The ApoM/S1P-complex: its role in vascular inflammatory disease and interaction with S1P-receptors

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The ApoM/S1P-complex: its role in vascular inflammatory disease and interaction with S1P-receptors

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Title and subtitle The apoM/S1P-complex: its role in vascular inflammatory disease and interaction with S1P-receptors

Abstrac

HDL is believed to be protective against cardiovascular disease (CVD) via the reverse cholesterol transport and anti-inflammatory actions in the vessel. Apolipoprotein M (apoM) is an apolipoprotein mainly associated with HDL. Recently ApoM was proven to be the main carrier of Sphingosine 1-phosphate (S1P) in circulation. S1P is a signaling phospholipid involved in the immune system, exerting most of its effects through signaling via 5-G-protein coupled receptors; $S1P_{1-5}$.

The aim of this thesis is to investigate the role of the apoM/S1P-complex in vascular inflammatory diseases such as atherosclerosis and sepsis. We also want to study the interaction between the apoM/S1P-complex and the S1P-receptors.

We developed a liquid chromatography-tandem mass spectrometry method for S1P-quantification in plasma and cell extracts. We found that plasma levels of S1P and apoM were decreased in sepsis, levels reflecting the severity of the disease. The apoM/S1P-complex contributes to the anti-inflammatory effects exerted by HDL as shown *in vitro* by its inhibiting potential of pro-inflammatory adhesion molecules on the endothelial surface and by its increment of the endothelial barrier function. Plasma levels of apoM and S1P in type-1-diabetes (T1D)- patients (who have increased risk of developing CVD) were not altered compared to healthy controls. However, HDL-particles from T1D showed decreased anti-inflammatory effects which was not related to reduced presence of apoM and S1P. The apoM/S1P-complex could interact with all S1P-receptors as shown by internalization of fluorescently labelled S1P-receptors overexpressed in HEK293-cells. Interestingly, extracellular levels of apoM and S1P could determine which receptor was available at the cellular surface.

In conclusion, our data suggest apoM and S1P to have a role in acute and chronic inflammation. Future research could help us clarify how the apoM/S1P-complex signals through the different S1P-receptors in different inflammatory disorders and hence contribute in developing new therapies against diseases in the vasculature.

Key words lipoproteins, apolipoproteins, phospholipids, sepsis, atherosclerosis

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Till mamma och pappa

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Curie

Content

List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I. Frej C., Andersson A., Larsson B., Jun GL., Norström E., Happonen KE., Dahlbäck B. Quantification of sphingosine 1-phosphate by validated LC-MS/MS method revealing strong correlation with apolipoprotein M in plasma but not in serum due to platelet activation during blood coagulation. *Anal Bioanal Chem. 2015, 407; 8533-42.*

II. Frej C., Linder A., Happonen KE., Taylor F., Lupu F. and Dahlbäck B. Sphingosine 1-phosphate and its carrier apolipoprotein M in human sepsis and in Escherichia coli sepsis in baboons. *J Cell Mol Med. 2016, 20; 1170-81.*

III. Ruiz M., Frej C., Holmér A., Guo L.J., Dahlbäck B. HDL associated Apolipoprotein M limits endothelial inflammation by delivering sphingosine 1-phosphate to the S1P1 receptor. *In revision Arteriosclerosis, Thrombosis and Vascular Biology.*

IV. Frej C., Mendez A., Ruiz M., Hughes TA., Dahlbäck B., Goldberg R. Shift in ApoM/S1P from dense to light HDL-particles in Type 1-diabetes associated with impaired anti-inflammatory effects of the HDL/apoM/S1P-complexes. *Manuscript.*

V. Frej C., Ruiz M., Happonen K., Dahlbäck B. Activation and internalization of GFP-tagged S1P receptors in stable HEK293-cells by the recombinant apoM/S1P-complex and apoM-containing HDL. *Manuscript.*

Abbreviations

A1GP	α1-acid glycoprotein
ABCA1	ATP-Binding Cassette Transporter
Apo	Apolipoprotein
ApoB-TG	Human ApoB Transgenic mice
CETP	Cholesterol Ester Transfer Protein
CAV	Coronary Artery Disease
CHD	Coronary Heart Disease
IHD	Ischemic Heart Disease
CVD	Cardiovascular Disease
eNOS	endothelial Nitric Oxide Synthase
EDHF	Endothelium Derived Hyperpolarizing Factors
GFP	Green Fluorescent protein
GPCR	G protein coupled receptor
HAEC	Human aortic endothelial cell
HDL	High Density Lipoprotein
HDL-C	HDL-Cholesterol
ICAM-1	Intercellular Adhesion Molecule-1
LC	Liquid Chromatography
LDL	Low Density Lipoprotein
LDL-C	LDL-Cholesterol
LDLR	LDL-Receptor
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemotactic Protein-1
MI	Myocardial Infarction
MO	Microorganism
MOF	Multi Organ Failure
MS	Multiple Sclerosis
MS/MS	Tandem mass spectrometry
MTP	Microsomal Triglyceride Transfer Protein
NK	Natural Killer Cell
NO	Nitric Oxide
RapoM	Recombinant apoM
RCT	Reverse Cholesterol Transport
ROS	Reactive Oxygen Species

PAF	Platelet Activating Factor
PDB	Prostaglandin D synthase
PON1	Paraoxonase-1
S1P	Sphingosine 1-phosphate
S1PR	S1P-Receptor
S1PL	S1P-Lyase
Sphk	Sphingosine kinase
TG	Triglyceride
VCAM-1	Vascular Cell Adhesion-protein-1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
VSMC	Vascular Smooth Muscle Cell
VTE	Venous thromboembolism
TF	Tissue Factor
TLR	Toll-like receptor
TGF-β	transforming growth factor β

Introduction

High Density Lipoproteins (HDL) circulate in the blood stream carrying a variety of lipids and proteins important in maintaining the vascular haemostasis. The protein cargo in HDL particles is believed to be protective against cardiovascular diseases via the reverse cholesterol transport and through anti-inflammatory actions on the endothelial surface. In diseases like sepsis and coronary artery disease, the endothelial functionality is altered and is thought to be a part of the pathogenesis behind these diseases. In sepsis, HDL levels are decreased and in cardiovascular disease, the HDL functionality is shifted towards a pro-inflammatory phenotype. This makes HDL and its protein cargo potential pharmaceutical targets in vascular inflammatory diseases. It is important to study through which mechanisms HDL regulates inflammation in order to understand the best approach to pharmacologically target HDL in different pathological conditions. Candidates targeting enzymes leading to increased HDL-cholesterol levels have failed in their ability to decrease the incidence of cardiovascular disease (1). Moreover, recently the concept of HDL as a protective agent in cardiovascular disease was challenged when individuals having high HDL-cholesterol levels were shown not to be more protected against cardiovascular diseases than those with normal HDL (2). This suggests that it is not the HDL cholesterol *per se* that is vasculo-protective, but instead insinuates the HDL proteome to be important. Adequate treatment and management of sepsis patients are difficult and recently several clinical trials have failed in improving the outcome for the patients (3).

Apolipoprotein M (apoM) was discovered in prof. Dahlbäck's lab in 1999 and was shown to be involved in the cardio-protective effects of HDL. More importantly, apoM was shown to carry Sphingosine 1-phospate (S1P), a potent signaling molecule involved in immune regulating functions. This thesis will describe what my research has contributed to in understanding the role of the apoM/S1P-complex in inflammatory vasculature diseases and its interaction with S1P-receptors. Here I will give an overview on what I have been working with during the last 5 years.

Lipoproteins

The fat content in the western diet consists of triglycerides (TGs), phospholipids, cholesterol, fat-soluble vitamins and long-chain fatty acids. Most of our fat intake comes from TGs, which are the dominant source (90 %) of fat in both plants and animals. TGs are digested in the intestinal lumen by lipases, which remove two fatty acids from every triglyceride molecule thus generating one monoglyceride and two free fatty acids. Lipids are not soluble in the aqueous milieu in the intestine. Amphipathic bile acids, synthesized in the liver and secreted from the gall bladder into the small intestine, interact with the surface of the large lipid droplets and create small micelles that form a stable emulsion. Free fatty acids are absorbed from the micelles and easily transported across the cell membrane by diffusion into enterocytes lining the intestinal lumen. Cholesterol is transported via specific active transporters, such as NPC1L1, ABCG5 and ABCG8 (4). In enterocytes, absorbed free fatty acids are resynthesized into TGs in the smooth endoplasmic reticulum and together with cholesterol and proteins arranged into lipoproteins called chylomicrons. **Chylomicrons** are large (up to 500-1000 nm) particles consisting of triglycerides and apolipoprotein B-48 (apoB48), which is the main structural protein element. Chylomicrons are secreted via endocytosis into the lymph system and further via the thoracic duct into the venous blood stream just before entering the heart. Chylomicrons binds to endothelial lipoprotein lipases (LPLs) located on luminal surface of the endothelial cells where the TGs again break down into free fatty acids, which can be absorbed by cells. Inside the cell, TGs are again reformed and stored or used as energy through mitochondrial oxidation. The remaining chylomicron, the chylomicron remnant, acquires apoE, lose apoC and concentrates cholesterol and are cleared by the liver via apoE-mediated mechanisms (5). The loss of apoCs is believed to terminate the action of the LPL since it requires apoC-II as a cofactor (6). ApoE can clear chylomicron remnants through high affinity binding to the hepatic LDL-receptor (LDLR), LDL receptor-related protein and finally via direct binding to hepatic membranes via heparan sulfate proteoglycans (5, 7).

The liver can produce TGs as energy source for tissues, which are secreted as **Very Low Density Lipoproteins** (VLDLs). VLDL is enriched in TGs and apoB100 is the main protein, but VLDL can also contain apoC-I, C-II, C-III and E (8). When entering the blood stream, VLDL is exposed to LPL, enabling tissues to

absorb free fatty acids (9). After LPL action on VLDL, **Intermediate density lipoprotein** (IDL) is formed, which is enriched in cholesterol and quickly transformed to **low density lipoproteins** (LDL). LDL particles are stable and consist of mainly cholesterol and they transport the majority of the cholesterol in human plasma. LDL is taken up by cells mainly via binding to the LDLR, which is ubiquitously expressed in humans, but particularly prevalent in the liver (10). A figure illustrating the different sizes and densities of lipoproteins is shown in Fig 1.

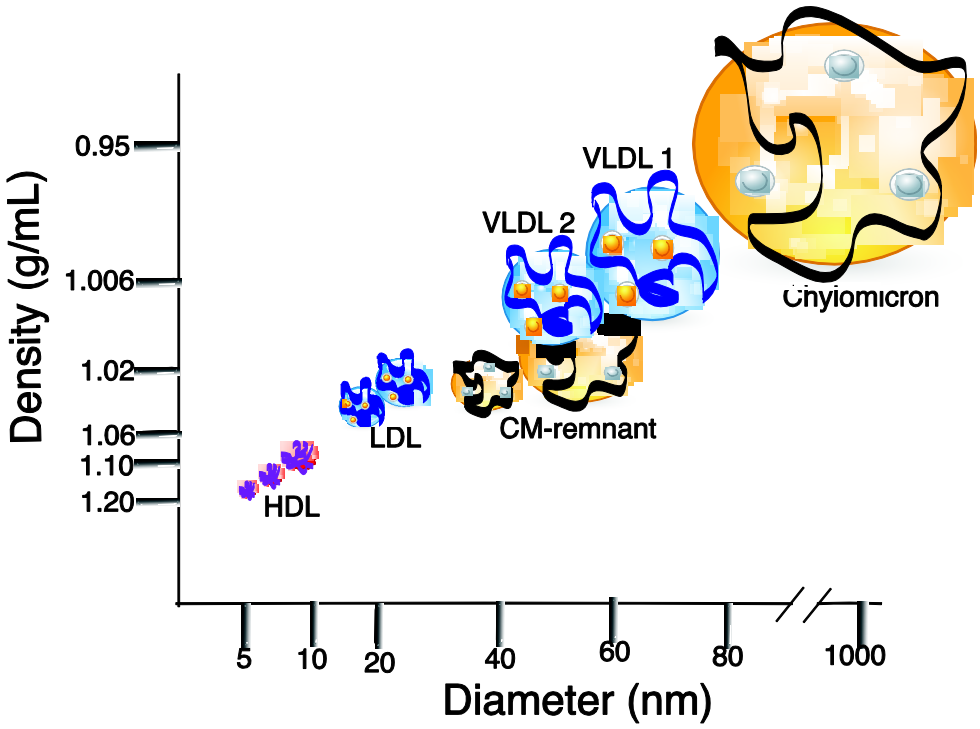

Figure 1: Lipoprotein subclasses

High Density lipoproteins (HDL) are produced by the liver (70-80 % of total HDL) and the intestine as small disc-shaped, lipid poor particles (11). HDL is the smallest lipoprotein (10 nm) with the highest density and can be sub-classified either by its protein content, density or size. The HDL population is highly diverse containing of up to 70 % of protein (by weight) and around 200 different proteins have been identified in the HDL proteome (12). The HDL-particle consists of an outer amphiphilic envelope composed of phospholipids (mainly phosphatidylcholine), un-esterified cholesterol and proteins covering an inner core of esterified cholesterol and TGs (13). ApoA1 is the main structural protein in

HDL and several apoA1 proteins arrange into a three dimensional structure, which stabilizes the lipids and proteins in HDL (14).

Based on its buoyant density, HDL can be divided by ultracentrifugation into three subgroups; the larger HDL_2 (1.063 g/mL <d<1.125 g/mL), the smaller HDL_3 (1.125 g/mL<d<1.210 g/mL) and the very small HDL_3 (> 1.210 g/mL) (15). Applying gradient gel electrophoresis, HDL can be separated into 5 subgroups based on its diameter; HDL2a (8.8-9.7 nm), HDL2b (9.7-12.9 nm), HDL3a (8.2 nm-8.8 nm), HDL3b (7.8-8.2 nm) and HDL3c (7.2-7.8 nm) (15). As disc-shaped HDL circulates in the blood stream, it absorbs cholesterol and phospholipids from peripheral macrophages via binding to the ATP binding cassette transporter (ABCA1) forming nascent HDL (pre βHDL). ABCA1 is a transcellular membrane protein ubiquitously expressed in the human body and binds to apoA1 in HDL enabling lipid secretion from cells to HDL (16). Cholesterol in nascent HDL is esterified by lecithin cholesterol acyltransferase (LCAT), which promotes the formation of mature spherical αHDL (17). Mature HDL can then further take up cholesterol via binding to ABCG1 or SR-B1 and transport cholesterol to the liver via binding to hepatic SR-B1 (reverse cholesterol transport, RCT). Alternatively, cholesterol can be transferred to VLDL/LDL via cholesterol ester transfer protein (CETP). HDL exchanges esterified cholesterol with TGs from VLDL and LDL via CETP, generating TG-rich HDL (18). HDL enriched in TGs is a substrate for LPL and subjects with TG-enriched HDL have an increased clearance of apoA1 and together this lead to the clearance of HDL from the circulation (19). As shown in transgenic animals and loss of function mutation in the human lipoprotein lipase-gene, endothelial lipase strongly regulates plasma HDL-levels (20, 21). A schematic picture of the lipoprotein metabolism is illustrated in Fig 2.

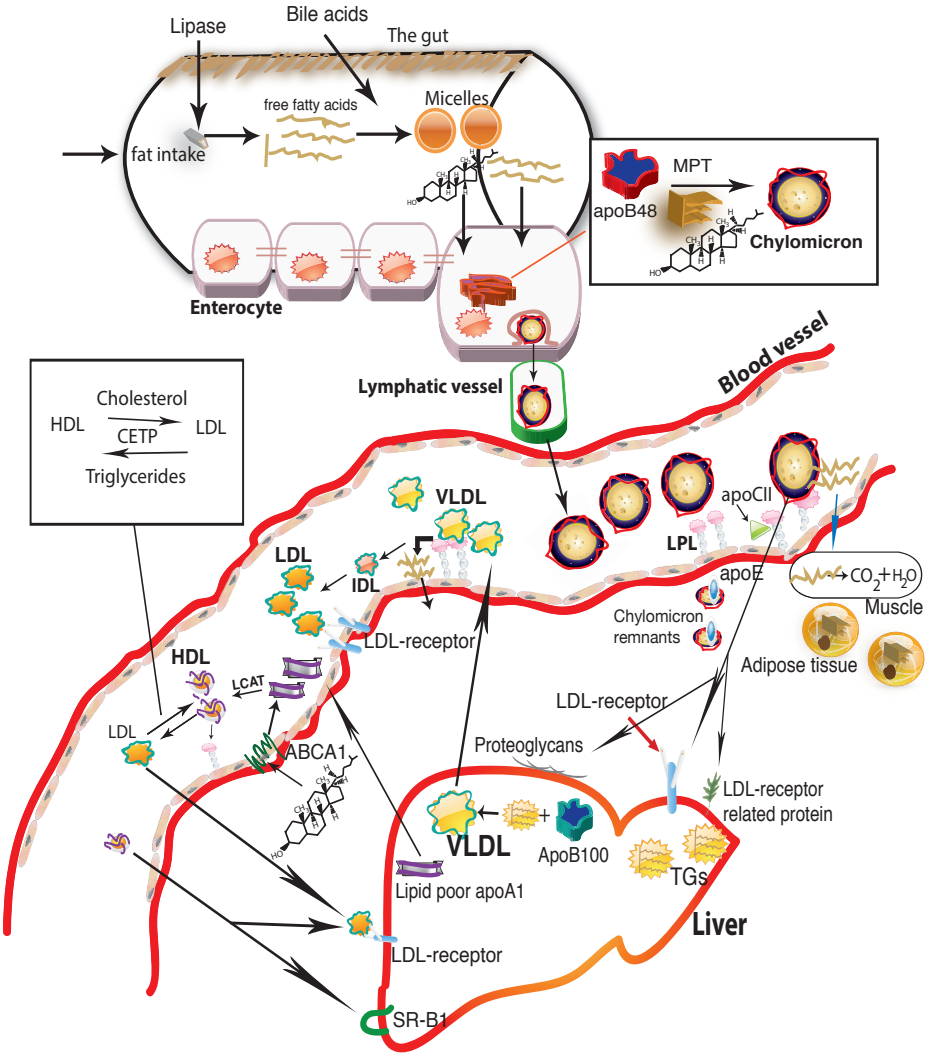

Figure 2: Lipoprotein Metabolism

Apolipoprotein M

The major functions of apolipoproteins in the circulation are i) formation of lipoproteins, ii) shaping and stabilizing the structure of lipoproteins, iii) providing functional properties to lipoproteins for example binding to the lipoprotein receptors and iiii) activate enzymes involved in lipoprotein metabolism (8). A description of the most important functions of the different apolipoproteins is enclosed in appendix 1.

