.2)</td>
<td>13.3 (6.3)</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Data are presented as means (SD) during 7 day fecal collection period post-infusion. P values are for unpaired Student’s T-test.

Discussion

This study is the first to demonstrate that in vivo tissue cholesterol efflux (TCE) is attenuated in patients with genetically determined low HDL-cholesterol. Carriers of mutations in APOA1 or ABCA1, with on average a 77% decrease in HDL-c levels as compared to controls, displayed a significant 19% reduction in TCE. In addition, in this small group of patients and controls we observed a surprisingly linear and independent association between TCE and HDL-c or apo A-I levels. This is the first unequivocal demonstration of the contribution of HDL-c to the centripetal transport of cholesterol from peripheral tissues in humans. Clearly, this relation was not proportional indicating the presence of alternative routes of non-HDL mediated RCT. The residual TCE and unaffected fecal sterol excretion in our patients with very low HDL-c levels indicate that non-HDL pathways compensate and/or contribute significantly to tissue cholesterol efflux and fecal elimination of cholesterol in humans. Upregulation of these compensatory pathways in our patients with genetically low HDL-c may have led to underestimation of HDL mediated TCE capacity.

Due to methodological complexity, few studies to date have successfully addressed tissue cholesterol fluxes in human. The stable isotope infusion method used in the present study was shown to reproducibly measure TCE in both animals and humans.
In vivo cholesterol efflux is reduced in genetic HDL deficiency

(Turner et al, co-submission). Here a 4-compartmental SAAM-II model was applied to optimally fit plasma 13C cholesterol enrichment data in order to calculate TCE, as well as fluxes of plasma FC to three additional compartments. The sizes of 2 additional pools interchanging FC with the plasma FC pool (V1) were calculated from measured cholesterol concentrations in the respective compartments. These include the FC pool in erythrocytes (RBC FC, V2) since considerable free cholesterol exchange exists between erythrocytes and plasma,23 and the plasma CE pool (V3), as esterification of plasma FC leads to irreversible plasma FC loss at steady-state. The fourth compartment in the SAAM-II analysis (V4) represents the rapidly exchanging and thereby rapidly equilibrating FC pool. This compartment was previously defined by Schwartz et al and includes hepatic cholesterol pools.20 Measurement of true tissue cholesterol efflux as postulated in RCT requires its independence from net hepatic cholesterol flux into plasma, which in its turn requires successful equilibration of the infused 13C-C tracer with hepatic cholesterol.3 Although we could not determine this equilibration in our study, studies in bile fistula patients, allowing for direct measurement of tracers in bile demonstrated that equilibration of FC within plasma lipoproteins and hepatic cholesterol pools occurs within hours.20 In our study, we found rapid exchange rated between plasma FC (V1) and V4 (k(1,4), Table 2), in support of rapid equilibration of both compartments, with no differences between carriers and controls.

Deployment of this 4-compartment model demonstrated impaired TCE in a unique population with genetically determined low plasma HDL-c levels. These in vivo data corroborate a series of previous in vitro and ex vivo studies, suggesting that apo A-I drives the first step of RCT. Plasma of apo A-I deficient mice24 and humans25, 26 had decreased capacity to accept cholesterol from a stable cell line compared to control plasma. Skin fibroblasts from carriers of mutations in ABCA1 effluxed less cholesterol compared to control fibroblasts.27 Finally, apo A-I and ABCA1 deficient mice displayed a reduced 3H-cholesterol flux from intraperitoneally injected 3H-cholesterol loaded macrophages through the plasma towards the liver and into the feces.24, 28 However, these results have been disputed by other studies, showing that in apo A-I and ABCA1 deficient mice, neither apo A-I nor ABCA1 determines centripetal cholesterol flux to either the liver or the feces.14, 15, 29

TCE was significantly reduced in carriers, but by only a third of the reduction in HDL-c, implying residual capacity and recruitment of compensatory mechanisms for TCE in humans with genetically low HDL-c. Tissue-derived cholesterol fluxes may in part be diverted via LDL-c that might take over the acceptor role in low-HDL states. Alternatively, ABCG1 might compensate and promote cholesterol efflux onto HDL particles. Interestingly, the activity of ABCG1 does not influence overall HDL levels30, 31, possibly indeed explaining the discrepancy between the magnitudes of reduction in HDL-c and in TCE observed in the carriers.

Of note, our kinetic model also permitted calculation of whole body esterification. This LCAT-mediated step is net unidirectional32 and was originally postulated as
a central element in the RCT concept, as the driving force for TCE.\textsuperscript{33} However, we found esterification fluxes similar to what was previously found in healthy controls.\textsuperscript{34} Interestingly, plasma cholesterol esterification fluxes did not differ between cases and controls, indicating cholesterol esterification independent of plasma Apo A-I levels. In line, LCAT gene therapy has been demonstrated to correct low HDL-c levels in mice with mutations in \textit{APOA1}\.\textsuperscript{35} Previous studies in humans have also suggested that plasma CE clearance is mostly mediated by apoB100-containing particles following CETP-mediated transfer, rather than by direct HDL-dependent CE removal.\textsuperscript{15, 36, 37} Our observation raises the question whether patients with a genetic LCAT deficiency display a reduced TCE. The current model could serve as a means to resolve this issue.

Fecal sterol excretion (FSE) measured by mass excretion of neutral sterols and bile acids as well as by \textsuperscript{13}C recovery in these fractions, was not significantly different between carriers and controls. In humans, equivocal data exist on the relation between HDL-c and fecal sterol excretion, showing decreased\textsuperscript{17} as well as normal\textsuperscript{14, 38-40} FSE in relatively small populations with genetically determined low HDL-c levels. Absence of a relation between HDL-c and FSE is corroborated by several studies in mice. ABCA1- and Apo A-I deficient mice were found to have normal FSE\textsuperscript{14, 15} and hepatobiliary cholesterol secretion and FSE were unaffected in ABCA1-deficient mice.\textsuperscript{13, 41} Furthermore, upregulation of individual steps in the RCT pathway did not affect fecal sterol excretion in mice.\textsuperscript{12} Although infusion of pro-apo A-I or rHDL increased FSE in 4\textsuperscript{18} and 6\textsuperscript{19} subjects, respectively, doubling HDL-c levels by CETP inhibition had no effect on FSE in 16 hypercholesterolemic individuals.\textsuperscript{42} In fact, another study reported a negative correlation between HDL-c levels and FSE in 63 healthy males.\textsuperscript{25} Our fecal mass excretion data are in line with these negative studies. In addition, our fecal \textsuperscript{13}C recovery results specifically show that fecal loss of \textit{plasma-derived} cholesterol is not primarily determined by plasma HDL-c levels and imply that other mechanisms contribute to whole-body cholesterol elimination. For instance, direct trans-intestinal cholesterol excretion (TICE) has been shown to contribute substantially to FSE in mice.\textsuperscript{43, 44} This non-biliary route of sterol excretion was shown to be largely independent of HDL as a cholesterol donor, as demonstrated in studies using ABCA1-/- and SR-B1-/- mice.\textsuperscript{43} Early intestinal perfusion studies imply the presence of TICE in humans\textsuperscript{45}, however, the extent of its contribution to human FSE in vivo remains to be elucidated. However, we cannot exclude that the by nature limited sample size, combined with the substantial variation in the fecal outcomes have resulted in a false negative finding.

Finally, our measurement of TCE and FSE does not quantify the efflux of cholesterol from the lipid laden macrophage via ABC-transporters to HDL for subsequent elimination into the feces\textsuperscript{3, 9}, as cholesterol in atherosclerotic plaques represents merely a minute fraction of the total exchangeable cholesterol pool size\textsuperscript{46, 47}. Assessment of this macrophage-specific RCT is currently confined to studies in mice.\textsuperscript{3}

In conclusion, carriers of mutations in \textit{ABCA1} and \textit{APOA1}, characterized by strongly reduced plasma HDL-c levels, present with a reduced tissue cholesterol efflux compared to unaffected controls. This strengthens the concept of apo A-I as a contributor to
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reverse cholesterol transport, considered the main atheroprotective mechanism of HDL. Indeed, increased atherosclerosis was found in carriers of mutations in both APOA1 and ABCA1. This first unequivocal demonstration of the contribution of HDL-c to the centripetal transport of cholesterol from peripheral tissues in humans holds promise for therapies aiming at an increase of plasma HDL-c levels and provides the means for in vivo assessment of the efficacy of RCT enhancing strategies.

Acknowledgements

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Reference List


Supplemental figure

Fecal sterol excretion and 13C-recovery plotted to plasma HDL-cholesterol

Scatter plots of fecal sterol excretion and 13C-recovery and HDL-cholesterol in carriers (closed symbols) and family controls (open symbols). Data are presented as means during 7 day fecal collection period post-infusion. None of the fecal parameters was significantly correlated with HDL-cholesterol.
Chapter 15

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Plasma Levels of 27-Hydroxycholesterol in Humans and Mice with Monogenic Disturbances of High Density Lipoprotein Metabolism

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g. Center of Integrated Human Physiology, University of Zurich, CH-8093 Zurich, Switzerland
Abstract

Secretion of 27-hydroxycholesterol (27OHC) from macrophages is considered as an alternative to HDL-mediated reverse transport of excess cholesterol. We investigated 27OHC-concentrations in plasma of humans and mice with monogenic disorders of HDL metabolism. As compared to family controls mutations in the genes for apolipoprotein A-I, ATP binding cassette transporter (ABC) A1 and lecithin:cholesterol acyltransferase (LCAT) were associated with reduced concentrations of both HDL-cholesterol and HDL-27OHC whereas mutations in the genes for cholesterylesterol transfer protein (CETP), scavenger receptor type BI and hepatic lipase were associated with elevated HDL concentrations of either sterol. Compared to family controls and relative to the concentrations of total 27OHC and cholesterol, lower 27OHC-ester but normal cholesterylesterol levels were found in HDL of heterozygous LCAT mutation carriers and nonHDL of heterozygous CETP mutation carriers. In family controls, LCAT activity and CETP mass were more strongly correlated with 27OHC-ester than cholesterylesterol concentrations in HDL and nonHDL, respectively. These findings suggest that the formation and transfer of 27OHC-esters are more sensitive to reduced activities of LCAT and CETP, respectively, than the formation and transfer of cholesterylesters. 27OHC plasma levels were also decreased in apoA-I-, ABCA1- or LCAT-knockout mice but increased in SR-BI-knockout mice. Transplantation of ABCA1- and/or ABCG1- deficient bone marrow into LDL receptor deficient mice decreased plasma levels of 27OHC. In conclusion, mutations or absence of HDL genes lead to distinct alterations in the quantity, esterification or lipoprotein distribution of 27OHC. These findings argue against the earlier suggestion that 27OHC-metabolism in plasma occurs independently of HDL.
Introduction

27-Hydroxycholesterol (27OHC) is a metabolite of cholesterol formed by the mitochondrial cytochrome P450 sterol 27-hydroxylase (CYP27), an enzyme particularly expressed in the vascular endothelium, macrophages and the liver [1-2]. Introduction of a hydroxyl group allows the otherwise hydrophobic cholesterol molecule to pass amphiphilic membranes more easily [2-3]. Because of these physicochemical properties, 27OHC has been postulated to be secreted from cells independently of transporters and extracellular lipoprotein acceptors and thereby to facilitate an alternative route for apolipoprotein (apo) A-I/ high density lipoproteins (HDL)-mediated transport of cholesterol from macrophages to the liver [2]. In the liver, 27OHC is an important intermediary product of the so-called alternative bile acid synthesis pathway which contributes ~10% to de novo bile acid biosynthesis [2]. In addition, 27OHC is an important ligand of at least two types of nuclear hormone receptors. It activates liver-X-receptors (LXR) alpha and beta which regulate the transcription of several genes involved in lipid and lipoprotein metabolism [4-5]. Most recently 27OHC was identified as the first endogenous selective estrogen receptor modulator (SERM). Both in vitro and in vivo 27OHC was found to modulate the transcriptional activity of estrogen receptors tissue-specifically either as an agonist or antagonist [6].

27OHC is the most abundant oxysterol in the circulation [4], while plasma levels of 27OHC have been found to correlate with the cholesterol content in atherosclerotic lesions and the severity of coronary artery disease [7]. Patients with genetic CYP27 deficiency suffer from cerebrotendinous xanthomatosis and develop premature atherosclerosis despite having normal levels of plasma cholesterol [2]. Taken together, 27OHC has been proposed to be an anti-atherogenic molecule [2, 8].

Since the classical reverse cholesterol transport is mediated by HDL, and because HDLs are important carriers of 27OHC in plasma [9], we investigated whether monogenic disorders of HDL metabolism affect plasma and lipoprotein concentrations of 27OHC. We used an LC-MS method which allows to quantify 27OHC in small plasma volumes (15-50 mL) [10]. Specifically, we analysed plasmas of patients with functionally relevant mutations or mice with knock-outs of the following genes: apoA-I which is the main protein component of HDL; ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) which mediate cholesterol and phospholipid efflux from cells to apoA-I and HDL, respectively; lecithin:cholesterol acyl transferase (LCAT) which esterifies cholesterol and thereby converts discoidal HDL precursors into mature spherical HDL particles; cholesterylester transfer protein (CETP, not expressed in mice) which exchanges cholesterylester of HDL against triglycerides of apoB-containing lipoproteins; scavenger receptor type B I (SR-BI) which mediates selective uptake of cholesterol esters in the liver and steroidogenic organs; and hepatic lipase (HL, only studied in humans) which hydrolyses triglyceride and phospholipids in HDL [11]. Through this analysis, we identified genetic variations in HDL
concentrations as well as the activities of ABCG1, LCAT and CETP as factors regulating 27OHC metabolism in plasma

Methods

Patient plasmas

Forty-one Dutch patients with functionally relevant mutations in the genes encoding for either LCAT, ABCA1, APOA1, SR-BI, CETP or HL as well as 41 unaffected family members were investigated. Most of the families and the underlying defects were described previously [12-16]. In addition, 4 Danish patients with mutations in APOA-I or CETP as well as 5 age- and sex-matched controls were included. The Medical Ethics Committee of the Academic Medical Center (AMC) in Amsterdam, The Netherlands, as well as the Danish Ethics Committee for Copenhagen and Frederiksberg, Denmark, approved all genetic and phenotypic studies described and all participants signed an informed consent to join the study. The characteristics of the study participants are shown in table 1.

Table 1. Characteristics of the mutation carriers and their unaffected family members.

<table>
<thead>
<tr>
<th>Mutated gene</th>
<th>Number of defective alleles</th>
<th>Mutation#</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DUTCH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOA1</td>
<td>0</td>
<td>p.L202P (c.605T&gt;C)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>p.L1056P (c.3167T&gt;C) or p.C1477R (c.4429T&gt;C)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>p.L1056P (c.3167T&gt;C, homozygote) or p.Q1038X (c.3112C&gt;T) + p.N1800H (c.5398A&gt;C) or p.C1477R (c.4429T&gt;C) + IVS25+1G&gt;C</td>
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<tr>
<td></td>
<td>2</td>
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</tr>
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<td>CETP</td>
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<td>IVS7+1 (G&gt;T)</td>
</tr>
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</tr>
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<td>p.S289F (c.866C&gt;T)</td>
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<td><strong>DANISH</strong></td>
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<tr>
<td>CETP</td>
<td>1</td>
<td>p.S349Y (c.1046C&gt;A)</td>
</tr>
</tbody>
</table>

Values represent mean±SD. * Not determined (ND) # Amino acid changes are localized on the basis of the entirely translated protein, that is including the signal peptides. To define the position within the mature protein, correct apoA-I by -24 amino acids, ABCA1 by -60 amino acids, LCAT by -24 amino acids, SR-BI by 0 amino acids, CETP by -17 amino acids, HL by -22 amino acids.
1. Fasting blood samples were collected after at least a 10-hours fast in the morning and EDTA-plasma was prepared through centrifugation of the blood at 3000 rpm for 10 min at 25°C by the study nurses of the AMC, Amsterdam, The Netherlands, and of the Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark. Aliquots were immediately frozen at -80°C until later use.

**Mouse plasmas**

All animal procedures were approved by the Bioethical committee of the Biomedical Research Institute of the Academy of Athens and Leiden University. All animal experimentations were in agreement with the ethical recommendation of the European Communities Council Directive (86/609/EEC). Knock-out as well as C57B/6 control mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on standard chow diets containing 5% fat (Harlan Teklad, Madison, WI, USA). Mice were fasted for 6 hours before the collection of blood samples. Samples from mutants and wild-type control mice were collected in EDTA-

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Cholesterol (mM)</th>
<th>HDL-cholesterol (mM)</th>
<th>NonHDL-cholesterol (mM)</th>
<th>Triglyceride (mM)</th>
<th>Number of smokers</th>
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<td>1.47±0.39</td>
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<td>ND*</td>
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</tr>
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<td>ND*</td>
<td>ND*</td>
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<td>0</td>
</tr>
<tr>
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<td>3.60±0.79</td>
<td>1.21±0.64</td>
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</table>
containing eppendorf tubes, kept in room temperature for 20 min and centrifuged to collect the plasma. All samples were stored in -80°C and transported in dry ice. Bone marrow transplantation was conducted by Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden University, The Netherlands [17]. Bone marrow transplant mice as well as their controls were fed with Western diet containing 0.25% cholesterol.

Quantification of 27OHC

The concentrations of 27OHC in total and apoB-depleted plasma were analysed using a dopant assisted-atmospheric pressure photoionization (DA-APPI) liquid chromatography-mass spectrometry (LC-MS) method as described previously [10]. For the quantification of unesterified 27OHC, the hydrolysis step was omitted and the sample volume was increased to 100 mL due to the very low endogenous level of free 27OHC (only ~10-20% of total 27OHC) [9].

For the determination of 27OHC in HDL, apoB-containing lipoproteins were removed from 100 mL human EDTA plasma by precipitation with dextran-sulfate-Mg²⁺ [18]. 27OHC concentrations assayed in apoB-depleted plasma were multiplied by 1.1 to correct for the dilution and to obtain the concentration of 27OHC in HDL. The difference between the concentrations of total 27OHC and HDL-27OHC was described as nonHDL-27OHC concentration. We have shown previously that this method recovers about 95% of 27OHC in the apoB-depleted fraction, as compared to density-gradient ultracentrifugation, and that nearly 90% of plasma 27OHC is contained in the lipoprotein fractions [9].

Quantification of other lipids, apolipoproteins, LCAT and CETP

Total plasma cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol, apoB, apoA-I, and apoA-II levels were measured with commercial kits (Wako, Neuss, Germany and Randox, Crumlin, UK) on a Cobas Mira autoanalyzer. Due to the limited sensitivity of the enzymatic homogenous assay, concentrations of HDL cholesterol as well as thereof derived measures (nonHDL cholesterol and the ratios of 27OHC/cholesterol in HDL and nonHDL) are not reported for patients with virtually complete HDL deficiency, namely the homozygotes and compound heterozygotes for mutations in ABCA1 or LCAT.

The unesterified cholesterol concentrations in the supernatant after precipitation of apoB-containing lipoproteins with polyethylenglycol were assayed using a procedure that has been developed in house. Briefly, a mixture of reagent (200 mL) containing 7.3 mg/mL homovanilic acid, 0.1 M 3-[N-Morpholine]propanesulfonic acid, 1.25 mM taurocholate, 1.35 U/mL cholesterol oxidase and 10 U/mL peroxidase was added to 30 mL sample and assayed using the FLUOstar Galaxy fluorometer (BMG Labtech, Offenburg, Germany). The assay was based on the reaction of cholesterol with oxygen to give hydrogen peroxide, which subsequently reacted with homovanilic acid to give a fluorescence emission at 450 nm wavelength. Cholesterylester concentrations were calculated as the differences of total and unesterified cholesterol concentrations.
LCAT activity and cholesterol esterification rates were determined using proteoliposomes containing radioactive cholesterol as the exogenous substrate assay and after equilibration of plasma with \(^3\)H-cholesterol as the endogenous substrate, respectively \cite{19}. CETP concentration was measured as described \cite{20}

**Statistical Analysis**

Statistical analyses were performed using Microsoft Excel (Microsoft, Redmont, WA, USA) and SPSS 17.0 (SPSS, Chicago, IL, USA). Normal distribution was tested using Kolmogorov-Smirnov test. Since the data distribution was normal, unpaired student-t test was used to test for statistical significances of differences between 2 groups (assuming equal variance). Significance of a correlation was tested from the Pearson’s correlation coefficient (r).

**Results**

*Effects of gender, age and statin treatment on 27OHC levels in humans*

Because of a strong effect of gender on plasma 27OHC levels \cite{9}, we stratified our data by sex. Because 27OHC concentrations in plasma are only mildly affected by age \cite{9}, we did not further adjust the data for age. About 35\% of all probands were treated with statins. However, when comparing family controls with and without statin treatment, this did not appear to affect plasma- and HDL- 27OHC concentrations: 0.47±0.07 mM vs. 0.50±0.15 mM, and 0.20±0.05 mM vs. 0.23±0.15 mM, respectively. Also other common medications including oral contraceptives, beta blockers, calcium channel blockers, angiotensin converting enzyme inhibitors as well diuretics were not associated with statistically significant differences of 27OHC concentrations in plasma, HDL or nonHDL, and we therefore did not stratify the data for statin or any other drug treatment.

*Effects of human inborn errors of HDL metabolism on 27OHC levels*

In table 2, we compared the concentrations of 27OHC in total plasma, HDL and nonHDL. As shown previously \cite{9}, we identified highly significant and similar positive correlations between 27OHC and cholesterol levels in total plasma, HDL and nonHDL fractions (see figure 1). The r-square values obtained by univariate regression analysis of data indicate that variations in total cholesterol, HDL cholesterol and nonHDL cholesterol explain 35\% to 40\% of the variance in plasma concentrations of total 27OHC, HDL-27OHC and nonHDL-27OHC, respectively. The correlation plots as well as the ratios of 27OHC to cholesterol (figure 1 and table 2) allowed us to identify those conditions in which 27OHC levels were altered beyond cholesterol levels.

Homozygous defects in LCAT and ABCA1 were associated with significantly increased 27OHC/cholesterol ratios in plasma (table 2). All other differences in plasma concentration of 27OHC between carriers of other gene defects and family controls were either not statistically significant or not consistent: Male but not female SR-BI mutation
carriers showed lower 27OHC plasma levels as compared to all male controls, but not as compared to unaffected male relatives from the same family. The significantly decreased levels of 27OHC plasma levels in male and female carriers of the apoA-I(p.L202P) mutant were not retrieved in individuals carrying the apoA-I(p.L168R) mutation (table 2).

Defects in \textit{LCAT}, \textit{ABCA1} and \textit{APOA1} were associated with decreases of HDL-27OHC levels. Conversely, defects that increase HDL-cholesterol levels also tended to be associated with an increase of HDL-27OHC levels (table 2 and figure 1B). However, this effect was statistically significant only for male CETP and HL mutation carriers. After normalisation for HDL-cholesterol levels, defects in LCAT showed consistent and statistically significant associations with increased HDL-27OHC/HDL-cholesterol ratios (table 2).

