



Universiteit
Leiden
The Netherlands

The role of apolipoprotein CI in lipid metabolism and bacterial sepsis

Berbée, J.F.P.

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Chapter 1

General Introduction

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Contents General Introduction

1. Lipid Metabolism	11
1.1. <i>Exogenous Pathway</i>	12
1.2. <i>Endogenous Pathway</i>	13
1.3. <i>Reverse Cholesterol Pathway</i>	13
2. Role of ApoE and ApoCI in TG-rich Lipoprotein Metabolism	14
2.1. <i>Synthesis, Structure, and Function of ApoE</i>	14
2.1.1. Synthesis and Structure of ApoE	14
2.1.2. Polymorphisms of ApoE	15
2.1.3. Functions of ApoE	16
2.2. <i>Synthesis, Structure, and Function of ApoCI</i>	17
2.2.1. Synthesis and Structure of ApoCI	17
2.2.2. Polymorphisms of ApoCI	18
2.2.3. Functions of ApoCI	19
2.3. <i>Role of ApoE and ApoCI in TG-rich Lipoprotein Processing</i>	19
2.3.1. Lipoprotein Lipase	20
2.3.2. Hepatic Lipase	21
2.3.3. TG-rich Lipoprotein Uptake Mechanisms	21
3. Role of ApoE and apoCI in Atherosclerosis	24
3.1. <i>The Pathogenesis of Atherosclerosis</i>	24
3.2. <i>ApoE in Atherosclerosis</i>	26
3.3. <i>ApoCI in atherosclerosis</i>	28
4. Role of ApoE and Other Apolipoproteins in Inflammation and Sepsis	29
4.1. <i>Infection, Sepsis, and Lipopolysaccharide</i>	29
4.2. <i>Host Response to Lipopolysaccharide</i>	32
4.3. <i>Lipid Metabolism in Inflammation and Sepsis</i>	33
4.4. <i>Lipoproteins and the Lipopolysaccharide Response</i>	34
4.4.1. Lipoprotein Constituents and the Lipopolysaccharide Response	36
4.4.2. ApoE and the Lipopolysaccharide Response	36
4.4.3. Other Apolipoproteins and the Lipopolysaccharide Response	38
5. Outline of this Thesis	39
6. References	40

Cardiovascular disease (CVD) is the primary cause of death in the Western world, accounting for up to 50% of all mortalities¹⁻³. Atherosclerosis is the main cause of CVD, and is considered a chronic inflammatory disease, characterized by the focal accumulation of cells, fibrous tissue, lipids and inflammatory blood constituents in the vessel wall^{4,5}. This results in narrowing of the arteries, which may subsequently cause clinical manifestations such as myocardial infarction and stroke. Several risk factors have been identified, such as dietary habits, age, gender, smoking, hypertension, stress, and physical inactivity^{3,6,7}.

Sepsis, another inflammatory disease, occurs when a subject is unable to successfully contain an infection with microorganisms. This uncontrolled infection will lead to an exaggerated inflammatory response by the host, with organ failure and finally septic shock or death as a result. A full panel of microorganisms, as bacteria, parasites, fungi, and viruses, can trigger the pathophysiological cascade leading to sepsis. Sepsis is the leading cause of death in medical and surgical intensive care units with mortality rates ranging from 15-80% in critically ill patients^{8,9}, and the incidence is still increasing, despite the development of new supportive therapies^{10,11}.

Atherosclerosis and sepsis are related to each other, in that in both diseases the immune system plays a central role. In both diseases the immune system serves initially as a protective factor, but in the same manner may initiate damaging processes. In atherosclerosis, it is a critical player in the repair of damaged tissues, whereas at the same time the atherosclerotic lesion develops. During infection, the immune system is critical to combat the infection, but is getting harmful when the infection cannot be contained and progresses into sepsis.

Several apolipoproteins, which are proteins on circulating lipid sphericals in the bloodstream, have been shown to be potent modulators of inflammatory processes¹²⁻¹⁷. The function of apolipoproteins in lipid metabolism, atherosclerosis, and sepsis will be outlined in more detail in this introduction.

1. Lipid Metabolism

Cholesterol and triglycerides (TG), the most common lipids of a diet, are of vital importance in many different cellular processes in the human body. Cholesterol is essential for biosynthesis of cellular membranes, steroid hormones, and bile acids. TG-derived free fatty acids (FFA) can be used as an energy source in cardiac and skeletal muscle or they can be stored in adipose tissue. Since cholesterol and TG are hydrophobic, they are packaged into water-soluble spherical particles for their transport in the circulation. These spherical particles

are composed of a lipid-rich core containing hydrophobic cholesteryl esters and TG surrounded by a polar surface monolayer of phospholipids, unesterified free cholesterol, and one or more amphipathic proteins termed apolipoproteins. These apolipoproteins facilitate the formation of lipoproteins, modulate the activity of enzymes and lipid transfer factors involved in lipoprotein remodelling in the circulation, and modulate receptor-mediated binding and endocytosis of lipoproteins and/or their remnants.

1.1. Exogenous Pathway

Dietary TG and cholesteryl esters that are absorbed in the intestine are packaged into chylomicrons, and are transported from the lymph to the blood circulation²³. Nascent chylomicrons are very large particles that consist mainly of TG but

Table 1. Physical properties, lipid and apolipoprotein composition of human plasma lipoproteins.

Properties	Chylomicron	VLDL	IDL	LDL	HDL
Source	Intestine	Liver	VLDL	VLDL	Liver+intestine
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Density (g/mL)	<0.96	0.96-1.006	1.006-1.019	1.019-1.063	1.063-1.210
Electrophoretic Mobility*	Origin	pre- β	slow pre- β	β	α
Composition**					
<i>Triglycerides</i>	87	54	27	11	10
<i>Phospholipids</i>	8	19	23	23	31
<i>Cholesteryl esters</i>	3	14	32	40	21
<i>Free cholesterol</i>	1	7	8	8	7
<i>Protein</i>	1	6	10	18	31
Apolipoproteins					
<i>ApoA</i>	AI, AII, AIV, AV	AV	-	-	AI, AII, AIV, AV
<i>ApoB</i>	B48	B100	B100	B100	-
<i>ApoC</i>	CI, CII, CIII, CIV	CI, CII, CIII, CIV	CI, CII, CIII, CIV	-	CI, CII, CIII, CIV
<i>ApoE</i>	E	E	E	-	E
Main function	Transport of exogenous cholesterol and TG	Transport of endogenous TG	Cholesterol transport to peripheral tissues	Cholesterol transport to peripheral tissues	Reverse cholesterol transport to liver

* According to electrophoretic mobility of plasma α - and β -globulins on agarose gel electrophoresis.

** Expressed as percentage of total weight.

Apo, apolipoproteins and TG, triglycerides. Modified from Gotto *et al.*¹⁸.

also phospholipids, cholesterol, cholesteryl esters, and apolipoproteins (e.g. apoAI, apoAIV, apoB48, and apoCs) (Table 1). Upon entering the circulation, these chylomicrons are processed by lipoprotein lipase (LPL), thereby delivering FFA to peripheral tissues such as adipose tissue (for storage into TG), and skeletal muscle and heart (as energy source). The resulting cholesterol-enriched core remnants are subsequently taken up mainly via apoE-specific recognition sites on hepatocytes, including the LDL receptor (LDLr), LDLr-related protein (LRP), heparan sulphate proteoglycans (HSPG), and possibly also Scavenger receptor BI (SR-BI)²⁴ (**Figure 1**).

1.2. Endogenous Pathway

Hepatocytes secrete cholesterol and TG packaged into VLDL. These lipids are either derived from incoming chylomicron remnants, IDL, LDL, and HDL, or from *de novo* synthesis^{25,26}. The formation of VLDL is described as a two-step process^{27,28}. In the first step, apoB100, the major structural apolipoprotein of VLDL, associates with the core lipids during formation of the particle. The microsomal TG transfer protein (MTP) catalyzes the transfer of lipids toward apoB100 and is in this way an essential link in the assembly of VLDL^{29,30}. Thereafter, the particle fuses with a lipid droplet to become a mature VLDL particle, which can be secreted into the blood^{27,31}. Nascent VLDL consists of TG, phospholipids, cholesteryl esters, cholesterol, and apolipoproteins (e.g. apoB100 and apoE) (**Table 1**). Upon entering the circulation the particle is further enriched with apoE and apoCs. These TG-rich VLDL particles serve, similarly as chylomicrons, as a source of FFA for extrahepatic tissues predominantly under fasting conditions. Hydrolysis of VLDL-TG by LPL results in the formation of IDL, which is partly taken up by the liver as mediated by apoE³². The remainder is extensively processed by LPL and hepatic lipase (HL) to become cholesterol-rich LDL with apoB100 as its sole apolipoprotein, which is recognized by the LDLr on the liver and peripheral tissues³² (**Figure 1**).

1.3. Reverse Cholesterol Pathway

To maintain cholesterol homeostasis, excess cholesterol in extrahepatic tissues is returned via HDL to the liver, which is classically known as the only organ capable of disposing cholesterol via the bile²². However, recent findings suggest that cholesterol is also secreted from the circulation directly into the intestine without the involvement of the liver (Groen AK, unpublished observations). In the liver and intestine, nascent discoidal HDL (HDL₃) is formed from apoAI and phospholipids³³. In the blood, discoidal HDL matures into spherical HDL (HDL₁) by acquisition of phospholipids from chylomicrons and VLDL via phospholipid transfer protein (PLTP), and cholesterol from the liver and peripheral tissues via

ATP binding cassette transporter AI (ABCA1), SR-BI, and ABCG1. The cholesterol is subsequently esterified by lecithin:cholesterol acyltransferase (LCAT) into cholesteryl esters, which can then be taken up by the liver, either directly via SR-BI³⁴, or indirectly via the LDLr, LRP, and/or HSPGs after transfer to VLDL and LDL in exchange for TG by the cholesteryl ester transfer protein (CETP)^{35,36} (Figure 1). It is important to note that rodents normally do not express CETP³⁷, and therefore in these species there is no bidirectional exchange of cholesteryl esters and TG between HDL and (V)LDL.

