1 General Introduction
Cardiovascular disease (CVD) is the first cause of death in the Western world and its prevalence is increasing in Eastern Europe and developing countries. Several factors such as diabetes, hypertension, cigarette smoking, obesity, and lipid abnormalities are associated with an increased risk of atherosclerosis development. The research described in this thesis was performed to gain more insight into lipid metabolism. Dietary triglycerides (TG) are absorbed by the intestine to end up in metabolically active tissues (e.g. heart or skeletal muscle) to serve as energy source or in adipose tissue for storage. Cholesterol is derived both from the diet and from de novo synthesis in the liver and is used for cellular membrane synthesis (most tissues) and as precursor for steroid hormones (produced by endocrine glands such as the adrenal cortex and the gonads) and bile acids (liver). For transport from the site of absorption or synthesis, via the blood circulation towards their destination, cholesterol and TG are packaged into lipoproteins. 

**1. Lipoproteins and Lipid Metabolism**

**1.1. Lipoprotein Classes**

Lipoproteins are complex, spherical particles that consist of a hydrophobic inner core of neutral lipids, which mainly consist of TG and cholesteryl esters (CE), and a polar outer shell, which is formed by proteins (e.g. apolipoproteins), cholesterol, and phospholipids (PL) (Fig. 1). Since lipoproteins constitute a heterogeneous population of particles, they are traditionally classified and separated according to their densities. The five main categories that are distinguished in human plasma are chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Table 1). These lipoprotein classes have individual functions in lipid metabolism as will be discussed in the next paragraphs and is schematically represented in figure 2.

**1.1.1. Chylomicron Metabolism**

Dietary lipids are absorbed in the intestinal lumen, and CE are packaged with TG into large, lipid-rich chylomicron particles, via microsomal triglyceride transfer protein (MTP)-mediated lipidation of apolipoprotein (apo)B-48. These particles, containing primarily apoB-48, apoAI, apoAII and apoAIV, are secreted into the lymph and subsequently enter the blood circulation. Here, the chylomicrons undergo several modifica-

![Figure 1. Schematic illustration of a lipoprotein particle. Reproduced with permission from http://www.peprotech.com. CE, cholesteryl esters; TG, triglycerides.](http://www.peprotech.com)
Table 1. Density, size, and composition of human plasma lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>Chylomicrons</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
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<tr>
<td>Density (g/ml)</td>
<td>&lt;0.95</td>
<td>0.95-1.006</td>
<td>1.006-1.019</td>
<td>1.019-1.063</td>
<td>1.063-1.210</td>
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<td>Diameter (nm)</td>
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<td>25-35</td>
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<td>Composition (% dry weight)</td>
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<td>Proteins</td>
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<td>TG</td>
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<td>IDL/VLDL</td>
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<td>Main function</td>
<td>Transport of exogenous TG and TC</td>
<td>Transport of endogenous TG</td>
<td>Transport of endogenous TG</td>
<td>Transport of endogenous TC</td>
<td>Reverse cholesterol transport</td>
</tr>
</tbody>
</table>

Table 1. Density, size, and composition of human plasma lipoproteins

Apo, apolipoproteins; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; TC, total cholesterol; PL, phospholipids. In this table only the main apolipoproteins are given. Modified from Wasan and Cassidy.210

Within the capillary beds of skeletal muscle, heart, and adipose tissue, their TG are hydrolyzed by the lipolytic action of lipoprotein lipase (LPL), thereby generating free fatty acids (FFA) that are used as energy source (muscle) or for storage (adipose tissue), respectively.

Upon entering the circulation, apoAIV rapidly dissociates from the chylomicrons. A small fraction of apoAIV becomes associated with HDL, while more than 90% of the apolipoprotein exists free in the circulation.5,6 Similarly, apoAI and apoAII are trans-
ferred to HDL, while the chylomicrons acquire apoCs (i.e. apoCI, apoCII, apoCIII) and apoE from circulating lipoproteins. Sequential delipidation steps, which causes depletion of the particles from TG and PL and enrichment in apolipoprotein such as apoE, result in the formation of chylomicron remnants.\textsuperscript{2,3} ApoE is a crucial factor for facilitating the subsequent rapid clearance of the remnants from the circulation by the liver via remnant receptors (e.g. low-density lipoprotein receptor [LDLr] and the LDLr-related protein [LRP]).\textsuperscript{7,8} The receptor-mediated internalisation of remnants may be preceded by the “secretion-recapture role” of apoE as was suggested by Ji et al.\textsuperscript{9} This process starts with the secretion of apoE by hepatocytes and its accumulation in the space of Disse. There, apoE binds to heparan sulfate proteoglycans (HSPG) that are abundantly present on the surface of hepatocytes. Remnants may initially be sequestered in the space of Disse by apoE-mediated binding to HSPG and subsequently be internalised via lipoprotein receptor-mediated clearance routes (i.e. LDLr and LRP). In addition, recent results suggest that, at least in mice, scavenger receptor class B type I (SR-BI) is involved in the hepatic uptake of chylomicron remnants, probably by functioning as an initial recognition site.\textsuperscript{10}