Finally, 27OHC levels and the ratio of 27OHC/cholesterol were increased in the nonHDL fractions of ABCA1 mutation carriers and female LCAT mutation carriers (table 2, figure 1C). By contrast, male heterozygotes for defects in apoA-I, SR-BI, CETP or HL had lower nonHDL-27OHC levels as compared to the unaffected male controls (table 2). In HL

<table>
<thead>
<tr>
<th>Mutated gene</th>
<th>Number of defective alleles</th>
<th>Number of participants</th>
<th>27OHC in total plasma (µM)</th>
<th>Ratio total-27OHC (µM) / total-cholesterol (mM)</th>
<th>27OHC in HDL (µM)</th>
<th>Ratio HDL-27OHC (µM) / HDL-cholesterol (mM)</th>
<th>27OHC in nonHDL (µM)</th>
<th>Ratio nonHDL-27OHC (µM) / nonHDL-cholesterol (mM)</th>
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<td>24</td>
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<td>Mutations causing decreased levels of HDL-cholesterol</td>
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<td>0.11±0.02**</td>
<td>0.36 **##</td>
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<tr>
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<td>Mutations causing increased levels of HDL-cholesterol</td>
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</tbody>
</table>

*, **, *** and #, ##, ### indicate statistically significant differences (p<0.05, p<0.01, p<0.001, respectively) as compared to „0” and Controls, respectively. * Not determined (ND)
27-hydroxycholesterol in monogenic HDL disorders

Effects of LCAT and CETP mutations on esterified 27OHC in humans

LCAT activity was decreased to about 75% of normal in female and male heterozygotes for LCAT defects (table 3). This 25% decrease in LCAT activity affected neither the esterification nor the cholesterylester/total cholesterol ratios in total plasma and HDL. By contrast, the percentage of 27OHC-ester was significantly decreased while the ratio of free-27OHC/free-cholesterol was significantly increased in both plasma and HDL of heterozygous LCAT mutation carriers as compared to unaffected family members. Regression analysis revealed a stronger correlation of LCAT activity with 27OHC-ester concentration than with cholesterylester concentration in HDL (r=0.6528, p<0.001 vs. r=0.0538, p<0.01, respectively; see supplemental figure 1). Interestingly, the percentage of esterified 27OHC was also significantly decreased in plasma and HDL of heterozygous apoA-I(p.L202P) carriers, but not of heterozygotes for the apoA-I(p.L168R) mutation.

Table 2. 27OHC levels and 27OHC/cholesterol ratios in plasma and lipoproteins in families with monogenic disturbances of HDL metabolism.

<table>
<thead>
<tr>
<th>Mutated gene</th>
<th>Number of defective alleles</th>
<th>Number of participants</th>
<th>27OHC in total plasma (µM)</th>
<th>Ratio total-27OHC (µM) / total-cholesterol (mM)</th>
<th>27OHC in HDL (µM)</th>
<th>Ratio HDL-27OHC (µM) / HDL-cholesterol (mM)</th>
<th>27OHC in nonHDL (µM)</th>
<th>Ratio nonHDL-27OHC (µM) / nonHDL-cholesterol (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>LCAT</td>
<td>0</td>
<td>21</td>
<td>24</td>
<td>0</td>
<td>0.43±0.10</td>
<td>0.60±0.14</td>
<td>0.09±0.01</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td></td>
<td>0.21±0.04</td>
<td>0.26±0.04</td>
<td>0.15±0.04</td>
<td>0.20±0.01</td>
<td>0.21±0.06</td>
<td>0.48±0.14</td>
<td>0.06±0.01</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td></td>
<td>0.10±0.04***</td>
<td>0.06*</td>
<td>0.16±0.02</td>
<td>0.31*</td>
<td>0.16±0.06</td>
<td>0.27</td>
<td>0.05±0.02</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>0.14±0.05*</td>
<td>0.16±0.04</td>
<td>0.23±0.01</td>
<td>0.06±0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20±0.04</td>
<td>0.23±0.05</td>
<td>0.12±0.01</td>
<td>0.19±0.02</td>
<td>0.18±0.05</td>
<td>0.27±0.06</td>
<td>0.06±0.01</td>
<td>0.09±0.01</td>
</tr>
<tr>
<td></td>
<td>0.14±0.02</td>
<td>0.18±0.08</td>
<td>0.16±0.05</td>
<td>0.19±0.07</td>
<td>0.26±0.05</td>
<td>0.40±0.10</td>
<td>0.09±0.02</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td></td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>0.29±0.06</td>
<td>0.27±0.06</td>
<td>0.17±0.02</td>
<td>0.25±0.05</td>
<td>0.13±0.03</td>
<td>0.36±0.09</td>
<td>0.05±0.02</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td></td>
<td>0.20±0.01*</td>
<td>0.22±0.04</td>
<td>0.22±0.06***</td>
<td>0.33±0.10***</td>
<td>0.27±0.16</td>
<td>0.34±0.15</td>
<td>0.08±0.04**</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td></td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.17±0.06</td>
<td>0.19±0.05</td>
<td>0.13±0.03</td>
<td>0.19±0.01</td>
<td>0.25±0.06</td>
<td>0.37±0.13</td>
<td>0.07±0.01</td>
<td>0.11±0.05</td>
</tr>
<tr>
<td></td>
<td>0.24±0.04</td>
<td>0.22±0.02</td>
<td>0.12±0.02</td>
<td>0.18±0.06</td>
<td>0.18±0.09</td>
<td>0.19±0.13*</td>
<td>0.07±0.03</td>
<td>0.06±0.04</td>
</tr>
<tr>
<td></td>
<td>0.22±0.03</td>
<td>0.30±0.06</td>
<td>0.15±0.03</td>
<td>0.22±0.02</td>
<td>0.20±0.07</td>
<td>0.46±0.12</td>
<td>0.06±0.02</td>
<td>0.12±0.03</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.33±0.12***</td>
<td>0.13</td>
<td>0.24±0.08</td>
<td>0.21</td>
<td>0.20±0.04</td>
<td>0.12***</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td></td>
<td>0.26</td>
<td>0.45*</td>
<td>0.15</td>
<td>0.10***</td>
<td>0.22</td>
<td>0.27</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>0.36±0.16</td>
<td>0.29±0.10</td>
<td>0.17±0.04</td>
<td>0.21±0.04</td>
<td>0.15±0.04</td>
<td>0.29±0.11</td>
<td>0.05±0.00</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td></td>
<td>0.45*</td>
<td>0.49±0.08***</td>
<td>0.15</td>
<td>0.30±0.06***</td>
<td>0.07</td>
<td>0.04±0.04***</td>
<td>0.02***</td>
<td>0.01±0.02 ***</td>
</tr>
</tbody>
</table>
Figure 1 Plots of 27OHC versus cholesterol concentrations in plasma (A), HDL (B) and nonHDL (C) of individuals with mutations in HDL genes and their unaffected relatives. The correlations and regression equations have been calculated on data from all unaffected family members ("Controls" of tables 1 and 2). Please note the biases towards elevated 27OHC concentrations in plasma, HDL and nonHDL of LCAT mutation carriers as well as the biases towards higher HDL-27OHC and lower nonHDL-27OHC concentrations in the carriers of HL mutations.
This finding is surprising because both in vitro and in vivo the p.L168R substitution rather than the p.L202P substitution was found to reduce the LCAT cofactor activity of apoA-I (V.I. Zannis, A. Tybjaerg Hansen, J. Albert Kuivenhoven; personal communication). No significant changes were observed in the degree of 27OHC-esterification in carriers of ABCA1 defects.

Heterozygosity for the Dutch CETP mutations was associated with decreased absolute and relative concentrations of 27OHC-esters in the nonHDL fraction but no differences in the HDL fraction (table 3). Despite half normal CETP concentration and CETP activity (16) the concentrations and percentages of cholesterylesters in HDL or nonHDL were not affected. Regression analysis revealed a significant correlation of CETP mass concentration with both absolute and relative 27OHC-ester concentrations in nonHDL ($r^2=0.337$, $p=0.0092$ and $r^2=0.276$, $p=0.029$, respectively), but insignificant correlations.

**Table 3.** Esterification of 27OHC and cholesterol in plasma and lipoproteins of LCAT and CETP mutation carriers and their unaffected family controls.

<table>
<thead>
<tr>
<th></th>
<th>LCAT mutation carriers</th>
<th>CETP mutation carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unaffected family members („0“)</td>
<td>Heterozygous mutants („1“)</td>
</tr>
<tr>
<td>Number (male/female)</td>
<td>5/3</td>
<td>8/3</td>
</tr>
<tr>
<td>LCAT activity (nmol cholesteryl ester/hour*ml)</td>
<td>8.37±1.21***</td>
<td>6.58±1.01***</td>
</tr>
<tr>
<td>CETP mass (mg/ml)</td>
<td>2.12±0.40</td>
<td>1.73±0.32*</td>
</tr>
<tr>
<td>% 27OHC-ester in plasma</td>
<td>92.3±6.7</td>
<td>89.8±4.3</td>
</tr>
<tr>
<td>% cholesteryl ester in plasma</td>
<td>87.7±2.4</td>
<td>88.4±1.4</td>
</tr>
<tr>
<td>Ratio 27OHC-ester (µM) / cholesteryl ester (mM) in plasma</td>
<td>0.16±0.04</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>Ratio unesterified 27OHC (µM) / unesterified cholesterol (mM) in plasma</td>
<td>0.09±0.06</td>
<td>0.15±0.08</td>
</tr>
<tr>
<td>% 27OHC-ester in HDL</td>
<td>88.4±4.4</td>
<td>79.8±10.6*</td>
</tr>
<tr>
<td>% cholesterol esters in HDL</td>
<td>80.9±3.0</td>
<td>82.5±3.6</td>
</tr>
<tr>
<td>Ratio 27OHC-ester (µM) / cholesteryl ester (mM) in HDL</td>
<td>0.22±0.06</td>
<td>0.21±0.07</td>
</tr>
<tr>
<td>Ratio unesterified 27OHC (µM) / unesterified cholesterol (mM) in HDL</td>
<td>0.12±0.04</td>
<td>0.28±0.20*</td>
</tr>
<tr>
<td>% 27OHC-ester in nonHDL</td>
<td>93.3±23.9</td>
<td>95.8±9.6</td>
</tr>
<tr>
<td>% cholesterol esters in nonHDL</td>
<td>92.5±1.4</td>
<td>91.4±2.0</td>
</tr>
<tr>
<td>Ratio 27OHC-ester (µM) / cholesteryl ester (mM) in nonHDL</td>
<td>0.13±0.06</td>
<td>0.14±0.05</td>
</tr>
<tr>
<td>Ratio unesterified 27OHC (µM) / unesterified cholesterol (mM) in nonHDL</td>
<td>0.14±0.19</td>
<td>0.10±0.10</td>
</tr>
</tbody>
</table>

*, ** and *** indicate statistically significant differences ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) between „1“ and „0“.
with absolute and relative cholesteryl ester concentrations in non-HDL ($r^2=0.077$ and $r^2=0.029$, respectively) (see supplemental figure 2).

**27OHC in mice with various knock-outs of HDL genes**

In normal murine plasma both cholesterol and 27OHC are almost exclusively carried by HDL (supplemental table). We therefore analyzed the different genetic mouse strains for total 27OHC plasma levels only. Compared to wild-type mice, apoA-I- or LCAT- knockout mice were characterized by more than 50% lowered plasma 27OHC concentrations, whereas SR-BI knockout mice had about 5-fold higher 27OHC levels. After normalization for total cholesterol, the differences remained statistically significant for LCAT and SR-BI knock-out mice only. In addition and compared to wild type mice, ABCA1 deficient mice showed a 140% increased 27OHC/cholesterol ratio ($p < 0.001$) in the presence of 25% lower 27OHC levels ($p < 0.05$, table 4). Interestingly, heterozygous ABCA1 knock-out mice showed a similar decrease in 27OHC plasma levels which did not result in a significantly altered 27OHC/cholesterol ratio (table 4) due to the equal decrease in cholesterol levels. To investigate the contribution of macrophage-derived 27OHC to plasma 27OHC levels, we also analyzed the plasmas of LDL receptor-deficient mice who received bone marrow from mice with knockouts of either ABCA1, ABCG, or both ABCA1 and ABCG1 (table 4). Notably in parallel with hypercholesterolemia, LDLR-/ mouse had 20-fold higher 27OHC concentrations and 2-fold higher 27OHC/cholesterol ratios than wild-type mice. Transplantation of ABCA1- or ABCG1- deficient bone marrow resulted in 45% and 23% decreases of 27OHC plasma levels as well as in significantly decreased 27OHC/cholesterol

<table>
<thead>
<tr>
<th>Mouse strains</th>
<th>Number of mice (n)</th>
<th>27OHC (µmol/L)</th>
<th>Cholesterol (mmol/L)</th>
<th>Ratio total-27OHC / total-cholesterol (µmol/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>28</td>
<td>0.15±0.08</td>
<td>1.81±0.55</td>
<td>0.08±0.04</td>
</tr>
<tr>
<td>APOA-I-/-</td>
<td>5</td>
<td>0.06±0.04*</td>
<td>1.24±0.43***</td>
<td>0.05±0.05</td>
</tr>
<tr>
<td>ABCA1-/-</td>
<td>6</td>
<td>0.11±0.06</td>
<td>0.68±0.50***</td>
<td>0.19±0.08***</td>
</tr>
<tr>
<td>ABCA1+/+</td>
<td>3</td>
<td>0.08±0.01</td>
<td>1.43±0.21</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>LCAT-/-</td>
<td>8</td>
<td>0.06±0.02**</td>
<td>2.28±1.08</td>
<td>0.03±0.01***</td>
</tr>
<tr>
<td>SR-BI-/-</td>
<td>10</td>
<td>0.96±0.32***</td>
<td>3.27±0.73**</td>
<td>0.29±0.10***</td>
</tr>
<tr>
<td>LDLR-/-: BMT wt</td>
<td>6</td>
<td>3.33±1.32</td>
<td>20.44±7.83</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>LDLR-/-: BMTABCA1-/-</td>
<td>6</td>
<td>1.88±0.26*</td>
<td>15.56±2.89</td>
<td>0.13±0.03*</td>
</tr>
<tr>
<td>LDLR-/-: BMTABCG1-/-</td>
<td>6</td>
<td>2.57±0.52</td>
<td>23.86±3.02</td>
<td>0.11±0.03*</td>
</tr>
<tr>
<td>LDLR-/-: BMTABCA1-/- * ABCG1-/-</td>
<td>9</td>
<td>1.11±0.25***</td>
<td>6.09±1.55***</td>
<td>0.19±0.07</td>
</tr>
</tbody>
</table>

*, **, *** indicate statistically significant differences ($p<0.05$, $p<0.01$, $p<0.001$, respectively) as compared to wild type mice or LDLR-/- mice transplanted with wild type bone marrow.
27-hydroxycholesterol in monogenic HDL disorders

ratios (p < 0.05 for ABCA1; p < 0.01 for ABCG1). Mice with bone marrow from mice with knock-outs of both ABCA1 and ABCG1 had even 66% lower mean 27OHC plasma levels, however, this was seen in the context of a slightly more pronounced 70% decrease in cholesterol levels so that the 27OHC/cholesterol ratio was rather increased.

Discussion

27OHC plays an important physiological role as an intermediary product of cholesterol removal from specific tissues and the entire organism as well as an agonist and modulator of liver-X- and estrogen receptors, respectively [9, 21]. We have previously shown for normolipidemic individuals that about 50%, 30% and 5% of circulating 27OHC in healthy individuals are recovered in HDL, LDL and VLDL, respectively [9]. However, the plasma concentration of 27OHC is 10'000-fold lower than that of cholesterol (0.5 mmol/L versus 5 mmol/L on average). It is even considerably lower than the particle concentration of HDL (on average 20 mmol/L) and LDL (on average 2 mmol/L) so that only a minority of lipoprotein particles carries 1 or more molecules of 27OHC. The very low plasma and lipoprotein concentrations of 27OHC, the significant correlations between 27OHC and cholesterol concentrations in plasma and lipoproteins [9] as well as the higher water-solubility of 27OHC compared to cholesterol [3] raised the question whether 27OHC is passively equilibrated among the various lipoprotein classes or specifically metabolized in plasma similar to cholesterol, that is by established transporters, enzymes, lipid transfer proteins and receptors. Some general support for the latter assumption could already be derived from the enrichment of 27OHC relative to cholesterol in HDL (on average 47±7% of plasma 27OHC instead of 33±6% plasma cholesterol [9] and from the 10% higher percentages of 27OHC-esters relative to cholesterylesters in plasma, HDL and LDL [9]. We here further explored this question by analysing 27OHC concentrations in plasma as well as HDL- and nonHDL-fractions of patients and mice with distinct monogenic disorders of HDL metabolism.

In the present study correlations between 27OHC and cholesterol concentrations in plasma and HDL were similar or even stronger than those reported by us previously, namely 0.59 instead of 0.54 in total plasma, and 0.64 instead of 0.34 in HDL (figure 1 and [9]). Independently of the metabolic or genetic origin, differences in HDL cholesterol appear to explain up to 40% of the variation in HDL-27OHC levels. As a consequence, functionally relevant mutations in apoA-I, ABCA1 and LCAT that decrease HDL-cholesterol were associated with reduced concentrations of 27OHC in HDL. Likewise, HDL deficient mice with knock-outs of apoA-I or LCAT presented with decreased 27OHC plasma levels. Conversely, 27OHC or HDL-27OHC plasma levels were strongly elevated in SR-BI knock-out mice as well as in male carriers of mutations in the genes of CETP, SR-BI and HL. The reason for the absent effect of the HDL-cholesterol increasing mutations on 27OHC levels in women might be related to the strong regulation of HL and SR-BI by estrogens [22-23], since 27OHC is a modulator of estrogen receptor activity [9, 21]. One may speculate
that this interaction of 27OHC and estrogens in the regulation of SR-BI and HL leads to differences in the metabolism of cholesterol and 27OHC between males and females. At first sight, in both humans and mice with inborn errors of HDL metabolism, HDL-27OHC levels appeared simply to change passively in parallel with HDL-cholesterol levels. However, in the light of the previously suggested concept that 27OHC may represent an HDL-independent pathway of reverse cholesterol transport (2) our findings in patients with defects in ABCA1, LCAT or CETP as well as mice with either systemic or macrophage specific knockouts of ABCA1 and ABCG1 deserve special attention.

**ABCA1 and ABCG1**

27OHC secretion has been suggested to represent an alternative pathway to HDL- and apoA-I-mediated cholesterol efflux by which macrophages protect themselves from cholesterol overload [2]. However, this hypothesis was generated based on experiments that had been performed before the discovery of ABCA1 and ABCG1 as important cholesterol efflux pumps and their involvement in 27OHC efflux has not yet been tested directly. ABCA1 and ABCG1 were however shown to mediate the efflux of two other oxysterols, namely 25-hydroxycholesterol and 7-ketocholesterol, respectively [24-25]. The current data show normal 27OHC plasma levels in the presence of decreased cholesterol levels and hence increased 27OHC/cholesterol ratios in both ABCA1 deficient Tangier patients and ABCA1 knock-out mice suggesting that 27OHC is secreted independently from ABCA1. However, transplantation of bone marrow from ABCA1- or ABCG1-knockout mice significantly decreased 27OHC/cholesterol ratios. The mice which received bone marrow of mice with a combined knock-out of ABCA1 and ABCG1 showed the most prominent decrease of both 27OHC and cholesterol levels so that the 27OHC/cholesterol ratio was not decreased. Nevertheless, we interprete the data as an indication that both ABCA1 and ABCG1 contribute to 27OHC-efflux from macrophages. Our contradiction to the concept of an HDL-independent reverse cholesterol transport pathway mediated by 27OHC in humans is also supported by the enrichment of 27OHC relative to cholesterol in HDL, the strong correlation between HDL-cholesterol and HDL-27OHC in the normolipidemic human population as well as the parallel changes of HDL-cholesterol and HDL-27OHC observed in almost all human heterozygotes for HDL-related gene defects. Although not proven by us directly, our findings rather suggest that 27OHC secretion involves HDL-mediated efflux pathways that are supported by ABCA1 or ABCG1 [26].

**LCAT**

In both genders of humans, mutations in LCAT caused relatively less pronounced decreases in HDL-27OHC than HDL-cholesterol levels in comparison with unaffected family members, the entire control population as well as other genetic low HDL conditions. This finding is surprising because about 90% of 27OHC in normal HDL but only about 80% of 27OHC in HDL of heterozygous LCAT mutation carriers is esterified. The elevated 27OHC/cholesterol ratio in LCAT mutation carriers is hence the result of a
relatively high concentration of unesterified 27OHC, rather than of esterified 27OHC. It thus appears that reduced LCAT activity increases the capacity of HDL particles to accommodate unesterified 27OHC, for example as the consequence of a higher content in phosphatidylcholine or an increased number of small HDL particles.

In both plasma and HDL, the proportions of 27OHC-esters in total 27OHC are about 10% higher than the proportions of cholesterylesters in total cholesterol (table 3 and [9]). Moreover, heterozygous LCAT mutation carriers showed reduced 27OHC-ester/total-27OHC ratios, but normal cholesterylester/total cholesterol ratios, in HDL. Finally, LCAT activity showed a stronger correlation with 27OHC-ester concentrations than with cholesterylester concentrations in HDL ($r^2=0.426$, $p \leq 0.001$ vs. $r^2=0.290$, $p \leq 0.01$, respectively; see supplemental figure 1). Together the data thus suggest that 27OHC is more readily esterified by LCAT and more sensitive to impairments of LCAT activity than cholesterol. Our observational data do not allow any conclusion on the biochemical basis for the higher sensitivity of 27OHC towards LCAT. One reason could be differences in the reaction kinetics (affinity and/or maximal reaction velocity) of LCAT towards 27OHC and cholesterol, so that reduced LCAT activity in mutation carriers affects 27OHC-esterification more strongly than cholesterol esterification. Importantly, 27OHC has a second free hydroxyl-group in the side chain in addition to the 3b-hydroxyl-group of the sterol which, by contrast to free hydroxyl-groups of other oxysterols, can also be esterified by LCAT [27]. Therefore, in theory, the stronger impact of LCAT activity on 27OHC-esterification may reflect the abundance of different 27OHC-ester species. Unfortunately our method did not allow for a direct and differentiated quantification of 27OHC-esters. We rather estimated the concentrations of all 27OHC-esters from the differences between total and unesterified 27OHC which were measured after and before saponification, respectively. We therefore cannot rule out that the higher concentration of 27OHC-esters relative to cholesterol esters in normal controls reflects the abundance of additional 27OHC-ester species and that the decreased 27OHC-ester formation in LCAT mutation carriers results from a specific loss of function towards the 27-hydroxyl-group. However, LCAT was reported to esterify the 27-hydroxyl-group only after esterification of the 3b-hydroxyl-group, so that 27OHC-esters were postulated to occur as either 3b-hydroxyl-monoesters or 3b/27-dihydroxyl esters only [27]. If so, our finding of decreased 27OHC-esters in heterozygous LCAT mutation carriers cannot be explained by a selectively reduced activity to esterify the 27-hydroxyl-group.

**CETP**

Both in the nonHDL fraction (this study) and LDL [9], more than 90% of 27OHC is esterified. To our knowledge the origin of these esters has not yet been explored directly. In analogy to cholesterylesters, 27OHC-esters of apoB containing lipoproteins may originate either from intrahepatic production by acyl-CoA:cholesterol acyltransferase (ACAT) and subsequent secretion together with VLDL [28-29] or from LCAT-mediated production either directly on apoB-containing lipoproteins or indirectly on HDL and
subsequent CETP-mediated transfer. ACAT has been shown to esterify most oxysterols with much less efficacy than cholesterol [28] but its contribution to 27OHC-esterification has not been explored. Unlike in HDL, the percentage of 27OHC-esters in non-HDL was not altered by heterozygous defects in LCAT suggesting that LCAT does not play a rate limiting role for 27OHC-ester formation in LDL. Alternatively, since many of the LCAT mutants analysed by us were found in patients with fish-eye disease [14, 15, 19], these mutants may have retained beta-LCAT activity, that is the ability to esterify cholesterol in apoB-containing lipoproteins [14, 15, 19]. Most interestingly and informative, however, heterozygosity for CETP defects was associated with reduced absolute and relative concentrations of 27OHC-esters in the non-HDL fraction but normal concentrations in HDL. This suggests that CETP mediates the transfer of 27OHC-esters between HDL and apoB-containing lipoproteins. This binding and transport capacity of CETP has not yet been proven directly for 27OHC-esters but for 25-hydroxycholesterol esters [30]. Compared to cholesterylester transfer, the efficacy of 27OHC-ester transfer appears to be even more sensitive to changes in CETP activity because apoB-containing lipoproteins of heterozygous CETP mutation carriers contained normal concentrations and percentages of cholesteryesters. Furthermore, CETP mass concentrations showed statistically significant correlations with absolute and relative concentrations of 27OHC-esters in non-HDL but no significant correlations with either absolute or relative cholesterylester concentrations in non-HDL (see supplemental figure 2).