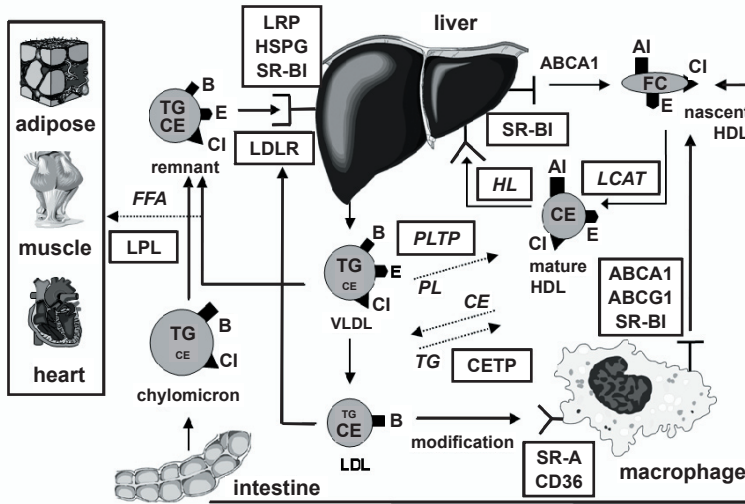


Figure 1. Lipoprotein metabolism. See text for explanation. AI, apolipoprotein AI; ABCA1/ABCG1, ATP-binding cassette transporter AI or GI; B, apolipoprotein B; CI, apolipoprotein CI; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; E, apolipoprotein E; FC, free cholesterol; FFA, free fatty acids; HSPG, heparan sulphate proteoglycans; LCAT, lecithin:cholesterol acyltransferase; LDLR, LDL receptor; LRP, LDL receptor-related protein; PL, phospholipids; PLTP, phospholipid transfer protein; SRA, scavenger receptor class A; SR-BI, scavenger receptor class B type I; TG, triglycerides. Modified from Berbée *et al.*³⁸.

2. Role of ApoE and ApoCI in TG-rich Lipoprotein Metabolism

2.1. Synthesis, Structure, and Function of ApoE

In 1973, Shore and Shore³⁹ identified apoE as a component of TG-enriched VLDL with a relatively high arginine content compared to other apolipoproteins known at that time, and referred to this protein as ‘arginine-rich protein’. Consistent with the nomenclature of the other known apolipoproteins (apoA, apoB, apoC, and apoD) Utermann suggested the designation ‘apoE’ in 1975⁴⁰.

2.1.1. Synthesis and Structure of ApoE

The *APOE* gene, located on human chromosome 19 in the *APOE/APOC1/APOC4/APOC2* gene cluster, is 3.7 kb in length and contains four exons and three

introns⁴¹. The primary product of the *APOE* gene is a 317 amino acid prepeptide that gives rise to the 299 amino acid mature protein by cotranslational cleavage of an 18-amino acid signal peptide⁴². This 34.2 kDa apoE protein is synthesized in most organs, including the liver, spleen, lung, adrenal, ovary, kidney, and muscle, primarily by macrophages and in the liver also by hepatocytes^{43,44}. ApoE is not expressed in the intestine. In the circulation apoE is mainly present on chylomicrons, VLDL, and HDL (**Table 1**) at total plasma levels of about 4-7 mg/dL^{45,46}. The mouse *apoe* gene encodes a 285 amino acid mature protein, which has 70% homology with the human apoE protein⁴⁷. Prediction of the secondary structure using the rules of Chou and Fasman⁴⁸, showed that the predicted structures of human and mice apoE are nearly identical with α -helical regions comprising two-thirds of the protein in 14 areas, and β -sheets comprising ~10% of the protein in three areas⁴⁷.

In the absence of lipids, apoE self-associates as a tetramer over a wide concentration range^{50,51}. In contrast, self-association does not occur on lipid surfaces⁵². ApoE contains two domains that are joined by a protease-susceptible hinge region^{50,53}. Thrombin digestion of apoE yields two fragments of which the 10 kDa C-terminal fragment harbours the lipid-binding domain, whereas the LDLr-binding domain (residues 139-153) is situated in the 22-kDa N-terminal fragment⁵⁴⁻⁵⁶ (**Figure 2**). The C-terminal fragment contains three predicted α -helical regions, of which the third (amino acids 268-289) is critical for tetramer formation as well as lipoprotein association⁵⁷. The N-terminal domain contains an antiparallel four-helix bundle, with the LDLr-binding domain is located in helix 4. The unusually high content of basic amino acids (Arg, Lys, His) within this LDLr-binding domain is important for binding to the LDLr, as has been demonstrated by chemical modifications^{58,59}. Furthermore, apoE contains two heparin-binding sites of which one is located within the LDLr-binding site⁶⁰. ApoE also interacts with HSPGs, which are suggested to be involved in both the secretion of apoE by hepatocytes and macrophages, as well as in the binding of lipoprotein-bound apoE⁶¹.

2.1.2. Polymorphisms of ApoE

APOE has three common alleles known as $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ ⁶². This polymorphism results in six genotypes, three heterozygote ($\epsilon 2/\epsilon 3$, $\epsilon 3/\epsilon 4$, $\epsilon 2/\epsilon 4$) and three homozygote ($\epsilon 2/\epsilon 2$, $\epsilon 3/\epsilon 3$, $\epsilon 4/\epsilon 4$). In a typical Caucasian population, the frequency of the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles are approximately 8%, 80%, and 12%, respectively^{46,63}. The encoded isoforms are distinguished from each other at two polymorphic sites: apoE2 (Arg¹¹², Arg¹⁵⁸), apoE3 (Cys¹¹², Arg¹⁵⁸), and apoE4 (Cys¹¹², Cys¹⁵⁸)^{62,64} (**Figure 2**). These isoforms differ in terms of their association with the various lipoproteins^{65,66} and binding affinity for cell surface receptors

(e.g. LDLr)⁶⁷⁻⁷⁰ and cell surface binding sites (e.g. HSPGs)^{71,72}. While apoE3 and apoE2 are preferentially located on HDL, apoE4 preferentially interacts with large lipoproteins such as VLDL^{65,66}. Furthermore, apoE2 exhibits lower affinity for the LDLr as compared to apoE3⁶⁷⁻⁷⁰, resulting in dramatically reduced clearance of apoE and higher plasma apoE levels^{46,63,73-75}. It is suggested that as a response the liver up-regulates the LDLr, resulting in lower cholesterol levels. Conversely, apoE4 is cleared more efficiently than apoE3, resulting in lower apoE levels, and concomitantly higher cholesterol levels^{46,63,75}.

Next to these common $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles, several rare *APOE* variants have been reported (reviewed by Greenow *et al.*⁷⁶). Most of these mutations are associated with hyperlipidemia (e.g. hypertriglyceridemia, hypercholesterolemia, type III hyperlipoproteinemia) as a result of defective LDLr binding⁷⁷⁻⁸² or as a result of apoE-deficiency⁸³⁻⁸⁵.

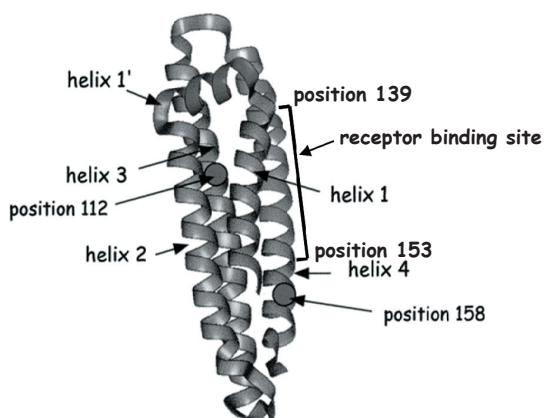


Figure 2. Ribbon model of the antiparallel N-terminal four-helix bundle of apoE. Highlighted are positions 112 and 158, which are either Arg or Cys residues in apoE2 (Arg¹¹², Arg¹⁵⁸), E3 (Cys¹¹², Arg¹⁵⁸), and E4 (Cys¹¹², Cys¹⁵⁸) isoforms (see text). The receptor-binding domain is located in helix 4 (residues 139-153). A short helix, helix 1', links helices 1 and 2. Adapted and modified from Weers *et al.*⁴⁹.

2.1.3. Functions of ApoE

ApoE is one of the most extensively studied apolipoproteins and appears to have numerous functions. The major role of apoE is the transport of (dietary) lipids within the blood circulation and determining the receptor-mediated uptake of these lipids as discussed above, and will be discussed in more detail in **section 2.3**. Furthermore, apoE has been proposed to play a role in intracellular lipid redistribution^{86,87}, VLDL-secretion⁸⁸, LPL inhibition⁸⁹ reverse cholesterol transport⁹⁰, atherosclerosis (discussed in **section 3.2**), and immunomodulation (discussed in **section 4.4.2**). In addition, apoE may have a role within the central nerve system. The *APOE4* gene is associated with familial and sporadic forms

of late-onset Alzheimer's disease, a neurodegenerative disorder associated with progressive dementia⁹⁰⁻⁹². The reduced ability of the brain to respond to damage in $\epsilon 4$ carriers associated with not only the rate of progression and/or age of onset of Alzheimer's disease, but possibly also with other neurodegenerative disorders (e.g. Parkinson's disease, amyotrophic lateral sclerosis), as well as coma's length following traumatic brain injuries⁹³⁻⁹⁵. Interestingly, the *APOE4* polymorphism shows strong linkage-disequilibrium with the *Hpa* I polymorphism in the *APOC1* promoter^{45,96,97}, which is also associated with risk for Alzheimer's disease⁹⁸. The consequence of this linkage-disequilibrium on causality for the above mentioned neurodegenerative disorders remains to be determined.

2.2. Synthesis, Structure, and Function of ApoCI

In the mid sixties, early seventies, three human apoCs were identified and characterized, apoCI⁹⁹⁻¹⁰², apoCII⁹⁹⁻¹⁰¹ and apoCIII¹⁰¹. They were initially referred to as apo-Val (later corrected to apo-Ser), apo-Glu and apo-Ala, respectively, as designated by their carboxyl terminal amino acids. These apoCs are often portrayed as members of one consistent protein family, because of their similar distributions among lipoprotein classes, their low molecular weights, and coincident purification. In 1995, Allan *et al.*¹⁰³ identified and characterized apoCIV as a fourth member of this human apoC-family, which was first discovered in mice¹⁰⁴. ApoCIV is less well studied than the other three apoCs. It is undetectable in human plasma¹⁰³, and to date no major modulating role for apoCIV could be identified. The role of the other three apoCs, in particular as significant modulators of lipoprotein metabolism, has been extensively reviewed^{105,106}. ApoCII is known as an essential cofactor of TG lipolysis by LPL¹⁰⁷⁻¹¹¹, whereas apoCIII is primarily known as the main endogenous inhibitor of LPL¹¹²⁻¹¹⁷. The function of apoCI will be outlined in more detail below.