1.1.2. VLDL Metabolism

The liver, in its turn, generates VLDL particles, consisting of cholesterol (derived from internalized remnants or \textit{de novo} synthesis) and TG (derived from remnant uptake, plasma FFA uptake, or lipogenesis) via MTP-mediated lipidation of apoB, which are subsequently secreted into the circulation.\textsuperscript{2,3} Whereas VLDL in humans is formed by lipidation of apoB-100, mouse VLDL results from lipidation of both apoB-100 and apoB-48.\textsuperscript{11} Nascent VLDL mainly carries apoB and small amounts of apoCs, apoE and the recently identified apoAV.\textsuperscript{12} Similarly to chylomicrons, TG in VLDL are hydrolyzed by LPL, thereby generating FFA, while VLDL is processed into VLDL remnants (i.e. IDL). These particles become enriched in apoCs and apoE and will be partly cleared from the plasma via the hepatic remnant receptors (i.e. LDLr and LRP). The remainder are processed further, which leads to loss of TG, PL, apoCs and apoE from the particles. This results in the formation of the relatively CE-rich LDL particles, characterized by one apoB-100 molecule as the sole protein constituent.\textsuperscript{2,3}

ApoB-100 is a ligand for the LDLr. About 70% of the LDLr activity is concentrated in the liver, leading to the hepatic uptake of LDL. Part of the remaining LDL is taken up in extrahepatic tissues for maintenance of membrane integrity and for the production of steroids in steroidogenic tissues (e.g. adrenal cortex and gonads).\textsuperscript{13} Besides being crucial for cholesterol delivery to peripheral tissues, LDL also has adverse atherogenic properties. LDL can infiltrate the vascular wall, where subsequent modification of LDL in the vascular wall via e.g. oxidation causes activation of the endothelium and an inflammatory response. This process leads to recruitment of monocytes from the circulation, which enter the vascular wall, and can differentiate into macrophages. These macrophages scavenge modified LDL via scavenger receptors that include scavenger receptor A (SRA) and CD36, thereby accumulating lipids, mainly cholesterol, and become foam cells.\textsuperscript{14} Foam cell formation is considered to be the initial stage in atherosclerosis development.\textsuperscript{15}
1.1.3. HDL Metabolism - Reverse Cholesterol Transport
Steroidogenic tissues (i.e. adrenal cortex and gonads) and skin cells are able to degrade low levels of cholesterol, however other non-hepatic peripheral tissues do not have this ability. To maintain cholesterol homeostasis, excess cholesterol from extrahepatic tissues (e.g. lipid-laden macrophages within the arterial wall) can be transported back to the liver to be secreted into the bile as neutral sterols and bile acids. This process is termed reverse cholesterol transport (RCT) and crucially involves apoAI and HDL.

Free cholesterol and phospholipids from the macrophage are transported to lipid-poor apoAI via the ATP-binding cassette (ABC) A1 transporter, thereby forming nascent discoidal HDL (nHDL). Subsequently, the enzyme lecithin:cholesterol acyl transferase (LCAT) esterifies the free cholesterol, to generate cholesteryl esters and transform nascent HDL into larger, mature HDL particles (mHDL). ABCG1, ABCG4 and SR-BI then further facilitate the efflux of cholesterol from cells to the mature HDL.

HDL-CE may then be transported to the liver via two pathways. First, cholesteryl ester transfer protein (CETP), which is expressed in species such as humans, rabbits, and hamsters, but not in mice and rats, transfers CE from HDL towards apoB-containing lipoproteins (e.g. VLDL, LDL and IDL) that are subsequently cleared from the circulation to the liver. Second, HDL-CE can be selectively taken up via ABC transporters or via SR-BI. CETP may also be involved in the direct uptake of HDL-CE into the liver, since administration of the CETP-inhibitor torcetrapib to mice that were treated with an adenovirus expressing CETP did not completely normalize HDL-CE levels. Therefore, cell-associated CETP might be directly involved in the hepatic uptake of HDL-CE.