In conclusion, mutations in several HDL genes lead to general alterations in the quantity of 27OHC in HDL and plasma as well as specific changes in the esterification and lipoprotein distribution of 27OHC. In general, changes in the concentration of HDL cholesterol are paralleled by equidirectional changes of HDL-27OHC levels indicating that HDL acts as a passive acceptor and transporter of 27OHC and to explain up to 40% of the variation in HDL-27OHC levels. More specifically our data from mice with macrophage specific knock out of ABCA1 and ABCG1 refutes the notion that 27OHC is effluxed from macrophages by HDL-independent pathways. In addition esterification and transfer of 27OHC (esters) appear to be more sensitive towards changes in the activities of LCAT and CETP than cholesterol. It hence appears that reverse transport of 27OHC is even more strongly influenced by HDL metabolism than cholesterol. In view of the role of 27OHC as a tissue specific modulator of transcription factors, future studies will have to show whether these specific aspects in the plasma metabolism and transport of 27-hydroxycholesterol have implications for health and disease.

Acknowledgements

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References


Chapter 15


Supplemental Figure 1. Correlations of LCAT activity with the concentrations of 27OHC-esters (A) and cholesterylesters (B) in heterozygous LCAT mutation carriers and their unaffected family members (N = 20).

Correlations of LCAT activity with the concentrations of 27OHC-esters (A) and cholesterylesters (B) in heterozygous LCAT mutation carriers and their unaffected family members (N = 20).
Supplemental Figure 2 Correlations of CETP mass concentration with the concentrations of 27OHC-esters (A) and cholesterylesters (B) in nonHDL of family controls without any mutation in HDL genes (N = 20).

Supplemental Table Comparison between the plasma and HDL concentrations of 27OHC and cholesterol in mice with knock-outs of apoA-I and SR-BI. “+/+”=wild-type controls, “+/−”=heterozygous knockouts, “−/−”=homozygous knockouts.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Number of mice (n)</th>
<th>27OHC concentration (µM)</th>
<th>Cholesterol concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>plasma</td>
<td>HDL</td>
</tr>
<tr>
<td>+/-</td>
<td>3</td>
<td>0.13±0.04</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>APOA-I-/-</td>
<td>2</td>
<td>0.08±0.00</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>SR-BI+/-</td>
<td>3</td>
<td>0.20±0.06</td>
<td>0.20±0.04</td>
</tr>
<tr>
<td>SR-BI-/-</td>
<td>3</td>
<td>1.11±0.12</td>
<td>1.08±0.32</td>
</tr>
</tbody>
</table>
Chapter 16

Submitted for publication

Plasma levels of sphingosine-1-phosphate and apolipoprotein M in patients with monogenic disorders of HDL metabolism

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§ both act as equal senior authors
Abstract

**Background:** The majority of sphingosine-1-phosphate (S1P) and apolipoprotein (apo) M is transported by HDL. ApoM has been suggested to bind S1P. Because little is known about the regulation of plasma S1P levels, we tested the hypothesis that plasma HDL-cholesterol levels or apoM determine plasma S1P levels.

**Methods:** S1P and apoM were quantified in plasmas of 48 patients with seven different monogenic disorders of HDL metabolism and their 51 unaffected relatives.

**Results:** Compared to family controls, S1P levels were decreased by 20% to 45% in heterozygous carriers of mutations in **APOA1**, **LCAT** or **ABCA1**, and by 50% to 65% in carriers of two defective alleles in **LCAT** or **ABCA1**. Mutations in **CETP**, **SCARB1**, **LIPC**, or **LIPG** did not significantly affect S1P concentrations. In family controls, S1P levels correlated significantly with HDL-cholesterol only in individuals with apoA-I plasma concentrations below the median. By contrast, concentrations of apoM correlated significantly with HDL-cholesterol levels of unaffected family controls but were not significantly altered by heterozygous mutations in HDL genes except 15% decreases and increases in heterozygotes for mutations in LCAT and LIPC, respectively. In HDL-deficient patients with no functional ABCA1 or LCAT, apoM plasma concentrations were lowered by about 40%. ApoM and S1P levels were not significantly correlated in family controls.

**Conclusion:** S1P plasma levels are limited by low levels of HDL-cholesterol or apoA-I levels but not apoM.
Introduction

Sphingosine-1-phosphate (S1P) is produced by the degradation of ceramide into sphingosine which is subsequently phosphorylated by sphingosine kinase. (1,2) As the ligand of at least five different G-protein coupled receptors, S1P exerts many biological activities. (1,2) Erythrocytes are the main source (~90%) of S1P found in plasma (3,4) while platelets and endothelial cells are considered as important contributors of the remaining ~10%. (3-5) Plasma S1P concentrations vary from ~0.2 mmol/L up to ~2 mmol/L and are 20- to 100-fold higher than the $K_d$ value of its receptors. (1,2) Circulating S1P is transported by high-density lipoproteins (HDL, ~50-70%), albumin (~30%), low and very-low density lipoproteins (LDL and VLDL, respectively; together <10%) (3). Importantly, particle concentrations of LDL and HDL in normolipidemic plasma are about 2 and 20 fold higher, respectively, than the S1P plasma concentration indicating that only the minority of lipoprotein particles carries one or more molecules of S1P. Despite its presence on only a minority of HDL particles, S1P is suggested to be responsible for many protective effects of this lipoprotein class. (3-6) For example, HDL-S1P inhibits the migration of vascular smooth muscle cells, promotes vasodilation by stimulating endothelial nitric oxide production, and promotes endothelial cell growth and survival by inducing cell proliferation and migration as well as tube formation and by inhibiting apoptosis (3-6). In addition, HDL-S1P inhibits reactive oxidative species production and interferes with the recruitment of monocytes and lymphocytes into the intima of the arterial wall by inhibiting endothelial cell expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in response to TNF-a. By contrast, intracellular S1P appears to exert adverse effects such as stimulating the expression of VCAM-1 and ICAM-1, mediating pro-inflammatory effects of cytokines, and inducing vasoconstriction. (3-6)

Apolipoprotein (apo) M is a 25 kDa member of the lipocalin family, which like S1P has a plasma concentration of approximately 1 mmol/L and is also mainly associated with HDL (7-9). ApoM was found to inhibit LDL oxidation and to increase the capacity of HDL to induce cholesterol efflux from macrophage foam cells by forming preβ-HDL, an important initial extracellular acceptor of cell-derived cholesterol (9,10). In line with these anti-atherogenic functions, apoM was found to reduce atherosclerosis in LDL-receptor knock-out mice (10,11). Interestingly, the X-ray crystallographic analysis of recombinant apoM revealed the presence of a fatty acid binding pocket within the eight-stranded antiparallel β-barrel, which is the structural hallmark of lipocalins (12). Subsequent ligand binding studies using wild-type and mutant apoM isoforms provided evidence that apoM can bind S1P with higher affinity than fatty acids and with an IC50 of 0.9 mmol/L, which is in the range of physiological S1P plasma concentrations (12). To date, little is known on the regulation of S1P levels in plasma. Since the majority of S1P is carried by HDL and since apoM has been proposed to bind S1P within HDL, we set out to test the hypothesis that plasma concentrations of HDL-cholesterol or...
apoM determine the concentration of S1P in plasma. We therefore analysed S1P and apoM levels in total and apoB-depleted plasmas of patients with monogenic disorders of HDL metabolism. Specifically, we examined patients with functional mutations in the genes encoding the following proteins: apolipoprotein A-I (apoA-I), which is the main protein component of HDL; ATP-binding cassette transporter A1 (ABCA1), which mediates cholesterol and phospholipid efflux from cells to apoA-I; lecithin:cholesterol acyl transferase (LCAT), which esterifies cholesterol and thereby converts nascent HDL into mature HDL; cholesteryl ester transfer protein (CETP), which exchanges cholesteryl ester from HDL with triglycerides from apoB-containing lipoproteins; scavenger receptor type B class 1 (SR-BI), which mediates selective uptake of cholesterol esters into the liver and steroidogenic organs; hepatic lipase (HL encoded by \textit{LIPC}), which hydrolyses triglycerides.

Table 1. Characteristics of the study participants. Values are presented as mean ± SD. The apolipoprotein levels in mg/dL were converted into mM based on apoA-I and apoB-100 (28,300 and 512,000, respectively).

<table>
<thead>
<tr>
<th>Mutated gene</th>
<th>Number of defective alleles</th>
<th>Mutation</th>
<th>Male/Female</th>
<th>Age (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>0</td>
<td>p.L202P (c.605T&gt;C)</td>
<td>1/2</td>
<td>27 ± 14</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>p.C1477R (c.4429T&gt;C)</td>
<td>3/3</td>
<td>57 ± 11</td>
</tr>
<tr>
<td>ABCA1</td>
<td>0</td>
<td>p.L1056P (c.3167T&gt;C) or p.C1477R (c.4429T&gt;C)</td>
<td>3/3</td>
<td>57 ± 11</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>p.L1056P (c.3167T&gt;C, homozygote) or p.Q1038X (c.3112C&gt;T) + p.N1800H (c.5398A&gt;C) or p.C1477R (c.4429T&gt;C) + IVS25+1G&gt;C</td>
<td>1/2</td>
<td>53 ± 10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>p.T147I (c.440C&gt;T) + V333M</td>
<td>0/2</td>
<td>69 ± 4</td>
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<tr>
<td>SR-BI</td>
<td>0</td>
<td>p.P297S (c.889C&gt;T)</td>
<td>4/5</td>
<td>54 ± 19</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3/4</td>
<td>45 ± 22</td>
<td></td>
</tr>
<tr>
<td>CETP</td>
<td>0</td>
<td>p.P297S (c.889C&gt;T)</td>
<td>2/1</td>
<td>36 ± 16</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2/1</td>
<td>39 ± 18</td>
<td></td>
</tr>
<tr>
<td>HL (LIPC)</td>
<td>0</td>
<td>2/1</td>
<td>45 ± 19</td>
<td></td>
</tr>
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<td></td>
<td>1</td>
<td>3/1</td>
<td>45 ± 15</td>
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</tr>
<tr>
<td>EL (LIPG)</td>
<td>0</td>
<td>3/7</td>
<td>41 ± 21</td>
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<tr>
<td></td>
<td>1</td>
<td>6/4</td>
<td>45 ± 21</td>
<td></td>
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</tbody>
</table>

* ND: not determined
# Amino acid changes are localized on the basis of the entirely translated protein, that is including the signal peptides. To define the position within the mature protein, correct apoA-I by -24 amino acids, ABCA1 by -60 amino acids, LCAT by -24 amino acids, SR-BI by 0 amino acids, CETP by -17 amino acids, HL by -22 amino acids, EL by 0 amino acids.
triglyceride and phospholipids in HDL; and endothelial lipase (EL encoded by \textit{LIPG}), which preferentially hydrolys HDL-phospholipids. (13) Mutations in either \textit{APOA1}, \textit{ABCA1} or \textit{LCAT} lower HDL cholesterol levels while mutations in \textit{CETP}, \textit{SCARB1}, \textit{LIPC} or \textit{LIPG}(14) increase HDL cholesterol levels.

**Methods**

**Participants and Plasma Collection**

Forty-eight Dutch patients with functional mutations in the genes encoding either apoA-I (\textit{APOA1}), ABCA1, LCAT, CETP, HL (gene: \textit{LIPC}), EL (gene: \textit{LIPG}) or SR-BI (gene: \textit{SCARB1}) as well as 51 unaffected family members were investigated. Most of the respective defects have been described previously (15-23). The as yet undescribed p.R322C, mutation

<table>
<thead>
<tr>
<th>Mutated gene</th>
<th>Number of defective alleles</th>
<th>Male/Female</th>
<th>Age (year)</th>
<th>Cholesterol (mM)</th>
<th>HDL-cholesterol (mM)</th>
<th>Triglyceride (mM)</th>
<th>ApoA-I (mM)</th>
<th>ApoB (mM)</th>
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</thead>
<tbody>
<tr>
<td>APOA1</td>
<td>0</td>
<td>1/2</td>
<td>27 ± 14</td>
<td>4.59 ± 0.68</td>
<td>1.16 ± 0.06</td>
<td>1.09 ± 0.29</td>
<td>57.57 ± 4.68</td>
<td>1.89 ± 0.30</td>
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<tr>
<td>ABCA1</td>
<td>0</td>
<td>4/4</td>
<td>44 ± 20</td>
<td>4.39 ± 0.89</td>
<td>1.47 ± 0.39</td>
<td>0.95 ± 0.22</td>
<td>56.44 ± 10.18</td>
<td>1.64 ± 0.24</td>
</tr>
<tr>
<td>LCAT</td>
<td>0</td>
<td>5/3</td>
<td>49 ± 9</td>
<td>4.96 ± 0.86</td>
<td>1.33 ± 0.38</td>
<td>1.29 ± 0.66</td>
<td>60.24 ± 7.63</td>
<td>1.84 ± 0.47</td>
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<tr>
<td>CETP</td>
<td>0</td>
<td>2/1</td>
<td>36 ± 16</td>
<td>4.14 ± 0.51</td>
<td>1.30 ± 0.56</td>
<td>0.97 ± 0.28</td>
<td>56.04 ± 11.67</td>
<td>1.74 ± 0.38</td>
</tr>
<tr>
<td>HL</td>
<td>0</td>
<td>7/3</td>
<td>45 ± 19</td>
<td>5.23 ± 0.99</td>
<td>1.61 ± 0.54</td>
<td>1.45 ± 0.15</td>
<td>56.67 ± 10.89</td>
<td>2.51 ± 0.48</td>
</tr>
<tr>
<td>EL</td>
<td>0</td>
<td>3/7</td>
<td>41 ± 21</td>
<td>5.43 ± 1.28</td>
<td>2.05 ± 0.51</td>
<td>1.37 ± 0.82</td>
<td>63.01 ± 16.67</td>
<td>1.86 ± 0.47</td>
</tr>
</tbody>
</table>

*ND: not determined

Amino acid changes are localized on the basis of the entirely translated protein, that is including the signal peptides. To define the position within the mature protein, correct apoA-I by -24 amino acids, ABCA1 by -60 amino acids, LCAT by -24 amino acids, SR-BI by 0 amino acids, CETP by -17 amino acids, HL by -22 amino acids, EL by 0 amino acids.
causes a 38% loss of LCAT activity when using a proteoliposome substrate assay. A frameshift mutation in \textit{LIPG} was anticipated to be functional since the deletion resulted in a premature truncation of the enzyme (p.L130fsX165).

The Medical Ethics Committee of the Academic Medical Center in Amsterdam, The Netherlands, approved all genetic and phenotypic studies described and all participants signed informed consent to join the study. The characteristics of the study participants are shown in Table 1. Fasting blood samples were collected after at least a 10h fast in the morning and EDTA-plasma was prepared through centrifugation of blood at 3000 rpm for 10 min at 25°C. Aliquots were immediately frozen at -80°C until later use.

\textit{Quantification of lipids and apolipoproteins}

Total plasma cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol, apoB, apoA-I, and apoA-II levels were measured with commercial kits (Wako, Neuss, Germany and Randox, Crumlin, UK) on a Cobas Mira autoanalyzer. Due to the limited sensitivity of the enzymatic homogenous assay, concentrations of HDL cholesterol as well as thereof derived measures (nonHDL cholesterol and the ratios of S1P per mM HDL-cholesterol and nonHDL-cholesterol) are not reported for patients with virtually complete HDL deficiency, namely the homozygotes and compound heterozygotes for mutations in \textit{ABCA1} or \textit{LCAT}.

Plasma ApoM levels were measured with an ELISA provided by Roche (Basel, Switzerland). In brief, 96-well nunc Maxisorp plates (Nunc A/S, VWR International AG, Dietikon, Switzerland) were coated with primary apoM antibody in PBS (5µg/mL) over night at 4°C. The plates were blocked in buffer containing 1% BSA and washed with PBS-Tween (0.05%). Plasma samples were diluted 1:2000 in blocking buffer. Bound apoM was detected with horseradish peroxidase coupled secondary antibody (1.5µg/mL) and TMB (3,3',5,5'-tetramethylbenzidine, Sigma Aldrich Chemie GmbH, Buchs, Switzerland) as the substrate. Absorbance was measured at 450 nm. The standard curve was prepared from recombinant human ApoM covering a range from 250 ng/mL to 0.1 ng/mL. The sigmoidal standard curve was fitted by nonlinear regression analysis and ApoM concentrations were calculated in µmol/L. The lowest detectable apoM concentration was 0.3µM and the inter-assay CV was 3.9%.

\textit{Lipoprotein Fractionation}

Lipoproteins were isolated from pooled plasmas of 2 healthy blood donors (Zürcher Blutspendedienst, Zurich, Switzerland) by stepwise ultracentrifugation at 59’000 rpm and 15°C using a Beckman Coulter Optima L90K ultracentrifuge (Beckman Coulter, Brea, CA, USA).\(^{(24)}\)

For the determination of S1P- and apoM-concentrations in HDL, apoB-containing lipoproteins were removed from 100 mL EDTA plasma by precipitation with dextran-sulfate – Mg\(^{2+}\) (0.9 g/L dextran sulfate and 45 mmol/L Mg\(^{2+}\) as the final concentration
The S1P and apoM concentrations in the LDL/VLDL fraction were calculated by subtracting S1P and apoM levels, respectively, measured in apoB-depleted-plasma, from S1P and apoM, respectively, measured in total plasma.

Quantification of S1P

Samples were prepared by protein precipitation without any further lipid extraction. In short, an aliquot of 25 mL total or apoB-depleted plasma was added to 200 mL methanol (MeOH) as well as 25 mL, 1.09 nM C17-S1P (Avanti Polar Lipids, Alabaster, AL, USA) in MeOH as internal standard (IS). The mixture was vortexed immediately each time after the addition to MeOH or methanolic solution to minimize anisotropic precipitation. The mixture was then centrifuged at 10,000 g for 10 min at 4°C. The precipitated proteins were discarded and the supernatant was directly injected into LC-MS.

To prepare a S1P-free sample matrix for blanks, calibrators, and quality control (QC), activated charcoal was added to EDTA plasma from a healthy volunteer (100 mg charcoal per mL plasma) and mixed for at least 12 hr. The mixture was then centrifuged (10,000 g, 15 min at RT) a few times until the supernatant was clear. Charcoal and the bound S1P were removed from the supernatant by sequential passaging through filters with pores of 0.45 mm and 0.22 mm diameter. S1P (Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in DMSO/concentrated-HCl (100/2 v/v) to make 0.28 µM stock solutions. Standards were prepared by further dilution of the stock solution in MeOH (0.06, 0.14, 0.28, 0.57, 1.42, 2.27 nM). Standards (25 mL), IS (25 mL) and MeOH (175 mL) were added to the charcoal-treated plasma (25 mL) and processed as described above. A calibration curve was constructed by plotting the S1P/IS peak area ratios and the concentrations of the S1P standards. The S1P concentration in samples was derived from the S1P/IS peak area ratios relative to the calibration curve. The calibration curve was obtained for each series of samples which was analysed within one day. Two quality controls (QC1 and QC2) were prepared using S1P standards in MeOH (0.45 and 1.14 nM) and assayed in the beginning and in the end of each sample series. Blank samples (blanks) for baseline correction were prepared by adding IS (25 mL) to charcoal-treated plasma (25 mL) and MeOH (200 mL), then processed as in “Sample Preparation”. Blanks were assayed before each run of calibrators and samples to correct for any remaining carryover in the system.

The LC-MS system consisted of an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) with cooled sample trays (4°C), a Rheos 2000 HPLC pump (Flux Instruments, Basel, Switzerland) and a TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic conditions for reversed-phase separation of S1P have been modified from Schmidt H et al. (26) We used a Nucleosil C18 HD column (125x2 mm, 100 Å, 5 mm) which was connected to a Nucleosil C18 HD guard column (8x4 mm, 100 Å, 5 mm) and heated at 30°C with a HotDog 5090 column oven (ProLab Instruments, Reinach, Switzerland). The mobile phase A was MeOH/THF/water (2/2/3) and the mobile phase B was MeOH/THF (1/1). Gradient elution was started with 100% mobile phase A for the first 0.6 min. Mobile phase B was increased to 100%
within 4.4 min and then kept constant for 4 min. The column was then re-equilibrated with 100% mobile phase A for 15.5 min. S1P and IS eluted at retention time (t_r) ~7.7 min. The injection volume was 5 mL. Carryover of S1P into the injection system was minimized by washing the syringe 12 times with MeOH/acetone/2-propanol (1/1/1) containing HCOOH 0.1% and 3 times with acetone/MeOH/water (2/2/1) after every injection. After each sample run, the column was flushed with MeOH at least twice. In addition, the column was flushed with MeOH/aceton/2-propanol (1/1/1) after injection of highly concentrated samples or calibrators to further minimize carryover of S1P into the column. Ionisation was performed using electrospray ionization (ESI). Detection was in the positive mode, monitoring [M+H]^+ ions using Selective Reaction Monitoring (SRM) for the transition of m/z 380.2 à 264.2 (16 V, 1 mTorr) for the target analyte (S1P).

<table>
<thead>
<tr>
<th>Mutated gene</th>
<th>Number of defective alleles</th>
<th>Number of participants</th>
<th>S1P in total plasma (µM)</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>Controls</td>
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<tr>
<td>Defects affecting HDL maturation</td>
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<tr>
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<td>Defects affecting HDL catabolism</td>
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<td></td>
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Controls = all unaffected relatives, „0” = unaffected relatives from specific mutation, „1” = heterozygous mutant carriers, „2” = homozygous or compound heterozygous mutant carriers. Symbols indicate levels of statistical significance for differences between mutant carriers and unaffected relatives and all controls, respectively.: * and #: P < 0.05; ** and ##: P < 0.01; *** and ###: P < 0.001. ND: not determined.
and m/z 366.2 ± 82.06 (34 V, 1 mTorr) for the IS. Spray voltage was 5000 V, skimmer offset was 10 V and ion transfer capillary temperature was 150°C. Further ionisation and detection parameters were optimized by tuning the system with S1P standards. Data analysis was performed on XCalibur 2.0.7 SP1.

Our method of S1P quantification had the following quality characteristics: Recoveries of 105.7% and 103.7% were found for charcoal-treated plasma spiked with 0.14 mM and 0.57 µM S1P, respectively. At concentrations of 0.06 mM, 0.14 mM and 0.57 µM, the intra-day imprecision amounted to 10.9%, 1.8% and 6.3%, respectively, and the inter-day imprecision to 10.2%, 8.2% and 3.9%, respectively. At the same concentrations, the intra-day accuracy amounted to 111.3%, 98.5% and 100.9%, respectively, and the inter-day accuracy to 103.2%, 92.35% and 95.2%, respectively.
Statistical Analysis

Statistical analyses were performed using Microsoft Excel (Microsoft, Redmont, WA, USA) and SPSS 17.0 (SPSS, Chicago, IL, USA). Normal distribution was tested using Kolmogorov-Smirnov test. Since the parameters of interest were normally distributed, unpaired student-t test was used to test statistical significances for the differences between 2 groups (assuming equal variance). Statistical significance of a correlation was tested from the Pearson’s correlation coefficient (r).

Results

Lipoprotein distribution of S1P in normolipidemic blood donors

In line with previous reports (3, 28), about 60% of S1P circulating in plasma was recovered in HDL. S1P in LDL and VLDL accounted for only 8.1% and 3.3%, respectively, of plasma S1P. The remainder, i.e. nearly 30% of S1P was recovered in lipoprotein-depleted serum (LPDS). After precipitation of apoB-containing lipoproteins with dextran-sulfate - Mg2+, 85.3% of S1P instead of 88.5% S1P expected from the ultracentrifugation data were recovered in the supernatant (containing S1P in HDL and LPDS).

