Apolipoprotein M – Studies of Structure and Function

Ahnström, Josefin

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Studies of Structure and Function

Josefin Ahnström

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LUND UNIVERSITY
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Abstract
Apolipoprotein M (apoM), a 25 kDa plasma protein, is found in all major lipoprotein classes, although the majority is found in high density lipoproteins (HDL). ApoM has been suggested to be involved in the formation of and adding to the antiatherogenic functions of HDL, but its function is still not completely known.

ApoM has been suggested to be a lipokalin. By studies in vitro using recombinant apoM we were able to show that apoM shares the ligand-binding abilities of lipokalins by being able to bind retinol and its two metabolites, all-trans retinoic acid and 9-cis retinoic acid. After successful crystallization trials we managed to determine the 3D structure of recombinant apoM expressed in E.coli. ApoM displays the typical lipocalin fold, i.e. characterized by an 8-stranded antiparallel β-barrel enclosing an internal ligand-binding pocket, flanked by a α-helix. The ligand-binding pocket in apoM can be subdivided into a hydrophilic upper part and a hydrophobic lower part. ApoM was crystallized as a complex with either myristic acid or glycerol-1-myristate in the hydrophobic pocket showing that apoM can work as a fatty acid binding protein. We were also able to detect a binding of D-sphingosine and sphingosine-1-phosphate in ligand-binding studies. The physiological importance of the binding is not known.

Mature apoM is circulating with a retained signal peptide. By constructing apoM with a cleavable signal peptide we were able to show that the signal peptide is necessary for the protein's ability to associate to lipoproteins. We were also able to show that, in contrast to apoM with a cleavable signal peptide, full-length apoM was accumulated in stably transfected HEK293 cells, expressing apoM, unless serum was present. The addition of extra cellular HDL or co-expression of HDL was enough to completely restore the apoM expression.

We have in a previous study found a marked positive correlation between plasma apoM and total cholesterol levels in healthy individuals. To investigate whether plasma apoM levels predict the risk of coronary heart disease, apoM was measured in plasma from subject later developing CHD and healthy controls from two prospective case-control studies, FINRISK ´92 and Copenhagen City Heart Study. In conditional logistic regression analyses, apoM was not a predictor of CHD events. We found positive correlations for apoM with both apoA-I and apoB.

Key words: Apolipoprotein, lipocalin, crystallization, signal peptide, signal peptidase, HDL, coronary heart disease

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Man can't help hoping even if he is a scientist.
He can only hope more accurately.
--Karl A. Menninger

CONTENTS

LIST OF PAPERS

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

I. ***Ahnström J.,*** *Faber K., Axler O. and Dahlbäck B.* Hydrophobic ligand-binding properties of the human lipocalin apolipoprotein M. *J Lipid Res* 2007. 48: 1754–1762.

II. *Sevvana M.*,* ***Ahnström J.*,*** *Egerer-Sieber C., Dahlbäck B. and Muller Y.A.* Serendipitous fatty acid binding reveals the structural determinants for ligand recognition in apoM. *Submitted manuscript.*

III. *Axler O.,* ***Ahnström J.,*** *and Dahlbäck B.* Apolipoprotein M associates to lipoproteins through its retained signal peptide. *FEBS Lett* 2008. 582(5): 826–828.

IV. ***Ahnström J.,*** *Axler O. and Dahlbäck B.* HDL stimulates ApoM secretion. *Submitted manuscript.*

V. ***Ahnström J.*,*** *Axler O.*, Jauhiainen M., Salomaa V., Havulinna A., Ehnholm C., Frikke-Schmidt T., Tybjaerg-Hansen A., and Dahlbäck B.* Levels of apolipoprotein M are not associated with the risk of coronary heart disease in two independent prospective case-control studies. J Lipid Res 2008. 49(9): 1912–1917.

*Authors contributed equally to this work.

