Cardiovascular disease (CVD) is the leading cause of death in the Western world. Myocardial infarction and stroke are the result of a compromised blood flow which may result from cholesterol accumulation in the vessel wall due to high plasma levels of LDL cholesterol. High plasma levels of HDL cholesterol, however, are inversely associated with CVD. This is commonly ascribed to a concept called "reverse cholesterol transport" a mechanism by which the HDL particle takes up cholesterol from the vessel wall for subsequent clearance by the liver. Therefore, increasing HDL cholesterol may decrease atherosclerosis and reduce cardiovascular disease. In the first part of this thesis, we aimed to unravel the relationship between HDL cholesterol and atherosclerosis.

Furthermore, HDL cholesterol exerts effects beyond the vessel wall. In the second part of this thesis, we investigated the effects of cholesterol on hormone synthesis, hematopoiesis and inflammation. This advances our understanding of human physiology and aids the prediction of the effects of HDL cholesterol increasing therapies.
HDL cholesterol: atherosclerosis and beyond

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HDL cholesterol: atherosclerosis and beyond
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voor mijn ouders
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General introduction
and outline of the thesis

Based on
“Genetics of HDL-c A Causal Link to Atherosclerosis?”
Current Atherosclerosis Reports 2013;15:326

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HDL and atherosclerosis

Cardiovascular disease (CVD) is the leading cause of death worldwide. Prospective epidemiological studies have consistently shown an inverse association between high-density lipoprotein cholesterol (HDL-c) levels and the risk for CVD. Low-density lipoprotein cholesterol (LDL-c) lowering by statins is the cornerstone in CVD prevention. Even when target levels for LDL-c have been reached, HDL-c levels are still predictive of major cardiovascular events. These findings have led to the widely accepted idea that raising HDL-c is a promising target for lowering CVD risk. This resulted in a quest to gain insight in HDL metabolism and identify therapeutic targets for HDL-c increasing therapies.

The main mechanism by which HDL exerts its atheroprotective effects is considered to be reverse cholesterol transport (RCT). In this process, HDL acts as acceptor of cholesterol from peripheral tissues such as the vessel wall and transport the cholesterol back to the liver for subsequent biliary excretion.

Surprisingly, different types of HDL-c increasing therapies have failed. Despite significant increases in HDL-c levels, studies investigating nicotinic acid (AIM-HIGH, HPS-2thrive) and CETP inhibitors (Dalcetrapib, Torcetrapib) have been discontinued for futility or increased risk for mortality. This has cast doubt on the causal role of HDL-c in atherogenesis. At this moment, it is widely questioned whether the association between HDL-c and risk for atherosclerotic events represents a causal relationship, or HDL-c is merely a biomarker for CVD.

The advance of genetic analyses has provided the means to study this over the last few years. Plasma lipid and apolipoprotein levels are highly heritable, with evidence from twin studies showing 40-60% heritability of plasma HDL-c. A major part of our present understanding of HDL metabolism originates from studies in patients with monogenic HDL disorders. These family studies, however, have not provided a definite answer to the question whether low HDL-c is directly related to an increased risk of atherogenesis. This is related to the fact that the number of affected individuals is small. Furthermore, in most cases it is difficult if not impossible, to account for referral bias. In this regard, Genome Wide Association (GWA) and Mendelian randomization studies may be a better tool to evaluate the role of HDL-c in atherogenesis. The first major results generated by such studies suggested that genetically defined alterations in HDL-c levels do not hold predictive value. These results further stirred up the HDL controversy.

A reason for the discrepant data may pertain to the fact that a large proportion of the heritability is not explained by common variants. Recent GWA studies show that only 10-12% of the heritability is attributable to common variants. Even with a conservative interpretation of these estimations, a large proportion of the molecular landscape underlying HDL-c levels is still unknown. Furthermore, a large proportion of the inconsistencies between the outcomes of epidemiological, intervention and genetic studies is likely related to the fact that HDL-c levels are closely linked to metabolic and environmental factors that are also associated with CVD risk. Last, the determination of plasma HDL-c has been shown to be highly polygenic, leaving studies assuming a monogenic aetiology oversimplifications.
Part I – plasma hDL cholesterol, ABC transporters and atherosclerosis

Chapter 2 assesses the relative contributions of HDL-c and its constituent protein: apolipoprotein (Apo) A-1 to CVD risk prediction and their epidemiological consistency in a prospective cohort of 17,000 individuals.

Molecular causes of low HDL-c

ATP-binding cassette transporters

Chapter 3 provides an overview of the role of ABC transporter dependent cholesterol efflux pathways in macrophages, hematopoietic stem and progenitor cells (HSPCs) or platelet progenitors. In the chapters thereafter, we focus on one specific ABC transporter: ABCA1. ABCA1 is a key protein in the regulation of cholesterol and phospholipid transfer from peripheral tissue to apoA-1. This interaction with apoA-1 forms the initial step in reverse cholesterol transport. Several studies showed a direct relationship between atherosclerosis and cholesterol efflux potential, suggesting an association between ABCA1 function and atherosclerosis in humans.

The key role of ABCA1 in HDL metabolism was established by the discovery that homozygosity or compound heterozygosity for loss of function mutations in ABCA1 results in Tangier disease, which is characterized by near absent HDL-c and apoA-1. Heterozygous ABCA1 mutation carriers display half-normal plasma HDL-c. Cholesterol efflux assays are used to assess whether ABCA1 mutations result in impaired function of ABCA1. Functional mutations in ABCA1 result in a defective transfer of lipids onto apoA-1, which leaves apoA-1 prone to rapid clearance from the circulation and disrupts the formation of nascent HDL particles.

By measuring the intima media thickness, Van Dam and co workers showed a correlation between cholesterol efflux potential and the extent of atherosclerosis in ABCA1 mutation carriers. Although premature atherosclerosis has been reported in these patients, elderly patients without any signs of CVD have also been described, despite extremely low HDL-values.

Several studies have reported an association between ABCA1 variants and CVD risk, but this association is not consistently associated with HDL-c. Other studies report contrasting conclusions on the association between ABCA1 variants and CVD risk.

In Chapter 4, novel mutations in ABCA1 and their consequences for cholesterol efflux are reported. Chapter 5 we investigated whether carriers of ABCA1 mutations that result in impaired cholesterol efflux capacity and low HDL-c levels, exhibited more atherosclerosis than non-carriers as assessed by 3Tesla MRI of the carotid arteries, whereas chapter 6 reports whether ABCA1 mutation carriers have increased arterial stiffness as assessed by pulse wave velocity (PWV).

Regarding applicability of ABCA1 increasing therapy in CVD prevention, Liver X receptors (LXR) and micro RNA 33 (miR-33) have been investigated. ABCA1 and a member of the same superfamily ABCG1 are considered the key players for cholesterol efflux from macrophages. Liver X receptors (LXRs) and micro RNA 33 (miR-33) control important parts of this process and have recently emerged as attractive therapeutic targets from animal studies. Exciting anti-atherogenic and anti-inflammatory effects of LXR agonists have
been observed in mice, however, these results were overshadowed by a concurrent rise in triglycerides and increased incidence of hepatic steatosis. Much work is currently going on in tackling these side-effects by developing molecules with more target specificity. MicroRNA-33 (miR-33), has also been shown to decrease ABCA1 and ABCG1 gene expression. In mice and primates, miR-33 inhibition was associated with an increase in HDL-c, cholesterol efflux potential and a decrease in VLDL-associated triglycerides.

Altogether, there is ample evidence for a role for ABCA1 in atherosclerosis. However, whether ABCA1 constitutes an attractive therapeutic target in CVD prevention remains to be established.

**Apolipoprotein AI**

Apolipoprotein AI (apoA-I) is the major apolipoprotein in HDL. ApoA-I rapidly acquires cholesterol and phospholipids through the interaction with membrane bound ABCA1. This early lipidation is necessary for the production of small nascent HDL. Both gain and loss of function mutations in ApoA1 have been described. Some are reported to result in hereditary amyloidosis which in turn has been suggested to accelerate atherosclerotic plaque formation.

In humans, homozygosity for loss of function mutations in apoA-1 gives rise to near absent HDL-c, similar to Tangier disease. However, this phenotype does not per se translate into an increased risk for CVD. To date, approximately 33 patients have been described with a homozygous deletion of apoA-1, characterized by undetectable apoA-1 and extremely low HDL-c levels. Strikingly, less than half of these patients had a history of CVD, but it should be emphasized that most of these patients were under 50 years of age. Interestingly, Wada and co workers reported a 67 year old subject (ex-smoker, 20 pack years) with total absence of apoA-1 and very low HDL-c levels, who had no CVD and performed a normal exercise treadmill stress test, further questioning a role for apoA-I in atherosclerosis. However, these low-HDL-c case studies should be interpreted with great caution, since a considerable referral and publication bias might be at stake.

Numerous variants have been reported for apoA-1 of which some were associated with an increased CVD risk. In Chapter 7 we report the consequences of a rare case of combined deficiency of ABCA1 and APOA1.

Based on the characteristics of apoA-1, synthetic analogues have been developed, commonly referred to as “apoA-1 mimetic peptides”. These peptides have been shown to mediate cholesterol efflux in vivo and to exert anti-inflammatory and anti-oxidative effects.

**Molecular causes of high HDL-c**

**Apolipoprotein C3 (apoCIII)**

ApoCIII resides on both HDL and apoB-containing lipoprotein particles and influences lipid metabolism by inhibiting lipoprotein lipase (LPL) mediated lipolysis. Furthermore, apoCIII inhibits hepatic uptake of apoB-containing lipoproteins, enhances catabolism of HDL particles and monocyte adhesion to vascular endothelial cells, and activates inflammatory signalling pathways. Through these actions ApoCIII plays a crucial role in HDL-c and triglyceride metabolism. Heterozygous APOC3 mutation carriers typically display 50%
of normal apoCIII levels and lower fasting and postprandial serum triglycerides. This apparently favourable lipid profile has been shown to be associated with less coronary artery calcification and lower CVD risk. In Chapter 8, we present two novel mutations in APOC3 and the biochemical consequences of heterozygosity for these mutations.

The notion that apoC3 mutations result in an atheroprotective lipid profile has given great impetus to the development apoCIII antisense therapy and a phase II antisense intervention trial ((ISIS-APOCIII Rx. Clinical Trials.gov: NCT01529424) has been started.

Scavenger receptor class B type 1 (SR-B1)

This protein was first discovered in mice, where the Scarb1 gene encodes Scavenger receptor class B type 1 (SR-B1). The presumed atheroprotective effects of SR-B1 are attributed to the ability to promote cholesterol efflux from tissues as well as the selective uptake of cholesteryl esters from HDL via liver cells expressing SR-B1.

For a long time, evidence supporting a role for SR-B1 in human lipoprotein metabolism was largely absent. However, recently Vergeer and co workers reported a functional mutation in SR-B1 in humans that was associated with significantly higher HDL-c levels and a reduced cholesterol efflux capacity in macrophages. No difference in carotid artery intima-media thickness or number of cardiovascular events between carriers and non-carriers was found, however, the study sample was small and likely underpowered.

In Chapter 9, two additional SR-B1 variants, associated with high HDL-c values are reported. Detailed data on these mutations show an impaired ability to bind HDL and a diminished selective uptake of cholesteryl esters.

Taken together, evidence for a role of SR-B1 in humans has recently emerged. High HDL-c levels in knockout rodent studies are linked to a striking increase in atherosclerosis. Furthermore, the newly discovered SR-B1 variants in humans, associated with high HDL-c, are thought to be related to a decrease in cholesterol efflux potential. Evidence supporting a direct link with atherosclerosis in humans is lacking, and a beneficial effect of raising HDL-c by means of SR-B1 inhibition does not seem likely.

Cholesterylester Transport Protein (CETP)

CETP is secreted primarily by liver and adipose tissue into the circulation. The protein transfers cholesteryl esters from HDL particles to VLDL particles and chylomicrons in exchange for triglycerides. Approximately ten mutations have been discovered in the CETP gene, mainly in the Japanese population, where CETP-variants are found in 50% of individuals with high HDL-c. In homozygous carriers of a splicing defect in the CETP gene, HDL-c levels were reported to be exceedingly high while LDL-c levels were at the lower end of the normal distribution.

Although early reports on the correlation between CETP variants and CVD risk have been conflicting, more recent reports support a beneficial association with cardiovascular endpoints in large studies.

Therefore, expectations for the novel group of CETP inhibitors were high. However, the ILLUMINATE trial, investigating the CETP inhibitor torcetrapib, was terminated prematurely due to an increase in cardiovascular event rate, which was attributed to
off-target toxicity. More recently, the trial on another CETP inhibitor, dalcetrapib, was terminated prematurely for futility. These two events have stirred the controversy on the benefit of CETP inhibitors and HDL-c raising therapies in general.

Having witnessed a series of conflicting reports on the applicability of HDL-c increasing agents in CVD prevention, chapter 10 provides an overview of the therapeutic efficacy and possibilities of apoA-I mimetic peptides.

Chapter 11 subsequently focuses on the developments around CETP inhibitors and addresses the expectations for the future.

Part II - HDL cholesterol beyond atherosclerosis
Reverse cholesterol transport has been mostly used to explain how HDL may provide atheroprotection. However, anti-inflammatory, anti-thrombotic, anti-oxidant and anti-microbial functions have also been ascribed to HDL. Furthermore, HDL has been implicated in steroidogenesis. Here, we provide an introduction to the role of HDL-c in adrenal steroidogenesis, hematopoiesis and inflammation, subject of Part III of this thesis.

HDL cholesterol and adrenal steroidogenesis
Adrenal steroidogenesis is pivotal for survival in humans. Cholesterol constitutes the substrate for steroid hormone synthesis. Although the adrenal gland is equipped with multiple pathways to secure a continuous cholesterol supply, three-quarters of all cholesterol needed for steroidogenesis is derived from plasma lipoproteins.

LDL and HDL can be taken up from the blood stream into cells via the LDL receptor (LDL) or the SR-B1 receptor (HDL). Both receptors are expressed on adrenal cells. Whereas a role for LDL derived cholesterol in human adrenal steroidogenesis has been described in small cohorts, the role of HDL derived cholesterol in adrenal steroidogenesis is sparsely investigated.

In mice, Hoekstra and co workers reported impaired adrenal stress response in mice lacking SR-B1 compared to control mice, lending support to a role for HDL-c as cholesterol donor in vivo. In humans, we recently showed that adrenal function was compromised in patients with a functional mutation in the gene encoding SR-B1. Collectively, these findings strongly suggest that cholesterol delivery to the adrenal via the HDL-c - SR-B1 pathway is pivotal for adrenal steroidogenesis.

In Chapter 12, adrenal function is investigated in Lcat knockout mice, characterized by very low HDL-c. LCAT is a plasma enzyme that esterifies cholesterol on lipoprotein particles. Human homozygous LCAT mutation carriers display near absent HDL cholesterol levels, whereas heterozygous carriers typically display half-normal levels of HDL-c.

Chapter 13 subsequently assesses the influence of plasma HDL-c levels on adrenal steroidogenesis in male carriers of ABCA1 or LCAT mutations and male subjects with low HDL-c without underlying genetic defect. In Chapter 14, adrenal steroidogenesis in female ABCA1 and LCAT mutation carriers is investigated.

Chapter 15 addresses the importance of the other major lipoprotein for adrenal steroidogenesis: LDL. We for the first time assessed adrenal function in a large cohort of
patients with mutations in the LDL receptor and in \textit{APOB}, resulting in impaired uptake of LDL derived cholesterol by the adrenal gland.

**Cholesterol efflux and hematopoiesis**

The role of inflammation in atherosclerosis is an emerging field. Inflammatory cells, in particular monocytes regarded as prominent players in atherosclerosis,\textsuperscript{83-85} Mice with a knockout of \textit{Abca1} and \textit{Abcg1} in cells of hematopoietic origin displayed a marked expansion of hematopoietic stem and progenitor cells (HSPCs), monocytosis, neutrophilia and systemic foam cell and myeloid cell infiltration of various organs, contributing to atherogenesis.\textsuperscript{32,86}

In chapter 16, the hypothesis is tested that in the presence of hypercholesterolemia, ApoA-1 and HDL would act to reduce HSPC proliferation and monocytosis. This was investigated on the basis of \textit{Ldlr}^{−/−}/\textit{Apoa1}^{+/−} mice and monocyte counts in children with a defective LDL receptor.

**HDL and inflammation**

The intriguing paradigm that atherosclerosis is an inflammatory process, initiated by the deposition of cholesterol in the arterial wall, has gained momentum in recent years and is now widely acknowledged.\textsuperscript{87} Whereas a link between ABC transporters and inflammation is reported in murine models,\textsuperscript{32,85,88} the mechanisms linking cholesterol disturbances to increased inflammation in humans are not well understood. Recent studies have reported a role for defective cholesterol efflux pathways in increased inflammation monocytes and macrophages, as well as in increased production of inflammatory cells such as monocytes and neutrophils.\textsuperscript{85,86} Deficiency of \textit{Abca1} and/or \textit{Abcg1} is associated with a pro-inflammatory phenotype in mouse peritoneal macrophages as well as in the macrophages of atherosclerotic plaques.\textsuperscript{88-90}

In Chapter 17, it is for the first time assessed whether human \textit{ABCA1} mutation carriers are characterized by increased inflammation.
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General introduction and outline of the thesis
PART I
PLASMA HDL CHOLESTEROL,
ABC TRANSPORTERS
AND ATHEROSCLEROSIS
The Association of High Density Lipoprotein -Cholesterol versus Apolipoprotein A-I with Risk of Coronary Heart Disease
The EPIC-Norfolk Prospective Population Study

Submitted for publication

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Abstract

Background
Plasma high-density lipoprotein cholesterol (HDL-c) and apolipoprotein A-I (apoA-I) levels are both strongly and inversely associated with coronary heart disease (CHD) risk. However, the contribution of apoA-I to risk stratification, over and above HDL-c, is unclear. In light of recent negative outcome data of Mendelian randomization approaches and HDL-c raising event studies, this issue is at the forefront of the current debate surrounding the HDL-c hypothesis. We studied the associations between plasma levels of HDL-c and apoA-I either alone or combined with risk of CHD events and cardiovascular risk factors among apparently healthy men and women.

Methods
HDL-c and apoA-I levels were measured among 17,661 participants of the EPIC-Norfolk prospective population study. Hazard ratios for CHD events and distributions of risk factors were calculated by quartiles of HDL-c and apoA-I.

Findings
Both HDL-c and apoA-I quartiles were strongly and inversely associated with CHD risk. Risk factors including age, male sex, body mass index, HbA1c, non-HDL-c, triglycerides, apolipoprotein B, systolic blood pressure and C-reactive protein were lower with ascending HDL-c quartiles (p<0.001). Also, within each apoA-I quartile, higher HDL-c levels were associated with lower CHD risk (p<0.001). In contrast, the above-mentioned risk factors were unexpectedly higher with ascending apoA-I levels (p<0.001) and CHD risk was higher with ascending apoA-I levels in the highest HDL-c quartiles.

Interpretation
Whereas both HDL-c and apoA-I quartiles per se were inversely associated with CHD risk, our findings demonstrate that apoA-I levels do not offer predictive information over and above HDL-c. In fact, within the highest HDL-c quartiles, higher apoA-I levels associate with a higher prevalence of CHD risk, possibly due to the higher prevalence of cardiovascular risk factors in association with higher apoA-I levels.
Introduction
Prospective epidemiological studies have consistently shown that plasma levels of high-density lipoprotein cholesterol (HDL-c) are inversely associated with coronary heart disease (CHD) risk. However, the biological foundation of this association is controversial. First, Mendelian randomization studies have shown us that single nucleotide polymorphisms (SNPs) in loci only affecting plasma HDL-c levels do not associate with CHD risk. Second, the biological mechanism underlying the supposed antiatherogenic properties of HDL-c is widely considered to be “reverse cholesterol transport”, a process by which HDL transports phospholipids and free cholesterol from peripheral cells towards the liver for subsequent biliary secretion. Despite decades of research, this pathway has not been unequivocally demonstrated in humans. Third, the inverse association between HDL-c and CHD risk has drawn attention to HDL-c as a pharmacological target to reduce CHD risk. In spite of this, there is currently no evidence in man that increasing HDL-c leads to CHD event reduction.

Combined, these findings give rise to the notion that HDL-c itself might not play a causative role in protection against atherogenesis and emphasize the possibility that a parameter closely related to HDL-c might constitute a better predictor of CHD risk. By virtue of its superior reflection of the underlying biological effect, this marker might replace HDL-c as a target for pharmaceutical intervention. Apolipoprotein A-I (apoA-I), the major constituent of HDL particles, might be such a parameter. Similar associations with CHD risk have been reported for apoA-I as for HDL-c, and antioxidant, anti-inflammatory, antithrombotic and nitric oxide promoting properties have been ascribed to apoA-I.

Prospective studies focusing on the comparison of the associations of apoA-I and HDL-c with risk of future CHD events, however, have shown conflicting results. Whether the assessment of apoA-I in CHD risk prediction has additional value compared to HDL-c, has not been elucidated so far.

A similar issue has been addressed for LDL-c and apolipoprotein B (apoB). In statin treated patients, apoB levels were shown to have superior predictive value compared to LDL-c levels. LDL and HDL, however, differ greatly with respect to the number of apolipoproteins per particle. Each LDL particle contains one apoB molecule, and consequently, the ration LDL-c/apoB is merely a reflection of the number and composition of the LDL particles. A low-normal LDL-c level, combined with high apoB levels, for example, has been shown to reflect a relative abundance of small-dense LDL particles, which have been shown to be pro-atherogenic. The opposite (high LDL-c and low apoB levels) reflect large buoyant LDL particles, which have been shown to exert less pro-atherogenic properties.

In contrast, apoA-I levels are not synonymous with HDL particle numbers since large HDL contains more apoA-I molecules than their small counterparts. As a consequence HDL-c/apoA-I can not be used as a proxy for particle size or composition.

It is unclear to which extent HDL-c and apoA-I levels are correlated. Current therapeutic strategies focus on increasing HDL-c and/or apoA-I levels, but it is not known which composition of HDL particles holds the largest predictive value. It is therefore important to establish whether apoA-I levels per se add to risk prediction over and above
levels of HDL-c. Furthermore, since therapeutic strategies to upregulate apoA-I in the liver are currently under investigation, this issue has significant clinical relevance for novel therapies that aim to reduce CHD risk.

It was our objective to study the associations between plasma levels of HDL-c and apoA-I either alone or combined, with risk of coronary heart disease (CHD) events and cardiovascular risk factors among apparently healthy men and women. We pursued this objective in the European Prospective Investigation into Cancer (EPIC) Norfolk cohort, a large prospective population study based in the United Kingdom.

Methods

Study design

The EPIC-Norfolk study is a prospective population study of 25,639 male and female residents of Norfolk, United Kingdom, aged between 39 and 79 years. The recruitment process, study design, and population characteristics have been published previously. The study was approved by the Norfolk Local Research Ethics Committee and complies with the declaration of Helsinki. All participants gave written informed consent.

All individuals have been flagged for mortality at the UK Office of National Statistics, with vital status ascertained for the entire cohort. Death certificates were coded by trained nosologists according to the International Classification of Diseases 10th revision (ICD-10). In addition, hospitalized participants were identified by using their unique National Health Service number through data linkage with the East Norfolk Health Authority (ENCORE) database, which identifies all hospital contacts throughout England and Wales for residents of Norfolk. Participants were identified as having developed CHD during follow-up if they had a hospital admission and/or died with CHD as the underlying cause during follow-up. CHD was defined as ICD-10 codes I20-I25 (which includes myocardial infarction, angina, and other ischemic heart disease). We report results with follow-up to March 31st 2008, after a mean of 12.7 ± 2.3 years. Previous validation studies in this cohort indicate a high specificity of case ascertainment.

Biochemical analyses

Blood samples were drawn at the baseline visit in either fasting or nonfasting state. Samples were processed for assay at the Department of Clinical Biochemistry, University of Cambridge, or stored at −80°C. In the entire population study, serum lipids were analysed for total cholesterol, HDL-c and triglyceride on a RA-1000 analyser (Bayer Diagnostics, Basingstoke, UK). HDL cholesterol levels were measured after precipitation of non-HDL particles with DSBmT and peroxidase. Low-density lipoprotein cholesterol (LDL-c) levels were calculated with the Friedewald formula. Serum apoA-I and apolipoprotein B (apoB) levels were measured using rate immunonephelometry (Behring Nephelometer BNII, Marburg, Germany) with calibration traceable to the International Federation of Clinical Chemistry primary standards. Researchers and laboratory personnel had no access to identifiable information and could identify samples by number only.
Statistical analysis

We evaluated the distribution of cardiovascular risk factors across quartiles of HDL-c and apoA-I. Metabolic syndrome was defined as previously described. C-reactive protein (CRP) and triglycerides (TG) plasma levels showed a skewed distribution and were log-transformed prior to analysis. P-values for trend across quartiles of HDL-c and apoA-I were assessed in an unadjusted model using the Jonckheere-Terpstra test. Cox proportional hazards models were used to calculate hazard ratios and corresponding 95% confidence intervals (95%CI) for CHD risk by quartiles of HDL-c and apoA-I, using the lowest quartile as a reference and event free survival per quartile was depicted in Kaplan-Meier curves. Unadjusted regression models were used (model 1), as well as regression models adjusting for sex, age, smoking, body mass index, systolic blood pressure, diabetes mellitus, apoB, log-transformed CRP (model 2) and log-transformed TG (model 3). In addition, we calculated hazard ratios and corresponding 95%CIs for combined quartiles of HDL-c and apoA-I. Linear regression analysis was used to assess correlations between HDL-c and apoA-I per HDL-c/apoA-I quartile. Analyses were undertaken using SPSS (version 18.0).

Results

A complete dataset was available for a total number of 17 661 individuals. This subset did not differ in any of the relevant baseline characteristics from the subset for whom data were missing. A total of 2226 (12.6%) participants experienced a CHD event during follow-up. Baseline characteristics by quartiles of HDL-c and apoA-I are shown in tables 1 and 2, respectively.

Risk of coronary heart disease across quartiles of HDL cholesterol and apolipoprotein A-I

Hazard ratios for risk of CHD by HDL-c and apoA-I quartiles and CHD risk are shown in table 3 and figure 1. Among individuals in the top HDL-c quartile the hazard ratio for CHD events was 0.34 (95% CI 0.30-0.39) compared to those in the bottom HDL-c quartile (p for linear trend <0.001). In a fully adjusted model, the hazard ratio for CHD was 0.69 (95% CI 0.59-0.80) for those in the top versus bottom quartile (p for linear trend <0.001).

Individuals in the top quartile for apoA-I had a hazard ratio for CHD events of 0.55 (95% CI 0.49-0.62, p for linear trend <0.001) using an unadjusted model. In a fully adjusted model the hazard ratio was 0.75 (95% CI 0.66-0.86, p for linear trend <0.001).

Table 4 shows the distribution of participants, CHD event rates, and hazard ratio and corresponding 95% CI across HDL-c and apoA-I quartiles. In the majority of study participants (n=9478, 54%), HDL-c quartiles corresponded with apoA-I quartiles. However, for 8183 participants, the HDL-c quartile and apoA-I quartile were discordant.

Compared to study participants in the bottom quartiles for both HDL-c and apoA-I, those in the second, third and top quartiles for both HDL-c and apoA-I had a HR of 0.69 (95% CI 0.59-0.81), HR 0.69 (95% CI 0.59-0.82), and HR 0.57 (95% CI 0.48-0.67), respectively. Those in the lowest HDL-c quartile and the top apoA-I quartile were not at lower CHD risk.
By contrast, those in the top HDL-c quartile but the bottom apoA-I quartile were at lower CHD risk (HR 0.47 95% CI 0.28-0.78), and this hazard ratio was even lower than the participants having both high HDL-c and apoA-I levels (HR 0.57 95% CI 0.48-0.67). Within each apoA-I quartile, higher HDL-c levels were associated with lower CHD risk. By contrast, within each HDL-c quartile, we did not find a consistent association between apoA-I and CHD risk. Interestingly, in the top two HDL-c quartiles, higher apoA-I levels were not associated with lower CHD risk, but rather a higher CHD risk.

HDL-c and apoA-I levels were positively correlated in all 16 subgroups (supplementary table 1).

### Table 1. Baseline characteristics by HDL cholesterol quartiles

<table>
<thead>
<tr>
<th>HDL cholesterol quartiles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5031</td>
<td>3595</td>
<td>4444</td>
<td>4591</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.0±0.1</td>
<td>1.3±0.1</td>
<td>1.5±0.1</td>
<td>2.0±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age, years</td>
<td>59.6±9.1</td>
<td>59.3±9.1</td>
<td>59±9.1</td>
<td>58.8±9.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex</td>
<td>71% (3572)</td>
<td>51% (1834)</td>
<td>35% (1555)</td>
<td>19% (872)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.3±3.7</td>
<td>26.5±3.67</td>
<td>25.9±3.8</td>
<td>24.9±3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus % (n)</td>
<td>3.0% (149)</td>
<td>2.1% (75)</td>
<td>1.8% (79)</td>
<td>1.2% (55)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.4±1.0</td>
<td>5.3±0.8</td>
<td>5.3±0.8</td>
<td>5.2±0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>136±18</td>
<td>136±18</td>
<td>134±18</td>
<td>133±19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>83±11</td>
<td>83±11</td>
<td>82±11</td>
<td>81±11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.0±1.2</td>
<td>6.2±1.1</td>
<td>6.2±1.1</td>
<td>6.3±1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>4.0±1.0</td>
<td>4.1±1.0</td>
<td>4.0±1.0</td>
<td>3.7±1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-HDL cholesterol, mmol/L</td>
<td>5.0±1.2</td>
<td>4.9±1.1</td>
<td>4.7±1.1</td>
<td>4.2±1.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.0 (1.5-2.7)</td>
<td>1.7 (1.2-2.2)</td>
<td>1.4 (1.0-1.9)</td>
<td>1.1 (0.8-1.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apolipoprotein A-I, mg/dL</td>
<td>129±22</td>
<td>148±23</td>
<td>161±26</td>
<td>184±30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apolipoprotein B, mg/dL</td>
<td>100±25</td>
<td>99±24</td>
<td>96±24</td>
<td>92±23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>2.0 (0.9-4.3)</td>
<td>1.6 (0.8-3.6)</td>
<td>1.6 (0.8-3.5)</td>
<td>1.3 (0.6-2.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>83% (4176)</td>
<td>70% (2517)</td>
<td>63% (2800)</td>
<td>56% (2571)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcohol intake, g/day</td>
<td>3.1 (0.8-9.7)</td>
<td>3.4 (0.8-10.5)</td>
<td>4.7 (0.8-10.9)</td>
<td>5.2 (0.9-11.8)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are shown as mean ± standard deviation, percentage (number), or median (interquartile range). P for Jonckheere Terpstra test across categories. HDL indicates high-density lipoprotein; LDL indicates low-density lipoprotein.

(HR 0.87 95% CI 0.54-1.40). By contrast, those in the top HDL-c quartile but the bottom apoA-I quartile were at lower CHD risk (HR 0.47 95% CI 0.28-0.78), and this hazard ratio was even lower than the participants having both high HDL-c and apoA-I levels (HR 0.57 95% CI 0.48-0.67). Within each apoA-I quartile, higher HDL-c levels were associated with lower CHD risk. By contrast, within each HDL-c quartile, we did not find a consistent association between apoA-I and CHD risk. Interestingly, in the top two HDL-c quartiles, higher apoA-I levels were not associated with lower CHD risk, but rather a higher CHD risk.

HDL-c and apoA-I levels were positively correlated in all 16 subgroups (supplementary table 1).

### Distribution of risk factors across quartiles of HDL cholesterol and apolipoprotein A-I

The distribution of risk factors across HDL-c and apoA-I quartiles is presented in table 5. There were significant trends across the majority of the HDL-c and apoA-I quartiles for
age, percentage males, body mass index, HbA1c, non-HDL-c, triglycerides, apoB and systolic blood pressure and CRP. The characteristics of the individuals at the extremes of the distribution were fundamentally different; the group defined by high HDL-c and low apoA-I levels consisted of relatively healthy subjects compared to the group defined by low HDL-c and high apoA-I levels, where an enrichment of traditional risk factors was observed.

**Discussion**

In this study, we present data on the associations between plasma levels of HDL-c and apoA-I, cardiovascular risk factors and risk of CHD in the EPIC-Norfolk cohort. As expected, both HDL-c and apoA-I were strongly and inversely associated with the risk of future CHD. It is noteworthy, however, that these associations were not readily interchangeable. We found that within each apoA-I quartile, higher plasma HDL-c levels were consistently associated with lower CHD risk. There was, however, no consistent

<table>
<thead>
<tr>
<th>Table 2. Baseline characteristics by apolipoprotein A-I quartiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein A-I quartiles</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
</tr>
<tr>
<td>Age, years</td>
</tr>
<tr>
<td>Male sex</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
</tr>
<tr>
<td>Diabetes mellitus % (n)</td>
</tr>
<tr>
<td>HbA1c, %</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
</tr>
<tr>
<td>Non-HDL cholesterol, mmol/L</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
</tr>
<tr>
<td>Apolipoprotein A-I, mg/dL</td>
</tr>
<tr>
<td>Apolipoprotein B, mg/dL</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
</tr>
<tr>
<td>Alcohol intake, g/day</td>
</tr>
</tbody>
</table>

Data are shown as mean ± standard deviation, percentage (number), or median (interquartile range). P for Jonckheere Terpstra trend test across categories. HDL indicates high-density lipoprotein; LDL indicates low-density lipoprotein.
reverse association between apoA-I quartiles and CHD risk within each HDL-c quartile. In fact, in the two highest HDL-c quartiles, apoA-I levels were, unexpectedly, positively associated with CHD risk and the lowest CHD risk was observed in subjects with HDL-c levels in the highest and apoA-I levels in the lowest quartile.

A number of studies have shown that HDL-c and apoA-I levels are inversely associated with CHD risk. Results with regard to the relative contribution of the parameters to CHD risk, however, have not been published, possibly concealing the superiority of one predictor over the other. In most subjects, HDL-c and apoA-I levels are closely correlated, as exemplified by the finding that HDL-c and apoA-I quartiles were concordant in more than half of the participants studied. This concordance might explain the findings in previous studies that HDL-c and apoA-I have a similar association with risk of cardiovascular events. However, in the higher HDL-c quartiles, the association between apoA-I quartiles and CHD risk was positive rather than inverse. This positive association between CHD risk and apoA-I quartiles in the highest HDL-c quartiles has not been reported before. It contradicts the results from the INTERHEART, in which the association between apoA-I...

<table>
<thead>
<tr>
<th>HDL cholesterol quartiles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1.1 mmol/L</td>
<td>963/5031</td>
<td>479/3595</td>
<td>465/4444</td>
<td>319/4591</td>
<td></td>
</tr>
<tr>
<td>1.1-1.4 mmol/L</td>
<td>1.00</td>
<td>0.67 (0.60-0.74)</td>
<td>0.52 (0.47-0.58)</td>
<td>0.34 (0.30-0.39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1.4-1.7 mmol/L</td>
<td>1.00</td>
<td>0.81 (0.73-0.91)</td>
<td>0.77 (0.69-0.87)</td>
<td>0.61 (0.53-0.71)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥ 1.7-5.9 mmol/L</td>
<td>1.00</td>
<td>0.85 (0.76-0.95)</td>
<td>0.83 (0.74-0.94)</td>
<td>0.69 (0.59-0.80)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3b. Risk of coronary heart disease events by apolipoprotein A-I quartiles

<table>
<thead>
<tr>
<th>Apolipoprotein A-I quartiles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 135 mg/dL</td>
<td>701/4325</td>
<td>624/4514</td>
<td>494/4403</td>
<td>407/4419</td>
<td></td>
</tr>
<tr>
<td>135-154 mg/dL</td>
<td>1.00</td>
<td>0.81 (0.73-0.90)</td>
<td>0.66 (0.59-0.74)</td>
<td>0.55 (0.49-0.62)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>154-177 mg/dL</td>
<td>1.00</td>
<td>0.77 (0.69-0.86)</td>
<td>0.74 (0.65-0.83)</td>
<td>0.69 (0.61-0.79)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≥ 177 mg/dL</td>
<td>1.00</td>
<td>0.79 (0.71-0.89)</td>
<td>0.78 (0.69-0.88)</td>
<td>0.75 (0.66-0.86)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are shown as hazard ratios and corresponding 95% confidence intervals for the risk of future coronary heart disease events. Hazard ratios were calculated by quartile, using the lowest quartile as reference category. Model 1 indicates an unadjusted regression model. Model 2 is adjusted for sex, age, smoking, body mass index, systolic blood pressure, apolipoprotein B and C-reactive protein. Model 3 is adjusted for the variables in model 2 and in addition for triglycerides.
and CHD risk was stronger than for HDL-c. However, the INTERHEART study was not a prospective study and did not assess hazard ratios for CHD risk by quartiles of apoA-I and HDL-c. The Emerging Risk Factors Collaboration (ERFC) reported similar predictive value of apoA-I and HDL-c levels. This is probably due to the inextricable relationship between HDL-c and apoA-I which prevents apoA-I from overcoming the very strong inverse association between HDL-c levels and cardiovascular risk in large cohort studies.

Our findings are in contrast with a previous report on the EPIC Norfolk population study, in which apoA-I has been shown to be associated with increased risk of major

Table 4. Risk of coronary heart disease events by HDL cholesterol and apolipoprotein A-I quartiles

<table>
<thead>
<tr>
<th>HDL cholesterol quartiles</th>
<th>ApoA-I quartiles</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>572/2923</td>
<td>77/615</td>
<td>36/476</td>
<td>16/302</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>0.86 (0.67-1.09)</td>
<td>0.61 (0.43-0.85)</td>
<td>0.47 (0.28-0.78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>301/1605</td>
<td>214/1658</td>
<td>97/978</td>
<td>12/273</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.91 (0.79-1.05)</td>
<td>0.69 (0.59-0.81)</td>
<td>0.65 (0.52-0.81)</td>
<td>0.35 (0.19-0.63)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>72/397</td>
<td>143/1039</td>
<td>199/1924</td>
<td>80/1043</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.88 (0.68-1.12)</td>
<td>0.75 (0.63-0.91)</td>
<td>0.69 (0.59-0.82)</td>
<td>0.59 (0.46-0.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>18/97</td>
<td>45/283</td>
<td>133/1066</td>
<td>211/2973</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.87 (0.54-1.40)</td>
<td>0.93 (0.68-1.26)</td>
<td>0.81 (0.67-0.99)</td>
<td>0.57 (0.48-0.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.41</td>
<td>0.18</td>
<td>0.005</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as number of coronary heart disease events / total number of study participants and hazard ratios and corresponding 95% confidence intervals. Hazard ratios were calculated using those in the bottom quartiles for both HDL cholesterol and apolipoprotein A-I as reference category. HDL indicates high-density lipoprotein; ApoA-I indicates apolipoprotein A-I.
Table 5. Baseline characteristics by HDL-c and apoA-I quartiles

<table>
<thead>
<tr>
<th>ApoA-I quartiles</th>
<th>HDL cholesterol quartiles</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Apolipoprotein A-I, mg/dl</td>
<td>1</td>
<td>116±17 (2909)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>143±5 (1605)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>163±6 (397)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>189±14 (97)</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1</td>
<td>0.93±1.13 (2932)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.03±0.09 (1605)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.04±0.09 (397)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.03±0.10 (97)</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ApoA-I/HDL-c ratio</td>
<td>1</td>
<td>1.26±0.25 (2932)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.40±0.14 (1605)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.58±0.16 (397)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.86±0.34 (97)</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age, years</td>
<td>1</td>
<td>59.1±9.1 (2932)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>60±9.0 (1605)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>60.7±9.0 (397)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>61.1±8.6 (97)</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex</td>
<td>1</td>
<td>75.5 (2214/2932)</td>
</tr>
<tr>
<td>2</td>
<td>67.5 (1083/1605)</td>
<td>57.0 (945/1658)</td>
</tr>
<tr>
<td>3</td>
<td>52.9 (210/397)</td>
<td>44.7 (464/1039)</td>
</tr>
<tr>
<td>4</td>
<td>46.4 (45/97)</td>
<td>40.6 (115/283)</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

| Body mass index, kg/m² | 1   | 27.1±3.6 (2926) | 26.2±3.7 (614) | 25.4±3.7 (475) | 24.7±3.5 (302) | <0.001 |
| 2       | 27.3±3.7 (1600) | 26.2±3.4 (1657) | 25.5±3.5 (977) | 25.1±4.0 (273) | <0.001 |
| 3       | 28±4.2 (396)    | 27.1±3.9 (1039) | 26±3.8 (1923)  | 24.7±3.3 (1042) | <0.001 |
| 4       | 28.3±3.8 (97)   | 27.3±3.9 (283)  | 26.5±3.8 (1064) | 24.9±3.5 (2967) | <0.001 |
| p-value | <0.001 | <0.001 | <0.001 | 0.28 |

| HbA1c, % | 1   | 5.4±0.9 (1059) | 5.2±0.7 (318) | 5.3±0.8 (341) | 5.2±0.8 (228) | 0.001 |
| 2       | 5.4±1.0 (615)  | 5.3±0.8 (476) | 5.3±1.0 (238) | 5.2±0.8 (140) | <0.001 |
| 3       | 5.7±1.2 (207)  | 5.3±0.8 (479) | 5.3±0.8 (737) | 5.2±0.7 (304) | <0.001 |
| 4       | 6±1.5 (54)     | 5.6±0.7 (166) | 5.3±0.7 (574) | 5.3±0.7 (1499) | <0.001 |
| p-value | 0.001 | <0.001 | 0.34 | 0.01 |

| Non-HDL cholesterol, mmol/L | 1   | 4.9±1.1 (2932) | 4.6±1.1 (615) | 4.5±1.2 (476) | 4±1.0 (302) | <0.001 |
| 2       | 5.2±1.1 (1605) | 4.9±1.1 (1658) | 4.4±1.1 (978) | 4.1±1.1 (273) | <0.001 |
| 3       | 5.3±1.2 (397)  | 5.1±1.1 (1039) | 4.7±1.1 (1924) | 4.3±1.2 (1043) | <0.001 |
| 4       | 5.6±1.2 (97)   | 5.2±1.1 (283)  | 5±1.1 (1066)  | 4.3±1.1 (2973) | <0.001 |
| p-value | <0.001 | <0.001 | <0.001 | <0.001 |
Table 5. Continued

<table>
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<th>ApoA-I quartiles</th>
<th>HDL cholesterol quartiles</th>
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<th>3</th>
<th>4</th>
<th>p-value</th>
</tr>
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<tbody>
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<td>Triglycerides, mmol/L</td>
<td>1</td>
<td>2.0 (1.4-2.7) (2932)</td>
<td>1.4 (1.0-1.9) (615)</td>
<td>1.3 (1.0-1.8) (476)</td>
<td>1.0 (0.8-1.4) (302)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.1 (1.5-2.7) (1605)</td>
<td>1.6 (1.1-2.1) (1658)</td>
<td>1.2 (0.9-1.6) (978)</td>
<td>1.0 (0.8-1.4) (273)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.3 (1.7-2.9) (397)</td>
<td>1.9 (1.4-2.4) (1039)</td>
<td>1.4 (1.0-1.8) (1924)</td>
<td>1.1 (0.8-1.4) (1043)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.4 (1.7-3.1) (97)</td>
<td>2.1 (1.5-2.7) (283)</td>
<td>1.6 (1.2-2.2) (1066)</td>
<td>1.1 (0.9-1.5) (2973)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein B, mg/dl</td>
<td>1</td>
<td>96±25 (2871)</td>
<td>79±23 (577)</td>
<td>66±19 (415)</td>
<td>59±16 (241)</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td>2</td>
<td>106±23 (1601)</td>
<td>101±22 (1653)</td>
<td>94±21 (975)</td>
<td>76±21 (271)</td>
<td>&lt;0.001</td>
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<tr>
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<td>3</td>
<td>109±22 (394)</td>
<td>105±23 (1035)</td>
<td>99±22 (1915)</td>
<td>92±22 (1038)</td>
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<tr>
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<td>4</td>
<td>116±23 (95)</td>
<td>109±23 (282)</td>
<td>105±22 (1059)</td>
<td>96±22 (2958)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p-value</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>1</td>
<td>135±18 (2925)</td>
<td>132±17 (613)</td>
<td>131±17 (476)</td>
<td>131±18 (302)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>138±18 (1601)</td>
<td>136±18 (1658)</td>
<td>132±18 (975)</td>
<td>130±18 (273)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>139±17 (397)</td>
<td>138±18 (1037)</td>
<td>135±18 (1920)</td>
<td>131±19 (1042)</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>4</td>
<td>142±18 (97)</td>
<td>140±20 (283)</td>
<td>138±19 (1061)</td>
<td>134±19 (2968)</td>
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<tr>
<td>p-value</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>1</td>
<td>1.8 (0.8-3.8) (2897)</td>
<td>1.1 (0.5-2.4) (595)</td>
<td>0.9 (0.4-1.9) (451)</td>
<td>0.7 (0.3-1.4) (276)</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>2.1 (1.1-4.1) (1587)</td>
<td>1.5 (0.8-3.2) (1635)</td>
<td>1.2 (0.6-2.6) (970)</td>
<td>1.0 (0.5-2.3) (265)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.4 (1.3-4.2) (390)</td>
<td>1.9 (1.0-3.7) (1024)</td>
<td>1.5 (0.8-3.2) (1899)</td>
<td>1.0 (0.5-2.0) (1024)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.2 (1.6-5.7) (96)</td>
<td>2.2 (1.2-4.9) (280)</td>
<td>2.0 (1.0-4.0) (1050)</td>
<td>1.4 (0.7-2.9) (2931)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p-value</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>84.0 (2464/2932)</td>
<td>58.9 (362/615)</td>
<td>35.9 (171/476)</td>
<td>26.8 (81/302)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82.2 (1319/1605)</td>
<td>77.5 (1285/1658)</td>
<td>77.9 (762/978)</td>
<td>53.1 (145/273)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>78.6 (312/397)</td>
<td>68.3 (710/1039)</td>
<td>66.1 (1271/1924)</td>
<td>72.2 (753/1043)</td>
<td>0.54</td>
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</tr>
<tr>
<td></td>
<td>85.6 (83/97)</td>
<td>61.5 (174/283)</td>
<td>56.1 (598/1066)</td>
<td>53.1 (1580/2973)</td>
<td>&lt;0.001</td>
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</tr>
<tr>
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<td>0.97</td>
<td>0.30</td>
<td>0.07</td>
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</tr>
<tr>
<td>Alcohol intake, g/day</td>
<td>6.9±10.7 (2830)</td>
<td>7.4±10.4 (539)</td>
<td>7.3±11.4 (464)</td>
<td>8.5±11.4 (297)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.3±12.1 (1554)</td>
<td>8.5±13.0 (1616)</td>
<td>9.3±12.5 (947)</td>
<td>9.4±12.9 (268)</td>
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<tr>
<td></td>
<td>7.5±11.1 (385)</td>
<td>8.6±13.9 (1007)</td>
<td>8.8±13.6 (1874)</td>
<td>9.5±13.8 (1009)</td>
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<tr>
<td></td>
<td>10±14.7 (90)</td>
<td>8.2±12.9 (276)</td>
<td>8.8±13.3 (1036)</td>
<td>9.8±13.6 (2876)</td>
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<td></td>
</tr>
<tr>
<td>p-value</td>
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<td>0.36</td>
<td>0.59</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For continuous variables data are shown as mean ± standard deviation or in case of skewed distribution, as median (interquartile range). In each cell the number between parentheses represents the total number of observations on which the summary estimate was based. For categorical variables, data are presented as percentage, and between parentheses number and total. P-value is for Jonckheere Terpstra trend test across categories. HDL indicates high-density lipoprotein; apoB indicates apolipoprotein B; ApoA-I indicates apolipoprotein A-I.
coronary events (MCE), retaining significance after simultaneous correction for HDL-c and apoB. However, in the high apoA-I quartiles of the distribution, we find high levels of apoB which may account for the increased CHD risk. Correction for apoB as done in the previous paper will therefore decrease the hazard ratios in the top apoA-I quartiles. Furthermore, an inappropriate student’s t test was used. In agreement with our study, when assessing the HDL-c and apoB corrected hazard ratios for MCE with increasing apoA-I subgroups, hazard ratio’s initially go down, after which they go up again. The finding of high apoB in the highest apoA-I quartiles also impedes comparison of our data with another analysis in the Epic Norfolk population study, which concludes that the predictive value of the apoB-apoA-I ratio was higher than the total cholesterol-HDL cholesterol ratio. Since no data are presented for apoA-I alone, the findings might be driven by the higher apoB levels in subjects with high apoA-I.

Our results strongly indicate that the predictive value of apoA-I, if anything, does not outperform the predictive value of HDL-c. In fact, the positive association between apoA-I quartiles and CHD risk establishes that apoA-I, overall, is not a better predictor of CHD than HDL-c.

The prevalence of CHD risk factors across quartiles of HDL-c and apoA-I confirms our observations above. First, we noticed a robust enrichment of risk factors in participants within the lowest HDL-c but highest apoA-I quartile. In other words, subjects with a combined phenotype of low HDL-c and high apoA-I levels are characterized by high levels of TG, apoB, and CRP, and high prevalence of hypertension and metabolic syndrome. Second, we observed that whereas HDL-c quartiles are inversely correlated with all analyzed cardiovascular risk factors, people in the highest apoA-I quartiles within each HDL-c quartile are characterized by a higher prevalence of risk factors compared to those in the lowest apoA-I quartile; age, BMI, TG, systolic blood pressure, CRP, prevalence of metabolic syndrome and percentage males were consistently higher in participants in the top apoA-I quartile compared to those in the lowest quartile, irrespective of the HDL-c quartile. This phenomenon has so far not been described in the literature. The unexpected association between established cardiovascular risk factors and apoA-I was possibly not acknowledged to date, because these associations were masked by the very close relationship between apoA-I and HDL-c.