Cholesterol that has returned to the liver can be re-used for lipoprotein assembly or can be excreted. The major pathway for elimination of cholesterol, either as neutral sterol or as bile acid, is mediated by the ABC transporters G4, G5 and G8 and results in secretion into the bile.

HDL particles are anti-atherogenic, partly because of their role in RCT but also due to antioxidative, anti-inflammatory, antithrombotic, and antiapoptotic properties.

1.2. Lipoprotein Remodelling Proteins
The proteins involved in the remodelling of lipoproteins during their residence in the circulation, can be categorized in lipolytic enzymes (e.g. endothelial lipase [EL], hepatic lipase [HL] and LPL) and neutral lipid transfer proteins (e.g. phospholipid transfer protein [PLTP] and CETP). Two of these proteins, LPL and CETP, will be described in more detail. While LPL is crucially involved in triglyceride metabolism and appears to be a causal factor in obesity, CETP represents a main link between (V)LDL and HDL metabolism by facilitating neutral lipid exchange between these lipoproteins.

1.2.1. Lipoprotein Lipase
LPL is a glycoprotein belonging to the gene family of lipases, which includes HL, EL and pancreatic lipase. It is mainly synthesized in parenchymal cells of tissues that
utilize FFA for energy or storage purposes (e.g. cardiac and skeletal muscles, and white adipose tissue). The VLDLr functions as an intracellular chaperone protein that allows LPL to be secreted from the cells to the capillary endothelial surface, where it is anchored by interactions with HSPG. Dimeric LPL is activated by its co-factor apoCII, that is present on its substrates (e.g. VLDL, chylomicrons), to facilitate lipolysis (i.e. hydrolysis of TG within the lipoprotein core to generate glycerol and FFA). The FFA that are generated are subsequently taken up by underlying tissues either via passive diffusional uptake or via a protein-facilitated component (e.g. VLDLr, CD36 or FA translocase, FA transport protein).

The LPL-mediated lipolysis is a prerequisite for the chylomicron and VLDL particles to be removed from the circulation. The LPL-mediated hydrolysis of TG is influenced by several apolipoproteins. Both apoCII and high amounts of apoE inhibit the lipolysis, whereas recent data indicate that apoAV increases LPL activity either directly or indirectly via still unresolved mechanisms. Lipolytically active dimeric LPL can
dissociate into monomeric LPL, which results in reduced affinity for HSPG and allows LPL to dissociate from the vascular wall and to travel through the circulation bound to lipoproteins. It is proposed that, in addition to apoE, LPL can mediate the lipoprotein binding to HSPG, after which the complex is transferred to lipoprotein receptors. As such, LPL is involved in remnant clearance via both its lipolytic function in the hydrolysis of triglycerides and its non-enzymatic bridging function.44-46

Heterozygous LPL mutations that are associated with a reduction or loss of LPL activity (i.e. Asp9Asn, Gly188Glu) are associated with an increased risk for development of combined hyperlipidemia and atherosclerosis.47 Also patients with defects in the structure or production of apoCII display hypertriglyceridemia and are indistinguishable from patients with LPL deficiency.48 Mice that are deficient for LPL have extremely elevated triglyceride levels and die within the first day after birth, although the exact cause of death is still to be elucidated.49 Deficiency for FFA uptake by CD3650,51 and overexpression of LPL-inhibitor apoCIII52 also lead to hypertriglyceridemia in mice. Interestingly, human APOC1 transgenic mice also develop hypertriglyceridemia,53 but the effect of apoCI on LPL activity is unresolved as yet.

LPL activity appears a crucial determinant for the development of obesity. Mice in which LPL activity is reduced by deficiency for LPL, CD36, or the LPL-chaperone VLDLR, are protected against diet-induced obesity.49,51,54 Similarly, APOC1 transgenic mice are resistant to the development of diet-induced obesity.55 On the other hand, mice with increased LPL activity as caused by apoCIII-deficiency are more prone to develop diet-induced obesity.56