Discovery of a new apolipoprotein

Apolipoprotein M (apoM) was discovered in 1999 by prof. Dahlbäck's laboratory (22). After isolation and delipidation of lipoproteins from lipid-rich human plasma, Xu and Dahlbäck performed N-terminal sequencing of proteins present in the lipoprotein fraction. A new sequence was discovered, which did not match any known protein when analysed by BLAST searches. However, several human-expressed sequence tags were found to match, which lead to the successful cloning and sequencing of apoM. ApoM was analysed by western blot using specific antibodies raised in rabbits against amino acid sequences corresponding to the identified sequence of apoM. ApoM was shown to be decreased in lipid-free plasma and mostly associated with HDL and hence was defined as a new apolipoprotein (22). The N-terminus did not contain any peptidase cleavage site and apoM was secreted in the presence of microsomes, suggesting that a retained signal peptide was used to anchor into the lipoproteins phospholipid monolayer (as illustrated in Fig 3). This was further confirmed in a study by Axler *et al.* where recombinantly expressed apoM (rapoM) lacking the signal peptide did not co-elute in a gelfiltration system with human HDL as did wild type rapoM (23).

ApoM, a member of the lipocalin family

Via molecular modeling and subsequently via X-Ray crystallography, apoM was initially shown to structurally belong to a ligand-binding family of proteins, called lipocalins (24, 25). One characteristic feature highly preserved in this protein family is the presence of an eight-stranded antiparallel β-barrel forming a

hydrophobic pocket, in which small hydrophobic ligands can bind (26). This was discovered to be true also for apoM, which upon crystallization was shown to carry a myristic acid, a serendipitous finding, which resulted in the identification of sphingosine 1-phosphate (S1P) as the natural ligand of apoM (25). ApoM contains 6 cysteines, which forms three disulfide bridges attaching the N-terminus and the C-terminus to the protein core (25). Upon secretion from HEK293 cells, apoM is glycosylated at one of its two possible glycosylation sites; Asn135. ApoM in isolated HDL from human plasma is partially glycosylated as revealed by two bands upon immunoblotting using specific apoM- antibodies (22). Searches for homologous structures via protein databases found that apoM was mostly related to a lipocalin called human α1-acid glycoprotein (A1GP), the complement protein C8γ and to prostaglandin D synthase (PDB) (25). A1GP is increased in acute coronary syndrome and high levels are associated with an increased risk of type 2-diabetes (27). Patients with atrial fibrillation who have high levels of PDB, have increased bleeding and higher mortality compared to those with low PDB levels (28). The complement protein C8γ is involved in complement-mediated cell lysis but its main function is unknown (29). Thus these structurally related proteins do not seem to have similar functions as apoM, which is believed to be an anti-inflammatory molecule as discussed later.

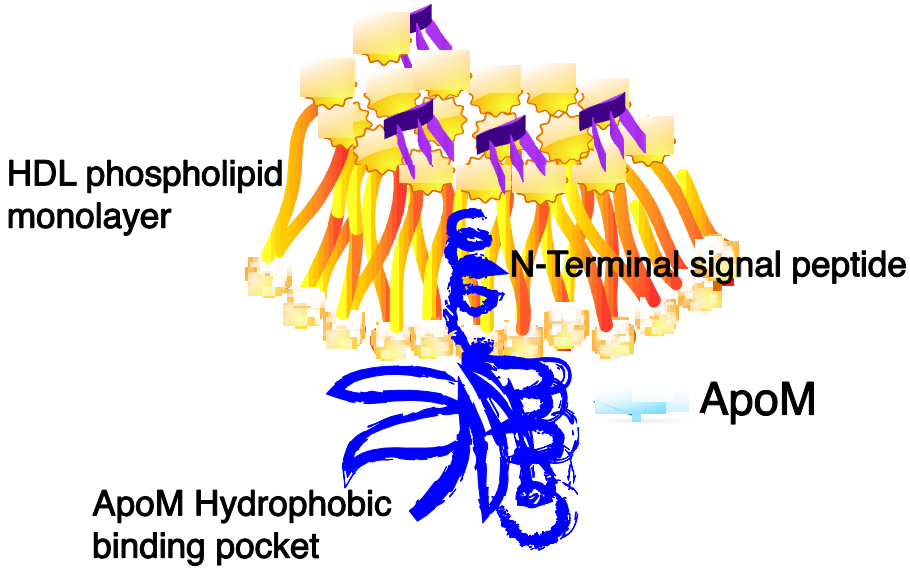

Figure 3: ApoM binding to the phospholipid monolayer in HDL via its signal peptide

ApoM distribution

Human apoM is mainly produced by the liver and is secreted into plasma where 5 % of the HDL particles contain apoM. It is also expressed in the kidney from where it is believed to be secreted into the urine and reabsorbed via binding to the Megalin receptor in the kidney proximal tubule cells (22, 30). However, the involvement of the LDL-receptor related protein chloride-proton exchanger CIC5 in tubular apoM/S1P- reabsorption was shown in $Cicn5^{-/-}$ mice, which had increased levels of apoM and S1P in the urine (31). ApoM is well preserved among different species (rabbit, cow, dog, mouse and rat) indicating an essential vital role of apoM (22) and we found that similarly to humans, baboons also express apoM in the liver and kidney (paper II).

ApoM in lipoproteins and lipoprotein metabolism

ApoM is mostly associated with HDL in human plasma where apoM is present in HDL-fractions corresponding to densities between 1.07-1.21 g/L and sizes of 8 nM or 10-12 nM particles (HDL_2 and HDL_3-particles) (32). In addition, apoM-associated lipoprotein particles contain apoJ, apoA1, apoA-II, apoC-I, apoC-II and apoC-III. ApoM is present to a smaller extent on LDL-particles and LDL containing apoM particles are slightly smaller than LDL particles lacking apoM (32). Interestingly, HDL containing apoM can protect both HDL and LDL from oxidation better than HDL lacking apoM (32). When the same experiment was done with LDL, LDL containing apoM was not more anti-oxidative compared to LDL lacking apoM (32).

Murine ApoM in mice

The *in vivo* role of apoM in lipoprotein metabolism has mostly been studied in mice. These studies have provided important information about the potential role of apoM in lipoprotein metabolism. However, one should be aware of that there are several factors in mice lipoprotein metabolism that are different from the human lipoprotein metabolism. For example, in mice cholesterol circulates mainly as HDL-cholesterol (HDL-C) whereas in human it is mainly as LDL-cholesterol (LDL-C). Mice do not develop atherosclerosis uncompelled but need to be genetically modified by knockdown of either LDL-R or apoE or both to develop human-like atherosclerosis (33). In genetically modified mice it takes months to develop atherosclerosis whereas it takes years in humans. Mice do not express CETP, which is an important protein in the human lipoprotein metabolism (33).

Unlike human apoM, murine apoM is not glycosylated (34). However, similarly to the human counterpart it is mostly associated with HDL to which it is bound with the retained signal peptide (34). It shares 80 % of the amino acid sequence with human apoM. ApoM is expressed from day 10 in the mouse embryo (34). In apoE$^{-/-}$ mice feed a chow diet, apoM is associated with HDL but when changing the diet to high fat and high cholesterol diet, apoM shifts towards the VLDL-sized particles (34). Whether this shift has any functional relevance for apoM remains to be elucidated. In apoA1$^{-/-}$ mice, plasma apoM is decreased by 33 % despite no alteration in liver apoM mRNA expression. This suggest that there is an altered translation, secretion or clearance pattern of apoM when apoA1 is not present (34).

Human ApoM in mice

To study functions of human apoM in the lipoprotein metabolism, human apoM transgenic mice (ApoM-Tg) and apoM-deficient mice (apoM$^{-/-}$) have been used (35). In addition, apoM metabolism has been investigated in several transgenically modified lipoprotein mouse models such as; LDLR$^{-/-,}$ apoE$^{-/-,}$ human ApoB transgenic mice (apoB-Tg), apoA1$^{-/-}$ and mice defective in LDL receptor-related protein (Lrp$^{n2/n2}$) (36). Upon overexpression of apoM, both total cholesterol and TG levels are increased whereas they are decreased when depleting apoM (37). Mice which have reduced apoM-expression in the liver have reduced HDL-C levels (~25 %) and overexpression of apoM results a in two-fold increase of HDL-C (37). The fraction of apoM in VLDL/LDL is increased compared to controls in LDLR$^{-/-}$ mice although most of apoM is still found in the HDL-fractions (36). Injecting apoM-enriched HDL into LDLR$^{-/-}$ mice results in a quick transfer of apoM to VLDL/LDL but this does not occur in WT-mice (36). Clearance of VLDL/LDL from the circulation is decreased upon injection of VLDL/LDL particles enriched in apoM compared to the clearance VLDL/LDL depleted of apoM, which is also observed for HDL (118)(38). Humans carrying LDLR-mutations or apoB-100-mutations have increased apoM plasma levels compared to noncarriers (38). This indicates that LDL and apoB are inovolved in apoM metabolism. Moreover, the presence of apoM on lipoproteins decreases lipoprotein elimination which prolongs the circulation time, hence indicating that it is most favourable for us when apoM is associated with HDL. Besides its role in the lipoprotein metabolism, the role of apoM has been a mystery and the main physiological ligand for apoM was for a long time unknown. A revolutionary finding regarding the role of apoM was the discovery that it is the main carrier of signaling phospholipid S1P in circulation (25, 39).

Sphingosine 1-phosphate

The first evidence for the existence of S1P was in 1973 when it was found that labeled sphingosine, the precursor of S1P, formed phosphorylated products in platelets (40). Later on in the 80's, sphingolipids gained a lot of interest when it was discovered that sphingosine simulated cell growth via inhibiting protein C (41). S1P was then proven to be involved in the sphingosine-mediated intracellular release of Ca^{2+} and to induce proliferation of fibroblasts (42). Since then, extensive research has been carried out and S1P is now known to be involved in immune cell trafficking, mediating endothelial permeability, stimulating survival and inhibiting apoptosis of endothelial cells and regulating bone homeostasis (43, 44). S1P may exert intracellular actions, however, most of its signaling properties are mediated from the extracellular space through 5 different G-coupled receptors (GPCRs); $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$. S1P in plasma is mainly produced by platelets, erythrocytes and endothelial cells, but cells like mast cells and macrophages can also produce S1P (45-47). Both erythrocytes and platelets express S1P-producing enzymes, whereas they lack expression of S1P-degrading enzymes (46, 48). The extracellular secretion of S1P from erythrocytes and platelets is believed to be mediated via ATP-mediated transport whereas secretion of S1P from endothelial cells has been shown to be regulated via the S1P transporter spinster homolog 2 (Spns2) (49-52). Platelets release S1P from two pools, one which is readily available constitutively releasing S1P and one which is present in α-granules and requires platelet activation for release (53). It was recently discovered that S1P is carried by apoM in HDL (60 %) and by albumin (40 %) in the circulation (39). Here I describe S1P metabolism and the present findings about S1P in inflammation and regulation of vascular tone.

S1P metabolism

Sphingolipids are structural components in cellular membranes and they are highly abundant in membrane microdomains such as lipid rafts and caveloae, suggesting a role in cellular signaling and transmembrane trafficking (54). The most studied sphingolipids are ceramide, sphingosine and S1P. Ceramide is composed of a N-acetylated sphingosine (C18) backbone and is a key player in the phospholipid

metabolism. Ceramide is formed de novo via condensation of serine and palmitoyl-CoA by serine palmitoyl-transferase to form 3-ketosphinganine which is further reduced to sphinganine and finally via N-acetylation transformed into ceramide (55). Ceramide can also be generated via degradation of sphingomyelin where sphingomyelin is hydrolysed by sphingomyelinases (SMases), which is thought to be the most abundant way to generate ceramide (56). Finally, ceramide can be generated by catabolism of complex phospholipids which are broken down to sphingosine which regenerate ceramide via re-acylation by ceramide synthase (55). In contrast to S1P, ceramide has pro-apoptotic effects and can induce cell cycle arrest (227).

Ceramide is produced as a response to stress due to factors such as TNFα, IL-1 and UV-light, which activate sphingomyelinases leading to release of ceramide (57). In frozen tissue sections isolated from different areas of the brain investigating alterations in gene expression in dementia and alzheimer, genes involved in *de novo* synthesis of ceramide were shown to have increased expression, which could contribute to neurodegenerative effects via increased apoptosis (58).

S1P is generated via breakdown of ceramide by ceramidases, resulting in sphingosine. Sphingosine is phosphorylated by two specific kinases, sphingosine kinase 1 (sphk1) and sphk2 in order to generate S1P. S1P can then be degraded reversibly to sphingosine via de-phosphorylation by specific S1P-phosphatases (Spp1 and Spp2) and unspecific sphingolipid phosphatases, or irreversibly to hexadecenal and ethanolamine-1-phosphate by S1P Lyase (S1PL) (43, 59). A description of the S1P-metabolism is illustrated in Fig 4. Ceramide is pro-apoptotic whereas S1P is anti-apoptotic. Thereby enzymes involved in the ceramide-sphingosine-S1P rheostat determine the levels of their respective sphingolipid and hence control the fate of the cell (60).

Sphk1 and Sphk2

Sphk1 and Sphk2 originate from different genes, i.e *SPHK1* and *SPHK2* and are highly conserved between species (61). SPHK2 encodes for a protein larger than SPHK1 enclosing an additional 236 amino acids.

Despite sphk1 and sphk2 exerting the same reaction, i.e. phosphorylation of sphingosine, they are localized in different subcellular compartments, expressed by different organs and have different cellular effects. Murine Sphk1 is expressed mostly in the spleen, lung and liver, whereas murine sphk2 is mostly expressed in the liver and heart. Human sphk2 is expressed in kidney, liver and brain (62).

Sphk1 is localized in the cytosol and its activation promotes cell survival and proliferation via intracellular production of S1P as shown in fibroblasts, T-cells and endothelial cells (63, 64). Upon activation, sphk1 is translocated to the cell membrane where it preferentially binds phosphatidylserine or filamin A. (65, 66). The binding between filamin A and sphk1 seems to be important for the cytoskeleton rearrangement effects exerted by these proteins such as lamellipodia formation and cell migration.

In contrast, sphk2 is targeted to the nucleus via its nuclear localization signal (NLS) sequence, where it inhibits DNA synthesis and induces cell cycle arrest (67). Sphk1 and sphk2 are important for embryonic neurogenesis and angiogenesis. Thus, mice lacking both the enzymes die at E12.5 due to extensive bleeding and exencephaly (cranial tube defect) (68). The pathogenic mechanism behind the undeveloped nervous system was an increment of apoptosis in the neuroepithelium in the mesencephalon (midbrain) and rhombencephalon (hindbrain) (68).

The two kinases have shown be involved in the inflammatory response via intracellular production of S1P (69). They show opposite involvement in inflammatory diseases, however published data are contradictive. In a murine collagen induced arthritis model, knockout of sphk1 resulted in a less aggressive phenotype with lower amounts of pro-inflammatory cytokines, whereas the opposite was seen in sphk2 $^{-/-}$ mice (70). In contrast, knocking out sphk2 in kidney ischemia-reperfusion injury worsened the histological damage and kidney function, which was not seen in sphk1$^{-/-}$ mice (71). Moreover, sphk1 was more important in the degranulation and antigen oriented migration of mast cells compared to sphk2, however both kinases were important for cytokine release in this cell type (72). In paper II we observed a strong increase in sphk1 transcription already 6-8 hours post *E.coli* i.v. infusion in baboons whereas sphk2 transcription was inhibited. These results suggest that both sphks are involved in the immune response. However, sphk1$^{-/-}$ mice developed a similar inflammation response as wild type in a peritonitis model (69). Moreover, in a recent study, none of the sphks were required for NF-κB activation and subsequent cytokine release in murine macrophages (73) indicating that the kinases may have varying functions in different cells.

S1P-Lyase

S1P-lyase (S1PL) is the only enzyme irreversibly degrading S1P, making it important in regulating intracellular levels of S1P. S1PL is located in the endoplasmic reticulum as an integral membrane protein. S1PL catalyses the

degradation of S1P in the cytosol via cleavage of the C_{2-3} carbon bond, leading to the formation of hexadecenal and ethanolamine phosphate (74, 75). S1PL is a 63.5 KDa protein encoded by the *SGPL1* gene. Murine S1PL is highly expressed in the small intestine and thymus whereas modest expression is found in liver, kidney, lung, stomach and virtually no expression in platelets (75). Even though the expression is low in the liver, the hepatic activity is equally strong as in the spleen, which has high expression (75). Studies of S1PL in human tissue revealed high expression in liver and kidney whereas no expression was found in platelets and erythrocytes (76). The importance of S1PL in liver function was shown in S1PL deficient mice, which had hyperlipidemia with increased levels of VLDL, LDL, HDL, serum TGs as well as increased S1P serum levels (77). These results suggest that hepatic S1PL is involved in regulating circulating S1P-levels as well as cholesterol metabolism.

Through the activity of S1PL, S1P levels are kept low in tissues creating a S1P-gradient between tissues and blood. This gradient is important for lymphocyte egress from secondary lymphoid organs (78). Mice treated with an S1PL-inhibitor have a 100-fold increase in S1P in lymphoid tissues whereas S1P in the circulation is normal (78). This interruption of the S1P-gradient leads to lymphopenia in the circulation and accumulation of lymphocytes in the thymus (78). In contrast to Sphk1 and 2, $S1PL^{-/-}$ mice are viable upon birth, however they are smaller and have a shorter life span (29 days) compared to their littermate controls (79). $S1PL^{-/-}$ mice have virtually no circulating T-lymphocytes and very low levels of B-lymphocytes. However, their total leukocyte levels are normal due to a compensatory increase in neutrophils and monocyte numbers (79). Targeting S1PL in mice results in decreased severity of collagen induced arthritis and currently pharmaceutical companies are developing pharmaceutical compounds targeting S1PL in multiple sclerosis (80). These results suggest that S1PL is an important regulator of immune cell trafficking mainly via controlling S1P levels in lymphoid organs. However, suppression of S1PL activity by administration of S1PL-inhibitor to wild type mice or using $S1PL^{+/-}$ mice it was showed that S1PL inhibition in LPS induced lung injury reduced the release of IL-6 and protected the endothelial barrier via increased S1P-levels in lung tissue (81).