ApoA-I is the major apolipoprotein of HDL particles and serves both structural and functional roles in HDL metabolism. The number of apoA-I molecules per HDL particle can range from one to five. This variation in lipoprotein composition has been proposed to determine the efficiency of reverse cholesterol transport, and could therefore be associated with CHD risk. For example, lipid-poor and apoA-I rich pre-beta HDL particles have been shown to be pre-eminent acceptors of cholesterol and are known to be strongly and inversely associated with CHD. On the other hand, the inverse association between larger HDL particles and CHD risk is less pronounced, which could be partly explained by their lower anti-oxidant and efflux capacity. Since incorporation of more apoA-I molecules into one HDL lipoprotein inevitably leads to a substantially larger particle, this could...
be a second explanation for the increase in CHD risk with ascending apoA-I quartiles. Furthermore, when speculating about the etiology of the increased CHD risk in the high apoA-I/ high HDL-c quartiles, two metabolic pathways need to be discussed. First, with ascending apoA-I quartiles, alcohol intake increases. Second, the increased prevalence of metabolic syndrome observed in the high apoA-I quartiles, has been described in some reports to coincide with high plasma levels of corticosteroids such as glucocorticoids and androgens, which in turn have been associated with high levels of apoB and apoA-I.

These explanations imply that individuals in the high HDL-c/high apoA-I quartiles are individuals with an abnormal metabolic phenotype, characterized by overproduction of apoB as well as apoA-I.

In recent studies, low HDL particle number in the presence of high HDL-c levels was not associated with reduced CHD risk. Furthermore, a high level of pre-β-HDL, which is characterized by a relatively high apoA-I/HDL-c ratio, was positively associated with CHD. Together with our investigations, these studies emphasize the complexity of HDL metabolism and its relationship to CHD and suggest that the anti-atherogenic functions of HDL may not be accurately reflected in either HDL-c or apoA-I measurements.

The descriptive nature of our study precludes us from further definition of the exact nature of the observed relationships, but it is astounding to witness the perfect association between almost every imaginable risk factor and HDL-c levels. HDL-c is the perfect reflection of a healthy cardiovascular risk profile, but, given the recent Mendelian randomization data, might not be the causal factor we think it is.

Limitations
Several aspects of this study merit attention when interpreting the results. First, although HDL-c and apoA-I measurements were available in a large set of study participants, these measurements do not inform us about the apolipoprotein content of HDL particles and the intraindividual range of apoA-I molecules per particle. Second, samples were not drawn in a fasting state. The fact that HDL-c and apoA-I are not strongly affected by a meal does not exclude the possibility of spurious associations. Third, CHD events were based on ICD-coding for hospitalizations and death certificates, and not adjudicated specifically for the purpose of this study. Lastly, the small numbers of participants and CHD events in the extremes of the HDL-c and apoA-I distributions warrant caution when interpreting the hazard ratios for these groups. Although the data from this study indicate that HDL-c may be more strongly associated with CHD risk than apoA-I, more factors have to be taken into account upon implementation in clinical practice. An important advantage of assessing apoA-I as opposed to HDL-c is the fact that it is not influenced by prandiality. However, the combination of apoA-I being a more costly measurement and an inferior predictor of CHD may tip the balance in the direction of HDL-c.

In conclusion, we show that both HDL-c and apoA-I are inversely correlated with CHD risk. Our findings demonstrate that apoA-I levels do not offer predictive information over and above HDL-c. In fact, within the highest HDL quartiles, higher apoA-I levels associate with a higher prevalence of CHD risk, possibly due to the higher prevalence
of cardiovascular risk factors in association with higher apoA-I levels. This could be a relevant finding in light of the current development of apoA-I increasing strategies, but the possibility exists that this phenomenon is based on a relatively small portion of individuals with abnormally high alcohol intake or abnormal metabolic features.

Acknowledgements
The authors would like to thank the participants, general practitioners and staff of the EPIC-Norfolk cohort study. We are grateful to the laboratory teams of the Medical Research Council Epidemiology Unit for the co-ordination of the cohort-wide biochemistry measurements.

Conflicts of interest
None

Funding sources
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Reference List


## Supplementary Data

### Supplementary table 1. Correlation between apolipoprotein A-I and HDL cholesterol by apolipoprotein A-I and HDL cholesterol quartiles

<table>
<thead>
<tr>
<th>ApoA-I quartiles</th>
<th>HDL cholesterol quartiles</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<td></td>
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<td>0.06</td>
<td>0.54</td>
<td>0.31</td>
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<td>0.20</td>
<td>0.66</td>
<td>0.002</td>
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</tbody>
</table>

Correlation coefficient is Spearman’s ρ. P-values are calculated by linear regression. HDL indicates high-density lipoprotein; ApoA-I indicates apolipoprotein A-I.
ABC Transporters, Atherosclerosis and Inflammation

Circulation Research; In Press

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4Haematopoiesis and Leukocyte Biology, Baker IDI Heart and Diabetes Institute, Melbourne, Australia
Abstract

While recent genome wide association studies have called into question the causal relationship between HDL cholesterol levels and CVD, ongoing research in animals and cells has produced increasing evidence that cholesterol efflux pathways mediated by ATP Binding Cassette (ABC) Transporters and HDL suppress atherosclerosis. These differing perspectives may be reconciled by a modified HDL theory that emphasizes the anti-atherogenic role of cholesterol flux pathways, initiated in cells by ABC transporters. ABCA1 and ABCG1 control the proliferation of hematopoietic stem and multipotential progenitor cells (HSPCs) in the bone marrow, and HSPC mobilization and extramedullary hematopoiesis in the spleen. Thus activation of cholesterol efflux pathways by HDL infusions or liver X receptor (LXR) activation results in suppression of HSPC mobilization and extramedullary hematopoiesis, leading to decreased production of monocytes and neutrophils, and suppression of atherosclerosis. In addition, macrophage-specific knockout of transporters has confirmed their role in suppression of inflammatory responses in the arterial wall. Recent studies have also shown that ABCG4, a close relative of ABCG1, controls platelet production, atherosclerosis and thrombosis. ABCG4 is highly expressed in megakaryocyte progenitors, where it promotes cholesterol efflux to HDL and controls the proliferative responses to thrombopoietin. Reconstituted HDL (rHDL) infusions act in an ABCG4 dependent fashion to limit hypercholesterolemia-driven excessive platelet production, thrombosis and atherogenesis, as occurs in human myeloproliferative syndromes. Activation of ABC transporter dependent cholesterol efflux pathways in macrophages, HSPCs or platelet progenitors, by rHDL infusion or LXR activation remain promising approaches to the treatment of human athero-thrombotic diseases.
**Introduction**

Plasma levels of High Density Lipoprotein (HDL) correlate inversely with the incidence of cardiovascular disease (CVD).\(^1\)\(^-\)\(^3\) However, Mendelian randomization studies\(^4\) and failed clinical trials involving HDL raising agents\(^5\)\(^-\)\(^7\) have called into question whether HDL has a causal relationship to atherosclerosis. Nonetheless, a large body of evidence indicates infusion or overproduction of HDL,\(^8\)\(^-\)\(^10\) as well as upregulation of cholesterol flux pathways by liver X receptor (LXR) activation,\(^11\) or targeting miR-33 have anti-atherogenic effects.\(^12\) The role of the ATP binding cassette transporters, ABCA1 and ABCG1, mediating cholesterol efflux\(^13\)\(^-\)\(^15\) and anti-inflammatory effects\(^16\)\(^,\)\(^17\) is central to this body of evidence. ABCA1 plays a major role in HDL formation, and was originally discovered in Tangier Disease (TD) patients, who display a loss of ABCA1 function and near absent HDL levels.\(^18\)\(^-\)\(^20\) ABCA1 mediates cholesterol efflux to lipid free apolipoproteins such as apoA-I and apoE, but not to large HDL particles.\(^15\) ABCG1 was shown to mediate cholesterol efflux from macrophages to HDL particles, but not to lipid-free apolipoproteins.\(^13\)\(^,\)\(^14\) ABCA1 and ABCG1 thus have complementary roles in mediating cholesterol efflux to HDL.\(^21\)

While originally implicated in macrophage cholesterol efflux,\(^22\)\(^,\)\(^23\) more recent studies have shown that these transporters have important functions in many parts of the hematopoietic system,\(^24\) in HDL formation by ABCA1 mediated cholesterol efflux from several tissues,\(^25\)\(^-\)\(^27\) and in the modulation of insulin secretion from pancreatic β-cells.\(^28\) The role of ABCA1/G1 in macrophages, HSPCs, and T-cells has been recently reviewed,\(^29\) as have the potential of HDL-increasing therapies and the role of macrophage cholesterol efflux pathways,\(^22\)\(^,\)\(^30\)\(^-\)\(^32\) and modifications that make HDL dysfunctional in terms of its anti-atherogenic properties.\(^33\) This review will focus on the mechanisms of ABCA1/G1 induced cholesterol efflux, on the role of ABCA1 in HDL formation, and the role of ABCA1/G1 in macrophage inflammation, endothelial function, HSPC proliferation, HSPC mobilization, and atherogenesis. Also cardiovascular risk in Tangier Disease (TD) patients will be discussed. In addition, the role of ABCG4, an ABC transporter that was recently identified to mediate cholesterol efflux from megakaryocyte progenitor cells (MkPs) to HDL,\(^34\) will be reviewed.

**Mechanisms and roles of ABCA1 and ABCG1 mediated cholesterol efflux**

ABCA1 is primarily localized in the plasma membrane of cells.\(^15\) ApoA-I and other apolipoproteins such as apoE can bind directly to cell surface ABCA1.\(^35\)\(^,\)\(^36\) Since lipid free apoA-I binds cholesterol relatively poorly, phospholipid efflux to apoA-I is considered to be essential for cholesterol efflux to apoA-I.\(^15\)\(^,\)\(^35\) The direct interaction of apolipoproteins with ABCA1 is of key importance to ABCA1-mediated cholesterol efflux; however, the detailed molecular mechanisms are still elusive and several models have been proposed.\(^37\)\(^,\)\(^38\) Using a novel single-molecule fluorescence tracking technique, Ueda and colleagues have shown that lipid efflux to apoA-I involves the ATPase-dependent conversion of mobile ABCA1 monomers into immobile homo-dimers in the plasma membrane: the model proposes that
ABCA1 monomers translocate lipids at the plasma membrane and form dimers; only the dimers can bind apoA-I, with one molecule of apoA-I binding to each ABCA1 molecule in the dimer. ApoA-I is subsequently lipitated and a discoidal HDL particle containing two apoA-I molecules is formed. The lipidation of apoA-I promotes its dissociation from the ABCA1 dimer, which facilitates conversion to ABCA1 monomers, that again can translocate lipids. This model contrasts with earlier models which proposed that the lipid translocating activity of ABCA1 led to an excess of phospholipids and cholesterol in the outer leaflet of the plasma membrane, membrane bulging and interaction with apoA-I to generate a nascent HDL particle. In addition to acting at the cell surface, ABCA1 can be internalized and there is evidence that the internalization and trafficking of ABCA1 is functionally important in mediating cholesterol efflux from intracellular cholesterol pools, especially in cells that ingest large amounts of lipids such as macrophages.

ABCA1 mediated cholesterol efflux to apoA-I is essential for HDL formation. Abca1−/− mice and TD patients who carry a homozygous mutation for ABCA1 leading to a loss of function, show nearly absent plasma HDL levels. Studies in tissue specific ABCA1 knockout mice showed that hepatocyte deletion resulted in ~80% decrease of plasma HDL cholesterol levels, while enterocyte deletion resulted in ~30% decrease and adipocyte deletion ~15%. In these studies, wild-type mice and not Abca1lox/lox mice were used as controls, thus a potential role of reduced ABCA1 expression in non-targeted tissues cannot be completely ruled out. ABCA1 expression in macrophages and other hematopoietic cells does not contribute to plasma HDL cholesterol levels. ABCA1 is transcriptionally induced by LXR which is activated in response to cellular oxysterol accumulation. Treatment of mice with LXR agonists leads only to a moderate increase in HDL levels, possibly reflecting upregulation of ABCA1 expression in the intestine, but not in the liver. Recently, it has also been shown that micro RNA (miR) 33 and miR 144 suppress hepatic ABCA1 expression. Activation of the farnesoid X receptor (FXR) in the liver increases the expression of miR144, leading to decreased ABCA1 protein and reduced HDL plasma levels. This implies that bile acids regulate plasma HDL levels through a FXR-miR144-ABCA1 pathway in hepatocytes. Along with the upregulation of SR-BI by bile salts, the down-regulation of hepatocyte ABCA1 by bile salts/FXR/miR144 in the postprandial state may lead to an increase in reverse cholesterol transport from the basolateral side of the hepatocyte into bile. A similar concept was originally proposed by Rader and colleagues based on the finding that probucol treatment led to downregulation of hepatocyte ABCA1 but increased reverse cholesterol transport across the hepatocyte. This was proposed as an explanation for anti-atherogenic actions of probucol despite lowering of HDL cholesterol levels.

In contrast to ABCA1, ABCG1 mediates cholesterol efflux to HDL particles but not to lipid-free apolipoproteins. In addition, ABCG1 promotes efflux of certain oxysterols such as 7-keto-cholesterol from cells to HDL, decreasing their toxic effects on cells, while both ABCA1 and ABCG1 can promote efflux of 25-hydroxycholesterol. Abcg1−/− mice showed defective macrophage cholesterol efflux to HDL and, when challenged with a
high fat diet, developed prominent macrophage foam cell accumulation in various tissues especially the lung.\textsuperscript{13} However, \textit{Abcg1}\textsuperscript{−/−} mice do not show alterations in plasma lipoprotein levels, leading some to question whether ABCG1-mediated cholesterol efflux to HDL has physiological importance.\textsuperscript{57, 58} Nonetheless, macrophage ABCA1 and ABCG1 make additive contributions to macrophage reverse cholesterol transport (RCT), strongly supporting an \textit{in vivo} role for ABCG1 in macrophage cholesterol efflux with subsequent transport via the plasma compartment to the liver and feces.\textsuperscript{59, 60} The lack of impact of ABCG1 expression on plasma lipoprotein levels may reflect the fact that its expression in hepatocytes is very low, with most hepatic expression reflecting contributions of Kupffer and endothelial cells.\textsuperscript{61} Accordingly, recent studies have shown almost complete disappearance of hepatic ABCG1 expression in mice with macrophage-specific knockout of ABCG1.\textsuperscript{16}

ABCG1 also promotes efflux of choline-phospholipids, particularly sphingomyelin (SM), from transfected cells to HDL.\textsuperscript{14, 62} ABCG1-mediated cholesterol efflux to HDL is defective in cells lacking ceramide transferase which transports ceramide from its site of synthesis in the ER to the Golgi, where SM biosynthesis is completed.\textsuperscript{63} It was shown recently that both SM and cholesterol stimulate ATPase activity of purified ABCG1 incorporated into liposomes,\textsuperscript{64} suggesting that these lipids are direct substrates of ABCG1. While ABCA1 directly binds apoA-I, ABCG1 expression does not affect the binding of HDL to cells.\textsuperscript{65} ABCG1 increases the availability of cholesterol to a variety of extracellular lipoprotein acceptors, including HDL and LDL.\textsuperscript{14} Similarly, ABCG4, the closest relative of ABCG1 does not bind HDL but promotes efflux of cholesterol onto HDL.\textsuperscript{14} Recent studies with specific antibodies have shown that ABCG4 is found predominantly in the trans-Golgi, where it may act indirectly to influence plasma membrane cholesterol content and HDL-mediated cholesterol efflux.\textsuperscript{34}

In contrast to ABCG4, the authentic cellular localization of ABCG1 is still unknown, reflecting the lack of suitable specific antibodies. Available studies have largely relied on overexpression of tagged versions of ABCG1, an approach which is notoriously prone to artefacts; for example, the localization of caveolin in endosomes was recently shown to be an artefact of overexpression.\textsuperscript{66} Wang \textit{et al.}\textsuperscript{65} reported localization of ABCG1 to plasma membrane, Golgi and recycling endosomes in transfected 293 cells. Using biotinylation, trypsin digestion and Western blotting, ABCG1 was detected in the plasma membrane of macrophages, especially when ABCG1 expression was increased by LXR activation.\textsuperscript{65} Macrophage deficiency of ABCG1 led to suppression of \textit{Ldlr} and \textit{Hmgcr} expression relative to wild-type cells and increased cholesteryl ester (CE) formation by ACAT, even in the absence of acceptors in the media to promote cholesterol efflux. This suggested redistribution of cholesterol from plasma membrane to the ER, leading to suppression of cholesterol biosynthetic genes, independent of cholesterol efflux.\textsuperscript{65} While several laboratories have reported localization of overexpressed ABCG1 in plasma membrane,\textsuperscript{63, 67} Tarling and Edwards\textsuperscript{58} could not detect ABCG1 in plasma membrane in their overexpression system, and found predominant localization to endosomes.\textsuperscript{58} Consistent with the observations of Wang \textit{et al.},\textsuperscript{65} the increased ABCG1 expression led to an increase in the mature form of SREBP-2 and upregulation of SREBP-2 target genes. It was proposed that ABCG1 may
facilitate the movement of sterols away from the ER, thus increasing SREBP-2 processing and relieving the sterol mediated inhibition of SREBP-2 processing. Studies using a fluorescent cholesterol derivative cholestatrienol in Abca1−/−Abcg1−/− macrophages demonstrated that ABCA1 and ABCG1 jointly promote movement of sterol from the inner to the outer leaflet of the plasma membrane. Thus, in the absence of the transporters there is increased accumulation of sterol on the inner leaflet of the plasma membrane. The increased availability of sterol on the cytosolic surfaces of the plasma membrane or intracellular organelles membranes likely promotes rapid diffusion to the ER, leading to sterol-mediated regulatory events.

While the precise cellular localization of ABCG1 remains unknown, a unifying hypothesis to explain its cellular effects is that ABCG1 promotes the “flopping” of sphingomyelin, cholesterol and certain oxysterols across various cellular membranes, possibly including the Golgi, endosomes and plasma membrane. The depletion of sterol of the membrane cytosolic surface creates a cholesterol and oxysterol chemical gradient that leads to diffusional removal of sterols from the ER and the anticipated regulatory events. Whether ABCG1 acts directly in the plasma membrane, or like ABCG4 acts intracellularly to influence plasma membrane lipid organization and cholesterol availability to HDL, the net result is increased availability of sterols at the cell surface, where they can be picked up by HDL.

Even though mouse and human ABCG1 are highly conserved, a recent study suggested that human ABCG1 does not mediate cholesterol efflux from macrophages to HDL, as suppression of ~80% of ABCG1 accomplished by siRNA in human macrophages seemed not to reduce cholesterol efflux. An independent study carried out under similar conditions showed that ~80% decreased expression of ABCG1 decreased cholesterol efflux to HDL by ~50%. The reasons for the discrepant results are unclear. However, levels of net cholesterol efflux were about 10-fold lower and no significant increase in CE in the media (suggesting that HDL did not contain active LCAT) was detected in the first study in contrast to the second study. Moreover, a third group also showed decreased cholesterol efflux to HDL in the context of specifically decreased ABCG1 expression in human macrophages.

As pointed out above, macrophage Abca1 and Abcg1 expression are transcriptionally regulated by LXR, LXRs are activated by certain oxysterols, and also sterols such as desmosterol, a precursor of cholesterol in the cholesterol biosynthetic pathway. Oxysterols are formed after cells take up sterols, through enzymatic reactions with cholesterol hydroxylases. The most important LXR activating oxysterols are thought to be 20S-, 22R-, 24S-, 25-, and 27-hydroxycholesterol, and 24(S)-, 25-epoxycholesterol as demonstrated in vitro and in vivo. In addition to mediating cholesterol efflux, ABCA1/G1 also mediate the efflux of the LXR activator 25-hydroxycholesterol, thus controlling the intracellular levels of sterols and oxysterols that activate LXR and maintaining cellular cholesterol homeostasis.

Chapter 3
ABCA1, ABCG1 and HDL suppress TLR-mediated inflammatory signaling

While it is clear that macrophage foam cell formation and macrophage inflammation are both central processes in atherogenesis, the detailed mechanisms linking these processes remain incompletely understood. There is strong evidence that ABCA1, ABCG1 and, HDL act to suppress inflammatory signaling via TLRs. While ABCA1 and ABCG1 function as ATP-binding cassette transporters, HDL is a lipoprotein that transports cholesterol and phospholipids from peripheral cells to the liver. Elicited peritoneal macrophages from mice with knockouts of Abca1 and/or Abcg1 showed increased expression of inflammatory cytokines and chemokines when challenged with ligands for TLR2, 3 or 4. Compared to wild-type cells, Abca1/g1 knockout macrophages showed increased cell surface levels of TLR4/MD-2 complexes and increased signaling via the MyD88 pathway in response to LPS (Figure 1A). The increased cell surface TLR4/MD-2 may reflect decreased internalization in response to LPS. Transporter deficiency was associated with increased plasma membrane cholera toxin B binding. Effects of transporter deficiency were exaggerated by cholesterol loading, and abrogated by cholesterol removal. This suggests that the accumulation of cholesterol led to the formation of ordered plasma membrane lipid raft domains, supporting increased levels of TLR4/MD-2 signaling complexes (Figure 1A).

Most studies showing increased inflammatory responses of transporter deficient macrophages have employed lipid A or LPS, and thus relevance to inflammation in plaques could be questioned. However, recent studies have confirmed increased inflammatory gene expression in Abca1−/−Abcg1−/− macrophages isolated from atherosclerotic plaques. Also, increased inflammatory gene expression in splenic Abca1−/−Abcg1−/−macrophages was observed, in particular of M-csf and Mcp-1, contributing to increased MCP-1 and M-CSF plasma levels. Surprisingly, in a recent study from the Glass laboratory, in vivo cholesterol loading of peritoneal macrophages was associated with suppression of inflammatory gene expression. This was linked to concomitant accumulation of desmosterol, an LXR activator, and induction of LXR target genes including Abca1 and Abcg1. The authors speculated that in the milieu of the atherosclerotic plaque, factors exogenous to macrophage foam cells must induce inflammation, overcoming the anti-inflammatory effects of LXR activation. Earlier studies provide strong clues that relevant exogenous factors likely include modified forms of LDL acting via pattern recognition receptors, such as TLR 4, 6 and CD36 to activate inflammatory signaling.

A key property of TLR inflammatory signaling is transrepression of the expression of LXR target genes, mediated by Interferon Regulatory Factor 3 (IRF-3) (Figure 1A). Thus, it is likely that in the normal inflammatory plaque milieu, expression of Abca1 and Abcg1 is relatively suppressed. Indeed while carrying out atherosclerosis regression studies, the Fisher laboratory noted that plaque macrophage Abca1 expression is initially low but becomes rapidly induced when the atherosclerotic segment is transplanted into a low plasma cholesterol environment. Although there is limited information, one study suggested very low expression of ABCA1 in human atherosclerotic plaques. Overall, there may be a balance between activity of TLRs and LXRs within macrophages of atherosclerotic plaques; the levels of expression of ABCA1 and ABCG1 may have a
central role in suppressing the activation of TLRs by modified LDL and other factors
(Figure 1A and 1B). Based on this model, one successful therapeutic approach would be
to decrease TLR responses most obviously by decreasing plasma LDL levels, or perhaps
LDL modifications that induce TLR signaling. In addition, targeting specific aspects of the
inflammatory response could be beneficial, such as IL-1 antagonism,\(^{87}\) or antagonism of
signaling downstream of the IL6R, which has been implicated in CHD in a recent meta-
analysis.\(^{88}\) However, a challenge to these latter approaches is redundancy in inflammatory
pathways and potential immunosuppression.\(^{89}\) Macrophage-specific targeting of LXR/RXR
would appear to be an ideal approach leading to induction of cholesterol efflux pathways
and suppression of inflammatory responses (Figure 1B).

HDL is also able to suppress innate inflammatory responses mediated by TLR signaling.
In part this may be mediated by promotion of cholesterol efflux via ABCA1/G1 (Figure
1B).\(^{17}\) However, higher concentrations of HDL are still able to suppress inflammation quite
potently even in macrophages lacking ABCA1 and ABCG1.\(^{90}\) A particular role of HDL in

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Figure 1. Crosstalk between TLR4 activation and LXR, and ABC transporter expression in
macrophages. A. minimally modified LDL (mmLDL) or lipopolysaccharide (LPS) activates TLR4,
leading to 1) IRF-3 mediated transrepression of LXR and 2) MyD88 mediated NFκB activation
in the nucleus (white circle). As a consequence ABCA1/G1 expression is reduced and cholesterol
(shown as black dots) accumulates in lipid rafts in the membrane, which enhances TLR4 surface
expression, thus amplifying TLR4 signaling, and increasing inflammatory gene expression.
B. LXR is activated, thus activating mRNA transcription of ABCA1 and ABCG1, which mediate cholesterol
efflux to HDL. As a consequence, less cholesterol accumulates in the membrane, decreasing TLR4
surface expression. LXR also transrepresses NFκB target gene activation. Both processes reduce
inflammatory gene expression.
suppressing Type 1 interferon responses mediated by TRIF signaling from endosomes has been suggested.\textsuperscript{91} Thus, various strategies to increase HDL may have benefit on plaque inflammation, in part by promotion of cholesterol efflux via ABCA1/G1, but also likely by incompletely understood mechanisms that may operate independently of cholesterol efflux.

**Tangier disease and cardiovascular risk**

TD patients are homozygous ABCA1 mutation carriers who display a loss of function of the ABCA1 protein and near absent HDL levels.\textsuperscript{18-20} Heterozygous ABCA1 mutation carriers have \textasciitilde{}50\% decreased HDL levels.\textsuperscript{18-20} Based on their HDL phenotype, increased CVD was expected in ABCA1 mutation carriers. However, the reports on CVD in TD patients are variable. Whereas some individuals with TD display striking premature atherosclerotic CVD,\textsuperscript{92-94} other TD patients appear to be spared.\textsuperscript{92, 94} The contradictory findings on the association of TD with atherosclerosis could be explained by reduced plasma LDL levels in TD patients,\textsuperscript{92, 94} as well as a compensatory increase in expression of ABCG1,\textsuperscript{95} and modification of the complex atherogenic response by other genetic and environmental factors. Mechanisms reported to underlie the increased atherosclerosis in TD patients include decreased ABCA1-mediated cholesterol efflux to a residual level of \textasciitilde{}20-30\%,\textsuperscript{35, 96} monocyte and neutrophil activation as assessed by expression levels of CD11b,\textsuperscript{97, 98} without effects on blood monocyte/neutrophil levels,\textsuperscript{99} and endothelial dysfunction.\textsuperscript{100} In heterozygous ABCA1 carriers either increased CVD or no CVD phenotype has been reported.\textsuperscript{94, 101-103} It has been suggested that the lack of CVD phenotype in some heterozygotes is due to a higher level of residual cholesterol efflux compared to the heterozygotes with increased CVD.\textsuperscript{102} This is supported by a study in ABCA1 heterozygotes where there was an inverse correlation between cholesterol efflux and carotid atherosclerotic plaque burden as assessed by carotid-Intima Media Thickness (c-IMT).\textsuperscript{103} A recent study showed increased atherosclerosis in ABCA1 heterozygotes as assessed by carotid MRI,\textsuperscript{101} which is a more specific method for measuring atherosclerotic plaque burden than c-IMT,\textsuperscript{104} thus further corroborating the increased atherosclerosis in ABCA1 heterozygotes.

Conflicting results have been reported for the correlation of CVD effects with ABCA1 missense variants associated with partial loss of function and moderate effects on cholesterol efflux and HDL levels. In a Mendelian randomization approach in a prospective cohort comprising \textasciitilde{}9000 individuals, heterozygosity for the ABCA1 mutation K776N led to a two-to-three times higher risk of ischemic heart disease.\textsuperscript{105} Furthermore, five SNPs in ABCA1 (V771M, V825I, I883M, E1172D, R1587K) were shown to predict risk of ischemic heart disease in a cohort of 9259 individuals.\textsuperscript{106} However, the same group reported more recently that heterozygosity for four loss-of-function mutations (P1065S, G1216V, N1800H, R2144X) was not associated with a higher risk of ischemic heart disease in three prospective cohorts comprising 56,886 individuals.\textsuperscript{107} It must be noted however, that only small decreases in HDL, of \textasciitilde{}28\% as opposed to \textasciitilde{}50\% in previously reported ABCA1 heterozygotes, were observed.\textsuperscript{94, 101-103, 107} Also the residual cholesterol efflux was substantial (74-79\% for P1065S and G1216V and 48-49\% for N1800H and R2144X for
homozygous mutations compared to controls), whereas in TD patients there was only 20-30% residual cholesterol efflux. Additionally, LDL levels were reduced by ~25%, probably offsetting the effects of reduced HDL on CVD. Thus, the conflicting results in these studies could be related to inclusion of relatively mild ABCA1 mutations as well as offsetting effects of reduced LDL cholesterol levels.

In a meta-analysis of genome-wide association studies (GWAS), single nucleotide polymorphisms (SNPs) near the ABCA1 gene have been associated with HDL and total cholesterol levels, but not with cardiovascular risk. Although these studies enjoy the benefit of huge statistical power, some caution is merited in the interpretation of findings. The effects of SNPs on HDL is often very small, possibly resulting in an underestimation of the association of the SNPs with cardiovascular risk. Most SNPs result in modest changes in HDL, within the normal range, whereas the largest effect of HDL on cardiovascular risk is expected in the lowest regions of the HDL distribution, which are typically underrepresented in GWAS. Also the small effect sizes of SNPs on HDL levels introduce the possibility of confounding effects related to lifestyle, medication or ethnicity.

There is much less known concerning the association of ABCG1 with cardiovascular risk in humans. One ABCG1 variant (g.376C→T) leading to a partial loss of function (~40%) has been identified, which was associated with an increased risk for myocardial infarction and ischemic heart disease in a combined cohort from the Copenhagen Ischemic Heart Disease and the Copenhagen City Heart Study.

Role of ABCA1 and ABCG1 in atherosclerosis: studies in mouse models

Studies on the roles of ABCA1 and ABCG1 in atherosclerosis are summarized in Table 1, and illustrated in Figure 2. Whole body ABCA1 deficiency in mice on a pro-atherogenic Apoe<sup>-/-</sup> or Ldlr<sup>-/-</sup> background does not increase atherosclerotic lesion area, probably due to the markedly decreased (~65-73%) LDL cholesterol levels. Hepatic ABCA1 deficiency, leading to ~50% decreased HDL levels and only ~30% decrease in LDL levels, increases atherosclerosis ~75% in Apoe<sup>-/-</sup> mice.

Bone marrow transplantation studies were carried out to study the role of hematopoietic ABCA1 in atherogenesis. Bone marrow Abca1 deficiency moderately increased atherosclerosis in Apoe<sup>-/-</sup> or Ldlr<sup>-/-</sup> mice. While these studies were interpreted as showing that macrophage ABCA1 deficiency was pro-atherogenic, a macrophage specific ABCA1 knockout showed no effect on atherosclerotic lesion area. These findings suggested that bone marrow ABCA1 deficiency in non-macrophage hematopoietic cells could be contributing to accelerated atherosclerosis.

The role of haematopoietic Abcg1 in atherogenesis was investigated in three different studies in Ldlr<sup>-/-</sup> mice transplanted with Abcg1<sup>-/-</sup> bone marrow on a Western type diet (WTD). Whereas one study showed increased atherosclerosis in mice transplanted with Abcg1<sup>-/-</sup> bone marrow, bone marrow Abcg1 deficiency decreased atherosclerosis in the two other studies. The reason for the discrepancy in the outcomes of these studies is
Table 1. Atherosclerosis studies in mouse models deficient in Abca1 or Abcg1 expression or both.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Mouse model and Genetic background</th>
<th>Diet and Time on diet</th>
<th>(V)LDL chol</th>
<th>HDL chol</th>
<th>Lesion area</th>
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<tr>
<td>Aiello et al.115</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;Abca1&lt;sup&gt;−/−&lt;/sup&gt; DBA/BL6</td>
<td>Chow, 12 wks</td>
<td>~50%↓</td>
<td>100%↓</td>
<td>-</td>
</tr>
<tr>
<td>Aiello et al.115</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;Abca1&lt;sup&gt;−/−&lt;/sup&gt; DBA/BL6</td>
<td>0.15% chol, 20% fat, 17 wks</td>
<td>~65%↓</td>
<td>100%↓</td>
<td>-</td>
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<td>Aiello et al.115</td>
<td>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;Abca1&lt;sup&gt;−/−&lt;/sup&gt; DBA/BL6</td>
<td>Chow, 20 wks</td>
<td>~50%↓</td>
<td>100%↓</td>
<td>-</td>
</tr>
<tr>
<td>Aiello et al.115</td>
<td>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;Abca1&lt;sup&gt;−/−&lt;/sup&gt; DBA/BL6</td>
<td>0.15% chol, 20% fat, 20 wks</td>
<td>~73%↓</td>
<td>100%↓</td>
<td>-</td>
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<tr>
<td>Brunham et al.45</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;AlbCreAbca1&lt;sup&gt;fl/fl&lt;/sup&gt; BL6</td>
<td>Chow, 12 wks</td>
<td>~30%↓</td>
<td>~50%↓</td>
<td>~75%↑</td>
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<td>Aiello et al.115</td>
<td>Apoe&lt;sup&gt;−/−&lt;/sup&gt;Abca1&lt;sup&gt;−/−&lt;/sup&gt; BM → Apoe&lt;sup&gt;−/−&lt;/sup&gt; DBA/BL6</td>
<td>Chow, 12 wks</td>
<td>-</td>
<td>-</td>
<td>~50%↑</td>
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<tr>
<td>Van Eck et al.116</td>
<td>Abca1&lt;sup&gt;−/−&lt;/sup&gt; BM → Ldlr&lt;sup&gt;−/−&lt;/sup&gt; BL6</td>
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<td>~14%↓</td>
<td>-</td>
<td>~60%↑</td>
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<td>Brunham et al.45</td>
<td>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;LysmCreAbca1&lt;sup&gt;fl/fl&lt;/sup&gt; BL6</td>
<td>Chow, 12 wks</td>
<td>-</td>
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<tr>
<td>Baldan et al.118</td>
<td>Abcg1&lt;sup&gt;−/−&lt;/sup&gt; BM → Ldlr&lt;sup&gt;−/−&lt;/sup&gt; BL6</td>
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<td>Out et al.117</td>
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<td>Chow, 12 wks</td>
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<td>~14%↓</td>
<td>-</td>
<td>~60%↑</td>
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<td>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;LysmCreAbca1&lt;sup&gt;fl/fl&lt;/sup&gt; BL6</td>
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<td>~31%↓</td>
<td>-</td>
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<td>Chow, 12 wks</td>
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<td>Chow, 12 wks</td>
<td>~20%↓</td>
<td>-</td>
<td>-</td>
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<td>Chow, 12 wks</td>
<td>~2.2-fold↑</td>
<td>-</td>
<td>-</td>
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<td>Yvan-Charvet et al.133</td>
<td>Abca1&lt;sup&gt;−/−&lt;/sup&gt;Abcg1&lt;sup&gt;−/−&lt;/sup&gt; BM → Ldlr&lt;sup&gt;−/−&lt;/sup&gt; DBA/BL6</td>
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<td>~19-fold↑, Il-1, Il-6, Mcp1, Mip1α mRNA↑</td>
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<td>Out et al.121</td>
<td>Abcg1&lt;sup&gt;−/−&lt;/sup&gt; Abca1&lt;sup&gt;−/−&lt;/sup&gt; BM → Ldlr&lt;sup&gt;−/−&lt;/sup&gt; BL6</td>
<td>Chow, 12 wks</td>
<td>~73%↑</td>
<td>-</td>
<td>-</td>
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<td>Westerterp et al.126</td>
<td>Abcg1&lt;sup&gt;−/−&lt;/sup&gt; Abca1&lt;sup&gt;−/−&lt;/sup&gt; BM → Ldlr&lt;sup&gt;−/−&lt;/sup&gt; BL6</td>
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<td>Chow, 20 wks</td>
<td>~73%↑</td>
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<td>Chow, 20 wks</td>
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still not completely clear. A later study by the same group found no effect of bone marrow Abcg1 deficiency in atherosclerosis, leading to the proposal that different results could be related to the time of the diet feeding. The decreases in atherosclerosis were attributed to increased oxidized LDL (oxLDL)-induced Abcg1−/− macrophage apoptosis and increased expression of ABCA1 and increased apoE secretion in Abcg1−/− macrophages. A subsequent study suggested that the oxLDL-induced apoptosis in Abcg1−/− macrophages was due to the accumulation of oxysterols such as 7-ketocholesterol. 7-Ketocholesterol is a major component of oxLDL and is found in atherosclerotic plaques. ABCG1 mediated the efflux of 7-ketocholesterol to HDL, protecting macrophages from oxLDL-induced apoptosis. The combined deficiency of Abcg1 and Apoe in hematopoietic tissues was associated with reduced atherosclerosis compared to Apoe−/− controls, increased susceptibility of macrophages to oxysterol-induced apoptosis and a marked increase in macrophage apoptosis in lesions.

ABCG1 is also highly expressed in human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs), where it mediates cholesterol and 7-ketocholesterol efflux to HDL. HAECs and HUVECs show almost no expression of ABCA1 and no cholesterol efflux to lipid free apoA-I. However, studies in AECs subjected to laminar shear flow conditions showed upregulation of LXRα expression and induction of its target genes Abca1 and Abcg1, thus suggesting that under conditions that simulate the in vivo environment of ECs, both ABCA1 and ABCG1 may be highly expressed. Overexpression of human ABCA1 in mouse endothelium decreased atherosclerosis, concomitant with decreased mRNA expression of pro-atherogenic Cxcl-1 and tumor necrosis factor superfamily 10 in the aorta. Surprisingly, HDL levels were increased by ~40% in these mice, potentially caused by a 2.6-fold increase in EC cholesterol efflux to apoA-I compared to controls.

In contrast to the results with hematopoietic Abcg1 deficiency, vascular Abcg1 deficiency resulting from transplantation of wild-type BM in Abcg1−/−Ldlr−/− mice resulted in accelerated atherogenesis compared to Ldlr−/− controls transplanted with wild-type BM. Vascular Abcg1 deficiency was associated with decreased endothelium-dependent vasorelaxation. The increased atherosclerosis was thus likely due at least in part to decreased NO-bioavailability due to endothelial Abcg1 deficiency. NO has an atheroprotective role in ECs in part by decreasing the expression of adhesion molecules and pro-inflammatory cytokines that enhance monocyte adhesion. Abcg1−/− ECs have been shown to exhibit increased secretion of MCP-1 and IL-6 as well as increased surface expression of ICAM-1 and E-selectin concomitant with a 4-fold increase in monocyte adhesion (Figure 2, step D). The decreased eNOS activity upon Abcg1 deficiency may have been due in part to accumulation of oxysterols and cholesterol. 7-Ketocholesterol accumulation in endothelial cells was shown to generate reactive oxygen species that combined with NO to form peroxynitrite, which disrupts eNOS dimers that are required for its activity. Cholesterol accumulation enhanced the inhibitory interaction between eNOS and caveolin-1 (cav-1), leading to decreased eNOS activity.

While endothelial ABCG1 thus appeared to have a major role in preserving eNOS activity, endothelium-dependent vasorelaxation was also mildly decreased in Abca1−/− mice on a
Figure 2. Contribution of ABCA1 and ABCG1 deficiency to atherogenesis. A. *Abca1/-Abcg1/-* haematopoietic stem and progenitor cells (HSPC) show increased proliferation, stimulating monocyte production. B. *Abca1/g1* deficient mice show enhanced BM HSPC mobilization into the blood and organs, including the spleen. HSPC accumulation in the spleen leads to enhanced monocyte production. C. *Abca1/-Abcg1/-* monocytes and macrophages in the spleen show increased expression of M-CSF and MCP-1, increasing M-CSF and MCP-1 plasma levels and monocyte production in the BM and monocyte release from the BM, respectively. D. *Abcg1/-* endothelium and E. *Abca1/-Abcg1/-* macrophages in the atherosclerotic plaque show increased foam cell formation and cytokine levels, which enhances monocyte infiltration. All processes contribute to atherosclerotic lesion formation. Figure artwork by Derek Ng.

cholesterol-rich diet, and further decreased in *Abca1^-/-Abcg1^-* mice compared to *Abcg1^-/-* mice on the same diet, suggesting that endothelial ABCA1 may also play a role in preserving endothelial function and have an athero-protective role in addition to endothelial ABCG1.

**Studies in mice with combined Abca1/g1 deficiency**

**Atherosclerosis studies**

In general, *Abca1^-/-Abcg1^-* mice display more dramatic phenotypes than *Abca1^-/-* or *Abcg1^-/-* mice, reflecting the fact that ABCA1 and ABCG1 have overlapping functions and display mutual compensation. To study the role of ABCA1/G1 in atherogenesis in vivo, *Ldlr^-/-* mice were transplanted with *Abca1^-/-Abcg1^-* bone marrow. On a high-cholesterol, bile
salt diet, mice with \textit{Abca1^{-/-}Abcg1^{-/-}} bone marrow deficiency displayed markedly increased atherosclerosis, compared to mice transplanted with wild-type, \textit{Abca1^{-/-}}, or \textit{Abcg1^{-/-}} bone marrow.\textsuperscript{133} \textit{Abca1^{-/-}Abcg1^{-/-}} macrophages showed a \textasciitilde70\% decrease in macrophage cholesterol efflux to HDL,\textsuperscript{133} increased inflammatory gene expression, and increased free cholesterol or oxLDL induced apoptosis.

In another transplantation study of BM into \textit{Ldlr^{-/-}} mice and Western diet feeding did not lead to an increase in atherosclerosis compared to the control group.\textsuperscript{121} However, in contrast to the earlier study,\textsuperscript{133} cholesterol levels were decreased by about 75\% in the \textit{Abca1^{-/-}Abcg1^{-/-}} bone marrow recipients, suggesting susceptibility to atherosclerosis at a lower threshold of plasma cholesterol levels than seen in control mice.\textsuperscript{121} As these mice were homozygously deficient for the \textit{Ldlr}, in contrast to heterozygous \textit{Ldlr} mice in the previous study, less VLDL/LDL cholesterol was being cleared by the liver than in the \textit{Ldlr^{-/-}} mice, and the VLDL/LDL may have been taken up by macrophages and monocytes deficient in \textit{Abca1/g1}.

Interestingly, \textit{Abca1^{-/-}Abcg1^{-/-}} mice exhibited a dramatic \textasciitilde5-fold increase in blood monocyte and neutrophil counts, as well as infiltration of the spleen, lung, liver and small intestine with myeloid cells including macrophage foam cells and neutrophils, a phenotype suggestive of a myeloproliferative syndrome.\textsuperscript{134} The increased blood leukocyte levels reflected a \textasciitilde5-fold expansion of the HSPC population in the bone marrow.\textsuperscript{134} This expansion was caused by enhanced proliferation probably due to increased cell surface expression of the common \(\beta\) subunit\textsuperscript{134} that is shared by IL-3, IL-5 and GM-CSF receptors.\textsuperscript{135, 136} Thus, the increased atherosclerosis in mice transplanted with \textit{Abca1^{-/-}Abcg1^{-/-}} BM may have been partly caused by HSPC expansion and the associated increased numbers of blood monocytes and neutrophils (\textit{Figure 2}, step A). Increased monocyte and neutrophil counts are well known to be associated with increased CVD in humans.\textsuperscript{137-139} Expression of the human \textit{APOA1} transgene reversed the accelerated atherosclerosis in \textit{Ldlr^{-/-}} mice transplanted with \textit{Abca1^{-/-}Abcg1^{-/-}} bone marrow concomitant with decreased expression of the common \(\beta\)-subunit, diminished proliferation of HSPCs, and reversal of monocytosis to the level of the control group.\textsuperscript{134} This suggested that markedly elevated ApoA-1 and HDL levels could act independent of ABCA1 or ABCG1 to promote cholesterol efflux from HSPCs and macrophages, presumably via passive diffusion or SR-BI facilitated efflux. Thus, in the setting of hypercholesterolemia, cholesterol efflux pathways mediated by ABCA1, ABCG1 and HDL act to suppress the proliferation of HSPCs and the resultant monocytosis and neutrophilia.

\textit{Apoe^{-/-}} mice were shown to have increased blood monocyte and neutrophil counts, especially when fed a high fat, high cholesterol diet.\textsuperscript{99, 140, 141} This was associated with expansion and proliferation of the HSPC population.\textsuperscript{99} ApoE is highly expressed on the surface of HSPCs, where it acts in an ABCA1/G1 dependent fashion to promote cholesterol efflux. \textit{Apoe^{-/-}} mice also have increased expression of the common \(\beta\) subunit on the surface of HSPCs, promoting HSPC proliferation, monocytosis and increased entry of monocytes into atherosclerotic plaques.\textsuperscript{99}

In order to assess the role of cholesterol efflux pathways in different populations of myeloid cells, we developed \textit{Abca1fl/flAbcg1fl/fl} mice. When crossed with the \textit{LysmCre} strain,
these mice displayed efficient (>95%) deletion of ABCA1 and ABCG1 in macrophages, but no deletion in HSPCs. Atherosclerosis was increased by 73% in in chow-fed Ldlr⁻/⁻ mice transplanted with LysmCreAbca1fl/flAbcg1fl/fl BM, in the absence of monocytosis or HSPC expansion. This result established a role for macrophage cholesterol efflux mediated by ABCA1/G1 in suppressing atherosclerosis. Analysis of lesional inflammatory gene expression by laser capture microdissection revealed increased expression of Mcp-1 and other inflammatory genes (Figure 2, step E), similar to observations in Abca1⁻⁻Abcg1⁻⁻ peritoneal macrophages treated with TLR4 ligands. In a parallel experiment, Ldlr⁻/⁻ mice transplanted with Abca1⁻⁻Abcg1⁻⁻ bone marrow, displayed a more pronounced 2.7 fold increase in atherosclerosis, in association with HSPC expansion and monocytosis. The more dramatic atherosclerosis phenotype in these mice suggested a role of cholesterol efflux pathways in HSPCs as well as in macrophages in the suppression of atherosclerosis.

When Ldlr⁻/⁻ mice transplanted with LysmCreAbca1fl/flAbcg1fl/fl BM were fed a high fat, high cholesterol diet, they showed ~2-fold increases in monocytes and neutrophils, concomitant with increased expression of M-CSF, Mcp-1, and G-csf in splenic Abca1⁻⁻ Abcg1⁻⁻ macrophages, and increased M-CSF, MCP-1, and G-CSF plasma levels. M-CSF and G-CSF stimulate granulocyte macrophage progenitor (GMP)-mediated monocyte and neutrophil production (Figure 2, step C). Increased HDL levels achieved by expression of the human APOA1 transgene reversed the increased M-CSF, MCP-1, and G-CSF, and the associated monocytosis and neutrophilia, indicating that HDL also suppresses inflammation independent of the ABC transporters. Thus, macrophage cholesterol efflux pathways mediated by ABCA1, ABCG1, and HDL suppress inflammation and the resulting monocytosis and neutrophilia.

**Extramedullary hematopoiesis**

Extramedullary hematopoiesis involves the mobilization of HSPCs from the bone marrow via the blood into the spleen and other organs. In Apoe⁻/⁻ mice, extramedullary hematopoiesis involving proliferation of Granulocyte Macrophage Progenitors (GMPs) has been shown to produce monocytes that infiltrate atherosclerotic plaques thus promoting lesion progression. Abca1⁻⁻Abcg1⁻⁻ mice also exhibited splenomegaly and extramedullary hematopoiesis; Abca1⁻⁻Abcg1⁻⁻ and Apoe⁻/⁻ mice displayed increased HSPC mobilization from the BM to the spleen (Figure 2, step B). Cell specific knockout models revealed that the mechanism underlying this phenomenon was increased IL-23 secretion from Abca1⁻⁻Abcg1⁻⁻ macrophages and dendritic cells as a result of upregulation of the TLR4 and TLR3 pathways in these cells. Whereas splenic Abca1⁻⁻Abcg1⁻⁻ macrophages and dendritic cells were shown to have a major contribution to IL-23 secretion, other Abca1⁻⁻ Abcg1⁻⁻ peripheral macrophages or dendritic cells, such as these in the adipose tissue, also may have contributed to the increased IL-23 levels in these cell specific knockout models. IL-23 secretion is also regulated by GM-CSF that signals through the common β subunit which is increased upon Abca1/g1 deficiency. IL-23 is known to initiate a signaling cascade leading to enhanced production of IL-17 by Th17 cells and G-CSF by bone marrow stromal cells thus directing GMPs in the BM towards neutrophil production rather than monocyte/
This subsequently decreases the abundance of osteoblasts and nestin+ mesenchymal stem cells that express CXCL12, which is a key retention ligand for CXCR4 on HSPCs. Thus the bone marrow niche is altered, decreasing its ability to retain HSPCs and HSPCs are mobilized to organs, including the spleen. Increasing HDL via the human APOA1 transgene, or by infusion of rHDL, suppressed HSPC mobilization in several different mouse models including Apoe−/− as well as mouse models of acute myeloid leukemia. The suppression of HSPC mobilization and extramedullary hematopoiesis represent additional potential therapeutic effects resulting from the activation of cholesterol efflux pathways. These may be particulary relevant in the setting of acute coronary syndromes, where sympathetic nervous system activation leads to mobilization of HSPCs, contributing to extramedullary hematopoiesis and atherogenesis.

**ABCG4 in thrombosis and atherosclerosis**

In contrast to the extensive studies on ABCA1 and ABCG1, relatively little is known about the function of ABCG4, a transporter highly homologous to ABCG1. Earlier studies demonstrate that ABCG4, like ABCG1, promotes cholesterol efflux to HDL when overexpressed in cultured cells. Both ABCG1 and ABCG4 are highly expressed in brain and promote efflux of cholesterol and other sterols to lipid poor discoidal HDL particles. Combined ABCG1 and ABCG4 deficiency result in increased levels of several oxysterols in the brain, in association with decreased cholesterol biosynthesis and repressed expression of several cholesterol response genes such as HMG-CoA reductase and the LDL receptor. Deficits in memory have been reported in Abcg4−/− mice. However, ABCG4 is not expressed in macrophages and ABCG4 deficiency does not affect macrophage cholesterol efflux.

In a recent study, it was found that ABCG4 was selectively expressed in megakaryocyte progenitors (MkPs), a type of progenitor cell in megakaryocyte/platelet lineage. Little ABCA1 or ABCG1 was expressed in these cells. In MkPs, ABCG4 staining co-localized with trans-Golgi markers. ABCG4 deficient MkPs showed defective cholesterol efflux to HDL and increased free cholesterol accumulation, with prominent accumulation in plasma membrane. Thus, even though localized in the Golgi, ABCG4 deficiency resulted in defective cholesterol efflux to HDL and an increase in cell cholesterol content including in the plasma membrane, consistent with studies suggesting segregation of sterol-rich plasma membrane domains in the trans-Golgi.