1.2.2. Cholesteryl Ester Transfer Protein
CETP is a glycoprotein that is mainly expressed by liver, spleen, and adipose tissue.57 Upon secretion into plasma, CETP becomes associated with all lipoprotein classes, but predominantly with HDL.58 CETP mediates the transfer of neutral lipids (i.e. TG and CE) between lipoproteins. This results in the net flux of CE from HDL towards apoB-containing lipoproteins (e.g. VLDL and LDL) in exchange for TG.59-61 In mice, which naturally lack CETP, the clearance of HDL-CE occurs almost exclusively via selective delivery to the liver via SR-BI.62,63 In rabbits, however, the clearance of HDL-CE from plasma is only partially mediated by the direct uptake of HDL-CE, as 25-70% is cleared after CETP-dependent transfer to apoB-containing lipoproteins.64,65 In humans, it has recently been shown that the CE output from plasma is even almost solely facilitated via apoB-containing lipoproteins, whereas selective and holo-particle uptake of HDL-CE by the liver could not be detected.66 This suggests that CETP-mediated CE transfer might constitute a major pathway in humans, with only a small contribution of selective HDL-CE uptake,66 which is in sheer contrast with the predominant involvement of SR-BI in selective uptake of HDL-CE in mice.

As a consequence of its role in the transfer of lipids, CETP is involved in the remodelling of lipoproteins just like lipases (e.g. LPL, HL, EL) and PLTP. The concerted actions of CETP, PLTP, and lipases facilitate the formation of small HDL particles67-69 that are involved in the cholesterol efflux from extrahepatic tissues, thereby increasing the RCT pathway. Subjects deficient for CETP have an increased proportion
of large HDL-particles that are enriched in CE and apoE.\textsuperscript{70-73} Thus, CETP may have anti-atherogenic properties by contributing to the remodelling of HDL, thereby facilitating the delivery of HDL-CE to the liver via apoB-containing lipoproteins.

However, by reducing anti-atherogenic HDL-cholesterol levels and increasing the cholesterol content in pro-atherogenic apoB-containing lipoproteins, CETP activity might be considered atherogenic. Indeed, genetic association studies in humans showed an association of the C629A promoter variant of CETP with higher CETP levels, lower HDL-cholesterol levels, and an increased progression of CVD.\textsuperscript{74} Likewise, increased CETP activity was associated with increased risk for CVD in subjects with elevated triglycerides.\textsuperscript{75} However, despite the fact that CETP deficiency is associated with increased levels of HDL cholesterol,\textsuperscript{71-73,76,77} increased CVD risk was found in studies of subjects with partial\textsuperscript{78} or complete\textsuperscript{79,80} loss of CETP. Since mice naturally lack the expression of CETP, transgenic mouse models that express human CETP have been used to gain more insight into the atherogenicity of CETP. Although studies in apoCIII-transgenic mice\textsuperscript{81} and LCAT-transgenic mice\textsuperscript{82} showed that human CETP expression reduced atherosclerosis, expression of CETP in mice that are hyperlipidemic because of attenuated hepatic uptake of apoE-containing lipoprotein remnants (i.e. apoE-deficient and LDLr-deficient mice) increased atherosclerosis.\textsuperscript{83}

Due to the pro- and anti-atherogenic properties of CETP and conflicting results from human and animal studies, the net effect of CETP on atherosclerosis development has been a subject of debate which has been extensively reviewed,\textsuperscript{61,84,85} but is still not clear.

1.3. Apolipoproteins

Apolipoproteins are generally composed of a series of class II amphipathic α-helices and play an important role in lipid metabolism by exerting various functions. They stabilize the lipoprotein particles (e.g. apoA, apoB, apoC), they serve as co-factors and modulators of enzymatic reactions (e.g. apoCs, apoAV), and they direct lipids to target organs by specific receptor interaction (e.g. apoA, apoB, apoE). The most abundant apolipoproteins, apoB-100\textsuperscript{86-89} and apoAI,\textsuperscript{88,90,91} have been extensively studied. The role of these apolipoproteins in lipoprotein metabolism are quite well-defined: apoB-100 regulates LDL metabolism by mediating the endocytosis of LDL by the LDL receptor, and apoAI is involved in HDL metabolism by mediating both the influx of cholesterol from peripheral cells into HDL and the efflux of cholesterol from HDL to the liver. However, the less abundant apolipoproteins apoCI, apoCIII, apoE, and apoAV seem to have overlapping functions in (V)LDL and HDL metabolism, as will be outlined below.

1.3.1. Apolipoprotein CI

The gene encoding for the smallest of the known apolipoproteins, \textit{APOC1}, is part of the \textit{APOE/APOC1/APOC2/APOC4} gene-cluster,\textsuperscript{93} located on chromosome 19 in humans.\textsuperscript{93-95} The gene is 4.7 kb in size and consists of 4 exons. \textit{APOC1} is primarily expressed by the liver, but is also found at low levels in lung, skin, spleen, adipose tissue, and brain.\textsuperscript{96} ApoCI is synthesized with a 26-residue signal peptide, which is cleaved cotranslationally in the rough endoplasmic reticulum, generating a mature protein of 57
amino acids. ApoCI consists of only two helices separated by a flexible hinge. The protein is subsequently secreted into the circulation where it is present at a high concentration of approximately 10 mg/dl, associated with chylomicrons, VLDL and HDL.