ABBREVIATIONS

ABCA1	ATP-binding cassette A1
ABCG1	ATP-binding cassette G1
apoA-I	Apolipoprotein A-I
apoA-II	Apolipoprotein A-II
apoB	Apolipoprotein B
apoB-48	Apolipoprotein B-48
apoB-100	Apolipoprotein B-100
apoC	Apolipoprotein C
apoD	Apolipoprotein D
apoE	Apolipoprotein E
apoM	Apolipoprotein M
Arg	Arginine
Asp	Asparagine
BMI	Body mass index
CCHS	Copenhagen City Heart Study
CETP	Cholesterol ester transfer protein
CHD	Coronary heart disease
CVD	Cardiovascular disease
Glu	Glutamine
HDL	High density lipoprotein
HDL-c	HDL-cholesterol
HEK293	Human embryonic kidney 293
HNF-1α	Hepatocyte nuclear factor 1-alpha
IC_{50}	Half maximal inhibitory concentration
IDL	Intermediate density lipoproteins
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein

LDL-c	LDL-cholesterol
LDLr	LDL-receptor
LPL	Lipoprotein lipase
LRH-1	Liver receptor homolog 1
LRP	LDLr-related protein
LTA	Lymphotoxin alpha
LXR	Liver X receptor
MHC	Major histocompatibility complex
MI	Myocardial infarction
MODY	Maturity onset of diabetes in the young
MUP	Major urinary protein
NGAL	Neutrophil-gelatinase associated lipocalin
PAF	Platelet-activating factor
PON-1	Paraoxonase-1
RBP	Retinol binding protein
SAA	Serum amyloid A
SNP	Single-nucleotide polymorphism
SR-B1	Scavenger receptor type B1
TNF	Tumor necrosis factor
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
Trp	Tryptophan
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein

INTRODUCTORY WORDS

This thesis will give you a short introduction to the lipid metabolism, the most common apolipoproteins and the lipocalin protein family. The focus will be on one of our apolipoproteins and lipocalins, apolipoprotein M (apoM), the protein I have been working with during my PhD studies. ApoM is a protein that was discovered only 10 years ago. In this thesis you will get a summary of what is known about apoM today, and what I have been spending the last four years doing.

LIPOPROTEINS AND THEIR METABOLISM

Fat is the main source of energy that is transported between different organs. As fat is insoluble in water it is transported in blood in the form of lipoproteins.[1,2] Lipoproteins are complexes formed by fat and specific proteins (apolipoproteins). Each particle consists of a hydrophobic core of mainly triglycerides and esterified cholesterol, surrounded by a shell of amphiphilic molecules, phospholipids, unesterified cholesterol and apolipoproteins (Figure 1).[1,2] The traditional definition of the lipoprotein classes is based on their density or, alternatively, their electrophoretic mobility.[1,3] When classified according to increasing density they are divided into chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL).[1-3] Because of the lack of standardized isolation procedures, the end products might not necessarily be identical from one laboratory to another. By electrophoretic criteria, serum lipoproteins may also be classified according to their mobility in electric fields. The correspondences to the ultracentrifugal classes are indicated in Table 1. Even though the lipoproteins are divided into different classes these are not distinct populations. All lipoproteins are parts of the same system with a constant exchange of contents and are being formed or degraded at different rates (Figure 2).[1]

Chylomicrons are large, triglyceride rich particles, formed in the intestinal mucosal cells, and are only present in the

circulation after food intake.[1,4] Their main apolipoprotein is apolipoprotein B-48 (apoB-48). The chylomicrons transport fat from the intestine to the tissues, where they are rapidly metabolized by lipoprotein lipase (LPL). The chylomicron remnants are readily taken up by the liver by the LDL-receptor (LDLr) and the LDLr-related protein (LRP).[4,5]