Bone marrow ABCG4 deficiency led to accelerated atherosclerosis and arterial thrombosis in hypercholesterolemic Ldlr−/− mice, in association with increased platelet counts, increased reticulated platelets, platelet/leukocyte complexes and platelet-derived microparticles, all with proven pro-atherosclerotic and/or pro-thrombotic properties. Abcg4−/− MkPs showed increased proliferation in response to thrombopoietin (TPO), the most important growth factor regulating megakaryocyte/platelet lineage development in vivo, and increased numbers of megakaryocytes in the bone marrow and spleen. There were increased levels of c-MPL, the TPO receptor, on the surface of Abcg4−/− MkPs and markedly enhanced increases in platelet counts in response to TPO injection.
The increased cell surface c-MPL levels in Abcg4−/− MkPs were due to blunting of the negative feedback regulation of c-MPL in response to TPO and involved a defective activation of LYN kinase and c-CBL E3 ligase. LYN kinase, a palmitoylated membrane protein, appears to act as a membrane cholesterol sensor. Increased membrane cholesterol in Abcg4−/− MkPs may increase LYN association with the membrane and decrease its tyrosine kinase activity in response to TPO, causing defective phosphorylation of c-CBL. This disrupts the negative feedback regulation of c-MPL and leads to increased platelet production.

Infusion of rHDL reduced MkP proliferation and platelet counts in wild-type mice but not in Abcg4−/− mice. The therapeutic potential of rHDL infusions in the control of platelet overproduction was exemplified by the finding that in a mouse model of essential thrombocythemia (ET) induced by bone marrow cell expression of a mutant form of c-MPL found in human subjects with ET, rHDL reduced the platelet count in mice receiving Abcg4+/− but not Abcg4−/− bone marrow cells. These studies link increased platelet production, initiated from its lineage progenitor cells, to accelerated atherosclerosis and arterial thrombosis.

Human GWAS studies have linked SNPs in or near the c-CBL gene to platelet count. Interestingly, ABCG4 is in tight linkage disequilibrium with c-CBL, and the SNPs associated with platelet counts could be influenced by expression of c-CBL and/or ABCG4. Together, these findings strongly support the human relevance of ABCG4 and the related mechanisms identified in mouse studies in regulation of megakaryopoiesis and platelet production. Increased platelet production is associated with an increased risk of arterial and venous thrombosis and athero-thrombosis in myeloproliferative syndromes such as essential thrombocythosis and myelofibrosis. In addition, there is some evidence that increased platelet production may precede the onset of acute coronary syndromes. These studies suggest that rHDL infusions or Lyn kinase activators such as Tolimidone may play a role in the suppression of platelet overproduction in these settings.

**Summary and Implications**

Since the discovery that mutations in ABCA1 were responsible for Tangier Disease, there has been a proliferation of studies demonstrating the role of cholesterol efflux pathways mediated by ABCA1, ABCG1, ABCG4 and SR-BI in atherogenesis. While confirming the importance of cholesterol efflux pathways in macrophage foam cell formation and inflammation, new roles for ABCA1/G1 in the control of HSPC and megakaryocyte progenitor proliferation, HSPC mobilization and extramedullary hematopoiesis have been discovered. These pathways control the production of monocytes, neutrophils and platelets. While studies have been largely done in mouse models, monocytosis, neutrophilia and parameters of platelet function have been associated with human athero-thrombotic disease, suggesting translational relevance. In contrast, human GWAS have called into question the causal relationship between SNPs associated with HDL-influencing genes and CHD, including for ABCA1. While it is undoubtedly true that not all interventions to raise HDL cholesterol levels in humans will be associated with protection, we suggest some caution in the interpretation of these studies, and conclude that genetic deficiency
of \textit{ABCA1} likely is associated with premature atherosclerosis. Thus, future therapies may be directed at cholesterol efflux pathways. One approach could be direct upregulation of \textit{Abca1/g1} by LXR activators. LXR agonists have been shown to have athero-protective effects, either directly in the vessel wall,\cite{11} or by regulating HSPC proliferation and the associated monocyte and neutrophil levels.\cite{99} These effects seem to be dependent, at least in part, on \textit{Abca1/g1} expression.\cite{11,99} Thus LXR agonists could constitute a potential anti-atherogenic therapy provided that their adverse effects on liver triglyceride metabolism, \textit{e.g.} hepatic steatosis,\cite{167} could be circumvented. Another approach could be infusions of rHDL. It was shown recently that injections of pegylated rHDL particles that have a prolonged circulation time, improve atherosclerotic lesions in mice, concomitant with reducing HSPC proliferation and monocytosis.\cite{8} Another study showed that injections of rHDL particles suppressed platelet counts, which was mediated by ABCG4.\cite{34} Elucidation of the pathway regulating the effects of ABCG4 on MkP TPO receptor expression has indicated that Lyn Kinase activators could be an alternative method to suppress platelet production especially in myeloproliferative neoplasms where athero-thrombotic risk is greatly increased. In the future intervention trials for HDL-directed therapies may take on a “personalized medicine” approach in which instead of taking all-comers who have been optimally treated with statins, individuals with persistent low HDL, high levels of atherogenic lipoproteins, patients with MPNs or an adverse genetic risk score may be targeted for treatment.

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**Disclosures**

Alan R. Tall is a consultant to Merck, Roche, Amgen, Arisaph and CSL.
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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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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 eight 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-\(\beta\) high-density lipoprotein (pre-\(\beta\) HDL). Through the action of lecithin:cholesterol acyltransferase (LCAT), this pre-\(\beta\) 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.\(^15,17\) In 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 ABCA1 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.

**Material 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 AI (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, 3000*g* 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. 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 deoxy 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 \textit{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 APOAIp.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.Wl747X) 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.C1941R). 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 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 (=Polymorphism Phenotyping)\(^2\) and SIFT\(^2\) software to predict functional significance of the 8 novel missense variations (see Supplementary Table 1). Four out of eight 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.

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

<table>
<thead>
<tr>
<th>Patients (gender, age)</th>
<th>Amino acid* (nucleotide*) change</th>
<th>TC</th>
<th>TG</th>
<th>LDL-c</th>
<th>HDL-c</th>
<th>Clinical manifestations of TD</th>
<th>CVD</th>
<th>Other relevant clinical data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Homozygotes</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Patient 1 (female, 42)</td>
<td>p.L1056P (c.3167T &gt; C)</td>
<td>2.4</td>
<td>0.9</td>
<td>1.99</td>
<td>&lt;0.10</td>
<td>Absent</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>p.W747X (c.5240G &gt; A)</td>
<td>1.76</td>
<td>1.93</td>
<td>0.52</td>
<td>0.1–0.3</td>
<td>Neuropathy, splenomegaly,</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>thrombocytopenia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 2 (male, 40)</td>
<td>p.S93L (c.1779C &gt; G)</td>
<td>4.4</td>
<td>1.4</td>
<td>3.6</td>
<td>&lt;0.10</td>
<td>Absent</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>p.E1253K (c.3757G &gt; A)</td>
<td></td>
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<tr>
<td><strong>Compound heterozygotes</strong></td>
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<td></td>
</tr>
<tr>
<td>Patient 4 (female, 63)</td>
<td>p.Q1038X (c.3112C &gt; T)</td>
<td>6.68</td>
<td>2.72</td>
<td>5.4</td>
<td>&lt;0.10</td>
<td>Absent</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>p.N1800H (c.5398A &gt; C)[32]</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Patient 5 (female, 28)</td>
<td>p.T1512M (c.4533C &gt; T)</td>
<td>4.42</td>
<td>1.83</td>
<td>3.46</td>
<td>0.1</td>
<td>Absent</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>p.N1800H (c.5398A &gt; C)[32]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p.O978X/O988G (c.2034delC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 6 (female, 17)</td>
<td>p.D575G (c.1724A &gt; G)</td>
<td>4.96</td>
<td>2.84</td>
<td>4.35</td>
<td>&lt;0.10</td>
<td>Absent</td>
<td>None</td>
<td>DM1</td>
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<tr>
<td></td>
<td>p.C1941R (c.582T &gt; C)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Heterozygotes</strong></td>
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<td></td>
</tr>
<tr>
<td>Patient 7 (male, 42)</td>
<td>p.S100C (c.299C &gt; G)</td>
<td>8.5</td>
<td>8.7</td>
<td>4.3</td>
<td>0.3</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 8 (male, 58)</td>
<td>p.E1172D (c.3516G &gt; C)[33]</td>
<td>6.4</td>
<td>2.7</td>
<td>4.1</td>
<td>0.9</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 9 (male, 35)</td>
<td>p.S1817F (c.5454C &gt; T)[17]</td>
<td>2.9</td>
<td>0.31</td>
<td>1.88</td>
<td>0.88</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 10 (male, 48)</td>
<td>p.C3477R (c.4429T &gt; C)[33]</td>
<td>2.01</td>
<td>1.4</td>
<td>0.92</td>
<td>0.46</td>
<td>N.A.</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td>Patient 11 (male, 68)</td>
<td>p.V1858A (c.5573T &gt; C)</td>
<td>4.9</td>
<td>3.78</td>
<td>2.41</td>
<td>0.75</td>
<td>N.A.</td>
<td>CAD</td>
<td>None</td>
</tr>
<tr>
<td>Patient 12 (female, 36)</td>
<td>p.N1800H (c.5398A &gt; C)[32]</td>
<td>4.6</td>
<td>1.2</td>
<td>4</td>
<td>&lt;0.10</td>
<td>N.A.</td>
<td>None</td>
<td>DM2, obesity</td>
</tr>
<tr>
<td>Patient 13 (male, 67)</td>
<td>p.R282X (c.844C &gt; T)[34]</td>
<td>3.2</td>
<td>1.21</td>
<td>2.14</td>
<td>0.51</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 14 (female, 42)</td>
<td>p.W424X (c.1272G &gt; A)</td>
<td>2.07</td>
<td>1.04</td>
<td>1.39</td>
<td>0.21</td>
<td>N.A.</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Patient 15 (female, 52)</td>
<td>N.A. – (IVS11−1G &gt; A)</td>
<td>5.51</td>
<td>3.51</td>
<td>3.28</td>
<td>0.56</td>
<td>N.A.</td>
<td>None</td>
<td>Hypothyroidism, hypertension</td>
</tr>
<tr>
<td>Patient 16 (female, 54)</td>
<td>N.A. – (IVS48+2T &gt; C)</td>
<td>3.29</td>
<td>1.92</td>
<td>1.94</td>
<td>0.49</td>
<td>N.A.</td>
<td>None</td>
<td>DM2, hypertension</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.
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.

**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. 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. 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 although only one study shows functionality of a mutation in the promoter region. 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\textsuperscript{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.\textsuperscript{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 to not 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\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{29,31} or apo A-I binding.\textsuperscript{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.
Reference List


Supplementary Figure 1. Localization of GFP-tagged ABCA1 WT and GFP-tagged ABCA1 mutant proteins in BHK cells.
Supplementary Figure 2. Monocyte cholesterol efflux to apo A-I in patient-derived monocyte-macrophages relative to monocyte-macrophages obtained from healthy volunteers. 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 10^6 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. Celles were then loaded overnight at 37°C with free [3H]cholesterol, by adding 0.5ml of labeling medium (RPML, Glutamax, 0.1% Pen/Strep, 0.2%)

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>Probably 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.
ABCA1 mutation carriers with low High Density Lipoprotein Cholesterol (HDL-C) are characterized by a larger atherosclerotic burden

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2Department of Experimental Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands
3Department of Radiology, Academic Medical Center, Amsterdam, The Netherlands
Abstract

Aims
Low HDL-C is a potent risk factor for cardiovascular disease (CVD). Yet, mutations in ABCA1, a major determinant of circulating HDL-C levels, were previously not associated with CVD risk in cohort studies. To study the consequences of low plasma levels of high-density lipoprotein cholesterol (HDL-C) due to ATP-binding cassette transporter A1 (ABCA1) dysfunction for atherosclerotic vascular disease in the carotid arteries.

Methods and results
We performed 3.0 Tesla magnetic resonance imaging (MRI) measurements of the carotid arteries in 36 carriers of high impact functional ABCA1 mutations and 36 normolipidemic controls. Carriers presented with 42% lower HDL-C levels ($P < 0.001$), a larger mean wall area ($18.6 \pm 6.0$ vs. $15.8 \pm 4.3$ mm$^2$; $P = 0.02$), a larger mean wall thickness ($0.82 \pm 0.21$ vs. $0.70 \pm 0.14$ mm; $P = 0.005$), and a higher normalized wall index ($0.37 \pm 0.06$ vs. $0.33 \pm 0.04$; $P = 0.005$) compared with controls, retaining significance after adjustment for smoking, alcohol consumption, systolic blood pressure, diabetes, body mass index, history of CVD, LDL-C, and statin use ($P = 0.002$).

Conclusion
Carriers of loss of function ABCA1 mutations display a larger atherosclerotic burden compared with age and sex-matched controls, implying a higher risk for CVD. Further studies are needed to elucidate the full function of ABCA1 in the protection against atherosclerosis. These data support the development of strategies to upregulate ABCA1 in patients with established CVD.
Introduction
Prospective studies have consistently shown that high-density lipoprotein cholesterol (HDL-C) is inversely correlated with cardiovascular risk. As a consequence, HDL-increasing strategies have been studied intensively to reduce the residual cardiovascular risk in statin-treated patients. The lack of specific and potent HDL-increasing compounds, however, has precluded us from answering the question whether HDL-C is a truly causal factor in atherogenesis. In fact, Briel et al reported the absence of a correlation between HDL-C increase and CVD risk in a recent meta-analysis comprising data from approximately 300,000 subjects having received lipid-modulating therapies, whereas low-density lipoprotein cholesterol (LDL-C) decrease was invariably associated with a decrease in cardiovascular risk. None of these lipid modulating therapies, however, had as its primary objective to raise HDL-C. The absolute increases in circulating HDL-C levels in these studies were modest, raising the possibility of a power problem of the analyses. Moreover, the lack of effect of CETP inhibition, and nicotinic acid has increased the concerns regarding HDL-C as a suitable target for the prevention of CVD, although these trials were troubled by either off-target effects or design issues. Combined with the fact that HDL-C levels are confounded by other risk factors, such as BMI, triglycerides and smoking, this has cast doubt on the causal role of the HDL particle in atherogenesis.

The most widely characterized mechanism by which HDL-C protects against atherosclerosis is reverse cholesterol transport (RCT). In this pathway, pivotal steps comprise the ABCA1 (adenosine triphosphate–binding cassette transporter A1)-mediated cholesterol efflux (GenBank No. AF275948) followed by esterification of cholesterol via lecithin-cholesterol acyltransferase (LCAT). Heterozygous ABCA1 mutation carriers are characterized by half-normal HDL-C levels. Schaefer et al. reported that 19% of ABCA1 heterozygotes and 45% of ABCA1 homozygotes had evidence of coronary artery or cerebrovascular disease, compared with 4% in the control population. In line, van Dam et al. reported the loss of efflux capacity in ABCA1 mutation carriers with a correspondingly increased carotid intima-media thickness (cIMT). In contrast, Frikke-Schmidt et al. reported that in 109 ABCA1 mutation carriers compared to 41,852 controls, the multifactorially adjusted OR for ischemic heart disease was 0.93 (95% CI: 0.53-1.62) for heterozygous ABCA1 mutation carriers compared with controls.

We therefore decided to assess the effects of loss of function ABCA1 mutations on atherosclerosis. We performed carotid 3.0 Tesla MRI as well as cIMT imaging in ABCA1 mutation carriers and controls. Functionality of each ABCA1 mutation was verified using in vitro cholesterol efflux assays.

Methods
Study design
Subjects with low HDL-C levels, defined as HDL-C < 5th percentile, were selected from a cohort of hypoalphalipoproteinemia patients and screened for ABCA1 mutations. Family members of ABCA1 mutation carriers were actively recruited. Carriers of functional
ABCA1 mutations (n=36) and controls (n=36) matched for age and gender were enrolled in this study. Index patients were excluded if CVD was present at the time of referral. Furthermore, blood was obtained from 36 unaffected family members for lipid analysis. All participants provided written informed consent. The study was conducted at the Academic Medical Center in Amsterdam, the Netherlands from March 2010 to November 2011. The study protocol was approved by the Institutional Review Board.

The presence of cardiovascular risk factors, use of medication and family history of CVD were assessed by a questionnaire. Blood pressures were measured using an oscillometric blood pressure device (Omron 705IT, Hoofddorp, the Netherlands). The BMI was calculated from weight and length. HOMA index was calculated as (glucose x insulin)/22.5. Blood was obtained after an overnight fast and stored at -80°C. Plasma cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were analysed using commercially available kits (Randox, Antrim, United Kingdom and Wako, Neuss, Germany). Plasma apolipoprotein AI and apolipoprotein B were measured using a commercially available turbidometric assay (Randox, Antrim, United Kingdom). All analyses were performed using the Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

Genotyping
Mutation detection was performed as published previously.21 In short, the sequence reactions were performed using a BigDye terminator ABI prism kit (Applied Biosystems, Foster City, CA, USA). Sequences were analysed with the Sequencher package (Gene Codes Co, Ann Arbor, Mi, USA).

Cholesterol efflux assays
ABCA1 mutation functionality was tested using skin fibroblasts (passage number 5–15), cultured in 24-well plates until 80% confluency. The cells were loaded with media containing 0.2% BSA, 30 ug/mL cholesterol and 0.5 uCi/mL 3H-cholesterol for 24 hours. After washing, cholesterol efflux was started by addition of 10 ug/mL apoA-I. After 4 h, the medium was collected and the amount of 3H-cholesterol was quantified by liquid scintillation counting. Cellular concentrations of 3H-cholesterol were measured after extraction of the cells with 2-propanol. The percentage efflux was calculated by dividing the counts in the efflux medium by the sum of the counts in the medium plus the cell extract.

Carotid magnetic resonance imaging
Scans were performed as described previously.22 In short, scans were obtained in a 3.0 Tesla Philips whole-body scanner (Philips, Best, the Netherlands), using a single-element microcoil (Philips, Hamburg, Germany). Ten slices were scanned of the distal 3.0 cm of the left and right common carotid artery. A total of 20 images were obtained per scan. Images were saved in DICOM format using standardized protocols.22,23 Quantitative image analysis was performed using semi-automated measurement software (VesselMass, Leiden University Medical Center, the Netherlands).24 One trained reader, with excellent scan-rescan and inraobserver variability analysed all the images using standardized
protocols for reading and rating images combined with dedicated semi-automated software,\textsuperscript{22,23} blinded for all data of the participants. The mean wall thickness (MWT), lumen area (LA), outer wall area (OWA), and total wall volume (TWV) were measured. The normalized wall index (NWI) was calculated as: NWI = MWA / OWA. The mean wall area is calculated as: MWA = meanOWA - meanLA. The prevalence of plaque components (PC) and total PC volume (mm\textsuperscript{3}) were also assessed. Plaque component was defined as an area with lower signal intensity within the arterial wall on a T1-weighted image, representing either lipid-rich tissue or calcification.\textsuperscript{25} The prevalence of PC was reported as percentage of the total number of images that showed PC. The volume of PC’s was reported as the sum of all PC volumes of all subjects per group. A total of 46 slides, corresponding to 3\% of the total, was excluded from the PC analysis due to insufficient quality.

\textbf{Carotid ultrasound imaging}

Carotid B-mode ultrasound scans of the left and right common, bulb and internal carotid arterial far walls were assessed as previously published.\textsuperscript{26} One reader analysed all the images, blinded for group and any other data of the participants. The ultrasound parameter was mean common carotid intima-media thickness (CcIMT), defined as the average far wall IMT of the left and right distal 1cm of the common carotid artery.

\textbf{Outcome parameters}

The NWI was the primary outcome parameter of the study. Secondary MRI outcome parameters were MWA (mm\textsuperscript{2}), MWT (mm) and TWV (mm\textsuperscript{3}). The secondary ultrasound parameter was CcIMT (mm). Plaque component analysis, expressed as PC prevalence and total PC volume (mm\textsuperscript{3}), was an exploratory endpoint.

\textbf{Statistical analysis}

Continuous variables are expressed as means ± standard deviations, unless otherwise specified. Possible differences in demographic, biometrical and biochemical parameters between carriers of \textit{ABCA1} mutations and controls were assessed using unpaired Student’s \textit{t}-tests, $\chi^2$ tests or Mann-Whitney \textit{U}-test, where appropriate. Differences in carotid imaging parameters between \textit{ABCA1} mutation carriers and controls were assessed using unpaired Student’s \textit{t}-tests, unless otherwise specified. In addition, a linear regression model was used, in which carriership, smoking, alcohol consumption, systolic blood pressure, diabetes, BMI, history of cardiovascular disease (CVD), LDL-C, and statin use were indicated as independent variables and NWI, MWA, MWT, TWV and CcIMT were indicated as dependent variables. The authors had full access to the raw data and take responsibility for its integrity.

\textbf{Results}

\textbf{Baseline characteristics}

\textit{ABCA1} mutation carriers from 14 separate families were included, comprising 2 homozygous, 2 compound heterozygous and 32 heterozygous patients. Subjects were carriers of the
following mutations: c.6401+2T>C, p.Ser930Phe, p.Ser824Leu, p.Arg587Trp, p.Thr929Ile, p.Asn935Ser, c.3535+1G>C, p.Asp571Gly, p.Asn1800his, p.Leu1056Pro, p.Gln1038Ter, c.1195-1G>C, p.Arg579Gln, p.Phe1760Valfs*21. Controls from the general population were matched for age and gender (Table 1). Carriers displayed a 16% lower total cholesterol (p=0.004; table 1), largely due to a 42% reduction of HDL-C levels (p<0.001). Apo B levels were higher by 10% in carriers (p=0.19), while carriers had 32% lower apo A-I levels (p<0.001; table 1). Other parameters were not significantly different (Table 1).

Lipid profiles were measured in 36 unaffected family members of ABCA1 mutation carriers, matched for age and gender. Total cholesterol (4.35±1.31mmol/l vs 5.23±1.10 mmol/l, p=0.003) and HDL-C (0.84±0.38 mmol/l vs 1.57±0.41 mmol/l, p<0.001) were lower in carriers compared to unaffected relatives, whereas LDL-C was comparable (3.11±1.04 mmol/l vs 3.23±0.92 mmol/l, p=0.60). Triglycerides were higher in carriers compared to unaffected relatives [1.06 (0.78-1.39) mmol/l vs 0.77 (0.57-1.06) mmol/l, p=0.002].

**Cholesterol efflux assays**

Fourteen mutations were found in the carriers. Five of these mutations have already been shown to have a significant impact on ABCA1 function (p.Asn1800his, p.Thr929Ile, p.Arg587Trp, p.Leu1056Pro, and p.Phe1760Valfs*21). The efflux capacity of the remaining nine mutations: p.Asn935Ser, c.3535+1G>C, p.Ser824Leu, p.Ser930Phe, p.Gln1038Ter, c.1195-1G>C, c.6401+2T>C, p.Asp571Gly and p.Arg579Gln are listed in figure 1. Cholesterol efflux capacity was assessed in heterozygotes, except for

![Figure 1](image)

**Figure 1.** Normalized cholesterol efflux from cultured skin fibroblasts to apolipoprotein A-I. Percentage efflux is shown as mean ± SD, n=3 separate experiments. Fibroblasts from a healthy control were used as a control.
Table 1. Characteristics in Carriers of \textit{ABCA1} Gene Mutations and Controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>\textit{ABCA1} mutation carriers (n=36)</th>
<th>Controls (n=36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>50.94 ± 15.56</td>
<td>50.91 ± 11.30</td>
<td>1.00</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>18 (50)</td>
<td>18 (50)</td>
<td>1.00</td>
</tr>
<tr>
<td>Body Mass Index (kg/m(^2))</td>
<td>26.03 ± 4.29</td>
<td>24.52 ± 3.04</td>
<td>0.90</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>13 (36)</td>
<td>12 (33)</td>
<td>0.15</td>
</tr>
<tr>
<td>Alcohol use (units per week)</td>
<td>6.31 ± 7.47</td>
<td>10.00 ± 6.33</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Medication use, n (%)

<table>
<thead>
<tr>
<th>Medication</th>
<th>\textit{ABCA1} mutation carriers (n=36)</th>
<th>Controls (n=36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statin</td>
<td>11 (28)</td>
<td>4 (11)</td>
<td>0.04</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>4 (11)</td>
<td>0 (0)</td>
<td>0.38</td>
</tr>
<tr>
<td>Niacin</td>
<td>2 (6)</td>
<td>0 (0)</td>
<td>0.54</td>
</tr>
<tr>
<td>Fibrate</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
</tr>
<tr>
<td>Aspirin</td>
<td>7 (19)</td>
<td>0 (0)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Blood pressure

<table>
<thead>
<tr>
<th>Parameter</th>
<th>\textit{ABCA1} mutation carriers (n=36)</th>
<th>Controls (n=36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic (mmHg)</td>
<td>139 (21)</td>
<td>131 (13)</td>
<td>0.05</td>
</tr>
<tr>
<td>Diastolic (mmHg)</td>
<td>81 (10)</td>
<td>80 (8)</td>
<td>0.84</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>9 (25)</td>
<td>7 (19)</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Glucose metabolism

<table>
<thead>
<tr>
<th>Parameter</th>
<th>\textit{ABCA1} mutation carriers (n=36)</th>
<th>Controls (n=36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>5.65 ± 1.52</td>
<td>5.47 ± 0.77</td>
<td>0.55</td>
</tr>
<tr>
<td>Fasting insulin (mU/L)</td>
<td>1.09 (1.09-6.01)</td>
<td>3.48 (1.09-5.65)</td>
<td>0.67</td>
</tr>
<tr>
<td>HOMA index</td>
<td>0.34 (0.25-1.33)</td>
<td>0.84 (0.26-1.36)</td>
<td>0.50</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>3 (8)</td>
<td>2 (6)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Lipid metabolism

<table>
<thead>
<tr>
<th>Parameter</th>
<th>\textit{ABCA1} mutation carriers (n=36)</th>
<th>Controls (n=36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.35 ± 1.31</td>
<td>5.20 ± 1.09</td>
<td>0.004</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.11 ± 1.04</td>
<td>3.41 ± 0.83</td>
<td>0.18</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>0.84 ± 0.39</td>
<td>1.44 ± 0.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.06 (0.78-1.39)</td>
<td>1.01 (0.68-1.40)</td>
<td>0.37</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/l/dL)</td>
<td>120.79 ± 41.10</td>
<td>109.19 ± 26.64</td>
<td>0.19</td>
</tr>
<tr>
<td>Apolipoprotein A-I (mg/dL)</td>
<td>106.21 ± 43.15</td>
<td>156.74 ± 25.68</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are indicated as mean ± SD unless otherwise indicated. Male sex, smokers, medication use, hypertension, diabetes: p for X\(^2\) test; for other parameters: p for student’s t-test. For HOMA index, fasting insulin and triglycerides we report median and interquartile range; P for Mann Whitney U test. 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.
mutation p.Gln1038Ter which was tested in a homozygous patient. Efflux measured in fibroblasts from a heterozygous p.Cys1477Arg carrier was used as a positive control since efflux capacity has consistently been shown to be impaired.\textsuperscript{19,27} One patient compound heterozygous for mutation p.Arg579Gln and p.Val771Met was included. In the view of contradictory statements on functionality of mutation p.Val771Met,\textsuperscript{31} we assumed that the major part of the efflux impairment of the p.Arg579Gln and p.Val771Met combination is attributable to p.Arg579Gln.

**Carotid MRI and ultrasound**

MRI data are shown in table 2. The NWI was significantly higher in carriers compared with controls (\(p=0.005\)) (figure 2). Adjustment for differences in smoking, alcohol consumption, systolic blood pressure, diabetes, BMI, history of cardiovascular disease (CVD), LDL-C, and statin use resulted in an even stronger statistical significance (\(p=0.002\)). The MWA, MWT and TWV were also significantly higher in carriers compared with controls (\(p=0.02, 0.005\) and 0.02 respectively) and retained significance after adjustment for the above mentioned risk factors (\(p=0.03, 0.002\) and 0.03 respectively). Plaque components (PC), related to lipid-rich tissue or calcification (PC prevalence) were 2.5 times more prevalent in carriers compared to controls (\(p=0.01\), figure 3) with a concomitant higher total PC volume (table 2).

Ultrasound CcIMT was not higher in carriers compared with controls.

**Table 2.** Carotid 3.0 Tesla MRI and B-mode Ultrasound Parameters for \(ABCA1\) mutation carriers and controls.

<table>
<thead>
<tr>
<th></th>
<th>(ABCA1) mutation carriers (n=36)</th>
<th>Controls (n=36)</th>
<th>(P^1)</th>
<th>Adjusted (P^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 Tesla MRI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NWI</td>
<td>0.37 (0.06)</td>
<td>0.33 (0.04)</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td>MWA (mm(^2))</td>
<td>18.6 (6.0)</td>
<td>15.8 (4.3)</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>MWT (mm)</td>
<td>0.82 (0.21)</td>
<td>0.70 (0.14)</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td>TWV (mm(^3))</td>
<td>1116 (363)</td>
<td>946 (255)</td>
<td>0.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Plaque Composition Analysis**

|                      |                                    |                 |         |                  |
|----------------------|                                    |                 |         |                  |
| PC presence (%)      | 32/670 (5)                         | 16/716 (2)      | 0.01    |                  |
| Total PC volume (mm\(^3\)) | 230.8   | 74.4 |                  |                  |

|                      |                                    |                 |         |                  |
|----------------------|                                    |                 |         |                  |
| B-Mode ultrasound    |                                    |                 |         |                  |
| CcIMT (mm)           | 0.67 (0.22)                        | 0.67 (0.16)     | 0.98    | 0.50             |

\(P^1\) for the unadjusted model, \(P^2\) for multivariate model adjusting for smoking, alcohol consumption, systolic blood pressure, diabetes, BMI, history of cardiovascular disease (CVD), LDL-C, and statin use. \(ABCA1\) is ATP-binding cassette transporter. NWI is normalized wall index, MWA is mean wall area, TWV is total wall volume, MWT is mean wall thickness, LA is lumen area, PC is plaque component. CcIMT is the mean common carotid intima media thickness. PC presence is reported as total number of images showing PC. Total PC volume is the sum of all PC volumes of all subjects per group.
Discussion

In the present study, we show that carriers of loss of function \textit{ABCA1} mutations exhibit more carotid artery wall thickening as assessed by MRI compared with age- and sex-matched controls. Both normalized wall index and mean wall area, as well as thickness and total wall volume were significantly higher in carriers compared with controls. These differences retained significance after adjustment for traditional risk factors. In support, plaque components were more prevalent in carriers compared to controls. Collectively, these findings support the concept that functional \textit{ABCA1} mutations, resulting in lower cholesterol efflux capacity, lead to more atherosclerotic vascular disease.

Our results show a higher burden of carotid atherosclerosis in \textit{ABCA1} mutation carriers. As expected, carriers presented with decreased cholesterol efflux capacity, which has been shown to be negatively correlated with atherosclerotic burden.\textsuperscript{32} Carriers also presented with more arterial wall thickening. Carotid artery wall thickening is associated with a higher risk
of cardiovascular events. In support, carriers were characterized by a higher prevalence of PCs. Recent data revealed that information on plaque composition in the carotid artery has a higher predictive value for CVD compared with a thickened cIMT per se.

In contrast, cIMT assessed using ultrasound was not different between carriers and controls. This discrepancy between carotid MRI and carotid ultrasound measurements most likely reflects the lower sensitivity and higher variability of cIMT measurements as compared with carotid 3.0-T MRI. We recently showed that heterozygous carriers of LCAT gene mutations also have more carotid atherosclerosis using MRI, whereas cIMT did not reveal any difference. It should be noted that carotid ultrasound measures the wall thickness in a two-dimensional way, whereas atherosclerosis is a three-dimensional disease. Consequently, carotid MRI has been shown to yield superior power compared with ultrasound measurements, enabling smaller sample sizes to detect differences in wall thickness.

The absence of an IMT difference contradicts earlier findings by van Dam et al. In their control group, IMTs were lower compared to our controls (0.63 vs 0.69 mm), whereas the IMTs in their ABCA1 mutation carriers were higher compared with our ABCA1 mutation carrier group (0.73 vs 0.67 mm). Yet, there are clear differences between these papers. Methodologically, van Dam used a composite IMT endpoint including carotid and femoral arteries, whereas we used the reproducible thickness measurement of the far wall of the common carotid artery only. In addition, the mean age was ~13 years lower in their control group compared with our controls. Taking into account an IMT progression of 0.0047 mm/year, the calculated IMT of their controls at the age of 51 (0.69 mm) approximates the value observed in our control group (0.67 mm). With respect to the lower mean IMT in our ABCA1 mutation carriers (0.67 vs 0.73), one-third (n=11) of our carriers used statins as compared with absence of statin use in the carriers reported by van Dam, whereas statins are known to reduce IMT progression by 0.02 mm within the first 6-12 months followed by a decreased IMT progression at a longer follow-up.

Genome-wide association studies (GWAS) revealed that ABCA1 correlates with HDL-C and total cholesterol, but not with incidence of CVD. A potential pitfall of GWAS, however, pertains to the fact that the effect of single nucleotide polymorphisms (SNPs) in a single gene on HDL-C levels is often small. This may result in an underestimation of the association between gene defects and CHD risk. Moreover, HDL is most strongly associated with CHD risk in case of low HDL-C levels. In GWAS, the extreme tails of HDL-C levels are typically underrepresented. In most cases, SNPs result in small changes in HDL-C levels within the normal range. Consequently, no effect of the SNP on CHD risk is observed. Moreover, it is often unknown whether SNPs give rise to biologically relevant changes in ABCA1 function. As a consequence, findings from GWAS regarding the effect of ABCA1 on CVD risk have only limited value, and cannot provide definite conclusions regarding gene function and associated CVD risk.

An alternative approach is the assessment of effects of genetic variation in ABCA1 using a Mendelian randomization approach as performed by Frikke-Schmidt and co-workers. In line with our results, they report that in a prospective cohort comprising approximately 9,000 individuals, heterozygosity for the ABCA1 mutation p.Lys776Asn
led to a two-to-three fold higher risk of ischemic heart disease. They also reported that three genetic variations in *ABCA1* predict risk of ischemic heart disease. In a more recent prospective cohort study they reported that heterozygosity for loss of function mutations was not associated with a higher risk of ischemic heart disease. Several factors may have contributed to the contrasting findings when comparing their results with our data. Firstly, the discrepancy may relate to methodological differences. Frikke-Schmidt and co-workers assessed functionality of the mutations using cholesterol efflux assays in transfected HeLa cells, mimicking homozygosity for the *ABCA1* mutation. An efflux capacity of 79% was considered indicative of compromised efflux capacity, whereas in case of homozygosity, efflux is expected to be 20% to 30%. This may have led to inclusion of non-pathogenic mutations. In contrast, the efflux assays in our study were performed using patients’ fibroblasts, representing the actual biology of a heterozygous *ABCA1* deficient cell. Since variability of the assay is considerable, we performed the experiments in triplicate. Inclusion of relatively mild mutations in the prospective cohort study of Frikke-Schmidt is further reflected by the fact that the HDL-C levels in carriers compared to controls is only 29% lower. HDL-C levels in the present study were 42% lower, which is in line with the earlier reported HDL-C levels in heterozygous *ABCA1* mutation carriers.

**Limitations**

Several aspects of our study deserve closer attention. First, we assessed a surrogate endpoint for CVD, which precludes us from drawing final conclusions with regards to cardiovascular event risk. The low incidence of mutation carriers makes it impossible to perform prospective outcome studies in *ABCA1* heterozygotes. In spite of the relatively low number of subjects, the present study does provide evidence of an adverse effect of *ABCA1* dysfunction on the arterial wall. Secondly, we cannot exclude the potential impact of indirect referral bias. Although we excluded all index cases referred for CVD from the analysis, a positive family history for CVD is a strong predictor for CVD. Hence, we cannot exclude that family members of affected probands are characterized by thicker carotid artery walls due to factors beyond their *ABCA1* mutation carriership.

**Clinical implications**

The present study shows that carriers of *ABCA1* mutations display more carotid atherosclerosis compared to controls implying a higher CVD risk. These findings suggest that early and aggressive CVD preventive measures are warranted in *ABCA1* mutation carriers. Collectively, our findings lend support to the concept that upregulation of ABCA1 is an attractive target for future CVD risk reduction.

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Conflict of interest

None.
Reference List


ABCA1 mutation carriers are characterized by increased arterial stiffness

Submitted for publication

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A.G. Holleboom, R. Duivenvoorden, E.S.G. Stroes
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Abstract

Objectives
Carriers of mutation in ATP-binding cassette transporter A1 (ABCA1) are characterized by life-long low plasma HDL-c levels and increased atherosclerosis. Evidence abounds on the role of ABCA1 in endothelial function in animal models, but no human data exist. Pulse wave velocity (PWV) is a reliable measure of arterial wall stiffness. PWV has been shown to be associated with ABCA1-mediated cholesterol efflux and endothelial function and constitutes an independent risk factor for cardiovascular disease. This prompted us to investigate whether PWV is increased in ABCA1 mutation carriers and to assess its correlations with carotid vessel wall thickness and HDL-c.

Methods
Lipid profiles, PWV and vessel wall thickness were assessed in 26 ABCA1 mutation carriers, 52 normolipidemic matched controls and 15 subjects with low HDL-c without underlying genetic defect.

Results
Plasma HDL-c was 42% lower in ABCA1 mutation carriers compared to normolipidemic controls. PWV was 21% higher in ABCA1 mutation carriers (p=0.007) compared to controls, independent of conventional risk factors. Furthermore, PWV correlated significantly with vessel wall thickness in both carriers and controls (p=0.002 and 0.007, respectively). PWV did not correlate with HDL-c (p=0.18 and 0.85, respectively).

Conclusions
This is the first evidence that ABCA1 mutation carriers are characterized by increased PWV, independent of HDL-c. This reflects their increased CHD risk and may constitute the first evidence of endothelial dysfunction in human ABCA1 mutation carriers. Given the correlations with vessel wall thickness, PWV may be a valuable, cost-effective, non-invasive addition to current CVD monitoring practice.
**Introduction**

Plasma high density lipoprotein (HDL) cholesterol is inversely correlated with coronary heart disease (CHD) risk.\(^1\) Carriers of mutation in the ATP-binding cassette transporter (ABC) A1 have dramatically decreased plasma levels of HDL-c. \(ABCA1\) mutation carriers have a loss-of-function of the \(ABCA1\) protein, which is responsible for the lipidation of lipid-poor apolipoprotein (apo) AI, and are characterised by increased atherosclerosis\(^2,3\) and CHD risk.\(^4,5\)

The increased CHD risk in \(ABCA1\) mutation carriers is thought to be attributable to decreased transport of vessel wall cholesterol to plasma HDL, a process known as reverse cholesterol transport (RCT).\(^6-8\) Indeed, homozygosity for \(ABCA1\) mutations, a condition known as Tangier disease, is characterized by foamy macrophages in both the vessel wall and other organs such as tonsils and spleen.\(^9\) Whether the pro-atherogenic phenotype in \(ABCA1\) mutation carriers is associated with functional changes of large arteries is unresolved to date.

Pulse wave velocity is a strong and independent predictor of CHD\(^10-12\) and constitutes the gold-standard for non-invasive measurement of arterial stiffness.\(^13\) PWV has emerged in recent years as a novel biomarker for predicting cardiovascular mortality and morbidity.\(^13\)

We have previously shown that PWV was increased in lecithin-cholesterol acyltransferase (\(LCAT\)) mutation carriers, another form of genetically low HDL-c, attributed to impaired RCT.\(^14\) Furthermore, a recent study reports an association between PWV and cholesterol efflux, as assessed by addition of participants’ plasma to an \(ABCA1\)-expressing cell line \textit{ex vivo}.\(^15\) Last, endothelial function is an important regulator of arterial stiffness. In animal models, \(Abca1\) has been shown to play a crucial role in endothelial function.\(^16\) However, whether this translates into increased PWV in human \(ABCA1\) mutation carriers, remains to be established.

We compared PWV as a measure of arterial stiffness in carriers of loss of function mutations in \(ABCA1\) to normolipidemic controls and low HDL-c subjects. In addition, we assessed the association between PWV and carotid ultrasound measurements and carotid magnetic resonance imaging to determine its association with anatomical large artery changes. We hypothesized that \(ABCA1\) mutation carriers have increased PWV as compared to matched controls.

**Methods**

**Study design**

Subjects with low HDL-c levels, defined as HDL-c < 5\(^{th}\) percentile, were selected from a cohort of hypoalphalipoproteinemia patients\(^17\) and screened for \(ABCA1\) mutations. Family members of \(ABCA1\) mutation carriers were actively recruited. In order to limit referral bias, we excluded family probands who were referred to our outpatient clinic with clinically manifest CVD. Unaffected family members were asked to participate as controls. Since an insufficient number of family members consented to participation, the control group was complemented with unrelated controls recruited by advertisement. Carriers of functional \(ABCA1\) mutations (n=26) were matched one on two to controls (n=52) for age and gender. Twenty six \(ABCA1\) mutation carriers were furthermore compared to a low HDL control group of 15 individuals without underlying genetic defect, matched for age, gender and plasma...
HDL-c. All participants provided written informed consent. The study was conducted at the Academic Medical Center in Amsterdam, the Netherlands from March 2010 to November 2011. The study protocol was approved by the Institutional Review Board.

Presence of cardiovascular risk factors, use of medication and family history of CVD were assessed by a questionnaire. Body mass index (BMI) was calculated from weight and length. Blood was obtained after an overnight fast and stored at -80 °C. Plasma cholesterol, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c) and triglycerides (TG) were analysed using commercially available kits (Randox, Antrim, United Kingdom and Wako, Neuss, Germany). Plasma apolipoprotein AI and apolipoprotein B were measured using a commercially available turbidometric assay (Randox, Antrim, United Kingdom). All analyses were performed using the Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

Genotyping
Mutation detection was performed as published previously. In short, the sequence reactions were performed using a BigDye terminator ABI prism kit (Applied Biosystems, Foster City, CA, USA). Sequences were analysed with the Sequencher package (Gene Codes Co, Ann Arbor, Mi, USA).

Blood pressure and PWV measurements
Participants visited the hospital after an overnight fast and were asked to refrain from smoking (if applicable) at least 3 hrs before the visit. All measurements were carried out in supine position after 15 min rest in a quiet, temperature-controlled room. The investigators were blinded for the genetic status of the participants. Brachial blood pressure was measured 3 times at 1- min intervals in supine position at the right arm after 15 min rest using a validated oscillometric device (Omron 705IT). 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 imaging
Carotid B-mode ultrasound scans of the left and right common, bulb and internal carotid arterial far walls were assessed as previously published. One reader analyzed all the images, blinded for group and any other data of the participants. The ultrasound parameter was mean common carotid intima-media thickness (CIMT), defined as the average far wall IMT of the left and right distal 1cm of the common carotid artery.

Carotid magnetic resonance imaging
Scans were performed in a random subset of study participants as described previously. In short, scans were obtained in a 3.0 Tesla Philips whole-body scanner (Philips, Best, the Netherlands), using a single-element microcoil (Philips, Hamburg, Germany). Ten slices
were scanned of the distal 3.0 cm of the left and right common carotid artery. A total of 20 images were obtained per scan. Images were saved in DICOM format using standardized protocols. Quantitative image analysis was performed using semi-automated measurement software (VesselMass, Leiden University Medical Center, the Netherlands). One trained reader, with excellent scan-rescan and intraobserver variability analyzed all the images using standardized protocols for reading and rating images combined with dedicated semi-automated software, blinded for all data of the participants. Lumen area (LA) and outer wall area (OWA) were measured. Normalized wall index (NWI) was calculated as: NWI = MWA / OWA. Mean wall area is calculated as: MWA = meanOWA - meanLA.

Statistical analysis
Continuous variables are expressed as means ± standard deviations (SD), unless otherwise specified. Possible differences in demographic, biometrical and biochemical parameters between carriers of ABCA1 mutations and controls were assessed using unpaired Student’s t-tests, Chi square tests or Mann-Whitney U-test, where appropriate. Differences in carotid imaging parameters between ABCA1 mutation carriers and controls were assessed using unpaired Student’s t-tests, unless otherwise specified. We chose to test correlations in the most conservative way, using nonparametric spearman’s rho coefficients and corresponding p-values. Difference between correlations were tested by means of a Fisher r-to-z transformation.

Results
Clinical characteristics
Baseline characteristics of study participants are listed in table 1. Twenty six ABCA1 mutation carriers from 14 separate families were included and compared to 52 normolipidemic controls, matched for age and gender, as well as 15 low HDL-c subjects. ABCA1 mutation carriers comprised two homozygous, two compound heterozygous and 21 heterozygous patients. Subjects were carriers of the following mutations: p.Leu1056Pro, c.3535+1G>C, c.6401+2T>C, p.Asn1800his, p.Phe1760Valfs*21, p.Cys1477Arg, p.Asp571Gly, p.Gln1038Ter, p.Thr929Ile, p.Arg587Trp, p.Asn935Ser and p.Arg579Gln. Cholesterol efflux impairment of all mutations has been previously published. CVD was more prevalent in ABCA1 mutation carriers than controls (p=0.03). Total cholesterol was 16% lower in ABCA1 mutation carriers (p=0.007), largely due to a 42% reduction in HDL-c (p<0.001), but was similar in low HDL-c subjects. Apolipoprotein AI was correspondingly decreased by 30% (p<0.001). Systolic blood pressure was higher in carriers compared to controls (p=0.04). Baseline characteristics were similar between ABCA1 mutation carriers and low HDL-c controls except for a trend towards higher triglycerides (p=0.07) and less males (p=0.09) in the low HDL control group.

Pulse wave velocity and vessel wall thickness in carriers of ABCA1 mutations and matched controls
PWV was increased in ABCA1 mutation carriers compared to controls (8.61±2.44 vs 7.14±1.34, p=0.007, table 2 and figure 1). Significance was retained after adjustment for
age, gender, BMI, history of CVD, statin use, smoking, diabetes and mean arterial pressure 
\( (p=0.007, \text{figure 1}) \). No gene-dose effect was observed when comparing homozygous and compound heterozygous \( \text{ABCA1} \) mutation carriers to heterozygous mutation carriers or controls. After sensitivity analysis, excluding the two and three carriers with highest PWV values, significance was retained \( (p=0.02 \text{ and } p=0.04 \text{ respectively}) \). In order to discern an \( \text{ABCA1} \) specific effect from an HDL-c effect, we also assessed PWV in subjects with low plasma HDL-c without an underlying genetic defect. PWV in \( \text{ABCA1} \) mutation carriers did not differ from low HDL-c subjects \( (8.61\pm2.44 \text{ vs } 7.98\pm1.80, p=0.39 \text{ (table 2)}) \).

Normalized wall index was increased in \( \text{ABCA1} \) mutation carriers compared to normolipidemic controls \( (p=0.007) \) but not compared to low HDL-c controls \( (p=0.15), \)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n=52)</th>
<th>( \text{ABCA1} ) mutation carriers (n=26)</th>
<th>( P^1 )</th>
<th>Low HDL controls (n=15)</th>
<th>( P^1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>46.6±11.7</td>
<td>49.7±16.2</td>
<td>0.34</td>
<td>48.7±10.8</td>
<td>0.84</td>
</tr>
<tr>
<td>Male sex, n (%)*</td>
<td>33 (64)</td>
<td>16 (62)</td>
<td>0.87</td>
<td>2 (13)</td>
<td>0.09</td>
</tr>
<tr>
<td>Body Mass Index (kg/m(^2))</td>
<td>25.8±3.5</td>
<td>25.1±3.1</td>
<td>0.37</td>
<td>26.7±3.1</td>
<td>0.41</td>
</tr>
<tr>
<td>Smokers, n (%)*</td>
<td>5 (10)</td>
<td>6 (23)</td>
<td>0.23</td>
<td>0</td>
<td>~</td>
</tr>
<tr>
<td>Diabetes, n (%)*</td>
<td>1 (2)</td>
<td>1 (4)</td>
<td>0.74</td>
<td>1 (8)</td>
<td>0.61</td>
</tr>
<tr>
<td>History of CVD n (%)*</td>
<td>2 (4)</td>
<td>5 (19)</td>
<td>0.03</td>
<td>2 (13)</td>
<td>0.63</td>
</tr>
<tr>
<td>Alcohol use n (%)</td>
<td>26 (79)</td>
<td>21 (81)</td>
<td>0.85</td>
<td>10 (83)</td>
<td>0.85</td>
</tr>
<tr>
<td>Statin use (%)*</td>
<td>6 (12)</td>
<td>7 (27)</td>
<td>0.09</td>
<td>5 (33)</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Blood pressure

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (mmHg)#</th>
<th>( \text{ABCA1} ) mutation carriers (mmHg)#</th>
<th>( P )</th>
<th>Low HDL controls (mmHg)#</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic (mmHg)#</td>
<td>129.8±12.4</td>
<td>136.5±13.4</td>
<td>0.04</td>
<td>135.4±11.6</td>
<td>0.79</td>
</tr>
<tr>
<td>Diastolic (mmHg)#</td>
<td>78.8±9.1</td>
<td>77.6±9.2</td>
<td>0.59</td>
<td>81.7±9.6</td>
<td>0.36</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>95.0±9.6</td>
<td>97.8±9.6</td>
<td>0.26</td>
<td>100.0±9.7</td>
<td>0.63</td>
</tr>
<tr>
<td>Hypertension, n (%)*</td>
<td>6 (13)</td>
<td>6 (25)</td>
<td>0.19</td>
<td>3 (27)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Lipid metabolism

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (mmol/L)</th>
<th>( \text{ABCA1} ) mutation carriers (mmol/L)</th>
<th>( P )</th>
<th>Low HDL controls (mmol/L)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.94±1.03</td>
<td>4.17±1.33</td>
<td>0.007</td>
<td>4.65±1.47</td>
<td>0.30</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.21±0.78</td>
<td>2.91±1.07</td>
<td>0.23</td>
<td>3.31±0.92</td>
<td>0.26</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.40±0.45</td>
<td>0.81±0.33</td>
<td>&lt;0.001</td>
<td>0.87±0.23</td>
<td>0.49</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)#</td>
<td>0.85 (0.69-1.38)</td>
<td>1.10 (0.72-1.59)</td>
<td>0.15</td>
<td>1.64 (1.38-2.39)</td>
<td>0.07</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dL)</td>
<td>108.1±24.54</td>
<td>111.6±40.69</td>
<td>0.67</td>
<td>113.10±26.45</td>
<td>0.91</td>
</tr>
<tr>
<td>Apolipoprotein A-I (mg/dL)</td>
<td>147.20±26.82</td>
<td>102.28±33.07</td>
<td>&lt;0.001</td>
<td>127.22±22.02</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Values are indicated as mean ± SD unless otherwise indicated. \( P \)-values are for student’s T-test unless otherwise indicated. \( P^1 \) compares carriers to controls; \( P^2 \) compares carriers to low HDL-C controls. * \( p \) for \( X^2 \) test. #: median and interquartile range; \( P \) for Mann Whitney U test.
while carotid ultrasound was not different between groups, which is in line with our earlier observations (p=0.62 and 0.64, respectively) (table 2).