Up to now, no apoCI polymorphisms have been identified in humans that result in functional apoCI variants. However, an HpaI polymorphism in the promoter region has been described, which results in the increased expression of the APOC1 gene (+57%), and is associated with elevated TG. To study the function of apoCI in lipid metabolism, mice were generated either lacking endogenous apoCI or overexpressing the human APOC1 gene. Whereas apoCI deficient mice did not have an obvious plasma lipid phenotype, APOC1 overexpression markedly increased the levels of plasma TC and TG. The observation in humans that apoCI levels are positively associated with plasma TG levels may thus be explained by a causal effect of apoCI. The apoCI-induced hyperlipidemia in APOC1 mice has initially been explained by interference of apoCI with the apoE-dependent hepatic uptake of remnants by the LDLr and LRP, and by the inhibition of binding of apoE-containing lipoproteins to the VLDLr as present on peripheral cells, thereby impeding remnant clearance. However, APOC1 mice show predominantly hypertriglyceridemia rather than hypercholesterolemia, which is not consistent with either of these proposed effects of apoCI. In addition, in vitro studies showed that apoCI is involved in the partial activation of LCAT, the inhibition of LPL, and the inhibition of HL. Most likely, apoCI interferes with the lipolytic function of LPL, thereby inducing hypertriglyceridemia and protecting against diet-induced obesity, but such an effect has not been shown yet in vivo.

Furthermore, human apoCI and the N-terminal fragment of baboon apoCI have been reported to inhibit CETP in vitro. Indeed, deficiency of apoCI in CETP transgenic mice resulted in increased CETP activity as compared to control mice. Similarly, human APOA1 overexpression in CETP transgenic rats leads to an increase in CETP activity possibly related to a reduction of the amount of apoCI associated with HDL.

1.3.2. Apolipoprotein CIII
The APOC3 gene is located in the APOA1/APOC3/APOA4/APOA5 gene-cluster located on chromosome 11 in humans and is primarily expressed in liver and intestine. After removal of the 20-amino acid signal peptide, the mature apoCIII protein (79 amino acids; 8.8 kDa) is secreted into the circulation where it is associated with chylomicrons, VLDL and HDL, and has a concentration of approximately 12 mg/dl.

ApoCIII plasma levels are positively correlated with plasma TG concentration. Indeed, human APOC3 transgenic mice display severely increased TG levels, whereas a reduction was observed in apoc3 deficient mice. Although a direct function on receptors has been postulated, the hypertriglyceridemia is most likely explained by the strong LPL inhibitory action of apoCIII that was demonstrated both in vitro and in vivo.

1.3.3. Apolipoprotein AV
In 2001, a novel apolipoprotein has been discovered independently in two research groups. Comparative sequence analysis of the mammalian APOA1/APOC3/APOC4
gene cluster between humans and mice identified a highly homologous sequence encoding an additional apolipoprotein, which was named apoAV. At the same time, apoAV was discovered as a protein that is highly upregulated in rats after a 70% hepatectomy. ApoAV is synthesized exclusively in the liver. After cleavage of its signal peptide, the 39 kDa mature protein (343 amino acids) is secreted into the circulation where it is present at low concentrations (approx. 125-180 ng/ml), mainly bound to chylomicrons, VLDL and HDL.

Apoa5 deficient mice displayed 4-fold increased plasma TG levels, whereas overexpression of human APOA5 in mice reduced TG by 65%. In addition, adenoviral expression of murine apoa5 dose-dependently decreased plasma TG up to 70%. In vitro studies suggested that the hepatic VLDL production may be decreased, which was confirmed in vivo. Strong evidence was found that apoAV facilitates the clearance of TG from the plasma by increasing the LPL-mediated TG hydrolysis, although it is not sure whether apoAV acts via direct activation of LPL or via an indirect pathway. Several mechanisms by which apoAV might enhance lipolysis have been postulated. 1) Since apoAV was shown to increase the binding of lipoprotein particles to HSPG, apoAV may bring the particles in close proximity of LPL thereby enhancing lipolysis. 2) Since apoAV is very hydrophobic, it penetrates more deeply into the lipoprotein particle than other lipoproteins, thereby potentially facilitating the access of LPL to TG, which are present in the core of the lipoprotein. Indeed, apoAV was shown to increase the LPL-mediated lipolysis in the absence of HSPG in vitro, although it should be noted that these effects were only obtained at high apoAV concentrations. 3) ApoAV might interact directly with LPL, thereby enhancing enzymatic activity, for example by stabilizing the LPL dimer. This is supported by the findings that both apoa5 deficient mice and humans that lack apoAV due to a mutation in apoAV that generates a truncated protein devoid of key functional domains have reduced levels of post-heparin LPL levels. So far, no experiments have been performed to address this possibility.