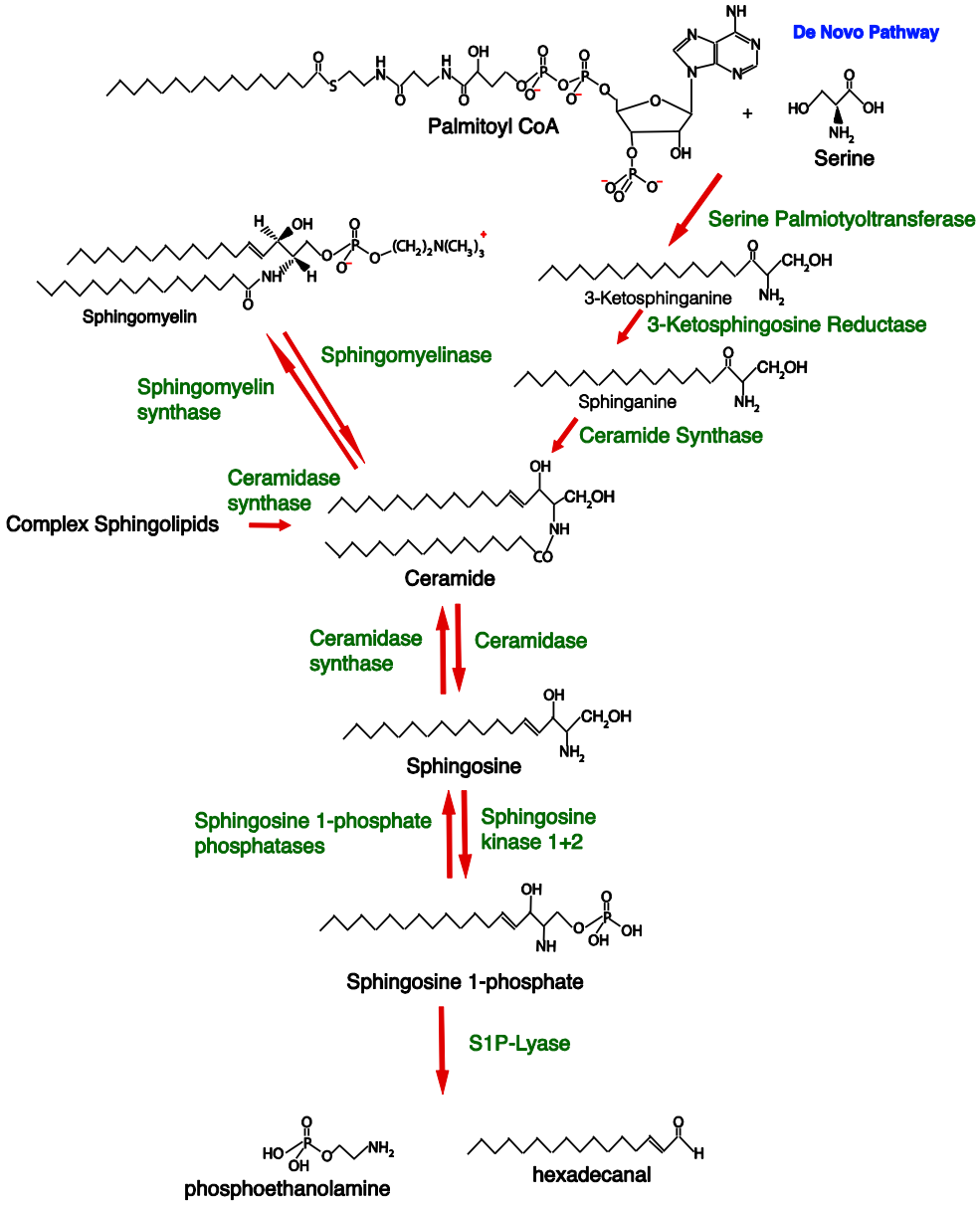

Figure 4: S1P metabolism

S1P receptors

GPCRs are one of the biggest receptor family in the human genome, consisting of up to 800 genes (82). GPCRs are made of 7 transmembrane domains with the C-terminus located in the intracellular space and the N-terminus towards the extracellular space. GPCRs can be grouped into 6 subgroups (A-F) based on their sequence homology and S1P-receptors (S1PRs) belongs to group A (82). The S1PR family was first named the endothelial differentiation gene (Edg) family of receptors. This name was based on the finding that Edg1 ($S1P_1$) was induced upon administration of growth inhibitors in endothelial cells (83). The Edg-receptors were named in the order they were discovered and later the name was changed to S1PR after the discovery of S1P as their major ligand (84, 85). S1P is important for many immune regulatory functions through signaling via its receptors. S1P activates Rac and Rho GTPases which are important for cytoskeleton rearrangement and regulates migration and chemotaxis of many cells of the immune and vascular systems (86). In addition, S1P can potentiate TLR4-mediated secretion of cytokines upon LPS stimulation in primary human gingival epithelial cells (87). Extensive research has been carried out in order to clarify the signaling effects of S1P through its receptor. How the effects of S1P in the vascular and immune systems are regulated by the different carries, i.e HDL and albumin, remains to be clarified. In the chapters presenting the respective receptors, I describe current findings about the S1P-singaling through each S1P-receptor which I have summarized in Fig 5.

$S1P_1$ is the most well studied receptor abundantly expressed in the human body. In a study in mice where relative expression of 353 GPCRs was evaluated, $S1P_1$ and $S1P_3$ stood out as ubiquitously and abundantly expressed (83). $S1P_1$ is important for vascularization and neurogenesis in mice (88). $S1P_1^{-/-}$ mice have reduced amounts of vascular smooth muscle cells (VSMCs), which result in an improperly matured vascular system that leads to embryonic death (between E12.5 and E14.5) due to excessive bleeding (88). S1P is also important for angiogenesis in adult mice and mature tissues. During the clotting process, S1P is released from platelets, making endothelial cells migrate and proliferate, enabling new vessel formation that supports the healing process during vascular thrombosis (89). As shown in paper I, S1P released from platelets is mainly bound to albumin suggesting a unique role of the different S1P-pools.

The chemotactic effect of S1P is abolished by pertussis toxin, which blocks signaling via the G_{ai}-protein, the signaling G-protein for $S1P_1$ (89, 90). HDL, which via apoM carries 60 % of plasma S1P was also able to induce endothelial cell migration that was dependent on the activity of endothelial lipase (91). $S1P_1$ is also important for immune cell migration. It is expressed by lymphocytes and

regulates their egress from secondary lymphoid organs (92). The S1P-gradient between lymph and blood is important for the trafficking of immune cells expressing the $S1P_1$ receptor. The $S1P_1$ receptor is located in the cell membrane at low S1P-levels and internalized at high S1P-levels (as shown in paper V), which enables T-lymphocytes to sense the S1P-gradient. A S1PR-antagonist named Fingolimod (FTY720) was the first oral drug against multiple sclerosis (MS), a disease where the immune system degrades the myelin sheet around neurons (93). Fingolimod degrades $S1P_{1,}$ which makes lymphocytes unable to egress from lymph nodes, thus inducing lymphopenia and inhibition of attacks of the myelin sheet, which are observed in MS (94, 95). The $S1P_1$/S1P-signalling axis in T-lymphocytes is also important for lymphocyte trafficking during an infection (96).

Further, $S1P_1$ is important for cellular barrier functions. The first evidence of this was shown by overexpressing $S1P_1$ in HEK293 cells, which lead to increased expression of P- and E-cadherin and increased cell aggregation (85). This was further supported by several studies. In human and bovine pulmonary artery cells, S1P was able to enhance the trans-monolayer electrical resistance (TEER) mainly via $S1P_1$ but also to some extent via $S1P_3$ and $S1P_2$ (97). $S1P_1$ induces endothelial barrier strengthening via assembling N-cadherin tight junction between endothelial cells and underlying mural cells (98). Administration of S1P in a cremaster muscle leakage model in rats could not rescue histamine-induced vascular leakage (99). However, the plasma level of the arterially injected S1P at a dose of 0.0019-0.38 mg/kg body weight was not measured (99). In the same model, administration of a $S1P_1$ agonist could significantly inhibit histamine-induced vascular leakage, whereas injection of a specific $S1P_2$ antagonist promoted protective effects of S1P (99). These data suggest that $S1P_1$ decreases endothelial barrier function whereas $S1P_2$ increases endothelial permeability.

Using $S1P_1$ GFP signaling reporter mice (where activation of $S1P_1$ leads to the expression a nuclear associated green fluorescent protein) it was observed that $S1P_1$ was activated under normal conditions in lymphoid and spleen endothelial cells. After administration of an $S1P_1$-agonist or LPS, activation was seen in vascular endothelial cells and hepatocytes (100). Using the same model, Galvani *et al*. showed that $S1P_1$ protein expression was enhanced in arterial endothelial cells upon an inflammatory challenge (101). $S1P_1$ was especially active in areas exposed to low shear stress, which are known to have increased susceptibility to atherosclerosis due to increased exposure time to pro-atherosclerotic molecules (102).

$S1P_2$ (Edg5) is also expressed in the endothelium and couples to G_i, G_q and in particular with $G_{12/13}$ –proteins (103). As discussed above $S1P_2$ has opposite effects on the endothelial barrier compared to $S1P_1$. It disrupts the barrier as shown by decreased assembly of N-cadherin at adherent junctions and inhibition of actin

polymerization upon overexpression of $S1P_2$ in human endothelial cells (104). $S1P_2$ also decreases TEER in endothelial cells mediated via downstream signaling through Rho-associated kinase (ROCK) and PTEN and reduced Rac activation (104). In mice, administration of an $S1P_2$ antagonist augments S1P mediated Rac activation and migration (105). In contrast to $S1P_1$, $S1P_2$ inhibits migration and proliferation via activation of Rho GTPase in fibroblasts, Chinese hamster ovary cells and hepatocytes (106-108).

$S1P_2^{-/-}$ mice are deaf at one month of age due to an impaired epithelial barrier in the primary vasculature in the cochlea (the inner ear) (109). The expression of $S1P_2$ is induced upon hypoxia in retinal vascular cells (110). When $S1P_2$ is present in the retina, an increased infiltration of immune cells is observed compared to wild type (110). Mice lacking $S1P_2$ are viable but when knocked out in combination with $S1P_1$ the mice die earlier than $S1P_1^{-/-}$ mice because of severe impaired development of the vascular branches (111). Taken together, $S1P_2$ inhibits cell migration, proliferation and disrupts cell barriers mainly via activation of the Rho GTPase.

$S1P_3$ (Edg3) signals via the same G-proteins as $S1P_2$ (103). $S1P_3$ mediates cell chemotaxis towards S1P via phosphoinositide 3-kinase and Rac activation (107). $S1P_3$ is important for VEGF-induced sprouting of endothelial cells as showed in vitro and ex vivo (112). $S1P_3$ also regulates coronary flow via activation of Rho in VSMCs (113).

Via signaling through $S1P_3$, S1P upregulates transcription of metalloproteinases 9 and mediates pro-invasive and migratory phenotypes in human breast epithelial cells (114). More evidence towards a pro-inflammatory role of $S1P_3$ was shown in a study by Mukarami *et al*. demonstrating that silencing of $S1P_3$ protected mice from developing severe pulmonary fibrosis independently of S1P levels in the Broncho-alveolar lavage fluid (115).

$S1P_3$ regulates the function of VSMCs and dendritic cells. Mice deficient in $S1P_3$ specifically in their dendritic cells were protected from kidney ischemia-reperfusion injury via expansion of immunosuppressive regulatory T-cells (116). In a murine sepsis model, inhibition of $S1P_3$ and PAR-1 receptor crosstalk restrained spreading of the inflammation (117). The $S1P_2$ and $S1P_3$ receptors can compensate for the loss of one of the receptors as shown in knock out mice models. In a myocardial ischemic reperfusion injury model, single knockout mice of $S1P_2$ and $S1P_3$ had similar infarct size as wild type. However, in double knockout mice the damaged area increased by 50 % (118). Interestingly, HDL was proven to reduce infarct size and reduce immune cell infiltration in the infarcted area via $S1P_3$ signaling (119). When investigating how the vasculature is developed when silencing the $S1P_1$, $S1P_2$ and $S1P_3$, the double $S1P_2$ $S1P_3$ knockout was not viable due to an increased pathologic morphology compared to single

knockout mice, which were viable (111). In conclusion, $S1P_3$ mediates pro-migratory effects via Rac-activation and upregulation of metalloproteinases. Moreover, $S1P_3$ exert pro-inflammatory effects in atherosclerosis and fibrosis, whereas is it protective in ischemic heart disease.

$S1P_4$ (Edg6) signals via G_i and $G_{12/13\ (120)}$. $S1P_4$ is mostly expressed in lymphoid tissue and regulates cell motility via Rho GTPase and induces cell rounding and the formation of stress fibers (120). $S1P_4$, in contrast to $S1P_1$, does not affect lymphocyte trafficking and $S1P_4^{-/-}$ mice have normal levels of circulating lymphocytes (121). However, dendritic cell migration and cytokine release are heavily affected in $S1P_4^{-/-}$ mice and they display dysfunctional T_H17-cell differentiation (121). $S1P_4$ deficiency potentiated T_H2-mediated immune responses and inhibited T_H1 related reactions suggesting $S1P_4$ to be involved in the regulation and direction of the immune response (121).

$S1P_5$ (Edg8) signals via G_i and G_{12}. $S1P_5$ is expressed in oligodendrocytes in the CNS and is expressed throughout the maturation of the myelin cell (122). In mature oligodendrocytes, S1P induces cell survival effects via Akt activation, whereas this was absent in cells from $S1P_5^{-/-}$ mice (122). $S1P_5$ is also expressed on natural killer (NK) cells and is important for their migration to inflamed tissues and homing to lymphatic tissues (123). NK-cells lacking $S1P_5$ accumulated in the lymph node suggesting $S1P_5$ to be important in the NK cell egress from secondary lymphatic organs (124). An interesting finding was that $S1P_5$ is expressed in human brain capillaries and is important for the brain endothelial brain barrier, as shown *in vitro* (125).

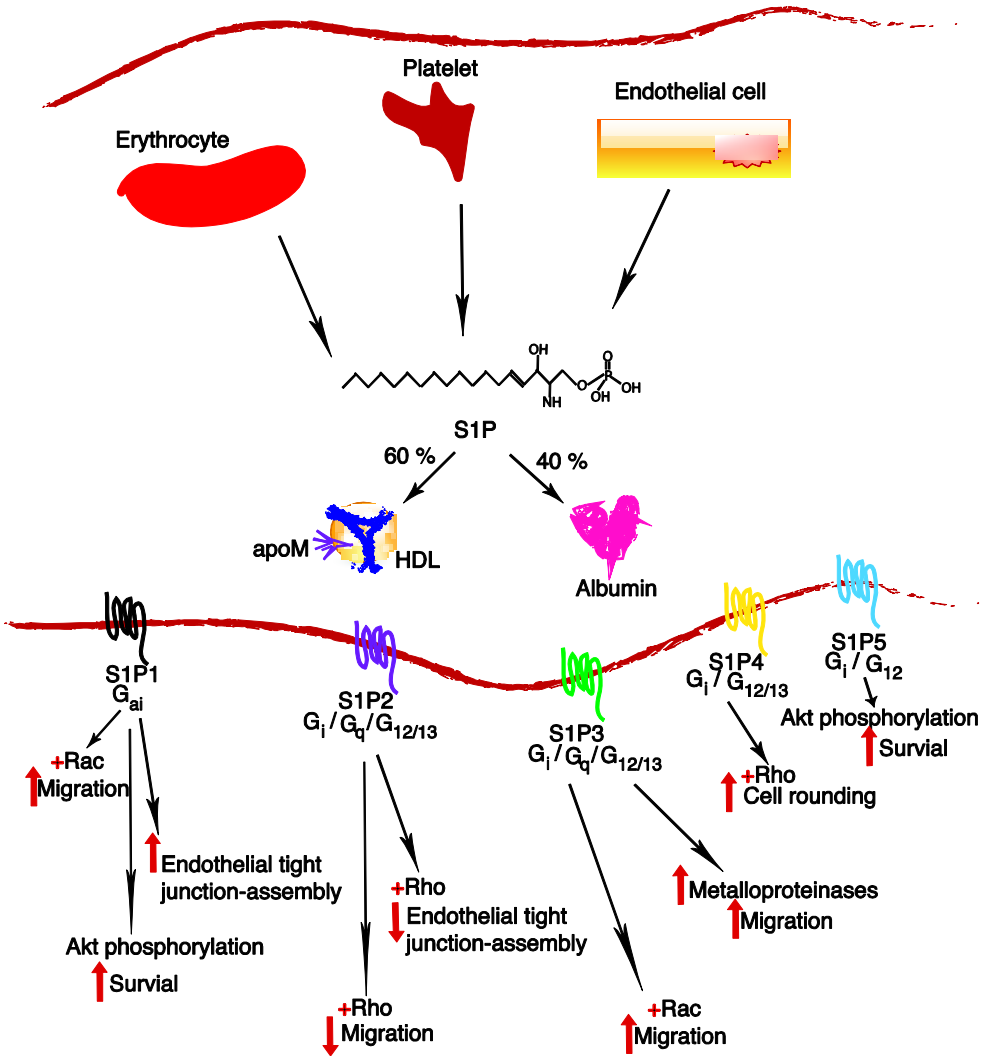

Figure 5: S1P signaling

The apoM/S1P-complex

The knowledge about the apoM/S1P complex and its function is limited due to the recent discovery of this constitution. The first evidence for the existence for the complex was the finding that S1P quenched the intrinsic fluorescence of recombinant expressed apoM (rapoM) (25). Later this was confirmed *in vivo* both in mice and humans as S1P co-eluted exclusively with HDL containing apoM and not with HDL depleted of apoM (39). Moreover, apoM$^{-/-}$ mice have 50 % lower plasma levels of S1P (39). Since the apoM/S1P complex is associated mainly with a lipoprotein, it is not surprising that lipoprotein metabolism regulates the circulating levels of the apoM/S1P-complex and vice versa. This was first shown in patients with monogenic disorders affecting HDL and LDL metabolism (126). Patients with heterozygous mutations in APOA1, LCAT and ABCA1 had 34 % and 12 % reduction of S1P and apoM respectively, whereas patients with two affected alleles had a 70 % and 48 % reduction, respectively (126). Interestingly, albumin-associated S1P was not affected in this study suggesting that lipoprotein metabolism only regulates the apoM-S1P pool. A study using both HepG2-cells and mice, showed that overexpression of hepatic apoM levels increased S1P in both liver tissue and in plasma, despite no increase in blood cell count (127). In another study, overexpression of hepatic apoM in mice lead to the formation of larger HDL particles enriched in apoM and S1P, however the HDL- concentration was not altered (128). This effect on HDL-size was shown to be dependent on the presence of the uncleaved signal peptide of apoM, as a mutated form of apoM lacking the signal peptide lead to formation of small nascent HDL-particles (129). Despite the presence of larger HDL particles, the macrophage cholesterol efflux was not altered (128). As mentioned, S1P is mainly produced by platelets and erythrocytes in plasma. S1P secretion from erythrocytes is increased in the presence of HDL isolated from apoM-TG mice compared to HDL isolated from apoM knockout mice (31). However, reconstituted HDL lacking apoM efficiently accepted S1P from erythrocytes whereas albumin did not, suggesting that S1P secretion from erythrocytes is not dependent on apoM but preferably release S1P in the presence of HDL compared to albumin (31). How apoM delivers S1P to the S1PRs is not understood. Recently, Zhang et al. proposed that spontaneous release of S1P is unlikely due to the high unbinding force between apoM and S1P (130). Instead they suggest based on molecular dynamic simulations that the release of S1P demands tight interaction with acceptor molecules, i.e the S1PRs. Further

experiments using expressed proteins are needed to confirm these preliminary findings.