**Figure 1.** Carotid-femoral pulse wave velocity in ABCA1 mutation carriers, normolipidemic controls and low HDL-c controls. p\(^1\) is unadjusted p-value; p\(^2\) is adjusted for age, sex, BMI, CVD, statin use, smoking, diabetes and mean arterial pressure.

**Correlation between PWV and vessel wall thickness**

PWV was significantly correlated with cIMT in carriers (p=0.62, p=0.003, figure 2a), as well as in low HDL-c controls (p=0.83, p<0.001) and normolipidemic controls (p=0.44, p=0.006, figure 2a). In line, PWV was significantly correlated with NWI in carriers (p=0.64, p=0.002, figure 2b) and normolipidemic controls (p=0.44, p=0.007, figure 2b). There was a trend towards a correlation between PWV and NWI in low HDL-c controls (p=0.54, p=0.07). There was no significant difference in the PWV-vessel wall thickness correlations between the groups.
Table 2. Pulse wave velocity and vessel wall thickness parameters in ABCA1 mutation carriers, normolipidemic controls and low HDL-c controls

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>ABCA1 mutation carriers</th>
<th>P1</th>
<th>Low HDL controls</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWV m/s</td>
<td>7.14±1.34</td>
<td>8.61±2.44</td>
<td>0.007</td>
<td>7.98±1.80</td>
<td>0.39</td>
</tr>
<tr>
<td>NWI</td>
<td>0.32±0.04</td>
<td>0.36±0.06</td>
<td>0.007</td>
<td>0.34±0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>cIMT (mm)</td>
<td>0.66±0.15</td>
<td>0.64±0.20</td>
<td>0.62</td>
<td>0.64±0.11</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Values are indicated as mean ± SD. P-values are for student’s T-test. PWV is pulse wave velocity, NWI is normalized wall index, cIMT is carotid intima media thickness. MRI data were available in 36 controls, 21 carriers and 12 low HDL-c controls. cIMT data were available in 38 controls, 21 carriers and 12 low HDL-c controls.

Correlation between PWV and HDL-c

PWV was not associated with plasma HDL-c levels in carriers (ρ=0.28; p=0.18, figure 3), low HDL-c controls (ρ=-0.18, p=0.53) nor in normolipidemic controls (ρ=0.03; p=0.85, figure 3). There was no significance difference in the PWV-HDL-c correlations between the groups.

Discussion

In this study, we show that aortic pulse wave velocity is increased in ABCA1 mutation carriers compared to controls, indicating increased arterial stiffness in ABCA1 mutation carriers. In addition, PWV correlated strongly with measures of carotid artery wall thickness.

The increased arterial stiffness in ABCA1 mutation carriers is in line with the study by Favari and co workers, showing that PWV is inversely correlated with ABCA1 dependent cholesterol efflux, measured as cholesterol efflux from J774 macrophages to serum from study participants. However, whether PWV is increased in ABCA1 mutation carriers, was never investigated. Previously, we showed that PWV is also increased in another group of subjects with a defect in the reverse cholesterol transport pathway downstream of ABCA1. In LCAT mutation carriers, esterification of nascent HDL particles is impaired, resulting in a lesser concentration gradient for cholesterol to flux from macrophage to HDL, resulting in more intracellular cholesterol. These findings may point to a detrimental role of intracellular cholesterol accumulation in vascular homeostasis, resulting in increased arterial stiffness.

The mechanism underlying increased PWV in ABCA1 mutation carriers may pertain to decreased endothelial function in response to reduced cholesterol efflux. In animal models, removal of the endothelium altered arterial stiffness. Terasaka and co workers showed that cholesterol accumulation in endothelial cells leads to impaired release of the vasodilator NO, due to an inhibitory interaction between caveolin 1 and endothelial NO synthase (eNOS). In line, blocking NO synthesis in humans increases arterial stiffness. Westerterp and co workers subsequently published that cholesterol accumulation in endothelial cells is associated with increased atherosclerosis in mice, possibly linking endothelial function to atherogenesis. In humans, endothelial function is inversely associated with arterial stiffness, suggesting that the increased PWV in ABCA1 mutation carriers is a reflection...
Figure 2. correlation between PWV and vessel wall thickness. a. correlation between PWV and intima media thickness (carotid ultrasound). b. correlation between PWV and normalized wall index (carotid MRI). a. carotid-femoral PWV plotted to carotid IMT. Continuous line indicates correlation between PWV and carotid IMT in controls (open symbols, n=38). Dashed line indicates correlation between PWV and carotid IMT in carriers (closed symbols, n=22). b. carotid-femoral PWV plotted to normalized wall index (NWI). Continuous line indicates correlation between PWV and NWI in controls (open symbols, n=36). Dashed line indicates correlation between PWV and NWI in carriers (closed symbols, n=22).

of decreased endothelial function due to cellular cholesterol accumulation. In line with this hypothesis, the slope of the correlation between NWI and PWV appeared to be steeper in ABCA1 mutation carriers than in controls, suggesting that arterial stiffness is caused by ABCA1 specific vascular alterations. This is further supported by the finding that there was no significant correlation between PWV and the most sensitive parameter of vessel wall thickness, MRI. Furthermore, the strong correlation between NWI and PWV was not found in for example Fabry patients, who are not characterized by decreased RCT. Alternatively, atherosclerosis has been suggested to directly cause arterial stiffness by inducing alterations
in the vessel wall. Our findings that NWI is increased in ABCA1 mutation carriers and strongly associates with PWV may be interpreted as supportive of this concept.

Interestingly, PWV did not correlate with plasma HDL-c levels. This is in line with the finding by Favari and co workers, that PWV correlates with ABCA1 mediated cholesterol efflux, independently of plasma HDL-c. Furthermore, this is in line with the absence of a difference between ABCA1 mutation carriers and low HDL-c controls. In addition, we and others have shown that in a cohort of LCAT mutation carriers, PWV was also found to be associated with vessel wall thickness, but not with plasma HDL-c levels. This notion supports the hypothesis that intracellular cholesterol accumulation due to ABCA1 deficiency underlies decreased endothelial function, resulting in increased PWV.

The finding that PWV was higher in ABCA1 mutation carriers, characterized by low plasma HDL-c, but did not correlate with plasma HDL-c concentration is intriguing. In line, PWV was not different in low HDL-c subjects compared to normolipidemic controls. Our limited sample size hampers definite conclusions, but our findings support the concept that ABCA1 deficiency and not plasma HDL-c levels underlies increased PWV. The finding of increased PWV in low HDL-c subjects compared to normolipidemic controls can be explained by the fact that cardiovascular risk factors are likely to be present in low HDL-c subjects without underlying genetic defect, whereas in ABCA1 mutation carriers, low HDL-c may be attributable to genetic deficiency alone.

Interestingly, no gene-dose effect was observed when comparing PWV in homozygous ABCA1 mutation carriers to heterozygous ABCA1 mutation carriers. This is likely due to the small sample size of homozygous/compound heterozygous carriers and may furthermore reflect the propensity of physicians to do everything within their means to reduce CVD risk in these patients with near-absent HDL-c. Alternatively, compensating mechanisms may have occurred in response to life-long exposure to extremely low HDL-c levels.

Figure 3. correlation between HDL-c and pulse wave velocity. Carotid-femoral PWV plotted to plasma HDL-c. Continuous line indicates correlation between PWV and carotid IMT in controls (open symbols). Dashed line indicates correlation between PWV and carotid IMT in carriers (closed symbols).
Several aspects of our study merit closer consideration. The sample size is relatively small, inherent to the low prevalence of ABCA1 mutation carriers in the general population. However, an important strength of our study is the individual matching of ABCA1 mutation carriers to controls. Although not significant, the percentage of males is lower in the group of low HDL-c subjects. PWV did not differ between males and females in any of our groups, indicating that gender did not significantly influence PWV in our cohort. Furthermore, systolic blood pressure was higher in ABCA1 mutation carriers compared to controls. However, the increased PWV in carriers retained significance after correction for systolic blood pressure, indicating that the increased PWV in ABCA1 mutation carriers is not attributable to the increased systolic blood pressure. Finally, the cross-sectional design of this study precludes us from answering whether increased PWV is causal or secondary to atherogenesis.

**Conclusion**

In this study, we provide the first evidence for the concept that ABCA1 mutation carriers are characterized by increased PWV, independent of HDL-c. This reflects their increased CHD risk and may constitute the first evidence of endothelial dysfunction in human ABCA1 mutation carriers. Given the correlations with vessel wall thickness, PWV may be a valuable, cost-effective, non-invasive addition to current CVD monitoring practice.

**Perspectives**

PWV is a reproducible, non-invasive and readily applicable proxy of vessel wall condition. Especially given its strong correlations with measures of vessel wall thickness in both study groups, the present work supports it to be a valuable technique to assess and monitor CVD risk in high risk patients. Furthermore, the finding that PWV is increased in ABCA1 mutation carriers, possibly due to a direct ABCA1-related effect on the vessel wall, opens the door for specific interventions aiming at specific ABCA1 upregulation in order to improve endothelial function. Given the finding that with conventional CVD risk reduction a substantial residual risk of 65-75% remains, implicates that a large population may benefit from such interventions. PWV would be the pre-eminent parameter for monitoring such therapy.

**Acknowledgements**

We are indebted to all study participants. The authors would like to thank C.A.M. Koch en J.F. Los for their assistance in expanding the pedigrees and J. Peter for his role in identification of ABCA1 mutation carriers.

**Sources of funding**

This work was supported by the Dutch Heart Foundation (grant numbers 2008B070 and 2009B027). Andrea Bochem is supported by fellowship WdL/HE/12-029 from the Saal van Zwanenbergstichting, the Netherlands.

**Conflicts of interest**

None.
Reference List


Cardiovascular event in a 36 year-old man with combined \textit{ABCA1} and apoA-I deficiency

\textit{Submitted for publication}

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A 36-year-old man was admitted because of acute chest pain. Upon diagnostic procedures, an acute myocardial infarction (AMI) was confirmed and a percutaneous coronary intervention (PCI) was performed with stent placement of a bare metal stent (BMS) in the ramus circumflex. After one week, an elective PCI with BMS placement in the right coronary artery was performed.

The patient reported to smoke. His blood pressure was 133/97 mmHg and BMI was 27.8 kg/m2. No murmurs over peripheral arteries, xanthomas, xanthelasmata or arcus lipoides were observed. Laboratory results were unremarkable except for a near absence of HDL-c (0.09 mmol/L).

The patient has two healthy brothers, with unknown HDL-c levels. His father experienced an AMI at the age of 48. His father’s brother had an AMI at the age of 50 and his father’s sister died from an unknown cause at the age of 50. His mother is 57 years-old and healthy except for diabetes and extremely low HDL-c levels (0.21 mmol/L) (figure C).

Genes involved in HDL-c metabolism were sequenced and both an ATP-binding cassette transporter A1 (ABCA1) mutation (p.Asn1800His;c.5398A>C) and an apolipoprotein AI (apoA-I) mutation (p.Leu202Pro;c.605T>C) (figure D) were identified. ABCA1 is essential for reverse cholesterol transport (RCT) by virtue of its ability to transport cholesterol from macrophages to lipid-poor apoA-I, the major protein constituent of the HDL particle. This combined molecular defect, which has not been described before, is consistent with the observed near absence of HDL-c and is likely to have constituted a substantial risk factor in this patient, since there was no abundance of other cardiovascular risk factors. Since specific HDL-c increasing therapy is not available yet, prevention focuses on modulating other risk factors such as LDL-c lowering.
Increased arterial stiffness in *ABCA1* mutation carriers.

Left coronary artery: occlusion in ramus circumflex (culprit lesion), no significant lesion in the left anterior descending branch; B. Right coronary artery: significant stenosis in mid portion with pre and post dilatation of the vessel; C. Patient’s pedigree: lipid profiles are in mmol/L, percentiles for age and gender between brackets; D. Mutations in *Apolipoprotein A-I* and *ATP-binding cassette transporter A1*. 
Two novel mutations in Apolipoprotein C3 underlie atheroprotective lipid profiles in families

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⁴Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, BC, Canada
Abstract

Objectives
Apolipoprotein C3 (APOC3) mutations carriers typically display high plasma high-density lipoprotein cholesterol (HDL-C) and low triglycerides. We set out to investigate the prevalence and clinical consequences of APOC3 mutations in individuals with hyperalphalipoproteinemia.

Methods and Results
Two novel mutations (c.-13-2A>G and c.55+1G>A) and one known mutation (c.127G>A;p. Ala43Thr) were found. Lipid profiles and apoCIII isoform distributions were measured. c.55+1G>A mutation carriers displayed higher HDL-C percentiles (35.6±35.8 vs 99.0±0, p=0.002) and lower triglycerides (0.51 (0.37-0.61) vs 1.42 (1.12-1.81) mmol/L, p=0.007) and apoCIII levels (4.24±1.57 vs 7.33±3.61 mg/dL, p=0.18). c.-13-2A>G mutation carriers did not display significantly different HDL-C levels (84.0±30.0 vs 63.7±45.7, p=0.50), a trend towards lower triglycerides (0.71 (0.54-0.78) vs 0.85 (0.85~) mmol/L, p=0.06) and significantly lower apoCIII levels (3.09±1.08 vs 11.45±1.06 mg/dL, p=0.003). p.Ala43Thr mutation carriers displayed a trend towards higher HDL-C percentiles (91.2±31.8 vs 41.0±29.7 mmol/L, p=0.06) and significantly lower triglycerides (0.58 (0.36-0.63) vs 0.95 (0.71-1.20) mmol/L, p=0.02) and apoCIII levels (4.92±2.33 vs 6.60±1.60, p=0.25).

Conclusion
Heterozygosity for APOC3 mutations results in high HDL-C and low triglycerides and apoCIII levels. This favourable lipid profile in patients with genetically low apoCIII levels holds promise for current studies investigating the potential of apoCIII inhibition as a novel therapeutic in CVD prevention.
Introduction

Large prospective epidemiological studies have consistently demonstrated a strong inverse relationship between plasma levels of high-density lipoprotein cholesterol (HDL-C) and the risk of cardiovascular disease (CVD). Furthermore, fasting as well as postprandial triglyceride (TG) levels have been shown to be associated with CVD risk. Plasma HDL-C and triglyceride levels are mechanistically closely and inversely linked. Apolipoprotein (apo) CIII plays a pivotal role in HDL-C and TG metabolism, by virtue of its lipoprotein lipase (LPL) inhibiting activity. In genome wide association studies (GWAS), genetic variants in APOC3 are both associated with high HDL-C levels as well as low triglyceride levels. Moreover, common variations in APOC3 have been shown to be associated with CVD risk. Pollin et al confirmed the importance of apoCIII as an independent risk factor for CVD in humans by showing that carriers of a null mutation in APOC3 exhibit 50% of normal plasma apoCIII levels, lower fasting and postprandial serum triglycerides and less subclinical atherosclerosis, as measured by the extent of coronary artery calcification.

Studies in patients with familial disorders of lipoprotein metabolism have led to significant advances in our understanding of HDL metabolism, including the identification of key players involved in HDL biogenesis, transport and modification such as ATP binding cassette transporter AI (ABCA1), apolipoprotein AI (APOAI), lecithin cholesterol acyl transferase (LCAT) and cholesteryl ester transfer protein (CETP). ApoAI, LCAT and CETP-based therapies could indeed modify lipid profiles in a favourable manner, especially in terms of HDL-C levels. However, none of these therapies have thus far shown a consistent beneficial effect on CVD outcome or atherosclerosis progression.

Whereas increasing plasma HDL-C by CETP inhibition does not lead to the desired CVD risk reduction, fibrates, both lowering triglycerides and increasing HDL-C, reduce CVD risk in subgroups characterized by high triglycerides. Noteworthy, apoCIII plasma levels are inversely associated with both plasma triglyceride levels and CVD risk. This indicates that in large populations, inhibition of apoCIII may be beneficial. In line with this, antisense inhibition of apoCIII induced reductions of plasma apoCIII and triglycerides in a recent phase I study. A phase II antisense intervention trial (ISIS-APOCIII Rx Clinical Trials.gov: NCT01529424) has been started.

We set out to study the consequences of novel mutations in APOC3 in an independent cohort. In this paper, we report the lipid profiles as well as apoCIII isoform distribution in carriers of two new APOC3 mutations and one previously published mutation.

Material and methods

Recruitment of the Study Family

The coding regions of APOC3 were sequenced in 80 unrelated probands with plasma HDL-C > 95th percentile for age and gender. Family members of APOC3 mutation carriers were recruited. Unaffected family members participated as controls. All participants provided written informed consent. The study was conducted at the Academic Medical Center in
Amsterdam, the Netherlands. The study protocol was approved by the Institutional Review Board of the Academic Medical Center in Amsterdam, the Netherlands.

**Mutation analysis**

Genomic DNA was extracted from 10 ml whole blood on an Autopure LS system according to the manufacturer’s instructions (Gentra Systems, Minneapolis, USA). *APOC3* was sequenced using filter-based hybridization capture as described. Mutations were validated using conventional Sanger sequencing. Primers were designed using Primer 3 software to cover the identified mutations in *APOC3* (NM_000041.1). An M13 tail was added to each primer (forward: 5’-GTTGTAAACGACGGCCACT-3’ and reverse: 5’-CACAGGAAACAGCTATGACC-3’) to facilitate DNA sequencing. Sequence reactions were performed as described. The coding DNA numbering starts at the ATG transcription start site according to the official nomenclature provided by the Human Genome Variation Society (www.hgvs.org/mutnomen).

**Patient characteristics**

Presence of cardiovascular risk factors, use of medication and family history of CVD were assessed by a questionnaire. Blood was obtained after an overnight fast and stored at -80 °C. Plasma cholesterol, low-density lipoprotein cholesterol (LDL-C), HDL-C and triglycerides were analysed using commercially available kits (Randox, Antrim, United Kingdom and Wako, Neuss, Germany). Since gender was (unequally distributed between carriers and family controls, HDL-C percentiles for age and gender are calculated. Plasma apoA-I, apoB and apoCIII (Randox, Antrim, United Kingdom) were measured using a commercially available turbidometric assay. All analyses were performed using the Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

**Fast performance liquid chromatography**

Individual patient plasma samples were fractioned using fast performance liquid chromatography (FPLC) analysis to determine lipoprotein particle size. In brief, 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 P-50 pump (Pharmacia Biotech, Uppsala, Sweden) was used for in-line cholesterol and TG (4-Amino-antiperine-peroxidase) PAP enzymatic reagent (Biomerieux, Marcy l’Etoile, France) addition at 0.1 mL/min. Ethylenediaminetetraacetic acid (EDTA) plasma was diluted 1:1 with Tris buffered saline and 60 μL sample/buffer mixture was loaded on a Superose 6 HR 10/30 column (GE Health care, Life sciences division, Diegem, Belgium) for lipoprotein separation at a flow rate of 0.31 mL/min.

**ApoCIII isoforms and distribution among lipoproteins**

Plasma apoCIII isoforms were separated with isoelectric focusing (IEF) gel electrophoresis on the Phast-gel system (Pharmacia) as previously described. Two Phastgel Dry IEF gels were hydrated with 5 ml solution containing 8M urea, 390 μl pharmalyte 4.2-4.9; 195 μl pharmalyte 2.5-5.0 for 1.5h at room temperature. Plasma was diluted 15 times with 0.9% NaCl followed by application on IEF gels. Samples were run on standard program 2 of the Phast system followed
by transfer to polyscreen polyvinylidene fluoride (PVDF) transfer membranes (Perkin Elmer NEF1002, Waltham, Massachusetts, USA), blocked with Odyssey blocking buffer (Westburg) for 1 h and incubated with rabbit anti human apoCIII (Abcam 21032; 1:2000) overnight at room temperature and after extensive washing stained for 1 h at room temperature with donkey anti rabbit IRdye 800CW (1:5000; Westburg, 926-32212, Leusden, the Netherlands). Blots were imaged on the Odyssey system (Licor, Westburg, Leusden, the Netherlands). IEF resolves apoCIII into three isoforms, apoCIII\(_0\), apoCIII\(_1\), and apoCIII\(_2\).

Distribution of apoCIII among HDL and TG-rich apoB-containing lipoprotein particles was analysed using immunoelectrophoresis using LpCIII hydragel (Sebia) according to the manufacturer’s manual.

**Statistical analysis**

Continuous variables are expressed as means ± standard deviations (SD), unless otherwise specified. Differences in demographic, biometrical and biochemical parameters between carriers of APOC3 mutations and controls were assessed using unpaired Student’s t-tests or Chi square tests, where appropriate. A p-value of 0.05 or less was considered statistically significant.

**Results**

**APOC3 Mutations**

In 5 of the 80 individuals (6%) we identified three mutations in APOC3 (NM_000040.1): Three heterozygous carriers of c.55+1G>A (rs138326449), one heterozygous carrier of c.-13-2A>G and one heterozygous carrier of c.127G>A (p.Ala43Thr, rs147210663). The p.Ala43Thr mutation was previously known as p.Ala23Thr. The new mutation nomenclature guidelines (http://www.dmd.nl/mutnomen/recs-prot.html) set the ATG start site as codon number 1. This explains the 20 amino acid difference compared to the old nomenclature. None of the mutations were found in 40 individuals with low HDL-C levels. The frequencies of these mutations in a population sample of 4000 individuals were very low (minor allele frequencies of 0.0019, 0.0001 and 0.0009, respectively). The c.55+1G>A (rs138326449) mutation leads to a change in a highly conserved nucleotide and disrupts the canonical splice site, leading to a predicted skipping of exon 2, which may affect the apoCIII protein. The p.Ala43Thr mutation is located in exon 3 and changes a highly conserved nucleotide leading to a change in amino acid 43. p.Ala43Thr is predicted to be deleterious by SIFT prediction software. The c.-13-2A>G mutation is located in intron 1, prior to the ATG start site in exon 2. This mutation is predicted to disrupt the splice site upstream of exon 2.

**Pedigrees**

Pedigrees A, B and C are shown in figure 1. Two of the c.55+1G>A mutation carriers were from families which could not be further expanded. Their pedigrees are not shown. Cosegregation of the variants with the lipid phenotype (high HDL-C/low triglycerides) was observed with the exception of one case of non-penetrance in pedigree C. There was one phenocopy in pedigree A.
Chapter 8

a. Pedigree A: c.-13-2A>G

b. Pedigree B: C.55+1G>A
Baseline characteristics

Baseline characteristics of $APOC3$ mutation carriers and family controls are shown in table 1. HDL-C levels in heterozygous carriers of p.Ala43Thr were not significantly different from controls (2.19±0.77 vs 1.43±0.32, p=0.10). However, HDL-C percentiles, correcting for the unequal gender distribution between carriers and family controls, showed a trend towards an increase (91.17±31.83 vs 41.00±29.68 mmol/L, p=0.06) whereas triglyceride levels were significantly lower (0.58 (0.36-0.63) vs 0.95 (0.71-1.20) mmol/L, p=0.02). There was no difference in HDL size between carriers and non-carriers (HDL peak: 50.07 ±0.43 vs 49.60 ±0.65 minutes retention time, p=0.26). HDL-C levels and HDL-C percentiles were higher in heterozygous c.55+1G>A mutation carriers compared to family controls (2.25±0.41 vs 1.16±0.26, p=<0.001 and 35.63±35.77 vs 99.00±0, p=0.002 respectively) and triglyceride levels were lower (0.51 (0.37-0.61) vs 1.42 (1.12-1.81) mmol/L, p=0.007).
HDL size did not differ (48.88±0.86 vs 49.79±0.63 minutes retention time, p=0.18). In c.-13-2A>G mutation carriers, neither HDL-C levels (1.80±0.24 vs 1.81±0.71), nor HDL-C percentiles (84.00±30.00 vs 63.67±45.74, p=0.50) or HDL size (49.76±0.53 vs 49.50±1.4 minutes retention time, p=0.75) were significantly different, whereas carriers displayed a trend towards lower triglyceride levels (0.71 (0.54-0.78) vs 0.85 (0.85-~), p=0.06).

**Plasma apoCIII isoforms and distribution among lipoproteins**

Plasma apoCIII levels were lower in all mutation carriers. This difference was significant for c.-13-2A>G (p=0.003), but did not reach significance for c.55+1G>A and p.Ala43Thr mutation carriers (p=0.18 and 0.25 respectively) (figure 2). None of the apoCIII isoform distributions differed from control values (figure 3). ApoCIII is present on HDL and TG-rich apoB-containing lipoproteins. The percentage of apoCIII in HDL did not differ for c.-13-2A>G (74±11 vs 90±13, p=0.17), c.55+1G>A (71 ±8 vs 78 ±28, p=0.69) or p.Ala43Thr (67±4 vs 64±13, p=0.78).

**Discussion**

We have identified two new mutations in *APOC3* (c.-13-2A>G and c.55+1G>A) and one previously identified mutation (c.127G>A; p.Ala43Thr). The mutations were found in five individuals with plasma HDL-C above the 95th percentile. The atheroprotective lipid profile often ascribed to *APOC3* mutation carriership is most pronounced in c.55+1G>A and p.Ala43Thr. C.-13-2A>G mutation carriers show a trend towards lower triglyceride levels and HDL-C percentiles. ApoCIII levels are significantly lower in c.-13-2A>G mutation carriers, but not in c.55+1G>A or p.Ala43Thr mutation carriers. ApoCIII isoform distribution did not differ between carriers and controls.

ApoCIII resides on both HDL and apoB-containing particles and acts on triglyceride metabolism by inhibiting lipoprotein lipase (LPL) mediated lipolysis. Moreover, apoCIII inhibits hepatic uptake of apoB-containing lipoproteins, enhances catabolism of HDL particles, enhances monocyte adhesion to vascular endothelial cells and activates inflammatory signalling pathways. Our findings are in line with the notion that heterozygosity for *APOC3* mutations is associated with a favourable lipid profile. Not all of the lipid parameters reached significance, probably due to the low number of study participants in each group.

Previously, Liu and co workers have reported that the mutation p.Ala43Thr (p.Ala23Thr) leads to less efficient lipid binding, resulting in degradation of free apoCIII. They report that the mutation is phenotypically associated with low plasma triglyceride levels in two of three mutation carriers. This is in agreement with our findings. Interestingly, however, apoCIII levels were substantially lower than in our p.Ala43Thr mutation carriers (1.75 vs 4.92 mg/dl). Triglyceride levels were in the same range (0.74 vs 0.58 mmol/l), whereas HDL-C levels were higher in our carriers (1.14 vs 2.19 mmol/l). These differences cannot be attributed to age, gender or BMI.

Sundaram and co workers set out to investigate the effect of the p.Ala43Thr mutation on lipid metabolism and report that in McA-RH7777 cells transfected with the mutant apoCIII protein,
fusion of lipid droplets with apoB in the VLDL assembly pathway is impaired. It was therefore postulated that the absence of a structural element within the N-terminal region of the apoCIII protein is responsible for the loss of function of apoCIII due to the p.Ala43Thr mutation.36

The c.55+1G>A and c.-13-2G>A are novel variants located respectively in intron 2 and 1 of APOC3 and affecting splice sites. Both mutations are predicted to disrupt a 5’ splicing site. To the best of our knowledge, the consequences of these mutations have not been reported. Plasma apoCIII levels decreased to a similar extent in c.55+1G>A and c.-13G>A mutation carriers as compared to p.Ala43Thr mutation carriers. Plasma HDL-C levels and triglycerides of c.55+1G>A mutation carriers are in the range of p.Ala43Thr carriers and show a strong atheroprotective phenotype. The atheroprotective phenotype is less pronounced in c.-13-2G>A mutation carriers. However, it is noteworthy that HDL-C levels in both c.-13-2G>A carriers and family controls are above the 85th percentile. A possible explanation pertains to the fact that the control group consists of females only, naturally displaying higher HDL-C levels, whereas the carrier group consists of two males and two females.

ApoCIII has three different isoforms characterised by zero, one or two sialic acid residues. ApoCIII, and apoC-III, inhibit lipoprotein lipase (LPL), and are therefore crucial for triglyceride clearance. However, apoC-III, has limited capacity to inhibit LPL and thus increases triglyceride clearance capacity.37,38 Mutations in N-acetylgalactosaminyltransferase 2 (GALNT2) have been shown to cause a reduction of ppGalNAC-T2, leading to posttranslational modification of apoCIII, increased plasma levels of apoCIII, decreased plasma levels of apoCIII, and apoCIII, and improved postprandial triglyceride clearance.38 These data have established that apoCIII isoforms are key regulators of triglyceride metabolism and potentially key players in atherogenesis. In our cohort, apoCIII isoform distribution does not differ between cases and controls, which indicates that post translational modification is not influenced by the mutations described in this study.

The differences in apoCIII between carriers and non carriers were not associated with differences in apoCIII distribution among HDL and TG-rich apoB-containing lipoproteins.

**Figure 2.** ApoCIII plasma levels. Data are shown as mean ± SD. P for student’s T-test.
### Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pedigree A</th>
<th>Pedigree B</th>
<th>Pedigree C</th>
<th>p-value</th>
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<td>carriers</td>
<td>p-value</td>
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<td><strong>Characteristics</strong></td>
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<td>Age (years)</td>
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<td><strong>Medication use, n (%)</strong></td>
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<tr>
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<tr>
<td>Presence of diabetes, n (%)</td>
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<tr>
<td><strong>Lipid metabolism</strong></td>
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<td>Total cholesterol (mmol/L)</td>
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<td>5.15±0.94</td>
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<td>LDL-cholesterol (mmol/L)</td>
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<td>HDL-cholesterol (mmol/L)</td>
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<td>HDL-cholesterol percentile</td>
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<td>Triglycerides (mmol/L)</td>
<td>0.85 *</td>
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<td>Apolipoprotein B (g/L)</td>
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<td>Apolipoprotein A-I (g/L)</td>
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<td>Apolipoprotein C3 (mg/dl)</td>
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<td>Apolipoprotein C3 in HDL (%)</td>
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<td>Apolipoprotein C3 in non-HDL (%)</td>
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<td>11±13</td>
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<td>Retention time VLDL (minutes)</td>
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<td>Retention time LDL (minutes)</td>
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<td>49.50±1.44</td>
<td>49.76±0.53</td>
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</table>

Values are presented as mean ± SD unless otherwise indicated. Male sex, smokers, medication use, history of cardiovascular disease, presence of hypertension, presence of diabetes: p for χ² test; for other parameters: p for student’s t-test.
**Table 1. Baseline characteristics**

<table>
<thead>
<tr>
<th>Pedigree A</th>
<th>controls</th>
<th>carriers</th>
<th>p-value</th>
<th>Pedigree B</th>
<th>controls</th>
<th>carriers</th>
<th>p-value</th>
<th>Pedigree C</th>
<th>controls</th>
<th>carriers</th>
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<td>46±19</td>
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<td>HDL-cholesterol percentile</td>
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<td>Triglycerides (mmol/L)</td>
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<td>Apolipoprotein C3 in HDL (%)</td>
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<td>Apolipoprotein C3 in non-HDL (%)</td>
<td>71±8</td>
<td>48.88±0.86</td>
<td>0.18</td>
<td>50.07±0.44</td>
<td>49.60±0.65</td>
<td>0.26</td>
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<td>Apolipoprotein A-I (g/L)</td>
<td>29±8</td>
<td>22±27</td>
<td>0.69</td>
<td>34±4</td>
<td>64±14</td>
<td>0.78</td>
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<td>Apolipoprotein A-I (g/L)</td>
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<td>Apolipoprotein C3 (mg/dl)</td>
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<td>0.26</td>
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Values are presented as mean ± SD unless otherwise indicated. Male sex, smokers, medication use, history of cardiovascular disease, presence of hypertension, presence of diabetes: p for \( \chi^2 \) test; for other parameters: p for student’s t-test. For alcohol intake and triglycerides, median and interquartile range is reported; P for Mann Whitney U test. *Since triglyceride levels are 1.02, 0.85 and 0.85 mmol/L, no interquartile range could be calculated.
Figure 3. ApoCIII isoform distribution. Data are shown as mean ± SD. None of the isoform distributions differed significantly from control values; p for student’s t-test.

In contrast, von Eckardstein and co workers report that in a family with a Lys58Glu mutation, carriership was associated with a larger decrease of apoCIII in VLDL (-85%) compared to HDL (-75%). Furthermore, we did not find a change in HDL particle size in our APOC3 mutation carriers in contrast to the finding of larger HDL particles in Lys58Glu carriers by von Eckardstein and co workers. These discrepancies may be due to inclusion of different mutations, affecting different regions of the APOC3 gene.
Elevated plasma levels of low density lipoprotein cholesterol (LDL-C) and triglycerides are important contributors to premature coronary heart disease.\textsuperscript{40-42} Whereas the role of LDL-C in cardiovascular disease (CVD) is established, the role of triglycerides is more controversial. This is partly due to its inextricable linkage to HDL-C, a biomarker inversely correlated to CVD. Furthermore, plasma triglycerides are associated with traditional cardiovascular risk factors such as obesity and diabetes, thereby constituting a source of potential confounding. However, the Adult Treatment Panel (ATP) III guidelines has defined the normal range of triglycerides as $<150$ mg/dL (1.69 mmol/L).\textsuperscript{43} Furthermore, the ATPIII recommends that non-HDL cholesterol (LDL+VLDL, be used as a secondary target of therapy, after LDL-C lowering. Genetic studies are widely considered a powerful tool to study the influence of a biomarker on endpoints, since confounding factors are circumvented and lifelong exposure to the biomarker is guaranteed. We used this approach and show that carriers of \textit{APOC3} mutations are characterized by high levels of plasma HDL-C and low levels of plasma triglycerides. The majority of evidence indicates that this apparently favourable lipid profile translates into a low CVD risk. There is ample evidence that apoCIII might be a suitable target in CVD prevention. Plasma apoCIII levels have been shown to be associated with coronary artery disease,\textsuperscript{44-46} myocardial infarction,\textsuperscript{47} coronary progression in niacin treated subjects,\textsuperscript{48} presence of lesions in lovastatin treated patients\textsuperscript{49} and cardiovascular disease in type II diabetic patients.\textsuperscript{50} In only one study, plasma apoCIII was inversely associated with CAD.\textsuperscript{51} The lack of power due the small size of our patient cohort precludes us from observing any effect of \textit{APOC3} mutation carriage on cardiovascular endpoints.

By confining our sequence efforts to participants with plasma HDL levels $>95^{th}$ percentile, we introduced a selection bias and may have missed \textit{APOC3} mutations not resulting in the expected phenotype. Furthermore, we present data on a small cohort of study participants. This is however inherent to family studies and the results observed are consistent within each group. Recruitment of family controls enhanced the power of the study. Last, given the linkage disequilibrium of APOC3 with APOA1 and APOA5, we cannot exclude the possibility that the lipid changes ascribed to APOC3 mutation carriage, are in fact attributable to APOA4 or APOA5,\textsuperscript{52} which are known to influence HDL and triglyceride metabolism,\textsuperscript{53} However, in this study we describe three rare variants leading to a loss of function of APOC3, indicating that changes in lipid profiles can be ascribed to altered APOC3 function. This is supported by the fact that interventions aimed at decreasing APOC3, result in an HDL-C increase and triglyceride decrease.\textsuperscript{21}

In summary, we present data on two novel mutations in \textit{APOC3}, as well as one previously identified mutation. Two of these three mutations show a clear effect on lipid profiles, accompanied by the relatively large decreases in plasma apoCIII, whereas the third mutation is associated with less pronounced changes in the lipid profile and smaller decrease in plasma apoCIII. This study suggests an inverse association between apoCIII and favourable lipid profiles and lends support to the study the effects of apoCIII lowering therapy.
Acknowledgements

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Reference List


Two novel mutations in \textit{APOC3}


Novel Mutations in Scavenger Receptor BI Associated with High HDL Cholesterol in Humans

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Abstract

The scavenger receptor class B, member 1 is a key cellular receptor for high density lipoprotein (HDL) in mice, but its relevance to human physiology has not been well established. Recently a family was reported with a mutation in SR-BI and high HDL cholesterol (HDL-c). Here we report two additional individuals with extremely high HDL-c (greater than the 90\textsuperscript{th} percentile for age and gender) with rare mutations in the gene encoding SR-BI. These mutations segregate with high HDL-c in family members of each proband and are associated with a 37\% increase in plasma HDL-c in heterozygous individuals carrying them. Both mutations occur at highly conserved positions in the large extracellular loop region of SR-BI and are predicted to impair the function of the SR-BI protein. Our findings, combined with the prior report of a single mutation in the gene encoding SR-BI further validate that mutations in SR-BI are a rare but recurring cause of elevated HDL-c in humans.
Introduction

The scavenger receptor class B, member 1 (SR-BI) is a high-affinity HDL receptor in mice that is highly expressed in the liver and steroidogenic tissues and is essential for HDL cholesterol uptake and excretion.\(^1\),\(^2\) In mice, targeted deletion of the Scarb1 gene that encodes SR-BI leads to significantly elevated levels of plasma HDL-c\(^3\) and impaired selective uptake of HDL-c by the liver and adrenal glands\(^4\). Conversely, overexpression of SR-BI in mice results in the disappearance of HDL-c from plasma.\(^5\),\(^6\) These and other data have established SR-BI as the major HDL receptor in the liver and other tissues and a key regulator of the reverse cholesterol transport pathway by which excess cholesterol is removed by HDL particles and delivered to the liver for excretion into bile. Consistent with its importance in reverse cholesterol transport,\(^7\) deletion of Scarb1 leads to increased atherosclerosis in mice deficient for ApoE or fed a Western-type diet.\(^8\),\(^9\)

Despite its established importance in mice, the role of SR-BI in HDL metabolism in humans is less clear. Common polymorphisms in SR-BI have been described with a mild effect on HDL-c levels.\(^10\)-\(^15\) In addition, in a large genome wide association study involving over 100,000 individuals, the minor allele of the rs838880 variant, located approximately 400 bp downstream of the gene encoding SR-BI, was associated with a small but statistically significant 0.016 mmol/L (~1.4%) elevation in HDL-c.\(^16\) However, such association studies do not identify causality between a variant and the phenotype of interest, and the variants identified have only very small effects on plasma HDL-c, leaving unanswered the question of the potential quantitative importance of SR-BI in human HDL metabolism.

Recently, Vergeer and colleagues identified a single loss-of-function mutation, P297S, in the gene encoding SR-BI that was associated with elevated HDL-c, platelet dysfunction, and adrenal steroid hormone deficiency.\(^17\) It is unknown if this mutation represents a unique event, or if there are other mutations in SR-BI that cause extreme high HDL-c. Additionally, the frequency of SR-BI mutations in individuals with high HDL-c is unknown. In this study we evaluated the hypothesis that rare sequence variants in the gene encoding SR-BI are a cause of high HDL-c levels in humans. We report two novel mutations in SR-BI associated with high HDL-c.

Material and methods

Patients

We identified 120 unrelated probands of Caucasian ancestry with plasma HDL-c concentrations greater than or equal to the 90\(^{th}\) percentile adjusted for age and gender. We also identified 80 individuals of Caucasian ancestry with HDL-c below the 10\(^{th}\) percentile and no other lipid abnormalities. The study protocol was approved by the Ethics Committees of the Academic Medical Center, Amsterdam and the University of British Columbia, Vancouver. All subjects provided written informed consent.
Lipoprotein Analysis
Lipoprotein measurement on fresh plasma was performed as described. Cholesterol and triglyceride levels were determined in total plasma and plasma at density d<1.006 g/mL obtained after preparative ultracentrifugation, before and after precipitation with dextran manganese.

Sequencing and Mutation Detection
The SR-BI gene was sequenced from genomic DNA in all probands using either standard fluorescent dye terminator chemistry (Seqwright, Houston TX) or next generation paired-end read sequencing (Illumina, San Diego CA). For standard sequencing, DNA primers were designed to flank SR-BI exons and adjacent intron and UTR sequence as defined in human genome hg18. APOA1, LCAT and ABCA1 coding exons were sequenced using similarly designed primer sets. Sequence changes were identified from data using Sequencher 4.7 (Ann Arbor, MI) and confirmed in dbSNP build 130 and 1000Genomes November 2010 data release. For next generation sequencing, sequence changes were identified by alignment of sequence data to the human genome (NCBI Build 36.1) using CASAVA v.1.7 software (Illumina, San Diego CA) and confirmed by standard sequencing. Nucleotide and amino acid positions are with respect to Ensemble transcript ENST00000415380. Mutations were genotyped in the family members of probands that carry mutations using standard sequencing techniques. Multiple sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Statistical Analysis
Differences in lipid and other values between mutation carriers and related controls were compared using a two-tailed student t-test and are reported as mean plus or minus standard deviation. A p value of 0.05 or less was considered statistically significant.

Results
We sequenced the gene encoding SR-BI in 120 unrelated probands with HDL-c levels greater than or equal to the 90th percentile for gender and age. We identified two novel missense mutations, S112F (nucleotide C588T) and T175A (nucleotide A776T), occurring in 1 separate individual each, for an overall rate of SR-BI mutations in individuals with high HDL-c of 1.2% in this cohort. In contrast, no novel SR-BI variants were found in 80 individuals with HDL-c less than the 10th percentile. Neither S112F nor T175A were identified in dbSNP or in the 1000genomes databases, indicating that these are novel, rare mutations.

Both S112F and T175A occur in the large extracellular loop of the SR-BI protein and affect residues highly conserved across multiple species (figure 1). The serine residue at position 112 is completely conserved in all mammalian species as well as the zebra fish, Danio rerio (figure 1B). The threonine residue at position 175 is conserved across mammals, but differs in Danio rerio where the orthologous position aligns to proline. Both mutations are predicted to be damaging by Polyphen. In addition, the substitution position specific evolutionary conservation score for T175A is −2.28, indicating a moderate prediction of a deleterious effect on protein function.
Figure 1. **A)** The location of mutations identified in this study and in the study by Vergeer and colleagues (17) are shown in a schematic of the SR-BI protein. All mutations identified to date occur in the large extracellular loop corresponding to the CD36 superfamily domain indicating that this is a critical functional domain. **B)** Multiple sequence alignment of the SR-BI protein from different species. The residues at which the S112F and T175A occur are highlighted. S112 is completely conserved across all available vertebrate sequences. T175 is conserved across all mammalian species and differs only in the zebra fish, *Danio rerio*.
We next genotyped the family members of these two probands to assess segregation of these mutations with high HDL-c. The T175A mutation segregates with the high HDL-c phenotype in the pedigree of this proband (figure 2A), such that individuals carrying the T175A allele have HDL-c levels greater than the 95th percentile (3 individuals) whereas no individuals without T175A have HDL-c levels above the 90th percentile (11 individuals). One individual heterozygous for the T175A mutation (ID II:04 in Figure 2A) had HDL-c at the 71st percentile. This individual has a history of other cardiovascular risk factors including hypertension and is on medication that may influence this phenotype (supplementary table 1). Similarly in the pedigree of the proband heterozygous for S112F, no individual without this mutation had elevated HDL-c (figure 2B) suggesting that this mutation is causal for high HDL-c in this family.

Interestingly, the mother of the proband in figure 2B (individual I:02) was found to carry S112F but has low HDL-c (15th percentile). This led us to hypothesize that this individual might carry a second mutation leading to low HDL-c. We therefore sequenced the LCAT, APOA1 and ABCA1 genes in this individual, three common genetic causes of low HDL-c. We identified a novel V2091I mutation in ABCA1 that occurs in the C-terminal region of the encoded protein – a region known to be highly susceptible to deleterious mutations. Moreover, this mutation segregates with low HDL-c in this pedigree, such that both individuals with V2091I had HDL-c less than the 20th percentile. Importantly, individual I:02 in figure 2B who is heterozygous for both the S112F mutation in SR-BI and the V2091I mutation in ABCA1 has low HDL-c, suggesting that ABCA1 mutations may be dominant to SR-BI mutations with respect to HDL-c levels. This individual also has a history of coronary artery disease and cerebrovascular disease.

Compared to family member controls, individuals heterozygous for mutations in SR-BI had a 37% increase in HDL-c (1.80 +/- 0.3 mmol/L vs. 1.31 +/- 0.2 mmol/L, p=0.002; table 1). No significant differences in triglycerides, LDL-c or body mass index (BMI) were observed between carriers and controls. Apart from individual I:02 in Figure 2B who carries both the SR-BI S112F and ABCA1 V2091I mutations, no mutation carrier or control had a history of coronary artery disease, cerebrovascular disease or peripheral vascular disease (supplementary table 1).

**Discussion**

The study of familial disorders of lipoprotein metabolism has led to significant advances in our understanding of HDL metabolism, including the identification of key proteins involved in HDL biogenesis, transport and modification such as ABCA1, APOA1, LCAT, and CETP. SR-BI plays a key role in hepatic HDL uptake in mice, but until recently the role of SR-BI in HDL metabolism in humans had not been well established. This is a crucial question, as strategies to therapeutically augment HDL levels or function will depend on an accurate understanding of the pathways of clearance of HDL from plasma.

Recently, Vergeer and colleagues reported a family in which a mutation in SR-BI, P279S, segregated with a high HDL-c phenotype. These authors also showed that carriers of P279S had reduced platelet function as well as evidence of adrenal insufficiency, suggesting a role for SR-BI-mediated uptake of cholesterol in these cell types. We used
Figure 2. A) Pedigree of the proband with T175A mutation in SR-BI. Below each individual is listed age in years, total plasma cholesterol (TC), triglycerides, HDL-c (and percentile) – all in mmol/L, body mass index and genotype with respect to T175A. B) Pedigree of the proband with S112F mutation in SR-BI. Below each individual is listed age in years, total plasma cholesterol (TC), triglycerides, HDL-c (and percentile) – all in mmol/L, body mass index, genotype with respect to SR-BI S112F and genotype with respect to ABCA1 V2091I mutation. Squares, Males; Circles, Females; Filled shape, HDL-c ≥90th percentile; empty shape, HDL-c <90th percentile; Arrow, proband.
a similar strategy of screening unrelated probands with very high levels of HDL-c (>90th percentile) and identified two novel mutations (S112F and T175A) in the gene encoding SR-BI, representing the 2nd and 3rd mutations described in this gene that underlie elevated HDL-c in humans. Together with the prior report17 there are now three different mutations reported in the gene encoding SR-BI in 23 individuals all of whom have extremely high HDL-c. In our cohort, the frequency of SR-BI mutations was 1.2%, indicating that SR-BI is an important, though infrequent, cause of high HDL-c.

We showed that both the S112F and T175A mutations segregate with the high HDL-c phenotype in the families of the probands, providing strong evidence that these mutations are causally related to high HDL-c. Moreover, no novel SR-BI variants were detected in 80 individuals with very low HDL-c, which additionally supports the interpretation that S112F and T175A are functional mutations that contribute to high HDL-c.

One individual (ID I:02) in the pedigree in figure 2B was heterozygous for both the S112F mutation in SR-BI as well as the V2091I mutation in ABCA1. This patient’s phenotype of low HDL-c is consistent with heterozygous deficiency for ABCA1.27 This suggests that ABCA1 mutations may be dominant to SR-BI mutations with respect to HDL-c. Although this notion requires testing in a larger number of individuals, this would be consistent with the roles of these proteins in HDL metabolism, namely ABCA1 acting on the initial step in HDL biogenesis and SR-BI acting on the downstream step of hepatic HDL uptake.28 This also highlights the importance of further investigation of individuals carrying a mutation but who manifest an unexpected phenotype.

We did not observe clinical evidence of accelerated atherosclerosis in SR-BI mutation carriers, consistent with prior reports.17 In mice, targeted deletion of SR-BI does not

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Lipid values are in mmol/L. Statistically significant differences are bolded. Individuals also carrying mutations in ABCA1 were excluded.
result in accelerated atherosclerosis in chow fed animals at 5-7 weeks of age. However in the context of apoE deficiency or feeding of a western-type diet, loss of SR-BI results in dramatically accelerated atherosclerosis. Thus it is possible that in humans, SR-BI mutations may result in accelerated atherosclerosis only when combined with a second atherogenic factor. Interestingly, the one individual we identified with an SR-BI mutation as well as a mutation in ABCA1 indeed had premature atherosclerotic disease manifesting as cerebrovascular disease at age 55 and coronary artery disease later in life (supplementary table 1). Consistent with this, combined deletion of macrophage SR-BI and ABCA1 in mice is associated with greater atherosclerotic burden than deletion of SR-BI alone.

Previous studies of SR-BI polymorphisms with small effects on protein function have suggested an association between SR-BI polymorphisms and BMI as well as LDL-c. We observed no effect of the S112F and T175A mutations on either BMI or LDL-c consistent, with data of Vergeer and colleagues. Overall these data suggest that SR-BI does not have a major influence on non-HDL lipid parameters or BMI.

Both of the mutations we describe as well the P279S mutation reported previously occur in the large extracellular loop corresponding to the CD36 cell adhesion superfamily domain. Whether mutations in other regions of this protein, such as the transmembrane domains, will also lead to impaired HDL uptake is unknown. In other proteins with multiple transmembrane and extracellular domains, such as ABCA1, mutations in the transmembrane regions occur very infrequently compared to mutations in the extracellular domains, and mutations in the transmembrane regions of SR-BI may be similarly infrequent. In mice, deletion of the C-terminal region of SR-BI required for PDZK1 binding leads to unstable expression of SR-BI at the cell surface, suggesting that mutations that effect this region of the protein may also be pathogenic.

In summary, we provide evidence for two novel mutations in the gene encoding SR-BI associated with elevated HDL-c in humans. Our data expand the number of documented human mutations in this gene to three and add further support for the concept of SR-BI as a physiologically relevant HDL receptor in humans.

Acknowledgements
This work was supported by Xenon Pharmaceuticals and the Dutch Heart Foundation (to JJK). MRH holds a Canadian Research Chair in Human Genetics and is a Killam Professor at the University of British Columbia. JJK is a recipient of the Lifetime Achievement Award (2010 T082) of the Dutch Heart Foundation.
Reference List

23. Brooks-Wilson A, Marcil M, Clee SM et al. Mutations in ABC1 in Tangier disease and


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**Figure 2A**

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All lipid values in mmol/L. HTN, hypertension. CABG, coronary artery bypass grafting. T2D, type 2 diabetes. TIA, transient ischemic attack. ASA, acetylsalicylic acid. nd, no data.
Apolipoprotein A-I mimetic Peptides

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Abstract

Objective
To review published data related to the potential applicability of apolipoprotein AI mimetic peptides.