S1P plasma levels in normolipidemic individuals

In the entire sub-cohort of family controls without any mutation in HDL genes, plasma levels of S1P amounted to 0.89 ± 0.34 mM, of which 73.1 ± 9.9 % could not be precipitated with apoB-containing lipoproteins and hence occurs in HDL and the lipoprotein-free fraction of plasma. Men (n=25) and women (n=26) had similar S1P concentrations in either plasma (0.83 ± 0.34 mM vs. 0.86 ± 0.35 mM), the supernatant after precipitation of apoB-containing lipoproteins (0.60 ± 0.31 mM vs. 0.60 ± 0.29 mM) or the calculated LDL/VLDL-fraction (0.26 ± 0.12 mM vs. 0.26 ± 0.12 mM). Regression analyses also showed the absence of statistically significant correlations between age and S1P levels in total (r=0.077) or apoB-depleted plasma (r=0.111). These results allowed us to compare S1P levels of patients and normolipidemic controls without stratification for age or gender.

S1P and apoM plasma levels in patients with inborn errors of HDL metabolism

As shown in Table 2, significantly decreased S1P plasma levels were observed in patients with low HDL-cholesterol levels due to mutations in LCAT, ABCA1 or APOA1. Compound heterozygotes for mutations in LCAT or ABCA1 showed the most prominent decreases in S1P plasma levels, namely -50% and -65%, respectively. The 20% to 30% decreased S1P plasma concentrations of heterozygotes for APOA1 or ABCA1 mutations as well as the 65% decreased S1P levels of compound heterozygotes for ABCA1 mutations were exclusively explained by the decreased S1P levels in the apoB-depleted fraction, whereas heterozygotes and compound heterozygotes for LCAT mutations also showed decreased S1P levels in the apoB-containing fraction of plasma. Remarkably, patients with mutations in SCARB1, CETP, LIPC or LIPG who have increased HDL-cholesterol levels,
ApoM levels in total plasma did not differ between heterozygous carriers of HDL cholesterol lowering mutations in APOA1 or ABCA1 and unaffected family controls but decreased gene-dose-dependently by 10% and 40% in carriers of one (P < 0.01 compared to all unaffected controls; not significant compared to own family controls) and two defective LCAT alleles (P < 0.001 to all unaffected controls; P < 0.01 to own family controls), respectively. Mutations in ABCA1 showed inconsistent associations with apoM plasma concentrations: ApoM levels were normal in all heterozygotes for ABCA1 mutations as well in one patient with a truncating nonsense mutation and a dysfunctional missense mutation in ABCA1 (1.3 mmol/L) who also had a high apoB (1.93 g/L) whereas two other compound heterozygous ABCA1 mutation carriers with rather low plasma levels of apoB (0.81/0.75 g/L, respectively) presented with 40% lower apoM levels. In the apoB depleted plasma fraction apoM levels were lowered in heterozygotes for mutations in APOA1 (-23% compared to all unaffected controls, P < 0.01 but only -11% when compared to the own family controls, not significant) and LCAT (-14% compared to both all unaffected controls and the own family controls, all P < 0.01) as well as in carriers of two defective alleles of LCAT (-40% compared to both all unaffected controls and the own family controls, all P < 0.01) or ABCA1 (below limit of quantification in two patients and 0.5 mmol/L in the third one). Heterozygous mutations in the genes of SCARB1, CETP or LIPG had no consistent and significant effects on apoM levels despite considerable increases in HDL-cholesterol and apoA-I concentrations. In contrast, heterozygosity for mutations in LIPC was associated with 15% to 20% higher apoM levels in both total and apoB-depleted plasma (P < 0.01 for comparisons with all unaffected controls).

Correlations of S1P with apoM levels and with other lipid parameters

Univariate regression analyses of data from the mutation-free family controls revealed inverse correlations of S1P levels in apoB-depleted plasma with total cholesterol, non-HDL-cholesterol, and apoB as the only ones with statistical significance. Interestingly, S1P levels in total plasma or apoB-depleted plasma did not correlate significantly with plasma concentrations of HDL-cholesterol, Apo A-I or apoM (table 4). Neither were the correlations of S1P levels in the VLDL/LDL fraction with non-HDL-cholesterol or apoB statistically significant (Table 4). Concentrations of apoM in both total and apoB-depleted plasma showed highly significant correlations with HDL-cholesterol (r = 0.42 and r = 0.5, both P < 0.01, respectively; figures 1A and 1B) and borderline significant correlations with apoA-I levels (r = 0.26, P < 0.07 and r = 0.348, P <0.05, respectively; figures 1C and 1D).

With the observation that S1P levels were reduced in patients with genetically low HDL-cholesterol but not increased in patients with genetically high HDL-cholesterol, we hypothesized that HDL limits S1P levels in plasma only up to a certain threshold.
concentration and repeated the regression analyses of the family controls in two subcohorts, stratified by the medians of either HDL-cholesterol (figure 2) or apoA-I (Figure 3).

In controls with HDL-cholesterol levels below the median (< 1.32 mmol/L), we did not see any significant correlation of S1P with concentrations of HDL-cholesterol (r = 0.179, n.s., figure 2A) or apoA-I (r = 0.215, n.s., figure 2C). Surprisingly, however, in controls with

<p>| Table 3. ApoM in patients with monogenic disturbances of HDL metabolism. Controls = all unaffected relatives, „0” = unaffected relatives from specific mutation, „1” = heterozygous mutant carriers, „2” = homozygous or compound heterozygous mutant carriers. Symbols indicate levels of statistical significance for differences between mutant carriers and unaffected relatives and all controls, respectively.: * and #: P &lt; 0.05; ** and ###: P &lt; 0.01; *** and ####: P &lt; 0.001. &lt; LOQ: below limit of quantification |</p>
<table>
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<tr>
<th>Mutated gene</th>
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<th>Number of participants</th>
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<th>ApoM in apoB-depleted fraction (mM)</th>
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| Table 4. Regression analyses of all cohorts (with and without mutation). Value represents correlation coefficient (r). * and ** indicate statistically significant Pearson's correlation coefficient (ps0.05 and ps0.01, respectively). |
|----------------|---------------------|---------------------|---------------------|---------------------|
|                | Total-S1P | apoB-depleted-S1P | LDL/VLDL-S1P |
|                | Non mutation carriers | All cohorts | Non mutation carriers | All cohorts | Non mutation carriers | All cohorts |
| Total-cholesterol | -0.1949 | -0.0141 | 0.2811** | -0.1034 | 0.0934 | 0.1327 |
| HDL-cholesterol   | 0.1414 | 0.1162 | 0.0316 | 0.1327 | -0.0689 | 0.0141 |
| nonHDL-cholesterol | -0.2366 | -0.0894 | -0.3225** | -0.2002* | 0.1353 | 0.1841 |
| ApoA-I            | 0.0447 | 0.0872 | -0.1342 | 0.0728 | 0.0327 | 0.0656 |
| ApoB              | -0.2683 | -0.1646 | -0.2966** | -0.2238* | 0.0837 | 0.0458 |
| ApoM              | -0.0300 | -0.0361 | 0.0933 | 0.0469 | -0.1118 | 0.0245 |
HDL-cholesterol above the median, the correlations of S1P in apoB-depleted plasma with HDL-cholesterol (r = -0.21, p = n.s., figure 2B) or apoA-I (r = -0.554, P < 0.05, figure 2D) became inverse. After stratification for plasma apoA-I levels, the subgroup of controls with apoA-I levels below the median (<54.2 mM or <1.53 g/L) showed statistically significant correlations of S1P levels in apoB-depleted plasma with HDL-cholesterol (r = 0.536, P = 0.0102; figure 3A). The correlation was inverse in controls with apoA-I levels above the median but did not reach statistical significance (r = -0.122; n.s., figure 3B). The correlations of S1P levels in apoB-depleted plasma with apoA-I plasma concentrations showed the same trend but were not statistically significant (figures 3C and 3D). Interestingly, in the subgroup of controls with apoA-I levels below the median, we also observed a strong inverse correlation between S1P in apoB-depleted plasma and non-HDL-cholesterol (r = -0.567, P < 0.01), which was not found in individuals with apoA-I levels above the median (r = 0.173, n.s.).

The effects of stratification of HDL cholesterol below or above the median or apoA-I levels on apoM were as follows: ApoM levels correlated with S1P levels neither in controls with HDL-cholesterol below the median (r = -0.164, n.s., figure 2E) nor in controls with HDL-cholesterol above the median (r = 0.126, p = n.s., figure 2F). ApoM levels correlated with S1P levels neither in controls with low apoA-I levels (r = 0.259, p = n.s., figure 3E) nor in controls with high apoA-I levels (r = 0.063, p = n.s., figure 3F).

**Discussion**

Plasma concentrations of S1P vary significantly among normolipidemic individuals, for example from 0.49 mM to 1.21 mM in the Dutch group of 47 normolipidimic individuals that was investigated in the current study. As yet, the determinants of S1P plasma concentrations are not well-explored. One obvious candidate is the plasma concentration of HDL particles, which induces S1P efflux from erythrocytes (27) and carries about 60% of S1P in plasma of normolipidemic individuals. In fact, Zhang et al. have previously reported very strong correlations of S1P levels with HDL-cholesterol (r = 0.82) or apoA-I levels (r = 0.91). (28) In contrast to these observations in a relatively small group of normolipidemic Japanese individuals (n=16), the current study shows a much looser and more complex relationships between HDL-cholesterol and S1P levels.

By investigating S1P levels in about 50 Dutch individuals with molecularly characterized monogenic errors of HDL metabolism and a comparably sized group of unaffected family members, we confirmed a significant positive correlation between S1P and apoA-I but only in individuals with relatively low plasma concentrations of HDL-cholesterol or apoA-I. Mutations in APOA1, ABCA1 or LCAT decreased plasma concentrations of HDL-cholesterol and apoA-I as well as S1P in an apparent gene-dose-dependent fashion: the lowest S1P levels were found in patients with HDL deficiency due to two mutated ABCA1 or LCAT alleles. Specifically, they presented with 30% and 50% of normal S1P levels in total...
plasma or 20% and 40% of normal S1P levels in apoB-depleted plasma, respectively. By contrast, mutations in CETP, SR-BI, LIPC or LIPG which increase plasma levels of both HDL-cholesterol and apoA-I, did not affect S1P levels in either total or apoB-depleted plasma. Furthermore, S1P levels in total or apoB-depleted plasma correlated significantly with HDL-cholesterol levels only in individuals who presented with apoA-I levels below the median, that is below 1.53 g/L. After stratification for HDL-cholesterol, we surprisingly observed a significant inverse correlation between S1P levels in apoB-depleted plasma and apoA-I plasma concentrations in control participants with HDL-cholesterol above the median (≥ 1.32 mmol/L). Taken together our data indicate that only at low plasma levels of HDL-cholesterol or apoA-I there exist a relation with S1P plasma concentrations. In view of the limitation of the relation of the putatively anti-atherogenic S1P with the lower concentration ranges of HDL cholesterol or apoA-I, it is interesting to note that also the inverse associations of HDL cholesterol or apoA-I with CHD risk are weakening or even disappearing in the higher concentration range (29).

Except for individuals with 2 defective ABCA1 alleles, all individuals had S1P/apoA-I ratios within the range of 0.004 to 0.034 µM S1P per µM apoA-I. Assuming that every HDL particle contains 2 to 4 molecules of apoA-I, only about 1 to 10% of HDL particles in plasma thus transports one S1P molecule in average. Thus, even heterozygosity for HDL-deficiency syndromes, which are characterized by approximately 60-70% of normal levels of HDL-cholesterol or apoA-I, does not limit the abundance of S1P in plasma. The reduced S1P concentration in plasmas of patients with HDL cholesterol lowering mutations in the genes of APOA1, ABCA1 or LCAT suggests that S1P is transported by specific HDL subclasses (which are critically lowered in patients with low HDL-cholesterol. In fact, Kontush et al reported that in normolipidemic subjects S1P is preferentially enriched in small dense and sphingomyelin-poor HDL3c particles (30). Since mutations in LCAT had the strongest negative effect on S1P levels in total plasma as well as in the different lipoproteins it is interesting to note that the HDL3c fraction has also been reported to be enriched in LCAT (31). Taken together, a loss of LCAT protein and/or activity appears to limit the capacity of lipoproteins to carry S1P.

Results from crystallographic and in vitro binding studies suggested that apoM binds S1P with high affinity (12). Therefore and because apoM occurs in plasma at a similarly low concentration like S1P (8) and because apoM like S1P has been predominantly found in small HDL3c particles (30,31), we tested whether apoM levels correlate with S1P levels in total and apoB-depleted plasma. However, heterozygosity for mutations in ABCA1 and APOA1, which was associated with significantly 30% to 45% lowered S1P levels, was not associated with changes in apoM levels consistently nor significantly either in total or apoB-depleted plasma. Mutations in LCAT led to gene-dose-dependent decreases in apoM levels in total and apoB-depleted plasmas, however, these were much less pronounced than those of S1P levels: Heterozygotes had 32% and 22% lower concentrations of S1P but only 9% and 14% lower concentrations of apoM in total and
apoB-depleted plasmas respectively. Carriers of two defective LCAT alleles had 53% and 62% lower concentrations of S1P but only 38% and 40% lower concentrations of apoM in total and apoB-depleted plasmas, respectively. Carriers of two defective ABCA1 alleles had 64% and 80% lower S1P levels compared to 40% and 80% lower apoM levels in total and apoB-depleted plasma, respectively. Conversely, mutations in LIPC were associated with statistically significant 10-15% elevations of apoM levels but no difference in S1P levels. Finally, we did not find any statistically significant correlation between S1P and apoM concentrations, neither in total nor in apoB-depleted plasma, neither in the entire study sample nor in subgroups obtained by stratification for medians of HDL-cholesterol or apoA-I concentrations. Taken together, the data suggest that in the physiological range the concentration of apoM is not regulating S1P concentrations in plasma or HDL. In agreement with a possible redundancy of apoM for transporting S1P in HDL in plasma, reconstituted HDL containing only apoA-I and phosphatidylcholine have been reported to induce significant dose- and time-dependent S1P efflux from erythrocytes in vitro (32). About 10% of S1P in normolipidemic plasma is transported by LDL and VLDL. Similar to HDL, the concentration of S1P in LDL and VLDL (about 0.2 mM) is about 10-fold lower than the concentration of apoB-containing particles (about 2 mM). Zhang and colleagues previously reported a strong correlation of S1P levels in LDL with plasma levels of LDL cholesterol or apoB in 16 individuals. (28) In our study, however, we did not observe any statistically significant correlation of S1P levels in apoB-containing lipoproteins with plasma concentrations of apoB or non-HDL-cholesterol. Rather by contrast, in controls with apoA-I levels below the median, we observed inverse correlations between S1P levels and apoB or total cholesterol concentrations as well as between S1P levels in the LDL/VLDL fraction and apoA-I concentrations. These inverse relationships may indicate the presence of an interaction of HDL particles and apoB-containing lipoproteins, by which in individuals with low levels of HDL-cholesterol or apoA-I, S1P is shifted to apoB-containing lipoproteins and partially lost from the plasma compartment. Despite this interaction in normolipidemic relatives of mutation carriers, only defects in the LCAT gene influenced S1P levels in apoB-containing lipoproteins so that S1P levels in VLDL and LDL were lower in homozygous or heterozygous mutant carriers than in unaffected family members. Like Dahlbäck and colleagues (8), we found a significant positive correlation between plasma levels of apoM and HDL cholesterol with coefficients of correlation of about 0.4. Surprisingly, however, inborn errors of HDL metabolism show no or little effect on apoM levels in either total or apoB-depleted plasma, except in completely HDL deficient individuals with two defective LCAT or ABCA1 alleles. This paradoxical finding corresponds with the previously reported negative correlation between apoM plasma levels and the fractional catabolic rate of apoA-I in obese individuals (33) since all defects studied by us change plasma concentrations of HDL cholesterol and apoA-I by either increasing (apoA-I, ABCA1, LCAT) or decreasing (CETP, LIPC, LIPG and SR-BI) the catabolism of apoA-I. It may hence be that apoM limits the catabolism of apoA-I, especially in situations which
otherwise cause enhanced catabolism of apoA-I, for example by its association with small HDL3 particles and preb-HDL (9,10,32,35), which are otherwise rapidly eliminated from the body by glomerular filtration. In fact, studies in apoM-overexpressing and apoM knock-out mice provided evidence that apoM contributes to the formation of preb-HDL (10). Also studies in patients with diabetes mellitus demonstrated the association of apoM with the concentration and formation of preb-HDL (34). However, more detailed experiments in HEK293 cells transfected with ABCA1 and apoM revealed that apoM is a limiting factor for the conversion of these nascent particles into larger preb-HDL but not for the ABCA1 dependent formation of small preb-HDL (35). In our observational studies the absence of functional ABCA1 or LCAT in compound heterozygous or homozygous mutation carriers resulted in a pronounced decrease of apoM levels in the apoB-depleted fraction of plasma that contains the residual HDL particles of these patients. Interestingly deficiencies of either LCAT or ABCA1 have little or no effect on the concentration of small preb1-HDL although this particle contains all apoA-I of ABCA1 deficient Tangier disease patients and large proportions of apoA-I of LCAT-deficient patients (36). The strongly reduced apoM concentrations of both ABCA1 and LCAT deficient patients hence support the findings of the aforementioned cell culture studies by Parks and colleagues (35) that apoM is needed for the conversion/processing rather than formation of nascent preb1-HDL particles. The little or not at all changed apoM concentrations in heterozygotes for mutations in LCAT or ABCA1 suggest that half normal activities of these factors (13,37) are sufficient to maintain normal apoM levels in apoB-free plasma. The involvement of apoM in preb-HDL processing rather than preb-HDL formation may also explain the absent or rather increased concentrations of apoM in total and apoB-depleted plasmas of patients with mutations in CETP, LIPC and LIPG which interfere with the regeneration of preb-HDL and are hence expected to lower apoM concentrations (13,38).

In conclusion, although only ≤5% of HDL particles contain S1P, both mildly and severely lowered concentrations of HDL or apoA-I due to mutations in APOA1, ABCA1 or LCAT are associated with reduced plasma S1P levels. By contrast, high concentrations of HDL-cholesterol and apoA-I because of mutations in CETP, SCARB1, LIPC or LIPG are not associated with changes in S1P levels in total or apoB-depleted plasma. It is possible that a subclass of HDL or a minor HDL-associated protein rather than HDL in general limits the abundance of S1P in HDL. Likely, this limiting factor is not apoM although apoM was previously found to bind S1P.

Acknowledgements
We wish to thank Kobie Los and Claartje Koch for their help to draw blood of the families with genetic disorders of HDL metabolism. In addition, we would like to thank Joram van Miert and Alinda Schimmel for their technical assistance.
Funding sources

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Figure 1: Correlations of HDL-cholesterol (A, B) and apoA-I concentrations (C, D) with concentration of apoM in total (A, C) or apoB-depleted plasma (B, D). Equations for linear regression; r & p: Pearson's correlation coefficient, p = probability value for significance of correlation. Regression and correlation analysis performed on controls only (black symbols); patients with mutations in genes affecting HDL are indicated in the figure by coloured symbols but are not incorporated in the statistical analyses. Regression and correlation outcomes are given in blue when statistically significant (P<0.05). (for color figure, see page 420)
Figure 2: Correlations of S1P levels in apoB-depleted plasma after stratification for the median of HDL-cholesterol (1.32 mmol/L) with plasma concentrations of HDL-cholesterol (A, B), apoA-I (C, D), HDL-apoM (E, F). Equations for linear regression; r & p: Pearson’s correlation coefficient, p = probability value for significance of correlation. Regression and correlation analysis performed on controls only (black symbols); patients with mutations in genes affecting HDL are indicated in the figure by coloured symbols but are not incorporated in the statistical analyses. Regression and correlation outcomes are given in blue when statistically significant (P<0.05) (for color figure, see page 421).
Figure 3: Correlations of S1P levels in apoB-depleted plasma after stratification for the median of apoA-I (54.2 mmol/L = 152 mg/dL) with plasma concentrations of HDL-cholesterol (A, B), apoA-I (C, D), HDL-ApoM (E, F). Equations for linear regression; r & p: Pearson’s correlation coefficient, p = probability value for significance of correlation. Regression and correlation analysis performed on controls only (black symbols); patients with mutations in genes affecting HDL are indicated in the figure by coloured symbols but are not incorporated in the statistical analyses. Regression and correlation outcomes are given in blue when statistically significant (P<0.05) (for color figure, see page 422).
Chapter 17

Reconstituted HDL Shortens Cardiac Repolarization

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Chapter 17

Abstract

Objective: We hypothesize that increasing high-density lipoprotein cholesterol (HDL-c) shortens cardiac repolarization.

Background: HDL-c is inversely associated with cardiovascular death including sudden cardiac death. The relation between HDL-c and repolarization of the heart is unexplored.

Methods: HDL-c was elevated with reconstituted HDL. Cardiac repolarization was studied by recording cardiac membrane potentials with the patch clamp technique from isolated rabbit cardiomyocytes that were superfused with rHDL. Infusions with rHDL (40 mg/kg bodyweight) were performed in healthy volunteers and in healthy volunteers and in patients with monogenetic disorders of HDL metabolism resulting in decreased levels of plasma HDL-cholesterol. Electrocardiograms were recorded to assess cardiac repolarization before and 24 hours after infusion with rHDL.

Results: rHDL as well as purified human apo A-I shortened repolarization of isolated rabbit cardiomyocytes by ~25% (p<0.05). rHDL infusion shortened the heart rate-corrected QT interval on surface electrocardiograms in all participants (p<0.001).

Conclusion: Reconstituted HDL shortens cardiac repolarization. These data provide evidence for a novel mechanism of HDL to reduce sudden cardiac death.
Introduction

Low plasma levels of HDL-c or apo A-I are independent predictors of increased cardiovascular morbidity and mortality (1). The inverse relation between these parameters has thus far been largely attributed to cholesterol depletion of the atherosclerotic plaque (2, 3), the first step in reverse cholesterol transport (4). Cardiovascular death is, however, often preceded by ventricular tachyarrhythmias. Prolongation of the cardiac repolarization time, which has been shown to predict sudden cardiac death (5-7), often underlies these rhythm disturbances. Elevated cholesterol has, besides its relation with accelerated atherogenesis, also been associated with the occurrence of ventricular fibrillation after myocardial infarction (8).

In the present study, we set out to study whether HDL exerts a direct effect on the cardiac repolarization, i.e. that increasing HDL shortens cardiac repolarization and the QT-interval. To test the hypothesis, we studied the effects of reconstituted (r) HDL in vitro, in healthy volunteers and in patients with monogenetic disorders of HDL metabolism resulting in decreased levels of plasma HDL-cholesterol.

Methods

Cellular study

This study was approved by the local institutional ethical committee. Animal care and handling conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). We used hearts of healthy New Zealand White male rabbits (N=3) to isolate midmural left ventricular cardiomyocytes as described (9). Cardiac action potentials were recorded using the amphotericin-B perforated patch-clamp technique at 37°C. Action potentials were elicited at 0.5 to 4 Hz by 3-ms, 1.5’ threshold current pulses through the patch pipette. Recordings were low-pass filtered with a cut-off frequency of 5-kHz, digitized 10-kHz, and corrected for liquid junction potentials(10). Bath solution contained Tyrode’s solution mmol/L: NaCl 140, KCl 5.4, CaCl2 1.0, glucose 5.5, HEPES 5.0; pH 7.4, pipette solution contained (mmol/L): K-glutamate 125, KCl 20, NaCl 5, HEPES 10, amphotericin-B 2.2; pH 7.2 (KOH). Data acquisition and analysis were accomplished using custom software.

rHDL consisting of apo A-I isolated from human plasma reconstituted with egg-yolk phosphatidylcholine (CSL111, CLS Australia) was applied at a concentration of 50 µg/mL until steady state was reached (approximately 5 minutes).