HDL, apoM and S1P in vascular inflammatory disease

In diseases in the vascular system like sepsis, atherosclerosis and diabetes there is an altered functionality in cells within the vascular wall. Endothelial dysfunction contributes to increased inflammation and pro-coagulant status in the vessel. Atherosclerosis is believed to be driven by a low grade chronic inflammation in the vasculature whereas sepsis is an acute activation of the innate immune response upon recognition of pathogens in the circulation. The pathogenic mechanisms behind atherosclerosis and sepsis are not completely understood, however, the diseases involves cells lining the vessel wall (131).

The vascular wall

The vascular wall is important in creating a membrane barrier which regulates the transport of water, gases, marcomolecules and cellular proteins between the blood and underlying tissues. The vascular wall is build up by three major layers; tunica intima, tunica media and tunica adventitia. Endothelial cells together with pericytes build up the primary layer of the basal membrane, which is called **tunica intima**. The endothelial cell, lining the luminal side of the vessel wall and coming in contact with all circulating molecules, is crucial in regulating blood homeostasis. The formation of the endothelial cell layer in the vascular wall begins with differentiation of precursor cells (angioblasts), which assemble in a vascular labyrinth (132). Different factors in circulation, such as vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR) 2 and basic fibroblast, stimulate the differentiation of angioblasts. These precursor cells are not only present during embryogenesis, but also in adult bone marrow and peripheral blood.

Endothelial cells release substances, such as prostacyclin, nitric oxide (NO) and endothelium derived hyperpolarizing factors (EDHF) in order to communicate and interact with its surrounding environment (133). EDHF and NO are important in regulating endothelial-mediated relaxation of the vascular wall which is irreversibly correlated with hypercholesterolemia in larger vessels (133, 134).

Endothelial cells express G-actin which forms F-actin polymers (135). F-actin binds nucleotides, which results in the F-actin polymerization activating ATPase. Activation of ATPase leads to actin re-arrangements, which is responsible for cell motility and protein transportation within the cell (136, 137). Rho GTPases are the main GTPases involved in cell motility. They belongs to the Ras superfamily and consist of Rho, Rac, Cdc42, TC10 and TCL (138). Rho and Rac kinases are involved in migration, motility and proliferation of vascular cells (139). Rho stimulates endothelial and vascular smooth muscle cell contractility by inhibiting NO production and via actin re-arrangements and is important in regulating the endothelial barrier function (139, 140). Rac regulates similar downstream effects as Rho and stimulates actin polymerization with subsequent cell migration (138). Adherent junctions and tight junctions between endothelial cells are important in creating the right vascular permeability. Tight junctions are located at the apical surface between endothelial cells and are made of a complex composition of proteins including occludins and claudins (141, 142). Adherent junctions are mainly composed by vascular endothelial (VE)- cadherin. Ve-cadherin connects endothelial cells via an extracellular domain and via intracellular binding to the catenin family of proteins (141). VE-cadherin is regulated by the Rho-GTPase family where Rho activation leads to disassembly and Rac to assembly of VE-cadherin with subsequent increased or decreased endothelial permeability, respectively.

Beneath the endothelial cell layer is the **pericyte** located which is believed to be a relative to the smooth muscle cell (143). Pericytes contribute to the building of the vascular basement membrane by inducing expression of connective protein such as fibronectin, nidogen-1, perlecan and laminin (144). Moreover, co-culturing of pericyte with endothelial cells results in up-regulation of integrins, compared to endothelial monocultures, however monocultures still express integrins (144, 145). Integrins are important for cell-cell and cell-extracellular contacts. Pericytes are in direct contact with endothelial cells via N-cadherin and β-cadherin adherent junctions in the basal membrane, which transfers contractile forces from the pericytes to the endothelial cell (146). N-cadherin is scattered throughout the pericyte-endothelial interaction surface and is absent without the presence of pericytes (147). In dermal scarring, pericytes transform to a collagen producing fibroblast (148). Separating the tunica intima from the underlying tunica media is the **internal elastic lamina** (149).

Tunica media is made of VSMCs, collagen fibers and elastic tissue. The main function of VSMCs is to mediate the contractile function to regulate blood flow and blood pressure (150). However, VSMCs are also important in the formation of new blood vessels, by stabilizing endothelial cells via producing extracellular matrix components such a collagen, elastin and proteoglycans, which also possess contractile properties (151). The contractile force of VSMCs is mostly due to the

organized cytoskeleton built up by smooth muscle α-actin, F-actin filaments, smooth muscle myosin heavy chain (MHC) and smoothelin (152, 153). In contrast to skeletal and cardiac muscles cells, VSMCs are not terminally differentiated and disperse a huge plasticity enabling phenotypic switching (154). VSMCs can switch to a proliferatory and migratory phenotype characterized by the presence of a more diffuse actin-network with loss of fibers and f-actin (155). Smooth muscle 22 alpha (SM22α), which is involved in regulating the equilibrium between G-actin and F-actin, inhibits the phenotypic switch in VSMCs (156). Genetic ablation of SM22α in mice leads to an increase in atherosclerotic area and increased presence of VSMC in the plaque (157).

Tunica adventitia is the outmost part of the vessel. It is primarily made of adventitial fibroblasts and collagen, which connects the blood vessel to the surrounding tissues (158). The adventitial fibroblast is the major producer of vascular reactive oxygen species (ROS) via NAPDH oxidase activation (158). ROS have many different effects on vascular cells, they can induce expression of endothelial adhesion proteins, reduce the availability of NO and induce apoptosis (159).

Lately it has become clear that tunica adventitia contains progenitor cells for macrophages, which cannot be replenished via the bone marrow or spleen (160). These macrophages are highly present in atherosclerotic plaques (161). Progenitor cells for endothelial cells and VSMCs (Sca-1^+) are also found in the adventitia (162, 163). VSMCs generated from Sca-1^+ progenitor cells are highly present in atherosclerotic plaques. These results suggest that monocyte chemotaxis from the circulation is not the only source of pathogenic cell accumulation in atherosclerotic plaques. The morphology of the vascular wall is illustrated in Fig 6.

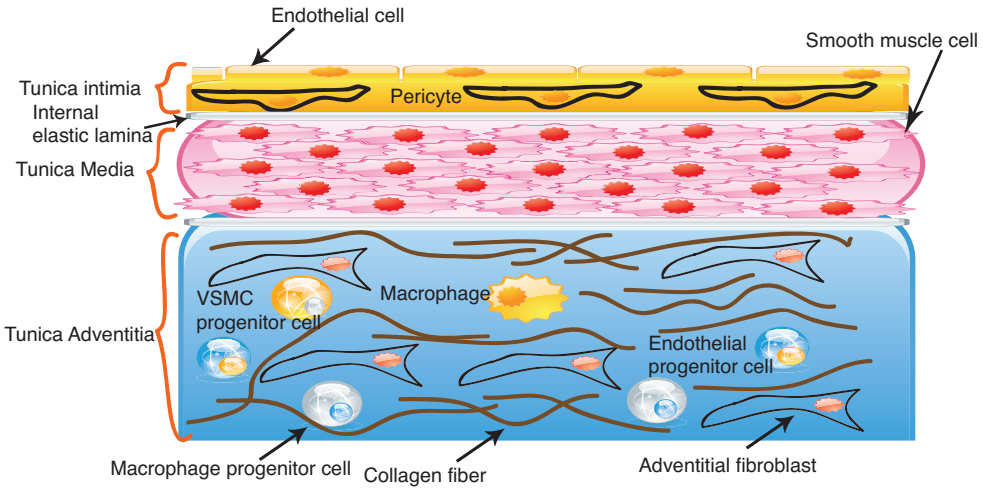

Figure 6: Morphology of the vascular wall

Atherosclerosis

The development of atherosclerosis can reside in cardiovascular diseases (CVD) like ischemic heart disease (IHD), coronary artery disease (CAD) and coronary heart disease (CHD). Atherosclerosis is believed to be driven by low grade chronic inflammation in the vasculature. Different inflammatory biomarkers have been associated with atherosclerosis (164). Higher levels of C-reactive protein in patients have been associated with increased incidence of cardiovascular disease (CVD) (165). Moreover, other circulating pro-inflammatory mediators have been associated with an increased risk of CVD, like serum amyloid A, soluble intercellular adhesion molecule type 1 (sICAM), IL-6, homocysteine, total cholesterol, LDL and apoB (166). In addition, several different immune cells are present in the atherosclerotic lesion for example macrophages, lymphocytes, dendritic cells, mast cells and neutrophils. Moreover, patients surviving sepsis have an increased risk of developing major cardiovascular events like ischemic stroke, hemorrhagic stroke, myocardial infarction (MI) and heart failure. The increased risk persisted up to 5 years post discharge from the hospital (167). These results suggest an inflammatory mechanism to be involved in the pathogenesis of atherosclerosis.

It is debated whether atherosclerosis starts with an inflammatory activation of endothelial cells, which leads to accumulation of LDL, or if it starts with an accumulation of LDL, which becomes pro-inflammatory and starts an inflammatory response in the endothelial cell. Lesion-prone areas in the arterial wall (e.g. the ascending aorta and the aortic arch) have 5-fold higher LDL levels

than lesion-resistant areas after 16 days of high fat diet in rabbits (168). Moreover, there are only minor traces of foam cells in the arterial walls, whereas most of the LDL is found intact in fatty streaks suggesting that a retention of LDL happens before or in parallel with the formation of foam cells (168). In serial sections isolated from human aortas with different stages of atherosclerosis, the early stages were characterized by the presence of lipid deposits followed by accumulation of macrophages at later stages (169). These results suggest that accumulation of lipids happens before the infiltration of immune cells occurs.

Modified LDL is believed to induce an injury to the endothelial cell thus initiating an inflammatory response (170). As a response to the injury, the endothelial cell upregulates adhesive molecules like E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion protein 1 (VCAM-1), which induces rolling and attachment of monocytes to the vascular endothelium and further their migration across the endothelial cell layer into the intima. Once in the intima, monocytes absorb oxidized LDL-particles and differentiate into macrophages and become foam cells. The importance of macrophages in the early development of atherosclerosis was shown in mice having 50 % lower circulating monocytes and reduced plaque development and a more stable plaque composition (171). Apoptosis of macrophages in fatty streaks (early stage of atherosclerosis development) was found to be beneficial. In contrast, macrophage apoptosis in the advanced lesion resulted in accumulation of apoptotic cell debris and increased lesion sizes and increased recruitment of circulating monocytes (172). The reason for this could be that early apoptosis of macrophages limits the accumulation of foam cells whereas when already formed, apoptosis of macrophages is not any longer protective. Oxidized LDL stimulates to increased VSMC proliferation and migration of VSMCs from the media into the intima of the artery, where they become a major part of the atherosclerotic lesion (173). VSMCs present in plaques can also originate from the bone marrow (174). When in the intima, VSMCs can differentiate to exhibit phenotypes of cell such as macrophages and mesenchymal stem cells (173, 175). Foam cells together with collagen and VSMCs build up a plaque covered by a fibrous cap which separates the plaque from the blood (176). Patients who suddenly died of ischemic heart disease and presented with coronary thrombosis had fibrous caps consisting of more macrophages and less VSMCs compared to those without thrombosis (177). The fibrous cap ruptures because metalloproteases, produced by entrapped macrophages, degrade extracellular matrix making the fibrous cap unstable (178). A necrotic core is formed in the deeper areas of the advanced plaque, which consists of lipids, dead cells and calcification (176). The presence of a necrotic core and a thin fibrous cap characterizes an unstable plaque which can easily rupture and release pro-thrombotic substances like TF leading to the formation of thrombi and an acute increased risk of stroke and MI. Actually, in a study by Davies in 1992, 73.3 % of

patients dying from a sudden coronary death had a recent coronary thrombotic lesion (179). An illustration showing the pathogenic morphology of an atherosclerotic plaque is illustrated in Fig 7.

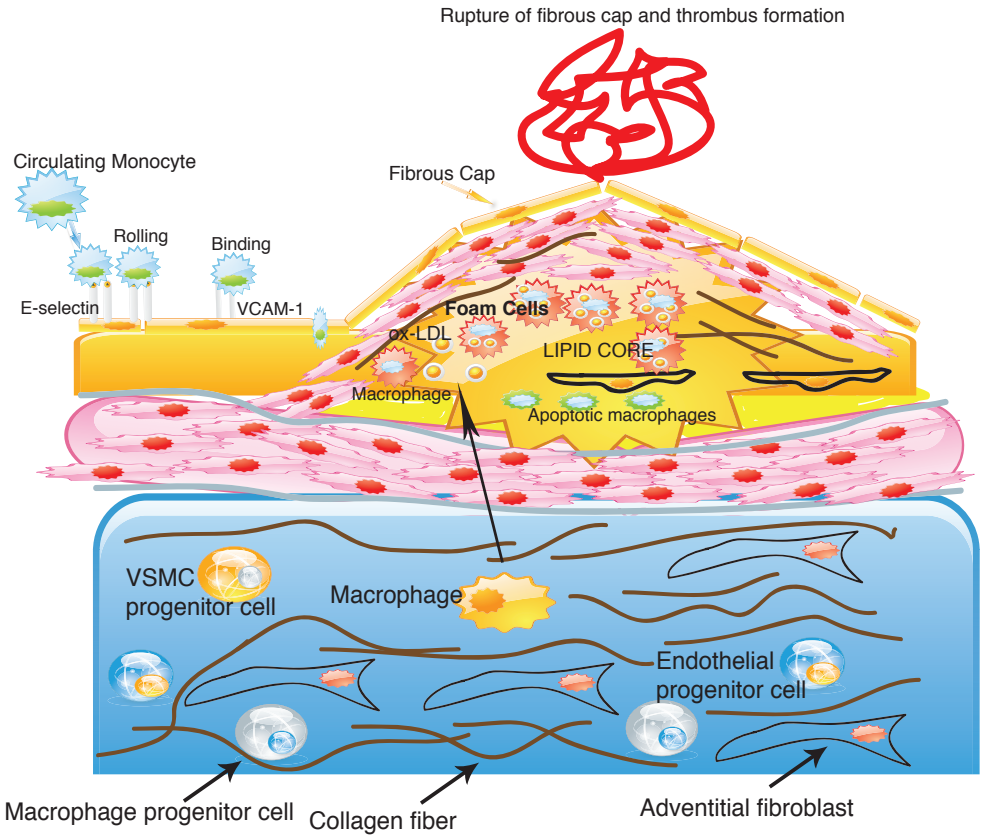

Figure 7: Morphology of an atherosclerotic plaque

The plasma level of LDL-C is an independent risk factor for the progression of CVD (180, 181). Genetically modified mice with increased LDL-C have increased atherosclerosis, whereas mice with normal LDL-C have no atherosclerosis (182, 183). The concentration and phenotype of circulating LDL is highly dependent on the secretion of VLDL. There are two forms of VLDL; VLDL1 and VLDL2 where VLDL1 is large and TG-enriched and VLDL2 is small and TG-depleted (184). VLDL1 is more pro-atherogenic and is present in patients with type-2-diabetes (185). Secretion of VLDL1 decreases HDL and increases the presence of small LDL-particles. Small LDL particles can more easily enter the arterial wall, are more easily oxidized and they have less affinity for the LDL-receptor, leading to decreased clearance and longer circulation time (8). The secretion of VLDL is

typically increased during acute inflammation due to stimulation of hepatic lipid synthesis by TNFα (186). However, LDL is in contrast decreased in sepsis and the oxidation of LDL is increased upon infection (187, 188).

HDL in atherosclerosis and endothelial functionality

HDL is believed to be atheroprotective mainly because of its role in RCT. Several clinical investigations have shown that low HDL-C or apoA1 are predictive risk factors of CVD (189-192). In addition, studies in rodents have shown beneficial effects on atherosclerosis by increasing the HDL-C (193, 194). HDL protects LDL from oxidation via human serum lactonase paraoxonase (PON1) that degrades lipid peroxidase and hence inhibits the formation of foam cells in the intima (195). Lately HDL has gained interest as a pharmaceutical target in order to lower the incidence of CVD by trying to increase the HDL-C in human plasma. Substances increasing HDL-C in humans such as niacin (196) and CETP-inhibitors remain to be proven protective in human clinical CVD events (13, 197, 198). Recently, a study showing that genetic variants leading to increased HDL-C did not correlate with a lowered risk of CVD suggests that it is not the HDL-C *per se* that is protective (2).