Apart from its role in TG metabolism, apoAV may also play a role in HDL metabolism because apoa5 deficient mice displayed increased HDL-cholesterol, whereas adenoviral APOA5 overexpression in mice dose-dependently reduced HDL-cholesterol levels. Further studies will be needed to resolve the mechanism(s) underlying the effect on HDL.

### 1.3.4. Apolipoprotein E

Just like apoCI, human APOE is part of the APOE/APOC1/APOC2/APOC4 gene-cluster, located on chromosome 19, and is mainly expressed in liver, macrophages and brain. After co-translational cleavage of an 18-amino acid signal peptide, the 299-amino acid mature protein circulates at plasma levels of approximately 2.5-5 mg/dl as a constituent of chylomicrons, VLDL, IDL and HDL.

High amounts of apoE have been shown to inhibit LPL-mediated lipolysis, thus resulting in increased plasma levels of triglycerides. However, physiological expression of apoE is crucial for mediating the binding and uptake of remnant particles via the LDLr, LRP and the VLDLr, thereby playing an important role in lipoprotein
metabolism. In addition, apoE has been suggested to be involved in the secretion-recapture process as was discussed in 1.1.1.

The human APOE gene is polymorphic, with three common alleles (E*2, E*3 and E*4) of which E*3 is the most frequent allele (70-85%). These isoforms differ with respect to the association with lipoproteins, binding affinity for the LDLr and interaction with HSPG.

ApoE*2 displays only about 1% of the binding affinity of apoE*3 to the LDLr. As a consequence, APOE*2 knockin mice display elevated levels of plasma TG and cholesterol. Simultaneously, apoE*2 accumulates in plasma, resulting in an increase in the apoE-mediated inhibition of LPL-mediated lipolysis, via reducing the interaction of particles with HSPG-bound LPL. Whereas in humans most of the homozygous APOE*2 carriers are normolipidemic, only about 10% develops type III hyperlipidemia. On the other hand, most of the subjects with type III hyperlipidemia are APOE*2 homozygotes. This indicates that certain additional factors are needed to develop the hyperlipidemia. The apoAV S19W polymorphism may constitute one of these factors. In this polymorphism, apoAV has a substitution at residue 19 in its predicted signal sequence, which was by in vitro experiments suggested to be required for the translocation across the endoplasmic reticulum membrane and the subsequent secretion into the circulation. In initial studies, the S19W rare allele has been associated with higher plasma TG levels. Strikingly, in a pilot study 6 out of 7 hypertriglyceridemic APOE*2/E*2 carriers the S19W polymorphism was found, which was later confirmed in a larger population, albeit that the effects were less dramatic (i.e. 53% in the APOE*2/E*2 carriers vs. 20% in the controls). These findings suggest that apoAV may be used to reverse type III hyperlipidemia in APOE*2/E*2 subjects, which will require further investigation.

Apart from the three common isoforms of apoE, several other rare variants are known, which are often associated with lipid disorders. For example, the APOE*3-Leiden mutation has a 7-amino acid tandem repeat outside the binding domain. However, the conformation of the receptor-binding domain is dramatically changed by the large insertion, leading to a binding defect of the protein to the LDLr (20-40% of apoE*3) and HSPG. APOE*3-Leiden has been implicated in Familial Dysbeta-lipoproteinemia (FD), which is characterized by accumulation of chylomicron and VLDL remnants.

To study the direct effect of the APOE*3-Leiden mutation on lipoprotein metabolism, mice have been generated that express the APOE*3-Leiden gene as well as the adjacent APOC1 gene. It appeared that the mice developed a moderate dyslipidemia, as explained by a reduced VLDL turnover. Because VLDL turnover was reduced, leading to the accumulation of apoB-containing lipoproteins, the lipoprotein profile of APOE*3-Leiden mice appeared to resemble that of humans. In addition, plasma cholesterol in these mice can be titrated by adjustment of dietary cholesterol intake and there is a clear relationship between plasma cholesterol and atherosclerosis severity. APOE*3-Leiden mice respond to lipid lowering therapies such as statins and fibrates. Despite these properties, the major difference in lipoprotein metabolism between mice and humans is the absence of CETP in mice. Since CETP is important in the cross-talk
between HDL and (V)LDL metabolism, expression of CETP in APOE*3-Leiden mice might enable us to study HDL metabolism in a model that resembles the human situation even more.