2.2.1. Synthesis and Structure of ApoCI

The human *APOC1* gene is located 4.3 kb downstream from the *APOE* gene on chromosome 19 in the same transcriptional orientation^{118,119}. The *APOC1* gene is about 4.7 kb in size and is primarily expressed in the liver, but also at low levels in a wide variety of other tissues, including lung, skin, and spleen, where it is primarily expressed by macrophages within these tissues¹¹⁸. ApoCI is synthesized with a 26-residue signal peptide, which is co-translationally cleaved, resulting in the formation of mature apoCI that consists of only 57 amino acids. With a molecular weight of 6.6 kDa mature apoCI is the smallest known apolipoprotein. The mouse *apoc1* cDNA contains an open reading frame encoding a protein of 88 amino acids, including a signal peptide of 26 amino acid residues, finally resulting in a mature apoCI of 62 amino acid residues¹²⁰. The mature mouse

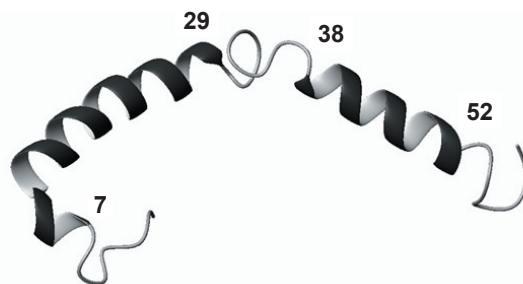


Figure 3. Structure of human apoCI. ApoCI consists of 2 α -helical structures, residues 7-29 (with a mobile hinge region involving residues 12-15) and residues 38-52, linked with a structurally unordered region (residues 30-37).

apoCI protein shares 67% homology with the human protein. Comparisons of amino acid sequence of apoCI from different species (human, baboon, mouse, rat, and dog) showed discrete regions with a high degree of conservation¹²⁰.

Just like apoCII and apoCIII, apoCI is mainly present on chylomicrons, VLDL and HDL (**table 1**), and circulates at levels in serum of about 8 mg/dL^{45,121}. Predominantly due to its high lysine content (*i.e.* 16 mol%), human apoCI has the highest isoelectric point of all apolipoproteins (pI 6.5), a feature which is often used for the purification of the protein from other apolipoproteins¹²²⁻¹²⁴. Human apoCI has a boomerang shape, it contains two amphipathic α -helices, the N-terminal helix (residues 7-29) and the C-terminal helix (residues 38-52), separated by an unordered flexible linker¹²⁵ (**Figure 3**). The N-terminal domain contains a mobile hinge involving residues 12-15. The hydrophobic side chains cluster on the nonpolar face of both helices, thus forming two discrete lipid binding sites in the N-terminal helix and one in the C-terminal helix. The C-terminal helix is tightly lipid-bound, while the N-terminal helix has lower lipid-binding affinity, but is more flexible and able to adjust to the lipoprotein size and composition¹²⁵. In other words, the C-terminal helix may act as a lipid anchor, while the N-terminal helix may be located more on the surface of the lipoprotein able to hinge off the lipid surface.

2.2.2. Polymorphisms of ApoCI

So far, no apoCI-deficient humans have been reported. Until recently, no human structural mutations had been reported as well, however Wroblewski *et al.*¹²⁶ recently described the first structural polymorphism of apoCI. This polymorphism involves the substitution of a tyrosine on position 45 for a serine, and could be found only in persons of American Indian, or Mexican ancestry, and not in individuals with ancestry of Europe, Africa and Asia. Within these American Indian, or Mexican ancestry about 14% of the individuals were carrier for this

mutation. Initial studies suggest that the S45 variant has higher preference for VLDL and lower preference for HDL as compared to normal apoCI, but additional studies are necessary to confirm these findings and to unravel whether this polymorphism also leads to functional changes of apoCI.

In contrast, a common *Hpa* I polymorphism has been described already two decades ago^{97,127,128}. This polymorphism is produced by a 4-bp CGTT insertion 317 bp upstream of the transcription initiation site of apoCI. *In vitro* studies showed that this polymorphism decreased the binding of a negatively acting transcription factor, leading to increased expression of apoCI⁹⁷. Follow up studies in human populations did confirm that circulating apoCI levels were (at least partly) dependent on the *Hpa* I polymorphism, however conflicting results have been obtained, as both negative and positive significant associations have been reported (even within the same study)^{45,121}. The results suggest that the biological impact of the *Hpa* I polymorphism is largely dependent on factors as gender, age, ethnicity, and hyperlipidemic state. As mentioned above, the *Hpa* I polymorphism is in almost complete linkage-disequilibrium with both the *APOE2* and *APOE4* alleles, but not the *APOE3* allele, which are located in the same gene cluster^{45,96,97}. Interestingly the *Hpa* I polymorphism has been associated with increased risk for Alzheimer's disease⁹⁸.

2.2.3. Functions of ApoCI

The first role ascribed to apoCI was by Havel *et al.*¹¹⁵, who showed that apoCI inhibited TG-hydrolysis by LPL. A few years later an inhibitory effect on HL activity was suggested as well¹²⁹, but these studies comprise *in vitro* findings and conformational *in vivo* studies were thus required. The inhibitory functions of apoCI on LPL and HL will be discussed in more detail in the next **section 2.3**. Others showed that apoCI was able to activate LCAT *in vitro*, resulting in increased formation of cholesteryl esters¹³⁰⁻¹³². Importantly, both *in vitro* and *in vivo* studies showed that apoCI interferes with the apoE-mediated binding and/or uptake of TG-rich lipoproteins by lipoprotein receptors (e.g. LDLr and LRP)¹³³⁻¹³⁶, which will be outlined in more detail in **section 2.3.3**. Furthermore, apoCI has been shown to inhibit CETP^{137,138}, and has been suggested to play a role in Alzheimer's disease⁹⁸, apoptosis of vascular smooth muscle cells¹³⁹, and atherosclerosis (the latter discussed in **section 3.3**).

2.3. Role of ApoE and apoCI in TG-rich Lipoprotein Processing

The metabolism of TG-rich lipoproteins, such as chylomicrons and VLDL, in the circulation is complex, and not yet fully understood. Indisputably, TG-rich lipoproteins are converted into lipoprotein remnants by size reduction via the hydrolysis of the core TG by lipases, primarily by LPL and HL. Subsequently,

these remnants are mainly taken up by the liver (~80%), but also by extrahepatic tissues, mediated via lipoprotein receptors. Most of the receptors participating in the uptake of TG-rich lipoproteins belong to the LDLr gene family (e.g. LDLr, VLDL-receptor (VLDLr) and LRP), but also binding sites outside this receptor family have been shown to be involved (e.g. HSPGs and SR-BI). Both apoE and apoCI have been proposed to play major roles in TG-rich lipoprotein processing.

2.3.1. Lipoprotein Lipase

As mentioned above, the hydrolysis of the core TG in chylomicrons and VLDL is an essential step in the processing, and the subsequent uptake, of these particles. The main enzyme responsible for this action is LPL, a member of a conserved lipase gene family, which included amongst others, HL, endothelial lipase, and pancreatic lipase¹⁴⁰. By hydrolyzing TG, LPL liberates fatty acids, which can be used either directly as an energy source by the muscle and heart, or indirectly via storage as TG in adipose tissues. LPL is expressed in virtually all tissues, and is most abundant in adipose tissue, heart, and skeletal muscle¹⁴¹⁻¹⁴³. LPL is not expressed in the adult liver¹⁴³. Active LPL consists of a homodimer of two non-covalently linked glycoproteins of equal size^{144,145}.

The role of LPL in lipid metabolism goes beyond the hydrolyzing properties of the enzyme. Once LPL is released from the cell membrane it circulates in plasma mainly as a monomer. As a monomer, LPL is able to enhance the binding and/or internalization of lipoproteins via the LDLr^{146,147}, LRP¹⁴⁸⁻¹⁵¹, VLDLr^{152,153} and HSPGs^{154,155}, most likely by bridging the lipoprotein particle directly to the receptor¹⁴⁸.

LPL requires apoCII as a co-factor to be catalytically active¹⁰⁷⁻¹¹¹. Also other apolipoproteins have been shown to influence the lipolytic activity of LPL. The main endogenous inhibitor of LPL is apoCIII¹¹²⁻¹¹⁷. Studies in transgenic mice and gene-targeted mice have documented the physiologic significance of the action of apoCIII in decreasing lipolysis¹⁵⁶⁻¹⁶¹. A few years ago, apoAV was discovered as a novel apolipoprotein^{162,163}. Recent work indicates that apoAV increases the LPL-mediated hydrolysis of TG by guiding VLDL and chylomicrons to HSPG-bound LPL^{164,165}. Since apoAV circulates in very low amounts in the human circulation (about 200-2000 ng/mL)^{166,167}, the physiological relevance of the LPL activation by apoAV requires further investigation.

Besides apoCIII and apoAV, also apoE and apoCI have been suggested to modulate the lipolysis of TG by LPL. ApoE was shown to directly stimulate LPL activity in the absence of apoCII^{168,169}, and was postulated to be required for the LPL-mediated metabolic conversion of VLDL into LDL¹⁷⁰. However, in the

presence of the co-factor apoCII, apoE effectively inhibits LPL-mediated lipolysis of TG-rich particles *in vitro*^{171,172} and *in vivo*⁸⁹. The physiologic importance of apoE-mediated inhibition of LPL is still subject of discussion. The role of apoCI in modulating the LPL-activity is much less described. A few decades ago, apoCI was shown to inhibit LPL *in vitro*^{115,173-176}. However, apoCI was not as efficient as apoCIII, leading apoCIII as the main focus of investigation. Eventually, in the nineties apoCI transgenic^{133,159,177} and knockout¹⁷⁸ mice were generated. The predominant hypertriglyceridemia in the apoCI transgenic mice^{133,159} is suggestive for apoCI-mediated inhibition of LPL *in vivo* as well; however no *in vivo* evidence has been reported so far.

2.3.2. Hepatic Lipase

Another enzyme that is postulated to be involved in remnant metabolism is HL. HL is primarily synthesized by hepatocytes, secreted, and mainly bound to the surface of parenchymal and hepatic endothelial cells associated with HSPG¹⁷⁹⁻¹⁸¹. The functional unit is a monomer in the liver, and may be a dimer in other tissues as the adrenal gland and ovary¹⁸². LPL and HL differ in their substrate preference and specificity. While LPL is mainly responsible for the hydrolysis of plasma TG, HL efficiently hydrolyzes phospholipids and has lower preference for TG¹⁸³⁻¹⁸⁷. In line with this, the preferred enzymatic substrates are IDL and HDL, but HL is also capable of processing chylomicrons and VLDL^{183,188-190}. HL does not have an absolute requirement for a cofactor in order to be enzymatically active, but the activity can be modulated by several apolipoproteins. The effects of HDL apolipoproteins on HL activity are well described *in vitro*^{129,191,192}. ApoAI^{191,192}, apoCI^{129,192}, apoCII^{129,193}, and apoCIII^{129,192} have been suggested to inhibit HL-mediated hydrolysis of TG, whereas for apoAII^{191,192,194} and apoE^{192,193,195,196} both inhibition and activation of HL-activity have been reported. apoE was suggested to activate HL-mediated hydrolysis of phospholipids in small HDL particles (with looser lipid packing), but not in larger VLDL particles (with tighter lipid packing), which could explain the inconsistencies found in earlier reports¹⁹⁷. The physiologic relevance of HL-inhibition by apoCI *in vivo* remains to be elucidated. Recently it was suggested that apoCI-mediated inhibition of HL is responsible for the hypertriglyceridemic phenotype in apoCI transgenic mice¹⁹⁸. Strikingly, HL-deficient mice do not show any sign of disturbed TG metabolism¹⁹⁹⁻²⁰¹, arguing against HL-inhibition as a major determinant of the observed hypertriglyceridemic phenotype in *APOC1* mice.