HDL exerts endothelial protective effects through several actions. It can inhibit the expression of pro-inflammatory adhesion molecules on the endothelial surface, stimulate endothelial NO-production and inhibit apoptosis (199, 200). Platelet-activating factor (PAF) is a pro-inflammatory mediator secreted during acute inflammation by activated platelets, leukocytes and endothelial cells, which activates monocytes and increases vascular permeability (201, 202). Two enzymes in HDL, PON1 and platelet-activating factor acetylhydrolase, were reported to inhibit monocyte binding to endothelial cells and inactivate PAF, respectively (199). However, HDL has altered phenotype in acute MI and can become pro-inflammatory instead (203)

ApoM in atherosclerosis

When HDL isolated from apoM-depleted mice is injected into C57BL/6 mice only a pool of larger HDL is formed in contrast to injection of wild type HDL where a smaller HDL-subfraction is formed over time (37). This small subfraction is believed to be preβ-HDL on which apoM is strongly present, suggesting apoM to be important in the formation of preβ-HDL (37). This was further supported in a study by Christoffersen *et al*., where generation of preβ-HDL was increased in

apoM-TG mice compared to controls (35). Interestingly, in one study, two-fold apoM overexpression on an $LDLR^{-/-}$ background lead to decreased atherosclerosis (35). In contrary in a second study overexpressing apoM two- or ten-fold on a $LDLR^{-/-}$ background, no inhibition in atherosclerotic plaque formation was observed, (36). The discrepancy between these studies can be explained by the gender differences as only female mice were used in the first study whereas only male mice were used in the second study. Interestingly, the female mice did not have a co-concomitant increase in VLDL/LDL cholesterol upon apoM-overexpression as did the male mice. This could explain the absence of atherosclerotic protection of apoM in male mice and the contradictive results between the two studies. Remarkably, two-fold increase of apoM- reduce oil-red stained lesions by 70 % compared to wild type and in $apoE^{-/-}$ mice, ten-fold increase of apoM reduce the atherosclerotic area by 60 % (36). These results strongly suggest apoM to be involved in anti-atherogenic effects in the vasculature.

The role of apoM in human cardiovascular disease is not clear. Genetic studies suggest that a defect in *APOM* associates with CHD, whereas plasma levels of apoM do not clearly correlate with CHD. The prevalence of three polymorphic sites in *APOM* is higher in Chinese CVD patients compared to controls and two of these sites confer lower expression of apoM as judged by luciferase assay (204, 205). In a Swedish population with recurrent venous thrombosis (VTE), an *APOM* polymorphism (rs805297) was correlated with increased incidence of VTE in men only, and plasma levels of apoM was reduced in men with VTE (206, 207).

Since apoM is important for the cardio-protective effects of HDL, one would expect it to correlate with CVD. However, in two large cohorts (n=255 and n=1865), there were no differences in apoM plasma levels between individuals who developed CVD and healthy subjects (208). In patients with critical limb ischemia or abdominal aortic aneurysm, plasma apoM was decreased but was not an independent risk factor (209, 210).

To conclude, apoM clearly protects against atherosclerosis in mice models. In humans, apoM plasma levels do not correlate with human CVD. The absent correlation between apoM and CVD could be explained by the presence of LDL-associated apoM, which might have altered properties and could gain pro-atherogenic characteristics. However, this needs to be further clarified.

S1P in atherosclerosis

S1P mediates different effects in atherosclerosis depending on through which S1PR it signals and on which cell type the S1PR is expressed. The $S1P_2$ receptor is involved in retaining macrophages in the atherosclerotic plaque as shown in $ApoE^{-}$

$^{/-}S1P_2^{-/-}$ mice having severely attenuated atherosclerosis due to less accumulation of macrophages and foam cell formation compared to $apoE^{-/-}$ mice (211). $S1P_3$ had similar effect as $S1P_2$ and was important for recruitment of monocytes and macrophages to the plaque in the aorta (212). However, accumulation of VSMCs was seen in $apoE^{-/-}S1P_3^{-/-}$ mice suggesting the overall atherosclerotic lesion volume not to be altered as compared in $apoE^{-/-}$ mice (212).

$S1P_3$ can induce mobilization of P-selectin on endothelial cells, thereby promoting leukocyte rolling and further atherosclerosis (213). In contrast, endothelial specific $S1P_1$ silencing blocked rolling via inhibition of cAMP with subsequent induction of sphk1 (213). The role of S1P-signaling in atherosclerosis has been largely evaluated by using FTY720, a non-specific S1PR-reverse agonist. Administration of FTY720 into $apoE^{-/-}$ mice reduced the plaque volume by 62.5 % mainly via reducing the amounts of infiltrating macrophages (214). The underlying mechanism was inhibition of thrombin-induced release of monocyte chemoattractant protein-1 (MCP-1) in isolated rat artery segments (214).

The role of S1P in human cardiovascular disease is not clear. A study investigating the correlation between S1P and aortic stenosis found that plasma S1P was a strong predictor of stenosis and rose with increasing severity of the disease (215). In contrary, a study by Sattler *et al*. observed a decrease in plasma S1P in patients with CAD compared to controls (216). This was further confirmed in patients with IHD having an inverse correlation between HDL-associated S1P (serum depleted of LDL and VLDL) and IHD occurrence (217). In patients with MI, the S1P plasma levels were decreased by 50 % both immediately and after 5 days post infarction (218). This was confirmed in a second study which was speculated to be due to decreased release of S1P from erythrocytes (219). HDL isolated from human plasma collected from patients with CAD had lower S1P content and decreased activation of known downstream S1P-signalling targets (Akt, Erk and eNOS) that could be restored with S1P-loading in HDL (220).

To conclude, in human atherosclerotic related diseases such as CVD and IHD, circulating S1P-levels are often decreased. S1P seems to affect atherosclerotic morphology mainly via regulating macrophage recruitment through $S1P_2$ and $S1P_3$ signaling. How low S1P-levels observed in human atherosclerotic related diseases affects its role in atherosclerotic pathogenesis remains to be clarified.

The apoM/S1P-complex in atherosclerosis and inflammation

Specific silencing of $S1P_1$ in endothelial cells resulted in enhanced expression of ICAM-1 and VCAM-1, whereas administration of HDL+apoM and not BSA+S1P to human endothelial cells suppressed the ICAM-1 expression (101). The relevance of apoM in S1P-mediated anti-inflammatory effects has been

investigated in apoM$^{-/-}$ mice. ApoM$^{-/-}$ mice exhibit 50 % less S1P in plasma and have increased extravasation of Evans blue in the lung indicating a lower endothelial barrier function in the lung (39). This was reversed by administration of an $S1P_1$-agonist (221) demonstrating the specificity for $S1P_1$. We have investigated through which receptor apoM-associated S1P regulates the expression of pro-inflammatory adhesion molecules on human endothelial cells and we could confirm that the apoM/S1P-complex signals through $S1P_1$ to exert endothelial protective effects (paper III).

In apoM$^{-/-}$ mice it was shown that the apoM/S1P-complex is expendable for lymphocyte trafficking. However, there was an increased proliferation of hematopoietic/lymphoid progenitor cells in the bone marrow of apoM$^{-/-}$ mice with increased presence of lymphocytes in the central nervous system (222). Moreover, this study showed that apoM-associated S1P inhibited lymphopoiesis via $S1P_1$ signaling, whereas albumin-associated S1P did not (222). In paper II we did not observe any correlation between S1P and white blood cell count in sepsis.

Sepsis

Sepsis occurs when microorganisms (MOs) enter the circulation and is characterized by an intensive and immediate activation of the innate immune system and the coagulation system and dysregulation of the endothelial barrier. Normally, the immune system protects us and its activation leads to killing and disarming invading MOs. However, when large amounts of MOs are present in the circulation, hyper activation of the immune and coagulation systems lead to uncontrolled actions damaging the host. Sepsis is defined as presense of MOs in the circulation with symtoms characterising systemic inflammatory response syndrome (SIRS) including hypothermia or hyperthermia, tachycardia, tachypnoea, high or low white blood cell count (223). A schematic figure demonstrating the acute phase reseponse in the vessel is illustrated in Fig 8.

MOs enter the circulation mainly via the respiratory tract during phnemonia, via the gastro-intestinal tract and the genito-urinary system during urinary tract infections or through primary blood infections (224). MOs contain antigens, such as lipopolysaccharide (LPS), lipoproteins, outer membrane proteins, flagellin, fimbriae, peptidoglycan and lipoteichoic acid but can also release toxins, such as heat-shock protein and DNA fragments. These antigens are recognized by pattern recognition receptors such as toll-like receptors (TLRs), nucleotide binding oligomerization domain (Nod1 and Nod2) and peptidoglycan recognition

receptors. Today, ten different TLRs have been identified and they are expressed on B- and T-lymphocytes, monocytes, granulocytes and endothelial cells (225). Binding of LPS to TLRs leads to activation of downstream intracellular adaptors such as myeloid differentiation protein (MyD88) and TIR receptor domain-containing adaptor protein inducing interferon β (TRIF), which leads to nuclear translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a cytokine transcription factor (226). Activation of immune cells via TLRs leads to excessive release of pro-inflammatory cytokines, such as tumour necrosis factor α (TNFα), interleukin-6 (IL-6) and IL-8 within 30-90 minutes after LPS recognition (226). The cytokine release leads to upregulation of adhesion molecules and attraction of immune cells into tissues. Neutrophils, accounting for 50-70 % of circulating leukocytes in humans, are recruted to damaged endothelial cells, bind to adhesion molecules and transmigrate into the tissue where they kill pathogens via degranulation, production of ROS and generation of neutrophil extracellular traps (NETs) (227). NETs trap and disarm microbes extracellularly. However, they can also be harmful to the host and cause obstructing of vessels leading to ischemia and organ failure (228). Activated neutrophils induce maturation of dendritic cells (DCs) making them produce cytokines, induce T-cell proliferation and activate natural killer cells (229).

Endothelial cells have important anticoagulant effects and regulates the coagulation status in the vessel via four main pathways; production of tissue-factor inhibitor (TFPI), generation of activated protein C (APC), production of tissue-type plasminogen activator to induce fibrinolysis and via production of NO inhibits platelet aggregation (3). All of these functions are impaired in sepsis leading to a pro-coagulant environment in the vessel. Endothelial cells express TLR4 and TLR2 and can syntesize Tissue Factor (TF), the main activator of the extrinsic coagualation pathway, upon activation by endotoxin, TNF and IL-1 (230, 231). TF is also found on neutrophils binding to monocyte derive microparticles or on activated monocytes (232). The formation of vascular thrombi upon infection is generated to protect us in order to enhance the entrapment and destruction of the pathogens and to limit their spread in the body (233). However, in systemic inflammation, the activation of the coagulation system occurs simultaneously in microvessels in the whole body leading to disseminated intravascular coagulopathy (DIC). DIC is characterized by massive thrombin formation, consumption of coagulation components, decreased fibrinolysis and extensive formation of thrombi in microvessels (233). DIC leads to the obstruction of microvessels with reduced blood flow to organs and subsequent development of organ failure (232). The consumption of coagulation components leads to a following high risk of bleeding.

Endothelial cells are important in maintaining a balanced barrier function which becomes heavily dysfunctional in sepsis (234). Re-arranged actin network in

endothelial cells occurs upon exposure of gram-negative bacteria due to the binding of thrombin to protease-activated receptor 1 (PAR-1) which leads to activation of G-protein $G_{12/13}$ and Rho-activation (235). Rho-activation leads to cell-contraction and cell-rounding with subsequent disruption of cell to cell contacts resulting in increased transport of albumin and macromolecules into the underlying tissues (17). This also leads to increased exposure of underlying tissues expressing large amounts of TF, exceeding the pro-coagulant status. Interestingly in later stages of sepsis, PAR-1 forms heterodimers with PAR-2 switching the signaling pathway to G_1 with subsequent Rac-activation and increased barrier function (3). The barrier protective effects exerted by both APC and thrombin are linked to co-activation of $S1P_1$, whereas thrombin induced barrier disruptive effects are associated with $S1P_3$ (3). Thrombin, together with VEGF, can induce the internalization of VE-cadherin, further increasing the endothelial permeability (236). The increased permeability is believed to be associated with multi-organ failure (MOF) in sepsis. However, interestingly histopathology findings in patients dying from MOF revealed that tissues are fairly normal characterized by a mild tissue oedema (3). However, even though the oedema was not extended, organ function still failed suggesting even a small leakage can be fatal for many organs.

As a response to the intense initial activation of the immune system, there is a following anti-inflammatory feedback mechanism. The production of anti-inflammatory cytokines such as IL-10, transforming growth factor β (TGF-β) and IL-13 is seen as a compensatory action which can lead to immunoparalysis and apoptosis of immune cells (228, 237). Ex vivo experiments revealed that blood from septic patients at the intensive care unit (ICU) had blunted TNFα-production in response to endotoxin and these patients were associated with longer ICU-stay and higher infection incidence (238, 239). A possible explanation could be the presence of soluble TLR receptors that has been reported to act as decoy receptors blocking TLR signalling (240).

Resolution of an inflammatory response was long believed to be a passive clearance of immune cells and cell debris but lately this hypothesis has been challenged with new evidence pointing towards an active process enabling tissues to restore into normal homeostasis. Lipoxins and resolvins program mononuclear cells to limit their entrance to inflamed tissues and activate macrophages to engulf apoptotic mononuclear cells (241). Metabolites from omega-3 are generated by leukocytes during resolution of the inflammatory process and these mediators increase phagocytosis and the transport of ingested leukocytes back to lymphatic tissues (242, 243). Resolvin E1 generated by human neutrophils inhibits superoxide production in response to TNF and inhibits >90 % of alveolar bone destruction in a rabbit periodontitis model (244, 245).

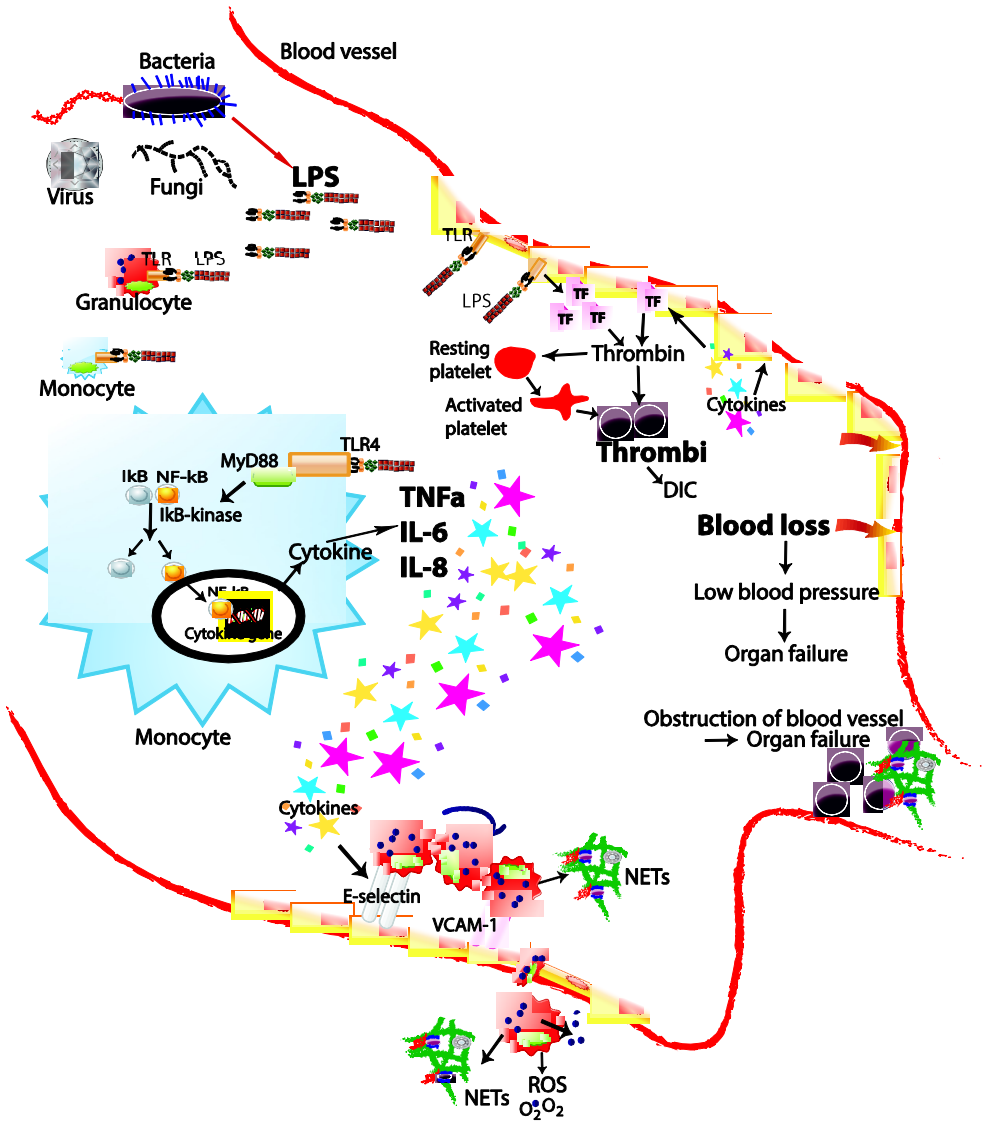

Figure 8: The acute phase response in a blood vessel

HDL in sepsis

HDL has antioxidant, anti-inflammatory and endothelial protective effects and is a part of the innate immune system (246-249). Levels of HDL are decreased in patients with sepsis and low levels correlate with increased mortality (250-252).

Patients having concentrations of HDL above 25 mg/dl have a 100 % survival rate and by using cutoffs of high HDL (>20 mg/dl) and low HDL (<20 mg/dl), HDL could predict the overall 30-day mortality with 92 % sensitivity, 80 % specificity and 83 % accuracy (252). ApoA1 might also be important in the acute phase response and human patients with sepsis have lower apoA1 levels, which are inversely correlated to survival (253). Moreover, injection of an apoA1-mimetic peptide decreased the inflammatory response and restored HDL levels in a cecal ligation and puncture rat model (254).

The mechanism behind the decreased HDL levels in sepsis is not clear but increased levels of the secretory phospholipase A_2 and cytokines have been shown to increase the catabolism of HDL in inflammation (255, 256). Recently Zou *et al*. reported a delta-HDL value to be an alternative prognostic marker to distinguish between gram-negative and gram-positive bacterial infection where patients with gram-negative bacterial infection had a much more severe decrease in HDL plasma levels during the first day of hospitalization (257).

HDL has several potential protective mechanisms in sepsis. HDL associates with LPS- binding protein and neutralizes LPS in the circulation (249). Administration of LPS to serum results in reduced buoyant density of LPS, indicating lipid-association of LPS, which was absent in delipidated plasma and restored with addition of HDL but not with addition of VLDL or LDL (258, 259). HDL inhibits the interaction between LPS and its receptors on monocytes thus preventing activation of immune cells and release of cytokines (260). HDL also promotes the clearance of LPS via SR-B1 by up to 4-fold in HEK-cells and 2-fold in primary hepatocytes (261). Lipoteichoic acid, which is generated by gram positive bacteria, activates immune cells via the TLR2/6 pathway. Lipoteichoic acid is mostly present in plasma as complex with HDL, which neutralizes its pro-inflammatory effects (262, 263). Moreover, HDL contains many proteins involved in immune regulation such as complement proteins (C3, C4A and C4B) and antimicrobial complexes made of haptoglobin-related protein, apolipoprotein L-I and apoA1 (264, 265).