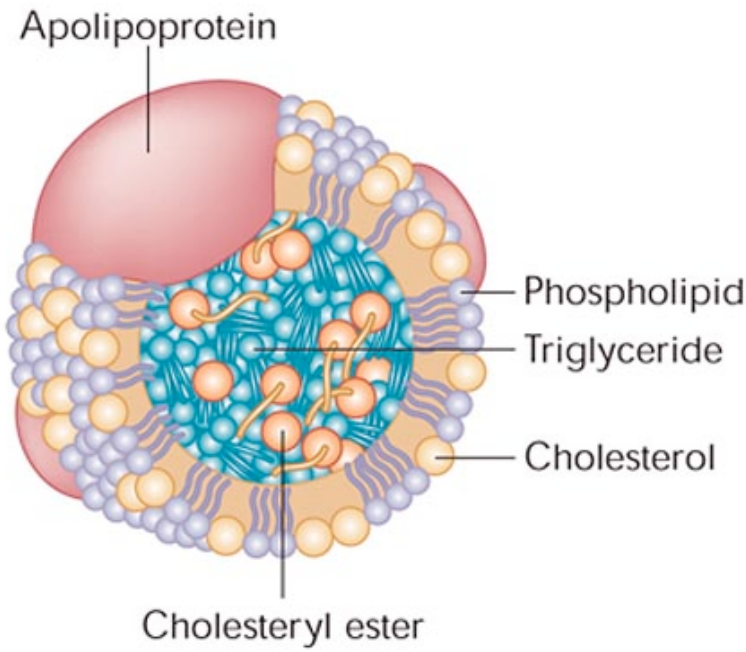

Figure 1. Lipoproteins are composed of a hydrophobic lipid core filled with esterified cholesterol molecules and triglycerides. Phospholipids, free cholesterol, and apolipoproteins form the outer surface of the lipoprotein particles. The image was originally published in Atlas of Heart Diseases: Cardiovascular Risk Factors by Gornik H., Plutzky J.[6] and is reprinted here with kind permission from Current Medicine Group LLC.

VLDL, IDL and LDL transport endogenous fat from the liver to the other tissues, mainly adipose tissues, heart and muscles.[2] VLDL is formed in the liver with apolipoprotein B-100 (apoB-100) as the main apolipoprotein. The half time of VLDL is, as for chylomicrons, very short. Most of the triglycerides are

taken up by the tissues, and the remaining content is forming an IDL particle that is quickly hydrolyzed to LDL.[4,5] In fact, the half time of IDL is so short (minutes) that the levels in plasma are normally almost undetectable.[1] LDL, in contrast to VLDL and IDL, has a fairly long half time (3-4 days). LDL are triglyceride-poor, cholesterol-rich particles and contain about 70% of the fat present in the circulation.[1] LDL transports cholesterol to tissues via the binding to the LDL receptors.[4]

HDL is a group of smaller, protein rich lipoprotein particles that contain apolipoprotein A-I (apoA-I) and/or apolipoprotein A-II (apoA-II). About 70% of the total HDL protein is apoA-I, and 20% is apoA-II.[7,8] HDL are heterogeneous, comprising several subpopulations of particles that vary in shape, size, density and surface charge. They contain around 25% of the cholesterol present in plasma.[1] HDL particles have a scavenger by taking up surface components from the other lipoprotein classes, but they are also crucial for the transport of excess cholesterol from peripheral tissues back to the liver, the reverse cholesterol transport.[8-13] Most HDL particles are spherical andα-migrating, and are called HDL_2 ($1.063<d<1.125$ g/ml) or HDL_3 ($1.125<d<1.21$ g/ml). HDL_2 are larger and contain more types of proteins than HDL_3.[1,8,14] There are also minor γ-migrating subpopulations of HDL particles containing only apolipoprotein E (apoE).[8,11,15] HDL can be formed either from the liver, intestine or from fragments of other lipoproteins.[10]

Table 1. Properties of the plasma lipoproteins.[1,3,11,15]

Parameter	Chylomicrons	VLDL	IDL	LDL	HDL
Electrophoretic mobility	Origin	Pre-β	β	β	Pre-β, α, γ
Density (g/ml)	<0.94	0.94-1.006	1.006-1.019	1.006-1.063	1.063-1.21
Main apolipoprotein	B-48	B-100	B-100	B-100	A-I
Main function	Transport of exogenous fat	Transport of endogenous triglycerides	Precursor for LDL	Transport of cholesterol	Reverse cholesterol transport