2.3.3. TG-rich Lipoprotein Uptake Mechanisms

Lipoprotein receptors like the LDLr, LRP, and VLDLr play crucial roles in lipid homeostasis by mediating the cellular uptake of primarily TG-rich lipoproteins.

These receptors belong to the LDLr gene family, which represents a class of endocytic receptors that is present in both vertebrate and non-vertebrate species. In the last years, these receptors have been identified and characterized²⁰²⁻²⁰⁴. The members of this family exhibit several distinct functional domains: 1) an amino-terminal ligand binding domain; 2) an epidermal growth factor precursor homology domain; 3) an O-linked sugar domain; 4) a transmembrane domain that is required for anchoring the receptor to the plasma membrane; and 5) a cytoplasmatic region with a signal (Asp-Pro-X-Tyr) for receptor internalization via coated pits²⁰⁵⁻²⁰⁷ (**Figure 4**).

2.3.3.1. LDLr

The LDLr (120 kDa) is the prototype of the LDLr family, is highly expressed in tissues that utilize lipoproteins, such as the liver and adrenals²⁰⁸, and recognizes both apoB100 and apoE²⁰⁹. The LDLr can contribute to the clearance of both chylomicrons and VLDL (remnants) *in vivo* in animals as well as in humans. The clearance of these particles is mainly mediated via apoE, which binds to the LDLr via its LDLr-binding domain (residues 139-153; **Figure 2**)^{58,59}. The LDLr also recognizes apoB100, the sole apolipoprotein of (mainly cholesterol-rich) LDL, and via this interaction the LDLr mediates the uptake of LDL from plasma (**Table 1**). ApoCI has been shown to inhibit the apoE-mediated hepatic uptake of TG-rich lipoprotein remnants by the LDLr, possibly by masking of the receptor binding domain of apoE¹³⁵, or via displacement of apoE from lipoprotein particles¹³⁶.

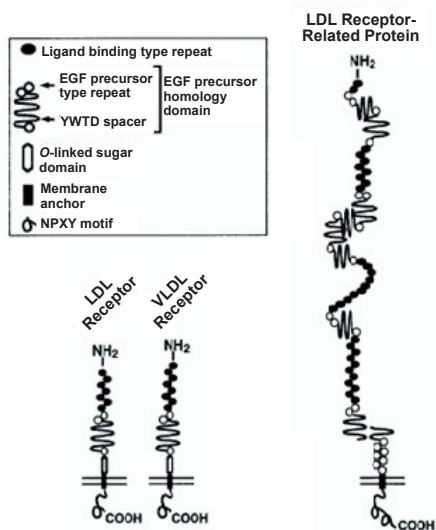


Figure 4. Schematic structures of several members of the LDLr family. See text for explanation. NPXY designates the tetraamino acid motif Asp-Pro-X-Tyr which directs the receptors into coated pits. EGF, endothelial growth factor. Adapted from Willnow *et al.*²⁰⁴.

2.3.3.2. LRP

Besides the LDLr, also LRP (*i.e.* LRP1) plays a role in TG-rich lipoprotein uptake. LRP (also known as the α 2-macroglobulin receptor) is the largest (*i.e.* 600 kDa) endocytotic receptor identified to date, and is expressed in a variety of tissues, such as the liver, intestine, lung, and brain, and in numerous cell types, such as fibroblasts, SMCs, monocytes/macrophages²¹⁰⁻²¹². LRP1 is a heterodimer consisting of a 515 kDa extracellular and an 85 kDa membrane anchored subunit²¹³, and recognizes >50 structurally and functionally different ligands^{214,215}, including apoE-containing lipoproteins²¹⁶⁻²¹⁹. Similarly as for the LDLr, apoCI also inhibits the apoE-mediated uptake by LRP, probably via the same mechanism¹³⁶. Studies from our group suggested that the inhibiting properties of apoCI towards LRP may exceed those towards the LDLr, because the apoCI-associated hyperlipidemia was substantially more pronounced on an LDLr-deficient background as compared to a wild-type background^{133,134}.

2.3.3.3. VLDLr

The member of the LDLr family that most closely resembles the LDLr is the VLDLr^{220,221}. The VLDLr enhances the binding and uptake of apoE-containing lipoproteins, such as chylomicrons, VLDL, and IDL, but not LDL^{152,220}. Similar as for the other members of the LDLr family, the apoE-mediated binding of TG-rich lipoproteins to the VLDLr is inhibited by apoCI¹³⁴. The VLDLr is most abundantly expressed in tissues active in FFA metabolism, such as the heart, skeletal muscle, and adipose tissue²²²⁻²²⁶, and only trace amounts are found in liver^{221,227}. Within these tissues the VLDLr is mostly localized in endothelial cells and SMCs of arteries and veins^{228,229}. Like LRP, the VLDLr is also a multiligand receptor, and is able to facilitate the uptake of fibrinolysis products¹⁵³ and extracellular matrix proteins²³⁰.

2.3.3.4. HSPG

HSPGs play also a role in TG-rich lipoprotein remnant clearance as part of the "HSPG/LRP pathway"²³¹⁻²³³. HSPGs are components of the extracellular matrix within the Space of Disse, as well as collagen, fibronectin, laminin, and elastin. HSPGs are heterogenous, with respect to their number of chains per polypeptide, chain length, and extent of postpolymeric modifications, such as N-acetylation, N-sulfation, and O-sulfation^{234,235}. Both *in vitro*^{61,236} and *in vivo*^{237,238} studies showed that HSPGs are involved in TG-rich lipoprotein clearance. It is envisioned that TG-rich lipoproteins may initially sequester within the Space of Disse through interaction with apoE bound to HSPGs, which are found on the microvilli of parenchymal cells^{61,239-241}. HSPGs are thought not to be involved in the actual ligand uptake process, but to transfer the TG-rich lipoproteins to

an internalizing receptor, such as the LRP and the LDLr²⁴², although a direct low affinity HSPG-mediated internalisation of TG-rich particles has also been described²⁴³.

2.3.3.5. SR-BI

Recently, SR-BI, which is well known for mediating selective uptake of cholesteryl esters from HDL without concomitant uptake of HDL protein²⁴⁴, was shown to accelerate chylomicron metabolism²⁴⁵. Similar as for HSPGs, SR-BI probably mediates the initial capture of chylomicron remnants by the liver, whereby the subsequent internalization can be exerted by additional receptors like the LDLr and LRP. The role of apoE and other apolipoproteins in this SR-BI-mediated pathway still has to be elucidated.

3. Role of ApoE and ApoCII in Atherosclerosis

Atherosclerosis is the main cause of CVD such as myocardial infarction and stroke, and accounts for up to 50% of all mortality in Western countries³. Atherosclerosis has traditionally been viewed to simply reflect the deposition of lipids within the vessel wall. Classically, elevated cholesterol and/or TG levels, and in particular high LDL-cholesterol and low HDL-cholesterol levels, are the principal risk factors of atherosclerosis. However, nowadays atherosclerosis is considered not only as a disease of the lipids, but also as a chronic inflammatory disease of the intima, slowly developing in time starting from childhood, resulting in additional risk factors such as high C-reactive protein (CRP) for example^{3,6,246}. Other risk factors for this multifactorial disease include age, gender, smoking, hypertension, stress, dietary habits, and physical inactivity^{3,6,7}.

3.1. The Pathogenesis of Atherosclerosis

Endothelial cells can be exposed to many forms of injury, including infectious, immunological, chemical, radiation and mechanical injury, which has an impact on their cellular structure and function^{3,7}. As a result, markers such as vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and selectins (e.g. E-selectin and L-selectin) are expressed. These adhesion molecules attract monocytes, which start rolling on the endothelium leading to their attachment and infiltration into the intima, initiating the formation of the lesion or plaque²⁴⁷⁻²⁴⁹ (**Figure 5**). The presence of macrophage colony-stimulating factor in the vessel wall mediates the maturation of the infiltrated monocytes into macrophages, which are scavenging and antigen-presenting cells that secrete cytokines, growth-regulating molecules, chemokines, proteases, reactive oxygen radicals, and other inflammatory molecules^{250,251}. These macrophages

may initially serve as a protecting factor, by playing a critical role in the repair and damaging processes while the lesion develops. However, scavenging of modified LDL results in lipid-laden macrophages, called foam-cells, which subsequently results in the formation of a fatty streak. This fatty streak is still completely reversible, but may progress into an advanced lesion by the influx of additional monocytes and T-cells, depending on the balance of proatherogenic and anti-atherogenic factors⁴. The resulting proatherogenic micro-environment in the lesion by the increased inflammation and tissue damage, stimulates migration of fibroproliferative vascular smooth muscle cells (SMCs), derived from the underlying media or circulating progenitor cells, to the endothelium to form a protective fibrous cap^{3,252}. These SMCs are also capable of accumulating cholesterol and contribute to the foam cell formation. Further progression of the plaque includes the accumulation of foam cells and the formation of a lipid core. Also other immunocellular components as T-and B-cells, mast cells, natural killer cells, neutrophils and dendritic cells, present in the advanced atherosclerotic lesion are able to modulate the progression of the lesion. Subsequently, macrophage death by apoptosis or necrosis, as a consequence of cholesterol-toxicity, inflammatory cytokines, oxidative stress, and growth factor depletion, contributes to the formation of a necrotic core^{253,254}. At this point the advanced fibrous lesion consists of a fibrous cap that covers a core of foam cells, macrophages and other inflammatory cells, SMCs, extracellular lipids, and a necrotic core. The plaque is stable when a uniform thick fibrous cap is formed. On the other hand,

