Apolipoproteins A-I and A-V as gene therapeutic targets to intervene in lipid metabolism
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General introduction and outline of thesis
Apolipoproteins combine with lipids to form lipoproteins

Apolipoproteins make up the main protein component of lipoproteins, i.e. the spherical macromolecules in blood that enable transportation of hydrophobic lipids through this watery compartment. These specific proteins combine with free cholesterol, phospholipids, cholesteryl esters and triglycerides to form lipoproteins consisting of a non-polar lipid core (mainly triglycerides and cholesteryl esters) and an outer layer of phospholipids and apolipoproteins (see Fig. 1 for a schematic representation). Human plasma contains a variety of typical apolipoproteins that are represented by four main groups; A (A-I, A-II, A-IV, A-V), B (B48, B100), C (C-I, C-II, C-III), and E and several proteins that are named apolipoproteins but are distinctively different in structure and function (e.g. apoD, F, H, J, M and L). The latter group of proteins has not been studied in this thesis and will not be further discussed in this introduction. The specific combinations of lipids and apolipoproteins define the size, density and, ultimately, the function of the different lipoprotein classes. While the different lipoprotein classes are first off categorized by density, i.e. from chylomicrons through very-low density lipoproteins (VLDL) and low-density lipoproteins (LDL) to high-density lipoproteins (HDL), this distinction also co-segregates their specific apolipoprotein composition and function in lipid metabolism (Table 1).

![Figure 1. Schematic representation of a typical lipoprotein with its main components](image-url)
Table 1. Lipoprotein classes and characteristics

<table>
<thead>
<tr>
<th>Lipoprotein class</th>
<th>Density (g/mL)</th>
<th>Main apolipoproteins</th>
<th>Role in lipid metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>&lt; 0.93</td>
<td>B48, A-I, A-II, A-IV</td>
<td>Lipoprotein that is synthesized and secreted from the intestine to transport mainly TG to the liver and peripheral tissues.</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.93 – 1.019</td>
<td>B48, C-II, C-III, E</td>
<td>Lipoprotein in FCT that is secreted from the liver to transport TG and cholesterol to peripheral tissues.</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019 – 1.063</td>
<td>B100, E</td>
<td>Lipoprotein that is formed after lipolysis of TG in VLDL and IDL.</td>
</tr>
</tbody>
</table>


The general property of apolipoproteins that enables them to bind and transport lipid is that they have both hydrophilic and hydrophobic (amphipathic) properties. Despite this common feature, apolipoproteins are a heterogeneous class of proteins consisting of 2 main categories. Most of the exchangeable apolipoproteins (i.e. A-I, A-II, A-IV, A-V, C-I, C-II, C-III and E) are encoded by a large multi-gene family, whereas the other group contains the non-exchangeable apolipoproteins apoB100 and apoB48, which are products of a single gene. The exchangeable apolipoproteins are in general relatively small (57-371 amino acid residues for apoC-I and apoA-IV, respectively) and are composed of repeated amphipathic α-helical regions. ApoBs, on the other hand, are much larger (the mature apoB100 molecule is composed of 4536 amino acid residues) and contain stretches of hydrophobic amino acid residues as well as amphipathic β structure that give them their lipophilic character. In addition, these β structures (specifically of residues 37-41) have very recently been shown to cause the unique non-exchangeable nature of apoBs.
Apolipoproteins are key players in lipid metabolism

Lipid metabolism encompasses the regulated distribution of cholesterol and triglycerides (TG), combined referred to as lipids, throughout the body. Two main directions of transport can be distinguished: Transport from the intestine and the liver to peripheral tissues (forward cholesterol transport; FCT) and the transportation of lipids from the periphery to the liver (reverse cholesterol transport; RCT). Apolipoproteins are critically involved in most of the steps that can be distinguished in these lipid transport pathways:

