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

Based on
“Genetics of HDL-c A Causal Link to Atherosclerosis?”
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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.14,22,23

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-1.21-23 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.14 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.24,25 Several studies have reported an association between ABCA1 variants and CVD risk, but this association is not consistently associated with HDL-c.26-29 Other studies report contrasting conclusions on the association between ABCA1 variants and CVD risk.30,31 In Chapter 4, novel mutations in ABCA1 and their consequences for cholesterol efflux are reported. Chapter 5 investigates 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,15 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.32 Liver X receptors (LXRs)33,34 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 \textit{ABCA1} and \textit{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 \textit{ABCA1} in atherosclerosis. However, whether \textit{ABCA1} constitutes an attractive therapeutic target in CVD prevention remains to be established.

\textbf{Apolipoprotein A1}

Apolipoprotein A1 (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 ApoAI have been described. Some are reported to result in hereditary amylodoidosis which in turn has been suggested to accelerate atherosclerotic plaque formation. In humans, homozygosity for loss of function mutations in apoA-I 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 \textit{ABCA1} and \textit{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 \textit{in vivo} and to exert anti-inflammatory and anti-oxidative effects.

\textbf{Molecular causes of high HDL-c}

\textit{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 \textit{APOC3} mutation carriers typically display 50%
of normal apoCIII levels and lower fasting and postprandial serum triglycerides.\textsuperscript{53,54} This apparently favourable lipid profile has been shown to be associated with less coronary artery calcification\textsuperscript{55} and lower CVD risk.\textsuperscript{56} 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\textsubscript{Rx}, Clinical Trials.gov: NCT01529424) has been started.\textsuperscript{61,62}

Scavenger receptor class B type 1 (SR-B1)

This protein was first discovered in mice, where the \textit{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 \textit{SR-B1} in humans that was associated with significantly higher HDL-c levels and a reduced cholesterol efflux capacity in macrophages.\textsuperscript{57} 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 \textit{SR-B1} variants, associated with high HDL-c values are reported.\textsuperscript{58} Detailed data on these mutations show an impaired ability to bind HDL and a diminished selective uptake of cholesteryl esters.\textsuperscript{59}

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 \textit{SR-B1} variants in humans, associated with high HDL-c, are thought to be related to a decrease in cholesterol efflux potential.\textsuperscript{57,60} 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 \textit{CETP} gene, mainly in the Japanese population, where CETP-variants are found in 50\% of individuals with high HDL-c.\textsuperscript{68} in homozygous carriers of a splicing defect in the \textit{CETP} gene, HDL-c levels were reported to be exceedingly high while LDL-c levels were at the lower end of the normal distribution.\textsuperscript{67}

Although early reports on the correlation between CETP variants and CVD risk have been conflicting,\textsuperscript{61,62} more recent reports support a beneficial association with cardiovascular endpoints in large studies.\textsuperscript{63-66}

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 athoprotection. However, anti-inflammatory, anti-thrombotic, anti-oxidant and antimicrobial functions have also been ascribed to HDL. Furthermore, HDL has been implicated in steriodogenesis. 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 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. Mice with a knockout of Abca1 and 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.

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 Ldlr−/−/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. Whereas a link between ABC transporters and inflammation is reported in murine models, 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. 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.

In Chapter 17, it is for the first time assessed whether human ABCA1 mutation carriers are characterized by increased inflammation.
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