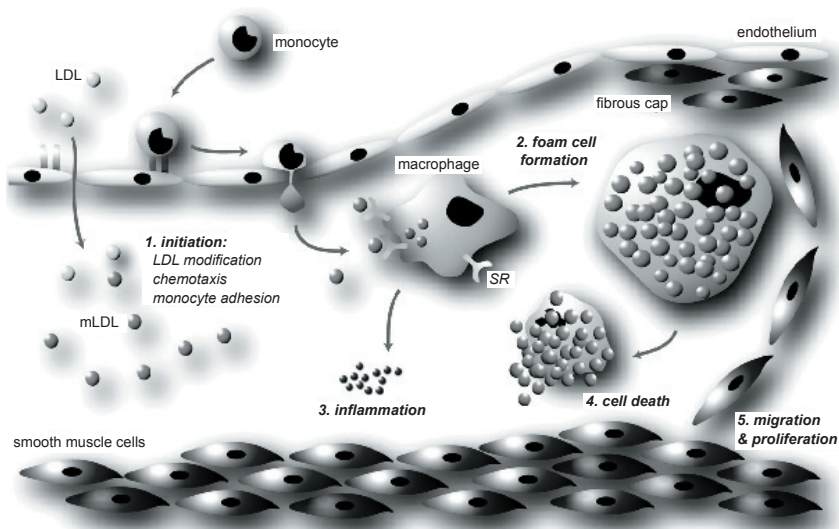


Figure 5. Schematic overview of atherogenesis from early to advanced atherosclerotic lesion formation. See text for explanation. mLDL, modified LDL; SR, scavenger receptor. Adapted from De Winther *et al.*²⁵⁶.

continuous influx and activation of macrophages, releasing metalloproteinases and other proteolytic enzymes in the plaque can result in thinning of the fibrous cap^{3,252,255}. The concomitant instable cap can lead to rupture of the plaque, which in worst case can occlude the vessel and result in cardiovascular events such as myocardial infarction and stroke.

3.2. ApoE in Atherosclerosis

A major focus of recent research on CVD has been to understand the molecular basis of atherosclerosis in detail, and has resulted in the identification of a key, but complex, role for apoE in this process. ApoE has been ascribed many anti-atherosclerotic functions (summarized in **Figure 6**), of which its central role in the regulation of lipid metabolism (discussed in **section 1 and 2**) is probably the most important and can be attributed to several actions: 1) Uptake and degradation of lipoprotein remnants by the liver^{79,80,257}, 2) stimulation of the reverse cholesterol transport^{80,258,259}, and 3) activation of enzymes involved in HDL-metabolism such as LCAT²⁶⁰ and possibly HL (discussed in **section 2.3.2**). These above functions of apoE in lipid metabolism can all be considered as anti-atherogenic mechanisms. However, apoE may also have a pro-atherogenic function in lipid metabolism by: 1) stimulating the hepatic VLDL production^{88,261}, and 2) activating CETP²⁶², which is considered a pro-atherogenic lipid transfer protein²⁶³⁻²⁶⁵. Since mice normally lack expression of CETP³⁷ this is not relevant in mice, but may be of importance in the human situation.

Additional anti-atherosclerotic functions of apoE have been identified which are mostly anti-inflammatory of nature. ApoE expressed by macrophages inhibits platelet aggregation by interacting with a specific cell surface receptor, the apoE receptor 2 (apoER2), initiating a signalling cascade leading to activation of nitric oxide (NO) synthase and the subsequent decrease in NO^{266,267}. In addition, via the same mechanism apoE inhibits VCAM-1 expression on endothelial cells²⁶⁸. Furthermore, apoE inhibits T-cell activation and proliferation²⁶⁹⁻²⁷¹, and SMC migration and proliferation as induced by platelet-derived growth factor and oxidized LDL²⁷²⁻²⁷⁴. ApoE may prevent the accumulation of oxidized LDL by inhibiting lipid oxidation²⁷⁵⁻²⁷⁷, and is suggested to inhibit endothelial cell proliferation by modulating the availability of cytokines and growth factors retained in the pericellular proteoglycan matrix²⁷⁸. Data also support an anti-inflammatory role for apoE in suppressing acute inflammation by lipopolysaccharide (LPS)^{15,279-281} or bacteria (e.g. *Listeria monocytogenes*²⁸², *Klebsiella pneumoniae*^{280,283} as will be discussed in **section 4.4.2**, which is likely to also have anti-atherogenic consequences.

In contrast to these anti-inflammatory properties, apoE has also proinflammatory properties, which thus may aggravate atherosclerosis

development. Van den Elzen *et al.*¹⁶ showed that apoE is involved in enhancing the presentation of lipid antigens by dendritic cells. ApoE captures lipid antigens in the circulation, and, via an LDLr-mediated uptake route, these lipid antigens are subsequently presented on the surface of dendritic cells (also discussed in **section 4.4.2**).

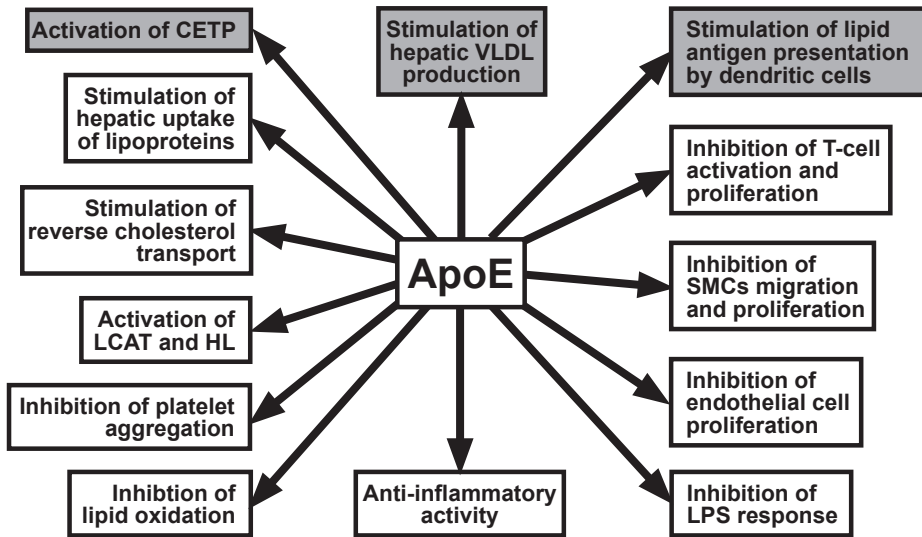


Figure 6. Proposed anti- (white boxes) and pro- (grey boxes) atherogenic roles of apoE. See text for explanation. CETP, cholesteryl ester transfer protein; HL, hepatic lipase; LCAT, lecitin:cholesterol acyltransferase; SMC, smooth muscle cell. Modified from Gilnow *et al.*⁷⁶.

Most of the understanding of the role of apoE in atherosclerosis as described above has been generated by the use of apoE-deficient mice. Disruption of the *apoe* gene in mice is associated with hypercholesterolemia and spontaneous, severe atherosclerosis that can be further enhanced by cholesterol feeding²⁸⁴⁻²⁸⁶. The development from the fatty streak to the advanced plaque in this model is to a certain extent similar as in the human plaque development, and, therefore, this model is widely used in atherosclerotic research.

In humans, apoE-deficiency (characterized by less than 1% of the normal apoE concentration in plasma) is associated with hyperlipidemia, mainly high VLDL, IDL, and LDL levels^{83,85,287-292}, increased lipid storage in monocyte/macrophages and phenotypic expression of xanthomas (massive foam-cell accumulation) early in life^{83,85,291}, and premature development of atherosclerosis is^{83,85,291}. Interestingly, heterozygous apoE-deficient subjects have normal lipid levels, although apoE concentrations are only approximately half of normal

levels⁸⁵. Thus, only the nearly complete deficiency of apoE will result in increased risk to develop atherosclerosis.

A number of studies have investigated the impact of the common human apoE2, apoE3, and apoE4 isoforms on cardiovascular research²⁹³⁻²⁹⁶. These isoforms have distinct effects on lipid metabolism (discussed in **section 2.1.2**). ApoE2 is associated with higher plasma apoE levels, but lower cholesterol levels as compared to apoE3 subjects^{46,63,73-75}, whereas in apoE4 subjects lower plasma apoE levels and higher cholesterol levels are found^{46,63,75}. The results on the association of the $\epsilon 2$ allele with CVD have yielded conflicting results; both harmful and protective associations have been found²⁹³⁻²⁹⁶. On the other hand, the $\epsilon 4$ allele has been consistently associated with an increased risk of CVD²⁹³⁻²⁹⁶. Importantly, irrespective of the *APOE* genotype, high plasma apoE levels are associated with increased cholesterol levels^{63,73}. This may indicate that in humans high plasma apoE levels are associated with increased CVD irrespective of the *APOE* genotype, but studies addressing this hypothesis have not yet been reported.

3.3. ApoCI in Atherosclerosis

The role of apoCI in atherosclerosis has been far less studied than the role of apoE. It appears that researchers only recently started to really appreciate the significant role of apoCI in lipid metabolism and started studies on the role of apoCI in atherosclerosis development. ApoCI has potent hyperlipidemic effects by inhibiting the hepatic apoE-mediated uptake of (atherogenic) remnant particles, and possibly also by inhibiting the processing of TG-rich lipoproteins by LPL (discussed in **section 2**). Besides its hypertriglyceridemic effects, *in vitro* studies have indicated that apoCI may also promote plaque rupture by inducing apoptosis of aortic SMCs, via recruitment of neutral sphingomyelinase¹³⁹. In contrast apoCI may also possess anti-atherosclerotic properties by promoting cholesterol efflux from macrophages via ABCA1²⁹⁷, possibly via stabilisation of ABCA1. In addition, apoCI is the main endogenous inhibitor of CETP, which is a very promising anti-atherosclerotic characteristic^{122,138}. Inhibition of this proatherogenic lipid transfer factor increases circulating HDL levels²⁹⁸⁻³⁰⁰ and subsequently may decrease atherosclerosis risk.

Expression of human apoCI in mice, that naturally do not express CETP³⁷, aggravated atherosclerosis development¹⁹⁸. To investigate the potentially important anti-atherosclerotic characteristic of apoCI as an inhibitor of CETP, studies using human CETP transgenic mice have been performed. Initial studies showed that human apoCI expression in CETP transgenic mice resulted in decreased specific CETP activity, but simultaneously increased total CETP mass as compared to

their controls³⁰¹. Since the overall CETP activity was probably enhanced, this led to an even aggravated proatherogenic lipoprotein profile, with decreased HDL levels and markedly increased VLDL and LDL levels, indicating that the potential anti-atherogenic properties of apoCI are overruled by its proatherogenic properties in this model. However, this has to be confirmed in humans, since data on association of plasma apoCI levels with CVD or clinical endpoints are still lacking. However, human studies did reveal that the apoCI content of TG-rich lipoproteins in the postprandial state predicts early atherosclerosis in normolipidemic healthy men³⁰²⁻³⁰⁴. This proatherogenic effect of apoCI is most likely a result of reduced processing of the postprandial TG-rich lipoproteins by lipases and inhibited receptor binding, and the concomitant delayed uptake of (atherogenic) TG-rich lipoproteins, as evident from experimental studies (see also **section 2**). Recently, Kwiterovich *et al.*³⁰⁵ described the presence of an elevated large HDL particle enriched in apoCI, in infants of lower birth weight and younger gestational age. Although this apoCI-enriched particle disappears soon after birth, these infants have increased risk of CVD in adulthood³⁰⁶. The molecular mechanism behind this association remains to be elucidated.