Study participants

The institutional Internal Review Board approved the study and all participants gave written informed consent. rHDL was infused in 12 individuals with a wide range of HDL-c plasma and apo A-I concentrations, consisting of healthy volunteers (n=3), heterozygotes
for mutations in either apo A-I (n=2) or lecithin: cholesterol acyltransferase (LCAT; n=5), or homozygotes for mutations in ATP binding cassette transporter A1 (ABCA1; n=2). Table 1 provides information on the participants. rHDL was infused at a dose of 40 mg/kg bodyweight over a 4 hours period without adverse effects. Electrocardiograms (ECGs) were recorded before and 24 hours after infusion. Blood was drawn before and 24 hours after infusion. Total plasma cholesterol, triglycerides, HDL-c, LDL-c, apoB, apoA-I, and apoA-II levels were measured with commercial kits (Wako, Neuss, Germany and Randox, Crumlin, UK) on a Cobas Mira autoanalyzer. QT intervals were measured at the intersect of the tangent of the end of the T-wave with the baseline in 3 consecutive beats in lead V5 by two independent researchers, and corrected for heart rate using the Bazett formula (QTc) (11). In 3 LCAT-patients saline was infused as a control.

Statistics
Data are presented as mean±sem and were analyzed using a paired t-test or a Signed Rank Test (P<0.05 is considered statistically significant).

Results

Cellular study
Figure 1A shows superimposed examples of a cardiac action potential under control conditions (arrow 1), in the presence of rHDL (arrow 2) and after a washout of rHDL (arrow 3) at a pacing cycle length of 1 s. Figure 1B demonstrates a statistically significant shortening of the action potential duration at 20%, 50% and 90% of repolarization after 5 minutes of superfusion (10 myocytes obtained from 3 hearts). The plateau amplitude (defined at action potential amplitude measured 60 ms following the upstroke) was significantly decreased (P<0.05, paired t-test), but the action potential upstroke velocity (Vmax) remained unaltered (Figure 1B). The maximum diastolic potential was also unchanged (not shown). The action potential shortening was observed over the entire range of tested pacing cycle lengths (0.5-4 Hz-Figure 1C). For the experiment we used rHDL at the lowest concentration with a maximum effect (arrow-Figure 1D). Superfusion with purified apo A-I (50 μg/mL, the lowest but still physiological apo A-I concentration observed in human plasma) caused a decrease of the APD90 (at 1 Hz) from 236±14.6 to 205±17.2 (P<0.05, paired t-test) similar to the rHDL superfusion.

Human study
Upon rHDL infusion, baseline apo A-I levels rose from 87±13.0 to 107±12.0 mg/dL (p=0.001-Figure 2A) and QTc decreased from 394±8.7 to 371±7.5 ms (p=0.001-Figure 2B). rHDL infusion resulted in a similar effect on QTc in all individuals studied independent of baseline apo A-I/HDL-c values (individual data in Figure 2C and D). Infusion with vehicle (saline solution) in 3 LCAT patients did not affect QTc (before infusion: 391±8.0
Reconstituted HDL shortens cardiac repolarization

349 ms and 392±6.3 ms following infusion). These data indicate that there are no time- and infusion-dependent effects related to the infusion procedure.

Other lipid parameters are summarized in table 2. HDL-c levels rose and total cholesterol, LDL-c and apo B levels decreased following the rHDL infusion in all subjects. Heart rate was significantly decreased following rHDL infusion in all participants. There were no differences in any of the other electrocardiographic parameters.

Discussion

This study shows for the first time that rHDL has a direct effect on cardiac repolarization. rHDL shortens the action potential duration in isolated cardiomyocytes over a wide range of pacing frequencies in vitro. Since pure apo A-I induced identical effects it is unlikely that (bioactive) phospholipids in the rHDL preparation contributed to this beneficial effect. rHDL infusion also shortened the QTc interval in 12 participants independent of baseline apo A-I levels. Interestingly, similar in vivo effects were observed in patients...
with complete ABCA1 deficiency, indicating that ABCA1-mediated lipidation of apo A-I is apparently not required for the observed effect. The difference in heart rate following the rHDL infusion in the participants may have been due to direct electrophysiological effects of rHDL on the sinus node or may have been due to other variables (stressors). We therefore corrected the QT interval using the Bazzet’s formula. Although this procedure may have underestimated the QT interval at slower heart rates, the recapitulation of these results in the animal study in which we were able to reconstruct the entire restitution curve, without the need for rate-correction, demonstrates that this has not played a major role.
The applied rHDL dose in humans is identical to that used in previous studies and was well-tolerated (12, 13). Because infusion of phosphatidylcholine as a ‘vehicle-control’ is potentially toxic and ethically not justified, we have used infusion of saline as a control (3). A potential role of phosphatidylcholine cannot be ruled out. The newly identified role of rHDL may have clinical implications in that QTc prolongation is associated with an increased risk of ventricular arrhythmias and is also reported as an independent risk factor for sudden cardiac death (6, 14-16). rHDL infusions have been successfully used in both animal studies and in clinical trials to elevate apo A-I and HDL-c levels (17, 18) but the long-term effect of this intervention is unknown and its feasibility as an antiarrhythmic drug in clinical practice remains to be determined. The current data underscore the need for future studies into the antiarrhythmic effect of apo AI-elevating strategies (19) in high-risk patients.

Conclusion

These data suggest a new role of HDL in the prevention of sudden arrhythmic death.

Acknowledgements

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Reference list


Chapter 18

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TNFα induces ABCA1 through NF-κB in macrophages and in phagocytes ingesting apoptotic cells

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Abstract

Recent evidence suggests that tumor necrosis factor α (TNFα) signalling in vascular cells can have antiatherogenic consequences, but the mechanisms are poorly understood. TNFα is released by free cholesterol-loaded apoptotic macrophages, and the clearance of these cells by phagocytic macrophages may help to limit plaque development. Macrophage cholesterol uptake induces ATP-binding cassette (ABC) transporter ABCA1 promoting cholesterol efflux to apolipoprotein A-I and reducing atherosclerosis. We show that TNFα induces ABCA1 mRNA and protein in control and cholesterol-loaded macrophages and enhances cholesterol efflux to apolipoprotein A-I. The induction of ABCA1 by TNFα is reduced by 65% in IκB kinase β-deficient macrophages and by 30% in p38α-deficient macrophages, but not in jun kinase 1 (JNK1)- or JNK2-deficient macrophages. To evaluate the potential pathophysiological significance of these observations, we fed TNFα-secreting free cholesterol-loaded apoptotic macrophages to a healthy macrophage monolayer (phagocytes). ABCA1 mRNA and protein were markedly induced in the phagocytes, a response that was mediated both by TNFα signalling and by liver X receptor activation. Thus, TNFα signals primarily through NF-κB to induce ABCA1 expression in macrophages. In atherosclerotic plaques, this process may help phagocytic macrophages to efflux excess lipids derived from the ingestion of cholesterol-rich apoptotic corpses.
Introduction

ABCA1 belongs to the ATP-binding cassette (ABC) transporter superfamily (1, 2) and promotes efflux of cholesterol and phospholipids from cellular membranes to apolipoprotein A-I (apoA-I) (3). In macrophages, oxysterol-activated liver X receptor (LXR) (4) and retinoid X receptor form a heterodimer that binds to a direct repeat 4 sequence (5, 6) located in the proximal promoter of the ABCA1 gene, resulting in increased gene transcription and increased cholesterol and phospholipid efflux to apoA-I (7). Bone marrow transplantation studies have shown that the expression of ABCA1 in macrophage foam cells has antiatherogenic consequences (8, 9).

Atherosclerosis represents an inflammatory reaction in the arterial wall, initiated by the retention of lipoprotein lipids (10,11). One of the most studied inflammatory cytokines is tumor necrosis factor α (TNFα), which is active in both human and rodent atherosclerotic plaques (10, 12). Many of the inflammatory properties of TNFα suggest that TNFα signalling is proatherogenic (13). Paradoxically, other studies have shown that signalling by means of the TNFα receptor I (p55) may have an overall atheroprotective effect (14) and moreover that TNFα-signalling through NF-κB in macrophages and vascular smooth muscle cells may be antiatherogenic (14–17). However, the mechanisms of atheroprotective effects of TNFα signalling in macrophages are not well understood. In this work, we show that TNFα induces ABCA1 through NF-κB in macrophages and in phagocytes ingesting apoptotic cells, revealing a previously undescribed antiatherogenic mechanism of TNFα signalling in macrophages.

Results

TNFα Induces ABCA1 mRNA and Protein Expression in Mouse Peritoneal Macrophages

We first investigated whether TNFα can alter expression of different ABC transporters potentially involved in lipid efflux from cells, i.e., ABCA1, ABCA7 (18), and ABCG1 (19, 20). We treated mouse peritoneal macrophages for 24 h with increasing doses of TNFα (0–50 ng/ml). As shown in Fig. 1A, TNFα up-regulated ABCA1 mRNA levels in a concentration-dependent manner up to 4-fold (50 ng/ml) (P < 0.01). ABCA7 was slightly increased by TNFα, but only at higher doses (20–50 ng/ml). In contrast, ABCG1 mRNA was repressed by TNFα treatment. No signs of cellular apoptosis or necrosis were detected by TUNEL or other assays even at the highest dose (data not shown), as expected because TNFα does not usually induce apoptosis unless NF-κB signalling is impaired (21).

Fig. 1B shows the time course of the response of ABCA1, ABCA7, and ABCG1 mRNAs to TNFα (10 ng/ml). ABCA1 mRNA was increased by ≈2.5-fold (P < 0.05) at 2–6 h and by ≈4-fold at 16–24 h (P < 0.01). ABCA7 was slightly increased by TNFα at later time points (2-fold; P < 0.05; 24 h), whereas ABCG1 mRNA was repressed (0–24 h). A similar induction of ABCA1 by TNFα was observed in bone-marrow-derived macrophages.
cultured in the presence of macrophage-colony stimulating factor (see below) and in human THP-1 macrophages (data not shown). In similar experiments, we monitored the levels of ABCA1 protein (Fig. 1C and D). Treatment of mouse peritoneal macrophages with TNFα resulted in an increase in ABCA1 protein in a dose- and time-dependent manner, with a 4-fold induction at 50 ng/ml (Fig. 1C) and at 24 h (dose = 50 ng/ml) (Fig. 1D). These changes were comparable in magnitude with the increases in ABCA1 mRNA (Fig. 1).

**Fig. 1.** TNFα regulates ABC transporter expression in mouse peritoneal macrophages.

Thioglycollate-elicited macrophages were treated with increasing concentrations of TNFα (0–50 and 0–100 ng/ml, respectively) in DMEM containing 10% FBS for 24 h (A and C) or with TNFα (10 ng/ml) (B and D) for the indicated times. (A and B) RNA extracts were prepared from cultured cells, and mRNA levels were analyzed by Taqman real-time PCR, as described in Materials and Methods. The average copy numbers of ABCA1, ABCG1, or ABCA7 were normalized to housekeeping gene expression. Results are expressed as fold induction (FI) as compared with untreated controls (control = 1 arbitrary unit) ± SEM. ***, P < 0.001; **, P < 0.01; *, P < 0.05. (C and D) Protein extracts were prepared. (Lower) The ratio of ABCA1 to β-actin is expressed as a percent of untreated controls. In each experiment, treatments were performed in duplicate, and values shown represent the average of two different cell preparations. (Upper) shows a Western blot and is representative of one experiment.
Mechanisms of the Induction of ABCA1 by TNFα

TNFα is known to induce the expression of genes via a number of different signalling pathways, notably the NF-κB pathway (22, 23) and various mitogen-activated protein kinase (MAPK) signalling pathways (24, 25). We initially used inhibitors to assess the role of these pathways in the induction of ABCA1 by TNFα, as shown in Fig. 6, which is published as supporting information on the PNAS web site. We used different inhibitors of NF-κB signalling.

The most common heterodimer of NF-κB consists of p50 and p65 subunits. SN50, an inhibitor of the nuclear translocation of the p50 subunit of NF-κB, caused a significant increase (60%; P < 0.05) in the induction of ABCA1 by TNFα, whereas the control peptide SN50M had no effect (Fig. 6). MG-132 and CAPE reduced or eliminated this response by 80% (P < 0.01) and 35% (P < 0.01), respectively. (Fig. 6) These experiments could indicate differential roles of p65 and p50 in the induction of ABCA1. However, we must consider that p65 and p50 inhibitors may have nonspecific effects; thus, we cannot be sure whether they truly have differential roles. We also used inhibitors to evaluate signalling via the MAPK pathways i.e., extracellular signal-regulated kinase (ERK), jun kinase (JNK), and p38-MAPK pathways (Fig. 6). Whereas ERK and JNK inhibitors had no effect, the p38-MAPK inhibitor SB202180 caused a 35% reduction (P < 0.01) in the TNFα response. Thus, the inhibitor experiments suggest a possible involvement of NF-κB and p38-MAPK signalling pathway in the induction of ABCA1 by TNFα.

To more clearly define the signalling pathways involved in this response, we next carried out experiments using macrophages from mice deficient in key molecules involved in the different signalling pathways. TNFα induction of ABCA1 was slightly increased in macrophages from JNK1−/− (P < 0.05) or JNK2−/− (not significant) mice (Fig. 2A and B). Although there may be redundancy in the JNK1 and JNK2 signalling pathways (26) together with the inhibitor data (Fig. 6), this result suggests that JNK signalling does not contribute in a positive fashion to the induction of ABCA1 by TNFα. To assess the p38-MAPK signalling pathway, we used macrophages deficient in p38α. As shown in Fig. 2C, the increase of ABCA1 by TNFα was reduced by 30% (P < 0.05) in the p38α-deficient macrophages as compared with the wild-type (WT) control. TNFα induction of ABCA1 was well preserved in LXRα/β−/− macrophages (Fig. 2D), indicating that TNF does not act via LXR to induce ABCA1. IKKβ phosphorylates IκBα, leading to its ubiquitination and proteasomal degradation and releases p65 for nuclear translocation (22). The induction of ABCA1 by TNFα was markedly reduced (by 65%) in IKKβ−/− macrophages (Fig. 2E). In comparison, the response of the well known NF-κB target gene metalloprotease-9 (MMP-9) to TNFα was reduced by 35% (P < 0.01) in the IKKβ−/− cells (Fig. 2F). In contrast, induction of ABCA1 by the LXR activator TO-901317 (abbreviated TO-1317) was intact in these cells, ruling out a nonspecific effect on the ABCA1 promoter. The data shown in Fig. 2 were obtained at the 5-h time point. A separate set of experiments carried out 12 h after addition of TNFα showed that the induction of ABCA1 by TNFα was reduced by 80% at 12 h (data not shown). Given that the deletion of IKKβ is only ≈75% efficient (27),
Fig. 2. NF-κB and p38-MAPK, but not JNK, mediate the increase of ABCA1 mRNA by TNFα.

(A, B, E, and F) Bone-marrow-derived-macrophages from JNK1−/− (A), JNK2−/− (B), and IKKβF/F and IKKβΔ/Δ (E and F) were cultured in DMEM containing 10% FBS and macrophage-colony stimulating factor (20 ng/ml) for 5–9 days, before addition of TNFα (20 ng/ml).  (C and D) Peritoneal macrophages from WT, p38αΔ/Δ (C), or LXRα/β-/- (D) mice were cultured as in Fig. 1 and treated with TNFα (20 ng/ml). RNA was extracted 5 h later, and ABCA1 or MMP-9 mRNA was analyzed by Taqman and corrected for housekeeping genes as described in Fig. 1. Results are expressed as fold induction (FI) compared with the control (no treatment). ***, P < 0.003; **, P < 0.01; *, P < 0.05. Each graph represents two or three different cell preparations, except for p38, which was conducted in one cell preparation. All experiments were performed in triplicate wells.
these experiments indicate a principal role of NF-κB signalling in the induction of ABCA1 by TNFα and a secondary role of p38-MAPK signalling in this response. Analysis of the ABCA1 promoter by MATINSPECTOR (Genomatix Software, Munich) suggested potential NF-κB binding sites in the proximal promoter region. However, TNFα did not induce a response after transfection of the human ABCA1 1-Kb promoter-luciferase in RAW macrophages or in primary mouse peritoneal macrophages (data not shown). This finding could indicate that there is a NF-κB binding site(s) elsewhere in the gene or that the effects of NF-κB are indirectly mediated.

**TNFα Induces ABCA1 and Cholesterol Efflux in Cholesterol-Loaded Macrophages**

We next determined whether induction of ABCA1 by TNFα would occur under conditions of relevance to atherogenesis. Thus, we examined the effects of TNFα in macrophages that had been cholesterol loaded with acetyl-low density lipoprotein (AcLDL) or treated with the synthetic LXR activator (TO-1317). Peritoneal macrophages were treated with submaximal doses of TNFα and TO-1317 (0.1 μM) or AcLDL (50 μg/ml) simultaneously for 24 h. As shown in Fig. 3A, the increase of ABCA1 mRNA resulting from treatment with TNFα and AcLDL (3.6-fold; P<0.0001) or TNFα and TO-1317 (8.6-fold; P<0.001) was at least additive, as compared with TNFα alone (1.9-fold), AcLDL alone (1.5-fold), or TO-1317 alone (5.6-fold). ABCG1 mRNA also was induced by AcLDL (1.4-fold) or TO-1317 (3.0-fold). However, TNFα or TNFα in combination with TO-1317 or with AcLDL had no

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**Fig. 3. Effect of TNFα, AcLDL, and LXR activator on the induction of ABCA1 mRNA and effect of TNFα on cholesterol efflux.**

Thioglycollate-elicited macrophages were cultured as described in Fig. 1. (A) An LXR agonist (TO-1317) (0.1 μM) or AcLDL (50 μg/ml) and TNFα (20 ng/ml) were added to the cells simultaneously in DMEM containing 10% lipoprotein-deficient serum (LPDS). ABCA1 and ABCG1 mRNA were analyzed as described in Materials and Methods. Results are expressed as fold induction (FI) compared with untreated controls ± SEM. ***, P < 0.0001; **, P < 0.001; *, P < 0.01. (B) Cells were labelled in DMEM and 10% LPDS containing [3H]cholesterol or [3H]AcLDL, with or without TNFα (50 ng/ml), for 18 h. Cholesterol efflux was calculated as described in Materials and Methods. Results are expressed as a percent of cholesterol efflux ± SEM. Cholesterol efflux to apoA-I was measured in triplicate or quadruplicate, and experiments were performed in three different cell preparations. *, P < 0.05

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additional effect on ABCG1 mRNA. TNFα and AcLDL, and TNFα and TO-1317, increased ABCA1 protein in a more than additive manner as well (data not shown). The mechanism of the apparent cooperation between LXR and TNFα (NF-κB) signalling in the induction of ABCA1 is unknown.

To assess cholesterol efflux, peritoneal macrophages were loaded with free-cholesterol ([3H]cholesterol) or cholesterol incorporated into AcLDL ([3H]AcLDL) overnight in the presence or absence of TNFα (50 ng/ml), and the efflux of cholesterol to apoA-I was measured during a subsequent 4-h incubation (Fig. 3B). As reported in ref. 28, AcLDL loading resulted in a higher level of cholesterol efflux (shown in Fig. 3B, open bars) than loading with cholesterol. TNFα increased cholesterol efflux by 2.5-fold \( (P < 0.05) \) when macrophages were loaded with [3H]cholesterol and 1.7-fold \( (P < 0.05) \) after loading with [3H]AcLDL. These studies show that ABCA1 induced by TNFα is functional in terms of cholesterol efflux, both in basal and cholesterol-loaded cells.

**Increase of ABCA1 During Phagocytosis of Apoptotic Cells Is Inhibited by Neutralizing Anti-TNFα Antibodies (Abs)**

Macrophages undergo apoptosis in atherosclerotic plaques, in response to loading with free cholesterol or oxidized lipids (12, 29). Apoptotic macrophages may be taken up by other phagocytic macrophages or, if not cleared in this way, may undergo postapoptotic necrosis, inciting an inflammatory response (30–32). Free cholesterol-induced macrophage apoptosis involves the unfolded protein response and leads to MAPK activation and release of inflammatory cytokines, notably TNFα (33). To further explore the potential physiological relevance of our observations, we next asked whether ABCA1-induction by TNFα could occur during macrophage phagocytosis of TNFα-secreting, free cholesterol-induced apoptotic macrophages (FC-AMs). In this model of phagocytosis, mouse peritoneal macrophages are loaded with free-cholesterol for 18 h [using 100 μg/ml AcLDL in the presence of acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor] to induce apoptosis, the FC-AMs are then fed to a monolayer of healthy macrophages (phagocytes) for a time period, and the monolayer is washed and analyzed for mRNA or protein responses. The phagocytes have been shown to ingest only apoptotic cells with high efficiency, and the washing procedure removes all uningested apoptotic and nonapoptotic cells from the monolayer (54). Moreover, the phagocytes show evidence of NF-κB activation, possibly in response to TNFα synthesized by the FC-AMs or the phagocytes themselves (33). Peritoneal macrophages were incubated with the FC-AMs, in the presence of neutralizing anti-TNFα or control Abs for 8 h. In this model of phagocytosis, uptake of apoptotic cells occurs rapidly (as early as 15 min) and the phagocytic index is high (up to 40%). However, preliminary time-course studies indicated that the induction of ABCA1 and the effect of TNF Abs required more prolonged incubation, and thus phagocytes were examined after 8 h of incubation. ABCA1 mRNA in the phagocytes was increased by ≈4-fold upon incubation with apoptotic cells, and this increase was suppressed by ≈40\% \( (P < 0.05) \) in the presence of anti-TNFα Abs (Fig. 4A).
This reduction of the response by TNFα Abs was reproduced in four separate experiments. A control IgG Ab for anti-TNFα had no effect on the induction of ABCA1 in phagocytes (data not shown). In a control experiment, we showed that ABCA1 was not increased in macrophages that were fed with nonapoptotic macrophages (Fig. 4A). Fig. 4B shows that ABCA1 protein also was increased (3.5-fold) in phagocytes after incubation with FC-AMs, and this increase was suppressed by ≈60% (P < 0.02) by neutralizing anti-TNFα Abs. These results show a marked induction of ABCA1 mRNA and protein in phagocytes after ingestion of FC-AMs and demonstrate that a major part of this response can be attributed to TNFα. It is unlikely that apoptosis of phagocytes themselves contributed to the induction of ABCA1 because Feng and Tabas (34) have shown that FC-induced apoptosis in macrophages markedly reduced ABCA1 protein levels.

![Chart showing ABCA1 induction in phagocytes](chart.png)

**Fig. 4. Neutralizing anti-TNFα Abs suppress the induction of ABCA1 that occurs during phagocytosis of apoptotic cells by peritoneal macrophages.**

Peritoneal macrophages were incubated with FC-AMs (A and B) or with non-apoptotic macrophages (NAP-MAC) as a control (A) with or without anti-TNFα Ab (10 μg/ml), phagocytes were washed vigorously to remove any bound but undigested apoptotic cells, and RNA extracts (A) or protein extracts (B) were prepared from the phagocytes 8 h later. mRNA data were normalized to housekeeping genes, and protein data were normalized to β-actin levels and are expressed as fold induction (FI) as compared with the controls that did not receive FC-AMs or NAP-MAC (control = 1 arbitrary unit). *, P < 0.05. The graph represents the average of three experiments conducted in three different cell preparations.