2. Lipid Modulating Therapies

To improve atherogenic lipoprotein profiles, thereby aiming to reduce cardiovascular risk, several classes of lipid-modifying drugs are available. These include bile acid-binding resins (e.g. cholestyramine, colestipol, colesevalam), which bind bile acids in the gastrointestinal tract to prevent re-uptake, cholesterol-absorption inhibitors (e.g. ezetimibe), fibrates (e.g. fenofibrate, clofibrate, gemfibrozil, bezafibrate) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (e.g. atorvastatin, rosuvastatin, simvastatin). The most widely prescribed drugs are the statins and fibrates, which will be discussed in more detail in the following paragraphs.

2.1. Statins

Statins are inhibitors of HMG-CoA reductase, the rate-determining enzyme in hepatic cholesterol biosynthesis, which converts HMG-CoA into the cholesterol-precursor mevalonate. Initially, semisynthetic fungal derivatives were used as HMG-CoA reductase inhibitors (e.g. simvastatin, pravastatin). Later, synthetic statins were designed (e.g. rosuvastatin, atorvastatin). The members in this class of drugs share a similar structure resembling the HMG-moiety that plays an important role in the inhibition of the enzyme. In the liver, the lowering of cholesterol biosynthesis results in depletion of hepatic intracellular cholesterol. To compensate for the depletion in intracellular cholesterol, LDLr receptor expression levels are increased to facilitate the uptake of plasma LDL and its precursors VLDL and IDL. At the same time, hepatic apoB-100 production is decreased, thereby reducing the synthesis and secretion of VLDL. These statin-mediated actions result in a reduction in plasma LDL-cholesterol, which in hypertriglyceridemic subjects often are accompanied with reductions in TG levels.

Indeed, a meta-analysis of 25 studies enrolling nearly 70,000 subjects with CVD indicated that statins reduce LDL-cholesterol levels up to 40%, which was associated with a reduction of CVD mortality by 23%. Apart from the effects on LDL-cholesterol, statins also decreased plasma TG and increased plasma HDL-cholesterol by about 5-15%. Although clinical studies indicate that a reduction in CETP activity may be involved in the statin-induced increase in HDL, this has not been firmly established as a causal factor yet. In addition, statins have been reported to exert a wide range of pleiotropic effects that may contribute to their beneficial actions. These include vasodilation, plaque stabilization, and antithrombotic, antioxidant, anti-proliferative and anti-inflammatory actions.

2.2. Fibrates

By mimicking the structure and function of FFA, fibrates are peroxisome proliferator-activated receptor α (PPARα) agonists. PPARα is mainly expressed in the
liver, and upon activation it translocates from the cytoplasm to the nucleus, where it heterodimerizes with retinoid X receptor (RXR). This complex subsequently binds to specific peroxisome proliferator response elements (PPREs) in a range of target genes, thereby either activating or repressing their transcription.

The hypotriglyceridemic effect of fibrates is based on increased lipolysis of TG-rich lipoproteins in plasma via a PPARα-mediated increase in hepatic LPL expression and decrease in hepatic apoCIII expression. Indeed, fibrate administration is associated with increased plasma LPL activity levels and a reduction in apoCIII synthesis in humans. In addition, fibrates reduce VLDL-TG production through enhanced FFA catabolism (via an increase in genes of the β-oxidation pathway) and reduced FFA production (via downregulation of acetyl CoA carboxylase).

Although the role of plasma TG as CVD risk factor has long been controversial, strong evidence has been provided that elevated TG levels indeed are correlated with increased risk. This is illustrated by a meta-analysis of 17 population-based, prospective studies, enrolling 46,413 men and 10,864 women, where TG were found to be an independent risk factor for CVD. In subsequent years this has been confirmed in the Prospective Cardiovascular Münster (PRO-CAM) study and the Copenhagen Male study, both prospective studies that only included men without evidence of myocardial infarction at entry.