4. Role of ApoE and Other Apolipoproteins in Inflammation and Sepsis

Sepsis is a major cause of morbidity and mortality. It affects approximately 700,000 people annually and accounts for about 210,000 deaths per year in the United States^{10,11}. In fact, it is the leading cause of death in medical and surgical intensive care units^{8,9}. Due to advances in medical practice and technology, as the use of invasive equipment, implantation of prosthetic devices, and administration of corticosteroids and other immunosuppressive agents to patients with organ transplants or inflammatory diseases, the incidence is still rising at rates between 1.5% and 8% per year^{10,11}.

4.1. Infection, Sepsis, and Lipopolysaccharide

Sepsis is currently viewed as a complex dysregulation of the inflammatory response arising when the host is unable to successfully contain an infection with microorganisms, as bacteria, parasites, fungi, and viruses^{11,307,308}. Infection with microorganisms first results in a proinflammatory response, during which proinflammatory cytokines (*e.g.* tumor necrosis factor- α (TNF α), interleukin-1 (IL-1), IL-6, and IL-12) are produced to effectively respond to the infection³⁰⁹⁻³¹¹. This first proinflammatory response is crucial to combat the bacterial infection in the early phase (**Figure 7**). When this initial proinflammatory response is inadequate and the invading microorganisms multiply, the proinflammatory

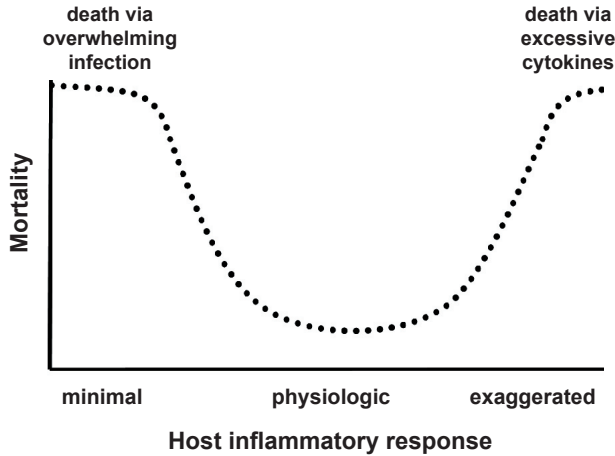


Figure 7. The U-shape relationship between the host inflammatory response and mortality. See text for explanation. Adapted from Cross *et al.* (Cross AS, *International Endotoxin Society Meeting, Kyoto, Japan, 2004*).

response enhances and develops into a systemic inflammatory response syndrome (SIRS). This response is counteracted by the compensatory anti-inflammatory response syndrome (CARS), during which IL-4, IL-10, IL-13, and other cytokines are produced. The correct balance of SIRS and CARS, as well as the intensity of these responses greatly influences host survival³⁰⁹⁻³¹¹. Imbalance between these responses can result in host damage (**Figure 7**). If the balance is shifted towards SIRS, excessive proinflammatory cytokine production will cause direct host damage. A shifted balance towards CARS will result in increased proliferation of the infection, eventually leading to an excessive proinflammatory response. Thus, an efficient proinflammatory response is crucial to prevent rapid multiplication of the invading microorganism and to surmount the first phase of infection, whereas in a later phase a high proinflammatory response is often harmful and may lead to tissue damage and organ failure.

Most cases of sepsis are caused by bacteria. The occurrence of Gram-positive sepsis increased over the last decades and accounts for 30-50% of all cases, whereas the incidence of Gram-negative sepsis is somewhat lower, but still accounts for 25-30% of all sepsis cases^{307,308,312,313}. While Gram-positive bacteria contain a number of immunogenic cell wall components (e.g. M protein), in addition to often highly deleterious exotoxins, such as lipoteichoic acid (LTA) and peptidoglycan, Gram-negative bacteria share LPS as their main pathogenic component^{314,315}. In fact, injection of LPS alone causes the same clinical features as can be seen in patients with Gram-negative sepsis³¹⁶. LPS is essential for the growth and structural integrity of the bacteria³¹⁷⁻³¹⁹, and, incorporated in the

bacterial membrane, activation of the immune cells is poor³²⁰. However, the release of LPS from the membrane during both cell division and death, exposes the toxic lipid A moiety to immune cells, evoking an immunological response^{321,322}. The LPS molecule consists of 4 different parts: 1) lipid A, 2) the inner core, 3) the outer core, and 4) the O-antigen (**Figure 8**)^{319,322,323}.

The lipid A moiety is the toxic part of LPS. It is the lipid component of LPS and consists of 6 or more fatty acid residues linked to 2 phosphorylated glucosamine sugars. Despite the common architecture, lipid A of different bacterial origin varies in their fine structure. These variations are: 1) the acylation pattern; 2) length of the fatty acid residues; 3) the presence of 4-amino-deoxy-L-arabinose and/or phosphoethanolamine linked to the phosphor groups on the glucosamine sugars; and 4) the number of fatty acids (most bacteria contain 6 fatty acid residues).

The inner core of LPS consists of two or more 2-keto-3-deoxyoctonic acid (KDO) sugars linked to the lipid A glucosamine. To these KDO sugars, 2 or 3 heptose (L-glycero-D-manno-heptose) sugars are linked. Similar as for lipid A, the sugars of the inner core are also unique to bacteria. Re-LPS is the smallest LPS molecule produced by Gram-negative bacteria, and consists of lipid A with 1 or 2 KDO sugar units.

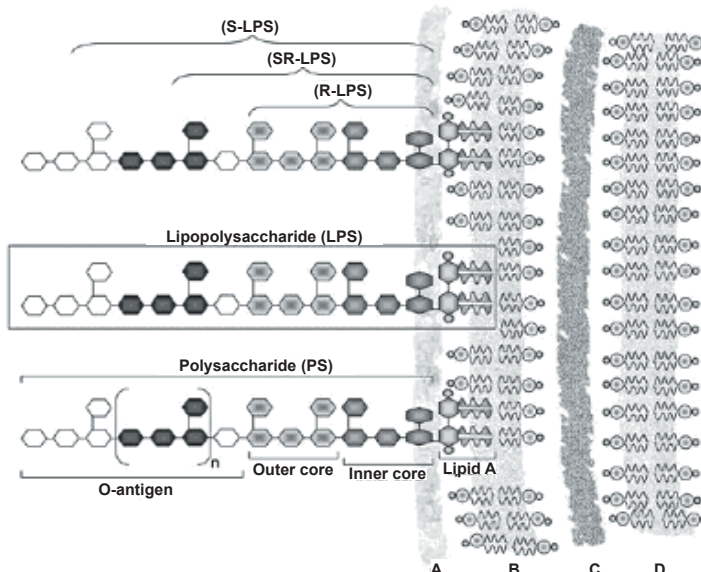


Figure 8. General simplified overview of lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria. See text for explanation of the LPS components. Some bacterial species contain an outer capsule that protects the bacterium from host defenses such as complement, lysis, and phagocytosis (A). Outer lipid bilayer with LPS which is approximately 8 nm in width (B). Peptidoglycan layer (C). Inner bilipid membrane (D). S-LPS, smooth LPS; SR-LPS, semi-rough LPS; R-LPS, rough LPS. Adapted from Dixon *et al.*³²⁴.

The outer core of LPS is more variable than the inner core and consists of common sugars. In most cases it is 3 sugars long with 1 or more covalently linked sugars as side chains. Serotypes of LPS that consist of lipid A, and the complete inner and outer core are denoted Ra-LPS, whereas the Rb- and Rc-LPS serotypes only contain a part of the outer core.

The O-antigen is attached to the outer core and is composed of repeating units of common sugars. The O-antigen extends from the bacterial surface, and is also immunogenic. The interspecies and interstrain variations in the composition and length of the O-antigen are huge, and can vary from 0 to 40 repeating units, but in general consist of 20-40 repeating units. O-antigen-containing LPS differ from O-antigen-lacking LPS by their smooth appearance on agar plates, and are therefore indicated as S-LPS, whereas O-antigen-lacking LPS have a rough (R) appearance.

4.2. Host Response to Lipopolysaccharide

The immune response to LPS and Gram-negative bacteria consists of the innate and adaptive immunity^{325,326}. The innate immunity is evolutionary ancient, whereas the adaptive immunity provides specific recognition and immunological memory. The innate immunity is the first line of defense. In particular the mononuclear cells (*i.e.* monocytes and macrophages), neutrophils, and mast cells are of great significance, since these cells are activated by LPS to differentiate rapidly into short-lived effector cells to combat infection^{325,327-329}. Immature dendritic cells which are specialized antigen-presenting cells involved in innate immunity, present internalized LPS on their surface to activate T-cells and to provide instructions about the nature of the microbe^{325,328-333}. For complete activation of T-cells, dendritic cells need to deliver co-stimulatory signals, such as CD80 and CD86. The activated T-cells then start the adaptive immune response, in which cytotoxic T-cells, B-cells, and macrophages serve as effector cells. Macrophages play a major role in immunity, since these effector cells are thus involved in both the innate and adaptive immunity, as described above.

An important family of innate immune receptors are the Toll-like receptors (TLRs), named after the homologous Toll protein in *Drosophila melanogaster*³³⁴. These receptors can discriminate between different pathogens and self-antigens. To date, 10 different TLRs are described in humans and mice, which differ from each other in ligand specificities, expression patterns, and in the target genes they can induce³³⁵⁻³³⁸. Strict discrimination between pathogens and self-antigens by TLRs is required for a TLR response, because they play a crucial role in deciding whether to respond or not to an invading microorganism³³⁹.