**ABCA1 Induction in Phagocytes Is Decreased in LXRα/β−/− Mice: Joint Role of TNFα and LXR in Induction of ABCA1 in Phagocytes**

Because the phagocytes are ingesting a cholesterol-rich meal, it seemed likely that LXR induction of ABCA1 also could be involved in the response. To assess the specific role of LXR, we carried out similar experiments using phagocytes from LXRα/β−/− mice, in which
the sterol induction of ABCA1 is abolished (35). WT or LXRα/β-/- macrophage monolayers were incubated with WT apoptotic cells for 8 h, the phagocytes were harvested, and RNA was extracted. The increase of ABCA1 mRNA by apoptotic cells was diminished by 60% in LXRα/β-/- phagocytes compared with control WT littermates (Fig. 5A). The mRNA of ABCG1, another LXR target gene, was modestly increased by the FCAMs (Fig. 5B). This effect was reduced in LXRα/β-/- cells. ABCA7, which is not regulated by LXR ligands (18) and only slightly induced by TNFα (Fig. 1B), was not induced by FC-AMs in either WT or LXRα/β-/- cells (Fig. 5C). These data show that ABCA1 induction in phagocytes is partially mediated by LXR. Nonetheless, there was considerable ABCA1 expression remaining in the LXR-deficient mice in the presence of apoptotic cells. We hypothesized that the remaining induction of ABCA1 mRNA levels was due to the effect of TNFα. To test this hypothesis, we investigated the effect of neutralizing anti-TNFα Abs on ABCA1 in LXRα/β-/- phagocytes compared with control WT littermates (Fig. 5). Neutralizing TNFα Abs added to phagocytes in the presence of apoptotic cells decreased ABCA1 mRNA by a similar amount in both WT and LXRα/β-/- mice cells and reduced ABCA1 mRNA almost to the control level in the LXRα/β-/- cells. In contrast, ABCG1 and ABCA7 mRNA levels were not significantly changed by neutralizing anti-TNFα Abs. The small residual response in LXRα/β-/- cells treated with TNFα Abs could represent incomplete neutralization of TNFα or other factors inducing ABCA1 in this model of phagocytosis. These experiments indicate a major role for both TNFα and LXR in the induction of ABCA1 in phagocytes.

**Fig. 5. ABCA1-induction is decreased in LXRα/β-/- phagocytes: joint role of TNFα and LXR in induction of ABCA1 in phagocytes.**

Thioglycollate-elicited peritoneal macrophages were isolated from LXRα/β-/- mice and their control WT littermates and cultured as described in Materials and Methods. Macrophage monolayers (WT or LXRα/β-/-) were incubated with WT FC-AMs in the presence or absence of anti-TNFα Ab (10 μg/ml), and RNA extracts were performed, as described in Fig. 4. ABCA1 (A), ABCG1 (B), and ABCA7 (C) mRNA were analyzed by real-time quantitative PCR, and data are shown normalized for housekeeping genes. *, *P < 0.05; **, **P < 0.056. The experiment was conducted in two different cell preparations; the graphs show the average of triplicates of wells of one representative experiment.

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Discussion

TNFα acting via NF-κB induces ABCA1 expression in macrophages. Macrophage ABCA1 expression is markedly upregulated during phagocytosis of cholesterol-induced apoptotic cells, reflecting both the effects of TNFα and LXR activation in the phagocytes. These studies show an important link between inflammatory signals and lipid efflux pathways in macrophages as they phagocyte apoptotic cells. It is likely that the induction of ABCA1 by TNFα and LXR facilitates the resolution of inflammatory lesions and represents a beneficial response of the phagocyte in the context of atherosclerosis. The findings are consistent with the emerging view that NF-κB signalling is involved both in the formation and in the resolution of inflammatory lesions (36). There was also a lesser role of p38-MAPK in the induction of ABCA1 by TNFα. p38-MAPK may contribute to NF-κB activation through phosphorylation of p65 (37) or indirectly through phosphorylation and activation of other factors that synergize with NF-κB (38–40).

The induction of ABCA1 by TNFα may be relevant to atherosclerotic plaques in which apoptotic free cholesterol-loaded macrophages may be cleared by healthy macrophages (41,42). ABCA1 and ABCG1 both could contribute to the efflux of cholesterol from the phagocytes after ingestion of the cholesterol-enriched apoptotic corpses. ABCG1 promotes cholesterol efflux to high-density lipoprotein (HDL) but has minimal effects on phospholipid efflux (20). In contrast, ABCA1 enhances both cholesterol and phospholipid efflux from macrophages to lipid-poor apolipoproteins. In addition to efflux of cholesterol and native phospholipids, the induction of ABCA1 could help the phagocyte to deal with oxidant stress and reactive oxygen species generation, subsequent to the ingestion of oxidized lipids in phagocytes. Macrophages play an important role in the clearance of apoptotic corpses that contain large amounts of oxidized phospholipids (43), and ABCA1 has been suggested to increase efflux of oxidized lipids from cells to apoA-I (44) limiting the formation and the nucleation of minimally oxidized low-density lipoprotein (LDL) (45), thereby potentially protecting against atherosclerosis. More generally, our study shows a positive interaction between inflammation and lipid metabolism. The cooperation of TNFα and LXR in the induction of ABCA1 contrasts with the well documented antiinflammatory transrepression of a number of TNFα target genes by LXR activation (46–48).

LXR activation inhibits lipopolysaccharide (LPS)- or TNFα-activated MMP-9 in macrophages (46). However, unlike ABCA1 these genes are not direct targets of LXR. In an elegant study, Castrillo et al. (47) also showed that LPS inhibits LXR-induced ABCA1 gene expression in macrophages, in a process that involves TLR4 and IRF-3 mediated signalling events. These pathways are not activated by TNFα, which may explain the different responses to LPS and TNFα. This result illustrates the different responses of ABCA1 in a pathologic process involving LPS compared with its physiologic induction in phagocytes ingesting apoptotic cells. Our studies likely have relevance to the role of TNFα in atherogenesis. Some recent studies have suggested that TNFα signalling via
NF-κB may be atheroprotective (14–16). Although some other studies have suggested a proatherogenic role of TNFα signalling, this role may depend on the cell type involved as well as the specific receptors and signalling pathways downstream of TNFα. Thus, there seems to be abundant evidence for a proatherogenic role of TNFα signalling in endothelial cells (49, 50). In contrast, TNFα signalling via NF-κB in macrophages and smooth muscle cells may have antiatherogenic consequences. Idel et al. (17) have shown that an atheroprotective locus on chromosome 10 in the C57Bl6 mouse contains the A20 gene, which is involved in downmodulating NF-κB signalling. Compared with the FVB variant, an amino acid change in the B6 A20 gene is associated with more sustained NF-κB signalling in response to TNFα in vascular smooth muscle cells. By using a macrophage-specific deletion of IKKB similar to that used in the current study (Fig. 2), Kanters et al. (16) showed that inhibition of NF-κB activation in macrophages increases atherosclerosis in LDL receptor−/− mice. By using the same strategy to achieve macrophage-specific knockout of NF-κB signalling, we show that TNFα induction of ABCA1 is virtually abolished in these cells. Together, this information suggests that TNFα-induction of macrophage ABCA1 by NF-κB is likely to be antiatherogenic.

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Materials and Methods

Materials
The LXR activator, TO-1317, was obtained from Sigma. Tritium-labeled cholesterol ([1,2-3H]cholesterol) was purchased from PerkinElmer Life Sciences. Rabbit anti-ABCA1 Ab was from Novus Biologicals (Littleton, CO); mouse anti-β-actin, horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit Abs were from Sigma. Recombinant mouse TNFα, recombinant mouse macrophage-colony stimulating factor, goat anti-mouse TNFα Ab, and control IgG were from R & D Systems. AcLDL was from Biomedical Technologies (Stoughton, MA). ApoA-I was from Biodesign International (Kennebunkport, ME).

Animals and Cell Culture
Male C57BL_6J mice were purchased from The Jackson Laboratory. LXRα/β−/− mice and their control WT littermates were kindly provided by David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas) (51). Peritoneal macrophages were
obtained from mice injected intraperitoneally with thioglycollate (52). Bone marrow-derived macrophages were isolated and cultured in DMEM/10% FBS supplemented with macrophage-colony stimulating factor (20 ng/ml) and antibiotics for 5–9 days before the experiment. IKKβF/F and IKKβΔ/Δ, JNK1± and JNK1−/− bone-marrow-derived macrophages are described in ref. 27. Macrophages deficient in p38α (p38αfloxflox) were obtained from p38αfloxflox (p38αF/F) mice crossed with LysMCre/C57BL6J mice (53).

**Cholesterol Efflux**

Macrophages were incubated with AcLDL and [3H]cholesterol in the presence or absence or TNFα (50 ng/ml), then incubated with apoA-I in the presence or absence of TNFα for 4 h. The cells were lysed in 0.5 ml of 0.1M sodium hydroxide containing 0.1% SDS at room temperature, and the radioactivity in the cell lysates and medium was quantified. To obtain apoA-I-specific efflux, radioactivity in apoA-I-free medium was subtracted. Cholesterol efflux was calculated as the percent of radioactivity released from cells into the medium relative to the total radioactivity in cells and medium.

**RNA Analysis**

Total RNA was isolated from mouse peritoneal macrophages by using the RNeasy Minikit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Real-time quantitative PCR assays were performed as described in ref. 52. All samples were analyzed for β-actin, 36B4 expression, or 18S RNA in the same run as ABCA1, ABCG1, ABCA7, or MMP-9. The sequences of the probes and primers can be provided by the authors upon request.

**Western Blot Analysis**

Protein extracts were prepared in modified radioimmunoprecipitation assay buffer as described in ref. 52. Equal amounts of protein (15 g) were fractioned on a 4–15% gradient SDS/polyacrylamide gel, then transferred to a nitrocellulose membrane, incubated with anti-ABCA1 and anti-β-actin Abs, and processed with horseradish peroxidase-conjugated secondary Ab using SuperSignal West Pico Chemiluminescent substrate (Pierce). The band intensity was analyzed with IMAGEQUANT (Amersham Pharmacia). Data were normalized for β-actin expression.

**Generation of Apoptotic Cells**

Con A or methyl-BSA elicited macrophages from female C57BL_6J mice were cultured in DMEM containing 10% FBS (Mediatech, Washington, DC), antibiotics, and 20% L cell-conditioned medium. The cells were incubated with 100 μg/ml AcLDL and 10 μg/ml acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor 58035 (Sigma) for 16–20 h to induce early apoptosis as described in ref. 33. Apoptosis was confirmed by Annexin V staining. In a typical experiment, 30–40% of the population was apoptotic with ≈5% necrosis. These
FC-AMs were collected, resuspended into XVIVO10 medium (BioWhittaker), and added to the phagocytes. The ratio of phagocytes to apoptotic cells was ≈1:5.

**Phagocytosis of Apoptotic Cells**

The phagocytes were incubated with the FC-AMs or control nonapoptotic macrophages for 8 h in the presence or absence of 10 μg of anti-TNFα Ab or control IgG Ab in X-VIVO10 medium. Phagocytes then were washed vigorously with the medium to remove any bound but undigested apoptotic cells, and RNA or protein was extracted from the phagocytes, as described earlier. In preliminary experiments, the interaction of fluorescently labeled FC-AMs with phagocytes was examined by confocal microscopy. We found that ingestion of FC-AMs by the phagocytes occurred as early as 15 min after incubation (54).

**Statistics**

All results are expressed as the means ± SEM unless otherwise mentioned. Statistical significance was determined by using the Student t test. $P < 0.05$ was considered significant.
References


TNFα induces ABCA1 through NFκB
Chapter 19

Summary, general discussion and concluding perspective
Summary, general discussion and concluding perspective

There is unequivocal evidence of an inverse association between plasma high-density lipoprotein cholesterol (HDL-c) concentrations and the risk of cardiovascular disease (CVD), a finding that has led to the hypothesis that HDL protects from atherosclerosis.1-3

As an introduction to this thesis, chapter 1 reviews the experimental evidence for this “HDL hypothesis”. In vitro studies suggest that HDL has a wide range of anti-atherogenic properties but validation of these functions in humans is absent to date. A significant number of animal studies and clinical trials support an atheroprotective role for HDL, however, most of these findings were obtained in the context of marked changes in other plasma lipids. Finally, genetic studies in humans have not provided convincing evidence that HDL genes modulate cardiovascular risk. Taken together, despite a wealth of information on this intriguing lipoprotein, future research remains essential to prove the HDL hypothesis right.

Because HDL metabolism is complex and closely interrelated with lipids and other lipoproteins, several questions can be asked:

- Does HDL itself actually and causally protect from atherosclerosis?
- What are the underlying molecular mechanisms?
- Which process in HDL metabolism should be targeted to achieve the desired therapeutic potential?

To advance our understanding of these matters, we studied HDL metabolism in patients with monogenic disorders that affect receptors, enzymes and other proteins that are essential to this macromolecule, as further outlined in chapter 2. The advantage of this approach is a reduction in the complexity of HDL metabolism because only one factor at time is altered, in a stable way, whilst at the same time studying human pathophysiology, thus not turning to in vitro reflections of HDL biology.

With monogenic disorders as a cornerstone, we have addressed the questions in the HDL field by conducting a range of studies, from infusion protocols based on the tracer dilution principle and kinetic modelling, to validation of a gene locus found in genome wide association studies (GWAS) through the identification of a linking molecular mechanism by the use of next generation, mass spectrometry and molecular studies of protein glycosylation: from Model to Mechanism.
Part 1 – Mutations in HDL candidate and GWAS genes

Twin studies have indicated that 50% of the variation of HDL-c levels is genetically determined and in fact, tens of different genes have been reported to affect HDL cholesterol or HDL function and this number is still increasing. Chapter 3 reviews the current literature on the genetics of HDL. In many cases, it is unclear how the respective gene product affects HDL metabolism. In particular, genome-wide association studies have identified several novel loci to be linked to HDL, but studies in mice, and in patients with rare variants will be required to validate these findings and identify the molecular link with HDL metabolism.

In 1996, scavenger receptor class B type I (SR-BI) was discovered to be a major HDL receptor in mice. In Chapter 4 we describe a non-synonymous missense mutation SR-BI in a Dutch family, resulting in a proline to serine substitution (P297S). In mice, SR-BI acts as a receptor for HDL and is involved in protection from atherosclerosis, macrophage cholesterol homeostasis, preservation of platelet function and the adrenal uptake of plasma cholesterol as a substrate for steroidogenesis. Carriers of the P297S mutation were found to have high levels of HDL-c. Carriers of the mutations exhibit impaired macrophage cholesterol efflux, but we did not identify increased carotid intima-media thickness. We did identify altered platelet aggregation, and lower levels of adrenal steroidogenesis. Together, these data support a clinically relevant role for SR-BI in cholesterol homeostasis in man. For the first time, this study also shows that HDL has an unanticipated role delivering cholesterol to the adrenals for steroidogenesis. This aspect merits consideration in the development of HDL-increasing drugs to reduce CVD.

Genome-wide association studies have linked variation in GALNT2, the gene encoding ppGalNac-T2, to variation in HDL-c and triglycerides in the general population. ppGalNAc-T2 is an enzyme responsible for the initiation of O-linked glycosylation of proteins. In chapter 5 we describe a molecular mechanism that can explain the association between GALNT2 and variation in plasma lipid levels. Carriers of a mutation in GALNT2 have increased HDL-c, decreased triglycerides, improved triglyceride hydrolysis upon oral fat challenge, and show defective glycosylation of apolipoprotein C-III. Apolipoprotein C-III is an established inhibitor of lipoprotein lipase, the main enzyme responsible for triglyceride hydrolysis in human plasma. Ex vivo and in vitro experiments confirm decreased inhibition of LPL, and decreased enzymatic function of the mutant ppGalNAc-T2.

This study describes I) a pathway through which a gene identified through GWAS affects human lipid metabolism, II) a novel congenital disorder of glycosylation, III) a direct specific role on of the ppGalNAc-transferases, which other functions in human physiology remain largely unknown, and IV) that glycosylation of apolipoprotein C-III affects its role in plasma triglyceride lipolysis. The study constitutes a first link between...
the cardiovascular and glycosylation research fields; supported by next generation sequencing and mass spectrometry, we aspire to study dyslipidemia in heterozygous and homozygous carriers of mutations in CDG genes to increase our insight in human lipid metabolism.

To better understand the molecular pathology of low plasma HDL-c levels, we sequenced the ABCA1 gene in 78 patients that were referred to our clinic with very low HDL-c levels. In chapter 6, we describe the identification of an unexpected high number of ABCA1 gene variants (n=19), of which 14 had not been described earlier. By in vitro as well as confocal imaging experiments, we evaluated whether the newly identified mutations were functional and could explain the low HDL-c phenotype of the respective patients. The identification of so many new carriers of ABCA1 defects enables further studies into the debated role of ABCA1 in atherosclerosis and also into its role in hematopoiesis and leucocytosis reported in mice as well as its potential role in adrenal steroidogenesis.

In conclusion, chapters 4 and 5 illustrate that studying rare loss of function mutations in families increases our understanding of candidate genes that have been discovered in mice and genome-wide association studies. To study the prevalence of rare loss of function mutations not only clarifies the molecular pathology of HDL disorders, but through identification of carriers of these mutations, enables initiation of further studies into of human HDL metabolism and its relation to atherosclerosis.

Part 2 - LCAT and its clinical consequences

Through hydrolyzing fatty acids at the sn-2 position of phospholipids and transferring these to free cholesterol, thereby generating cholesteryl esters, LCAT catalyses the maturation of HDL: nascent discoidal particles mature into larger spherical HDL.

In a similar effort to study the ABCA1-related molecular pathology of low HDL-c (chapter 6), chapter 7 describes the frequency and effects of LCAT gene mutations in 98 patients with a low HDL-c. 28 of these patients (29%) had mutations in LCAT that were shown to cause loss of enzyme function in vitro. Combined, these studies indicate that routine sequencing of ABCA1 and LCAT in referred low HDL-c patients in The Netherlands improves insight in the pathophysiology of low HDL-c. LCAT is considered a target for pharmaceutical drugs to reduce atherosclerosis. The identification of so many carriers of LCAT mutations enables further studies into the unresolved relation between LCAT and atherosclerosis through adequately powered imaging studies of subclinical atherosclerosis. In addition, patients could enrol in future clinical trials aimed at increasing LCAT by enzyme or gene therapy (in preclinical development).
In chapter 8 we studied the relationship between plasma levels of LCAT protein, lipid metabolism, and risk of coronary artery disease (CAD) in the general population. On the basis animal studies and studies of patients with LCAT deficiency, we hypothesized that low plasma levels of LCAT would be associated with low HDL-c levels. In this prospective population study, however, LCAT protein levels were not associated with HDL-c levels. In addition, no association of LCAT levels with risk of CAD was found. When studying men and women, we identified opposite trends. In women, LCAT levels were positively associated with CAD risk while the reverse appeared true for males. We can only speculate that the discrepancy may be related to the possibility that plasma LCAT concentration is not rate-limiting for the formation of HDL in the general population (with a very low prevalence of LCAT mutations). These results demonstrate the difficulty of translating findings from inherited metabolic disorders (LCAT deficiency) to the general population and vice versa.

In chapter 9 we present a case of three siblings homozygous for a new mutation in the LCAT gene that leads to loss of a disulfide bond in the enzyme. Consequentially, their plasma is deficient of LCAT enzyme, and all show signs of classical familial LCAT deficiency, a rare congenital metabolic disorder: HDL deficiency, early signs of corneal opacification, and proteinuria as a sign of renal damage, even at the early age of three. In the eldest patient, the proteinuria was stably reduced by treatment with an ACE inhibitor and thiazide diuretic, and until enzyme replacement becomes available, we recommend this symptomatic treatment to prevent renal insufficiency in FLD patients. In addition, lipid lowering therapy and – recently - methylprednisolone have been reported to decelerate the progression towards end stage renal disease in these patients.

In the subsequent three chapters, we have studied arterial wall thickness (chapter 10, 11) and stiffness (chapter 12) in carriers of LCAT mutations. Chapter 10 describes a case control study of heterozygous LCAT mutation carriers. With the use of B-mode ultrasound, we found increased carotid intima media thickness (cIMT) as a marker of increased atherosclerosis in these patients. Interestingly, Calabresi et al. reported an opposite conclusion from a matched case control study: carriers of LCAT mutations have a gene-dose dependent decrease in cIMT compared to controls. The expanded cohort of LCAT mutation carriers (chapter 7) allowed us to study atherosclerosis in these carriers in more detail in order to solve this discrepancy: as detailed in chapter 11 and 12, we performed a second study in our center, this time matching cases and controls for age. In addition to cIMT, we applied 3.0 T MRI technology and a dynamic assessment of arterial stiffness to investigate the arterial condition in these patients. As shown in chapter 11, carotid wall thickness assessed by MRI was increased in carriers. There was, however, no significant difference in cIMT in this study which may be related to high statin use in the carriers in recent years. Chapter 12 furthermore shows that arterial stiffness assessed by carotid-femoral pulse wave velocity was increased in carriers. In both groups, arterial stiffness was strongly correlated to carotid wall thickness assessed by both IMT and MRI.
Together, these three studies indicate increased atherosclerosis in carriers of LCAT gene mutations in the Netherlands. We are currently collaborating with prof. Calabresi to re-analyze our combined cIMT data sets to hopefully solve the apparent discrepancy between their IMT study and our studies.

Through its ability to function as a phospholipase A₂, LCAT is proposed to have anti-oxidant properties. It is not known whether this function is relevant to lipid oxidation in humans. In chapter 13, we therefore studied lipid oxidation in carriers of LCAT mutations. We report a decrease in activities of three anti-oxidative enzymes, i.e. LCAT, paraoxonase 1 and platelet-activating factor acetyl hydrolase, an increase in oxidized phospholipids on apolipoprotein B-containing particles and a decrease in the potential of HDL to reduce oxidation on low-density lipoprotein (LDL) particles, although overall lipid oxidation was not increased in plasma of patients with LCAT mutations. Together these findings suggest that alternative mechanisms, such as clearance of oxidized LDL by CD36 and other scavenger receptors, may be stronger determinants of circulating oxidized LDL and lipid oxidation than these HDL-associated anti-oxidative enzymes.

In conclusion, both the increased atherosclerosis in carriers of LCAT gene defects in our center and the severity of the renal pathology observed in patients suffering from complete LCAT deficiency support the development of LCAT enhancing and/or replacing therapies. Our molecular understanding of LCAT has come to such level that predominantly clinical studies are needed now to answer the chief remaining questions:

- Can the difference between familial LCAT deficiency and fish eye disease indeed explain the opposite conclusions of the Dutch and Italian ultrasound studies on carotid atherosclerosis in carriers of LCAT mutations?

- Can familial LCAT deficiency and the renal pathology secondary to this disease be adequately treated with recombinant enzyme or gene replacement therapy?

- Would LCAT therapy reduce cardiovascular risk in mutation carriers, bearing in mind that extreme overexpression of LCAT in mice actually increased the progression of atherosclerosis and that we did not observe a relation of LCAT with HDLc or CAD risk in the general population, in which LCAT enzyme levels are within normal range (chapter 8)?
Part 3 - HDL function: beyond plasma cholesterol concentration

In order to define a causal role for HDL in atheroprotection the proposed mechanisms that are brought forward need to be studied in detail. As a matter of fact, HDL has been bestowed with a plethora of functions that are potentially atheroprotective, but their relevance in human pathophysiology is largely unknown. The development of assays of HDL function which are reproducible and applicable in larger clinical studies (that study HDL modulating compounds has presented a great difficulty to this field of research. The complexity of HDL metabolism and its functions are directly illustrated by the diversity of topics in the final part of this thesis.

Atheroprotection by HDL is generally considered to be mediated by reverse cholesterol transport (RCT) from peripheral tissues to cholesterol excretion via the liver. In humans, however, little is known about in vivo cholesterol fluxes through this pathway. In chapter 14, we therefore investigated whether RCT cholesterol fluxes were altered in patients with genetic HDL deficiencies and found that tissue cholesterol efflux is reduced in carriers of mutations in APOA1 or ABCA1, substantiating that HDL contributes to tissue cholesterol efflux in humans. The residual tissue cholesterol efflux and unaffected fecal sterol excretion in these patients indicate, however, that non-HDL pathways contribute significantly.