Assembly and secretion of lipoproteins

First of all, apolipoproteins are crucial for the proper assembly and secretion of lipoproteins. In FCT the central apolipoproteins in lipoprotein formation are apoB48 for intestinal chylomicron production\(^5\) and apoB100 for hepatic VLDL production\(^6,7\), respectively. Assembly of both lipoproteins starts with the lipidation of the respective apoBs by microsomal transfer protein. The formation of HDL particles, the central lipoprotein of the RCT, starts with the lipidation of apoA-I which results in the formation of discoidal phospholipid-rich nascent HDL particles which can subsequently mature into spherical HDL by further acquisition of lipids.\(^8\) Lipoproteins are formed intracellularly and require secretion into the circulation, a process that requires the involvement of apolipoproteins. For example, intestinal chylomicron secretion was shown to be severely perturbed by inhibiting apoA-IV production in mice.\(^9\) Likewise, hepatic VLDL secretion in apoE deficient mice was strongly inhibited which suggests that apoE plays a role here.\(^10,11\)

Further acquisition of lipids by lipoproteins

Apolipoproteins can also act as essential co-factors in further lipid acquisition. In HDL metabolism for example, interaction of apoA-I with adenosine-triphosphate binding cassette transporter A1 (ABCA1) allows for cellular transfer of unesterified cholesterol and phospholipids onto HDL.\(^12,13\) Subsequently, apoA-I is a required co-factor for lecithin-acyltransferase (LCAT)-mediated esterification of this cholesterol\(^14\), allowing the transfer of cholesteryl ester into the hydrophobic core of the maturing HDL particle.
Unloading of lipids and removal of lipoproteins

The delivery of cholesterol from lipoproteins to peripheral tissues involves for a significant part binding to (cell membrane) receptors, whereby apolipoproteins can act as ligands. In FCT, VLDL and LDL bind to the LDL receptor through apoB100 and apoE, or to the VLDL receptor and LDL related receptor through apoE. This binding is followed by receptor-mediated endocytosis. In RCT, scavenging receptor B1 (in humans also named CLA-1) interacts with apoA-I on HDL, which accounts for selective uptake of cholesterol by tissue cells, predominantly hepatic and steroidogenic cells. TG, in contrast to cholesterol, need to be hydrolyzed prior to cellular uptake. The majority of circulating TG, carried mainly in chylomicrons and VLDL, are hydrolyzed by the enzyme lipoprotein lipase (LPL) with apolipoprotein C-II acting as an essential co-factor. Regulation of this LPL-mediated hydrolysis occurs in part through additional apolipoproteins such as apoC-I, apoC-III, apoA-IV and apoA-V.

Thus, the role of apolipoproteins extends well beyond an initially presumed role of providing structural integrity to lipoproteins, with critical involvement in virtually every step in lipid metabolism. Moreover, apolipoproteins have also been shown to be important molecules in processes that are not directly related to lipid metabolism, such as protective roles in inflammation and oxidation (apoA-I), or by acting in satiety signalling pathways (apoA-IV).

Defects in apolipoprotein functioning have a serious impact on lipid metabolism

Given their central role in lipid metabolism, it is not surprising that mutations in the genes encoding for apolipoproteins can have a strong impact on lipid metabolism with sometimes serious clinical implications (Table 2). For example, apoA-I deficiency causes HDL deficiency and an increased risk of premature coronary artery disease (CAD). With a mild effect on lipid metabolism, APOA2 gene mutations have on the other hand been implicated in (renal) amyloidosis and are possibly associated with an increased risk of CAD and type 2 diabetes mellitus. Subjects with complete deficiency of apoA-V suffer from severe hypertriglyceridemia, although additional causes seem to be necessary to evoke this phenotype. ApoA-V deficiency is rare, but clinical symptoms...
### Table 2. Effects of apolipoprotein mutations on plasma lipid profile and clinical consequences