TLR4, together with its indispensable unit MD2, is essential for the recognition and inflammatory response towards LPS³⁴⁰⁻³⁴⁴. This LPS-signalling receptor was

identified after cloning of the defective gene in the LPS-unresponsive C3H/HeJ and C57BL/10ScCr mice^{340,345,346}. Only few exceptions of LPS are known to be undetected by TLR4, these are the LPS of *Porphyromonas gingivalis*³⁴⁷ and *Leptospira interrogans*³⁴⁸ bacteria. The LPS of these bacteria differ in structure from other Gram-negative bacteria, and are recognized by TLR2. TLR4, however, is not the sole receptor involved in LPS recognition. Transport of LPS in the circulation is mainly mediated by LPS-binding protein (LBP), which catalyzes the movement of free LPS or LPS from the outer membrane of Gram-negative bacteria directly to HDL and other lipoprotein particles³⁴⁹, but also to CD14^{350,351}. CD14 is either expressed on the surface of myelomonocytic cells as a glycosyl phosphatidylinositol (GPI)-anchored molecule (membrane CD14, mCD14), or is present in the circulation as a soluble molecule (sCD14)³⁵². CD14 then delivers the LPS to TLR4. CD14 appears not to be essential for LPS responses, but probably has a role in their amplification³⁵³.

LPS processing by LBP and sCD14 does not necessarily result in LPS responses, but also in LPS clearance. Transfer of LPS to lipoproteins can result in neutralization and subsequently clearance via the liver^{349,354-356} (discussed in **section 4.4**). Therefore, both LBP and CD14 have a dual role in LPS responses; they not only trigger LPS responses, but can also terminate them³⁵⁷⁻³⁵⁹, which might explain the complex phenotypes of mice that lack these molecules. For instance, LBP-deficient mice are susceptible to Gram-negative bacterial infection, but are resistant to experimentally LPS-induced shock³⁴⁹, while LPS-stimulated TNF α production *in vivo* is not impaired³⁶⁰. Similarly, CD14-deficient mice are highly resistant to LPS- and Gram-negative bacteria-induced shock³⁶¹. Surprisingly, these mice also show reduced circulating bacteria levels, suggesting a role for CD14 in facilitating the dissemination of Gram-negative bacteria³⁶². LPS clearance by CD14 might attenuate local inflammation and hereby could permit bacterial replication.

4.3. Lipid Metabolism in Inflammation and Sepsis

Accumulating evidence indicates that lipoprotein metabolism is strongly influenced by inflammation, infection, and sepsis³⁶³. In human sepsis, plasma TG are increased³⁶⁴⁻³⁶⁷, phospholipids are maintained at near normal levels³⁶⁴, while total cholesterol is decreased^{364,366-369}. The decrease in cholesterol, caused by a reduction in cholesteryl esters, and not unesterified cholesterol³⁶⁴, can be attributed to reductions of LDL³⁶⁵⁻³⁶⁷ and mainly HDL^{365-367,369}. The decrease in HDL is accompanied by a loss of mainly large apoAI-containing particles, an almost total loss of apoCI, and an increase in apoE-containing HDL, which does not contain significant amounts of apoAI, apoAII, or apoCI³⁶⁵. In addition, apoAI-containing HDL also shows an increased content of the inflammation-associated

isoforms of SAA, which may inhibit the selective uptake of HDL-cholesteryl esters by SR-BI³⁷⁰. Also, plasma LBP is strongly increased, albeit that the disposition of LBP in the circulation (*i.e.* HDL versus apoB-containing lipoproteins) remains quite controversial. Initially, LBP was suggested to be bound to HDL based on removal of LBP from plasma by anti-apoAI immunochromatography³⁷¹. However, more recent studies have indicated that LBP is mainly associated with apoB-containing lipoproteins as evidenced by co-migration during separation of lipoproteins by electrophoresis^{365,372}. Intravenous infusion of a single dose of LPS into healthy volunteers can mimic many of the effects observed in septic patients, including a transient increase in plasma FFA and TG, and a decrease in cholesterol, LDL-cholesterol and apoB³⁷³.

The mechanisms underlying the effects of sepsis on lipid metabolism have been mainly derived from studies in animals, by mimicking the septic conditions as seen in patients. Injection of LPS or bacteria into rats leads to a marked hyperlipidemic response, caused primarily by an increase of VLDL-TG³⁷⁴. Low doses of LPS increase the hepatic VLDL production, as related to an increased hepatic synthesis of TG³⁷⁵ and cholesterol³⁷⁶. In contrast, high doses of LPS inhibit VLDL clearance³⁷⁵, which may be related to a decrease in plasma LPL activity^{377,378}, VLDL-apoE content³⁷⁹, or hepatic LDLr expression³⁸⁰. In addition, LPS has been shown to affect a wide range of HDL-associated apolipoproteins, plasma enzymes, lipid transfer proteins, and receptors that are involved in plasma HDL metabolism. Besides a decrease in HDL-associated apoAI and an increase in HDL-associated SAA, apoAIV, apoAV, apoE³⁶³, and apoJ^{381,382}, LPS affects LCAT³⁷⁸, HL³⁸³, PLTP³⁸⁴, CETP³⁸⁵, macrophage ABCA1³⁸⁶, macrophage SR-BI³⁸⁷, and hepatic SR-BI³⁸⁸, as summarized in **Figure 9**. Since PLTP is decreased in LPS-treated mice³⁸⁴ and increased in human sepsis³⁶⁵, the actual significance of some of these findings for human sepsis remains to be established. Nevertheless, despite some potential species' differences, both sepsis and LPS thus affect plasma lipoprotein levels by modulating lipoprotein production and clearance through their effect on apolipoproteins, lipolytic enzymes, lipid transfer factors, and lipoprotein receptors^{363,389}.

4.4. Lipoproteins and the Lipopolysaccharide Response

In addition to the effects of LPS and sepsis on lipid metabolism, all lipoprotein classes have been demonstrated to bind LPS. LPS, when added to whole human normal blood, mainly binds to HDL (60%), in addition to LDL (25%) and VLDL (12%)^{364,390}. In septic blood, in which HDL levels are decreased, LPS binding shifts towards VLDL³⁶⁴. Biophysical studies showed that LPS interacts with HDL through its lipid A moiety, in particular involving the diglucosamine-

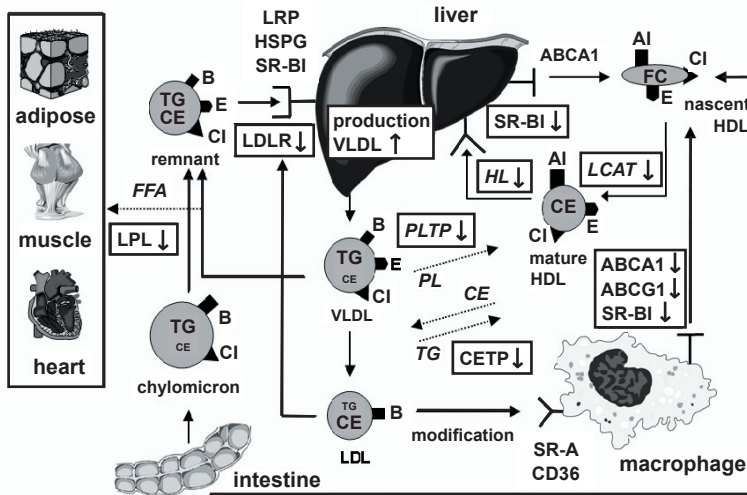


Figure 9. Effect of LPS on plasma lipoprotein metabolism. Animal studies have demonstrated that LPS affects the metabolism of VLDL, LDL, and HDL by influencing many processes involved in their formation, secretion, and clearance, as shown by the arrows in the boxed items. See text for explanation. AI, apolipoprotein AI; ABCA1/ABCG1, ATP-binding cassette transporter AI or GI; B, apolipoprotein B; CI, apolipoprotein CI; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; E, apolipoprotein E; FC, free cholesterol; FFA, free fatty acids; HSPG, heparan sulphate proteoglycans; LCAT, lecithin:cholesterol acyltransferase; LDLR, LDL receptor; LRP, LDL receptor-related protein; PL, phospholipids; PLTP, phospholipid transfer protein; SRA, scavenger receptor class A; SR-BI, scavenger receptor class B type I; TG, triglycerides. Modified from Berbée *et al*⁸⁸.

phosphate region³⁹¹. However, the mode of interaction may strongly depend on the composition of HDL, especially with respect to its apolipoprotein pattern, which is drastically altered in inflammation and sepsis (as discussed in the previous **section 4.3**). The binding of LPS to lipoproteins modulates the biological response to LPS, as demonstrated *in vitro* and in rodents^{392,393}. Both chylomicrons and VLDL were able to protect mice and rats against a lethal dose of LPS^{354,393}. In fact, chylomicrons were able to prevent septic death resulting from cecal ligation and puncture (CLP) in rats³⁹⁴. TG-rich lipoproteins appeared to redirect LPS to hepatocytes^{354,355}, where LPS was secreted into the bile in a de-activated form, as shown in rats³⁵⁶. The cholesterol-rich lipoproteins LDL and HDL can also bind LPS and neutralize its biological activity³⁹². Transgenic mice expressing human apoAI, resulting in a 2-fold elevated plasma HDL level, exhibited lower cytokine levels and improved survival rates after LPS challenge as compared to control mice³⁹⁵. Based on the LPS-neutralizing properties of HDL, it was hypothesized that reconstituted HDL (rHDL), built from phospholipids and apoAI, may constitute a valuable therapeutic agent in the protection and treatment of septic shock. Indeed, rHDL reduced LPS-induced TNF α production in mice³⁹⁵, rabbits^{396,397}, and dogs³⁹⁸. In humans, infusion of rHDL reduced the

LPS-induced release of TNF α , IL-6, and IL-8, while the release of inhibitors of proinflammatory cytokines (*i.e.* IL-1 receptor antagonist, soluble TNF receptors, and IL-10) were only marginally affected³⁹⁹.

4.4.1. Lipoprotein Constituents and the Lipopolysaccharide Response

The amphiphilic structure of LPS would suggest that LPS associates with lipoproteins merely by intercalation of the highly lipophylic lipid A moiety into the particle's phospholipid shell. Indeed it has been shown that the ability of the various lipoprotein classes to neutralize the bioactivity of LPS *in vitro* depends on their phospholipid content and not on their content of cholesterol or TG⁴⁰⁰. LPS can be directly transferred into phospholipids by LBP in the presence⁴⁰¹ or absence⁴⁰² of sCD14. The LBP-induced intercalation of LPS into phospholipid liposomes may also be enhanced by HDL³⁹¹. Moreover, infusion of phospholipid-rich lipid emulsion has been shown to improve survival in a porcine model of septic peritonitis, as related to an increased phospholipid content of lipoproteins⁴⁰³. Finally, *in vitro* studies, in which LPS was added to the serum of septic patients, has shown that the distribution of lipoprotein-bound LPS among the major lipoprotein classes paralleled the phospholipid content of those classes³⁶⁴.