Lipoprotein-independent secretion of 27-hydroxycholesterol (27OHC) from macrophages has been proposed to offer an alternative to HDL-mediated reverse transport of excess cholesterol (RCT). In chapter 15, we investigated 27OHC concentrations in plasma of humans and mice with monogenic disorders of HDL metabolism. Compared to family controls, carriers of mutations in APOA1, ABCA1 and LCAT had reduced HDL-c and HDL-27OHC whereas mutations in CETP (encoding cholesterol ester transfer protein, CETP), SCARB1 (encoding SR-BI) and LIPC (encoding hepatic lipase) were associated with elevated HDL cholesterol and 27OHC levels. The data furthermore suggest that the formation and transfer of 27OHC-esters are more sensitive to reduced activities of LCAT and CETP respectively, than the formation and transfer of cholesterol esters. 27OHC plasma levels were also decreased in APOA1-, ABCA1- or LCAT-knockout mice but increased in SCARB1-knockout mice. Together, these data argue against the hypothesis that 27OHC-metabolism in plasma occurs independently of HDL.

In plasma, HDL is the major carrier of both sphingosine-1-phosphate (S1P), a bioactive sphingolipid, and also of apolipoprotein M (apo M), which is involved in the formation of nascent HDL particles. Apo M has been proposed to bind S1P within HDL. In chapter 16, we tested the hypothesis that plasma HDL-c or apo M determine plasma S1P levels. We found that both mildly and severely lowered HDL-c or apo A-I due to mutations in APOA1, ABCA1 or LCAT are associated with reduced S1P levels. By contrast, high levels of
HDL-c and apo A-I due to mutations in CETP, SCARB1, LIPC or LIPG were not associated with changes in S1P levels. It is possible that a subclass of HDL or a minor HDL-associated protein, rather than HDL in general, limits the abundance of S1P in HDL. Because apo M levels were not correlated with S1P levels in family controls, and not as pronouncedly reduced as levels of S1P in heterozygotes and carriers of two defective alleles of APOA1, ABCA1 and LCAT, this limiting factor likely is not apo M, although apo M was previously proposed to bind S1P.

Chapter 17 provides a novel mechanism by which HDL might protect from cardiovascular death including sudden cardiac death. Cardiovascular death is often preceded by ventricular tachyarrhythmias. Prolongation of the cardiac repolarization time, which has been shown to predict sudden cardiac death, often underlies these rhythm disturbances. We hypothesized that increasing HDL shortens cardiac repolarization. We found that reconstituted HDL (rHDL) as well as purified human apo A-I shortened repolarization of isolated rabbit cardiomyocytes. In addition, rHDL infusion shortened the heart rate-corrected QT interval on surface electrocardiograms of patients with genetically low HDL-c as well as controls. These data establish that reconstituted HDL shortens cardiac repolarization, an effect that should be further evaluated in future studies with rHDL infusion in patients with acute coronary syndrome.

In chapter 18 we studied a potentially antiatherogenic consequence of tumor necrosis factor α (TNFα) signalling in vascular cells. TNFα is released by free cholesterol-loaded apoptotic macrophages, and the clearance of these cells by phagocytic macrophages may help to limit plaque development. Macrophage cholesterol uptake induces ATP-binding cassette transporter A1 (ABCA1) to promote cholesterol efflux to apolipoprotein A-I, potentially reducing atherosclerosis. We show that TNFα induces ABCA1 mRNA and protein in control and cholesterol-loaded macrophages and enhances cholesterol efflux to apolipoprotein A-I through intracellular signalling via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). To evaluate the potential pathophysiological significance of these observations, we fed TNFα-secreting free cholesterol-loaded apoptotic macrophages to a healthy macrophage monolayer (phagocytes). ABCA1 mRNA and protein were markedly induced in the phagocytes, a response that was mediated both by TNFα signalling and by liver X receptor activation. In atherosclerotic plaques, this process may help phagocytic macrophages to efflux excess lipids derived from the ingestion of cholesterol-rich apoptotic corpses.

In conclusion, this third and final part of my thesis presents some answers to the many and diverse questions concerning HDL function. An important step in the development of HDL enhancing compounds would be to study their effect on reverse cholesterol transport in humans. The in vivo measurement of tissue cholesterol efflux described in chapter 14 might facilitate such evaluations. In such studies, the effect of HDL modulating
compounds on cardiac repolarization could also be evaluated, although molecular studies into the underlying mechanism are equally called for to possibly more accurately modulate this function in the future. In addition to chapter 14, chapter 18 also underscores the importance of HDL-mediated cholesterol efflux, but here at the level of the macrophage, the key cellular player in atherosclerotic lesions. Crosstalk between cellular cholesterol overloading, cholesterol efflux, and cytokine signalling likely are at the heart of the atherosclerotic process where HDL-modulating strategies should be focused.
Concluding perspective

Currently, the hypothesis that HDL would protect from atherosclerosis is no longer simply expected to be confirmed. The increase in mortality caused by the CETP inhibitor torcetrapib and its off-target toxicity (the compound raised levels of HDL-c but also of aldosterone, and blood pressure)\textsuperscript{13, 14} and the absence of a higher risk of CVD in carriers of \textit{ABCA1} mutations with a lower HDL-c in large prospective studies\textsuperscript{15} tampered optimism.

In the next few years, positive outcomes of phase III clinical trials with anacetrapib\textsuperscript{16} and dalcetrapib (ClinicalTrials.gov Identifier: NCT01059682), CETP-inhibitors that do not affect aldosterone nor blood pressure would certainly change emotions around again by confirming the HDL hypothesis in patients with CVD. Nevertheless, this relation will always be confounded by the LDLc and TG-lowering effect of these compounds.

Such positive outcomes would be an example of how clinical science can be ahead of in depth molecular understanding of lipid biology, and as a result we would still be left with an association between an increase in HDL-c and a reduction of CVD risk. We would, however, still not know whether HDL actually carries excess cholesterol from atherosclerotic lesions through the circulation for hepatic excretion into bile, the classical concept of how HDL would protect. An actual pathophysiological explanation will need to be uncovered through advances in molecular biology together with translational studies, which subsequently may truly unlock the therapeutic potential of HDL.

In the studies presented in this thesis, we have attempted to critically evaluate the HDL hypothesis in families with genetic HDL disorders. In this way we found novel aspects of its metabolism in humans: as in mice, SR-BI proved to be a physiologically relevant HDL receptor. In another study we could establish a molecular link between the research fields of lipid and glycosylation disorders. We show that ppGalNAc-T2 affects lipolysis of plasma triglycerides through glycosylation of apo C-III. Reduced function of both SR-BI and of ppGalNAc-T2 leads to elevated HDL-c levels in patients carrying mutations in the encoding genes. These proteins could therefore possibly form targets for future therapies.\textsuperscript{17} The above mentioned studies also demonstrate that families with rare genetic variants are very helpful to establish the relevance of candidate genes identified in large association studies and/or in studies in animals.

We furthermore redefined LCAT, an enzyme crucial to HDL maturation, as a potential therapeutic target. In future years, studies with recombinant enzyme or gene replacement therapy will evaluate whether targeting LCAT to modulate HDL metabolism is beneficial.

Finally, we helped validating an \textit{in vivo} assessment of tissue cholesterol efflux by demonstrating that patients with a genetically low HDL-c have reduced cholesterol efflux
in vivo. This finding supports the concept of HDL-mediated reverse cholesterol transport in man. Future translational studies should aim to develop a measure of macrophage-specific cholesterol efflux applicable in the human setting, potentially in concert with the exciting development of HDL-based contrast agents for MR imaging of atherosclerotic plaques. These synthetic nanoparticles home to plaque macrophages and are capable of inducing cholesterol efflux.\textsuperscript{18} Combining these approaches has the potential to truly test the concept of HDL-mediated RCT out of atherosclerotic plaques. As the ultimate measure of HDL function, this would also allow to properly address the effect of various HDL-modulating compounds.

In conclusion, we did not prove the HDL hypothesis \textit{correct} in this thesis, but we certainly prove it to be very much \textit{alive}.

Onno Holleboom
Amsterdam, March 16 2011
Reference List


Samenvatting voor niet-ingewijden
Volgens Fact Sheet No. 317 van wereldgezondheidsorganisatie WHO (januari 2011) vormen hart- en vaatziekten (HVZ) op dit moment de belangrijkste doodsoorzaak ter wereld.\(^1\) 60 jaar geleden lieten Barr et al. zien dat er een sterk omgekeerd verband bestaat tussen de hoogte van het cholesterol in je bloed dat in high-density lipoproteins (HDL-c) zit, en het risico dat je loopt om HVZ te ontwikkelen.\(^2\) In verschillende grote bevolkingsonderzoeken kwam dit omgekeerde verband steeds opnieuw terug en het bleek onafhankelijk te zijn van andere factoren die de kans op HVZ beïnvloeden, zoals leeftijd, geslacht, rookgedrag en bloedwaarden van low density lipoprotein cholesterol (LDL-c).\(^3,4\) Deze bevindingen leidden tot de hypothese dat HDL direct beschermt tegen atherosclerose ofwel slagaderverkalking, het ziekteproces van slagaders dat ten grondslag ligt aan HVZ: HDL werd bekend als ‘het goede cholesterol’. Verhoging of verbetering van het HDL-cholesterol met daartoe geschikte medicijnen zou HVZ en de sterfte hieraan kunnen terugdringen. Sindsdien heeft dit concept artsen, biologen, epidemiologen en andere wetenschappers gedreven om de HDL-stofwisseling nader te onderzoeken. Hierdoor is onze kennis over HDL sterk toegenomen, maar is ook duidelijk geworden dat dit metabolisme complex is. Er zijn vele moleculen bij betrokken en deze zijn onderling met elkaar verbonden. Dat maakt het niet eenvoudig om de drie fundamentele vragen in dit onderzoeksveld te beantwoorden:

- Beschermt HDL zelf, direct, tegen hart- en vaatziekten?
- Wat zijn de onderliggende metabole werkingsmechanismen van deze bescherming?
- Welk proces in het HDL-metabolisme moeten we aangrijpen om therapieën te ontwikkelen die het risico op HVZ verlagen?

Als een inleiding op dit proefschrift, geeft hoofdstuk 1 een overzicht van het experimentele bewijs voor de “HDL-hypothese”: de gedachte dat HDL beschermt tegen HVZ. Ten eerste komt uit experimenteel onderzoek in het laboratorium naar voren dat HDL vele verschillende eigenschappen heeft die mogelijk bescherming geven tegen atherosclerose, maar er is tot nu toe geen solide bewijs dat deze functies in het menselijk lichaam daadwerkelijk bescherming tegen HVZ bieden. Ten tweede ondersteunen meerdere studies die gebruik maken van diermodellen een rol voor HDL in de bescherming tegen HVZ. Ten derde ondersteunt ook een aantal klinische onderzoeken met HDL-modulerende medicijnen bij mensen deze rol. Echter, deze bevindingen werden verkregen in de context van belangrijke veranderingen in andere plasma-lipiden dan HDL-cholesterol. Tot slot leveren ook genetische studies geen overtuigend bewijs dat HDL-genen bij de mens de kans op hart- en vaatziekten direct beïnvloeden. Er is dus veel bekend over dit intrigerende lipoproteïne, maar nader onderzoek blijft noodzakelijk om de HDL-hypothese definitief te toetsen.
Om dichter bij een antwoord op bovengenoemde vragen te komen, hebben we in dit proefschrift de HDL-stofwisseling onderzocht bij patiënten met monogenetische aandoeningen (aandoeningen die door een verandering in één gen worden veroorzaakt) van receptoreiwitten, enzymen en andere eiwitten die essentieel zijn voor het HDL-macromolecuul. Een nadere uiteenzetting hiervan staat in hoofdstuk 2. Deze aanpak heeft als groot voordeel dat de complexiteit van het HDL-metabolisme gereduceerd wordt, omdat 1 factor, namelijk de functie van slechts 1 eiwit, tegelijk veranderd is bij deze patiënten; niet tijdelijk, maar permanent (levenslang). Tegelijkertijd bekijkt je direct de HDL-stofwisseling in de mens: je neemt dus niet je toevlucht tot reflecties van HDL-biologie in een reageerbuis.

Met deze monogenetische stoornissen als uitgangspunt hebben we getracht een stap dichterbij een antwoord op bovenstaande vragen te komen door verschillende studies uit te voeren, variërend van een infusieprotocol gebaseerd op het tracer-dilutie-principe en kinetische modellering, tot de identificatie van een nieuw molecular mechanisme door middel van high throughput sequencing, massaspectrometrie en moleculaire studies van eiwitglycosylatie: van Model tot Mechanisme.

Dit proefschrift bestaat uit drie delen. In het eerste deel bestuderen we mutaties (veranderingen) in genen die voor HDL-gerelateerde eiwitten coderen, en hoe deze veranderingen van invloed zijn op de HDL-stofwisseling. In het tweede deel is er speciale aandacht voor lecithin:cholesterol acyl transferase (LCAT), 1 van deze HDL-gerelateerde eiwitten. LCAT is een enzym dat cruciaal is voor de maturatie van HDL-deeltjes: het zorgt ervoor dat cholesterolmoleculen als het ware verankerd worden in het HDL-deeltje. We onderzoeken de gevolgen van mutaties in het gen dat codeert voor dit enzym. In het derde deel van dit proefschrift onderzoeken we niet alleen simpelweg de cholesterolconcentratie van HDL-deeltjes, maar kijken we verder door een aantal functies van HDL in de humane homeostase te bestuderen.

Deel 1 – Mutaties in HDL-kandidaatgenen en GWAS-genen

Onderzoek bij tweelingen geeft aan dat 50% van de variatie in HDL-cholesterol in het bloed genetisch bepaald is. Van tientallen genen is gerapporteerd dat ze HDL-cholesterolconcentraties of HDL-functie beïnvloeden, en dit aantal neemt nog steeds toe. Hoofdstuk 3 geeft een overzicht van de huidige literatuur die betrekking heeft op de genetica van HDL. In veel gevallen is het onduidelijk hoe het eiwit dat door een bepaald gen gecodeerd wordt, de HDL-stofwisseling beïnvloedt. In het bijzonder dienen hier de genome-wide association studies genoemd te worden. Deze studies hebben verschillende nieuwe genetische loci gevonden die samenhangen met HDL, maar muisonderzoek en onderzoek bij patiënten met zeldzame genetische varianten is nodig om deze bevindingen te valideren en de moleculaire link met het HDL-metabolisme te identificeren.
In 1996 wees muisonderzoek uit dat scavenger receptor class B type I (SR-BI) een belangrijke HDL-receptor is bij muizen. In hoofdstuk 4 beschrijven we een missense mutatie in het SR-BI-gen in een familie van Nederlandse afkomst. Deze mutatie resulteert in substitutie van een proline voor een serine (P297S) in de SR-B1 receptor. In muizen fungeert SR-BI als een receptor voor HDL, met name op de lever, en is betrokken bij de bescherming tegen atherosclerose, cholesterolhuishouding van macrofagen en behoud van bloedplaatjesfunctie en de opname van cholesterol uit het bloed door de bijnier als substraat voor de aanmaak van steroidhormonen. We onderzochten deze functies van SR-B1 in de bovenbeschreven familie en vonden dat dragers van de P297S-mutatie hoge bloedwaarden van HDL-cholesterol hadden. Macrofagen van mutatie-dragers bleken een gestoorde efflux van cholesterol te hebben, maar we vonden geen toegenomen intima-media dikte van de halsslagaders van de dragers (een indicator van atherosclerose). Wat we wel vonden, was een veranderde aggregatie van bloedplaatjes, en lagere aanmaak van steroidhormonen bij de mutatie-dragers. Samengenomen laten deze bevindingen zien dat er een klinische relevante rol van SR-B1 in de cholesterolhuishouding van de mens is. We laten hier ook voor de eerste keer zien dat HDL een onverwachte rol speelt bij het leveren van cholesterol aan de bijnieren voor de aanmaak van steroidhormonen. Dit laatste aspect moet meegenomen worden bij de ontwikkeling van HDL-verhogende medicijnen om HVZ terug te dringen.

Associatiestudies die het hele menselijke genoom bekijken (GWA-studies), hebben variatie in GALNT2, het gen dat codeert voor ppGalNAc-T2, gelinkt aan variatie in HDL-cholesterol en triglyceriden in de algemene bevolking. ppGalNAc-T2 is een enzym dat verantwoordelijk is voor het begin van O-linked glycosylatie van eiwitten. In hoofdstuk 5 beschrijven we een moleculair mechanisme dat de associatie tussen GALNT2 en variatie in lipidenconcentraties in plasma kan verklaren. Wij vonden dat dragers van een mutatie in GALNT2 een verhoogd HDL-cholesterol en verlaagde triglyceriden hebben, en na een orale vetbelasting een verbeterde afbraak van triglyceriden. Bovendien toonden we aan dat dragers een abnormale glycosylatie van apolipoproteïne C-III hebben. Apolipoproteïne C-III is een bekende remmer van lipoproteïne lipase (LPL), het belangrijkste enzym dat verantwoordelijk is voor de hydrolyse van triglyceriden in humaan plasma. Ex vivo en in vitro experimenten bevestigen de verminderde remming van LPL door abnormaal geglycosyleerd apo C-III. Tot slot laten we zien dat recombinant gekloneerd mutant ppGalNAc-T2 een verminderde enzymatische functie heeft. Samenvattend beschrijft deze studie I) een mechanisme waarmee een GWAS-gen het menselijk lipidenmetabolisme beïnvloedt, II) een nieuwe congenitale stoornis van de glycosylatie, III) een directe specifieke rol van 1 van de ppGalNAc-transferases (de functies van de meeste andere ppGalNAc-transferases moeten namelijk nog opgehelderd worden) en IV) dat glycosylatie van apolipoproteïne C-III van belang is voor zijn rol in de plasma-lipolyse van triglyceriden. De studie legt voor het eerst een verband tussen erfelijke lipidenstoornissen en glycosylatiestoornissen. We stellen voor
om dyslipidemie te onderzoeken in heterozygote en homozygote dragers van mutaties in andere glycosylatiegenen, om meer te leren over het lipidenmetabolisme.

Om de moleculaire pathologie van lage plasmawaarden van HDL-cholesterol beter te begrijpen, hebben we de erfelijke code van het ABCA1-gen (coderend voor het transmembraan-eiwit ABCA1 (ATP binding cassette protein A1)) geanalyseerd bij 78 patiënten die naar onze kliniek verwezen waren met zeer lage plasmawaarden van HDL-cholesterol. In hoofdstuk 6 beschrijven we de identificatie van een onverwacht groot aantal variaties in het ABCA1-gen (n=19), waarvan er 14 nog niet eerder beschreven waren. Met experimenten in vitro en confocaal-microscopie tonen we aan dat de nieuw gevonden mutaties tot functionele defecten van het ABCA1-eiwit leiden. Met dit bewijs kan het lage HDL-cholesterol bij de betreffende patiënten verklaard worden. De identificatie van zoveel nieuwe dragers van gen-defecten van ABCA1 maakt in de toekomst nieuw onderzoek mogelijk naar de omstreden rol van ABCA1 bij het ontstaan van atherosclerose, als naar de rol van ABCA1 in hematopoiese (de aanmaak van nieuwe bloedcellen en –plaattjes) en het ontstaan van leukocyrose zoals recent is beschreven bij muizen. Bovendien is er een mogelijke rol voor ABCA1 bij de aanmaak van steroidhormonen in de bijnier.

Concluderend, laten hoofdstuk 4 en 5 zien dat zeldzame mutaties die overerven in families en tot verlies van eiwitfunctie leiden, ons begrip vergroten van de functie van HDL-genen die geïdentificeerd zijn in onderzoek met muizen of genome-wide association studies. Onderzoek naar de prevalentie van zeldzame loss of function mutaties verheldert niet alleen de moleculaire pathologie van HDL-stoornissen. Het stelt ons ook in staat om, door het vinden van nieuwe mutatiedragers, het menselijke HDL-metabolisme en de relatie met atherosclerose nader te onderzoeken.

Deel 2 - LCAT en zijn klinische gevolgen

LCAT stimuleert de aanmaak van cholesterol-esters, door de hydrolyse van vetzuren op de sn-2 positie van fosfolipiden te katalyseren en deze over te dragen aan vrije cholesterolmoleculen. Di gebeurt met name op HDL-deeltjes. LCAT stimuleert hiermee de maturatie van deze deeltjes: nascente schijfvezelige deeltjes matueren tot grotere sferische deeltjes.

In een vergelijkbare exercitie als waarmee we de ABCA1-gerelateerde moleculaire pathologie van laag HDL-cholesterol onderzochten (hoofdstuk 6), beschrijft hoofdstuk 7 de frequentie en de gevolgen van mutaties in het LCAT-gen bij 98 patiënten met een laag HDL-cholesterol. 28 van deze patiënten hadden een mutatie in het LCAT-gen. We laten zien dat deze mutaties leiden tot verlies van enzymatische functie van LCAT in vitro. Samen laten hoofdstuk 6 en 7 zien dat het routinematig analyseren van de sequentie van
**Samenvatting voor niet-ingewijden**

*ABCA1* en *LCAT* bij Nederlandse patiënten die verwezen werden vanwege een laag HDL-cholesterol, ons inzicht in de pathofysiologie van verlaagd HDL-cholesterol daadwerkelijk verbetert. *LCAT* wordt beschouwd als een aangrijpingspunt voor de ontwikkeling van geneesmiddelen om atherosclerose te behandelen. De identificatie van zoveel nieuwe dragers van *LCAT*-mutaties maakt nader onderzoek naar de onopgehelderde relatie tussen *LCAT* en atherosclerose mogelijk via adequaat gepowerde imagingstudies van subklinische atherosclerose. Hiernaast zouden patiënten kunnen participeren in toekomstige klinische onderzoeken die erop gericht zijn om *LCAT* te verhogen door middel van enzym- of gentherapie (nu in preklinische ontwikkeling).

In **hoofdstuk 8** onderzochten we de relatie tussen de hoogte van de concentratie van het *LCAT*-eiwit in het bloed, het lipidenmetabolisme, en de kans op HVZ in de algemene bevolking. Gébaseerd op onderzoek bij dieren en bij patiënten met *LCAT*-deficiëntie, hypothetiseerden we dat lage bloedwaarden van *LCAT* geassocieerd zouden zijn met lage bloedwaarden van HDL-cholesterol. In deze prospectieve studie bleken *LCAT*-eiwitwaarden echter niet geassocieerd met HDL-cholesterolwaarden. Ook vonden we geen associatie van *LCAT*-waarden met het risico op HVZ. Aparte analyse van dit verband voor mannen en vrouwen toonde tegengestelde trends. Bij vrouwen waren *LCAT*-waarden positief geassocieerd met HVZ- risico, terwijl het omgekeerde op leek te gaan voor mannen. We kunnen slechts speculeren dat de discrepantie tussen deze studie in de algemene bevolking en eerder onderzoek bij patiënten met *LCAT*-deficiëntie gerelateerd is aan de mogelijkheid dat *LCAT*-bloedwaarden niet snelheidsbepalend zijn voor de vorming van HDL in de algemene bevolking (met een zeer lage prevalentie van *LCAT*-mutaties). Deze bevindingen illustreren dat kennis verkregen door studie van aangeboren metabole stoornissen (in casu *LCAT*-deficiëntie) niet zomaar naar de algemene bevolking vertaald kunnen worden, en vice versa.