<table>
<thead>
<tr>
<th>Apolipoproteins (gene)</th>
<th>OMIM</th>
<th>Effect of mutations on plasma lipid parameters</th>
<th>Clinical consequences associated with mutations</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I (APOA1)</td>
<td>107680</td>
<td>HDL ↓</td>
<td>Increased risk of premature CAD and systemic amyloidosis.</td>
<td>23,24</td>
</tr>
<tr>
<td>ApoA-II (APOA2)</td>
<td>107670</td>
<td>Marginal effect on plasma lipids</td>
<td>Systemic amyloidosis, possible association with increased risk of CAD and Type 2 DM</td>
<td>26-28</td>
</tr>
<tr>
<td>ApoA-IV (APOA4)</td>
<td>107690</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoA-V (APOA5)</td>
<td>606368</td>
<td>TG ↑</td>
<td>Xanthomas, hepatosplenomegaly and risk of pancreatitis</td>
<td>29-31</td>
</tr>
<tr>
<td>ApoB (APOB): Mutations inhibiting secretion of apoB</td>
<td>107730</td>
<td>TG ↓, TC ↓</td>
<td>Acanthocytosis, intestinal fat malabsorption, failure to thrive, fatty liver</td>
<td>33</td>
</tr>
<tr>
<td>ApoB (APOB): Mutations affecting the LDL receptor binding domain</td>
<td></td>
<td>TC ↑</td>
<td>Increased risk of coronary artery disease</td>
<td>33</td>
</tr>
<tr>
<td>ApoC-I (APOC1)</td>
<td>107710</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoC-II (APOC2)</td>
<td>207750 and 608083</td>
<td>TG ↑</td>
<td>Pancreatitis, xanthomas, corneal clouding</td>
<td>32,34, 35</td>
</tr>
<tr>
<td>ApoC-III (APOC3)</td>
<td>107720</td>
<td>TG ↓, HDL ↑</td>
<td>Indications of reduced risk of CAD</td>
<td>36,37</td>
</tr>
<tr>
<td>ApoE (APOE)</td>
<td>107741</td>
<td>TC ↑, TG ↑</td>
<td>Xanthomas, increased risk of CAD and peripheral atherosclerosis</td>
<td>38</td>
</tr>
</tbody>
</table>

OMIM is the Online Mendelian Inheritance in Man-database. HDL: high-density lipoprotein, TG: triglycerides, TC: total cholesterol, CAD: coronary artery disease, Refs; references and references therein.
include xanthomas, hepatosplenomegaly and risk of pancreatitis as seen in patients suffering from LPL deficiency. Mutations in the APOB gene can have opposite effects based on the location of the mutation. Mutations can lead to a decreased or complete inhibition of apoB secretion, resulting in hypolipidemia. Clinical features associated with hypolipidemia are fat malabsorption, failure to thrive, neuropathy and fatty liver. Mutations that affect the LDL receptor binding domain of apoB, however, prevent uptake of apoB-containing particles and, as a consequence, these patients suffer from hypercholesterolemia and present with the same, although generally less severe, clinical symptoms as patients with defects in the LDL receptor (familial hypercholesterolemia). As indicated above, apoC-II is an essential co-factor for LPL-mediated TG hydrolysis and apoC-II deficiency thus results in functional LPL deficiency with its associated features. Biochemically this entails extremely elevated plasma TG levels which can cause eruptive xanthomas and, most seriously, recurrent and life-threatening pancreatitis. Interestingly, mutations in the APOC3 gene appear to be (cardio)protective due to alleviation of LPL inhibition, which is associated with decreased plasma TG and increased HDL levels. Finally, mutations in the APOE gene inhibit clearing of remnant lipoproteins, which is associated with increased plasma levels of both TC and TG, a phenotype classified as Type III Hyperlipidemia. Besides xanthomas, this condition most notably leads to a strongly increased risk of atherosclerosis in the coronaries as well as the peripheral vasculature.