On the other hand, it has also been reported that LPS aggregates do not intercalate into phospholipid membranes in a non-specific hydrophobic manner⁴⁰⁴, indicating the necessity for proteins for association of LPS with lipoproteins. Although protein-free, TG-rich emulsion particles are able to bind LPS as shown by mobility shift assay on an agarose gel, the interaction between the emulsion particles and LPS is not strong enough to prevent dissociation of LPS in the blood¹⁵. Pre-incubation of high doses of emulsion particles with LPS before intravenous injection into rodents did not affect the kinetics of LPS¹⁵ nor the proinflammatory reaction to LPS²⁸¹ (**Figure 10**). Likewise, continuous infusion of a commercial protein-free, TG-rich, lipid emulsion (*i.e.* Intralipid) did not attenuate inflammatory responses to LPS in humans, albeit that plasma TG levels were 9-fold elevated prior to LPS administration⁴⁰⁵.

4.4.2. ApoE and the Lipopolysaccharide Response

The data thus indicate that the interaction between LPS and lipoproteins may not be mediated by simple intercalation of LPS into the amphiphilic phospholipid bilayer of lipoproteins, and that lipoprotein-associated apolipoproteins are important for the LPS binding properties of lipoproteins. Indeed, it was found that emulsions enriched with apoE, or even lipid-free apoE, avidly bind to LPS as evidenced by co-localization of ¹²⁵I-LPS and apoE upon agarose gel electrophoresis¹⁵. As with TG-rich lipoproteins³⁵⁴⁻³⁵⁶, apoE-enriched, TG-rich, emulsion particles, but not protein-free, emulsion particles, were able to prevent the association of LPS with

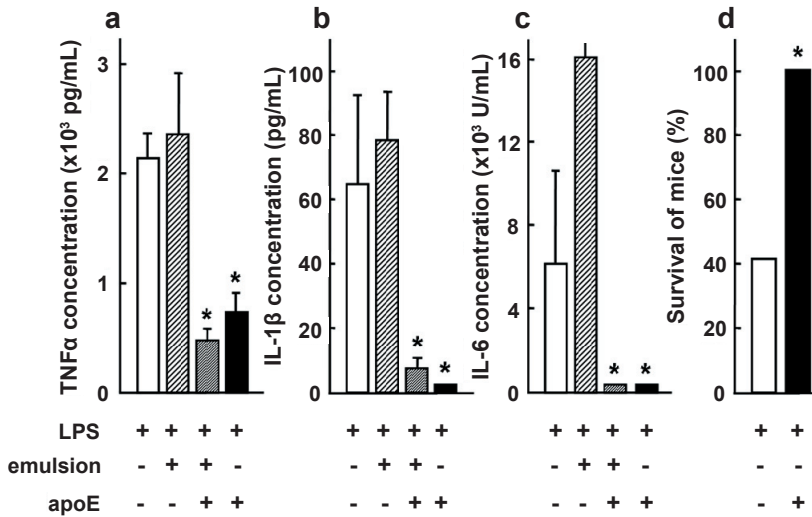


Figure 10. Effect of apoE on the biological response to LPS. ReLPS (*Salmonella Minnesota*; 25 μ g/kg) was intravenously injected into rats, in the absence or presence of protein-free emulsion particles (20 mg triglycerides/kg) and/or human apoE (800 μ g/kg). Concentrations of TNF α (A), IL-1 β (B), and IL-6 (C) were determined in plasma samples taken at 60, 90, and 120 min, respectively. Data are means \pm SEM (n=3). Alternatively, LPS (150 ng/kg) was intravenously injected with or without apoE (25 μ g/kg) into mice (n=7 per group) that were sensitized to the effects of LPS by intraperitoneal injection of 20 mg of D-galactosamine, and survival of mice was determined over a 3-day period (D). Adapted from Van Oosten *et al.*²⁸¹. Asterisks indicate significant differences as compared to the effects of LPS alone ($P < 0.05$).

macrophages in the liver (*i.e.* Kupffer cells) and spleen, and enhance the uptake of LPS by hepatocytes¹⁵. Strikingly, the same effect was observed with lipid-free apoE¹⁵, indicating that apoE may be a key lipoprotein component to mediate the protective effect of lipoproteins in endotoxemia and sepsis. Indeed, subsequent studies confirmed that apoE-emulsions and lipid-free apoE, but not protein-free emulsions, largely inhibited LPS-induced serum levels of proinflammatory mediators TNF α , IL-1 α , and IL-6. In fact, apoE was able to protect mice against LPS-induced mortality (**Figure 10**)²⁸¹.

Accordingly, apoE-deficient mice showed increased plasma levels of TNF α ²⁷⁹⁻²⁸¹, IL-6²⁷⁹, IL-12²⁷⁹, and interferon- γ (IFN γ)²⁷⁹ on intravenous challenge with LPS, and increased plasma TNF α levels after intravenous *K. pneumoniae* injection²⁸⁰. Concomitantly, apoE-deficient mice were more susceptible to death from intravenous LPS or *K. pneumoniae*²⁸⁰, the latter accompanied by increased bacterial outgrowth in their organs²⁸³. Since lipoprotein levels are severely increased in the apoE-deficient mice, as evident from 8-10-fold elevated plasma cholesterol levels, this indicated that increased lipoprotein levels *per se* do not necessarily lead to LPS neutralization. Interestingly, hypercholesterolemic LDLr/apobec-1 double knockout showed similar responses as wild-type mice²⁷⁹,

whereas for LDLr single knockout mice even opposite results were reported as compared to apoE-deficient mice⁴⁰⁶. Since these mice accumulate apoE-rich lipoproteins in their plasma, it is tempting to speculate that protection of these mice against endotoxemia is at least partly mediated by apoE.

Besides attenuating inflammatory responses by direct binding to LPS, apoE may also exert direct immunomodulatory effects by initiating a signalling cascade in macrophages, thereby down-regulating macrophage activation after exposure to a variety of structurally diverse stimuli^{279,407-409}. Recently, an unexpected proinflammatory property of apoE was described. Van den Elzen *et al.*¹⁶ showed that apoE was able to deliver lipid antigens via LDLr-mediated uptake into endosomal compartments in antigen presenting dendritic cells. Via this route, apoE mediates the presentation of serum-borne lipid antigens to initiate immunological response to these antigens. Since dendritic cells are capable of presenting LPS, a lipid antigen, on their surface to activate T-cells³³¹⁻³³³, apoE may also stimulate the presentation of LPS by dendritic cells. However, if so, the anti-inflammatory effects of apoE overrule this proinflammatory action²⁷⁹⁻²⁸¹.

4.4.3. Other Apolipoproteins and the Lipopolysaccharide Response

Besides apoE, other apolipoproteins have now also been shown to modulate the response to LPS, either by direct binding to LPS or by modulating the function of LPS-responsive cells. For example apoAI was shown to inhibit the LPS-induced release of cytokines by monocytes³⁹². Recently, apoAI was reported to bind LPS¹³, reduce cytokine levels in serum^{12,17,410}, and improved survival rates after LPS challenge^{12,13,17}. In addition, apoAI-deficient mice show increased LPS-induced serum levels of cytokines (*e.g.* TNF α , IL-1 β , IL-4, IL-6, IL-10), but this could also be attributable to the almost complete lack of circulating HDL in these mice⁴¹⁰. The effects of apoAI may be partially explained by direct effects of apoAI on LPS-responsive cells, since both apoAI^{17,411,412} and apoAII⁴¹² have been reported to down-regulate neutrophil adhesion, oxidative burst and degranulation. Another explanation for the *in vivo* findings with apoAI was proposed by Massamiri *et al.*⁴¹³, who showed that apoAI facilitates binding of the LBP-LPS complex to HDL. Blocking apoAI with antibodies directed against specific epitopes within apoAI reduced binding of LBP to HDL. In addition to apoAI itself, also apoAI mimetic peptides (*i.e.* L-4F) reduces LPS-induced cytokines, chemokines, and adhesion-molecules by macrophages *in vitro*⁴¹⁴.

Recently, it has been shown that apoAIV reduced the inflammatory response during experimental acute colitis (*i.e.* infection of the colon), presumably involving the inhibition of P-selectin-mediated leukocyte and platelet adhesive interactions^{414,415}. Moreover, Recalde *et al.*^{14,414} showed that apoAIV strongly inhibits monocyte activation as evident from a strong reduction in LPS-induced

TNF α production, while no effect of apoAI was detected under the applied experimental conditions^{14,414}. Although the mechanism remains unclear as yet, expression of human apoAIV in hyperlipidemic apoE-deficient mice reduced the development of LPS-stimulated atherosclerosis, as related to a reduced production of proinflammatory cytokines¹⁴.

5. Outline of this Thesis

In the first part of this thesis the role of apoCI in lipid metabolism will be further addressed. The severe hypertriglyceridemia, as observed in human apoCI transgenic mice^{133,159,416}, can not be completely explained by only the inhibitory effects of apoCI on the apoE-mediated uptake of TG-rich lipoproteins. Therefore, in **chapter 2** we aimed at elucidation of the main mechanism responsible for this apoCI-mediated severe hypertriglyceridemic phenotype. In **chapter 3**, we studied whether this main hypertriglyceridemic effect of apoCI was dependent of the VLDLr and/or apoCIII.

In the second part of this thesis the role of apoE in CVD in humans will be investigated. Human studies on apoE and CVD mortality have only focused on the role of the *APOE* genotype. However, independent of this genotype, plasma apoE levels are positively correlated with plasma cholesterol levels, indicating that irrespective of the *APOE* genotype high plasma apoE levels may be associated with increased CVD. Since the relation between plasma apoE levels and CVD mortality has not been studied yet, we investigated the association between plasma apoE levels and CVD independent of the *APOE* genotype in the Leiden 85-plus Study in **chapter 4**.

Finally, since we found by sequence alignment analysis that apoCI contains a putative LPS-binding domain in its C-terminal domain, we studied in the third part of this thesis the role of apoCI in Gram-negative sepsis. In **chapter 5** we assessed whether apoCI is indeed able to bind LPS, and whether apoCI is able to modulate the biological response towards LPS and survival in a murine Gram-negative sepsis model. These studies are extended in **chapter 6**, where we studied the structure-function relationship of apoCI with respect to modulating LPS by using apoCI-derived peptides. Since Barlage *et al.*³⁶⁵ showed that apoCI was virtually absent from HDL during human sepsis, we studied in **chapter 7** the time course of plasma apoCI levels in severely septic patients and correlated these plasma apoCI levels with survival. In **chapter 8** we investigated whether high plasma apoCI levels could protect against mortality from infection in the Leiden 85-plus Study, a prospective population based follow-up study.

The results obtained from these studies as well as implications for future research are discussed in **chapter 9**.

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