ApoM and S1P in sepsis

APOM is located in a cluster of genes involved in the immune system and hence apoM could also be a mediator of the inflammatory response. The first indication of this was found in 2007 where Feingold *et al.* showed *in vitro* and *in vivo* a decrease of apoM upon pro-inflammatory stimuli (266). However, one limitation with this study was the small number of human patients (n=6). To follow up this result in a larger cohort, Kumaraswamy *et al.* quantified apoM in plasma from 233

patients with different severity of sepsis and found that apoM levels were increasingly lower with increased severity of the disease (187). S1P is decreased in sepsis as recently showed in a study by Winkler *et al*. who quantified S1P in serum from 100 sepsis patients and 214 healthy controls (267). Moreover, patients with dengue-fever, a disease characterized with substantial plasma leakage, had lower S1P levels compared to controls (268). The importance of the apoM/S1P-complex in sepsis is not clear. In paper II we measured plasma levels of apoM and S1P in both human and non-human primate sepsis and found that both were decreased reflecting the severity of the disease. S1P was decreased rapidly already within the first 4-6 hours making S1P a potential biomarker in sepsis. This will be further discussed under present investigation.

Diabetes

Type 1 diabetes (T1D) is characterized by abolished insulin production caused by an autoimmune assault on B-cells in the islet of Langerhans in the pancreas, whereas type 2-diabetes (T2D) is mainly due to insufficient usage of insulin in the body i.e insulin resistance (269). In 2015, the number of individuals having diabetes worldwide was estimated to 415 million people and the number is believed to rise in the nearest 20 years (270). The causative mechanisms behind the onset of T1D, which often occurs in teenage years, are unclear, however genetic as well as environmental factors and existence of co-autoimunity (co-existanse of other auto-immune diseases) are believed to be involved. In 90 % of the individuals who develop T1D, certain histocompatibility complex haplotypes are present as compared to 40 % in healthy individuals (271). Co-existence of auto-immune diseases, such as thyroid disease or coeliac disease, have been linked to T1D as well as environmental factors, such as viral infections, the hygiene-hypothesis (that we are too clean) and breast feeding versus formula milk, however further evidence are needed before links can readily be established (270).

T1D patients have an increased risk of CVD and the mechanism behind is unclear (272). The difference of CVD between T1D and T2D is that CVD occurs much earlier in T1D. Moreover, in T1D, women are affected by stroke equally much as men, whereas in T2D the incidence in men is higher. Patients with diabetes (non-defined type) have atherosclerotic plaques consisting of more lipids, macrophages and thrombus as compared to non-diabetic patients (273). Atherosclerosis in T1D specifically, is characterized by more widespread burden (more vessels affected) with increased severity of stenosis (274). One explanation of increased atherosclerosis in T1D could be the presence of increased pro-inflammatory

mediators (C-reactive protein, IL-6 and fibrinogen) in the circulation of T1D patients compared to controls (272). Another causative mechanism of atherosclerosis in T1D is endothelial dysfunction (275). Both hyperglycemia and hypoglycemia are associated with increased oxidative stress and reduced NO-production in endothelial cells as shown *in vitro* (276). Also, endothelial vasodilation was impaired *in vivo* in humans, as injection of methacholine induced stronger increase in blood flow in healthy subjects compared to individuals with insulin-dependent diabetes (277). These results suggest that there is an increased endothelial dysfunction in T1D, which could contribute to the increased risk of CVD associated with T1D.

HDL is often increased in T1D whereas it is decreased in T2D (278). Despite this, T1D-patients, especially women, are not more protected against CVD, suggesting altered properties of HDL in T1D. Indeed, HDL can lose its protective effects or gain dysfunctional properties, becoming pro-atherogenic and pro-inflammatory. The concept of dysfunctional HDL was first established in rabbits in 1995, when it was shown that HDL during acute inflammation lost apoA1, PON-1, PAF-AH and gained serum amyloid A (SAA). This HDL was much less effective in inhibiting LDL oxidation (279). Moreover, overexpression of LCAT in mice leads to increased HDL-C levels but surprisingly results in increased atherosclerosis (280). Dysfunctional HDL is further discussed in paper IV.

ApoM and S1P in diabetes

Recently, the composition of HDL was shown to be altered in T2D with lower levels of S1P and subsequently decreased protection of HDL against oxidative stress in cardiomyocytes (281). More evidence of alteration in the HDL phosphosphingolipidome in diabetes was observed in T1D patients having reduced S1P levels in both HDL_2 and HDL_3 particles compared to controls (282). Diabetic aortas express more ICAM-1 and VCAM-1 than healthy aortas and 100 nM S1P could inhibit this increased expression *ex vivo* (283). ApoM is known to be decreased in T2D, however alterations of plasma levels of apoM and S1P in T1D are not known (284). Furthermore, the importance of the apoM/S1P-complex and its functionality in T1D has not been studied. In paper IV we quantified plasma levels of apoM and S1P in T1D patients and healthy controls. Moreover, we investigated the anti-inflammatory effects of different HDL-subpopulations isolated from T1D patients and controls. We found that there was no difference in plasma levels of apoM and S1P between controls and T1D-patients. The functionality of T1D-HDL was altered and this was not due to reduced levels of apoM and S1P. This is further discussed in next chapter.

Present Investigation

The aims of this study were to explore the role of the apoM/S1P complex in its interaction with S1P-receptors and in vascular inflammation. As shown by Galvani *et al*., the apoM/S1P-complex increases the assembly of VE-cadherin adherent junctions via signaling through $S1P_1$ (39). In addition, apoM is strongly reduced in sepsis with plasma levels negatively correlating with disease severity (187). This got us engaged to further investigate the anti-inflammatory role the apoM/S1P-complex and its interaction with the S1PRs. In order to quantify S1P in patient cohorts and in different preparations for cell-experiments, we developed an S1P-quantification method in collaboration with the clinical chemistry department at Skåne University Hospital as described in paper I. In paper II, we used this method to quantify plasma S1P in the same human sepsis-cohort as used for apoM. In addition, we investigated the plasma levels of apoM and S1P in a non-human primate sepsis model where we could study alterations of apoM and S1P in plasma and tissues over time. In paper III, we further investigated the mechanism behind the anti-inflammatory role of the apoM/S1P-complex *in vitro* by studying its potential to inhibit pro-inflammatory adhesion molecules on the surface of primary human endothelial cells (HAECs). As the apoM/S1P-complex was shown to have anti-atherogenic properties in our experiments, we were interested in analyzing the importance of apoM and S1P in a group of patients with known increased risk of developing atherosclerosis and CVD; patients with diabetes (paper IV). In paper V, we aimed to investigate if the apoM/S1P-complex could interact with all of the S1PRs. In order to do this, we created stable cell clones expressing $S1P_{1\text{-}5}$ coupled to a green fluorescent protein (GFP) and followed the internalization pattern of the S1PRs upon addition of S1P carried by different cargos.

S1P quantification

S1P is a zwitterion meaning it has both positive and negative charges within the molecule but a formal neutral charge at neutral pH. In addition, S1P has a hydrophobic C18-carbon tail with a hydrophilic inorganic phosphoric acid head, making it amphipathic. These properties make S1P difficult to separate and handle in chromatographic systems since it will affect intermolecular forces between S1P

and the stationary/mobile phases. S1P was first quantified by using thin layer chromatography (TLC), however these methods had poor recovery and complex sample preparations (285, 286). Later liquid chromatography (LC) was introduced to increase resolution and automation. Today, LC coupled to tandem mass spectrometry (MS/MS) is the most commonly used method to quantify S1P (287). The powerful MS/MS-methodology has very high selectivity and high resolution due to its possibility to select and separate molecules with specific mass down to 1 Da.

In paper I we describe a LC-MS/MS method, which we developed in order to quantify S1P in plasma and cell extracts (see schematic overview in Fig 9). We tried different LC-columns like HILIC, C_8 and C_{18}, however all gave distinct peak tailing, which is a sign of carry over. Carry over of S1P between injections has been described in the literature and was solved with derivatization of the amino-group or repeated wash-steps between each injection (288). However, this is time consuming and can cause unspecific binding that will interfere with the quantification of S1P. We solved it with a C_{18} column from Waters that consisted of a positively charged stationary phase at low pH. The potential explanation behind the absence of peak tailing with this column could be repelling forces between the amino-group and the stationary phase which both were positively charged the low pH in our mobile buffer. An internal standard (IS) is used to compensate for experimental variations during the quantification and is added during the extraction procedure. We started using a S1P-homolog with a C_{17} aliphatic chain, which was the most used internal standard at that time. However, S1P:C_{17} had shorter retention time than S1P (3.4 and 3.8 minutes respectively) due to the shorter aliphatic chain. We investigated ion suppression by matrix components in the extract at those particular retention times and found ion-suppression at the S1P:C_{17} elution time. This can induce unstable results and hence we tried deuterium-marked S1P (d7:S1P) as IS instead. D7:S1P co-eluted with S1P and hence removed the problem with ion-suppression.

In this paper we also established protocol for blood sample preparation for S1P quantification. Since S1P is produced by both platelets and erythrocytes, S1P can be secreted into blood upon sample collection. We came to the conclusion that plasma anticoagulated with citrate, lithium-heparin or EDTA and serum from the same individual contained different amount of S1P. Serum had particularly high S1P-levels, which was due to activated platelets that released S1P. In addition, S1P-concentrations in plasma were dependent on the centrifugation force applied. S1P decreased between 300*g* x 15 minutes and 2000*g* x 10 minutes due to removal of platelets, however from 2000*g* x 20 min to 20000*g* x 20 minutes S1P was to our surprise increased. We speculated that this was due to a re-organization of erythrocytes due to increased centrifugation forces as showed in a study by Cines *et al*. (289). Finally, we discovered that the S1P released from activated platelets

was mainly bound to albumin. If this was due to a saturation of apoM of due to the lack of an active transporter of S1P into apoM, needs to be clarified.

To conclude, we have established a robust S1P-quantification method and developed a handling protocol of blood samples ensuring accurate S1P-quantification. Quantification of S1P in blood samples is a delicate process since both erythrocytes and platelets can secrete S1P upon blood sampling. Platelets are easily activated for example by cold temperatures (290) and great care has to be considered in sample handling to ensure proper comparability between patient and control groups. Identical sampling protocol including type of plasma-anticoagulant and centrifugation force should be applied to all samples included in a study where S1P-levels are compared between groups. S1P-quantification in serum should be avoided, as this could be a measure of platelet function rather than actual S1P-concentration in the circulation. A drawback of the LC-MS/MS technique is the limited availability for most labs. However, future quantification methods more available such as ELISAs, needs to be further proven reliable in the complex quantification of S1P in order to replace the robust LC-MS/MS technique (291).

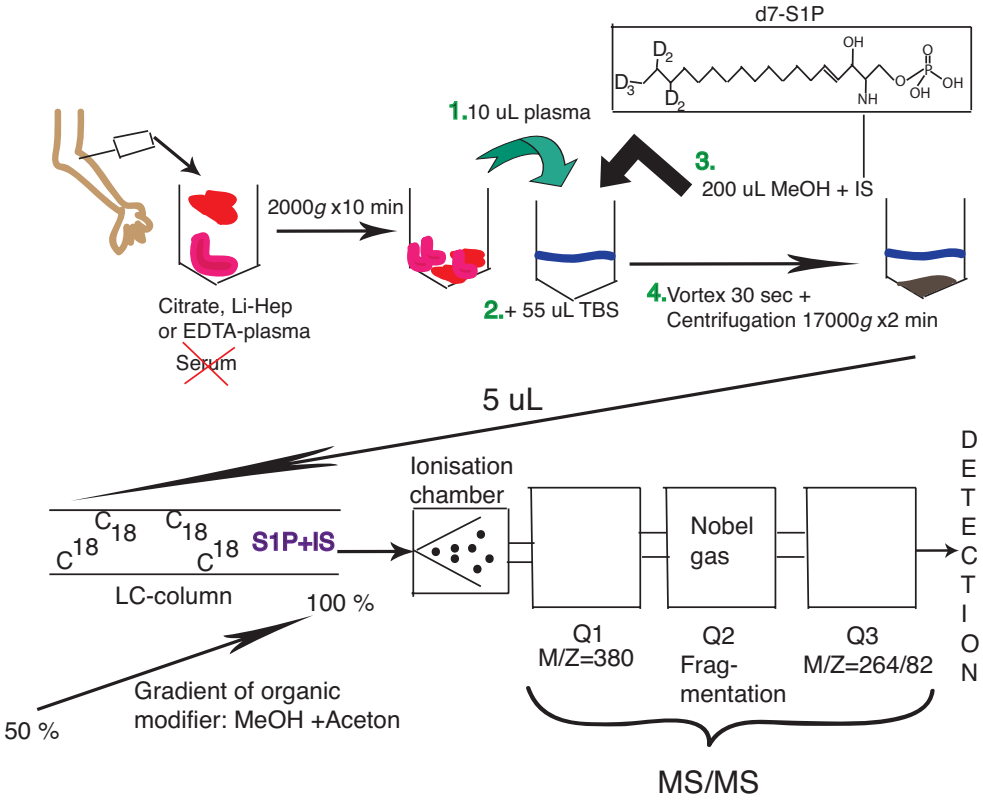

Figure 9: LS-MS/MS quantification of S1P

ApoM and S1P decrease in sepsis

In sepsis, the endothelial cell layer becomes highly permeable, leading to leakage of blood into tissues with subsequent low blood pressure and organ failure. The apoM/S1P-complex is important in mediating anti-inflammatory and endothelial protective effects (39, 101). It can through signaling via $S1P_1$ increase the assembly of VE-cadherin and increase the endothelial barrier function (39). Hence, it is interesting to study the role of apoM and S1P in sepsis. Plasma levels of apoM were shown to correlate with disease severity in human sepsis patients (187). By the time this study was carried out we did not have access to an S1P-quantification method. **In paper II** we used the LC-MS/MS method developed in paper I to quantify S1P in the same patient-cohort as used for apoM and found that S1P was decreased with increased severity of the disease, similarly to apoM. In addition, we had access to archived samples from a unique well-defined sepsis model in baboons developed at Oklahoma medical research center in the USA by Fletcher Taylor and Florea Lupu. They had infused different doses of *E.coli* into baboons, which subsequently developed different severities of sepsis and followed them by collecting samples over time. The mRNA expression of apoM in both liver and kidney was strongly decreased after 12 hours post *E.coli* infusion. We found that plasma levels of both S1P and apoM were decreased in relation to the severity of the disease and that S1P was decreased already within the first 6-8 hours, which was not seen for apoM. These results suggest that the loss of apoM is not the reason per se for the decrease in S1P. S1P correlated strongly with circulating platelets, hence we believe that the loss of S1P in our model was due to platelet senescence. Erythrocytes, which have been proposed to be the main source of circulating S1P, were decreased at later timepoints and did not correlate strongly with S1P levels. A recent study investigating the importance of erythrocytes and platelets as individual S1P sources revealed that both were needed for recovery after sepsis, whereas they had overlapping functions for the vascular development (292).

To conclude, our results show that the apoM/S1P complex is heavily decreased in sepsis reflecting the severity of the disease. Moreover, the transcription of apoM is turned off in the liver early in the sepsis time course. Since S1P is an important regulator of the endothelial barrier and function of mural cells, the decrease in apoM/S1P may lead to vascular dysfunction and could hence be a potential future pharmacological target in sepsis. It is intriguing to speculate in why the apoM-production is turned off in the liver and why plasma S1P is decreased already after 6 h despite strong presence of apoM and erythrocytes. S1P has opposing effect on the endothelial barrier depending on which receptor its signals through. $S1P_1$ increases endothelial barrier function whereas $S1P_2$ is barrier-disruptive (104). It is possible that S1P signals more through $S1P_1$ at low S1P levels and more through

$S1P_2$ at high S1P levels as we suggest in paper V. Hence it could be beneficial to reduce S1P-levels during the acute phase response. To investigate if the reduced apoM/S1P-levels in sepsis are protective or causative, wild type, $apoM^{-/-}$ and apoM-Tg mice could be challenged with different doses of bacteria to induce different severity of sepsis. If the $apoM^{-/-}$ mice had worse outcome and if the apoM-TG mice had increased survival or vice versa, it could give us a clue about whether the observed decreased plasma levels of apoM and S1P in sepsis are regulated in order to limit the inflammation or if it is a pathological consequence in sepsis. In addition, after induction of sepsis the S1P-levels could be restored by administration of S1P carried by different carriers in order to rule out carrier-specific effects and to evaluate whether restoring plasma S1P could rescue the septic phenotype. To investigate how the S1P-receptors mediate the endothelial permeability during sepsis, one could use endothelial specific knockouts of $S1P_{1-3}$, induce sepsis and inject Evans Blue and analyze leakage out in the tissues. To pharmacologically modulate the S1P/S1PR signaling pathway in sepsis, one would need to develop specific receptor inhibitors targeting only vascular S1P receptors. This because the S1PRs mediate trafficking of immune cells between tissue and circulation, which could be disrupted if targeting S1PRs generally. This is not favorable in sepsis since the presence of white blood cells disarms the invading MOs and prolonged lymphopenia is associated with increased mortality in sepsis (293). In addition, S1PRs have unique downstream effects and exert tissue specific signaling, demonstrating the need of receptor specific targeting in diseases to avoid unwanted side-effects. The inhibitory effect on formation of soluble VCAM-1 (sVCAM-1) by apoM and S1P is not known. If apoM and S1P have additional inhibitory effects on sVCAM-1, it could be important in sepsis as circulating levels of sVCAM-1 are associated with MOF and increased mortality (294).