**Treatment of apolipoprotein disorders**

The two main drug classes used to date to correct abnormal plasma lipid levels are statins and fibrates. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase, a key enzyme in cholesterol synthesis. Inhibiting this enzyme results both in a decreased hepatic cholesterol secretion as well as in an up-regulation of the LDL receptor to maintain cellular cholesterol homeostasis. These effects cause marked reductions of plasma cholesterol levels. However, they only have very modest effects on HDL-cholesterol and TG levels, which render them ineffective to treat subjects with mutations in apolipoproteins As, Bs and Cs. Fibrates are amphipathic carboxylic acids that activate peroxisome proliferator-activated receptors (PPARs), resulting in altered gene expression of many key players in lipid metabolism such as increased expression of LPL, apoA-I and apoA-II and reduced expression of apoC-
III. As a result, these drugs primarily decrease plasma TG and increase HDL-cholesterol. Since fibrates act through the up-regulation of gene expression they are not effective in case of the presence of dysfunctional gene copies. Taken together, both drug classes are mostly ineffective in patients who suffer from complete apolipoprotein deficiencies.

**Gene therapy as a means to correct apolipoprotein deficiency**

Therapeutic options for complete apolipoprotein deficiency are thus limited to protein therapy or gene therapy. Although strategies of administering apolipoproteins (apoC-II and apoA-I or mimetic peptides thereof) have been shown to have beneficial effects, the fact that these patients need lifelong correction makes a strategy in which a single injection can give long-term expression of therapeutic proteins very attractive. Indeed, the delivery of functional DNA copies to cells can provide the body with the means to produce the encoded protein for a long time, possibly lifelong. The field of gene therapy is very diverse but most approaches make use of vectors such as viruses to deliver DNA copies encoding the desired therapeutic protein in somatic cells. Perhaps the most promising viral vectors to date are adeno-associated viral (AAV) vectors. Derived from a non-pathogenic virus and containing no viral genes as recombinant vectors, they have an excellent safety profile in experimental animals as well as in humans. The ongoing discovery of new AAV serotypes, the design of stronger and tissue-specific promoters, and the development of self-complementary vectors has recently, strongly increased both the obtainable expression levels as well as the safety of these vectors. Further attempts to reduce the impact of the capsid proteins on the host immune system make AAV vectors promising to be used for diverse clinical applications. These AAV features form the basis for the choice of AAV for gene delivery in this thesis which focuses on apolipoproteins involved in HDL and TG metabolism, i.e. apoA-I and apoA-V, respectively.
Apolipoprotein A-I as a pharmaceutical target to reduce atherosclerosis

ApoA-I is a 243 amino-acid protein which, after synthesis, is secreted from the liver and the intestine. ApoA-I is the main protein component of HDL (60-70%) and plays an important role in HDL biogenesis, stability and metabolism. It is critically involved in 3 steps of HDL-mediated RCT (facilitating ABCA1 mediated cholesterol efflux to HDL, stimulating HDL maturation by inducing LCAT-mediated cholesterol esterification and enabling hepatic HDL-cholesterol uptake by acting as a ligand for SR-B1). In addition, apoA-I has other athero-protective properties such as acting as anti-inflammatory and anti-oxidative agent, and by recruiting endothelial progenitor cells. Given its central role in HDL metabolism, apoA-I deficiency is invariably associated with strongly reduced HDL-cholesterol levels. Despite the strong epidemiological evidence of an inverse correlation between HDL-cholesterol levels and cardiovascular risk in general, the association of apoA-I deficiency with increased cardiovascular risk has proven difficult to demonstrate due to the limited number of carriers of apoA-I mutations. However, a recent study by Hovingh and co-workers shows the expected increase of cardiovascular risk in subjects carrying an apoA-I gene mutation. Conversely, the specific increase of apoA-I in mice increases HDL levels and leads to a reduction in atherosclerosis progression.