Recent findings
Despite a wealth of information on HDL-c levels and risk for CVD, little evidence is present to suggest that raising HDL-c levels per se will result in CVD risk reduction. Rather, increasing HDL functionality might be a more successful strategy to reverse the process of atherosclerosis. In as such, apoAI mimetic peptides, either in single or tandem formulation, hold great promise. Evidence gathered over the last years has provided insight in the extent to which mimetics influence several cardio metabolic pathways. ApoA-I mimetics have shown to have anti-inflammatory, antioxidant, and antiatherogenic effects. Direct comparisons between different mimetics have provided insight in factors influencing the differential beneficial consequences of these peptides.

Data derived from recent studies suggest that mimetics might also gain their position as a therapeutic intervention in the treatment of septicaemia, transplantation rejection, diabetes and other auto-immune diseases.

Summary
This review provides a summary of the current literature on the potential application of apoAI mimetics as therapeutic agents. There is increasing evidence that these mimetics should be considered as a promising supplement to current strategies. Results from human studies addressing the in vivo effects of the different apoAI mimetics are eagerly awaited.
Introduction

Since one of the first reports in 1951, a plethora of prospective epidemiological studies have shown that plasma HDL-c levels and the risk for cardiovascular disease (CVD) are inversely related. The magnitude of the association is quantified in a widely cited meta-analysis showing that a 1 mg/dl increase of HDL-c level is associated with a 2–3% decrease in CVD risk.

A number of mechanisms by which HDL-c may influence the process of atherosclerosis has been described. Fielding and Fielding demonstrated in 1982 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). RCT is defined as the uptake of cholesterol from peripheral cells by lipid-poor apoA-I and HDL, and the subsequent delivery to the liver for excretion into the feces. The function of HDL as a atheroprotective particle extends well beyond this role in cholesterol transport; it has also been found to have anti-apoptotic, antiinflammatory and antithrombotic capacities.

Cardiovascular disease remains a major cause of morbidity and mortality, despite interventions to decrease the number or severity of known risk factors. As a consequence, HDL-c increasing therapy is considered a suitable target for CVD prevention. Indeed, infusion of exogenous HDL has been shown to result in improvements in hallmarks of atherosclerosis, such as endothelial function, coronary atheroma volume and plaque morphology. These studies, however, were small scaled and should be regarded as a proof of concept that an increase in HDL-c does change biomarkers of atherosclerosis. Repetitive infusions of HDL are not considered a feasible therapy for the wide scaled burden of CVD; it would require large resources to produce considerable quantities of this lipoprotein. Consequently, large efforts have been put in the strive for orally administered HDL-c increasing medication. Several of these are available (i.e. fibrates or nicotinic acid derivates). Most of these drugs, however, do not exclusively raise HDL, but have beneficial effects on other risk factors as well, and as such, the contribution of the HDL-c increase per se has been impossible to delineate.

However, efforts in the field of HDL therapeutics have resulted in the recent development of Cholesteryl ester transfer protein (CETP) inhibitors. This class of drugs induces significant HDL-c increases. The ILLUMINATE trial, in which 15.000 individuals were randomized to either statin or statin combined with the CETP inhibitor torcetrapib, did show increased mortality amongst the torcetrapib treated patients, despite an impressive (>100%) increase in HDL-c. The result is counterintuitive, and the underlying mechanism has not been fully elucidated, but off target effects of the torcetrapib molecule are the most likely explanation to date.

After Illuminate, a subtle shift in the HDL-atherosclerosis paradigm has occurred. Whereas in the past the main focus was to increase HDL-c levels per se, the concept has changed to increase HDL functionality.

With this new paradigm kept in mind, apolipoproteinA-I (apoA-I) should be considered an attractive target. apoA-I is the structural protein of the HDL particle and it plays a crucial role in many of the beneficial effects of HDL. Studies in rodents have established that apoA-I does play a role in atherosclerosis, although the magnitude of the effect differed amongst the studies, which might be in part attributable to the genetic background of the animals and additional
manipulations used in these models (reviewed in Vergeer et al\textsuperscript{17}). The fact that apoA-I is a crucial protein for HDL generation is exemplified by the finding that apoAI KO mice have 75\% lower HDL-c levels. These apoA-I knockout mice, however, do not show increased susceptibility to atherosclerosis.\textsuperscript{18} In an atherogenic background, such as an LDL receptor null mice fed with an atherogenic diet, apoA-I deficiency results in markedly increased atherosclerosis.\textsuperscript{19}

A combined knockout apoA-I and human apoB transgenic mice model also showed increased atherosclerosis when fed a Western diet.\textsuperscript{20}

Overexpression of hApoA-I in atherosclerosis prone animal models do show uniform results. Hepatic overexpression of human apoAI has been shown to reduce the extent of atherosclerosis in mice on pro-atherogenic diets\textsuperscript{21} and these findings have been reproduced in different animal models.\textsuperscript{22,23}

Clearly, these data point towards apolipoprotein AI as a very attractive interesting target and the finding that human carriers of mutations in the apoA-I gene do suffer from increased atherosclerosis strongly supports this view.\textsuperscript{24}

The development of ApoA-I mimetic peptides
Apolipoprotein AI is a relatively large protein, comprising of 243 amino acids in 10 amphipathic alpha helices, of which most are separated by a proline residue. Due to its size, apoAI would require parenteral administration. As a consequence, it was compulsory to design smaller mimetic peptides, without losing the lipid binding and antiatherogenic capacities of these molecules. In 1985, Anantharamaiah and coworkers produced an 18 aminoacid containing peptide folded into an alpha-helix.\textsuperscript{25} The peptide did not have direct sequence homology to either one of the helices of the apoAI protein, but the secondary structure of apoAI was replicated. This so called 18A peptide was found to have similar properties to the apoA-I helices in terms of charge distribution and lipid-binding capacity. Cellular efflux-inducing capacity of 18A was shown to be similar to apoA-I in an assay where cultured mouse fibroblasts were used.\textsuperscript{26} In addition, like apoA-I, 18A was shown to activate Lecithin:Cholesterol Acyltransferase (LCAT), a pivotal enzyme in HDL maturation. As such, 18A was thus shown to have quite similar properties in lipid metabolism as apoA-I.

In an attempt to optimize the peptide, stability was improved by replacing the existing nonpolar amino acids on 18A with phenylalanine (F) residues. The increasing number of F residues (resulting in 2F, 3F, 4F, 5F, 6F and 7F, reflecting the number of F residues) did result in increased lipid affinity and hydrophobicity; 6F and 7F were the most hydrophobic, but these peptides were shown to lose the affinity to bind phospholipids, and in subsequent experiments the main focus has been on the 4F and 5F peptides.

Peptides can either be synthesized from L-aminoacids (i.e. L-4F) or D-aminoacids (D-4F). \textit{In vitro} properties have been shown to be similar for both isomers,\textsuperscript{27,28} but the L isomer is significantly more prone to proteolysis compared to the D isomer,\textsuperscript{28} since mammalian enzymes recognize peptides made from L-aminoacids, but not from D-aminoacids.

In addition to lipid binding and hydrophobicity, peptides have been tested for their antiinflammatory and antioxidant characteristics. A commonly described method is the one
described by Navab and coworkers, who have used a model in which LDL-c induced activation of monocyte chemotactic activity was tested in an in vitro arterial wall coculture model.

In these models mimetic peptides are compared with each other and with apoA-I. Attempts to mimick apoAI in all its anti-atherogenic effects have thusfar not been successful, but mimetics have been shown to be superior to apoA-I when it comes to specific characteristics. 4F, for example, was shown to bind oxidized lipids with orders of magnitude higher affinity compared to apoA-I. This finding might explain why these peptides have biological effects despite low plasma concentrations. The variation in effects between peptides and apoA-I also suggests that “mimetics” might not be a proper nomenclature.

**Tandem helical mimetic peptides**

As said above, apoA-I contains multiple helices linked by a proline residue. Single helix peptides have been shown to be efficient antiatherogeneic molecules, but tandem helical mimetic peptides have been created in an attempt to further increase efficacy by more closely mimicking the apoA-I peptide.

In a direct comparison, Wool and coworkers showed that symmetrical peptides, composed of two 4F peptides linked by a proline or alanine residue, were more efficient in a cholesterol efflux from lipid-loaded murine macrophages compared to the single 4F peptide. Copper-mediated oxidation of purified mouse LDL was inhibited by the 4F peptide, but the tandem peptides increased oxidation, clearly emphasizing the variability of effects between the single and tandem peptides in in vitro assays.

Recently, 22 bihelical apoA-I mimetic peptides were investigated in vitro for their capacity and specificity of cholesterol efflux, and their inhibiting effects on inflammation and LDL-oxidation. In this comprehensive first systematic analysis of multiple structural modifications none of the peptides tested were found to be equally effective in all antiatherogenic functions.

Moreover; the anti-inflammatory, antioxidant and efflux capacities were found to be related to differential structural features of the peptides. For efflux efficiency and specificity, for example, mean hydrophobicity, charge, size and angle of the link between two helices were crucial, whereas for antioxidant properties the presence of cysteine and histidine residues was important. The latter has also been proposed as a potential explanation for the increased antioxidant property of apoA-I Milano, a genetic variant known to be associated with reduced risk for CVD.

The fact that none of the peptides tested could outcompete the beneficial functions of apoAI in all facets of antiatherogeneity suggests that different portions of the apoAI peptide might be involved. As a consequence, mimicking one part of apoAI does not necessarily result in an overall beneficial property of the designed apoAI mimetic peptide. A combination of different mimetic peptides, harboring various beneficial effects, might therefore be a prerequisite to mimick the full spectre of antiatherogeneic characteristics of apoAI.

One could also envision that tailored therapy can be achieved by the combination of different peptides.
ApoA-I mimetics in animal studies

The initial in vivo studies addressing the effect of apoA-I mimetics were performed by Garber and coworkers. In this study, C57Bl/6J mice were put on an atherogenic diet and 5F was administered daily by intraperitoneal injection at a dose of 20 microg/day for 16 weeks. Mice treated with PBS or murine apoAI (50microgram/day) were used as controls. Lipids and lipoproteins were not significantly altered upon 5F injections and administration of 5F was found to be non toxic. Importantly, no antibodies to the injected materials were observed. The aortic atherosclerotic lesion area was significantly less (44% reduction) in 5F treated mice compared to mice receiving placebo or murine apoA-I. HDL isolated from 5F injected mice was shown to be superior to HDL derived from PBS and apoAI treated mice in terms of reduction of monocyte chemotaxis and lipid hydroxyperoxidase formation. This, in combination with the finding that lipid levels were not changed by 5F injection, shows that functionality of the HDL pool was enhanced by 5F.

In subsequent in vivo studies, the main focus has been on 4F. This is in part due to the fact that D-4F can be administered orally in drinking water or by gavage. In apoE knockout mice as well as in LDL-r KO mice, D-4F was shown to decrease the aortic root lesions by more than 70%, despite very low bioavailability and low plasma concentrations. The marked reduction in atherosclerotic lesions occurred independent of changes in total plasma or HDL-cholesterol. However, administration of D-4F in the drinking water resulted in a substantial change of HDL from a pro- to an anti-inflammatory particle, emphasizing that D-4F therapy induced a qualitative rather than a quantitative effect on HDL. In a subsequent study, this suggestion was confirmed by showing that administration of D-4F caused a change in HDL distribution towards pre-beta HDL (a fraction known for its efflux efficacy), and that it increased HDL associated paraoxonase activity.

Low doses of D-4F and pravastatin work synergistically on HDL-c level, HDL function and atherosclerosis prevention in an apoE null mice model. This might be relevant for future clinical perspectives.

Bielicki and co workers published the beneficial effect of a new HDL mimetic single helix peptide (ATI-5261) in LDL-r KO mice and apoE null mice on a 13-18 week during high-fat Western diet. Daily intraperitoneal injections of ATI-5261 (30 mg/kg) for 6 weeks reduced atherosclerosis, as judged by lesion area covering the aorta, by 30% and 45% in LDL-R -/- and apoE -/- mice respectively. Interestingly, one single intraperitoneal injection of ATI-5261 was found to increase reverse cholesterol transport from macrophage foam-cells to feces over 24-48 h.

Emerging perspectives for apoA-I mimetics

The effects of apoA-I mimetics is not restricted to dyslipidemic mice models. In a recent study, Vaziri and coworkers showed the benefit of L4F (5 mg/kg s.c. 3 times weekly for 4 weeks) on inflammation and oxidative stress in a chronic kidney disease (CKD) rat model. L4F attenuated a large number of the markers for inflammation and oxidation (such as NAD(P)H oxidase subunits, COX-2, 12-lipoxygenase, MCP-1, PAI-1, myeloperoxidase)
in the thoracic aorta without altering plasma lipids. Compared to WT mice, ApoE-deficient mice have been shown to suffer significantly more from signs of renal dysfunction such as proteinuria, tubulo-interstitial inflammation, and mesangial expansion. Oral D-4F administration was recently found to have favorable effects on all of these characteristics. Further studies in CKD patients are awaited to address whether apoAI mimetics hold promise for atherosclerosis regression and preservation of kidney function.

Another patient population that might benefit from apoA-I mimetics are septic patients. Apolipoproteins and HDL have been shown to beneficially change the inflammatory response to lipopolysaccharide (LPS), and a recent study showed that intraperitoneal injection of 4F significantly blunted the hypotensive response to LPS (LPS treated rats: 34% decrease, LPS and 4F treated rats: 17% decrease in systolic blood pressure). In order to unravel the underlying mechanism, aortic ring segments from LPS-treated rats were isolated. These ex vivo studies showed a reduced contractile response to phenylephrine in aortae of LPS-treated rats compared to controls, and this reduced contractility was reverted by 4F via nitric oxide synthase 2 (NOS2) downregulation. The concentration of circulating endotoxin was significantly reduced in 4F treated rats, and interestingly, HDL-c levels increased in the LPS+4F treated animals (38 to 45mg/dl). Similar to other studies, an HDL-c reduction was found in LPS+vehicle treated rats (38 to 28mg/dL).

The findings of this study are in line with previous studies showing that the apoA-I mimetic 4F significantly inhibits the induction of pro-inflammatory mediators by LPS in cultured endothelial cells. Interestingly, 4F was shown to reduce 24hour mortality in LPS-treated rats (60% mortality in LPS and 10% in LPS+4F, total 40 animals).

In addition, transplant-associated vasculopathy is reverted upon intraperitoneal D-4F administration in a mouse heart transplant model. The authors hypothesize that this is partly mediated through D-4F induced HemeOxygenase-1 upregulation.

In a mouse lupus model L-4F was not only shown to reduce atherosclerosis progression, but it also resulted in a reduction of IgG anti-dsDNA, proteinuria and glomerulonephritis, suggestive of protective effect on lupus-like disease. Beneficial vascular effects have also been described in a systemic sclerosis model.

Administration of L-4F (2mg/kg/d) to ob/ob mice reduced adiposity, improved insulin sensitivity, and improved glucose tolerance, which might be related to apoAI mimic induced increase in uncoupling protein 1 (UCP1) mRNA and protein levels as well as the stimulation of AMPK phosphorylation in brown adipocytes in culture.

Other favorable effects of mimetics have shown to be a reduction of platelet aggregation, an increase of cognitive function in an Alzheimer’s mice model and prevention of fibrosis after onset of steatohepatitis.

**ApoA-I in human studies**

Based on all intriguing beneficial findings in animal studies, apoA-I mimetics hold promise for human therapy. Apart from its anticipated efficacy, apoA-I mimetics have more positive characteristics: they are safe, well tolerated and relatively inexpensive compared...
to rHDL. Moreover, the size of the peptide does implicate that administration might be possible in an oral formulation, which is a prerequisite for a long term treatment in large numbers of patients and modifications for optimal oral delivery are in development.

The first clinical trial of oral D-4F was performed in CAD and high risk patients who received a single dose of 30, 100, 300, or 500 mg of D-4F (n = 8 for each dose) or placebo (n = 8) under fasting conditions. Ten additional patients received 500 mg (n = 8) or placebo (n = 2) with a low-fat meal. The Tmax was shown to be 30 minutes and D-4F was detectable in plasma at all dosages. The single dose was well tolerated and shown to be safe. No effect on lipids and lipoproteins was found, but the anti-inflammatory index, assessed by comparing the ability to inhibit LDL-induced monocyte chemotactic activity in cultures of human aortic endothelial cells, increased in the highest doses compared to placebo. Additional studies focussing on these vascular effects are expected.

**Other apoA-I based therapies.**

Apart from apoA-I mimetics, several other apoA-I based therapies have emerged and these have reached different stages of research. Full-length apoA-I, recombinant HDL, apoAI enhancers and active delipidation are amongst these therapies. Results from studies with these are eagerly awaited.

**Conclusion**

Recent studies have increased our understanding of the effects of apoA-I mimetics. All peptides studied have been shown to induce beneficial effects on oxidation, inflammation and cholesterol efflux and combining different peptides might be a prerequisite to establish the full spectrum of possible beneficial effects.

Whereas the focus for apoA-I mimetics has traditionally been directed towards atherosclerosis, recent studies have shown effects in several other disease states. These provocative findings do require further investigations in carefully designed clinical trials in humans.
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The promise of Cholesteryl Ester Transfer Protein (CETP) inhibition in the treatment of cardiovascular disease


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Abstract

There is a strong need to reduce the risk of cardiovascular disease (CVD) beyond the use of statins that lower low-density lipoprotein cholesterol (LDL-c). The inverse relationship of high-density lipoprotein cholesterol (HDL-c) with cardiovascular disease suggests HDL-c raising therapy as a novel target. This review discusses the role of HDL-c in atherogenesis as well as the promise of cholesteryl ester transfer protein (CETP) inhibition in CVD prevention. While genetic studies show conflicting results on correlations between HDL-c and CVD, experimental studies have yielded sufficient encouraging data to proceed with the development of HDL-c raising strategies. CETP inhibition has been shown to successfully increase HDL-c levels in man. However, the first CETP inhibitor tested in phase III trials increased mortality possibly due to torcetrapib-specific vasopressor effects. More recently, dalcetrapib did not show an effect on CVD outcome while raising HDL-c by 30%, thereby refuting the HDL-c hypothesis. Anacetrapib and evacetrapib are currently tested in phase III clinical trials and have not shown adverse effects thus far. Both compounds not only increase HDL-c by 29-51%, they also decrease LDL-c (36-41%) and anacetrapib lowers Lp(a) (17%). Combined, these effects are anticipated to decrease CVD risk and the results will be revealed in 2017.
Introduction
Cardiovascular disease (CVD) is the leading cause of death worldwide. Low-density-lipoprotein cholesterol (LDL-c) is a direct contributor to the initiation and progression of atherosclerosis, the underlying chronic disorder of CVD. As a consequence, LDL-c lowering strategies provide the basis for therapeutic efforts aimed at lowering CV-risk; statins reduce major cardiovascular event risk by 25% for every 1 mmol/L LDL-c decrease. However, even when target levels for LDL-c are reached, a substantial residual risk of 65-75% remains which emphasizes the need for additional therapeutic strategies on top of statins.

Large epidemiological surveys have consistently shown that HDL-c is inversely correlated with cardiovascular risk. Every mg/dl increase of HDL-c levels has been reported to be associated with a 2-3% decreased CVD risk. Even when target levels for LDL-c have been reached, HDL-c levels are still found to be predictive of major cardiovascular events. Furthermore, in a post hoc analysis comprising 1455 patients from 4 prospective studies, the HDL-c increase associated with statin therapy was also found to independently predict risk reduction. Moreover, in a meta-analysis of twenty randomized controlled trials (RCTs), statin therapy did not alter the relationship between HDL-c and CVD. These findings have led to the widely accepted idea that raising HDL-c is a promising target to lower CVD risk. By accepting HDL-c increase as a therapeutic target, however, one adheres to the assumption that HDL-c is a causal factor in atherogenesis (for review see ). This assumption has been criticized in the light of recent observations supporting the idea that HDL-c levels merely constitute a biomarker for a pro-atherogenic milieu rather than a causal factor. The profound impact of a wide variety of risk factors such as metabolic syndrome, smoking, inflammation and physical inactivity on HDL-c concentration confounds the relation between HDL-c and CVD. In this scenario, HDL-c can be seen as a sensitive indicator of an adverse CV-risk profile, rather than a protective partaker in the course of atherogenesis. In support, genetically determined high HDL-c was not found to be associated with decreased CVD risk while elevated HDL-c has also been shown to have the capacity to turn into a pro-atherogenic factor. This review will discuss the role of HDL-c in atherogenesis as well as the promise of cholesteryl ester transfer protein (CETP) inhibition in CVD prevention.

Search strategy
We searched for articles on CETP in both animal models and humans, using search terms ‘CETP’, ‘murine’, ‘atherosclerosis’, ‘atherogenesis’, ‘rabbit’, ‘HDL-c’. With regards to the human studies, we searched for articles covering CETP genetics, genome wide association studies (GWAS), studies reporting on CVD risk and articles describing the effects of pharmaceutical CETP inhibition. For this, we used search terms ‘human’, ‘CETP’ ‘genetics’ ‘CETP mutation’ ‘atherosclerosis’, ‘atherogenesis’, ‘cardiovascular’ ‘torcetrapib’, ‘dalcetrapib’, ‘anacetrapib’ and ‘evacetrapib’.

The promise of CETP inhibition
HDL and atherosclerosis

Animal studies
Wild-type mice have no CETP, carry most of their cholesterol in HDL and do not suffer from atherosclerosis even when put on high fat/cholesterol diets. However, after introduction of CETP in mice, HDL-c drops and the mice become prone to atherosclerosis.19 Already in 1991, raising HDL-c through overexpression of human apolipoprotein AI (apoA-I; an integral part of HDL) in mice was shown to protect against atherosclerosis.20 Crossing these transgenic mice with atherosclerosis prone strains also resulted in atheroprotection21,22 and even plaque regression.23 In addition, gene therapeutic approaches using apoA-I have shown clear beneficial effects.24

However, the relation between HDL-c plasma levels and atherogenesis in experimental models is not straight forward.

Raising HDL-c through overexpression of e.g. lecithin:cholesterol acyltransferase (LCAT) was shown to exert different effects on atherosclerosis dependent on the animal models and mouse strain crosses studied.25-28 Also modulation of hepatic lipase activity has provided conflicting results,29-31 while loss of scavenger receptor class B1 (SRB1), leading to a stark increase of HDL-c was associated with increased atherosclerosis.32 Finally, modulation of ATP binding cassette transporter A1 (ABCA1) activity has also provided ambiguous data.33-35

Human studies
While epidemiological evidence that low HDL-c associates with increased CVD risk is irrefutable, evidence from genetic studies is equivocal. Mutations in APOA1 have consistently been reported to be associated with atherosclerosis.36-39 However, this is not the case for mutations in other major HDL genes such as ABCA1 and LCAT.40-45 In addition, common ABCAI, LCAT and LIPG gene variants were associated with HDL-c levels but not with the incidence of coronary artery disease,41,46,47 while for LIPC increased HDL-c was even associated with an increased risk of ischemic heart disease.48-50 Furthermore, GWAS have consistently reported that all major single nucleotide polymorphisms (SNPs) associated with high LDL-c levels correlate with CVD risk51 while this is not the case for HDL-c.14,53 Using an allele scoring system consisting of 14 SNPs that exclusively associate with HDL-c, Voight et al recently also showed no association with CV risk.15

Hence, apart from APOAI, other genes in HDL metabolism have failed to provide convincing and consistent evidence in support of HDL-c as a causal factor in atherogenesis.

HDL-c concentration versus HDL function
While HDL is best known for its central role in reverse cholesterol transport (RCT),52 it has also been found to exert a plethora of anti-atherogenic effects.53 The latter comprise anti-inflammatory effects, direct stimulation of endothelial nitric oxide availability and hence improvement of endothelial function54-58 as well as stimulation of endothelial repair.57,59 A possible explanation for the wide variety of effects associated with HDL-c is provided by the observation that the heterogeneous pool of HDL particles carries large numbers of metabolically active proteins and enzymes with a range of different functions.60-63 Thus,
HDL is probably not only a passive carrier of cholesterol. Interestingly, recent studies have demonstrated that in patients with overt CVD, HDL-c has lost its association with eNOS activation, anti-inflammatory action and endothelial regenerative capacity. These findings challenge the concept of simply targeting an increase in HDL-c concentration in CVD prevention strategies. In addition, it has been reported that ‘dynamic’ HDL-c-flux may behave independent from ‘static’ HDL-c concentrations. In fact, an increased HDL-c plasma concentration may even indicate a decreased capacity of the RCT pathway, as can best be exemplified by the situation in SRB1 deficiency, in which the hepatic uptake of HDL cholesterol is compromised. Although there is a strong interest to focus on HDL function, the epidemiological evidence simply points at HDL-c concentration and there is thus far no evidence that HDL function parameters are correlated with CVD in prospective studies.

Collectively, these data imply that raising HDL-c as a target to decrease the CVD risk is a challenging concept, which remains to be established. In view of the complexity of the HDL metabolism, the endpoints need to be validated for each HDL-c raising strategy. This complexity is corroborated by the results of human clinical trials, in which the impact of HDL-c increase on CVD risk reduction has been disappointing. Nicotinic acid and fenofibrate did not offer CVD protection, although design could be related to these failures. Also trials using apoA-I mimetics aimed at improving HDL function have not been uniformly successful thus far. Collectively, these data emphasize the need to provide solid clinical evidence that raising HDL-c actually does decrease CVD risk. However, raising only HDL-c is not easily achieved with the currently used regimens as will be discussed below.

**CETP inhibition and CVD risk**

CETP is a protein that is secreted by the liver, adipose tissue and macrophages. In plasma, CETP accommodates the transfer of cholesterol esters from HDL to (V)LDL in exchange for triglycerides. This reaction drives a decrease of cholesterol in HDL and an increase of cholesterol in LDL, especially when triglyceride levels are elevated. Thus, CETP inhibition retains cholesterol in HDL whilst decreasing the cholesterol content of the atherogenic apoB fraction. The central role of CETP in HDL metabolism was illustrated by Inazu and co workers who reported that genetic CETP deficiency causes very high HDL-c levels (up to 4.24 mmol/L) while a less marked decrease of LDL-c levels was observed. Furthermore, in rabbits, which are naturally characterized by a high level of CETP activity, it has been shown that inhibiting CETP through inhibitors or antisense strategy significantly attenuates atherogenesis.

**CETP genetics**

Whereas the association between functional *CETP* mutations and plasma HDL-c levels is irrefutable, the association with cardiovascular endpoints is more complex. Curb and co workers did not find decreased coronary heart disease in carriers of deleterious *CETP* mutations, neither did Moriyama and co workers, while the Honolulu Heart Study even showed increased coronary heart disease in male Japanese-American mutation carriers.
On the other hand, Kakko and co-workers reported that carriers of a mutation resulting in increased CETP activity displayed increased atherosclerosis.\textsuperscript{85} In the Multi-Ethnic study of Atherosclerosis, alleles associated with high CETP activity were also associated with increased coronary artery calcium score, a reliable surrogate for CVD risk.\textsuperscript{86} CETP was also associated with coronary risk in GWAS.\textsuperscript{87} The latter was corroborated in the Copenhagen City Heart Study, showing that CETP gene variation leading to a 14% HDL-c increase was associated with a hazard ratio of 0.74 for ischemic heart disease.\textsuperscript{88} Similar findings were reported in the Women’s Genome Health study.\textsuperscript{89} In a meta-analysis comprising more than 100,000 subjects, the relation between decreased CETP and decreased CVD was further corroborated,\textsuperscript{90} reporting a 5% CV-risk reduction for 3 commonly reported CETP polymorphisms, all of which were associated with lower CETP activity and higher HDL-c levels. Finally, plasma levels of CETP were also shown to be associated with risk of coronary artery disease, predominantly in individuals with high triglyceride levels.\textsuperscript{91} These data on CETP genetics clearly differ from those obtained in studies regarding \textit{ABCAI}, \textit{LCAT} and \textit{LIPC} and \textit{LIPG} as described above. This apparent discrepancy has been partly attributed to the fact that variation in the CETP gene does not only modulate HDL-c levels but has also marked effects on both LDL-c and triglyceride levels.\textsuperscript{15}

In summary, there is sufficient evidence supporting the continuation of programs in humans to test the impact of CETP inhibition on CV-risk.

\textbf{Differences between CETP inhibitors}

Torcetrapib, anacetrapib and dalcetrapib all work through promoting the formation of a complex between CETP and HDL.\textsuperscript{92} Still, the four CETP inhibitors which are or have been tested in humans do show clear structural differences, resulting in different mechanisms of action. Torcetrapib and anacetrapib give rise to a larger increase in plasma HDL-c than dalcetrapib. Whereas torcetrapib and anacetrapib can be regarded true CETP inhibitors, dalcetrapib has been described to be a CETP modulator,\textsuperscript{93} pertaining to the fact that torcetrapib and anacetrapib are 3,5-bis-trifluoromethyl-benzenes\textsuperscript{92,94} while dalcetrapib is a benzenethiol derivative. Whereas torcetrapib and anacetrapib treatment results in larger HDL particles and promote the formation of HDL-cETP complexes,\textsuperscript{92,93} dalcetrapib at lower dosages leads to a smaller increase in HDL particle size.\textsuperscript{92} Furthermore, the CETP-lipoprotein complex in case of torcetrapib is unable to efficiently exchange neutral lipids between different lipoprotein particles. In contrast, dalcetrapib decreases CETP activity by facilitating a specific interaction between cystein 13 of CETP and the benzenethiol moiety of dalcetrapib.\textsuperscript{79} Still, torcetrapib, anacetrapib and dalcetrapib all induce tight binding of CETP to HDL, indicating that these inhibitors work through promoting the formation of a complex between CETP and HDL.\textsuperscript{92} The exact inhibitory effects of evacetrapib (BAY60-5521) have not been fully elucidated.\textsuperscript{95}

\textbf{Important ‘safety’ lessons learned from torcetrapib}

Torcetrapib was the first CETP inhibitor that was tested in phase III clinical trials. Despite a 70% HDL-c increase and a 25% LDL-c decrease in 15067 patients with overt CV disease,
the drug unexpectedly caused a significant increase in death. To explain these findings, attention focused on off-target effects. A major worry pertains to the acute vasopressor effect of torcetrapib which also occurred in species that by nature do not have CETP, such as mouse, rat and dog. Mechanistically, this pressor effect has been attributed to an effect of torcetrapib on the adrenal glands, resulting in increased secretion of aldosterone and corticosterone. Since the latter occurred also in animal models lacking CETP, these effects were independent of the CETP pathway. Moreover, torcetrapib also increased the expression of renin-angiotensin-aldosterone and endothelin-related genes in the vessel wall as well as the adrenal glands of spontaneous hypertensive rats, possibly also contributing to an enhanced vasopressor response. In patients, torcetrapib has been associated with decreased potassium and increased sodium and bicarbonate levels with a concomitant increase in serum aldosterone level. In the ILLUMINATE study, there was an increased risk of death in patients treated with torcetrapib whose reduction in potassium or increase in bicarbonate was greater than the medium change. Since the use of torcetrapib has also been associated with increased progression of intima media thickness, it likely that the blood pressure is related to the adverse effect of this compound. Collectively, these observations provide strong evidence that off-target effects of torcetrapib have contributed to its failure. On the other hand, it cannot be ruled out that CETP inhibition results in HDL particles that do not function normally with slower turnover rates than normal and that the observed inverse relationship between HDL-c and CV-risk reflects an epiphenomenon. Interestingly, the increased mortality in the treatment group cannot be attributed to cardiovascular deaths only. More patients in the treatment group died from cancer and infection. This finding increased the interest in the role of HDL-c in cancer and inflammation, however, no definite explanation has been found to date.

**Absence of aldosterone effect of other CETP inhibitors**

The findings from the torcetrapib trial gave rise to an increased interest in blood pressure effects of this class of drugs. Dalcetrapib had no effect on aldosterone synthase or aldosterone production in adrenal cell lines nor did it increase blood pressure or renin-angiotensin-aldosterone related gene expression in spontaneously hypertensive rats. In addition, blood pressure profiles did not show clinically relevant changes during 12-week studies with no dose-related trends. The dal-VESSEL study, assessing effects of dalcetrapib on endothelial function, blood pressure, inflammatory markers and lipids, further established the safety and tolerability of dalcetrapib. Also anacetrapib had no effect on aldosterone and corticosterone secretion by adrenal cells and human safety data from this trial have confirmed this. Finally, evacetrapib has no effect on aldosterone and corticosterone secretion by adrenal cells either.

**Efficacy of dalcetrapib**

In a phase IIb RCT study (the Dal-PLAQUE study), magnetic resonance imaging was used to analyse vessel wall structure and 18F-fluoro-deoxyglucose (FDG)-PET-CT to measure vessel wall inflammation. Dalcetrapib, administered for 2 years, increased HDL-c by 27% without
affecting LDL-c and triglycerides in 64 patients. In the active arm, a non-significant reduction in carotid vessel wall inflammation at six months and a reduced total vessel wall area at 24 months were observed. All other primary outcome parameters were not significantly different.\textsuperscript{115}

In the dal-OUTCOMES trial, 15,600 patients who had suffered from an acute coronary event were enrolled, regardless of their HDL-c level, to test the hypothesis that dalcetrapib would reduce CV morbidity and mortality on top of LDL-c lowering therapy.\textsuperscript{116,117} The primary efficacy measure was time to first occurrence of CVD, death, nonfatal acute myocardial infarction, unstable angina requiring hospital admission, resuscitated cardiac arrest or atherothrombotic stroke. On May 7th 2012, Roche announced that the trial had been terminated (http://www.roche.com/media/media_releases/med-cor-2012-05-07.htm). Importantly, whereas the ILLUMINATE trial was discontinued due to excess mortality in the treatment group, the dal-OUTCOMES trial was terminated on the basis of futility. Although there were no adverse safety signals, the Data Safety Monitoring Board concluded that there was virtually no chance of yielding a positive result. Taking into account that the trial was powered to reveal a benefit of an anticipated 30% HDL-c increase without significant effects on LDL-c and triglycerides, it is fair to state that dalcetrapib does not offer the CVD risk reduction expected on the basis of epidemiological evidence for the HDL hypothesis.\textsuperscript{7,8}

Following the disaster of the torcetrapib program and the disappointment of the dalcetrapib program, the bar for future CETP inhibitors has once again been raised. There is however one crucial, differentiating effect of anacetrapib and evacetrapib versus dalcetrapib. While dalcetrapib had no effect on LDL-c, both anacetrapib and evacetrapib lower LDL-c by 36-41% in addition to a much larger HDL-c increase (129-151%) (see table 1). In addition, anacetrapib lowers Lp(a) by 17%.\textsuperscript{113,114} Irrespective of changes in HDL-c levels, a 40% decrease of LDL-c (and a 30% decrease in Lp(a) levels for anacetrapib) would be expected to offer a direct CVD benefit in patients.

**Awaiting evidence for Anacetrapib**

In the DEFINE study, 1623 high risk patients with an HDL-c below 1.6 mmol/L were included and randomized to anacetrapib 100mg per day versus placebo.\textsuperscript{113} After 6 months, a 151% increase in HDL-c, a 45% decrease in LDL-c and a 24% reduction in Lp(a) were observed. Although this study was not powered for endpoints, fewer patients in the anacetrapib group underwent revascularization (8 vs. 21; \(P=0.001\)).\textsuperscript{113} Based on the absence of evidence for off-target toxicity and the marked reduction of LDL-c and Lp(a), Merck continued their endpoint study ‘REVEAL’ (Randomized Evaluation of the Effects of Anacetrapib through Lipid-modification; clinicaltrials.gov ID NCT01252953). This study aims to include 30,000 subjects randomized to anacetrapib 100mg daily or placebo on top of LDL-c lowering therapy with a predicted follow up of 5 years. The outcome of this study is due in 2017.

**Emerging evidence for Evacetrapib**

After 3 months, a 129% increase in HDL-c was accompanied by a 36% decrease in LDL-c in patients randomized to the 500mg evacetrapib dose.\textsuperscript{118} No data on effect on Lp(a) were provided. To date, no endpoint study has been announced for evacetrapib.
Summary

Raising HDL-c levels as a target in CVD prevention is at present no longer considered to be the crown prince after statin-induced LDL-c reduction. The intertwine of HDL-c with a wide array of pro-atherogenic risk factors, comprising overweight, inactivity, hypertriglyceridemia and smoking makes it difficult to translate lessons from epidemiology to a potential benefit of a pharmacological HDL-c-increase.

Caution with raising HDL-c as a therapeutic target has grown following a general lack of genetic evidence as well as the absence of a correlation between HDL-c increase and CVD benefit in a meta-analysis of lipid-modulating randomized controlled trials. Although measures of HDL-functionality have been proposed to shed light on the ‘rocky road’ of HDL-c raising strategies, there is no prospective evidence for the relevance of HDL-functionality on CVD outcome. Hence, to date we are left with studying the impact of any agent mediating an increase in HDL-c through CV endpoint studies.

For CETP inhibition, the picture is equally blurred, although recent genetic evidence would support this intervention. Clinical trials with CETP inhibitors have thus far not been able to show benefit. Whereas for torcetrapib it can be argued that off target effects have contributed to increased CV mortality, the absence of a benefit in the dal-OUTCOMES study unambiguously shows the lack of a significant CV-benefit through an isolated, CETP-inhibitor induced HDL-c increase. The clearly divergent effects of novel potent CETP-inhibitors, which also significantly lower LDL-c, Lp(a) and non-HDL-c by 30-40%, however, provide a solid rationale to continue with clinical trials. However, one should realize that these compounds can not answer the question whether CETP-inhibition-induced HDL-c increase protects against CVD risk.

Acknowledgements

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Disclosures

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Table 1. Cholesteryl-ester transfer protein inhibitors in clinical investigation

<table>
<thead>
<tr>
<th>Name</th>
<th>HDL-c increase</th>
<th>LDL-c reduction</th>
<th>Triglyceride reduction</th>
<th>Lp(a) reduction</th>
<th>Study duration (wks)</th>
<th>development phase</th>
<th>reference</th>
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<tbody>
<tr>
<td>Torcetrapib</td>
<td>61%</td>
<td>20%</td>
<td>11%</td>
<td>Not reported</td>
<td>24</td>
<td>phase III: discontinued</td>
<td>101</td>
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<tr>
<td>Dalcetrapib</td>
<td>40%</td>
<td>0</td>
<td>10%</td>
<td>Not reported</td>
<td>24</td>
<td>phase III</td>
<td>117</td>
</tr>
<tr>
<td>Anacetrapib</td>
<td>51%</td>
<td>41%</td>
<td>7%</td>
<td>17%</td>
<td>76</td>
<td>phase III</td>
<td>113</td>
</tr>
<tr>
<td>Evacetrapib</td>
<td>29%</td>
<td>36%</td>
<td>11%</td>
<td>Not reported</td>
<td>12</td>
<td>phase II</td>
<td>118</td>
</tr>
</tbody>
</table>

HDL-c is high-density lipoprotein cholesterol; LDL-c is low-density lipoprotein cholesterol; Lp(a) is lipoprotein(a).

The promise of CETP inhibition
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PART II
HDL CHOLESTEROL
BEYOND ATHEROSCLEROSIS
Lcat deficiency in mice is associated with a diminished adrenal glucocorticoid function


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Abstract

Background
In vitro studies have suggested that high-density lipoprotein (HDL) and apolipoprotein B-containing lipoproteins can provide cholesterol for synthesis of glucocorticoids. Here we assessed adrenal glucocorticoid function in lecithin-cholesterol acyltransferase (Lcat) knockout mice to determine the specific contribution of HDL-cholesteryl esters to adrenal glucocorticoid output in vivo.

Methods and Results
Lcat knockout mice exhibit an 8-fold higher plasma free cholesterol-to-cholesteryl ester ratio (P<0.001) and complete HDL-cholesteryl ester deficiency. Apolipoprotein B-containing lipoprotein and associated triglyceride levels are increased in Lcat knockout mice as compared to C57BL/6 controls (+44%; P<0.05). Glucocorticoid producing adrenocortical cells within the zona fasciculata in Lcat knockout mice are devoid of neutral lipids. However, adrenal weights and basal corticosterone levels are not significantly changed in Lcat knockout mice. In contrast, adrenals of Lcat knockout mice show compensatory upregulation of genes involved in cholesterol synthesis (HMGCR; +516%; P<0.001) and acquisition (LDLR; +385%; P<0.001) and a marked 40-50% lower glucocorticoid response to adrenocorticotropic hormone exposure, endotoxemia, or fasting (P<0.001 for all).

Conclusion
Our studies show that HDL-cholesteryl ester deficiency in Lcat knockout mice is associated with a 40-50% lower adrenal glucocorticoid output. These findings further highlight the important novel role for HDL as cholesterol donor for the synthesis of glucocorticoids by the adrenals.
Introduction

The production and subsequent secretion of glucocorticoids by adrenocortical cells of the zona fasciculata is dependent on the availability of the steroidogenic precursor cholesterol. Unesterified cholesterol is converted to glucocorticoids through a series of side-chain modifications by cytochrome P450 enzymes and hydroxysteroid dehydrogenases. The intra-mitochondrial transfer of unesterified cholesterol by the enzyme steroidogenic acute regulatory protein (StAR) is considered to be the rate-limiting step in the basal synthesis of glucocorticoids. In vitro studies using isolated adrenocortical cells have suggested that both high-density lipoprotein (HDL) and apolipoprotein B (apoB)-containing lipoproteins are able to provide cholesterol as source for the synthesis of glucocorticoids. We and others have shown that under conditions where glucocorticoids are physiologically relevant, i.e. under stress, the exogenous uptake and intracellular processing of lipoprotein-associated cholesteryl esters becomes of crucial importance to maintain optimal adrenal glucocorticoid function in vivo. Probufol-induced depletion of plasma cholesterol associated with both high- (HDL) and low-density lipoproteins (LDL) in C57BL/6 wild-type mice is associated with a lower stress-induced glucocorticoid level. In addition, a defect in the hydrolysis of lipoprotein-associated cholesteryl esters in hormone-sensitive lipase (Hsl) knockout mice is associated with adrenocortical hypofunction. Furthermore, apolipoprotein A1 (Apoa1) knockout mice that virtually lack HDL particles and scavenger receptor BI (Scarb1) knockout mice that exhibit an impaired uptake of cholesteryl esters from HDL both show a parallel diminished adrenal glucocorticoid function. Combined, these findings suggest that the uptake of HDL-cholesteryl esters by the adrenals is essential to maintain optimal glucocorticoid production in vivo.

The HDL-associated enzyme lecithin-cholesterol acyltransferase (LCAT) mediates the synthesis of HDL-cholesteryl esters. Human subjects with a deleterious mutation on both alleles of the Lcat gene present with HDL deficiency, whereas heterozygotes typically have HDL cholesterol levels that are half of normal HDL cholesterol. Heterozygous and homozygous Lcat knockout mice show a similar dose dependent decrease in plasma HDL levels and thus represent a good mouse model to study the consequences of HDL-cholesteryl ester deficiency on general physiology. To delineate the quantitative contribution of HDL-associated cholesteryl esters to the adrenal glucocorticoid output, here we assessed adrenal glucocorticoid function in Lcat knockout mice.

Experimental procedures

Animals

Lcat knockout mice and C57BL/6 wild-type controls were bred in house and fed a regular chow diet ad libitum. Throughout the experiment both types of mice were housed in the same climate controlled stable with a 12h/12h dark-light cycle and handled identically. Age-matched 10-12 week old C57BL/6 mice (n=10) and Lcat knockout mice (n=8) were switched to a new cage and subsequently fasted overnight (~18h) before tail chop blood draws. After an additional two weeks, these mice were injected intraperitoneally with
200 µg human ACTH analogue (ACTH(1-24); tetracosactide) followed by tail blood draws at t=1, 2, 3 hours after ACTH exposure. Six week after the start of experiment, the mice received an intraperitoneal 50 µg/kg sub-lethal dose of lipopolysaccharide (LPS; Salmonella minnesota R595) followed by tail blood draws at t=1, 2, and 3 hours after LPS exposure and sacrifice and tissue harvest at 4 hours after LPS exposure. Before all three types of stress, mice were bled through tail chop to obtain an average basal plasma corticosterone value of each separate mouse. An additional group of 12 week old Lcat knockout (n=4) and C57BL/6 mice (n=11) was subjected to overnight fasting (18h) and subsequently sacrificed for tissue harvesting. Animal care and procedures were performed in accordance with the national guidelines for animal experimentation. All protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

**Plasma lipid analyses**
Plasma concentrations of free cholesterol, cholesteryl esters, and triglycerides were determined using enzymatic colorimetric assays. The cholesterol distribution over the different lipoproteins in plasma was analyzed by fractionation of 50 µl pooled plasma of each mouse genotype using a Superose 6 column (3.2 x 30 mm, Smart-system, Pharmacia). Free cholesterol and cholesteryl ester content of the effluent was determined using enzymatic colorimetric assays.

**Adrenal neutral lipid visualization**
Seven micrometer cryosections were prepared on a Leica CM3050-S cryostat. Cryosections were routinely stained with Oil red O for neutral lipid visualization. Nuclei were detected using a hematoxylin stain.

**Plasma hormone analysis**
Corticosterone levels in plasma were determined using the corticosterone ³H RIA Kit from ICN Biomedicals according to the protocol from the supplier.

**Plasma tumor necrosis factor-α (TNF-α) analysis**
TNF-α protein levels were determined in plasma by ELISA (OptEIA kit, BD Biosciences Pharmingen, San Diego, CA) using the standard protocol.

**Real-time quantitative PCR**
Gene expression analysis was performed essentially as described. Equal amounts of RNA were reverse transcribed and subsequently real-time quantitative PCR analysis was executed on the cDNA using an ABI Prism 7500 apparatus (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Beta-actin and GAPDH were used as housekeeping genes for normalization.

**Data analysis**
Statistical analysis was performed using Graphpad Instat software (San Diego, USA, http://www.graphpad.com). Normality of the experimental groups was confirmed using the
method of Kolmogorov and Smirnov. The significance of differences was calculated using
a two-tailed unpaired t-test or two-way analysis of variance (ANOVA) where appropriate.
Probability values less than 0.05 were considered significant.

Results

**Lcat knockout mice exhibit isolated HDL-cholesteryl ester deficiency**

In accordance with a prominent role for LCAT in the esterification of free cholesterol,\(^{16}\) Lcat knockout mice exhibited an almost complete absence of cholesteryl esters (-86%;
P<0.001) in plasma with unchanged plasma free cholesterol levels (table 1). As a result,
the free cholesterol to cholesteryl ester ratio was 8-fold higher (P<0.001) in plasma
from Lcat knockout mice as compared to C57BL/6 wild-type controls (table 1). FPLC
lipoprotein analysis revealed that both free cholesterol and cholesteryl esters levels in
C57BL/6 wild-type mice were primarily associated with the HDL-fraction (65% and
78%). As anticipated, virtually no cholesteryl esters were present in the HDL fraction in
plasma of Lcat knockout mice, while the non-HDL cholesteryl ester content was essentially
unaffected (figure 1). The HDL-associated free cholesterol level was also markedly lower
Lcat knockout mice as compared to C57BL/6 mice (figure 1). In contrast, the level of free
cholesterol associated with apoB-containing lipoproteins was 2.9-fold higher in plasma
of Lcat knockout mice (figure 1). In line with an increased amount of triglyceride-rich
apoB-containing VLDL and LDL particles circulating in plasma of Lcat knockout mice, as
previously already noted by Sakai et al,\(^{13}\) we detected a significantly higher level of plasma
triglycerides (+44%; P<0.05) in Lcat knockout mice (Table 1).

**Lcat knockout mice show a diminished adrenal corticosterone output**

The basal secretion of glucocorticoids by adrenals in mice is relatively low and is generally
assumed to be independent of the acquisition of extracellular cholesterol pools since
endogenous de novo production of cholesterol from acetyl-CoA should be sufficient to
maintain basal levels. However, although the effect failed to reach statistical significance
(P=0.073), we did observe a marked decrease in basal plasma levels of corticosterone - the
primary glucocorticoid circulating in rodents - in response to Lcat deficiency (96±20 ng/
ml for Lcat knockout mice vs 148±20 ng/ml for C57BL/6 mice).

Table 1. Plasma lipids in wild-type C57BL/6 and LCAT knockout (LCAT KO) mice

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>LCAT KO</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol (FC; mg/dl)</td>
<td>21±1</td>
<td>20±2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cholesteryl esters (CE; mg/dl)</td>
<td>50±2</td>
<td>7±1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total cholesterol (TC; mg/dl)</td>
<td>71 ± 3</td>
<td>27 ± 2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FC/ CE ratio</td>
<td>0.41±0.01</td>
<td>3.31±0.57</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>93±4</td>
<td>134±18</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
Activation of the hypothalamus-pituitary-adrenal axis results in the secretion of glucocorticoids by the adrenals at levels that effectively activate downstream glucocorticoid receptor signalling, an essential part of the body’s response to physiological stressors. As anticipated, adrenocortical cell activation upon the administration of a synthetic mimetic of the pituitary-derived hormone ACTH (tetracosactide; 200 µg i.p.) – a potent activator adrenal steroidogenesis – was associated with an acute rise in plasma corticosterone levels in C57BL/6 wild-type mice. Plasma corticosterone reached a plateau concentration at 1 hour after the tetracosactide injection, which remained maximal until 3 hours after the administration (figure 2A). Tetracosactide exposure also increased plasma corticosterone levels in Lcat knockout mice, however, the peak concentration of corticosterone in plasma of Lcat knockout mice was 37% lower than the level detected in C57BL/6 mice after 1 hour of tetracosactide exposure (365±31 ng/ml vs. 584±34 ng/ml; P<0.001; figure 2A). In contrast to wild-type mice, the concentration of corticosterone rapidly declined in Lcat knockout mice after 1 hour and returned to basal levels at the 3 hours (figure 2A). This suggests that the adrenals of Lcat knockout mice are only capable of realizing a short attenuated glucocorticoid response upon activation of the hypothalamus-pituitary-adrenal axis.