Fibrates are widely used to reduce hypertriglyceridemia, thereby reaching TG reductions, indicating lower levels of VLDL that exceed 50% in subjects with hypertriglyceridemia. In hypercholesterolemic patients TG reductions are generally less than 30%. Although it appears that the extent of TG-lowering by fibrates is dependent on the lipid phenotype and the fibrate used, a meta-analysis with data from 53 trials (16,802 subjects), including subjects with hypercholesterolemia, hypertriglyceridemia, type II diabetes mellitus and combined hyperlipidemia, clearly demonstrated a 36% reduction in TG. In addition to TG-reduction, fibrates exert a spectrum of lipid modulating actions, involving reduction of cholesterol in VLDL, VLDL remnants, IDL and LDL, and elevation of HDL-cholesterol by approximately 10%. Except for their lipid-modulating effects, fibrates also exert pleiotropic anti-inflammatory effects by downregulating the expression of genes encoding inflammatory cytokines and acute phase response proteins, as was recently reviewed. Altogether, the previously mentioned meta-analysis showed that fibrate administration is associated with a CVD risk reduction of 25%.

The mechanism underlying the fibrate-induced increase in HDL-cholesterol has been intensively studied. In contrast to humans, fibrates do not affect or even decrease HDL-cholesterol levels in mice. This effect may be attributed to the fact that, in contrast to the human APOA1 promoter, which contains a functional positive PPRE leading to increase APOA1 transcription, the murine apoA1 promoter contains a non-functional PPRE. However, since not all clinical studies show an increase in apoA1 plasma levels, it is likely that fibrates may increase HDL-cholesterol via apoA1-independent mechanisms. As was mentioned previously, a major difference between humans and mice is that mice do not express CETP. Whether the fibrate-induced increase in HDL-cholesterol depends on CETP expression has not yet been investigated.
3. Outline of this Thesis

Since lipid homeostasis presents an important factor in determining CVD risk, a thorough understanding of lipid metabolism is required to optimize lipid-lowering therapies. In the past decades this knowledge has increased tremendously, especially by the development of mice deficient or transgenic with respect to proteins in plasma lipid metabolism. In this thesis we aimed to expand our knowledge of the roles of apoCI, LPL and CETP in lipid metabolism, by performing studies using a combination of genetically modified (i.e. transgenic and knockout) mice, adenovirus-mediated gene expression, and in vitro studies.

Data obtained from studies in humans and mice have shown that plasma apoCI protein levels are positively correlated with combined hyperlipidemia, displaying the most pronounced effect on TG levels. Thus far, the apoCI-induced hyperlipidemia in APOC1 mice has been explained by mainly inhibition of receptor-mediated remnant clearance, but this does not satisfactorily explain the predominant hypertriglyceridemia. In chapter 2, human APOC1 expressing mice were used to elucidate the main underlying cause. We showed that the hypertriglyceridemia was predominantly caused by impaired LPL-mediated lipolytic conversion of VLDL-TG. Therefore, we further studied whether apoCI inhibits the LPL-mediated lipolysis via interaction with the VLDLr or with apoCIII in chapter 3. Our results showed that apoCI is a powerful inhibitor of LPL activity in vivo, independent of the VLDLr and apoCIII.

Chapter 4 focuses on the role of LPL in the apoE-receptor independent VLDL remnant clearance. This was studied by adenoviral-mediated gene transfer of human APOC1 and human LPL into mice that lack the three main apoE-recognizing receptors, i.e. LDLr, the VLDLr and hepatic LRP. We found that also in the absence of these receptors the remnant clearance depends on LPL. In chapter 5, we addressed whether increasing LPL activity can lead to reduction of the combined hyperlipidemia in APOE*2-knockin mice. These studies revealed that the hyperlipidemia in these mice can be ameliorated by a direct increase in LPL activity and by overexpression of apoAV, but not by deletion of apoCIII.

Since a major difference between mice and men regarding lipid metabolism is the expression of CETP and the atherogenicity of CETP is still under debate, we studied in chapter 6 the effect of human CETP expression in APOE*3-Leiden mice with a human-like distribution of cholesterol over lipoprotein particles. These results showed that CETP expression has a major impact on the cholesterol distribution between lipoproteins and represents a clear pro-atherogenic factor in these mice. Since both statins and fenofibrate increase HDL-cholesterol levels in humans, whereas this is not observed in mice, we addressed the hypothesis that this increase in HDL-cholesterol could depend on CETP in chapter 7 and chapter 8. Therefore, mice on an APOE*3-Leiden background with or without CETP expression received a cholesterol-rich diet with or without fenofibrate or atorvastatin. Indeed, the mice with CETP displayed an increase in HDL-cholesterol, that was absent in the control mice, and could not be explained by differences in other genes involved in HDL-metabolism.


Chapter 9 discusses the results of these studies, together with the future perspectives.

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Chapter 1


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