In **hoofdstuk 9** presenteren we een casus van twee zussen en één broer die homozygot zijn voor een nieuwe mutatie in het *LCAT*-gen. Deze mutatie leidt tot het verlies van een zwavelbrug in het enzym. Hierdoor is het plasma van deze patiënten deficiënt voor het *LCAT*-enzym. Alledrie vertonen ze tekenen van klassieke *LCAT*-deficiëntie, een zeldzame aangeboren afwijkung van de stofwisseling: HDL-deficiëntie, vroege tekenen van hoornvliesvertroebeling, en proteinurie als uiting van nierschade, die zelfs al blijkt te bestaan op 3-jarige leeftijd. Bij de oudste patiënt werd de mate van proteinurie op een stabiele manier teruggebracht door behandeling met een ACE-remmer en een thiazidediureticum. Totdat enzymvervangingstherapie beschikbaar is, raden we deze symptomatische behandeling aan om nierinsufficiëntie te voorkomen bij patiënten met FLD. Daarnaast hebben lipidenverlagende therapie en – recent gepubliceerd – behandeling met methylprednisolon mogelijk een plek in deze symptomatische behandeling om de progressie naar eindstadium-nierziekte bij patiënten met FLD af te remmen.
In de volgende drie hoofdstukken hebben we arteriële wanddikte (hoofdstuk 10 en 11) en stijfheid (hoofdstuk 12) onderzocht bij dragers van mutatie in het LCAT-gen. **Hoofdstuk 10** beschrijft een case-control-onderzoek van patiënten die heterozygoot zijn voor een LCAT-mutatie. Door gebruik te maken van B-mode echografie, vonden we een toegenomen intima media thickness van de arteriae carotis (cIMT) als indicator van toegenomen atherosclerose bij deze patiënten. In het licht van deze bevindingen is het frappant dat Calabresi et al. een tegenovergestelde conclusie rapporteerden van een gematcht case-control-onderzoek: dragers van een LCAT-mutatie hebben een gen-dosisafhankelijke afname van cIMT in vergelijking met controle-deelnemers. Het uitgebreide cohort van dragers van een LCAT-mutatie (hoofdstuk 7) stelde ons in staat om atherosclerose bij deze dragers nader te onderzoeken, zodat we deze discrepancie kunnen oplossen: zoals uiteengezet in **hoofdstuk 11 en 12**, hebben we in het AMC een tweede studie uitgevoerd, waarbij we carriers en controle-deelnemers matchten voor leeftijd. Naast cIMT pasten we 3.0 T MRI technologie en een dynamische meting van arteriële stijfheid toe om de conditie van de arteriën bij deze patiënten te onderzoeken. Zoals uiteengezet in **hoofdstuk 11**, was wanddikte van de arteriae carotis bij dragers groter dan bij controle-deelnemers. Er was echter geen significant verschil in cIMT in dit onderzoek, wat mogelijk verklaard wordt door het frequente gebruik van statines onder dragers van een LCAT-mutatie in de laatste jaren. **Hoofdstuk 12** laat zien dat arteriële stijfheid gemeten als de polsgolfsnelheid van arteria carotis naar arteria femoralis, groter was bij dragers van een LCAT-mutatie. In beide groepen was arteriële stijfheid sterk gecorreleerd aan wanddikte van de arteriae carotis gemeten met IMT en met MRI. Samen wijzen deze drie studies op toegenomen atherosclerose bij dragers van mutaties in het LCAT-gen in Nederland. We werken op dit moment samen met prof. Calabresi aan een heranalyse van onze gecombineerde IMT-datasets om tot een verklaring te komen voor de ogenschijnlijke discrepancie tussen haar IMT-studie en onze onderzoeken.

Via het vermogen te fungeren als een fosfolipase A2, is LCAT in staat om geoxideerde acylgroepen van fosfolipiden te hydrolyseren. Het is onbekend of deze functie relevant is voor de oxidatie van lipiden bij mensen. In **hoofdstuk 13** hebben we daarom lipidenoxidatie bestudeerd bij dragers van LCAT-mutaties. We laten een afname zien in de activiteiten van de drie HDL-geassocieerde enzymes met anti-oxidatieve eigenschappen, i.e. LCAT, paraoxonase 1 en platelet-activating factor acetyl hydrolase, een toename van geoxideerde fosfolipiden op apolipoprotein B-bevattende deeltjes en een afname van het vermogen van HDL om oxidatie op LDL-deeltjes te reduceren, hoewel de algehele oxidatie van lipiden in het plasma van patiënten met LCAT-mutaties niet verschillend was ten opzicht van controle-deelnemers uit dezelfde families. Samengenomen suggereren deze bevindingen dat alternatieve mechanismen, zoals de klaring van geoxideerd HDL door CD36 en andere scavenger receptoren, mogelijk belangrijker determinanten zijn van circulerend geoxideerd LDL en lipiden-oxidatie dan deze HDL-geassocieerde anti-oxidatieve enzymen.
**Concluderend** wordt de ontwikkeling van LCAT-stimulerende of –vervangende therapieën ondersteund door zowel de toegenomen atherosclerose in dragers van LCAT-gendedefecten in het AMC, als de ernst van de renale pathologie geobserveerd bij patiënten met complete deficiëntie van LCAT (FLD). Ons moleculaire begrip van LCAT is nu zodanig dat voornamelijk klinische studies nodig zijn om de belangrijkste overgebleven vragen te beantwoorden:

- Kan het verschil tussen familial LCAT deficiency en fish eye disease – bij FLD is er door compleet verlies van LCAT naast een laag HDL-cholesterol ook een lager LDL-cholesterol in het bloed, bij FED is er alleen een verlaagd HDL-cholesterol - de tegengestelde conclusies van de Nederlandse en Italiaanse echo-onderzoeken naar atherosclerose van de halsslagaders bij dragers van LCAT-mutaties verklaren?

- Kan familial LCAT deficiency en de renale pathologie die hierdoor ontstaat adequaat behandeld worden met recombinante enzymtherapie of gentherapie?

- Zou LCAT-therapie het cardiovasculair risico van dragers kunnen verlagen en geldt dat ook voor andere patiënten? Hierbij moet worden meegewogen dat sterke overexpressie van LCAT bij muizen juist een toename van atherosclerose gaf en dat we in de algemene bevolking, waarin bloedwaarden van het LCAT-enzym geen enorme variatie vertonen, geen relatie vonden tussen LCAT en HDL-cholesterol of HVZ-risico, (hoofdstuk 8).

Deel 3 – HDL-functie: voorbij cholesterolconcentratie in plasma

Om een causale rol voor HDL bij de bescherming van hart- en vaatziekten te definiëren moeten de voorgestelde werkingseigenheden in detail onderzocht worden. Aan HDL wordt een keur aan functies toegeschreven die mogelijk vaatbeschermend zijn, maar hun relevantie bij de pathofysiologie van atherosclerose bij mensen is grotendeels onbekend.

De ontwikkeling van assays van HDL-functie die reproduceerbaar zijn en toepasbaar in grotere klinische studies (die HDL-modulerende geneesmiddelen onderzoeken) is erg moeilijk gebleken. De complexiteit van het HDL metabolisme en van de mogelijk beschermende functies van HDL worden direct weerspiegeld door de verscheidenheid aan onderwerpen in dit laatste deel van dit proefschrift.

Het wordt algemeen aangenomen dat vaatbescherming door HDL gemedieerd wordt door reverse cholesterol transport (RCT) van perifere weefsels naar cholesterolexcretie via de lever. Bij mensen is echter weinig bekend over de rol van in vivo cholesterolfluxen in dit mechanisme. In **hoofdstuk 14** hebben we daarom onderzocht of cholesterolfluxen...
in RCT anders waren bij patiënten met genetische HDL-deficiënties. We vonden dat bij dragers van mutaties in APOA1 of ABCA1 de cholesterol-efflux uit de weefsels gereduceerd is, een bevinding die onderschrijft dat HDL bij mensen betrokken is bij cholesterol efflux uit de weefsels. De resterende cholesterol-efflux en de onveranderde uitscheiding van fecale sterolen bij deze patiënten laten echter zien dat andere mechanismen dan HDL in significante mate bijdragen aan de cholesterolfluxen.

Het is gepostuleerd dat lipoproteïne-onafhankelijke uitscheiding van 27-hydroxycholesterol (27OHC) uit macrofagen mogelijk een alternatief vormt voor het HDL-gemedieerde reverse transport van een overschot aan cholesterol (RCT).14 In hoofdstuk 15 hebben we 27OHC-bloedwaarden gemeten bij mensen en muizen met monogenetische stoornissen van de HDL-stofwisseling. Vergeleken met controle-deelnemers uit dezelfde families, hadden dragers van mutaties in APOA1, ABCA1 en LCAT een verlaagd HDL-cholesterol en een verlaagd HDL-27OH, terwijl mutaties in CETP (coderend voor cholesterol ester transfer protein, CETP), SCARB1 (coderend voor SR-B1) and LIPC (coderend voor hepatic lipase) samenhangen met hogere waarden van HDL-cholesterol en 27OH. Onze bevindingen suggereren dat respectievelijk de vorming en transfer van 27OH-esters gevoeliger zijn voor verminderde activiteit van respectievelijk LCAT en CETP, dan de vorming en transfer van cholesterol-esters. Plasmawaarden van 27OH waren ook verlaagd bij APOA1-, ABCA1- of LCAT-knockout muizen, maar toegenomen bij SCARB1-knockout muizen. Samengenomen weerspreken deze bevindingen de hypothese dat 27OH-metabolisme in plasma plaatsvindt op een HDL-onafhankelijke manier.

In plasma is HDL de voornaamste drager van zowel sfingosine-1-fosfaat (S1P), een bioactief sfingolipid, als apolipoproteïne M (apo M), dat betrokken is bij de formatie van nascente HDL-deeltjes. Eerder onderzoek wees op de mogelijkheid dat Apo M S1P bindt op het HDL-deeltje. In hoofdstuk 16 testten we de hypothese dat plasma-HDL-cholesterol of plasma-apo M de plasmawaarden van S1P bepaalt. We vonden dat zowel een matige als ernstige verlaging van het HDL-cholesterol of apolipoproteïne A-I ten gevolge van een mutatie in APOA1, ABCA1 of LCAT geassocieerd is met verlaagde S1P-waarden. Aan de andere kant waren hogere waarden van HDL-cholesterol en apolipoprotein A-I ten gevolge van mutaties in CETP, SCARB1, LIPC of LIPG niet geassocieerd met veranderingen in S1P. Het is mogelijk dat in plaats van HDL in het algemeen, een subklasse van HDL of een mineur HDL-geassocieerd eiwit het voorkomen van S1P op HDL beperkt. Omdat apo M-bloedwaarden niet gecorreleerd waren aan S1P-waarden bij familie-controles, en omdat ze niet zo duidelijk verlaagd waren bij heterozygoten voor genmutaties in APOA1, ABCA1 en LCAT, of in dragers van twee defecte allelen van ABCA1 en LCAT, is deze limiterende factor waarschijnlijk niet apo M.

Hoofdstuk 17 beschrijft een nieuw mechanisme waardoor HDL mogelijk beschermt tegen cardiovasculaire sterfte inclusief plotselinge hartdood. Cardiovasculaire sterfte wordt
vaak vooraf gegaan door bepaalde hartritmestoornissen: ventriculaire tachyarrhythmieën. Verlenging van de repolarisatietaal van het hart, waarvan is aangetoond dat het plotselinge hartdood voorspelt, ligt vaak ten grondslag aan de ritmestoornissen. We hypothetiseerden dat verhoging van HDL de repolarisatie van het hart verkort. We vonden dat zowel gereconstueerde HDL (rHDL) als gezuiverd humaan apo A-I de repolarisatie van geïsoleerde konijen- enomyocyten verkort. Daarnaast verkortte rHDL-infusie het voor hartsnelheid gecorrigeerde QT-interval op electrocardiogrammen van zowel patienten met erfelijk verlaagd HDL-cholesterol als bij controle-deelnemers. Deze bevindingen tonen aan dat HDL de repolarisatie van het hart kan verkorten, een effect dat nader geëvalueerd dient te worden in toekomstig onderzoek met rHDL-infusies bij patiënten met acute coronair syndroom.

In hoofdstuk 18 hebben we een mogelijk anti-atherogene consequentie van tumor necrosis factor α (TNFα) signallng in vasculaire cellen onderzocht. TNFα wordt afgegeven door macrofagen die volgeladen zijn met cholesterol en apoptose ondergaan. Het opruimen van deze cellen door fagocyterende macrofagen helpt mogelijk bij het beperken van de ontwikkeling van atherosclerotische plaques. Opname van cholesterol door macrofagen zet ATP-binding cassette transporter A1 (ABCA1) aan tot het faciliteren van cholesterol-efflux naar apolipoproteine A-I, waardoor atherosclerose mogelijk wordt gereduceerd. We laten zien dat TNFα ABCA1-mRNA en eiwit induceren in cholesterol-geladen en in controle-macrofagen en dat het cholesterol-efflux naar apolipoprotein A-I stimuleert door intracellulaire signallng via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Om het potentieel pathofysiologisch belang van deze observaties te onderzoeken, voerden we TNFα-secernerende apoptotische macrofagen geladen met vrij cholesterol aan een gezonde macrofaag-monolaag (de fagocyten). ABCA1-mRNA en –eiwit waren significant verhoogd in de fagocyten, een respons die gemedieerd werd door zowel TNFα signallng als door activatie van de liver X receptor. In atherosclerotische plaques helpt dit proces fagocyterende macrofagen om een overschot aan lipiden, afkomstig van de ingestie van cholesterolrijke apoptotische lichaampjes, kwijt te raken.

Concluderend geeft dit derde en laatste deel van mijn proefschrift enkele antwoorden op de vele en diverse vragen met betrekking tot HDL-functie. Een belangrijke stap bij de ontwikkeling van HDL-verbeterende geneesmiddelen zou zijn om hun effect op reverse cholesterol transport te onderzoeken. De in vivo meting van cholesterol-efflux uit de weefsels beschreven in hoofdstuk 14 zou zulke evaluaties mogelijk kunnen maken. In zulke onderzoeken zou dan direct ook de invloed van HDL-modulerende geneesmiddelen op de repolarisatie van het hart geëvalueerd kunnen worden, hoewel moleculaire studies naar het precieze mechanisme dat dit effect verklart, net zo belangrijk zijn. Naast hoofdstuk 14, onderstreep ook hoofdstuk 18 het belang van HDL-gemedieerde cholesterol-efflux, maar in dit onderzoek op het niveau van de macrofaag, de voornaamste cellulaire

Concluderend perspectief

Op dit moment wordt niet langer simpelweg verwacht dat de hypothese dat HDL beschermt tegen atherosclerose bevestigd zal worden. Het optimisme is getemperd door de toegenomen sterfte in een grote farmaceutische studie met de CETP-remmer torcetrapib en diens off-target toxiciteit (het proefmedicijn liet het HDL-cholesterol stijgen bij onderzoeksdeelnemers, maar ook het aldosteron en de bloeddruk)\textsuperscript{15, 16} en door de afwezigheid van een verhoogd risico op HVZ bij dragers van een $ABCA1$-mutatie met lage bloedwaarden van HDL-cholesterol in grote prospectieve onderzoeken.\textsuperscript{17}

In de aankomende jaren zouden positieve uitkomsten van grote klinische studies met anacetraripib\textsuperscript{18} en dalcetrapib (ClinicalTrials.gov Identifier: NCT01059682), CETP-remmers die geen effect hebben op aldosteron of bloeddruk, de emoties in het onderzoeksveld weer kunnen omdraaien door de HDL-hypothese te bevestigen bij patiënten met HVZ. Niettemin zou ook hierbij meegewogen moeten worden dat de interpretatie van de relatie tussen HDL en CVD altijd gecompliceerd zal worden door de gelijktijdige LDL-cholesterol- en triglyceriden-verlagende effecten van deze medicijnen.

Dergelijke positieve uitkomsten zouden een voorbeeld zijn van hoe klinische wetenschap vooruit kan lopen op een grondig moleculair begrip van lipidenbiologie; in feite zouden we nog steeds niet meer in handen hebben dan een associatie tussen een toename van HDL-cholesterol en een afname van het risico op HVZ. We zouden nog steeds niet weten of HDL daadwerkelijk het overschot aan cholesterol van atherosclerotische lesies door de circulatie naar de lever brengt voor uitscheiding in de gal, het klassieke concept van de manier waarop HDL zou beschermen. Een echte pathofysiologische verklaring zal voort moeten komen uit de moleculaire biologie, samen met translationele studies, waardoor vervolgens het ware therapeutische potentieel van HDL duidelijk wordt.

In de onderzoeken gepresenteerd in dit proefschrift, hebben we geprobeerd de HDL-hypothese kritisch te beschouwen aan de hand van families met genetische HDL-stoornissen. Hiermee hebben we nieuwe aspecten van de HDL-stofwisseling gevonden: net als bij muizen, bleek SR-BI een fysiologisch relevante HDL-receptor. In een volgend onderzoek tonen we een moleculaire link tussen de onderzoeksvelden van lipiden- en glycosilatiestoornissen. We laten zien dat ppGalNAc-T2 de lipolyse van plasma-triglyceriden beïnvloedt door glycosylatie van apolipoproteïne C-III. Gereduceerde functie van zowel SR-BI als ppGalNAc-T2 leidt tot verhoging bloedwaarden van HDL-cholesterol.
bij patiënten die mutaties dragen in de daarvoor coderende genen. Deze eiwitten vormen daarmee potentiële aangrijpingspunten voor toekomstige therapieën. De hierboven genoemde onderzoeken laten ook zien dat families met zeldzame genetische varianten zeer nuttig zijn om de relevantie van kandidaatgenen te onderzoeken die zijn geïdentificeerd in grote associatie-onderzoeken en/of in proefdierstudies. Ten tweede herdefiniëren we LCAT, een enzym dat cruciaal is voor de maturatie van HDL, als een mogelijk therapeutisch target. In aankomende jaren zullen onderzoeken met recombinant enzym of met gentherapie duidelijk maken of het aangrijpen van LCAT om het HDL-metabolisme te moduleren gunstige effecten heeft.

Tot slot hebben we bijgedragen aan de validatie van een in vivo-meting van weefsel-cholesterol-efflux door te laten zien dat patiënten met genetisch verlaagd HDL-cholesterol verminderde efflux van cholesterol in vivo hebben. De bevinding ondersteunt het concept van HDL-gemedieerde reverse cholesterol transport bij mensen. Toekomstige translationele onderzoeken zouden erop gericht moeten zijn om een methode te ontwikkelen die macrofaag-specifieke efflux van cholesterol meet en die geschikt is om toe te passen voor onderzoek bij mensen, mogelijk hand-in-hand met de spannende ontwikkeling van HDL-gebaseerde contrastmiddelen voor magnetic resonance imaging van atherosclerotische plaques. Deze synthetische nanodeeltjes zoeken plaque-macrofagen op en zijn in staat om cholesterol-efflux te induceren. Het combineren van deze benaderingen heeft het in zich om het concept van HDL-gemedieerde RCT uit atherosclerotische plaques echt te testen. Als de ultieme maat van HDL-functie zou een dergelijke techniek het ook mogelijk maken om de effecten van verschillende HDL-modulerende stoffen te onderzoeken.

Kortom, we hebben in dit proefschrift de juistheid van de HDL-hypothese zeker niet bewezen, maar we hebben wel laten zien dat deze hypothese springlevend is.

Onno Holleboom
Amsterdam, 16 maart 2011
Reference List

Ref Type: Internet Communication


Ref Type: Abstract


Samenvatting voor niet-ingewijden


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**Characterization of antioxidant/anti-inflammatory properties and apoA-I-containing subpopulations of HDL from family subjects with monogenic low HDL disorders.**  

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**Identification and characterization of novel loss of function mutations in ATP-binding cassette transporter A1 in patients with low plasma high-density lipoprotein cholesterol.**  

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TNFalpha induces ABCA1 through NF-kappaB in macrophages and in phagocytes ingesting apoptotic cells.


Dankwoord (acknowledgements)
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De patiënten en hun familieleden

(Co-)promotoren
“If I have seen a little further than others, it is by standing upon the shoulders of giants.”
*Isaac Newton after Bernard of Chartres (12th century)*

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Studenten:

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“If a child is to keep alive his inborn sense of wonder, he needs the companionship of at least one adult who can share it, rediscovering with him the joy, excitement and mystery of the world we live in.” Rachel Louise Carson, biologist/environmentalist
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Onno
Color section
Chapter 4, Figure 1. Pedigree used for linkage analysis and the obtained haplotype information. The red haplotype, including rs3782287 which is located in intron 7 of the SCARB1 gene, is present in all individuals with the high HDL cholesterol phenotype. Two recombination events upstream of, and very close to, the SCARB1 gene (observed in IV:1 and III:5) and one recombination event downstream of SCARB1 (observed in V:2) determined the borders of a ~30MB linkage interval.
Chapter 5, Figure 3. Molecular modeling of D314A mutation in ppGalNAC-T2 (a) Ribbon diagram of the human T2 structure (PDB ID 2ffu). Alpha helices (except as noted) are shown in orange, beta strands in blue and loop regions in gray. Residue D314 (green carbons) is at the N-terminus of an alpha helix (yellow ribbon) which lies on the side of the catalytic domain opposite to that of the substrate binding face. The enzyme is shown with bound EA2 peptide (white carbons), UDP (green carbons) and a manganese ion (yellow sphere). A portion of the lectin domain is shown in the upper right of the figure. The figure was created using CCP4mg software. (Potterton et al., 2004)

Chapter 6, Supplementary Figure 1: Localization of GFP-tagged ABCA1 WT and GFP-tagged ABCA1 mutant proteins in BHK cells.
Chapter 7, Figure 4: Molecular modelling of mutated residues in LCAT. Residues S205, D369 and H401 form the catalytic triad of LCAT. T147, R159 and R182: mutated residues – p.T147I, p.R159Q and p.R182C - identified in patients of the present study and characterized in a previous model (Peelman et al., 1998). I202 and L338: residues that are mutated – p.I202T and p.L338F - in patients identified in the present study that have not been modelled before.

Chapter 9, Figure 1. Ocular pathology in the proband with familial LCAT deficiency

Peripheral corneal opacification

Slit lamp examination showing opaque dots located centrally in the cornea
Chapter 15, Figure 1 Plots of 27OHC versus cholesterol concentrations in plasma (A), HDL (B) and nonHDL (C) of individuals with mutations in HDL genes and their unaffected relatives. The correlations and regression equations have been calculated on data from all unaffected family members ("Controls" of tables 1 and 2). Please note the biases towards elevated 27OHC concentrations in plasma, HDL and nonHDL of LCAT mutation carriers as well as the biases towards higher HDL-27OHC and lower nonHDL-27OHC concentrations in the carriers of HL mutations.
Chapter 16, Figure 1: Correlations of HDL-cholesterol (A, B) and apoA-I concentrations (C, D) with concentration of apoM in total (A, C) or apoB-depleted plasma (B, D). Equations for linear regression; r & p: Pearson’s correlation coefficient, p = probability value for significance of correlation. Regression and correlation analysis performed on controls only (black symbols); patients with mutations in genes affecting HDL are indicated in the figure by coloured symbols but are not incorporated in the statistical analyses. Regression and correlation outcomes are given in blue when statistically significant (P<0.05).
Chapter 16, Figure 2: Correlations of S1P levels in apoB-depleted plasma after stratification for the median of HDL-cholesterol (1.32 mmol/L) with plasma concentrations of HDL-cholesterol (A, B), apoA-I (C, D), HDL-apoM (E, F). Equations for linear regression; $r$ & $p$: Pearson’s correlation coefficient, $p$ = probability value for significance of correlation. Regression and correlation analysis performed on controls only (black symbols); patients with mutations in genes affecting HDL are indicated in the figure by coloured symbols but are not incorporated in the statistical analyses. Regression and correlation outcomes are given in blue when statistically significant ($P<0.05$).
Chapter 16, Figure 3: Correlations of S1P levels in apoB-depleted plasma after stratification for the median of apoA-I (54.2 mmol/L = 152 mg/dL) with plasma concentrations of HDL-cholesterol (A, B), apoA-I (C, D), HDL-ApoM (E, F). Equations for linear regression; r & p: Pearson’s correlation coefficient, p = probability value for significance of correlation. Regression and correlation analysis performed on controls only (black symbols); patients with mutations in genes affecting HDL are indicated in the figure by coloured symbols but are not incorporated in the statistical analyses. Regression and correlation outcomes are given in blue when statistically significant (P<0.05).