Endogenous glucocorticoids protect against sepsis and other inflammation-associated pathologies.17 In line with an essential role for glucocorticoids in the response to infection,

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Figure 1. Distribution of free cholesterol and cholesteryl esters over high-density lipoprotein (HDL) and non-HDL very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions in pooled plasma of wild-type C57BL/6 and LCAT knockout (LCAT KO) mice.
exposure to a sub-lethal dose of endotoxin (lipopolysaccharide [LPS]; 50 µg/kg i.p.) also induced a rapid increase in plasma corticosterone levels in C57BL/6 mice, which reached a plateau of 586±24 ng/ml at 2 hours after endotoxin exposure (figure 2B). Plasma corticosterone levels did rise in Lcat knockout mice upon endotoxin exposure (figure 2B). However, Lcat knockout mice displayed a more gradual increase up to 3 hours after LPS exposure (341±24 ng/ml), which was markedly lower as compared to the maximal level observed in C57BL/6 mice (593±31 ng/ml; P<0.001; figure 2B). Of note, the observed maximum plasma corticosterone levels (~600 ng/ml for C57BL/6 mice vs ~300 ng/ml for Lcat knockout mice) as well as the area-under-the-curve (AUC; ~1400 ng/ml.h for C57BL/6 mice vs ~800 ng/ml.h for Lcat knockout mice) were similar for either genotype after tetracosactide and endotoxin exposure. It is therefore suggested that both treatments induced a maximal acute adrenal steroid output, which is apparently 40-50% lower in Lcat knockout mice.

Since glucocorticoids through activation of the nuclear glucocorticoid receptor are important regulators of gluconeogenesis and glucose utilization, overnight fasting is associated with an obligatory stimulation of adrenal glucocorticoid secretion to overcome hypoglycemia.10,18,19 In line, after ~18 hours of fasting we observed 51% lower (P<0.001) corticosterone levels in Lcat knockout mice as compared to C57BL/6 controls (figure 2C). Combined, these findings suggest that HDL deficiency in Lcat knockout mice is associated with a diminished adrenal corticosterone output in response to stress.

**Adrenals of Lcat knockout mice are deprived of neutral lipids despite compensatory upregulation of genes associated with cholesterol acquisition**

No significant difference in the weight of the adrenals between Lcat knockout mice and C57BL/6 mice during endotoxemia, i.e. 4 hours after LPS exposure, or under fasting stress conditions was noted (figure 3A). Ng et al. have previously described that adrenals from Lcat knockout mice are under normal conditions deprived of cholesteryl esters.14 In our

![Figure 2](image-url)
group of Lcat knockout mice, this deficiency of neutral lipids in adrenocortical cells could be verified using Oil red O neutral lipid staining. As evident from figure 3B, specifically the glucocorticoid-producing adrenocortical cells within the zona fasciculata in Lcat knockout mice lack the intense Oil red O staining as seen in the C57Bl/6 mice.

The unesterified cholesterol pool used for steroidogenesis can be supplied by (1) endogenous synthesis of cholesterol in which HMG-CoA reductase catalyzes the rate-limiting step, (2) hydrolysis of stored cholesteryl esters, or (3) uptake of exogenous lipoprotein-associated cholesterol. To identify potential compensatory gene regulation, using quantitative real-time PCR, we measured gene expression levels in adrenals harvested from 18 hour fasted Lcat knockout mice and C57BL/6 wild-type controls (figure 3C). A 6-fold stimulation of HMG-CoA reductase (HMGCR; P<0.001) mRNA expression levels was detected in the adrenals of Lcat knockout mice. The relative expression levels of enzymes crucially involved in synthesis (acetyl-CoA acetyltransferase 1; ACAT-1) and hydrolysis

Figure 3. (A) Adrenal weights in wild-type C57BL/6 (n=10) and LCAT knockout (LCAT KO; n=8) mice that either suffered from sub-lethal endotoxemia or were fasted overnight for ~18 hours. (B) Representative Oil red O neutral lipid staining of cortical zones in adrenals from C57BL/6 and LCAT KO mice. ZG, zona glomerulosa; ZF, zona fasciculata. (C) Relative mRNA expression levels of cholesterol metabolism-associated genes in adrenals of LCAT KO mice (n=4) as fold compared to those found in C57BL/6 controls (n=11). *** P<0.001 vs C57BL/6 mice.
Diminished adrenal function in *Lcat* knockout mice

(hormone-sensitive lipase; HSL) of cholesteryl esters within the adrenals were unaffected. Adrenal scavenger receptor BI (SR-BI) expression was unaltered, while the LDL receptor (LDLR) expression level was significantly increased (+384%; *P*<0.001) in *Lcat* knockout mice. It thus seems that adrenals of *Lcat* knockout mice as compared to those of wild-type controls, probably as a compensatory response, attempt to synthesize more cholesterol and acquire increased amounts of LDL-cholesterol through receptor-mediated uptake.

**Lcat** knockout mice display hepatocyte but not leukocyte glucocorticoid insufficiency

The glucocorticoid receptor is highly expressed in leukocytes where it modulates inflammatory responses\(^\text{20}\) as well as in hepatocytes where it regulates glucose metabolism.\(^\text{21}\) Since leukocyte glucocorticoid insufficiency is associated with an enhanced endotoxemia-associated cytokine profile,\(^\text{22,23}\) we determined the effect of *Lcat* deficiency on the LPS-induced tumor necrosis factor-α (TNF-α) response. Plasma levels of the early response cytokine TNF-α were not significantly different at any time point measured after LPS (50 µg/kg i.p.) exposure (0-3 hours; *figure 4A*). It thus seems that *Lcat* knockout and C57BL/6 wild-type mice reach a similar (optimal) anti-inflammatory glucocorticoid receptor signalling level in leukocytes after LPS exposure.

As anticipated, *Lcat* mRNA expression was completely absent in livers of *Lcat* knockout mice (*P*<0.001; *figure 4B*). The relative expression level of the hepatocyte-specific glucocorticoid-induced gene APOA4 encoding apolipoprotein A4\(^\text{18}\) however, was also significantly reduced in livers of overnight fasted *Lcat* knockout mice (-66%; *P*<0.01; *figure 4B*). Furthermore, *Lcat* deficiency was associated with a marked 3.7-fold increase
(P<0.001; figure 4B) in the relative hepatic mRNA expression level of the glucocorticoid carrier protein corticosteroid binding globulin (CBG), whose gene expression level in hepatocytes is subject to negative feedback control by glucocorticoids.24 Thus, downstream glucocorticoid receptor signalling pathways appear to be relatively de-activated in hepatocytes within the liver of Lcat knockout mice. Combined, these findings suggest that Lcat knockout mice do not suffer from total body glucocorticoid insufficiency, but rather display hepatocyte-specific glucocorticoid insufficiency.

**Discussion**

Although it is widely acknowledged that both HDL and non-HDL apoB-containing lipoproteins can be used as source of cholesterol for the production of glucocorticoids by adrenocortical cells, the relative contribution of the separate lipoprotein classes to this process in vivo is not exactly known. In the current study, we assessed glucocorticoid function in Lcat knockout mice to investigate the contribution of specifically HDL-associated cholesteryl esters to adrenal steroidogenesis. Despite relatively high circulating non-HDL cholesterol levels and a compensatory upregulation of genes associated with adrenal cholesterol acquisition, Lcat knockout mice that lack HDL-cholesteryl esters are unable to execute a full glucocorticoid response (40-50% lower as compared to C57BL/6 wild-type controls) to three types of stress being tetracosactide administration, LPS exposure, and overnight fasting. The VLDL cholesteryl ester composition (saturation state) is significantly changed in Lcat KO mice,25 which can theoretically also affect the adrenal glucocorticoid output.26 However, isolation of VLDL fractions from pooled plasma of C57BL/6 and Lcat KO mice (n=13 each) using ultracentrifugation indicated that both types of mice do not have sufficiently high levels of VLDL to carry out in vitro steroidogenesis studies using H295R adrenocortical cells (data not shown). This suggests that any differences in the VLDL cholesteryl ester composition are unlikely to have a major impact on the glucocorticoid insufficiency phenotype observed in Lcat KO mice.

We thus consider our current findings clear proof that HDL-associated cholesteryl esters make an essential contribution to in vivo adrenal glucocorticoid production. Accordingly, data from studies in human LCAT deficient subjects further support this concept, since LCAT deficiency in humans is associated with a lower adrenal steroid output as measured by a decrease in the urinary excretion of 17-ketogenic steroids and 17-hydroxycorticoids.27

Our previous data from Scarb1 knockout mice that lack a functional HDL receptor indicated that disrupted adrenal uptake of cholesteryl esters from HDL is associated with glucocorticoid insufficiency as these mice display 1) a lowered hepatic glucocorticoid signalling and 2) an enhanced susceptibility to endotoxemia.9,10,28 In the current study we observed that Lcat knockout mice that completely lack HDL-cholesteryl esters also display diminished glucocorticoid signalling in hepatocytes as they exhibit a lowered hepatic APOA4 mRNA expression level and a compensatory upregulation of liver CBG expression. In line with a generally lower hepatic glucocorticoid signalling in Lcat knockout mice, Ng et al. have previously detected a lower relative expression level of the glucocorticoid-responsive gene PEPCK and a parallel decrease...
in plasma glucose levels in response to Lcat deficiency. In contrast to the change in hepatic glucocorticoid action in Lcat knockout mice, leukocyte-specific glucocorticoid signalling is apparently normal in these mice, as the susceptibility to endotoxemia is unaffected. Such a difference in cell-specific glucocorticoid sensitivity, i.e. high glucocorticoid receptor sensitivity in leukocytes and low glucocorticoid receptor sensitivity in hepatocytes has also been detected in our previous adrenalectomy and adrenal transplantation studies.

Total Scarb1 knockout mice thus display a more severe glucocorticoid insufficiency phenotype than that observed in Lcat knockout mice. SR-BI is the sole molecule involved in the selective uptake of HDL-associated cholesteryl esters. As both types of mice display hepatocyte-specific glucocorticoid insufficiency, we anticipate that the HDL / SR-BI interaction is essential to generate the bulk of corticosterone needed to effectively activate the glucocorticoid receptor in hepatocytes. However, the difference in leukocyte glucocorticoid insufficiency and endotoxemia susceptibility between the Lcat and Scarb1 knockout mice seems to rely on a HDL-cholesteryl ester independent corticosterone response. SR-BI knockout mice fail to increase their plasma corticosterone levels in response to LPS exposure, whereas the present study shows that Lcat knockout mice are able to increase – albeit to a lower extent – their plasma glucocorticoid levels in response to endotoxemia. Both our Lcat knockout mice and Scarb1 knockout mice display an increase in the adrenal relative expression level of the LDL receptor, the primary protein involved in the clearance of apoB-containing lipoproteins such as VLDL and LDL. Since previous findings by Kraemer et al. have suggested that the LDL receptor does not supply cholesterol the steroidogenic pathway, a qualitative role for the LDL receptor / apoB-lipoprotein interaction in the synthesis of this distinct corticosterone pool in Lcat knockout mice can be excluded. Importantly, SR-BI is also able to clear apoB-containing lipoproteins. Lcat knockout adrenals - in contrast to Scarb1 knockout adrenals - may thus actually acquire cholesteryl esters from VDL/LDL particles through receptor-mediated uptake by SR-BI, which is sufficient to maintain plasma glucocorticoid levels that effectively activate the glucocorticoid receptor in leukocytes (but not hepatocytes). Future studies with specific SR-BI mutants that are selective for either only HDL or non-HDL binding and the associated selective uptake, as identified by for example the group of Dr. Monty Krieger, will unequivocally show the possible contribution of the non-HDL / SR-BI interaction to total adrenal steroid production.

In conclusion, our studies show that HDL-cholesteryl ester deficiency in Lcat knockout mice is associated with a 40-50% lower adrenal glucocorticoid stress response and, as a result, a hepatocyte-specific glucocorticoid insufficiency phenotype. These findings further highlight the important novel role for HDL as cholesterol donor for the synthesis of glucocorticoids by the adrenals.

Acknowledgements
The authors wish to thank Dr. Jeroen C. Rijk from the RIKILT - Institute of Food Safety of Wageningen UR in The Netherlands for his experimental suggestions. This study was supported by TIPharma (Grant T2-110), the Dutch Heart Foundation (Grants 2007T056, 2008T070, and 2009B027) and the Landsteiner Foundation for Blood Transfusion Research (Grant 0912F).
Reference List

networks involving the neuroendocrine system, interleukin-1 and tumor necrosis factor-alpha. 


High-density lipoprotein as a source of cholesterol for adrenal steroidogenesis; a study in individuals with low plasma HDL-c

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Abstract

Introduction
Few studies have addressed the delivery of lipoprotein derived cholesterol to the adrenals for steroid production in humans. While there is evidence against a role for low-density lipoprotein (LDL) it is unresolved whether high-density lipoprotein (HDL) contributes to adrenal steroidogenesis.

Methods and results
To study this, steroid hormone profiles in urine were assessed in male subjects suffering from functional mutations in ATP-binding cassette transporter A1 (ABCA1; n=24), lecithin cholesterol:acyltransferase (LCAT; n=40) as well as in 11 subjects with low HDL-c without ABCA1/LCAT mutations. HDL-c levels were 39% lower in the ABCA1, LCAT and low HDL-c group, compared with controls (all p<0.001). In all groups with low HDL-c levels, urinary excretion of 17-ketogenic steroids was reduced by 33%, 27% and 32% compared with controls (all p<0.04). In 7 carriers of either type of mutation, ACTH stimulation did not reveal differences from normolipidemic controls.

Conclusion
This study shows that basal but not stimulated corticosteroid metabolism is attenuated in subjects with low HDL-c, irrespective of its molecular origin. These findings lend support to a role for HDL as cholesterol donor for basal adrenal steroidogenesis in humans.
Introduction

The synthesis and secretion of adrenal steroid hormones for regulating stress responses, electrolyte homeostasis, and maintenance of secondary sexual characteristics depends upon the availability of the precursor cholesterol. To secure a continuous cholesterol supply, the adrenal glands can synthesize cholesterol, metabolize intracellular esterified cholesterol or obtain cholesterol from circulating lipoproteins. Although exact data are lacking, plasma lipoproteins have been suggested to contribute more than 75% of all cholesterol required for adrenal steroidogenesis but the current literature gives little insight in associations between plasma lipoproteins levels and adrenal function in humans.

With respect to a role for low-density lipoprotein (LDL), it has been shown that LDL receptor deficient patients suffering from familial hypercholesterolemia display normal urinary excretion of both 17-hydroxycorticosteroids and 17-ketogenic steroid metabolites indicative of unaffected adrenal function. In addition, low or absent LDL-c in carriers of one or two defective APOB alleles respectively did not affect basal adrenal function either. Combined this suggests that LDL is probably not playing a major role in delivering cholesterol for steroid hormone production in humans. Associations with high-density lipoprotein cholesterol (HDL-c) have thus far only been studied in critically ill patients. In one study it has been shown that low HDL-c in such patients was associated with attenuated adrenal responses to synthetic ACTH. In support, others reported a high incidence of adrenal failure in critically ill individuals with liver disease, with HDL-c being the only variable predictive of adrenal insufficiency.

Using adrenal cells, it has been suggested that HDL is the preferred lipoprotein for cholesterol delivery to the adrenal gland and in accordance, scavenger receptor type B1 (SRB1) mediated cholesterol uptake from HDL has been shown to be the predominant source of cholesterol in mice. In line, it has been shown that mice lacking SRB1 display an impaired adrenal glucocorticoid stress response, lending support to a major role for HDL as cholesterol donor in mice. In humans, we also showed that adrenal function was compromised in individuals with a functional mutation in SCARB1, the gene encoding SRB1. While this study showed that cholesterol delivery to the adrenals via the HDL-SRB1 pathway is important for adrenal steroidogenesis in humans, it is unclear whether plasma HDL-c levels are associated with adrenal steroidogenesis in humans. To investigate this, we assessed basal and ACTH-stimulated adrenal cortical function in males with low HDL-c due to mutations in either ATP-binding cassette transporter 1 (ABCA1) or lecithin-cholesterol acyltransferase (LCAT) and in subjects with low HDL-c without mutations in ABCA1/LCAT as well as in normolipidemic controls. We hypothesized that in subjects with low HDL-c levels, adrenal function would be compromised irrespective of the molecular origin of the low HDL-c.

Methods

Recruitment of study participants

Male subjects with HDL-c levels below the fifth percentile were screened for mutations in ABCA1 and LCAT of which the functionality was assessed in previously published
For the current study, we enrolled 24 carriers of mutations in the \( ABCA1 \) gene. We furthermore enrolled 40 male carriers of mutations in the \( LCAT \) gene. In addition, subjects with similarly reduced HDL-c levels without mutations in \( ABCA1 \) and \( LCAT \) were included (n=11). As a control group, normolipidemic age matched male individuals were recruited by advertisement. None of the included individuals used medication interfering with steroid metabolism. The study was approved by the institutional review board of the Academic Medical Center, Amsterdam, The Netherlands and all participants provided written informed consent.

**Questionnaire and biochemical measurements**

Medical history, cardiovascular risk factors, use of medication and family history of cardiovascular disease were assessed using a questionnaire. Brachial artery blood pressures were measured using an oscillometric blood pressure device (Omron 705IT, Hoofddorp, the Netherlands). Hypertension was defined as 1) use of antihypertensive medication or 2) a systolic blood pressure at visit above 140 mmHg and/or diastolic blood pressure was above 90 mmHg.

Plasma was obtained after an overnight fast and stored at -80 °C. Total cholesterol, LDL-c, HDL-c and triglyceride levels were analyzed using commercially available enzymatic methods (Randox, Antrim, United Kingdom and Wako, Neuss, Germany) on a Cobas Mira autoanalyzer (Roche, Basel, Switzerland). Free cholesterol and total cholesterol were measured before and after precipitation of apoB-containing lipoproteins using phosphotungstic acid (Sigma) using a commercial available enzymatic assays (Diasys) on a selectra autoanalyzer (Sopachem). ACTH was determined by an immunoluminometric assay (Nichols Institute, Los Angeles, CA). Aldosterone was measured using a radioimmunoassay (Siemens, Los Angeles, USA).

**Baseline adrenal steroidogenesis**

Urinary excretion of steroid metabolites was analyzed by gas chromatography in 24-hour urine samples as previously described.\(^{22,23}\) Androsteron (A), etiocholanolon (E), dehydroepiandrosteron (D), 11-keto-androsteron (KA), 11-keto-etiocholanolon (KE), 11-hydroxy-androsteron (HA), 11-hydroxy-etiocholanolon (HE), pregnanediol (P2), Pregnaantriol (P3), 11-deoxytetrahydrocortisol (THS), tetrahydrocortison (THE), tetrahydrocortisol (THF) and allo-tetrahydrocortisol (ALLO) were measured as readout of adrenal steroidogenesis. A, E, D, KA, KE, HA and HE make up total 17-ketogenic steroids (17-KS), whereas THS, THE, THF, ALLO and P3 are the constituents of total 17-hydroxycorticoids (17-OHCS). In addition, urinary free cortisol was determined using solid-phase extraction-liquid chromatography- tandem mass spectrometry on a Symbiosis Pharma (Spark Holland, Emmen, The Netherlands) Quattro premier Tandem Mass spectrometer (Waters, Millford MA) system. Solid Phase extraction was performed on Oasis HLB cartridges (Waters, Millford, MA), chromatographic separation was achieved on a Waters Sunfire C18 column 3.5 \( \mu \text{m} \) 2.1 x 50 mm using ammonium acetate mM with 0.1% formic acid as mobile phase and acetonitrile as mobile phase B. Limit of detection 5 nmol/L, intra-assay variation <4%, total assay variation <7%.
Stimulated adrenal steroidogenesis
Random subgroups of seven ABCA1 and 7 LCAT mutation carriers consented to an ACTH stimulation study (co-syntropin or tetracosactin, 0.25 mg/ml, Novartis Pharma b.v., Arnhem, The Netherlands). After an overnight fast, participants underwent cosyntropin testing at 0900h. Two baseline blood samples were obtained, 15 minutes and 1 minute before administration of the 1μg cosyntropin bolus. Subsequent blood samples were drawn 30 minutes and 60 minutes after cosyntropin administration. Plasma cortisol levels were measured by enzyme immunoassay (Siemens Medical Solutions, Los Angeles, CA), and cortisol binding globulin (CBG) levels were measured with a commercial radioimmunoassay (Siemens Medical Solutions, Los Angeles, CA). Free cortisol levels were calculated using the method described by Coolens et al.24

Statistical analysis
Unpaired student's T-test was performed for analysis of continuous data with a normal distribution. In case of a skewed distribution, data were log-transformed prior to T-testing. Categorical data were assessed by χ²-testing. A p-value of <0.05 was considered statistically significant. A linear regression model was used to correct for differences in LDL-c and statin use.

Results
Population characteristics
We enrolled 24 and 40 carriers of loss of function mutations in either ABCA1 or LCAT. Two of the ABCA1 mutation carriers were compound heterozygotes while three of the LCAT mutation carriers were homozygotes. None of the participants was referred to our clinic for symptoms of adrenal dysfunction. Individuals were matched to male controls for age. In parallel, we also included subjects with equally low HDL-c levels without mutations in ABCA1 or LCAT. Demographic, clinical and biochemical characteristics of all low HDL-c groups as well as controls are listed in table 1. As expected, HDL-c levels were 39% lower in carriers of ABCA1 or LCAT mutations and in the low HDL-c group, compared with normolipidemic controls (p<0.001). LDL-c levels were 25% lower in carriers of ABCA1 mutations (p=0.003), 11% lower in carriers of LCAT mutations (n.s.) and 12% lower in the low HDL-c group (p=0.34 and p=0.39) compared with controls. Detailed analysis of the concentrations of cholesteryl esters and free cholesterol in the total cholesterol fraction and the isolated HDL-c fraction, in a subset of patients, is provided in table 2.

Compared with controls, hypertension was more prevalent among LCAT mutation carriers (p=0.01). Statin use was more prevalent in the ABCA1 and LCAT mutation carriers, compared with the control group. The low HDL-c group had similar baseline characteristics compared with the ABCA1 and LCAT groups.

Basal adrenal steroidogenesis
Compared with the control group, we identified lower 24 hour urinary excretion of 17-ketogenic steroids (17-KS) in carriers of mutations in ABCA1 (33%; p=0.003; figure 1a),
Table 1. Characteristics of male ATP-binding cassette transporter 1 (*ABCA1*) and Lecithin-cholesterol acyltransferase (*LCAT*) mutation carriers and matched male controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ABCA1 Carriers (n=24)</th>
<th>Controls (n=24)</th>
<th>Low HDL-c (n=11)</th>
<th>p value</th>
<th>p value</th>
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<td>Demographic</td>
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<td>Age -yr</td>
<td>45.2±20.6</td>
<td>45.5±18.5</td>
<td>44.6±12.0</td>
<td>0.96</td>
<td>0.91</td>
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<td>BMI (kg/m2)</td>
<td>25.3±3.3</td>
<td>25.1±5.2</td>
<td>26.3±8.8</td>
<td>0.92</td>
<td>0.81</td>
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<td>Current smoker (%)</td>
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<td>4 (17.4)</td>
<td>3 (27)</td>
<td>0.21</td>
<td>0.72</td>
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<td>Alcohol users - no (%)</td>
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<td>19 (79)</td>
<td>9 (82)</td>
<td>0.51</td>
<td>0.49</td>
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<td>Coronary artery disease-no (%)</td>
<td>7 (29.2)</td>
<td>1 (4.8)</td>
<td>4 (46)</td>
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<td>~</td>
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<tr>
<td>Diabetes mellitus - no (%)</td>
<td>3 (13.6)</td>
<td>0 (0)</td>
<td>1 (9)</td>
<td>0.07*</td>
<td>0.71*</td>
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<td>Hypertension - no (%)</td>
<td>7 (29.2)</td>
<td>3 (12.5)</td>
<td>1 (9)</td>
<td>0.16*</td>
<td>0.19*</td>
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<tr>
<td>Statin use - no (%)</td>
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<td>2 (8)</td>
<td>4 (33)</td>
<td>0.02*</td>
<td>0.84*</td>
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<td>Blood pressure (mmHg)</td>
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<td>132±13</td>
<td>127±11</td>
<td>0.34</td>
<td>0.29</td>
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<tr>
<td>Systolic</td>
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<td>0.29</td>
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<td>Diastolic</td>
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<td>Aldosterone (nmol/L)</td>
<td>0.20±0.14</td>
<td>0.23±0.12</td>
<td>0.22±0.19</td>
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<td>ACTH (ng/L)</td>
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<td>LDL cholesterol (mmol/L)</td>
<td>2.7±1.0</td>
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<td>HDL cholesterol (mmol/L)</td>
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<td>0.8±0.2</td>
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<td>Median</td>
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<td>1.2</td>
<td>1.5</td>
<td>0.65*</td>
<td>0.02*</td>
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<tr>
<td>Interquartile range</td>
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<td>0.8-1.9</td>
<td>1.3-2.1</td>
<td>0.65*</td>
<td>0.02*</td>
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<td>Urinary free cortisol (nmol/24h)</td>
<td>60.10±31.66</td>
<td>59.27±39.96</td>
<td>60.10±31.66</td>
<td>0.62*</td>
<td>n.a.</td>
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</table>

Blood pressure measurements were available in 21 *ABCA1* mutation carriers and 17 controls and in 34 *LCAT* mutation carriers and 26 controls. Plasma ACTH levels were available in 18 *ABCA1* mutation carriers and 18 controls and in 20 *LCAT* mutation carriers and 31 controls. Urinary cortisol levels were available in 22 *ABCA1* mutation carriers and 22 controls and in 35 *LCAT* mutation carriers and 36 controls. Values are means ± SD unless otherwise indicated. P for Student’s T-test.

*Triglycerides, plasma ACTH and urinary cortisol were log-transformed prior to T-test. # P for χ² test. No t-test was performed for history of coronary artery disease since referral bias was present. Partial overlap exists between the two control cohorts. N.a. stands for not available.

in *LCAT* (27%; p=0.01, figure 1b) and in the low HDL-c group (30%; p=0.04 and 34%; p=0.02; figure 1). These differences remained statistically significant after correcting for differences in plasma LDL-c and statin use using a linear regression model (p=0.01 and p=0.02 for carriers of *ABCA1* mutations or *LCAT* mutations vs. controls, respectively).

The mean 17-KS steroid excretion of 14.1 µmol/24hr in the *ABCA1* mutation carriers was below the reference values for 17-KS in the appropriate age group.23
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<td>0.75</td>
<td>78±11</td>
<td>0.90</td>
</tr>
<tr>
<td>Aldosterone (nmol/L)</td>
<td>0.20±0.14</td>
<td>0.23±0.12</td>
<td>0.55</td>
<td>0.22±0.19</td>
<td>0.87</td>
</tr>
<tr>
<td>ACTH (ng/L)</td>
<td>23.11±12.62</td>
<td>21.64±15.10</td>
<td>0.25</td>
<td>22.00±14.25</td>
<td>24.57±23.08</td>
</tr>
<tr>
<td>Total cholesterol mmol/L</td>
<td>3.9±1.2</td>
<td>5.2±1.3</td>
<td>&lt;0.001</td>
<td>4.5±2.1</td>
<td>0.27</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.7±1.0</td>
<td>3.6±0.9</td>
<td>0.003</td>
<td>3.2±1.8</td>
<td>0.08</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>0.8±0.3</td>
<td>1.3±0.4</td>
<td>&lt;0.001</td>
<td>0.8±0.3</td>
<td>0.12</td>
</tr>
<tr>
<td>Triglycerides mmol/L</td>
<td>1.0 (1.5)</td>
<td>1.2 (1.2)</td>
<td>~</td>
<td>1.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>0.7-1.5</td>
<td>0.8-1.9</td>
<td>~</td>
<td>1.3-2.1</td>
<td>~</td>
</tr>
<tr>
<td>Urinary free cortisol (nmol/24h)</td>
<td>60.10±31.66</td>
<td>59.27±39.96</td>
<td>0.62</td>
<td>50.33±36.34</td>
<td>57.81±35.19</td>
</tr>
</tbody>
</table>

Blood pressure measurements were available in 21 ABCA1 mutation carriers and 17 controls and in 34 LCAT mutation carriers and 26 controls. Plasma ACTH levels were available in 18 ABCA1 mutation carriers and 18 controls and in 20 LCAT mutation carriers and 31 controls. Urinary cortisol levels were available in 22 ABCA1 mutation carriers and 22 controls and in 35 LCAT carriers and 36 controls. Values are means ± SD unless otherwise indicated. P for Student’s T-test. *Triglycerides, plasma ACTH and urinary cortisol were logtransformed prior to T-test. # P for χ² test. No t-test was performed for history of coronary artery disease since referral bias was present. Partial overlap exists between the two control cohorts. N.a. stands for not available.

The lower levels of urinary17-hydroxy corticosteroids (17-OHCS) in both ABCA1 and LCAT mutation carriers as well as the low HDL-c group did not reach statistical significance (p=0.11 and p=0.15, respectively, *figure 1a-b). The full panel of steroid metabolites is presented in *figure 2a-b*. No gene-dose effect was observed when comparing the 2 compound heterozygous ABCA1 mutation carriers or the 3 homozygous LCAT mutation carriers to heterozygous carriers.
Free urinary cortisol was not significantly different between ABCA1 mutation carriers and controls (60.10±31.66 vs. 59.27±39.96 nmol/24h, p=0.62) or LCAT mutation carriers and controls (50.33±36.34 vs. 57.81±35.19, p=0.31).

Plasma levels of ACTH did not differ significantly between ABCA1 mutation carriers and controls (23.11±12.62 vs. 21.64±15.10, p=0.25) or LCAT mutation carriers and controls (22.00±14.25 vs. 24.57±23.08, p=0.81).

Adrenal response to cosyntropin
In a cosyntropin stimulation test, the cortisol response to physiological levels of ACTH is measured as an approach to assess adrenal cortical function. The peak serum cortisol response to ACTH was comparable in ABCA1 and LCAT mutation carriers and did not differ from controls (p=0.10 and p=0.87 respectively, figure 3a-b). Also peak plasma level of free cortisol, taking into account possible differences in cortisol binding globulin (CBG) levels, were not different. Plasma lipid levels did not differ significantly before and after cosyntropin testing (data not shown).

Discussion
The present study demonstrates that under normal conditions male individuals with low levels of HDL-c irrespective of molecular origin have significantly lower urinary excretion of total 17-KS compared with normolipidemic controls indicative of disturbed basal corticosteroid synthesis. A parallel reduction in hydroxycorticosteroid excretion in both groups did not reach statistical significance which may suggest a preferential adrenal pathway for HDL driven steroidogenesis. Mutations in either ABCA1 or LCAT did, however, not affect the response to ACTH while physical examination and a standardized questionnaire for signs of adrenal insufficiency did not reveal symptoms of clinically

### Table 2. Free cholesterol and cholesteryl ester values in a subset lecithin-cholesterol acyltransferase (LCAT) mutation carriers and controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=29)</th>
<th>Heterozygous carriers (n=29)</th>
<th>p value</th>
<th>Homozygous carriers (n=3)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol mmol/L</td>
<td>5.3±1.2</td>
<td>4.5±1.2</td>
<td>0.002</td>
<td>4.1±0.6</td>
<td>0.09</td>
</tr>
<tr>
<td>FC</td>
<td>1.56±0.35</td>
<td>1.4±0.4</td>
<td>0.04</td>
<td>2.1±0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>CE</td>
<td>3.65±1.04</td>
<td>3.0±0.9</td>
<td>0.01</td>
<td>2.0±0.6</td>
<td>0.01</td>
</tr>
<tr>
<td>FC/CE ratio</td>
<td>0.48±0.30</td>
<td>0.5±0.1</td>
<td>0.73</td>
<td>1.1±0.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Cholesterol after apoB precipitation</td>
<td>1.3±0.4</td>
<td>0.7±0.3</td>
<td>0.03</td>
<td>0.04±0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FC</td>
<td>1.78±0.71</td>
<td>0.1±0.1</td>
<td>0.13</td>
<td>0.0±0.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CE</td>
<td>0.7±0.2</td>
<td>0.6±0.2</td>
<td>0.02</td>
<td>0.04±0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FC/CE ratio</td>
<td>0.24±0.06</td>
<td>0.±0.12</td>
<td>0.50</td>
<td>1.1±0.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

P for Student’s T-test, compared with controls.
relevant adrenal dysfunction. These findings are in line with those in mice and tissue culture showing that the supply of plasma lipoprotein derived cholesterol is used for basal adrenal steroidogenesis but that this pathway is not able to respond to acute stress.\textsuperscript{27-29} For a proper quick response, endogenous adrenal cortisol reserves are secreted upon stimulation. Since steroidogenic tissues are rapidly depleted of cortisol following stimulation, replenishing is thought to occur via uptake of cholesterol from lipoproteins.

We previously reported that individuals with reduced SRB1 function displayed mild adrenal insufficiency in addition to a reduced urinary excretion of steroid hormones.\textsuperscript{16} Thus reduced SRB1 function on adrenal cells has a larger impact on adrenal steroidogenesis when compared with low levels of plasma HDL-c caused by reduced ABCA1 or LCAT function.

There were no differences in urinary excretion of free cortisol. However, particularly in the lower ranges the quantification of free cortisol has been shown to be less reliable as compared with the quantification of a full urinary steroid metabolites in diagnosing the presence of hypocortisolism.\textsuperscript{30,31}

Figure 1. 24-hour urinary steroid excretion in male \textit{ABCA1} and \textit{LCAT} mutation carriers compared with age-matched male controls. Data are presented as mean ± SD. \(P^1\) is the uncorrected p-value for Student’s T-test; \(P^2\) is the p-value corrected for LDL-c and \(P^3\) is the p-value corrected for statin use. 17-KS is 17-ketogenic steroids; 17-OHCS is 17-hydroxycorticosteroids.
Figure 2. Urinary steroid metabolites in male *ABCA1* and *LCAT* mutation carriers compared with age-matched male controls. Data are presented as mean ± SD.
In accordance with LCAT’s mechanism of action, cholesteryl ester concentration is lower in LCAT mutation carriers, with the most pronounced effect in homozygous carriers. Since adrenal steroidogenesis was not further decreased in homozygous carriers compared with heterozygous carriers, there is no indication that the adrenal gland has a preference for cholesteryl esters as a substrate for steroidogenesis. This is supported by the fact that a similar reduction in steroid metabolites was observed in subjects with low HDL-c not related to defects in LCAT.

Plasma levels of ACTH did not differ between the low HDL-c group and controls. The latter most likely reflects the fact that the decrease in urinary steroid metabolites is only mild, thereby precluding a compensatory ACTH increase. In line, none of the study participants reported signs of hypocortisolism.

We would like to discuss three limitations of our study. First, adrenal cells express ABCA1. Thus reduced adrenal ABCA1 expression could conceptually lead to accumulation of cholesterol in adrenal cells, compromising intra-adrenal signalling and steroidogenesis due to cholesterol toxicity as proposed for pancreatic beta-cell dysfunction in individuals carrying ABCA1 mutations. Although we cannot exclude this possibility, mutations in LCAT as well as low HDL-c in non-carrier individuals are associated with similar reductions in HDL-c and urinary steroid metabolite excretion, making an ABCA1 specific effect implausible.

A second limitation is that carriers of ABCA1 mutations showed significantly lower levels of plasma LDL-c levels in addition to low levels of HDL-c. This could mean that combined low levels of HDL-c and LDL-c account for the effects observed but as already discussed, a role for LDL-c in human adrenal corticoid production is unlikely. In line, statistical corrections for the observed reductions in LDL-c did not affect outcome. Third, in the urinary steroid metabolites, the 17KS were particularly decreased in carriers compared with controls. Since 17KS are metabolites derived largely from the androgenic pathway, this finding hints towards the preferential use of HDL-derived cholesterol in adrenal steroidogenesis.
studies are needed to dissect whether and to what extent HDL-derived cholesterol contributes to steroidogenesis in either adrenals or testicular steroid production.

In conclusion, we demonstrate that basal adrenal steroidogenesis is compromised in males with low levels of plasma HDL-c, establishing a role for HDL derived cholesterol in adrenal steroidogenesis in humans.

Acknowledgements
The authors would like to thank C.A.M. Koch en J.F. Los for their assistance in expanding the pedigrees and J. Peter for his help in the identification of \textit{ABCA1} and \textit{LCAT} mutations. The study was supported by three grants from the Dutch Heart Foundation (numbers 2009B027, 2008B070 and 2008T070). G.K. Hovingh and A.G. Holleboom are supported by Veni grants (project numbers 91612122 and 91613031 respectively) from the Netherlands Organisation for Scientific Research (NWO).

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Reference List


Adrenal function in females with low plasma HDL-c due to mutations in \textit{ABCA1} and \textit{LCAT}

Submitted for publication

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Abstract

Introduction
Adrenal steroidogenesis is essential for human survival and depends on the availability of the precursor cholesterol. Male subjects with low plasma levels of high density lipoprotein (HDL) cholesterol are characterized by decreased adrenal function. Whether this is also the case in female subjects with low plasma HDL-c levels is unresolved to date.

Methods and Results
15 female ATP binding cassette transporter A1 (ABCA1) and 14 female lecithin-cholesterol acyltransferase (LCAT) were included in the study. HDL-c levels were 38% and 41% lower in ABCA1 and LCAT mutation carriers compared to controls, respectively. Urinary steroid excretion of 17-ketogenic steroids or 17-hydroxy corticosteroids did not differ between 15 female ABCA1 mutation carriers (p=0.27 vs 0.30 respectively) and 30 matched normolipidemic controls or between 14 female LCAT mutation carriers and 28 matched normolipidemic controls (p=0.10 and 0.14, respectively). Cosyntropin testing in an unselected subgroup of 8 ABCA1 mutation carriers and 3 LCAT mutation carriers did not reveal differences between carriers and controls.

Conclusion
Adrenal function in females with molecularly defined low HDL-c levels is not different from controls. The discrepancy with the finding of impaired steroidogenesis in males with molecularly defined low HDL-c levels underscores the importance of gender specific analyses in cholesterol-related research.
**Introduction**

The adrenal gland plays a pivotal role in essential physiological processes such as the regulation of stress response, blood pressure and electrolyte homeostasis. Cholesterol is the substrate for adrenal steroidogenesis and is for 75% derived from plasma lipoproteins. However, whether the availability of lipoprotein derived cholesterol is a rate-limiting factor for adrenal steroidogenesis in humans is sparsely investigated to date.

The importance of low density lipoprotein (LDL) derived cholesterol for adrenal steroidogenesis has been studied in patients with familial hypercholesterolemia (FH), carrying mutations in the LDL receptor (LDLR). The adrenal gland takes up plasma cholesterol via endocytosis of the LDL receptor. In line with this notion, adrenal steroidogenesis is impaired in homozygous LDLR mutation carriers and homozygous apolipoprotein B (APOB) mutation carriers, suffering from genetically impaired binding of LDL to the LDL receptor. Heterozygous LDLR and APOB carriers, however, did not show any signs of impaired adrenal steroidogenesis. Together, these studies indicate that plasma lipoprotein derived cholesterol plays a role in adrenal steroidogenesis, but that LDL derived cholesterol does not constitute the major source of substrate for the adrenal glands.

High density lipoprotein (HDL) has been suggested to be the preferred lipoprotein for cholesterol delivery to the adrenal gland in adrenal cell lines and in murine models. In critically ill patients, low HDL-c levels are associated with impaired adrenal responses to synthetic ACTH. Furthermore, others reported a high incidence of adrenal failure in critically ill individuals with liver disease, with HDL-c being the only variable predictive of adrenal insufficiency.

Importantly, adrenal function was assessed in individuals with functional mutations in SCARB1, the gene encoding the HDL receptor SRB1, which is highly expressed on the adrenal gland. Heterozygous SRB1 mutation carriers display markedly decreased adrenal steroidogenesis. Furthermore, we recently demonstrated that male individuals with low plasma HDL-c levels are characterized by decreased adrenal function, further underlining the importance of HDL derived cholesterol for adrenal steroidogenesis. However, whether adrenal steroidogenesis is also impaired in females with low plasma HDL-c remains to be investigated.

We set out to assess adrenal function in female carriers of functional mutations in ATP-binding cassette transporter A1 (ABCA1) and lecithin-cholesterol acyltransferase (LCAT), typically displaying half-normal plasma levels of HDL-c. We hypothesized that in female subjects with low HDL-c levels, adrenal function is compromised irrespective of the molecular origin of the low HDL-c levels.

**Methods**

**Recruitment of study participants**

Subjects with HDL-c levels < 5th percentile were screened for mutations in ABCA1 and LCAT. For the current study, we enrolled 15 female carriers of mutations in the ABCA1 gene and 14 female carriers of mutations in the LCAT gene. Functionality of all mutations
was established in previously published studies.\textsuperscript{21-23} As a control group, normolipidemic age matched female individuals were recruited by advertisement. None of the included individuals used oral contraceptives or medication interfering with steroid metabolism. The study was approved by the institutional review board of the Academic Medical Center, Amsterdam, The Netherlands. All participants provided written informed consent.

\textbf{Questionnaire and biochemical measurements}
Medical history, cardiovascular risk factors and use of medication were assessed using a questionnaire. Brachial artery blood pressures were measured using an oscillometric blood pressure device (Omron 705IT, Hoofddorp, the Netherlands). Hypertension was defined as 1) use of antihypertensive medication and/or 2) a systolic blood pressure at visit above 140 mmHg and/or diastolic blood pressure above 90 mmHg.

Plasma was obtained after an overnight fast and stored at -80 ºC. Total cholesterol, LDL-c, HDL-c and triglyceride levels were analyzed using commercially available enzymatic methods (Randox, Antrim, United Kingdom and Wako, Neuss, Germany) on a Cobas Mira autoanalyzer (Roche, Basel, Switzerland). Aldosterone was measured using a radioimmunoassay (Siemens, Los Angeles, USA).

\textbf{Baseline adrenal steroidogenesis}
Urinary excretion of steroid metabolites was analyzed by gas chromatography in 24-hour urine samples as previously described.\textsuperscript{24,25} Androsteron (A), etiocholanolon (E), dehydroepiandrosteron (D), 11-keto-androsteron (KA), 11-keto-etiocholanolon (KE), 11-hydroxy-androsteron (HA), 11-hydroxy-etiocholanolon (HE), pregnanadiol (P2), Pregnaantriol (P3), 11-deoxytetrahydrocortisol (THS), tetrahydrocortison (THE), tetrahydrocortisol (THF) and allo-tetrahydrocortisol (ALLO) were measured as readout of adrenal steroidogenesis. A, E, D, KA, KE, HA and HE make up total 17-ketogenic steroids (17-KS), whereas THS, THE, THF, ALLO and P3 are the constituents of total 17-hydroxycorticoids (17-OHCS). In addition, urinary free cortisol was determined using solid-phase extraction-liquid chromatography- tandem mass spectrometry on a Symbiosis Pharma (Spark Holland, Emmen, The Netherlands) Quattro premier Tandem Mass spectrometer (Waters, Millford MA) system. Solid Phase extraction was performed on Oasis HLB cartridges (Waters, Millford, MA), chromatographic separation was achieved on a Waters Sunfire C18 column 3.5 μm 2.1 x 50 mm using ammonium acetate mM with 0.1% formic acid as mobile phase and acetonitrile as mobile phase B. Limit of detection 5 nmol/L, intra-assay variation <4%, total assay variation <7%.

\textbf{Stimulated adrenal steroidogenesis}
Unselected subgroups of 8 \textit{ABCA1} and 3 \textit{LCAT} mutation carriers consented to an ACTH stimulation study (co-syntropin or tetracosactin, 0.25 mg/ml, Novartis Pharma b.v., Arnhem, The Netherlands). After an overnight fast, participants underwent cosyntropin testing at 09.00h. Two baseline blood samples were obtained, 15 minutes and 1 minute before administration of the 1μg cosyntropin bolus. Subsequent blood samples were
drawn 30 minutes and 60 minutes after cosyntropin administration. Plasma cortisol levels were measured by enzyme immunoassay (Siemens Medical Solutions, Los Angeles, CA), and cortisol-binding globulin (CBG) levels were measured with a commercial radioimmunoassay (Siemens Medical Solutions, Los Angeles, CA). Free cortisol levels were calculated using the method described by Coolens et al.26

**Statistical analysis**

Unpaired student’s T-test was performed for analysis of continuous data with a normal distribution. In case of a skewed distribution, data were log-transformed prior to T-testing. Categorical data were assessed by χ²-testing. A p-value of <0.05 was considered statistically significant.

**Results**

**Population characteristics**

We enrolled 15 and 14 female carriers of loss of function mutations in *ABCA1* and *LCAT*, respectively. Three of the *ABCA1* mutation carriers were either compound heterozygous or homozygous, while two of the *LCAT* mutation carriers were homozygous. None of the participants was referred to our clinic for symptoms of adrenal dysfunction. Age-matched female family members were asked to participate as controls. As an insufficient number of family members volunteered, unrelated age-matched female controls were recruited by advertisement. Demographic, clinical and biochemical characteristics of all study participants are listed in *table 1*. As expected, HDL-c levels were 38% lower in carriers of *ABCA1* mutations and 41% lower in *LCAT* mutation carriers, compared to normolipidemic controls (p<0.001). Hypertension was more prevalent in *ABCA1* mutation carriers (p=0.002) and *LCAT* mutation carriers (p=0.02). Systolic and diastolic blood pressure was significantly higher in *LCAT* mutation carriers compared to controls (p=0.03 and p=0.03, respectively). Other parameters did not differ significantly.

**Basal adrenal steroidogenesis**

Twenty four hour urinary excretion of 17-ketogenic steroids (17-KS) or 17-hydroxycorticosteroids (17-OHCS) did not differ between carriers of mutations in *ABCA1* and controls (p=0.27 and 0.30, Figure 1a), or between *LCAT* mutation carriers and controls (p=0.10 and 0.14, Figure 1b). Urinary steroid excretions were within the normal range for both for 17-KS and 17-OHCS.25

The full panel of urinary steroid metabolites is presented in figure 2a-b.

No gene-dose effect was observed when comparing the three compound heterozygous/ homozygous *ABCA1* mutation carriers or the two homozygous *LCAT* mutation carriers to heterozygous carriers and controls.

**Adrenal response to cosyntropin**

In a 1 μg cosyntropin stimulation test, the cortisol response to physiological levels of ACTH is measured as a proxy of adrenal cortical function27,28. The peak serum cortisol response to ACTH was not different between *ABCA1* and *LCAT* mutation carriers and
Table 1. Characteristics of female ATP-binding cassette transporter 1 (ABCA1) and Lecithin-cholesterol acyltransferase (LCAT) mutation carriers and matched female controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ABCA1 Controls (n=30)</th>
<th>ABCA1 Carriers (n=15)</th>
<th>p value</th>
<th>LCAT Controls (n=28)</th>
<th>LCAT Carriers (n=14)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Demographic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age - yrs</td>
<td>48.1±14.8</td>
<td>47.4±14.5</td>
<td>0.88</td>
<td>41.4±17.4</td>
<td>41.7±15.2</td>
<td>0.99</td>
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<td>BMI (kg/m²)</td>
<td>24.8±4.0</td>
<td>25.7±7.4</td>
<td>0.59</td>
<td>24.7±4.3</td>
<td>25.1±5.9</td>
<td>0.79</td>
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<td>Current smokers - no (%)</td>
<td>4 (13)</td>
<td>2 (13)</td>
<td>1.00a</td>
<td>3 (10)</td>
<td>1 (25)</td>
<td>0.74a</td>
</tr>
<tr>
<td>Statin users - no (%)</td>
<td>3 (33)</td>
<td>6 (67)</td>
<td>0.10a</td>
<td>3 (38)</td>
<td>5 (63)</td>
<td>0.25a</td>
</tr>
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<td>Alcohol users - no (%)</td>
<td>19 (63)</td>
<td>8 (53)</td>
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<td>18 (62)</td>
<td>7 (50)</td>
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<td>Coronary artery disease - no (%)</td>
<td>1 (3)</td>
<td>4 (27)</td>
<td>~</td>
<td>1 (3)</td>
<td>0</td>
<td>~</td>
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<tr>
<td>Diabetes mellitus - no (%)</td>
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<td>1 (7)</td>
<td>~</td>
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<td>Hypertension - no (%)</td>
<td>2 (7)</td>
<td>7 (47)</td>
<td>0.002a</td>
<td>1 (3)</td>
<td>4 (29)</td>
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<td>Blood pressure (mmHg)</td>
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<tr>
<td>Systolic</td>
<td>121.1±16.9</td>
<td>136.1±24.5</td>
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<td>117.0±10.1</td>
<td>130.2±16.6</td>
<td>0.03</td>
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<tr>
<td>Diastolic</td>
<td>73.0±11.7</td>
<td>80.0±10.2</td>
<td>0.10</td>
<td>71.9±10.2</td>
<td>81.1±10.2</td>
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<tr>
<td>Aldosterone (nmol/L)</td>
<td>0.17±0.15</td>
<td>0.26±0.22</td>
<td>0.23</td>
<td>0.18±0.15</td>
<td>0.17±0.14</td>
<td>0.88</td>
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<tr>
<td>Cortisol Binding Globulin (mg/L)</td>
<td>68.0±40.8</td>
<td>65.3±2.5</td>
<td>0.86</td>
<td>68±40.8</td>
<td>73.3±41.3</td>
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<td>Cholesterol mmol/L</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>4.50±1.10</td>
<td>4.41±1.47</td>
<td>0.14</td>
<td>4.82±1.12</td>
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<td>LDL</td>
<td>3.23±1.03</td>
<td>3.20±1.08</td>
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<td>3.04±0.81</td>
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<td>HDL</td>
<td>1.43±0.34</td>
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<td>1.48±0.36</td>
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<td>Triglycerides mmol/L</td>
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<td>Median</td>
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<td>1.09</td>
<td>0.25</td>
<td>0.80</td>
<td>1.12</td>
<td>0.10</td>
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<tr>
<td>Interquartile range</td>
<td>0.65-1.28</td>
<td>0.71-1.63</td>
<td>0.60-1.21</td>
<td>0.68-1.64</td>
<td></td>
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</tbody>
</table>

Discussion

This study shows that adrenal steroidogenesis is not impaired in female individuals with low plasma HDL-c levels. This is in contrast with our earlier finding of lower basal urinary steroid
a. ABCA1

Figure 1. 24-hour urinary steroid excretion in male ABCA1 and LCAT mutation carriers compared to age-matched female controls. Data are presented as mean ± SD. P values for student’s t-test.