The apoM/S1P-complex is important for the anti-inflammatory effects of HDL

In paper III we investigate the role of the apoM/S1P complex in endothelial inflammation. As discussed above, endothelial dysfunction is believed to be an initial step in atherosclerosis and contribute to increased risk of CVD in diabetes. Upon activation by pro-inflammatory stimuli, endothelial cells upregulate their expression of pro-inflammatory adhesion molecules like E-selectin, ICAM-1 and VCAM-1 to attract circulating monocytes to the site of inflammation. Hence, the upregulation of these adhesion molecules is an early event in the pathogenesis of atherosclerosis and is thereby an interesting pharmaceutical target. HDL can inhibit pro-inflammatory adhesion molecule expression on the endothelial surface

(246) and the apoM/S1P-complex has been suggested to mediate these effects of HDL (101). Galvani *et al*. isolated HDL from wild type and apoM$^{-/-}$ mice which they applied on human endothelial cells *in vitro* and found that S1P carried on HDL was able to inhibit ICAM-1 expression, whereas albumin-associated S1P and HDL-depleted of apoM were less efficient (101).

In paper III we investigate if we could confirm the findings by Galvani *et al*. by using HDL isolated from human healthy donors to study if the anti-inflammatory effects exerted by HDL are due to the presence of apoM and S1P. We challenged HAECs with TNFα to induce an inflammatory response and to induce expression of the pro-inflammatory adhesion molecules E-selectin, VCAM-1 and ICAM-1 on the endothelial surface. To test if apoM and S1P could inhibit expression of these adhesion molecules, recombinant apoM (rapoM) loaded with S1P or HDL isolated from human plasma containing or lacking the apoM/S1P-complex were added to the cells together with TNFα for 4 hours incubation in a cell incubator. Expression of adhesion molecules on the endothelial surface was analyzed by flow cytometry. We found that rapoM loaded with S1P could significantly inhibit the TNF-induced expression of E-selectin and VCAM-1 but not ICAM-1 compared to rapoM only. More interestingly, HDL containing apoM or enriched in apoM inhibited expression whereas HDL lacking apoM did not. Through the use of S1PR-specific inhibitors against $S1P_1$, $S1P_2$ and $S1P_3$ we found that these protective effects mediated by the apoM/S1P-complex were through $S1P_1$ signaling.

We analyzed monocyte adhesion by calculating the amount of fluorescently labeled monocytes that were attached on the endothelial cell layer upon the treatment described above. Bound monocytes were quantified visually in a fluorescent microscope or by measuring total cell fluorescence in cell lysate by a TECAN reader. RapoM+S1P and total HDL were slightly more efficient in inhibiting monocytes compared to either rapoM or HDL-apoM. The absence of a stronger effect by the apoM/S1P-complex could be due to the presence of ICAM-1 expression which is known to be constitutively expressed on endothelial cells (295). Previously, S1P associated with HDL has been shown to be more efficient in keeping the endothelial barrier intact as compared to albumin-associated S1P, suggesting carrier-specific roles of S1P (101, 296). We could confirm these results as we found that S1P carried by rapoM at low S1P-concentration was more efficient in inhibiting VCAM-1 compared to S1P carried by albumin.

To investigate the role of apoM and S1P in their influence on the permeability of the endothelial cell layer, we used two different methods commonly used for this purpose. In one we measured electric resistance over a confluent cell layer (TEER) and in the other we measured transcellular efflux of large molecules (70 KDa). We found that rapoM+S1P and HDL+apoM could rescue the barrier as it increased TEER and decreased transcellular efflux as compared to rapoM and HDL-apoM.

Even though the presence of the apoM/S1P-complex could inhibit the expression of adhesion molecules, it could not completely suppress the TNFα-induced upregulation of these molecules and in addition, it did not have any effect on ICAM-1 expression. These results suggest that there are other factors in HDL contributing to its endothelial protective effects. PON1 is associated with the same HDL-particles as apoM and has several endothelial-protective effects like inhibiting monocyte adhesion on human endothelial cells via antioxidative effects on HDL (297). Moreover, PON1 has anti-apoptotic effects on endothelial cells as shown by lack of inhibitory effects on caspase-9 in HDL isolated from $PON1^{-/-}$ mice as compared to wild type HDL (298). ApoA1 is also important as shown in humans with cardiovascular symptoms. When they were injected with reconstituted HDL containing apoA1 and phosphatidylcholine they had lower expression of VCAM-1 and lower lipid content in their plaques as compared to controls in the placebo group (299).

To conclude, these results suggest that the apoM/S1P-complex contributes in mediating the anti-inflammatory and endothelial protective effects by HDL through $S1P_1$. The S1P/S1PR signaling system has many known effects in murine atherosclerosis. $S1P_1$ suppresses ICAM-1 and VCAM-1 expression on endothelial cells as shown *in vivo* whereas $S1P_2^{-/-}$ mice have greatly attenuated foam cell formation and reduced atherosclerosis (101, 211). $S1P_3^{-/-}$ mice have diminished leukocyte rolling on endothelial cells compared to wild type mice as visualised *in vivo* by intravital microscopy (213). Whether S1P associated with apoM in HDL mediates these observed effects through $S1P_2$ and $S1P_3$ remains to be clarified. Possibly a specific $S1P_1$-agonist specifically targeting $S1P_1$ in the endothelium could be a potential pharmaceutical target in the treatment of atherosclerosis. Investigating how the apoM/S1P-complex directs its signaling specificity to the different S1PRs could help us develop such a compound.

ApoM and S1P in Type 1-diabetes

Obesity in the young population is associated with subsequent development of T1D (300). T1D patients have increased media-intima thickness and increased risk of developing CVD despite increased levels of HDL (301, 302). This make T1D-patients an interesting group in which to study HDL-functionality. Plasma levels of S1P are increased in obese mice and men (303). Moreover, S1P is increased in mice with T1D whereas both apoM and S1P are decreased in T2D (281, 304). However, the role of the apoM/S1P complex in human T1D is unknown.

In paper IV we investigated the role of the apoM/S1P-complex in HDL-functionality in T1D patients compared to healthy controls. We quantified plasma

levels of apoM and S1P in human T1D patients and healthy controls by ELISA and LC-MS/MS, respectively and found no difference between the groups. Both apoM and S1P have been shown to be associated with HDL_2 and HDL_3 particles. To investigate in which HDL-particles the apoM/S1P-complexes are located in T1D, light, medium-dense and dense HDL-particles were isolated from T1D and control plasma by ultracentrifugation. Both men and women with T1D had much more cholesterol in their light HDL-particles compared to corresponding particles in controls. Moreover, apoM and S1P were mostly located in dense and medium-dense particles in controls whereas in T1D there was a shift towards light HDL-particles, especially in women. HDL is believed to lose its anti-atherogenic properties in patients with CAV (305) and hence we were interested in evaluating the HDL functionality in T1D. Different HDL-subfractions were either pooled to generate total HDL or kept separated in order to investigate the functionality of the different subpopulations individually. We evaluated HDL-functionality by measuring its inhibitory effects on pro-inflammatory adhesion molecule expression on the endothelial surface by flow cytometry as in paper III. When total HDL was added to HAECs there was no difference in HDL-functionality between T1D and control HDL. Interestingly, when adding HDL-subfractions separately based on the cholesterol distribution in plasma, light HDL-particles were less able to inhibit the expression of E-selectin and completely unable to inhibit VCAM-1 expression compared to denser particles in both T1D or controls. When adding the different subfractions containing the same amount of S1P, i.e. 100 nM, light HDL-particles were still completely unable to inhibit VCAM-1 expression.

To conclude, T1D patients, particularly women, have increased levels of cholesterol in their light particles and these particles are less anti-inflammatory compared to dense HDL-particles. Thereby, T1D-patients have more dysfunctional HDL in regards of its anti-inflammatory properties. The light HDL particles in T1D have higher levels of apoM/S1P-complexes, but despite this, these particles are still less anti-inflammatory. This shows that the light HDL-particles are not less anti-inflammatory due to lower amounts of apoM and S1P. The inhibitory effects by the apoM/S1P-complex in HDL is carried out through signaling via the $S1P_1$ receptor (101). As shown by our results, this interaction is impaired when apoM and S1P is present on lighter HDL particles. The underlying mechanism behind this could be sterical hindrance since these particles are large and hence the interaction with receptors on the endothelial surface could be aggravated. To investigate the mechanism behind the reduced anti-inflammatory effects by the lighter HDL-particles one could use reconstituted HDL made of different composition to rule out the influence of different proteins and lipids. One such approach was made by Ashby *et al*. who replaced apoA1 with apoAII in HDL_3 particles and found no alteration in the inhibition of VCAM-1 on human endothelial cells (306). In addition, delipidated apoA1 or phosphatidylcholine

separately did not have any inhibitory effect on VCAM-1, however when combined into spherical HDL-particles the inhibitory effect was restored (306).

One explanation of the altered apoM/S1P-funtionality in light HDL-particles could be altered specificity in the signaling with the different S1PRs. The different S1P-receptors mediate opposite effects in the regulation of pro-inflammatory adhesion molecules. $S1P_2$ increase the expression of VCAM-1 and ICAM-1 whereas $S1P_1$ inhibits its expression. Since endothelial dysfunction is believed to contribute to increased atherosclerosis and CVD in T1D, impaired delivery of S1P to $S1P_1$ could be one explanation behind endothelial dysfunction in T1D. Altered specificity between the apoM/S1P-complex and S1PRs when the complex is present on different HDL-subclasses is possible and a very interesting research question. It is possible that when apoM/S1P-complexes are present on light HDL-particles, the specificity shifts towards $S1P_2$ and hence a possible loss of anti-inflammatory effect via $S1P_1$ is gained. This could be tested by using methods described in paper V.

In conclusion, there were no alteration in plasma levels of apoM and S1P in T1D compared to controls. T1D patients, especially women, had more cholesterol and apoM/S1P-complexes in their light HDL-particles and despite this, light HDL-particles were less anti-inflammatory than dense particles. This suggests that T1D patients have more dysfunctional HDL and that this could be one explanation for the absent protection by increased HDL in T1D-patients. A simplified overview of the anti-inflammatory actions by the apoM/S1P-complex are illustrated in Fig 10.

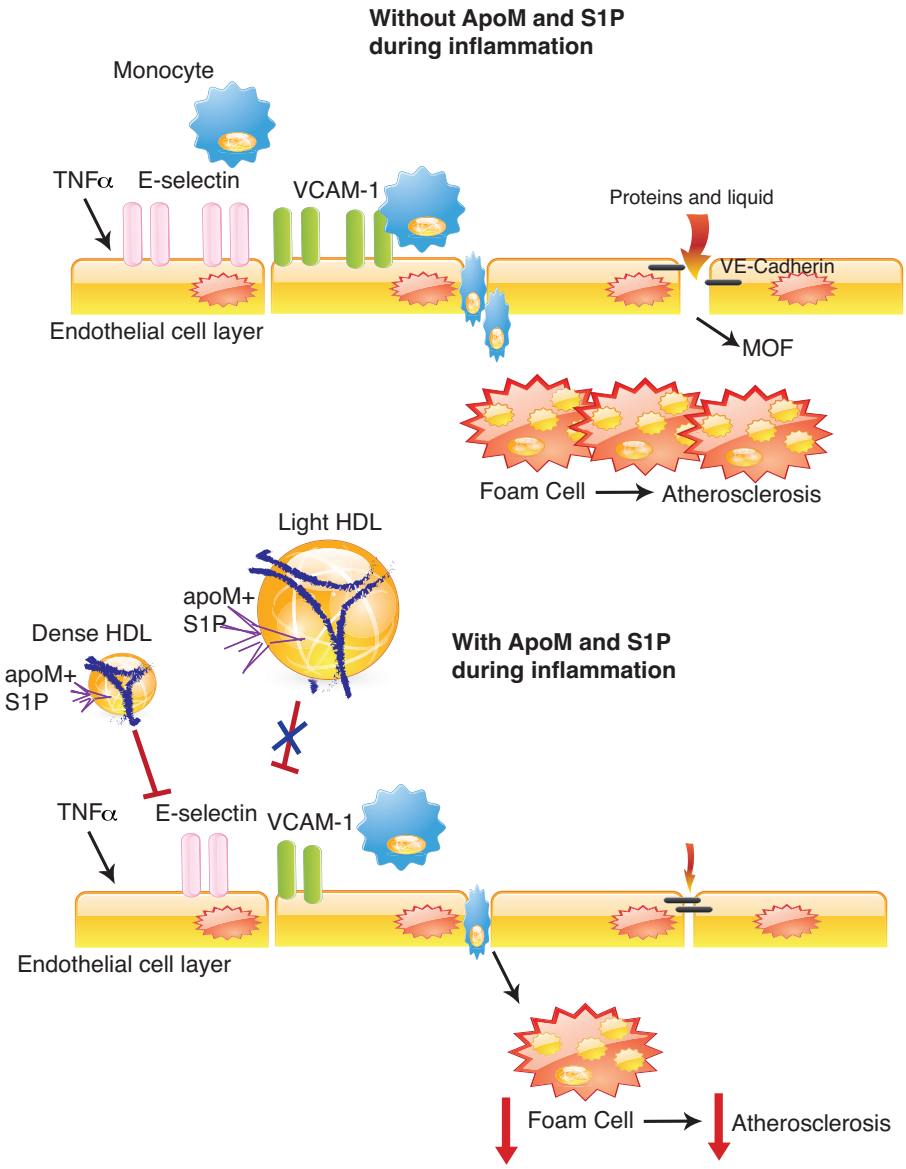

Figure 10: Actions of apoM and S1P in vascular inflammation

The apoM/S1P-complex and its interaction with S1P-receptors

S1P exerts its extracellular signaling via 5 GPCRs, $S1P_{1-5}$. These receptors are expressed in distinct cellular populations and in different organs in the human body. All S1PRs have been shown to internalize in response to the presence of extracellular S1P (120, 307-309). $S1P_1$ internalization and recycling are important for enabling lymphocyte egress from secondary lymphoid organs and to migrate towards the circulation. However, the importance and functionality of internalization and recycling pattern within the cell for the S1PRs are not well understood. Moreover, most of published internalization studies have been carried out by using unbound S1P. The role of S1P signaling through its different receptors when associated with apoM in HDL is not known.

In order to investigate the importance of apoM in delivering S1P to the S1PRs, we established stable cell clones of HEK293 cells expressing the five different S1P-receptors tagged with a GFP molecule **in paper V**. The coupling to a GFP-tag was done in order to follow the internalization pattern of the receptors by confocal microscopy. To investigate if rapoM+S1P could internalize all receptors and to study the time-dependent effect on receptor internalization, we added 900 nM rapoM+S1P to S1PR-GFP HEK293 cells and incubated them from 0-60 min. We fixed the cells with paraformaldehyde and analyzed receptor localization by confocal microscopy. All receptors were internalized by rapoM+S1P although with possibly different efficacy. The internalization of the S1PRs was differently regulated by time where $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ were mostly internalized after 20-30 minutes whereas $S1P_5$ was internalized after 40-60 minutes.

To quantify the effect of the S1P-dose on internalization, we established a method to isolate membrane-associated proteins using a non-cell permeable biotin. Biotinylated proteins were isolated with magnetic streptavidin-coated dynabeads and analyzed with SDS-PAGE gel followed by western blotting using a GFP-antibody. S1PR-GFP expressing cells were exposed to 0-900 nM recombinant apoM loaded with S1P followed by evaluation by confocal microscopy and cell surface biotinylation. Interestingly, $S1P_1$ and $S1P_2$ showed distinct results from one another. $S1P_1$ was internalized in a dose-dependent manner whereas $S1P_2$ was strongly internalized at low S1P levels (100 nM) but present at the cellular membrane at high S1P-levels (900 nM). $S1P_4$ and $S1P_5$ were not clearly affected in a dose-dependent manner by rapoM+S1P.

In order to evaluate the function of the apoM/S1P-complex when present on its different carriers, HDL was isolated from human plasma obtained from healthy volunteers through ultracentrifugation and subsequent gelfiltration. ApoM-

containing HDL was depleted from apoM via affinity chromatography with monoclonal antibodies against apoM (made in house). HDL was characterized by its size, and apolipoprotein content as evaluated by SDS-PAGE gel followed by silver staining. ApoM and apoA1 were quantified by ELISA, and S1P by LC-MS/MS. The addition of HDL containing apoM/S1P (150 nM S1P) resulted in internalization of all S1PRs, whereas HDL lacking the apoM/S1P complex retained the receptor at the cell surface. Also albumin containing similar amounts of S1P had similar effects as HDL+apoM/S1P.

To investigate if HDL could induce the same dose-dependent effects as rapoM+S1P, HDL containing 150, 300 and 900 nM S1P was added and the internalization was evaluated by confocal microscopy. HDL containing different S1P-concentrations revealed the same pattern for $S1P_1$ and $S1P_2$, as did rapoM+S1P, i.e $S1P_1$ was internalized dose-dependently and $S1P_2$ only at low S1P-levels. $S1P_4$ and $S1P_5$ were only slightly internalized by rapoM+S1P or HDL. These results can explain dose-dependent effects exerted by S1P as different doses regulate which S1PR is present on the cell surface. For example, low levels of S1P (1nM) induce substantial degranulation of human mast cell in vitro via the $S1P_2$ receptor (72) and high S1P concentrations (1 μM) induce release of pro-inflammatory cytokines from mast cells whereas low levels does not (72).

In conclusion, this paper shows that S1P carried by apoM can internalize all S1P-receptors although with different efficacy and at different concentration. Extracellular S1P-levels might regulate which receptors are present in the cellular membrane. Since the S1PRs mediate diverse intracellular signaling pathways, this finding could be important in order to understand how apoM/S1P controls S1P-signaling. These methods could be used in order to study the mechanism behind the observed effects in paper IV. Different sizes of reconstituted HDL could be added to the different S1PR-expressing cell clones and internalization could be measured. This could tell us if light HDL preferentially signals through $S1P_2$ and hence could explain the observed effects of decreased anti-inflammatory effects by light HDL-particles.

To conclude, our findings suggest that the apoM/S1P-complex is an important mediator in regulating endothelial protective effects. In sepsis, effective treatment is lacking and treatment-resistant low blood pressure is characteristic of septic shock. In atherosclerosis, recent clinical trials investigating compounds resulting in increased HDL-C levels failed in reducing the risk of CVD. ApoM and S1P could potentially be future pharmaceutical targets in these inflammatory diseases mediating increased endothelial barrier function and decreased expression of pro-inflammatory adhesion molecules. However, one should be aware of targeting the S1P/S1PR signaling is challenging as the receptors mediate opposing downstream effects in distinct cell populations.

Major findings

*Identical blood sampling protocol for all samples enrolled in a study in which S1P-quantification is exerted is of highest priority to ensure accurate comparison between groups.

* S1P secreted upon platelet activation is bound to albumin.