For individuals with deleterious apoA-I mutations, the introduction of functional apoA-I into the circulation represents one of the few methods to restore their atherogenic lipid profile. In particular heterozygote carriers of apoA-I mutations might benefit from such strategy in view of their severely increased risk of premature CAD combined with an ability to produce normal apoA-I. The latter property is very useful, because upon introduction of apoA-I, this molecule will not be recognized as a neo-antigen and thus prevents an immune response. Increasing apoA-I levels to increase HDL-cholesterol levels may also proof beneficial when considering the strong inverse association between HDL levels and risk of CAD in general. Since apoA-I represents the core protein of HDL metabolism, an increase of apoA-I generated in the liver (an endogenous production site) might be expected to result in an increase of functional, anti-atherogenic HDL.
Apolipoprotein A-V and its therapeutic potential in decreasing triglycerides

ApoA-V is the most recently discovered member of the of the APOA1/A4/C3/A5 gene cluster. Independently discovered in 2001 by 2 groups\textsuperscript{52,53}, it was found to be an important factor in TG regulation because overexpression of the human or murine APOA5 gene in mice resulted in a pronounced decrease of plasma TG\textsuperscript{20,52,54}, whereas mice lacking APOA5 displayed a 4-fold increase of plasma TG levels\textsuperscript{52,55}. Complete apoA-V deficiency in humans was also shown to be associated with strongly increased plasma TG levels\textsuperscript{29,31,56}. In addition, single nucleotide polymorphisms (SNPs) in the APOA5 gene have consistently been shown to be associated with increased plasma TG levels in the general population\textsuperscript{52,57-60}. In line with the strong positive association between plasma TG levels and risk of CAD\textsuperscript{61,62}, the alleles associated with elevated TG were also associated with an increased risk of CAD\textsuperscript{60,63-65}, although others could not demonstrate this association\textsuperscript{66-68}. With the association of plasma TG levels and risk of CAD in mind, apoAV may thus be an interesting therapeutic target, even beyond treatment of individuals with apoA-V deficiency.
Aim and outline of this thesis

The aim of this thesis was to develop an optimized gene therapeutic protocol (for eventual use in humans) to realize efficient and long-lasting expression of therapeutic proteins in the liver by means of AAV. It was decided to choose apoA-I as transgene of choice since this molecule could serve as a reporter as well as a potential therapeutic molecule. As a second protein with therapeutic potential, apoA-V was studied. The outline of these studies is given below:

Chapter 2 is a review which gives an overview of the reported gene therapeutic efforts to treat lipid disorders. In chapter 3 we describe the optimization of a gene therapeutic application using adeno-associated virus (AAV) to maximize expression of human (h) apoA-I in mice. Using the optimized AAV vector to drive hepatic hApoA-I expression, we subsequently set out to study the physiological effects of the use of this vector in the next two chapters. Chapter 4 examines whether the AAV-mediated expression of near-physiological levels of hApoA-I was sufficient to reduce atherosclerosis progression in LDLr-/- mice. In chapter 5 we describe an experiment in which we employed this AAV vector to drive hApoA-I expression in LDLr-/- mice having received a bone marrow transplantation with ABCA1-deficient cells to determine whether properties of apoA-I beyond cholesterol efflux could contribute to reducing atherosclerosis progression.

Chapters 6, 7 and 8 concern studies on apoA-V. Preceding the development of an AAV-application to express apoA-V, we first determined in chapter 6 whether apoA-V plasma levels were associated with increased risk of coronary artery disease (CAD) in a large prospective epidemiological study. Contrary to expectations, a positive correlation between plasma TG levels and apoA-V levels was found, which led us to examine this relationship closer in mouse models with variable TG levels in chapter 7. To study the impact of apoA-V mutations on lipid metabolism, we furthermore used adenovirus to overexpress human apoA-V variants that are associated with hypertriglyceridemia to examine their TG-lowering potential in chapter 8. Finally, in chapter 9, we reflect on our studies and translate the data into implications and recommendations for future studies.
References


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