Studies showing decreased adrenal steroidogenesis in murine models of compromised availability of HDL derived cholesterol were carried out in both males and females. These studies did not report gender specific effects. It should be taken into account, however, that cholesterol metabolism differs greatly between mice and men and, as a consequence, results derived from murine models are not necessarily reflecting human (patho)physiology. Human studies addressing this question are sparse and data about the distribution of males and females are lacking.
Figure 2. Urinary steroid metabolites in female ABCA1 and LCAT mutation carriers compared to age-matched female controls. Data are presented as mean ± SD. P values for student’s t-test.
Our findings constitute the first evidence of differential adrenal cholesterol handling in males and females. Based on the current data, we can only speculate about the mechanism underlying the differential effect of low plasma HDL-c levels in men and women. In murine models, there was no differential effect between male and female animals, and, therefore, experiments in these experimental models can not be expected to elucidate the gender-dependent findings in humans.

Plasma HDL cholesterol levels are gender specific, as reflected by different reference values for men and women. Furthermore, the correlation between HDL-c levels with age is negative in women, but positive in men. The correlation between HDL-c levels and cardiovascular disease has been described to be gender specific. For example, plasma levels of LCAT were associated with low HDL-c levels in men, but not in women. Moreover, plasma LCAT levels were associated with a surprisingly increased CHD risk in women, but not in men.

Gender-specific effects in adrenal function have also been described. Different reference values are used for urinary steroidogenesis in males and females. Furthermore, females have been shown to exhibit a stronger response to synthetic ACTH than males. However, neither explains the gender-specific findings in our cohort.

Figure 3. Peak serum cortisol increase after cosyntropin administration in female ABCA1 and LCAT mutation carriers versus female controls. Data are presented as mean ± SD. P values for Student’s T-test.
Several mechanisms may explain the different effects of low HDL cholesterol levels on adrenal steroidogenesis between men and women. First, women are characterized by higher levels of estradiol compared to men, which has shown to be essential in maintaining an adequate adrenal output. This may constitute an additional stimulatory pathway, which men lack, equipping women with sufficient adrenal stimuli to overcome the effects of low availability of substrate. Conversely, men display higher levels of androgens, which have shown to be associated with decreased adrenal function, indicating that men not only lack the stimulatory effects of estrogen on adrenal steroidogenesis, but have an inhibitory pathway instead. On top of low levels of HDL-derived cholesterol, this may compromise adrenal steroidogenesis in males, whereas females are relatively protected.

Furthermore, the gender-specific differential effects may pertain to differences in the immune system. Interleukin (IL) 6 is essential for the activation of the hypothalamic-pituitary-adrenal axis. IL6 expression is lower in males compared to females. Given the fact that male adrenal steroidogenesis is more strongly affected by plasma interleukin 6 (IL6) than female adrenal steroidogenesis, this additional lack of adrenal stimulation in low HDL males, may tip the balance to lower adrenal steroidogenesis in males, whereas females both have higher IL6 expression and less dependence of adrenal steroidogenesis on IL6 levels.

Interestingly, hypertension was more prevalent in both ABCA1 and LCAT mutation carriers, but seemed better regulated in ABCA1 mutation carriers. Increased prevalence of hypertension has been previously reported in LCAT mutation carriers. Given the absence of a difference in both plasma aldosterone levels and adrenal function, an adrenal component is unlikely to contribute to the increased hypertension in carriers.

A limitation of this study is the fact that moment in menstrual cycle was not recorded, nor was pre- or postmenopausal state. However, by closely matching study participants for age, the chance was minimized that these age-related circumstances influenced results. Furthermore, the study cohort was relatively small, inherent to studies in subjects with rare mutations. Considering the current data, however, we are confident that increasing the sample size is unlikely to change the results. Lastly, ABCA1 is expressed in the adrenal gland. However, the similar effects in LCAT and ABCA1 mutation carriers suggest that an effect specific to adrenal expression of ABCA1 is unlikely.

Alltogether, our data show that adrenal function in females with molecular defined low HDL-c levels is not different from controls. The discrepancy with the finding of impaired steroidogenesis in males with molecularly defined low HDL-c levels, underscores the importance of gender specific analyses in cholesterol related research.

Acknowledgements

The authors would like to thank C.A.M. Koch en J.F. Los for their assistance in expanding the pedigrees and J. Peter for his help in the identification of ABCA1 and LCAT mutations. This study was supported by three grants from the Dutch Heart Foundation (numbers 2009B027, 2008B070 and 2008T070). A.E.B. and M.M.M. are supported by a grant from Fondation LeDucq. G.K.H. and A.G.H. are supported by Veni Grants (project numbers 91612122 and 91613031, respectively) from the Netherlands Organisation for Scientific Research (NWO).
A role for LDL cholesterol in Cortisol Production in Humans

Submitted for publication

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Abstract

Background
Adrenal steroidogenesis is essential for survival in humans. Three-quarters of all cholesterol required for adrenal steroidogenesis is derived from plasma lipoproteins. We previously discovered that both low levels of circulating HDL-c as well as deficiency of the HDL-receptor impaired adrenal steroidogenesis. Here, we set out to investigate whether LDL derived cholesterol contributes to adrenal response to adrenocorticotropic hormone (ACTH) as a sensitive read-out of adrenal function in humans.

Methods
Cortisol response to 250 μg ACTH (cosyntropin) was measured in LDL receptor mutation carriers, apolipoprotein B mutation carriers as well as controls.

Findings
Cortisol response was lower in 123 male LDL receptor mutation carriers compared to 24 male controls (401.63±126.58 vs 793.75±116.91 nmol/L; p<0.001) and also in 64 female LDL receptor mutation carriers versus 25 female controls (429.24±135.15 vs 846.80±152.83 nmol/L; p<0.001). No differences in cortisol response became apparent between LDL receptor mutation carriers and apolipoprotein B mutation carriers.

Interpretation
These data support a role for LDL derived cholesterol in the adrenal response to stress in humans. Importantly, monitoring of adrenal function as a safety outcome measure might be indicated when aggressively lowering LDL cholesterol with novel therapeutics.
**Introduction**

Adrenal steroidogenesis is essential for homeostasis and survival in humans and depends on the availability of its precursor cholesterol. Although exact data do not exist, circulating plasma lipoproteins have been suggested to contribute more than 75% of all cholesterol required for adrenal steroidogenesis. Whereas HDL derived cholesterol is indeed important for adrenal steroidogenesis in humans, low plasma levels of HDL-c are not associated with the life threatening disease of adrenal insufficiency in otherwise healthy individuals. In contrast, robust data on the contribution of the other major plasma cholesterol source, low density lipoprotein (LDL), to the adrenal production of hormones, are lacking to date.

Familial hypercholesterolemia (FH) is an autosomal dominant disorder predominantly caused by mutations in the gene encoding the low density lipoprotein receptor (LDLR). FH patients are characterised by extremely high plasma levels of LDL cholesterol due to (partial) loss of function of the hepatic LDLR, resulting in decreased clearance of LDL-c. Next to hepatic cells, the LDLR is abundantly expressed on cells in steroid producing organs such as adrenals, testes and ovaries. Based on the putative role of the LDLR in steroidogenesis it has been postulated that adrenal response to ACTH might be decreased patients with FH, because of a decrease in functional LDL receptors, resulting in decreased LDL-c uptake into the adrenals. However, this hypothesis has only been tested in a handful of FH patients in the 1980’s.

LDL metabolism can be perturbed by mutations in the LDLR as well as in apolipoprotein B (APOB), the constituent protein of LDL particles and the protein that binds to the extracellular domain of the receptor. APOB mutations result in impaired binding of the LDL particle to the LDL receptor and thus, a defect in either the receptor (LDLR) or its ligand (apoB) might be hypothesized to have a similar effect on adrenal steroidogenesis. Illingworth and co workers did indeed show that in homozygous LDLR as well as in APOB mutation carriers, adrenal response to ACTH is impaired. An intriguing finding, since a complete deficiency of cholesterol uptake through the LDL pathway by the adrenal gland is likely in this situation. In contrast, the same investigators could not demonstrate any consequences for adrenal hormone production in heterozygous carriers of an LDLR or APOB mutation. The finding that cholesterol uptake via the LDL-receptor pathway is relevant in humans, has consequences for future drug development and the extremely low LDL-c levels attained by that therapy.

We therefore investigate whether LDL derived cholesterol plays a role in adrenal cholesterol uptake in humans. To this end, we assessed cortisol response to adrenocorticotropin hormone (ACTH) in heterozygous LDL receptor mutation carriers and APOB mutation carriers as well as in a control groups.

The clinical relevance of our experiment lies in the fact that novel therapies that lower LDL-c over and above statins, will significantly increase the number of patients reaching very low levels of LDL-c. It is therefore of consequence to the long-term safety of these therapies to understand the relationship between low levels of LDL-c and adrenal function.

We originally hypothesized that heterozygous LDLR and APOB mutation carriers would not have altered adrenal steroidogenesis. Here we present our results.
Methods

Study population

Patients, aged between 40 and 75 years (male) or between 45 and 75 years (female) with a functional LDLR or APOB mutation, were enrolled in the study. ACTH stimulation testing was performed at baseline. As a control group, we recruited healthy normolipidemic individuals matched for age and gender by advertisement. The study was approved by the Institutional Review Board of our hospital, and all participants provided written informed consent.

Adrenocorticotropic hormone (ACTH) testing

After an overnight fast, participants underwent ACTH testing at 0900 h. A peripheral IV catheter was placed in the antecubital vein. Two baseline blood samples were obtained, 15 minutes and one minute before administration of the ACTH bolus. Subsequent blood samples were drawn 30 minutes and 60 minutes after administration of 250 μg Cortrosyn® (cosyntropin, Amphastar Pharmaceuticals, Rancho Cucamonga, CA, USA).

Laboratory analyses

Lipid profiles were routinely measured. For plasma cortisol levels, samples were centrifuged for 15 minutes at 2000 rpm. The SST tubes were refrigerated and send to a central laboratory for analysis (Medical Research Laboratories International, Zaventem, Belgium) or measured by enzyme immunoassay (Siemens Medical Solutions, Los Angeles, CA, USA).

Results

Baseline characteristics

A total of 187 LDLR mutation and 24 APOB mutation carriers as well as 49 healthy controls were enrolled in this study. Normolipidemic individuals were recruited by advertisement and enrolled as controls. Data from males and females were analysed separately. Baseline characteristics are described in tables 1 and 2. Besides lipoproteins, other characteristics were similar between groups.

ACTH testing

Absolute cortisol response was significantly lower in 123 male LDLR mutation carriers compared to 24 matched controls (401.63± 126.58 nmol/L vs 793.75±116.91 nmol/L, p<0.001, figure 1) as well as relative cortisol response (120.30±75.54 vs 274.39±112.04, p<0.001, figure 1). This significant difference was also observed in 64 female LDLR mutation carriers compared to 25 female controls (absolute response: 429.24±135.15 nmol/L vs 846.80±152.83 nmol/L, p<0.001; relative cortisol response: 146.35±96.51 vs 225.29±75.25, p=0.01, figure 2). All values in controls were within the reference range.16,17

Furthermore, cortisol response was compared between LDLR mutation and APOB mutation carriers. Cortisol responses did not differ between male carriers (absolute response: 377.97±127.19 nmol/L vs 390.72 vs 164.81 nmol/L, p=0.73; relative response: 109.19±72.72 vs 122.17±115.80, p=0.55, figure 2a) or female carriers (absolute response:
A role for LDL in adrenal function

Figure 1. Cortisol response to cosyntropin testing in male controls vs male LDL receptor mutation carriers (a) and female controls vs female LDL receptor mutation carriers (b). Data are presented as mean ± SD. P for student's T-test.
Table 1. Baseline characteristics male and female controls vs LDL receptor mutation carriers

<table>
<thead>
<tr>
<th></th>
<th>Males controls (n=24)</th>
<th>LDLR mutation carriers (n=123)</th>
<th>p-value</th>
<th>Males controls (n=25)</th>
<th>LDLR mutation carriers (n=64)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>44·5±5·7</td>
<td>49·5±5·2</td>
<td>0·14</td>
<td>44·2±14·5</td>
<td>49·2±4·0</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>26·5±5·7</td>
<td>27·0±3·5</td>
<td>0·71</td>
<td>26·2±5·2</td>
<td>26·5±5·6</td>
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<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5·6±1·32</td>
<td>5·3±0·10</td>
<td>0·91</td>
<td>5·7±4·1</td>
<td>5·5±1·7</td>
<td>0·47</td>
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<td>LDL cholesterol (mmol/L)</td>
<td>4·0±1·4</td>
<td>3·4±0·84</td>
<td>0·006</td>
<td>4·0±0·86</td>
<td>3·5±1·10</td>
<td>0·10</td>
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<td>HDL cholesterol (mmol/L)</td>
<td>1·6±0·27</td>
<td>1·2±0·24</td>
<td>0·46</td>
<td>1·2±0·40</td>
<td>1·3±0·26</td>
<td>0·12</td>
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<td>Triglycerides (mmol/L)</td>
<td>1·35 (0·92-2·33)</td>
<td>1·22 (0·91-1·54)</td>
<td>0·42</td>
<td>1·18 (0·63-2·12)</td>
<td>1·13 (0·79-1·49)</td>
<td>0·64</td>
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<tr>
<td>Plasma cortisol (nmol/L)</td>
<td>337·92±149·40</td>
<td>382·51±97·04</td>
<td>0·17</td>
<td>420·20±171·12</td>
<td>379·66±164·47</td>
<td>0·30</td>
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</tbody>
</table>

Values are means ± SD, p-value for Student’s T-test. Triglyceride concentrations were log-transformed prior to T-test.

Table 2. Baseline characteristics LDL receptor mutation carriers vs APOB mutation carriers

<table>
<thead>
<tr>
<th></th>
<th>Males LDLr mutation carriers (n=186)</th>
<th>APOB mutation carriers (n=13)</th>
<th>p-value</th>
<th>Males LDLr mutation carriers (n=98)</th>
<th>APOB mutation carriers (n=11)</th>
<th>p-value</th>
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<tr>
<td>Age (yrs)</td>
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<td>53·2±10·6</td>
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<td>56·4±8·0</td>
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<td>BMI (kg/m²)</td>
<td>27·1±3·4</td>
<td>26·7±3·3</td>
<td>0·71</td>
<td>26·7±5·0</td>
<td>25·5±3·8</td>
<td>0·45</td>
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<td>Total cholesterol (mmol/L)</td>
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<td>5·28±0·73</td>
<td>0·66</td>
<td>5·6±0·95</td>
<td>5·3±0·68</td>
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<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3·5±0·85</td>
<td>3·39±0·60</td>
<td>0·58</td>
<td>3·5±0·88</td>
<td>3·37±0·55</td>
<td>0·57</td>
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<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1·2±0·26</td>
<td>1·29±0·30</td>
<td>0·33</td>
<td>1·4±0·45</td>
<td>1·48±0·40</td>
<td>0·96</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1·22 (0·88-1·57)</td>
<td>0·96 (0·83-1·55)</td>
<td>0·53</td>
<td>1·14 (0·82-1·49)</td>
<td>1·03 (0·67-1·23)</td>
<td>0·07</td>
</tr>
<tr>
<td>Plasma cortisol (nmol/L)</td>
<td>403·78±110·52</td>
<td>415·34±152·02</td>
<td>0·72</td>
<td>396·23±153·10</td>
<td>361·43±131·70</td>
<td>0·47</td>
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</table>

Values are means ± SD, p-value for Student’s T-test. Triglyceride concentrations were log-transformed prior to T-test.
Figure 2b. Cortisol response to cosyntropin testing in male LDLr mutation carriers vs male apoB receptor mutation carriers (a) and female LDLr mutation carriers vs female apoB receptor mutation carriers (b). Data are presented as mean ± SD. P for Student’s T-test.
420.69 nmol/L ± 147.42 vs 454.73 ± 112.40 nmol/L, p = 0.46; relative response 133.62 ± 87.04 vs 158.74 vs 102.95, p = 0.38, figure 2b).

**Discussion**

The data in our study point to a definite role for LDL derived cholesterol in adrenal steroidogenesis since both LDL receptor as well as APOB mutation carriers were characterised by a decreased response to synthetic ACTH when compared to controls. To our knowledge, this is the first time that adrenal response to ACTH has been assessed in a large cohort of individuals with impaired LDL-c uptake in tissues.

Our finding that cholesterol uptake via the LDLR plays a role in adrenal steroidogenesis supports the observations by Illingworth who showed impaired response to ACTH in homozygous carriers of LDLR mutations. However, the authors could not confirm these findings in heterozygous LDLR mutation carriers, which is likely due to inclusion of only four subjects. However, we find cortisol responses to be decreased in both heterozygous LDLR as well as APOB mutation carriers. These data in individuals with impaired cholesterol uptake in adrenal cells, as a consequence of two different defects, validate each other and point to a crucial role for the LDL derived cholesterol in adrenal steroidogenesis.

Plasma lipoproteins have been shown to supply 75% of the cholesterol necessary for adrenal steroidogenesis. However, the relative contribution of LDL and HDL derived cholesterol is unknown. HDL derived cholesterol has been shown to influence basal adrenal function, as reflected by urinary steroid metabolite excretion. However, adrenal response to ACTH was not decreased in low HDL-c subjects, but was lower in individuals with a deficiency of the HDL-receptor SRB1, as we showed recently. This indicates that other cholesterol sources than HDL can supply the adrenal gland with substrate for steroidogenesis in situations of stress. Our current finding that response to ACTH is indeed impaired in LDL receptor mutation carriers indicates that LDL derived cholesterol may constitute that cholesterol source and is, at least in part, responsible for adrenal steroidogenesis in situations of stress. Adrenal steroidogenesis has been extensively assessed in intervention trials aiming to reduce LDL-c in children with FH. While there is no effect of long-term statin therapy on maturation and growth, subtle differences were noted in the hypothalamic-adrenocortical axes, indicating a mild influence of circulating LDL-c on hormone production. These differences did not translate into clinical consequences. Nevertheless, it does seem prudent not to decrease LDL-c in children to extremely low levels, in line with US guidelines.

**Clinical implications**

The contribution of LDL derived cholesterol to acute adrenal steroidogenesis does not translate into clinical signs of adrenal insufficiency across a wide range of circulating LDL-c levels. Therefore, LDL-lowering according to the current guidelines for cardiovascular risk reduction is unlikely to have consequences for adrenal steroidogenesis. However, recent recommendations to decrease LDL-c as low as possible might have an effect on adrenal steroidogenesis in situations where adrenal hormones are of acute importance for
the individual. In heterozygous LDL receptor mutation carriers, with only half the adrenal uptake capacity for LDL-c, further lowering of available substrate might have untoward consequences. Our data also suggest that the assessment of adrenal function might be indicated in ongoing trials with novel and potent LDL-c lowering compounds that lower LDL-c to below physiological levels.

Limitations
All included patients were on statin therapy, but none of the controls. However, statins have been consistently shown not to influence adrenal function in adults. Due to a limited number of healthy volunteers who were willing to undergo ACTH testing, we enrolled a smaller number of controls relative to patients. However, the groups were well-matched and group size was addressed by our statistical analysis.

Interpretation
Our study is the first to address the role of LDL derived cholesterol in adrenal steroidogenesis in a large cohort with enough power to yield a statistically significant difference. The findings that adrenal steroidogenesis is impaired in heterozygous LDLR and APOB mutation carriers does not only increase our understanding of cholesterol metabolism in man, but it has also implications for current developments in cardiovascular prevention.

Conclusion
Our data support a role for LDL derived cholesterol in adrenal steroidogenesis in the acute setting. It is the first time that adrenal steroidogenesis was assessed in a large cohort of patients with a molecularly defined deficiency in cholesterol uptake in the adrenal gland. Our findings suggest that aggressive LDL lowering to very low LDL-c values, as recently advocated, might result in safety issues in terms of adrenal function. LDL-c lowering with novel and potent strategies should be accompanied by adequate safety testing in future clinical trials.
Reference List


Hypercholesterolemia and reduced HDL-c promote hematopoietic stem cell proliferation and monocytosis: studies in mice and FH children


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*These two authors contributed equally to this manuscript
Abstract

Introduction
Previous studies have shown that mice with defects in cellular cholesterol efflux show hematopoietic stem cell (HSPC) and myeloid proliferation, contributing to atherogenesis. We hypothesized that the combination of hypercholesterolemia and defective cholesterol efflux would promote HSPC expansion and leukocytosis more prominently than either alone.

Methods
We crossed \textit{Ldlr}^{-/-} with \textit{Apoa1}^{-/-} mice and found that compared to \textit{Ldlr}^{-/-} mice, \textit{Ldlr}^{+/-} / \textit{Apoa1}^{-+} mice, with similar LDL cholesterol levels but reduced HDL cholesterol (HDL-c) levels, had expansion of HSPCs, monocytosis and neutrophilia.

Results
\textit{Ex vivo} studies showed that HSPCs expressed high levels of \textit{Ldlr}, \textit{Scarb1 (Srb1)}, and \textit{Lrp1} and were able to take up both native and oxidized LDL. Native LDL directly stimulated HSPC proliferation, while co-incubation with reconstituted HDL attenuated this effect. We also assessed the impact of HDL-c levels on monocytes in children with familial hypercholesterolemia (FH) (n=49) and found that subjects with the lowest level of HDL-c, had increased monocyte counts compared to the mid and higher HDL-c levels. Overall, HDL-c was inversely correlated with the monocyte count.

Conclusion
These data suggest that in mice, a balance of cholesterol uptake and efflux mechanisms may be one factor in driving HSPC proliferation and monocytosis. Higher monocyte counts in children with FH and low HDL cholesterol suggest a similar pattern in humans.
Introduction

Atherosclerotic cardiovascular disease remains the major cause of morbidity and mortality in industrialized societies despite the advent of potent cholesterol lowering statin drugs and improvements in clinical care. Low HDL-c levels have long been known to be associated with CAD, especially in the setting of hypercholesterolemia. However, while the association of HDL-c with cardiovascular disease (CVD) is clear, the mechanisms by which HDL exerts its athero-protective effects have not been fully elucidated.

Studies in animals and humans support the hypothesis that HDL prevents atherosclerosis in part via its ability to promote cholesterol efflux from cells. Kehra et al. recently showed that impaired serum cholesterol efflux potential is associated with an increased burden of atherosclerotic disease, suggesting that HDL-mediated cholesterol efflux is a key anti-atherogenic property of HDL. This concept is also supported by bone-marrow transplantation studies showing increased atherosclerosis in hypercholesterolemic mice that have defective cholesterol efflux pathways as a result of knockout of the ATP-binding cassette transporters ABCA1 and ABCG1 in cells of hematopoietic origin. These mice also displayed a marked expansion of hematopoietic stem cells (HSPCs), monocytosis, neutrophilia and systemic foam cell and myeloid cell infiltration of various organs. Accelerated atherosclerosis and myeloid cell defects were reversed by transgenic overexpression of ApoA1. Murphy et al. have also recently shown that apoE, in particular, plays an important role in cholesterol efflux in HSPCs. Apoe--/-- mice fed a Western-type diet (WTD), develop HSPC and myeloid proliferation, monocytosis and increased numbers of monocyte/macrophages in atherosclerotic plaques. Infusion of reconstituted HDL was able to reverse HSPC and myeloid proliferation.

Surprisingly, while Apoe--/-- mice developed HSPC expansion and monocytosis on chow or WTD, ApoA1--/-- mice did not. Moreover, humans with Mendelian forms of HDL deficiency (LCAT deficiency, Tangier Disease) also did not display monocytosis. However, in both ApoA1--/-- mice and humans with genetically low HDL-c, plasma levels of LDL cholesterol are typically either normal or reduced, and so these findings do not preclude a detrimental effect of low HDL-c on HSPC proliferation in the setting of elevated levels of LDL cholesterol.

We hypothesized that in the presence of hypercholesterolemia, ApoA-1 and HDL would act to reduce HSPC proliferation and monocytosis. We therefore crossed ApoA1--/-- mice with Ldlr--/-- mice, and showed that the combination of marked hypercholesterolemia and low HDL-c was associated with HSPC proliferation and monocytosis. We also examined the mechanisms and impact of LDL uptake in HSPCs in vitro, and showed that proliferative effects of LDL were attenuated by HDL. Finally, we examined the relationship between elevated LDL-c levels and monocytosis in a cohort of children with familial hypercholesterolemia (FH) and confirmed that HDL-c is associated with lower monocyte counts in the setting of elevated LDL-c levels.
METHODS

Animals
WT (C57BL/6), Ldlr<sup>-/-</sup> and Apoa1<sup>-/-</sup> mice were purchased from The Jackson Laboratory. Ldlr<sup>-/-</sup> and Apoa1<sup>-/-</sup> mice were crossed to obtain Ldlr<sup>+/+</sup>/Apoa1<sup>-/-</sup> and Ldlr<sup>-/-</sup>/Apoa1<sup>+/+</sup> mice. For in vivo studies, mice at 8 weeks of age were placed on a Western type diet (21% milk fat, 0.2% cholesterol; catalog no. TD88137; Harlan Teklad) for 6 weeks. For in vitro studies, mice were maintained on a normal chow diet. The age of the animals ranged from 8-20 weeks.

Mouse cholesterol assays
Total cholesterol and HDL cholesterol were measured from the plasma of mice using the Cholesterol E or HDL-cholesterol E kit (from Wako Diagnostics) per the manufacturer’s instructions.

Mouse white blood cell counts
Absolute monocytes and neutrophils were determined by applying percentages from flow cytometry to total leukocyte count, which was obtained from freshly drawn blood (via tail bleed) in EDTA tubes and analyzed by automated cell counter (FORCYTE Veterinary Analyzer, Oxford Science Inc.).

Flow cytometry
Blood leukocytes: Blood monocytes and neutrophils were analyzed from whole blood collected in EDTA coated tubes by tail bleed. Blood was placed on ice (all processing on ice), lysed (BS pharm Lyse; BD Biosciences) and washed by centrifuging in HBSS (0.1% BSA, 5mM EDTA). As previously detailed<sup>10</sup> cells were stained with a cocktail of antibodies against CD45-APC-Cy7, Ly6-C/G (Gr-1)-PerCP-Cy5.5 (BD Biosciences-Pharminogen), and CD115-APC (eBiosciences). Monocytes were classified as CD45<sup>+</sup>, CD115<sup>+</sup>, and further subdivided into LyC-6<sup>hi</sup> and LyC-6<sup>lo</sup> subsets. Neutrophils were classified as CD45<sup>+</sup>, CD115<sup>-</sup>, LyC-6<sup>+</sup>. Samples were analyzed on an LSR-II (BD Bioscience).

HSPCs: Bone marrow (BM) was harvested from femurs and tibias of mice and lysed to remove RBCs. BM was resuspended in HBSS and incubated with an antibody cocktail which included antibodies to FITC labeled lineage markers (CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, and Ly-6G; all eBioscience), and stem cell markers Sca 1-Pacific Blue and ckit-APC-Cy7. HSPCs were identified as lineage marker negative, Sca1 positive, ckit positive (lin<sup>-</sup>, Sca1<sup>+</sup>, ckit<sup>+</sup>). Samples were analyzed on an LSR-II or FACSaria, when sorted.

HSPC in vitro proliferation
Mice were injected with EdU 18 hours before sacrifice. BM was harvested and processed and stained as described above. Cells were then fixed and permeabilized using BD Cytofix/Cytoperm solution for 20 minutes on ice, washed with BD Perm/Wash buffer, and stained with Alexa Flour-conjugated azides using Click-iT system (Invitrogen). Cells were analyzed on LSR-II and proliferation was quantified as percentage of EdU+ cells.
Real-time PCR analysis of HSPCs
For ex vivo studies, HSPCs were processed and stained in the same manner, and then sorted on FACS Aria, directly into RLT lysis buffer (Qiagen). For in vitro studies, cultured BM cells (see below for details) were removed from culture plates, washed and then placed in lysis buffer. In both cases, RNA was extracted using RNeasy Micro Kit (Qiagen) and cDNA was synthesized using SuperScript VILO (Invitrogen). Differences in mRNA levels were controlled using the reference gene m36B4.

LDL uptake assays
BM cells from chow fed animals were obtained and processed, as described above. BM from 2-3 animals was pooled together and plated onto 24 well plates in IMDM (Gibco) with penicillin, streptomycin and 10% lipoprotein deficient bovine calf serum (Biomedical Technologies Inc.) for 90 minutes to allow for separation of adherent cells. Non-adherent cells were then removed and cultured for stated time period in the presence of 10% IL-3 supplement media (BD Bioscience), 2ng/mL GM-CSF (R&D) systems and either BODIPY labeled human nLDL (Molecular Probes) or Dil labeled oxLDL (Biomedical Technologies). Cells were then collected, washed of serum and resuspended in HBSS (BSA, EDTA) and stained for HSPCs as described above, with the exception that lineage markers were APC labeled. Cells were analyzed on LSRII and uptake was determined by the mean fluorescent intensity of BODIPY or DiI in lin⁻, Sca1⁺, ckit⁺ HSPCs.

In vitro HSPC proliferation assays
As with LDL uptake assays, pooled BM cells were plated to remove adherence cells then cultured in IMDM with 10% LPDS, IL-3 and GM-CSF. Cells were then loaded overnight with designated concentration of human native LDL (Biomedical Technologies) +/- reconstituted HDL (rHDL; CSL-111 was provided by CSL Behring AG; CSL-111 is composed of human ApoA-1 and phosphatidylcholine from soybean in a 1:150 ratio). Cells were then collected, washed, fixed and permeablized (as described above) and finally stained with DAPI and analyzed on LSRII.

Human Studies
Heterozygous children with familial hypercholesterolemia were already enrolled in the Phase III clinical trial NCT01078675, “A Study to Evaluate Rosuvastatin in Children and Adolescents with Familial Hypercholesterolemia,” sponsored by Astra/Zeneca. Blood was collected after overnight fast, and automated blood count was performed to determine WBC counts and differentials. LDL-c, HDL-c and triglyceride levels were fasting values. Data used here are the pre-treatment baseline values. Institutional review boards of participating centers approved the protocol. Statistical analysis for all continuous variables in Table II was done by one-way analysis of variance (ANOVA). For gender, a chi-square test was used, and for statin use, a Fisher’s exact test. Triglycerides are reported as a median and interquartile range since this variable is not normally distributed.
Results

Hypercholesterolemia and reduced HDL-c promote monocytosis, neutrophilia and hematopoietic stem cell proliferation in mice

Previous studies showed that Ldlr<sup>−/−</sup> mice develop moderate monocytosis and neutrophilia when fed a WTD<sup>10</sup>. To assess if this effect is magnified in the setting of reduced HDL levels we crossed Ldlr<sup>−/−</sup> mice with Apoal<sup>−/−</sup> mice. Given that Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> mice have substantially reduced levels of non-HDL cholesterol,<sup>13,14</sup> when compared to Ldlr<sup>−/−</sup> mice, we bred Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> mice to avoid this potentially confounding effect. Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> mice had prominent hypercholesterolemia not differing from that in Ldlr<sup>−/−</sup> controls, as well as approximately 30% reductions in HDL-c levels (table 1). Notably, this moderate reduction in HDL-c levels was associated with a prominent increase in blood neutrophils and monocytes after six weeks on a WTD in Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> mice when compared to Apoal<sup>−/−</sup>, Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice (figure 1). Ly6-C<sup>hi</sup> and Ly6-C<sup>lo</sup> populations of monocytes were both significantly increased in Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> mice when compared to other genotypes (figure 1 B). A lower HDL to total cholesterol ratio across all mice genotypes also significantly correlated with higher blood monocyte percentage (figure 1C).

The increase in blood leukocytes seen in Ldl<sup>−/−</sup>/Apoa1<sup>−/−</sup> mice, paralleled an expansion of the HSPC population in the BM (figure 2A&B), and increased incorporation of EdU into HSPCs, consistent with increased HSPC proliferation (figure 2C). While Ldlr<sup>−/−</sup> mice also had significantly higher blood monocyte counts (and a trend toward higher neutrophils) when compared with Apoal<sup>−/−</sup>, Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice (figure 1), Ly6-C<sup>hi</sup> and Ly6-C<sup>lo</sup> populations of monocytes were both significantly increased in Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> mice when compared to other genotypes (figure 1 B). A lower HDL to total cholesterol ratio across all mice genotypes also significantly correlated with higher blood monocyte percentage (figure 1C).

This suggests that in the setting of marked hypercholesterolemia as seen in Ldlr<sup>−/−</sup> mice, reduced HDL-c levels are associated with HSPC proliferation and leukocytosis. In addition to increased myeloid proliferation, there was a trend towards increased atherosclerosis in Ldlr<sup>−/−</sup>/Apoa1<sup>−/−</sup> mice compared to Ldlr<sup>−/−</sup> mice (P=0.1), as measured by proximal aortic plaque area (figure 2C,D).

HSPCs have the capacity to take up both native and oxidized LDL

To better understand how HSPCs take up cholesterol we screened FACS-isolated HSPCs from WT and Ldlr<sup>−/−</sup> mice for expression of key receptors for LDL and modified forms of LDL. WT mice predominately express Ldlr, Scarb1 (Srb1), and Lrp1 with virtually no expression of CD36, Sra1 or Lox1, and with the exception of Ldlr, the Ldlr<sup>−/−</sup> mice had a similar pattern of gene expression (figure 3A). As expected from this receptor profile, in vitro loading with labeled nLDL and oxLDL showed that HSPCs are able to take up both native and modified LDL (figure 3C). Since SR-BI can mediate uptake of oxLDL,<sup>15</sup> this receptor may be mediating oxLDL uptake in these cells. Of note, no significant differences in expression levels of Abca1,
Table 1. Total cholesterol and HDL-cholesterol for mice after six weeks of high-fat Western diet.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Apoai&lt;sup&gt;−/−&lt;/sup&gt; (n = 10)</th>
<th>Ldlr&lt;sup&gt;+/−&lt;/sup&gt;/Apoai&lt;sup&gt;−/−&lt;/sup&gt; (n = 9)</th>
<th>Ldlr&lt;sup&gt;−/−&lt;/sup&gt; (n = 13)</th>
<th>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;/Apoai&lt;sup&gt;+/−&lt;/sup&gt; (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>263.7 ± 18.0 *</td>
<td>569.6 ± 41.9 *</td>
<td>1664.0 ± 82.6</td>
<td>1578 ± 119.3</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>16.8 ± 2.7</td>
<td>15.5 ± 2.1</td>
<td>36.0 ± 3.3 *</td>
<td>25.4 ± 1.6</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. * = P ≤ 0.05 compared with Ldlr<sup>+/−</sup>, Apoai<sup>−/−</sup>.
Abcg1 or Apoe were noted between WT and Ldlr<sup>−/−</sup> mice (figure 3B). Consistent with a major role of the LDLR in the uptake of LDL, there was saturable high affinity specific uptake of LDL in WT HSPCs that was largely abolished in Ldlr<sup>−/−</sup> HSPCs. Both WT and Ldlr<sup>−/−</sup> HSPCs showed significant levels of non-specific LDL uptake.

**LDL directly stimulates hematopoietic stem cell proliferation, which is opposed by HDL**

To assess the direct effects of HDL and LDL on HSPC proliferation, we looked at the response of HSPCs to differential loading of cholesterol <em>in vitro</em>. BM from Ldlr<sup>−/−</sup> mice was cultured in media with lipoprotein deficient serum in which HDL and LDL cholesterol concentrations were varied. LDL in the absence of HDL, increased HSPC proliferation in a dose dependent fashion (figure 4A). The addition of rHDL at potentially therapeutic concentrations had a potent effect in reversing proliferation (figure 4B). Of note, the commercial LDL preparation used in these studies may have contained small amounts of oxidized LDL, which could have contributed to proliferative effects.
and also hypercatabolism of abnormal LDL [14,25,26]. This is likely why, paradoxically, heterozygotes for TD and LCAT deficiency have increased atherosclerosis, while findings in homozygotes have been mixed [24,27,28]. The trend towards increased atherosclerosis in Ldlr−/+ mice in our study is consistent with other studies in mice showing that Apoa-1 deficiency in the setting of hypercholesterolemia contributes to development of atherosclerosis [21,22]. Of note, in the absence of low HDL, we still see a modest increase in blood monocytes and a trend towards increased blood neutrophils in hypercholesterolemic mice, but in this setting there is no parallel increase in BM HSPCs suggesting that there are also non-stem cell mediated mechanisms at play.

Our study provides some novel insight into the effect circulating lipoproteins have on modulation of HSPCs. We have shown that HSPCs are able to take up both native and oxidized LDL likely in part via LDLR and SR-B1. The relatively high expression of SR-BI in HSPCs, also suggests that it could be participating in cholesterol efflux, as well as ABCA1, ABCG1 and apoE. Previous studies have shown the importance of these latter cholesterol efflux pathways in regulating cholera toxin B binding to the plasma membrane, the cell surface expression of the common beta-subunit of the IL-3/GM-CSF receptor and proliferative responses to IL-3 and GM-CSF [9,10]. IL-3 is a key growth factor for HSPCs, while GM-CSF promotes differentiation of myeloid progenitors into granulocytes and monocytes. It remains to be seen if a similar pathway is utilized in the current model. We have also showed that LDL loading promotes HSPC proliferation, and that increasing rHDL concentrations to therapeutic levels reverse this effect in cells from Ldlr−/− mice. Of note, we have seen similar proliferative response in HSPCs from WT mice incubated with LDL (data not shown).

Overall, these findings suggest that a balance of uptake and efflux of cholesterol plays a role in HSPC and myeloid proliferation. However, we cannot exclude the possibility that the effects seen in these experiments may be mediated by non-efflux mechanisms. Furthermore, since we see that uptake of native LDL in Ldlr−/− mice is modest when compared to WT mice, we hypothesize that modified LDL may also be playing an important role in HSPC stimulation.

In conclusion, we show that HSPC expansion, and the resultant increase in circulating monocytes and neutrophils, occurs in the Fig. 2. Ldlr−/- ApoA1+/- mice on a WTD have expansion of HSPCs in the bone marrow and increased proliferation in vivo, as well as a trend towards increased atherosclerosis. (A&B) BM cells were isolated and analyzed by flow cytometry to identify percent HSPCs. (C) Prior to sacrifice, mice were injected with EdU. In vivo proliferation was determined by EdU incorporation via flow cytometry. *P<0.05 vs. all other genotypes. (D) Hearts were placed in paraffin, stained with H&E and plaque area was determined by averaging plaque at aortic root over serial sections for each animal. P=0.1. Data are mean with SEM, n=10-13.

HDL deficiency in the setting of hypercholesterolemia is associated with higher monocyte counts in children with Familial Hypercholesterolemia

In order to assess whether the higher monocyte counts observed in Ldlr−/-ApoA1+/- mice translate into a similar phenotype in humans, we analyzed baseline data previously collected for a trial of rosuvastatin in children with heterozygous familial hypercholesterolemia. The
Figure 3. WT and Ldlr−/− BM HSPCs take up nLDL and oxLDL. (A&B) HSPCs were isolated from BM by FACS, cDNA was prepared and mRNA expression was quantified by real-time PCR. Data is mean with SEM, *n* = 3-5. (C&D) Cultured HSPCs were loaded with increasing concentrations of BODIPY-nLDL alone and in presence of excess unlabeled nLDL (200 μg/mL) in WT and Ldlr−/− BM cells. Uptake was quantified by flow cytometry and data points are averages of mean fluorescence intensity (MFI) of BODIPY in HSPCs. (E) HSPCs were cultured in media with LPDS serum, IL-3 and GM-CSF in the presence of either BODIPY labeled nLDL or DiI labeled oxLDL. Uptake was determined by flow cytometry at 6 and 12 hours.
children were statin naïve or underwent a wash-out period. They had an average age of 12 and similar gender distribution (table 2). Patients were grouped by level of HDL-c, where low HDL-c was defined as ≤ 40mg/dL, mid HDL-c as 40-50mg/dL and high HDL-c as >50mg/dL. LDL cholesterol levels were elevated across the patient population, with an average concentration of 216mg/dL, and importantly were not statistically different among HDL groups. FH children with the lowest HDL-c levels, had significantly higher monocyte counts (figure 5A). In addition, there was a significant inverse correlation between HDL concentration and monocyte percentage (figure 5B).

**Discussion**

In this study, we have shown that the combination of hypercholesterolemia and low HDL levels are associated with increased proliferation and expansion of BM HSPCs and increased monocytosis and neutrophilia in mice. In a parallel translational study, we present evidence that this balance may be important in regulating monocytosis in humans too, as FH children with reduced HDL-c levels had increased monocyte counts compared to FH children with normal or higher HDL-c levels.

In humans, leukocytosis, and in particular, high monocyte counts are clearly associated with cardiovascular disease. In healthy people, increased monocyte count is associated with increased risk for cardiovascular events, ischemic stroke and cardiovascular mortality. Leukocytosis has also been implicated in the development of the accelerated atherosclerosis observed in heterozygous FH patients. In FH adults, low HDL-c levels have been associated with increased atherosclerotic plaque burden. Here we have the

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**Figure 4.** LDL directly stimulates HSPC proliferation, which can be reversed by reconstituted HDL. (A) BM from Ldlr−/− mice was cultured and loaded overnight with increasing nLDL concentration (µg/mL). In vitro cell cycling in HSPCs was determined by FACS using DAPI and is expressed by the percent of HSPCs in SG2M phase. nLDL increased HSPC proliferation in a dose dependent fashion and (B) co-incubation of LDL (50µg/mL) with rHDL potently reversed proliferation. Reconstituted HDL concentrations are reflective of protein. ^P<0.05 vs LDL alone.
2. Results

Ldlr<sup>−/−</sup> had prominent hypercholesterolemia not differing from that in Ldlr<sup>+/+</sup> controls, as well as approximately 30% reductions in HDL-C in both genotypes, they did not have a corresponding increase in triglycerides (Table 1). Low HDL was defined as ≤ 40mg/dL, mid HDL as 40-50mg/dL and high HDL as ≤ 50mg/dL. Percent monocyte was measured by automated blood count. Data is mean with SEM, n=49. *P <0.01 vs low HDL group. (B) HDL plotted with associated monocyte count for all individuals in cohort.

**Figure 5.** Children with familial hypercholesterolemia and low HDL have increased circulating blood monocytes. (A) Children with homozygous FH were grouped based on plasma HDL concentration. Low HDL was defined as ≤ 40mg/dL, mid HDL as 40-50mg/dL and high HDL as ≤ 50mg/dL. Percent monocyte was measured by automated blood count. Data is mean with SEM, n=49. *P <0.01 vs low HDL group. (B) HDL plotted with associated monocyte count for all individuals in cohort.

**Table 2.** Baseline characteristics of children with familial hypercholesterolemia.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Low HDL (n = 14)</th>
<th>Mid HDL (n = 19)</th>
<th>High HDL (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>10.9 ± 4</td>
<td>10.1 ± 3</td>
<td>8.1 ± 3</td>
</tr>
<tr>
<td>Male sex</td>
<td>8 (57%)</td>
<td>8 (42%)</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>Statin use *</td>
<td>5 (36%)</td>
<td>3 (16%)</td>
<td>0</td>
</tr>
<tr>
<td>Triglycerides †</td>
<td>0.94 (0.71–1.12)</td>
<td>0.85 (0.70–1.19)</td>
<td>0.61 (0.55–1.11)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>6.55 ± 1.9</td>
<td>7.24 ± 1.7</td>
<td>7.84 ± 1.0</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>5.18 ± 1.7</td>
<td>5.64 ± 1.6</td>
<td>5.85 ± 0.9</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>0.92 ± 0.13 **</td>
<td>1.16 ± 0.06 **</td>
<td>1.65 ± 0.29 **</td>
</tr>
<tr>
<td>Leukocytes (10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>5.64 ± 1.5</td>
<td>5.81 ± 1.2</td>
<td>5.74 ± 1.0</td>
</tr>
<tr>
<td>Neutrophils (percent)</td>
<td>48.5 ± 14</td>
<td>52.0 ± 9.9</td>
<td>47.9 ± 7.5</td>
</tr>
<tr>
<td>Lymphocytes (percent)</td>
<td>40.0 ± 14</td>
<td>37.1 ± 9.0</td>
<td>41.1 ± 8.1</td>
</tr>
</tbody>
</table>

*P = 0.03 by Fisher’s exact test, † median and interquartile range, ** P<0.001 vs. other two groups.
benefit of studying leukocytosis in a pediatric population, whose only medical condition is dyslipidemia, so increased monocytes in this group are less likely to be confounded. Thus, unlike studies in adults, leukocytosis is less likely to be reactive to established atherosclerosis, but rather can be considered as a factor contributing to its development.

In addition to leukocytosis and monocytosis, low HDL-c and ApoA-1 are also associated with increased CVD, but previous work in mice has shown an inconsistent relationship with atherosclerosis.\textsuperscript{14,19-22} This may be partly related to the finding of low levels of non-HDL-c in ApoA-1 deficient mice and humans,\textsuperscript{23,24} which is thought to be due to decreased intestinal absorption of cholesterol, and also hypercatabolism of abnormal LDL.\textsuperscript{14,25,26} This is likely why, paradoxically, heterozygous \textit{ABCA1} and \textit{LCAT} mutation carriers have increased atherosclerosis, while findings in homozygous carriers have been mixed.\textsuperscript{24,27,28} The trend towards increased atherosclerosis in \textit{Ldlr}^{-/-}/Apoa1^{+/-} mice in our study is consistent with other studies in mice showing that Apoa-1 deficiency in the setting of hypercholesterolemia contributes to development of atherosclerosis.\textsuperscript{21,22} Of note, in the absence of low HDL, we still see a modest increase in blood monocytes and a trend towards increased blood neutrophils in hypercholesterolemic mice, but in this setting there is no parallel increase in BM HSPCs suggesting that there are also non-stem cell mediated mechanisms at play.

Our study provides some novel insight into the effect circulating lipoproteins have on modulation of HSPCs. We have shown that HSPCs are able to take up both native and oxidized LDL likely in part via LDLR and SR-B1. The relatively high expression of SR-BI in HSPCs, also suggests that it could be participating in cholesterol efflux, as well as \textit{ABCA1}, ABCG1 and apoE. Previous studies have shown the importance of these latter cholesterol efflux pathways in regulating cholera toxin B binding to the plasma membrane, the cell surface expression of the common beta-subunit of the IL-3/GM-CSF receptor and proliferative responses to IL-3 and GM-CSF.\textsuperscript{9,10} IL-3 is a key growth factor for HSPCs, while GM-CSF promotes differentiation of myeloid progenitors into granulocytes and monocytes. It remains to be seen if a similar pathway is utilized in the current model. We have also showed that LDL loading promotes HSPC proliferation, and that increasing rHDL concentrations to therapeutic levels reverse this effect in cells from \textit{Ldlr}^{-/-} mice. Of note, we have seen similar proliferative response in HSPCs from WT mice incubated with LDL (data not shown).

Overall, these findings suggest that a balance of uptake and efflux of cholesterol plays a role in HSPC and myeloid proliferation. However, we cannot exclude the possibility that the effects seen in these experiments may be mediated by non-efflux mechanisms. Furthermore, since we see that uptake of native LDL in \textit{Ldlr}^{-/-} mice is modest when compared to WT mice, we hypothesize that modified LDL may also be playing an important role in HSPC stimulation.

In conclusion, we show that HSPC expansion, and the resultant increase in circulating monocytes and neutrophils, occurs in the setting of combined increases in LDL and low HDL levels. This finding underlines the close link between circulating lipoproteins and leukocytosis, both of which are important contributing factors to the development of atherosclerosis.
Acknowledgements

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Increased systemic and plaque inflammation in ABCA1 mutation carriers with attenuation by statins

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Abstract

Introduction
We previously demonstrated that subjects with functional ATP-binding cassette (ABC) A1 mutations have increased atherosclerosis, which has been attributed to the role of ABCA1 in reverse cholesterol transport. More recently, a pro-inflammatory effect of Abca1 deficiency was shown in mice, contributing to a pro-atherogenic state. In the present study, we evaluated whether ABCA1 deficiency is associated with pro-inflammatory changes in humans.

Material and methods
21 heterozygous and 5 homozygous ABCA1 mutation carriers as well as 21 matched controls were included in the study. PET-CT scanning was performed in a subset of carriers and controls to assess arterial wall inflammation (target-background-ratio, TBR). Plasma cytokines and mRNA expression of inflammatory genes in monocytes were measured in ABCA1 deficient monocytes/macrophages and in control macrophages exposed to plasma from ABCA1 mutation carriers.

Results
ABCA1 mutation carriers had a 21% higher TBR compared to controls (TBR; p=0.004). In carriers using statins TBR was 20% reduced compared to non-statin users (p=0.02). In plasma from ABCA1 mutation carriers, Tumor Necrosis Factor α (TNFα) and Monocyte Chemoattractant Protein-1 (MCP-1) levels showed a gene-dose dependent increase. Macrophages incubated with lipoprotein deficient plasma isolated from ABCA1 mutation carriers showed a gene-dose dependent increase in inflammatory cytokine mRNA expression.

Conclusion
Our data confirm a pro-inflammatory state in ABCA1 mutation carriers as reflected by increased circulating cytokines, most likely secondary to a cellular effect of ABCA1 deficiency. Our findings suggest that the increased inflammation documented in ABCA1 deficient cells and animal models is also present in humans. The increased inflammation in ABCA1 mutation carriers seems to be attenuated by statins, as shown by normalization of PET-CT and plasma cytokine levels.
Introduction

High-Density Lipoprotein Cholesterol (HDL-C) levels are inversely correlated with cardiovascular risk.\(^1\)-\(^3\) However, recent Mendelian randomization studies have called into question whether common variation in HDL cholesterol levels is causally related to cardiovascular disease (CVD) risk.\(^4\) The atheroprotective effects of HDL have traditionally been attributed to its role in reverse cholesterol transport (RCT). The ATP-binding cassette transporter A1 (ABCA1) transporter plays a crucial role in mediating cholesterol efflux from peripheral cells, including arterial wall macrophages, to lipid-poor apolipoprotein A1 (apoAI) or pre-β HDL particles.\(^5,6\) Homozygous \(ABCA1\) mutation carriers display near absent HDL-C levels, whereas heterozygous carriers are characterized by half-normal HDL-C. While single nucleotide polymorphisms (SNPs) in the \(ABCA1\) gene have variously been reported to have no impact on CVD,\(^7,8\) or to be associated with increased CVD risk,\(^9,10\) studies in \(ABCA1\) mutation carriers, displaying marked defects in cholesterol efflux and profound decreases in HDL levels, showed increased arterial wall thickness,\(^11,12\) and CVD risk\(^9,10\) in carriers compared to controls.