*The apoM/S1P-complex is decreased in sepsis reflecting the severity of the disease in both humans and non-human primates.

*The transcription of apoM is turned off in *E.coli* sepsis in the non-human primate liver.

*ApoM and S1P contribute in the anti-inflammatory effects exerted by HDL on the endothelial surface.

*ApoM and S1P plasma levels are not altered in T1D

*The apoM/S1P-complex shift towards light HDL-particles in T1D. These particles are unable to inhibit pro-inflammatory adhesion molecule expression on endothelial cells.

*The apoM/S1P-complex can induce internalization of all S1PRs, however with different efficacy.

*$S1P_1$ is dose-dependently internalized by rapoM+S1P and HDL, whereas $S1P_2$ is strongly internalized at lower levels and present at the cellular membrane at higher levels.

Populärvetenskaplig sammanfattning

Insidan av kärlväggen kläds av ett enkelt lager av endotelceller med underliggande motoriska celler och stödvävnad som fungerar som en barriär mellan blod och vävnad. Det innersta lagret, endotelcellerna, kommer i direkt kontakt med komponenter i blodet och utsöndrar molekyler för att kommunicera med underliggande celler. Endotelcellerna reglerar genomsläppligheten av vatten, gaser och proteiner till underliggande celler och utgör därmed en viktig funktion i kommunikationen mellan blod och vävnad. Hög-densitets-lipoproteiner (HDL) transporterar kolesterol och fett i blodet och anses skydda mot sjukdomar i blodsystemet. Dess huvudsakliga funktion är att transportera kolesterol från celler i kroppen till levern för vidare utsöndring i avföringen och anses på så sätt skydda från ansamling av kolesterol i kärlväggen (så kallad ateroskleros). HDL har utöver detta anti-inflammatoriska egenskaper och anses vara en del av den första försvarslinjen i immunsystemet. Till exempel så kan HDL neutralisera sjukdomsalstrande proteiner utsöndrade från bakterier och förhindra inflammatoriska förändringar i endotelceller. HDL kan även öka kontakten mellan endotelceller och på så sätt förhindra alltför stor genomsläpplighet. Apolipoproteiner utgör en stor del av HDLs komposition och är viktiga för många av HDLs effekter i den vaskulära väggen. Apolipoprotein A1 är det vanligaste proteinet och är oumbärligt för strukturen av HDL. ApoA1 utsöndras fritt från kolesterol och fett från levern som genom sin resa genom kroppen absorberar kolesterol från celler och formar moget HDL. HDL kan därmed finnas i olika storlekar eftersom det kan innehålla olika mängd kolesterol. Betydelsen av de olika storlekarna av HDL är inte klarlagt, men det finns vissa indicier som pekar på att det har olika effekt.

Ett nytt apolipoprotein blev upptäckt för ca 16 år sedan och blev kallat apolipoprotein M (apoM) efter att ha blivit upptäckt näst efter apolipoprotein L. ApoM visade sig vara associerat till största delen med HDL i humant plasma genom att binda med en icke-klyvbar peptid. ApoM tillhör en grupp proteiner som heter Lipokaliner. Lipokaliner karakteriseras av att de har en ficka där små fettsyror kan binda in och på sätt transportera fettsyror i kroppen. Genom djurstudier visade sig apoM vara involverat inom lipoprotein metabolismen och påverkade nivåer av både HDL och låg-densitets lipoproteiner (LDL). Ökning av apoM-nivåer i plasma ledde till minskad inflammation och ansamling av

kolesterol i kärlväggen. Även om dessa resultat indikerade på att apoM kan vara involverat i lipoprotein metabolismen, var den huvudsakliga rollen av apoM länge okänd. Det skulle dröja ända till 2011 då det visade sig att apoM var den huvudsakliga transportören av sfingosin 1-fosfat (S1P). S1P kan binda till 5 olika receptorer på cellens yta och kan på så sätt reglera olika effekter i cellen. Till exempel kan S1P reglera överlevnad, cell-delning och cell-rörlighet. S1P är involverat i immunförsvaret där den till exempel reglerar förflyttning av immunceller mellan lymfan och blodet. S1P kan också reglera genomsläppligheten hos endotelceller genom att påverka dess rörlighet och cell till cell kontakter. I blodet transporteras S1P till största delen av apoM i HDL men kan även transporteras av albumin. Hur de olika effekterna av S1P fungerar när det är bundet till apoM är till största del okänt.

I denna avhandling har vi studerat reglering av inflammatoriska effekter på humana endotelceller av S1P när det i huvudsakligen är bundet till apoM. För att kunna göra detta etablerade vi först en kvantifieringsmetod för S1P där vi kunde kvantifiera S1P i serum och plasma. S1P produceras av både röda blodkroppar och blodplättar, vilka kan utsöndra S1P vid provtagning. Detta gör det svårt att mäta S1P i blodet då aktiverade blodceller kan utsöndra S1P och ge felaktiga resultat. Vi etablerade ett protokoll för säker hantering av blodprover genom att utföra olika sorters stabilitets-experiment. Vi kom fram till slutsatsen att tillräcklig centrifugering av proverna var nödvändigt för att avlägsna alla blodplättar (ca 2000*g* i 10 minuter). När väl alla blodplättar var avlägsna var provet stabilt mot till exempel upprepade nedfrysnings-cykler. Den viktigaste slutsatsen var att behandla blodprover från kontroller och patienter identiskt eftersom många parametrar kunde påverka S1P-halterna i proverna.

Genom att använda denna metod kvantifierade vi S1P i plasma från patienter med olika svårighetsgrad av blodförgiftning (sepsis) och i arkiverade prover från icke-humana primater, också med olika svårighetsgrader av sepsis. Vi kunde även analysera transkription av gener i levern och njuren såsom apoM, apoA1, albumin och andra gener involverade i S1P-metabolismen. Vi fann att plasma nivåer av S1P och apoM var kraftigt minskade vid sepsis och minskningen reflekterade sjukdoms-graden. S1P i plasma sjönk tidigt, redan efter några timmar vilket innebär att S1P kan fungera som en markör för sepsis. Vi fann även att transkriptionen av apoM i både njuren men framförallt i levern var starkt hämmad redan 12 timmar efter att bakterier hade kommit in i blodet.

Vi var även intresserade av att undersöka om apoM/S1P-komplexet kunde förhindra inflammations-mekanismer involverade i ateroskleros. Vi utsatte humana endotelceller för inflammatorisk stimuli med eller utan närvaro av apoM och S1P. Sedan mätte vi antalet inflammatoriska molekyler, så kallade adhesions-molekyler, som är en del av patologin bakom uppkomsten av ateroskleros. Vi

mätte dessa på endotelcellens yta för att se om apoM och S1P kunde förhindra dess uppkomst. Vi fann att HDL innehållande apoM och S1P delvis kunde hämma förekomsten av inflammatoriska molekyler på ytan medan HDL utan apoM och S1P var oförmögen till det. Detta var också sant för upprätthållandet av endotelcells-barriären. Dessa resultat visar att apoM/S1P-komplexet är viktigt för anti-inflammatorisk verkan av HDL på endotelceller och att dess närvaro kan hjälpa till med att förhindra ateroskleros.

För att studera detta i en population som har ökad risk för ateroskleros och hjärt/kärl-sjukdom undersökte vi apoM och S1P i patienter med typ 1-diabetes (T1D). Vi kvantifierade apoM och S1P i plasma från 89 patienter med T1D och 42 hälsosamma kontroller. Vi fann att det inte var någon skillnad mellan dessa grupper med avseende på halter av apoM och S1P i plasma. T1D-patienter har ofta ökad koncentration av HDL i blodet, men är inte mer skyddade från hjärt/kärl-sjukdom. Detta gör dem till en intressant grupp att studera HDL-funktionalitet i eftersom deras HDL kan vara dysfunktionellt. Vi isolerade olika storlekar av HDL från plasma genom centrifugering och applicerade HDL från både T1D-patienter och kontroller på humana endotelceller via samma experimentella modell som beskrivet ovan. Vi fann att T1D-patienter hade mer kolesterol i deras lätta HDL-partiklar jämfört med kontroller. Även apoM/S1P-komplexet var förskjutet mot lätta partiklar i T1D jämfört med kontroller, särskilt för kvinnor. Trots att apoM och S1P fanns i lätta partiklar var dessa sämre i att hämma inflammatoriska adhesions-molekyler på endotelcellernas yta. Dessa resultat föreslår att T1D-patienter har mer HDL som inte kan hämma inflammatoriska processer. Detta kan vara en del av orsaken till en ökad risk för hjärt/kärl-sjukdom i T1D.

Det är väletablerat att S1P när det är buret till apoM kan signalera via en av S1P-receptorerna, $S1P_1$. Huruvida apoM-associerat S1P kan signalera genom övriga receptorer är inte känt. Vi undersökte detta genom att utrycka grön-fluorescerande S1P-receptorer som vi stimulerade med S1P bundet till apoM. Eftersom receptorerna var fluorescerande kunde vi följa receptorernas reaktion på olika stimuli. Vi såg att apoM-associerat S1P kunde interagera med alla S1P-receptorerna och att mängden tillsatt komplex kunde bestämma vilken receptor som var närvarande på cell-ytan.

För att sammanfatta så pekar våra resultat på att halter av apoM och S1P i blodet är starkt minskade under akut inflammation. Om detta är en patologisk påföljd av sepsis eller regleras för att påverka den inflammatoriska responsen återstår att klargöra. Vi såg också att apoM/S1P-komplexet är viktigt för de anti-inflammatoriska effekterna av HDL i kärlväggen och kan hämma inflammatoriska adhesionsmolekyler på endotelcellens yta och därmed hämma uppkomst av ateroskleros. När apoM och S1P är närvarande på stora HDL-molekyler (när de innehåller mycket kolesterol) försvinner delar av deras anti-inflammatoriska

effekter och denna sorts HDL-molekyler är mer frekventa i personer med ökad risk för hjärt/kärl-sjukdom. Slutgiltligen så visar våra studier att apoM-associerat S1P kan interagera med alla S1P-receptorer med olika effektivitet. Våra resultat visar att apoM och S1P är framtida potentiella farmaceutiska mål för utvecklandet av nya läkemedel inom vaskulära inflammatoriska sjukdomar.

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Appendix 1: Apolipoproteins

Apolipoprotein B100 (apoB100) is a 515 KDa protein responsible for assembling the non-HDL-C lipoproteins; VLDL, IDL and LDL (184). Every VLDL/LDL-particle contains one apoB100-molecule and hence it can be used to measure the amounts of VLDL/LDL-particles in plasma since the amount of cholesterol in these particles highly varies. VLDL is formed via stepwise intracellular lipidation of apoB100, which is dependent on the triglyceride pool stored in intracellular cytosolic lipid droplets (310, 311). ApoB100 is important for the binding of the VLDL/LDL-particle to the LDL-receptor (312, 313) although it binds with a relatively low affinity (314). ApoB100 is pro-atherogenic. It directly interacts with negatively charged proteoglycans in the arterial wall which are produced by smooth muscle cells upon pro-inflammatory stimuli like TGFβ (315). The apoB100 containing LDL-particle is taken up by monocytes which differentiates into macrophages and becomes foam cells, a major component in the atherosclerotic plaque (131).

Apolipoprotein B48 (apoB48) is important in the formation of chylomicrons in the enterocyte. As newly synthesized, apoB48 is lipidated by microsomal triglyceride transfer protein (MTP) in the endoplasmic reticulum forming nascent chylomicrons. ApoB48 is produced by the same gene as apoB100, but in the intestine a posttranscriptional enzyme, apobec-1, induces a stop-codon by replacing a single nucleotide (C→U) in the mRNA of apoB100 (316). ApoB48 lacks the LDL-receptor binding site.

Apolipoprotein A1 (apoA1, 28 KDa), the major protein in HDL, is the main substrate for the ATP-binding cassette transporter (ABCA1) which lipidates pre-β-HDL with cholesterol. ABCA1 protects apoA1 from rapid degradation as shown in patients with Tangiers disease (317). ApoA1 has a double α-helix structure, which is important for its high affinity for lipids. For apoA1 to adapt to the alteration in HDL-particle size, an inter-helical segment on the N-terminal domain has been identified to be important (318). Moreover, ApoA1 acts as a catalytic enzyme for CETP enabling the formation of spherical HDL (319).

ApoA1 is protective against cardiovascular disease as shown in both mice and humans. Overexpression of apoA1 in mice leads to increased levels of

HDL and reduced atherosclerosis and apoA1 knock-out mice have increased atherosclerosis (320-322). Low levels of apoA1 is correlated with increased risk of a primary stroke in humans (323). Moreover, plasma levels of apoA1 was superior to HDL levels in order to assess the severity or predict human coronary heart disease (CHD) (324, 325). Infusion of apoA1-containing reconstituted HDL into patients with manifested atherosclerotic lesions resulted in altered plaque phenotype with reduced lipid content, lower macrophage size and decreased presence of inflammatory markers (299). ApoA1 has potent anti-oxidative properties via association with hydroperoxyoctadecadienoic acid and hydroperoxyeicosatetraenoic acid, which could inhibit endothelial mediated oxidation of LDL (326).

ApoA1 also have neuroprotective effects assigned to its inhibitory effects on pro-inflammatory molecules and might have a therapeutic role in multiple sclerosis (327-329). Moreover, apoA1 is important in glucose metabolism. ApoA1-null mice had reduced glucose tolerance and reduced endurance capacity probably due to ATP-depleted synthesis in their mitochondria, which was restored in apoA1 transgenic mice (330).

Apolipoprotein A-II (apoA-II, 17.4 KDa) is produced by the liver and is the second most common protein in HDL (20 % by weight of total proteins in HDL). ApoA-II is believed to be shaped like a “double hairpin” wrapped around the HDL-particle (331). It is believed to be important for the stabilization of apoA1 and in creating the form of mature HDL (332). ApoA-II can indirectly inhibit the interaction between apoA1 and LCAT by displacing apoA1 on the HDL surface (333).

Apolipoprotein A-IV (apoA-IV, 45.4 KDa) is produced upon fat ingestion in the intestine where it is attached to chylomicrons. However, it is detached upon lipase action on chylomicrons. Acute increased intake of fat increased the secretion of ApoA-IV into the lymph. In contrast, chronic consumption or obesity is associated with reduced apoA-IV gene expression (334, 335). In the circulation, apo-IV is mostly in a lipid-free form (40-50 %) but it can also associate with HDL and chylomicron remnants. Overexpression of ApoA-IV in mice leads to reduced atherosclerosis suggesting apoA-IV to be a protective against CVD (336, 337).

Apolipoprotein A-V (apoA-V, 41.2 KDa) is synthesized in the liver and important in the clearance of TGs as shown by transgenic and knock-out apoA-V mice (338). ApoA-V enhances the activity of LPL and decreases TG levels in TG-rich lipoproteins (339).

Apolipoprotein C-I (apoC-I, 9.3 KDa) and **Apolipoprotein C-II** (11.2 KDa) are produced mainly in the liver and secreted in a lipid-free form but quickly associate with lipoproteins due to their high affinity for lipids (340). ApoC-I and apoAC-II inhibit uptake of apoE-containing VLDL by interacting with the binding of apoE to the low density lipoprotein receptor (LDLR-) related protein (LRP) (341). ApoC-II is a potent activator of LPL via its lipid binding domain (342-344).

Apolipoprotein C-III (apoC-III, 10.8 KDa) is important in the metabolism of TG-rich lipoproteins and is mostly associated with chylomicrons, VLDL and HDL. Upon endothelial LPL action on VLDL, apoC-III transfers mostly to HDL but is then transported back to newly synthesized VLDL (345, 346). ApoC-III prolongs the circulatory time for VLDL by inhibiting the action of endothelial LPL and in contrast to apoE it does not bind to the LDL-receptor (347). ApoC-III inhibits the action of LPL via direct interaction (348). ApoC-III levels strongly correlates with the levels of TGs in human plasma and increased TG levels are present in patients with CHD (349). Overexpression of apoC-III in mice leads to strongly increased levels of TGs and in apoC-III null mice, TGs are low (350, 351). Since ApoC-III inhibits the action of lipases, individuals with low levels of apoC-III have low TG levels due to a strong lipolysis (352).

Apolipoprotein D have a hydrophobic binding pocket and was found to be in complex with progesteron, cholesterol, bilirubin and arachidonic acid (353). The presence of the binding pocket makes it a structural member of the lipocalin family (353). In contrast to many other lipoproteins, the expression of apoD is not limited to the liver but is widely expressed in the human body (354). ApoD is important for proper function of the brain and humans and mice treated with antipsychotic drugs have increased plasma levels of apoD (355). ApoD is also increased in the brain after traumatic brain injury (356). ApoD is mainly bound to HDL in circulation and is enriched in HDL from patients with CVD compared to healthy controls (264). ApoD is increased in $apoE^{-/-}$ mice suggesting a role of apoD in atherosclerosis (357).

Apolipoprotein E (apoE) is a small protein (36 KDa) synthesized mainly in the liver and intestine. ApoE has anti-atherogenic properties and is mostly present on TGs-rich lipoproteins. ApoE has high affinity for the LDL-receptor (är det inte en related receptor?) which is important in the clearance of pro-atherogenic TGs-rich lipoproteins (e.g IDL and VLDL) (358). After uptake of lipoprotein remnants, apoE are re-used in an apoE-containing HDL-pool and hence HDL can be cleared via the LDL-receptor (359).

Apolipoprotein F (apoF, 35.4 KDa) is associated with LDL and HDL and has CETP inhibitory activity (360). Overexpression of apoF in mice lead to increased clearance of HDL and increased macrophage cholesterol efflux (361). However, depletion of apoF did not have any effects on HDL-C levels (362). In a study by Lagor *et a*l., apoF silencing resulted in reduced atherosclerosis despite no influence on circulating lipoprotein levels, suggesting pro-atherogenic properties of apoF (363).

Apolipoprotein J (apoJ, 52.4 KDa) also known as clusterin, is associated with dense HDL-particles containing PON-1 and apoA1. ApoJ has a regulatory role in the immune system through interactions with proteins such as immunoglobulins and complement factors (364).

Apolipoprotein L (apoL, 43.9 KDa) is associated with apoA1-containing particles in the density range between 1.123 and 1.21 g/mL and no free apoL is found in plasma (365). ApoL is in complex with trypanosome lyctic factor-1 (TLF-1) and forms pores in lipid bilayer membranes, which lyse bacteria and parasites (366). Hence, apoJ is important for the role of HDL in the innate immune defense.