The paradigm that a macrophage-dominated inflammatory process, that is initiated by the deposition of cholesterol-rich lipoproteins in the arterial wall, is central to atherosclerosis has been widely acknowledged.\(^13\) The molecular mechanisms linking defective cholesterol homeostasis to increased inflammation are not well understood. Recent studies have implicated defective cellular cholesterol efflux pathways in increased inflammatory gene expression in monocytes and macrophages, as well as the increased production of inflammatory cells such as monocytes and neutrophils.\(^14\) Deficiency of ABCA1 and/or ABCG1 is associated with a pro-inflammatory phenotype in mouse peritoneal macrophages as well as in the macrophages of atherosclerotic plaques.\(^14-17\) Whether ABCA1 deficiency in humans represents a pro-inflammatory state is presently unknown.

In the present study, we assessed whether \(ABCA1\) mutation carriers are characterized by pro-inflammatory changes of the arterial wall as assessed by PET-CT as well as a systemic pro-inflammatory state. \textit{In vitro} experiments were performed to determine whether the observed changes originate from plasma or a cellular component using plasma isolated from \(ABCA1\) mutation carriers.

Methods

Study participants

Subjects with low HDL-C levels, defined as HDL-C < 5\(^{th}\) percentile, were selected from a cohort of hypoalphalipoproteinemia patients\(^18\) and screened for \(ABCA1\) (GenBank No. AF275948) mutations. Family members of \(ABCA1\) mutation carriers were recruited. Carriers of functional \(ABCA1\) gene mutations and controls matched for age and gender were enrolled in this study. Body mass index (BMI) was calculated from weight and length. Hypertension was defined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg or use of antihypertensive medication. Blood was obtained after an overnight fast and stored at -80 °C. All participants provided written informed consent. The study protocol was approved by the Institutional Review Board at the AMC, The Netherlands.
Genotyping

Mutation detection was performed as published previously. In short, the sequence reactions were performed using a BigDye terminator ABI prism kit (Applied Biosystems, Foster City, CA, USA). Sequences were analysed with the Sequencher package (Gene Codes Co, Ann Arbor, MI, USA).

Plasma processing

Blood samples were collected from all subjects after 12-hour fasting. Plasma cholesterol, low-density lipoprotein cholesterol (LDL-C), HDL-C and triglycerides (TG) were measured using commercially available kits (Randox, Antrim, United Kingdom and Wako, Neuss, Germany). Plasma apolipoprotein AI and apolipoprotein B were measured using a commercially available turbidometric assay (Randox, Antrim, United Kingdom). All analyses were performed using the Cobas Mira autoanalyzer (Roche, Basel, Switzerland). For experiments with apoB depleted plasma, apoB-containing particles were precipitated from plasma by adding 100 μl of plasma to 40 μl of 20% polyethyleneglycol (PEG, Sigma P-2139 in 200 mM glycine, pH 10) solution. This mixture was incubated at room temperature for 15 min after which it was centrifuged at 4,000 rpm for 20 min. The supernatant was removed for use in experiments. This is referred to as PEG-plasma. For experiments with lipoprotein deficient plasma (referred to as LPDS), plasma from 10 controls or ABCA1 mutation carriers, or from 4 TD patients, was pooled, and LPDS was isolated by ultracentrifugation according to Havel and co workers. LPDS was then dialyzed against PBS for use in experiments.

Carotid 18F-FDG PET-CT

Carotid 18F-FDG PET-CT scanning was performed in 16 ABCA1 mutation carriers and 15 controls. Seven of the ABCA1 mutation carriers were using statin therapy. Scans were performed on a Gemini time-of-flight multidetector helical PET/CT scanner (4 min/bed position) (Philips, Best, the Netherlands) as previously published. Subjects fasted for at least 6 h before i.v. injection of 18F-FDG (200 MBq, 5.5 mCi). After 90 minutes of 18F-FDG circulation time subjects underwent PET/CT imaging according to a previously validated acquisition and reconstruction protocol. Mean and maximum standardized uptake values (SUV) were measured in both carotids at 5 mm intervals on axial slices. SUV is a widely used PET quantifier, calculated as a time-corrected and dose-corrected ratio of tissue radioactivity divided by body weight. Then, target to background ratio (TBR) was calculated from the ratio of arterial SUV of right and left common carotid artery compared with the background activity in the jugular veins. Both TBR_max, the mean of maximum SUV values and TBR_mean, the average of mean TBR values derived from every axial section of the vessel were calculated. Images were analysed by two experienced readers, blinded for patient data.

In vitro glucose uptake

Monocytes from controls and ABCA1 mutation carriers were treated with 100nM phorbol myristate acetate (PMA) to facilitate differentiation into macrophages. Cells were cultured
with 40 μM 2-NBDG (a fluorescently-labeled deoxyglucose analog) and washed. Cellular uptake 2-NBDG uptake was measured by means of quantitative flow cytometry.

**Plasma cytokine measurements**

Plasma levels of tumor necrosis factor α (TNFα), and monocyte chemoattractant protein-1 (MCP-1) were measured using ELISA (R&D systems, Minneapolis, USA).

**Carotid magnetic resonance imaging**

Scans were performed as described previously. In short, scans were obtained in a 3.0 Tesla Philips whole-body scanner (Philips, Best, the Netherlands), using a single-element microcoil (Philips, Hamburg, Germany). Ten slices were scanned of the distal 3.0 cm of the left and right common carotid artery. A total of 20 images were obtained per scan. Images were saved in DICOM format using standardized protocols. Quantitative image analysis was performed using semi-automated measurement software (VesselMass, Leiden University Medical Center, the Netherlands). One trained reader, with excellent scan-rescan and intraobserver variability analyzed all the images using standardized protocols for reading and rating images combined with dedicated semi-automated software, blinded for all data of the participants. Mean wall thickness (MWT), lumen area (LA) and outer wall area (OWA) were measured. Normalized wall index (NWI) was calculated as: NWI= MWA / OWA.

**mRNA expression levels**

Total RNA was extracted from human monocytes or macrophages using an RNeasy Micro or Mini kit (Qiagen), respectively. For monocytes, RNA was synthesized using SuperScript VILO and for macrophages using M-MLV (Invitrogen). mRNA levels of TNFα, IL-1β, MCP-1, IL-6, IL-8, and iNOS were assessed using qPCR on on a Stratagene Mx3000P (Agilent Technologies), and initial differences in RNA quantity were corrected for using the housekeeping gene cyclophilin.

**Experiments in THP-1 cells**

THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% pen-strep at 37°C in 5% CO₂. For experiments, cells were plated at a concentration of 5*10⁴ cells/ml and incubated with 100nM PMA for 72 h to facilitate differentiation into macrophages. Macrophages were then washed, and incubated o/n with RPMI 1640 medium containing 2.5% PEG-plasma or LPDS from controls, ABCA1 mutation carriers, or TD patients. Cells were then lysed and RNA was extracted, cDNA synthesized, and mRNA expression assessed as described above.

**Statistics**

All data are presented as means ± SEM. Student’s t-test was used to test for differences between two datasets. To define differences between more datasets, One-way Analysis of Variance (ANOVA) was used with a Bonferroni multiple comparison post-test. The criterion for significance was set at $P<0.05$. Statistical analyses were performed using GraphPad Prism version 5.01 (San Diego, CA, USA) and PASW statistics 18 (Chicago, IL, USA).
Results

Baseline characteristics

Baseline characteristics of study participants are listed in Table 1. 25 ABCA1 mutation carriers from 14 separate families were included, comprising 3 homozygous 2 compound heterozygous and 21 heterozygous patients. Homozygous and compound heterozygous subjects suffer from Tangier Disease (TD). Subjects were carriers of the following mutations: p.Leu1056Pro, c.3535+1G>C, c.6401+2T>C, p.Asn1800his, p.Ser930Phe, p.Phe1760Valfs*21, p.Ser824Leu, p.Gln1038Ter, p.Asn935Ser and p.Arg579Gln. Heterozygosity for these mutations has been shown to impair cholesterol efflux by 40 to 85 %.12,18,26-28

Twenty one controls from the general population were matched for age and sex; statin users were excluded from the control cohort (Table 1). Total cholesterol levels were 21% lower in ABCA1 mutation carriers (p=0.005), largely due to a 50% reduction in HDL-C (p<0.001). ApoAI was correspondingly decreased by 40% (p<0.001). Normalized wall index and mean wall thickness of the carotid arteries were increased in both statin using ABCA1 mutation carriers (p<0.001 and 0.006 respectively), as well as non-statin using ABCA1 mutation carriers (p<0.001 and 0.002 respectively, Supplementary figure 1), consistent with earlier reports.12 Other parameters were not significantly different (table 1).

Vascular PET-CT

PET-CT scanning was performed in a random subset of ABCA1 mutation carriers (n=16) and controls (n=15). In the whole group, TBR was not significantly different in ABCA1 mutation carriers compared to controls (data not shown). However, the average mean TBR of the left and right carotid was significantly higher in non-statin using ABCA1 mutation carriers compared to statin using ABCA1 mutation carriers (p=0.02 for left mean TBR; p=0.008 for right mean TBR, figure 1). After excluding the ABCA1 mutation carriers using statins, the right mean TBR was significantly higher in ABCA1 mutation carriers compared to controls (p=0.01, figure 1), whereas the left mean TBR showed a trend towards an increase (p=0.06, figure 1). Max TBR was significantly higher for the right carotid (p=0.004, figure 1), but not in the left carotid (p=0.12, figure 1). The inverse association between statin use and TBR signal was not observed on 3T-MRI (Supplementary Figure 1).

Since the TBR signal depends on glucose uptake in macrophages in the arterial wall29 and ABCA1 has been reported to have a role in glucose uptake,30 we evaluated a potential direct effect of ABCA1 expression on macrophage glucose uptake. However, macrophage-glucose uptake did not differ between ACBA1 mutation carriers and controls (Supplementary Figure 2), indicating that the differences in TBR signal cannot be explained by a direct effect of ABCA1 on glucose uptake.

Systemic inflammatory phenotype in ABCA1 mutation carriers

To assess whether the apparent inflammatory phenotype in the arterial wall of ABCA1 mutation carriers also manifested itself systemically, plasma cytokines were measured. Plasma levels of TNFα were significantly higher in homozygous ABCA1 mutation carriers
versus controls (figure 2A). In line with the PET-CT data, TNFα levels were significantly higher in non-statin using ABCA1 heterozygous mutation carriers compared to statin using heterozygous carriers (figure 2A). TNFα levels also appeared to be higher in the non-statin using homozygous carriers compared to statin using homozygous carriers. Plasma MCP-1 levels were dose dependently increased in heterozygous ABCA1 mutation carriers and homozygous ABCA1 mutation carriers compared to controls (figure 2B). In contrast to TNFα, no effect of statin use was observed (figure 2B). IL-1β levels in controls and heterozygotes were too low to detect, even using a high sensitivity IL-1β ELISA. Furthermore, circulating Tangier disease patients’ monocytes showed increased inflammatory gene expression (data not shown).

Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n=21)</th>
<th>ABCA1 mutation carriers (n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51.0±11.3</td>
<td>49.8±14.6</td>
<td>0.76</td>
</tr>
<tr>
<td>Male sex, n (%)*</td>
<td>9 (43)</td>
<td>11 (44)</td>
<td>0.94</td>
</tr>
<tr>
<td>Body Mass Index (kg/m2)</td>
<td>24.1±3.1</td>
<td>26.0±4.5</td>
<td>0.10</td>
</tr>
<tr>
<td>Smokers, n (%)*</td>
<td>2 (10)</td>
<td>4 (16)</td>
<td>0.52</td>
</tr>
<tr>
<td>Diabetes, n (%)*</td>
<td>1 (5)</td>
<td>2 (8)</td>
<td>0.66</td>
</tr>
<tr>
<td>Statin use (%)*</td>
<td>0</td>
<td>9 (36)</td>
<td>~</td>
</tr>
</tbody>
</table>

Blood pressure

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (mmHg)#</th>
<th>ABCA1 mutation carriers (mmHg)#</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic</td>
<td>129 (122-138)</td>
<td>139 (132-149)</td>
<td>0.75</td>
</tr>
<tr>
<td>Diastolic</td>
<td>80 (74-85)</td>
<td>82 (72-87)</td>
<td>0.74</td>
</tr>
<tr>
<td>Hypertension, n (%)*</td>
<td>3 (14)</td>
<td>1 (6)</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Lipid metabolism

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (mmol/L)</th>
<th>ABCA1 mutation carriers (mmol/L)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>5.39±0.92</td>
<td>4.26±1.60</td>
<td>0.005</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>3.49±0.79</td>
<td>3.09±1.21</td>
<td>0.19</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>1.53±0.40</td>
<td>0.76±0.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.01 (0.64-1.42)</td>
<td>1.10 (0.88-1.52)</td>
<td>0.38</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>110.65±21.44</td>
<td>124.89±45.15</td>
<td>0.23</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>161.99±19.87</td>
<td>97.13±53.34</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Vessel wall thickness

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>NWI</th>
<th>MWT (mm)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.32±0.03</td>
<td>0.38±0.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>0.66±0.11</td>
<td>0.85±0.21</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are indicated as mean ± SD unless otherwise indicated. P-value for student’s T-test, compared to control, unless otherwise specified. * p for X2 test. #: median and interquartile range; P for Mann Whitney U test. NWI is mean wall thickness; MWT is mean wall thickness.
ABCA1 deficiency and macrophage inflammation

We then investigated whether the decreased HDL levels and the increased inflammatory cytokines in the plasma from ABCA1 mutation carriers and TD patients could contribute to enhanced macrophage inflammation. We thus added LPDS from controls, heterozygous ABCA1 mutation carriers, and TD patients to THP-1 macrophages. LPDS from heterozygous ABCA1 mutation carriers increased TNFα, IL-1β, and MCP-1 mRNA expression, whereas LPDS from TD patients increased mRNA expression of almost all pro-inflammatory cytokines (TNFα, IL-1β, MCP-1, IL-8, and iNOS) (figure 3). These experiments suggest that the increased macrophage inflammation is caused by pro-inflammatory cytokines (e.g. TNFα) present in the LPDS fraction from ABCA1 mutation carriers or TD patients. Subsequently, we studied whether the lower HDL levels in ABCA1 mutation carriers could also affect the inflammatory response in macrophages. Incubation of THP-1 macrophages with apoB depleted plasma from TD patients led to a major increase in mRNA expression of all inflammatory cytokines measured (TNFα, IL-1β, MCP-1, IL-8, iNOS, and IL-6), compared to both controls and heterozygous ABCA1 mutation carriers. PEG-plasma from heterozygous ABCA1 mutation carriers did not affect macrophage inflammation compared to controls (supplementary figure 3). This suggests that the decreased HDL levels in TD patients also have a major contribution to the increased macrophage inflammation and that the ~50% lower HDL-C levels in ABCA1 mutation carriers could be sufficient to reverse the increased inflammatory response induced by the cytokines present in the LPDS fraction.
Increased inflammation in *ABCA1* mutation carriers

**Discussion**

*ABCA1* mutation carriers displayed both increased vessel wall inflammation as assessed by PET-CT as well as increased systemic inflammation, as reflected by a pro-inflammatory plasma cytokine profile and increased inflammatory gene expression in circulating monocytes. *In vitro* experiments revealed a pro-inflammatory effect of plasma from *ABCA1* mutation carriers, most likely secondary to increased levels of plasma cytokines. Both cellular *ABCA1* deficiency and reduced levels of plasma HDL likely contributed to increased monocyte and macrophage inflammatory responses. These data show for the first time that *ABCA1* deficiency in humans is associated with increased systemic and plaque inflammation, probably contributing to the increased atherosclerotic plaque volume that has been observed in *ABCA1* mutation carriers.\textsuperscript{11,12}

\textsuperscript{18}F-FDG PET/CT has emerged as a reliable technique to non-invasively visualize metabolic activity in the arterial wall in humans.\textsuperscript{31} Metabolic activity likely reflects the inflammatory state of the arterial wall, since the arterial uptake of FDG has been shown to correlate with circulating inflammatory biomarkers,\textsuperscript{32} inflammatory gene expression,\textsuperscript{33} etc.
CVD risk factors, as well as the number of plaque macrophages. We showed that the PET-CT signal in the arterial wall of non-statin using ABCA1 mutation carriers was increased compared to matched controls, paralleling an increase in vessel wall thickness. Although suspected based on studies in macrophages from Abca1-/- mice, this is the first confirmation of an in vivo role for ABCA1 in the suppression of inflammation in humans. The finding of increased vessel wall inflammation in ABCA1 mutation carriers is likely to contribute to their increased cardiovascular risk, since carotid arterial wall FDG uptake has been associated with increased cardiovascular risk, independent of the degree of stenosis.

Interestingly, the increased inflammatory status in the vessel wall of ABCA1 mutation carriers was also manifested systemically, as plasma levels of TNFα and MCP-1 were increased in a gene-dose dependent manner. This is consistent with previous reports, showing that ABCA1 suppresses secretion of IL-1β, IL-6 and TNF-α. Furthermore, mRNA expression of inflammatory markers was increased in Tangier patients’ circulating monocytes, consistent with a systemic pro-inflammatory state. These findings are also consistent with reports that plasma C-reactive protein levels are negatively associated with
ABCA1 mRNA levels in human peripheral monocytes. The cross-sectional design of this study precludes us from answering whether plaque inflammation is causal or secondary to the atherosclerosis. However, since the increases in inflammatory mediators in our study are secondary to genetic changes in ABCA1, this suggests the excessive plaque inflammation contributes to increased atherosclerotic burden.

The pro-inflammatory properties of plasma isolated from ABCA1 mutation carriers (figure 3) are likely due to increased circulating cytokines such as TNF-α and MCP-1. In addition, the lower levels of plasma HDL-C could contribute to the pro-inflammatory changes. However, as in mouse macrophages, knockdown of ABCA1 resulted in enhanced inflammatory gene expression in either the basal condition or after LPS stimulation. These effects have been attributed to increased plasma membrane lipid raft formation and increased cell surface levels and signalling of TLR4-MD2 complexes.

Impact of statin therapy
Our findings suggest a marked anti-inflammatory effect of statin treatment in humans as determined both by reduced PET-CT signals and decreased circulating levels of cytokines. Although effect of statins on PET-CT signal in atherosclerotic subjects is in line with previous publications, the ~16% decrease in TBR in statin users in our study is larger than the ~10% and ~9% decreases in other reports. This may be explained by the short treatment period of 3-6 months in these intervention trials versus long-term use in our patients or the increased inflammatory status of ABCA1 mutation carriers. The finding of a statin effect on PET-CT, but not on MRI underlines the capacity of PET-CT to visualize inflammation. Libby and colleagues have suggested a specific effect of statins to decrease the uptake of FDG-glucose by macrophages in a hypoxic plaque environment; a contribution of such an effect to our results certainly cannot be excluded. Notably, however, statins also decreased some systemic markers of inflammation strongly suggesting that the decreased PET-CT signal was indicative at least in part of decreased inflammation in plaque.

Conclusion
Our data confirm a pro-inflammatory state in ABCA1 mutation carriers as reflected by increased circulating cytokines, most likely secondary to a cellular effect of ABCA1 deficiency. Our findings suggest that the increased inflammation documented in ABCA1 deficient cells and animal models is also present in humans. The increased inflammation in ABCA1 mutation carriers seems to be attenuated by statins, as shown by normalization of PET-CT and plasma cytokine levels.

Acknowledgements
The research was supported by a grant from Fondation LeDucq. Part of the research was supported by a grant from the Netherlands Heart Foundation (2011-B019: generating the best evidenced based pharmaceutical targets for atherosclerosis (GENIUS)). M. Westerterp has received funding from The Netherlands Organization of Scientific Research (NWO VENI-grant 916.11.072).
Reference List

Increased inflammation in ABCA1 mutation carriers
Supplementary Data

Supplementary Figure 1. Vessel wall thickness in controls and ABCA1 mutation carriers. Vessel wall thickness was assessed by MRI in controls (n=21), ABCA1 mutation carriers without (n=16) and with (n=8) statin treatment. TBR denotes target to background ratio; NWI normalized wall index, and MWT mean wall thickness. Data are presented as mean ± SEM and p-values for student’s t-test are indicated.

Supplementary Figure 2. 2-NBD Glucose uptake in macrophages from controls and ABCA1 mutation carriers. Peripheral blood mononuclear cells were isolated from controls (n=9) and ABCA1 mutation carriers (n=9) and differentiated into macrophages. Uptake of the glucose analogue 2-NBDG was measured using flow cytometry. Each datapoint represents one condition. N.s. is non significant.
Supplementary figure 3. Effect of the apolipoprotein B depleted serum fraction isolated from controls, heterozygous and homozygous *ABCA1* mutation carriers on macrophage inflammation. THP-1 macrophages were incubated o/n with polyethylene glycol supernatants from controls (n=13), heterozygous *ABCA1* mutation carriers (n=13), or homozygous *ABCA1* mutation carriers (n=4). RNA was isolated and the mRNA expression of *TNFα*, *IL-1β*, *MCP-1*, *IL-8*, inducible nitric oxide synthase (*iNOS*), and *IL-6* were assessed, corrected for the housekeeping gene cyclophilin and normalized to control values. Data are presented as mean ± SEM. *P*<0.05, **P*<0.01, ***P*<0.001.
Summary, Interpretation and Future Perspectives

A. E. Bochem
This thesis addresses the metabolism of high density lipoprotein (HDL) and its role in atherogenesis, steroidogenesis, hematopoiesis and inflammation.

**Chapter 1** gives an overview of the evidence from genetic studies for a role of HDL-c in atherogenesis as an introduction to this thesis. Whereas epidemiological studies have unequivocally shown that HDL-c is inversely associated with risk for CVD, the failure of HDL-c increasing therapies cast doubt on the causal role of HDL-c in atheroprotection. Studies in monogenic disorders causing low HDL-c such as ABCA1, APOA-1 and LCAT could not provide a definite answer to the causality issue, nor could studies of disorders causing high HDL-c such as CETP, SR-BI and APOC3 deficiency. Genome Wide Association Studies (GWAS) have not shown an effect of SNPs affecting only HDL-c levels on CVD risk. The debate whether HDL-c is an actor in atherogenesis or a mere biomarker, is at the center of current cardiovascular research.

In **Part 1** of this thesis, we focus on the correlation between HDL metabolism, ABC transporters and atherosclerosis.

In **Chapter 2**, we show that plasma levels of apoA-I do not offer additional predictive value over plasma levels of HDL-c in the Epic-Norfolk Prospective Population Study. Interestingly, within the highest HDL-c quartiles, higher apoA-I levels associate with a higher prevalence of CVD, possibly due to the higher prevalence of cardiovascular risk factors in association with higher apoA-I levels. This could be a relevant finding in light of the current development of apoA-I increasing strategies.

In **Chapter 3**, the evidence for suppression and prevention of atherosclerosis by ABC transporters is reviewed. The atheroprotective effects of ABC transporters have long been attributed to their capacity to transport cholesterol from lipid-laden macrophages in the vessel wall to HDL for subsequent delivery to the liver for biliary excretion. However, by promoting cholesterol efflux, ABC transporters also control the proliferation and mobilization of hematopoietic stem and multipotential progenitor cells in the bone marrow and spleen. Furthermore, ABC transporters suppress inflammatory responses in the arterial wall and control platelet production and thrombosis. These processes likely play an important role in the development of CVD in humans.

**Chapter 4** describes the prevalence of **ABCA1** mutations in 78 subjects with HDL-c below the 10th percentile. Sixteen subjects were found to carry 19 variants in **ABCA1**. Functionality of the mutations was assessed by means of cellular cholesterol efflux potential. Seven out of eight missense mutations resulted in a significant loss of cholesterol efflux capacity. In **Chapter 5** we subsequently assessed whether the cholesterol efflux impairment of these mutations are associated with increased atherosclerosis as assessed by carotid ultrasound and the highly sensitive method of carotid 3T MRI. **ABCA1** mutation carriers were shown to have more atherosclerosis compared to controls. These data support the development of strategies to upregulate ABCA1 in patients with established cardiovascular disease. However, further studies are needed to elucidate which of ABCA1’s properties is responsible for these findings.

In murine models, Abca1 deficiency has been shown to lead to endothelial dysfunction. Pulse wave velocity is an independent risk factor for cardiovascular disease and is
associated with endothelial function. In Chapter 6 we show that \textit{ABCA1} mutation carriers not only have more atherosclerosis, but also display increased arterial stiffness as assessed by pulse wave velocity, independent of HDL cholesterol levels. Furthermore, we conclude that PWV may be a valuable, cost-effective, non-invasive addition to current CVD monitoring practice, given its strong correlations with vessel wall thickness parameters. Chapter 7 describes a unique case of combined deficiency of \textit{ABCA1} and \textit{APOAI} in a 36-year old patient who experienced a myocardial infarction. This combined molecular defect is consistent with the observed near absence of HDL-c in plasma. Since specific HDL-c increasing therapy is not available yet, treatment is limited to the modulation of other risk factors, including LDL-c lowering.

After studying the consequences of genetically determined low HDL-c for atherosclerosis, we addressed the consequences of genetically determined high HDL-c. In Chapter 8, we report the prevalence and clinical consequences of \textit{APOC3} mutation carriership in individuals with plasma HDL-c levels above the 95\textsuperscript{th} percentile. In five out of 80 individuals, an \textit{APOC3} mutation was found. We found two novel mutations and one previously reported mutation. Two of these three mutations show a clear effect on lipid profiles, accompanied by large decreases of plasma apoCIII, whereas the third mutation is associated with less pronounced changes in the lipid profile and a smaller decrease in plasma apoCIII. This study confirms an association between loss of apoCIII and favourable lipid profiles and lends support to apoCIII lowering therapy which is currently tested in a clinical trial. In Chapter 9, we sequenced the gene encoding SR-BI in 120 unrelated individuals with HDL-c above the 90\textsuperscript{th} percentile. We found two novel mutations in the gene encoding SR-BI. Our data expand the number of documented human mutations in this gene to three. The novel mutations segregate with high HDL-c within the family, indicating that the SR-BI receptor is a physiologically relevant HDL receptor in humans.

ApoA-I is the structural protein of the HDL particle and it plays a crucial role in many of the beneficial effects of HDL. Having investigated consequences of both low and high HDL cholesterol, we subsequently review the potential applicability of pharmacologically increasing apoA-I by means of apoA-I mimetic peptides in chapter 10. While genetic studies show conflicting results on correlations between HDL-c and CVD, experimental studies have yielded sufficient encouraging data to proceed with the development of HDL-c raising strategies. There is evidence that ApoA-I mimetic peptides may be a promising supplement to current strategies. However, results from human studies are sparse and more research in the human setting is needed. In Chapter 11, we review the promise of another class of HDL-c increasing agents in CVD prevention: CETP inhibitors. CETP inhibition has been shown to increase HDL-c levels in man. However, torcetrapib, the first CETP inhibitor tested in phase III trials resulted in increased mortality possibly due to apparent compound-specific vasopressor effects. More recently, dalcetrapib, another CETP inhibitor, did not show an effect on CVD outcome while raising HDL-c by 30\%, thereby refuting the HDL-c hypothesis. Anacetrapib and evacetrapib are currently tested in phase III clinical trials and have not shown adverse effects thus far. Both compounds not only increase HDL-c
by 29-51%, they also decrease LDL-c (36-41%) while anacetrapib lowers lipoprotein (a) by 17%. Combined, these effects are anticipated to decrease CVD risk and the results will be revealed in 2017. However, these compounds will not aid us in answering the question whether pharmacological increase of HDL-c leads to decreased CVD risk.

In part II of this thesis, we focus on the role of HDL in adrenal function, hematopoiesis and inflammation. In Chapter 12 we assessed adrenal function in Lcat knockout mice. In vitro studies have suggested that HDL and LDL can provide cholesterol for steroid hormone synthesis by the adrenal gland. We report that HDL deficiency in Lcat knockout mice, characterized by dramatically decreased HDL-c levels, is associated with a 40–50% lower adrenal steroid output. These findings highlight the important novel role for HDL as a cholesterol donor for the adrenal steroidogenesis in mice. In Chapter 13, we subsequently assessed adrenal function in male subjects with low HDL-c due to mutations in ABCA1 or LCAT, as well as in subjects with low HDL-c without underlying genetic defect. We show that basal adrenal steroidogenesis is decreased in male subjects with low plasma HDL-c, irrespective of its origin. These findings support a role for HDL as a cholesterol donor for basal adrenal steroidogenesis in humans. In contrast, Chapter 14 describes that adrenal steroidogenesis is not lower in female ABCA1 or LCAT mutation carriers. This gender-related discrepancy underscores the importance of gender specific analyses in cholesterol-related research.

Chapters 12 and 13 provide evidence for a role for HDL derived cholesterol in adrenal steroidogenesis. However, in homozygous subjects with extremely low HDL-c, substantial adrenal steroidogenesis was present. Furthermore, basal adrenal steroidogenesis but not adrenal response to ACTH was impaired in low HDL-c subjects, indicating that other pathways may supply the adrenal gland with cholesterol in acute settings. We therefore hypothesized that LDL provides the human adrenal gland with substrate for hormone synthesis. In Chapter 15 we therefore assessed cortisol response to ACTH in patients with impaired uptake of LDL derived cholesterol due to defective LDL receptor function or defective apolipoprotein B (the ligand for LDL binding to its receptor) and controls. Response to ACTH was lower in patients with impaired uptake of LDL derived cholesterol compared to controls. Our data support a role for LDL derived cholesterol in the adrenal response to stress in humans. Current therapeutics, aiming to lower plasma LDL cholesterol to below 1 mmol/L may jeopardize adrenal function.

In recent years, it has become clear that atherosclerosis is an inflammatory process. Previous studies have shown that mice with defects in cellular cholesterol efflux are characterized by hematopoietic stem cell (HSPC) and myeloid proliferation, leading to more circulating inflammatory cells, likely contributing to atherogenesis. In Chapter 16, we hypothesized that the combination of hypercholesterolemia and defective cholesterol efflux would promote HSPC expansion and leukocytosis more prominently than either alone. Our data suggest that in mice, a balance of cholesterol uptake and efflux mechanisms may be driving HSPC proliferation and monocytosis. Higher monocyte counts in children with FH and low HDL-cholesterol suggest a similar pattern in humans, possibly contributing to the atherosclerotic phenotype in hypercholesterolemic subjects.
Given recent findings in mice that defective cellular cholesterol efflux pathways increased inflammatory gene expression in monocytes and macrophages, as well as production of inflammatory cells, we hypothesized in Chapter 17 that human ABCA1 mutation carriers would be characterized by increased inflammation. We show that ABCA1 mutation carriers are characterized by pro-inflammatory changes of the arterial wall as assessed by PET-CT as well as a systemic pro-inflammatory state. In vitro experiments were performed to determine whether the pro-inflammatory phenotype originates from plasma or a cellular component using plasma isolated from ABCA1 mutation carriers. Our data confirm a pro-inflammatory state in ABCA1 mutation carriers as reflected by increased circulating cytokines, most likely secondary to a cellular effect of ABCA1 deficiency. Statins seem to exert beneficial anti-inflammatory effects, as shown by normalization of PET-CT and plasma cytokine levels.

**Interpretation and future perspectives**

Although epidemiological studies show that HDL-c is inversely associated with CVD risk, HDL-c increasing therapies have not resulted in lower incidence of CVD. This has cast doubt on the atheroprotective capacities of HDL. However, it has also increased interest in the roles of HDL in processes unrelated or indirectly related to atherosclerosis. This thesis does not answer the question whether HDL is an atheroprotective entity or not, but it provides pieces to the puzzle of HDL metabolism.

Studies in ApoAI (chapter 7), ApoCIII (chapter 8), SR-BI (chapter 9) and LCAT (chapter 12, 13 and 14) mutation carriers give us an insight into their specific effects on HDL metabolism. ABC transporter deficiency is a particularly interesting condition since it leads to both cholesterol accumulation and low plasma HDL-c (chapter 3, 4, 5, 6, 7, 13, 14, 17).

We show that ABCA1 mutation carriership is associated with increased atherosclerosis and increased arterial stiffness. The finding that ABCA1 mutation carriership does not uniformly result in an increased CVD event rate, underscores the complexity of HDL metabolism. Although we show that novel mutations in the genes encoding SR-BI and APOC3 are associated with favourable lipid profiles, the sample size and cross-sectional design of the study do not allow us to draw conclusions regarding CVD risk.

Despite setbacks, considerable effort is still put into HDL-c increasing strategies as reviewed in chapters 10 and 11. In order to predict their efficacy and side effects, it is important to oversee the whole range of effects that are mediated by HDL. In that regard, more insight is needed on the effects of HDL beyond atherosclerosis. The novel finding that low HDL-c is associated with decreased steroidogenesis in males is intriguing and is an example of how crosstalk between research fields can lead to important findings and an increased understanding of human physiology. Other examples include the importance of the lymphatic vessel route for reverse cholesterol transport and atherosclerosis, the role of cholesterol accumulation in cancer and macular degeneration, and the role of cholesterol efflux in haematopoiesis.

The roles of HDL and ABC transporters in inflammation form a particularly fascinating field. The paradigm that atherosclerosis is an inflammatory process, has been widely...
acknowledged. Our findings in chapter 17 constitute the first evidence for increased inflammation in \textit{ABCA1} deficiency in humans. The role of inflammation in atherosclerosis, as well as its importance in a range of other diseases, secures a strong research interest where crosstalk between researchers from different areas may turn out crucial.

In conclusion, HDL and ABC transporters are definitely actors of importance in multiple physiological processes in humans. The notion that HDL may not be the atheroprotective particle it was long assumed to be, elicited a queste to explain the inverse correlation between HDL-c and CVD risk by taking the broad spectrum of effects of HDL-c on various organ systems into consideration. This enabled a shift of interest and resources towards exploration of effects of HDL-c beyond reverse cholesterol transport and advanced the insight in human physiology. ABC transporters aid us in shedding light on the importance of cholesterol homeostasis in human physiology. The full range of effects of ABC transporters has not been elucidated to date, and more research is needed on their function in different tissues and organ systems.

Andrea Bochem
Amsterdam, June 27 2013
Reference List


Appendices

Nederlandse samenvatting
Dankwoord
Portfolio
CV
List of Publications

Het exacte mechanisme achter het mogelijk beschermende effect van HDL is onbekend. In dit proefschrift onderzoeken we de associatie tussen hoog of laag HDL-c en atherosclerose en hart –en vaatziekten. Naar aanleiding daarvan bespreken we wat we mogen verwachten van HDL-verhogende therapiën. Daarnaast hebben we onderzocht welke andere effecten HDL heeft, onder andere om bijwerkingen van HDL-verhogende therapiën te voorspellen.


In het eerste deel van dit proefschrift concentreren we ons op de correlatie tussen HDL metabolisme en atherosclerose.
Apolipoproteine A-I (apoA-I) is het eiwit waaruit HDL is opgebouwd. Het speelt een cruciale rol in veel van de gunstige effecten van HDL. In hoofdstuk 2 laten we zien dat de plasma concentratie van ApoA-I echter geen beter voorspeller is voor het optreden van hart- en vaatziekten dan de plasma concentratie van HDL-c in de EPIC-Norfolk Prospective Population Study. Sterker nog, in de hoogste HDL-c kwartieren correleert een hoog ApoA-I met een hogere prevalentie van hart- en vaatziekten. Dit zou verklard kunnen worden door een hoger voorkomen van risicofactoren voor hart- en vaatziekten in de groep met hoge ApoA-I concentraties. Dit is een belangrijke bevinding in het licht van de ontwikkeling van ApoA-I verhogende therapiën ter voorkoming van hart- en vaatziekten.

In hoofdstuk 3 geven we een overzicht van het bewijs dat ABC transporters atherosclerose kunnen voorkomen. De vaatwandbeschermende effecten van ABC transporters werden tot voor kort voornamelijk toegeschreven aan hun capaciteit om cholesterol uit de vaatwand naar HDL in het bloed te transporteren, waarna het cholesterol het lichaam verlaat met de ontlasting. Echter, recent is duidelijk geworden dat ABC transporters ook op andere manieren atherosclerose voorkomen. ABC transporters reguleren de proliferatie en mobilisatie van cellen in het beenmerg en de milt. Daarnaast onderdrukken ABC transporters ontsteking in de vaatwand en reguleren ze de productie van bloedplaatjes en de ontwikkeling van trombose. Deze processen spelen waarschijnlijk een belangrijke rol in de ontwikkeling van hart- en vaatziekten bij mensen.

HDL-c wordt voor een deel genetisch bepaald. In de volgende hoofdstukken belichten we cohorten met een genetisch laag HDL-c of een genetisch hoog HDL-c. In hoofdstuk 4 wordt het voorkomen van ABCA1-mutaties beschreven in 78 individuen met een HDL onder het tiende percentiel. Zestien studie deelnemers bleken drager van negentien varianten in ABCA1. De mate waarin de mutatie de functie van het eiwit beperkt, is onderzocht door middel cholesterol efflux experimenten. Zeven van acht missense mutaties resulteerden in een afgenomen functie van het eiwit. In hoofdstuk 5 hebben we vervolgens onderzocht of studie deelnemers met een afgenomen functie van het ABCA1-eiwit meer atherosclerose hebben. We hebben dit zowel onderzocht door middel van echografie van de carotiden als een nieuwe methode: vaatwanddiktemeting door middel van MRI. ABCA1-mutatiedragers bleken meer atherosclerose te hebben vergeleken met controles. Deze data ondersteunen de ontwikkeling van ABCA1-opregulatie in patiënten met hart- en vaatziekten. Echter, verder onderzoek is nodig om vast te stellen welke eigenschap van ABCA1 verantwoordelijk is voor deze bevindingen.

Verminderde werking van het eiwit Abca1 leidt tot verminderde werking van de cellen die de binnenkant van vaten bekleden (endotheeldysfunctie) in muismodellen. Pulse wave velocity is de snelheid waarmee golven zich door de vaten bewegen en is een risicofactor voor hart- en vaatziekten. Pulse wave velocity is geassocieerd met endotheeldysfunctie. In hoofdstuk 6 laten we zien dat ABCA1-mutatiedragers niet alleen meer atherosclerose, maar ook een hogere pulse wave velocity en dus een stijvere vaatwand hebben. Dit is onafhankelijk van HDL-c concentraties in plasma. Daarnaast concluderen we dat pulse wave velocity een waardevolle, kosteneffectieve, niet-invasieve toevoeging zou zijn aan de huidige monitoring van progressie van hart- en vaatziekten, gezien de sterke correlatie.
met vaatwanddiktemetingen. **Hoofdstuk 7** beschrijft een unieke casus van een man die op 36-jarige leeftijd een hartaanval kreeg. De man had geen risicofactoren voor hart–en vaatziekten. Echter, bij laboratoriumonderzoek werd een extreem laag HDL-c gevonden, wat bij nader onderzoek waarschijnlijk het gevolg bleek van mutaties in zowel *ABCA1* als *APOA1*. Het is voor het eerste dat deze combinatie van mutaties wordt beschreven. Het is aannemelijk dat het lage HDL-c een bijdrage heeft geleverd aan het vroege optreden van hart- en vaatziekten in deze patient. Aangezien er nog geen therapie bestaat om HDL-c te verhogen, concentreert de behandeling van deze patiënt zich op het moduleren van andere risicofactoren, waaronder het verlagen van LDL-c.

Na het bespreken van de gevolgen van genetisch laag HDL-c voor atherosclerose, bespreken we de gevolgen van genetisch hoog HDL-c. In **hoofdstuk 8** rapporteren we het voorkomen en de gevolgen van dragerschap van *APOC3* mutaties. In vijf van tachtig studieellemen met een HDL-c boven het 95e percentiel werd een *APOC3* mutatie gevonden. We vonden twee nieuwe mutaties en een eerder beschreven mutatie. Twee van deze drie mutaties zijn geassocieerd met een gunstig lipidenprofiel en een lagere plasma apoCIII concentratie. De derde mutatie is minder sterk geassocieerd met een gunstig lipidenprofiel en de plasma apoCIII concentratie is ook minder verlaagd. Deze studie bevestigt de eerdere bevinding dat weinig apoCIII geassocieerd is met een gunstig lipidenprofiel. Daarmee ondersteunen onze bevindingen de ontwikkeling van apoCIII verlagende medicijnen, welke op dit moment getest worden in mensen. In **hoofdstuk 9** is het gen dat codeert voor scavenger receptor class B type 1 (SR-BI) gesequenced in 120 individuen met een HDL-c boven het 90e percentiel. We vonden twee nieuwe mutaties in het gen dat codeert voor SR-BI. Daarmee hebben we het aantal bekende mutaties in dit gen verhoogd naar drie. De door ons gevonden mutaties segregeren met hoog HDL-c in de familie, hetgeen impliceert dat de SR-BI receptor een receptor van belang is in mensen.

Nadat we de gevolgen van hoog en laag HDL-cholesterol hebben onderzocht, geven we in **hoofdstuk 10** een overzicht van de mogelijk gunstige effecten van het verhogen van apoA-I door middel van apoA-I analogen. We concluderen dat hoewel genetische studies geen eenduidig antwoord geven op de vraag of HDL-c beschermt tegen hart–en vaatziekten, er voldoende bemoedigende data zijn uit experimentele studies om door te gaan met de ontwikkeling van apoA-I (en daarmee HDL) verhogende therapieën. Echter, er zal meer onderzoek in mensen gedaan moeten worden om het vaatwandbeschermende effect van apoA-I met zekerheid te kunnen vaststellen. In **hoofdstuk 11** bespreken we de belofte van een andere manier om HDL-c te verhogen: CETP-remming. De ontwikkeling van CETP-remmers verloopt niet zonder slag of stoot. Hoewel het remmen van CETP HDL-c verhoogt in mensen, bleek behandeling met CETP-remmer torcetrapib geassocieerd met een toename in sterfte. Dit is toegeschreven aan effecten specifiek voor torcetrapib en daarom is de ontwikkeling van andere CETP inhibitors voortgezet. Echter, dalcetrapib, een andere CETP-remmer, verhoogde HDL-c, maar leidde niet tot minder hart–en vaatziekten. Op dit moment worden CETP-remmers anacetrapib en evacetrapib getest in fase III trials. Aangezien beide middelen niet alleen HDL-c verhogen, maar ook LDL-c verlagen, wordt...
een gunstig effect verwacht op hart–en vaatziekten. Echter, de uitkomst van deze trials zal geen antwoord geven op de vraag of HDL-c verhoging leidt tot minder hart–en vaatziekten.

In het deel II van dit proefschrift concentreren we ons op de rol van HDL in andere processen dan atherosclerose, namelijk bijnierfunctie, productie van cellen in het beenmerg en ontsteking. In hoofdstuk 12 hebben we de bijnierfunctie van Lcat knockout muizen onderzocht. De bijnier gebruikt cholesterol om daaruit hormonen te produceren. In vitro studies hebben laten zien dat HDL en LDL cholesterol kunnen doneren aan de bijnier voor hormoonproductie. We laten zien dat extreem lage HDL-c concentraties in Lcat knockout muizen geassocieerd zijn met een verminderde bijnierfunctie. In hoofdstuk 13 hebben we vervolgens de bijnierfunctie bekeken van mannen met een laag HDL-c door mutaties in ABCA1 of LCAT, en in mannen met een laag HDL-c zonder onderliggend genetisch defect. We laten zien dat basale bijnierfunctie lager is in mannen met een laag plasma HDL-c dan in mannen met een normaal HDL-c, ongeacht door oorzaak van het lage HDL-c. Deze bevindingen ondersteunen het concept dat HDL een cholesterol donor is voor basale hormoonproductie door de bijnier in mannen. Echter, in hoofdstuk 14 laten we zien dat de bijnierfunctie van vrouwen met een laag HDL-c door mutaties in ABCA1 of LCAT niet lager is dan in vrouwen met een normaal HDL-c. Dit man-vrouw verschil onderstrept het belang van sexe-specifieke analyses in cholesterol gerelateerd onderzoek.

Hoofdstukken 12 en 13 laten zien dat cholesterol afkomstig van HDL een rol speelt in hormoonproductie door de bijnier. Echter, in patiënten zonder HDL-c produceert de bijnier nog steeds hormonen. Daarnaast was basale hormoonproductie verlaagd in mensen met laag HDL-c, maar reageert hun bijnier onveranderd op stress, geïnduceerd door toediening van het middel adrenocorticotropic hormon (ACTH). Daarom hypothetiseerden wij dat ook LDL substraat vormt voor hormoonproductie door de bijnier. In hoofdstuk 15 hebben we gekeken of de stress respons van de bijnier verminderd is in mensen die geen LDL in de bijnier kunnen opnemen door een defecte LDL receptor of door een defecte binding van LDL aan de LDL receptor. De stress respons van de bijnier bleek lager in patiënten die minder LDL-c kunnen opnemen in de bijnier. Dit geeft aan dat LDL-c een rol speelt in de respons op stress en is een belangrijke bevinding gezien de ontwikkeling van potente LDL-verlagende middelen die LDL-c onder de 1 mmol/L kunnen brengen. Behandeling met deze middelen zou adequate hormoonproductie door de bijnier in gevaar kunnen brengen.

In de afgelopen jaren is duidelijk geworden dat atherosclerose een ontstekingsproces is. Daarnaast is aangetoond dat muizen die geen cholesterol kunnen transporteren, proliferatie van beenmergcellen laten zien hetgeen leidt tot meer ontstekingscellen in het bloed die waarschijnlijk bijdragen aan de ontwikkeling van atherosclerose. In hoofdstuk 16 hypothetiseerden we dat de combinatie van hoog cholesterol en om onvermogen cholesterol te transporteren zou leiden tot proliferatie van beenmergcellen in een grotere mate dan een van beide factoren alleen. We laten zien dat in muizen die een balans tussen opname van cholesterol in de cel en transport van cholesterol uit de cel bepalend is voor het optreden van proliferatie van beenmergcellen. Veel opname van cholesterol en weinig transport van cholesterol gaat gepaard met een meer proliferatie van beenmergcellen een hogere
concentratie monocyten in het bloed. Ook laten we zien dat in kinderen met een hoog LDL cholesterol, hoge concentraties monocyten gepaard gaan met een laag HDL-cholesterol, waarschijnlijk een gevolg van weinig cholesterol transport. Dit mechanisme zou kunnen bijdragen aan de ontwikkeling van atherosclerose in mensen met hoog cholesterol.

Aangezien recentelijk is aangetoond dat muizen met een defect in het cholesterol transport meer genexpressie hebben van ontstekingsfactoren in monocyten en macrofagen en daarnaast meer ontstekingscellen produceren, hebben we in hoofdstuk 17 onderzocht of ABCA1-mutatiedragers, die immers ook minder cholesterol kunnen transporteren, gekenmerkt worden door meer ontsteking. We laten zien dat ABCA1-mutatiedragers zowel meer ontsteking hebben in de vaatwand als meer tekenen van ontsteking in het bloed. Middels in vitro experimenten laten we zien dat de verhoogde mate van ontsteking in ABCA1-mutatiedragers waarschijnlijk het gevolg is van een effect van ABCA1-deficiëntie op de cel. Statines lijken een ontstekingsremmend effect te hebben, gezien de normalisatie van ontsteking in de vaatwand en ontstekingsfactoren in het bloed.
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Web of Science 2010 0.4 ECTS
Crash Course (Bio) chemistry and Molecular Biology 2010 0.4 ECTS
Clinical Data Management 2010 0.2 ECTS
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Advanced Topics in Biostatistics 2011 2.1 ECTS
Mass Spectrometry, Proteomics and Protein Research 2011 2.1 ECTS
Clinical Epidemiology 2012 0.6 ECTS
DNA technology 2012 2.1 ECTS
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Presentations
ABCA1 mutation carriers with low High Density Lipoprotein Cholesterol (HDL-c) are characterized by a larger atherosclerotic burden Cardiovasculair Conference (CVC), Noordwijkerhout, the Netherlands 2012 0.5 ECTS
International Symposium on Atherosclerosis, Sydney, Australia Rembrandt Institute of Cardiovascular Science (RICS), Amsterdam, the Netherlands 2011 0.5 ECTS
Adrenal function in low HDL-C subjects Satellite meeting European Atherosclerosis Society Copenhagen, Denmark 2011 0.5 ECTS
High-density lipoprotein as a source of cholesterol for adrenal steroidogenesis; a study in individuals with low plasma HDL-c International Symposium on Atherosclerosis, Sydney, Australia Fondation LeDucq network meeting Lenzerheide, Switzerland 2012 0.5 ECTS
Lower adrenal steroid production in mice and humans with low HDL-cholesterol due to mutations in \( LCAT \)
European Atherosclerosis Society conference Lyon, France 2013 0.5 ECTS

Increased systemic and plaque inflammation in \( ABCA1 \) mutation carriers with attenuation by statins
Fondation LeDucq network meeting, Amsterdam, the Netherlands 2013 0.5 ECTS
European lipoprotein Club meeting Tutzing, Germany 2013 0.5 ECTS
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ATVB Early Career Networking Reception 2013 0.5 ECTS

Conferences
HDL Workshop on High Density Lipoproteins, Whistler, Canada 2010 1.25 ECTS
Dutch Atherosclerosis Society Conference, Ede, the Netherlands 2010 0.5 ECTS
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Academic Medical Center Amsterdam, the Netherlands 2010-2011
Curriculum Vitae

Andrea Bochem was born on February 3rd 1984 in Nijmegen, the Netherlands. She graduated from the Stedelijk Gymnasium Nijmegen in 2002 and started her medical training at the University of Amsterdam after successfully having completed one year of Economics and Law at the University of Utrecht. In 2007, she finished two research projects in Israel, one at the department of surgery at the Hadassah Medical Center in Jerusalem, the other at the department of cardiology at the Sourasky Medical Center in Tel Aviv. In that same period she did voluntary work at the “Jaffa Institute” in Jaffa and at “Physicians for Human Rights” in Tel Aviv and on the Westbank. Back in the Netherlands, she started clinical rotations including an internship in Hlabisa, a rural area of South Africa. During her clinical rotations, she had a prominent role within the faculty’s student council and became involved in research at the department of clinical genetics at the Academic Medical Center (AMC) Amsterdam. In November 2009, the day after graduating from medical school, she entered the PhD program at the Department of Vascular Medicine at the AMC Amsterdam under enthusiastic supervision of dr. G.K. Hovingh, dr. J.A. Kuivenhoven and prof. dr. E.S.G. Stroes. With the support of multiple grants she continued her research at Columbia University, New York, USA, in 2012-2013. At the laboratory of prof. dr. Tall, she was inspiringly supervised by prof. dr. A.R. Tall and dr. M. Westerterp. The results of her PhD program are presented in this thesis. In July 2013, Andrea started her specialty training in internal medicine at the AMC Amsterdam under supervision of prof. dr. J. B. L. Hoekstra.
List of Publications