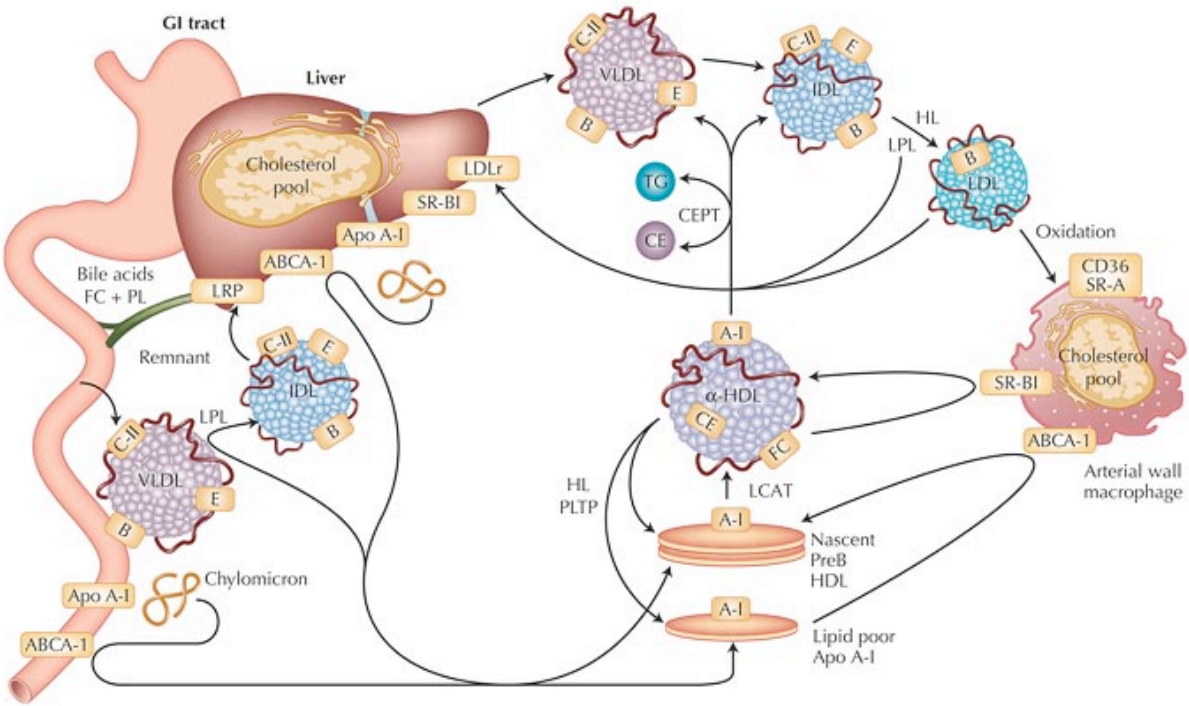

Figure 2. Schematic overview of the metabolism of the plasma lipoproteins and reverse cholesterol metabolism. The image was originally published in Atlas of Heart Diseases: Atherosclerosis by Brewer B.H.[16] and is reprinted here with kind permission from Current Medicine Group LLC.

Newly formed HDL particles are discoidal and are often called nascent HDL or pre-β-HDL. Discoidal HDL particles consist only of surface constituents arranged as a molecular bi-layer of phospholipids and unesterified cholesterol encircled by one or two molecules of apoA-I.[8,10,17,18] With help from various enzymes, pre-β-HDL particles acquire their lipid contents and formspherical HDL particles. Enzymes involved in these reactions include the ATP-binding cassette A1 (ABCA1), ATP-binding cassette G1 (ABCG1), scavenger receptor type B1 (SR-B1), lecithin:cholesterol acyltransferace (LCAT) and cholesterol ester transfer protein (CETP) (Figure 2).[1,8-11,13,19]

Lipoproteins in cardiovascular diseases

Cardiovascular disease (CVD) is the leading cause of death in the world.[20] The involvement of plasma cholesterol in the development and progression of atherosclerotic cardiovascular risk is well known and accepted. A positive relationship between LDL-cholesterol (LDL-c) and the risk of cardiovascular events has been observed in many large population studies, as has the inverse correlation for HDL-cholesterol (HDL-c).[21-28]

Atherosclerosis is a chronic inflammatory disorder occurring in the artery wall and is ultimately responsible for myocardial infarction, stroke and peripheral vascular disease. The molecular and cellular mechanisms for the pathobiological changes that lead to the disease are still poorly understood and several hypotheses have been articulated to explain the events that initiate atherogenesis.[29] A hallmark is the accumulation of cholesterol in arterial macrophages.[30,31] LDL is the main atherogenic lipoprotein in plasma. If the plasma concentration of LDL is high enough it does not only infiltrate but also accumulate in the arterial intima.[30,31] Modification of LDL, through oxidation or enzymatic attack in the intima, activates endothelial cells and evokes the immune system, causing an inflammation and the formation of an atherosclerotic plaque.[21,30,32-34] HDL is antiatherogenic, both by being anti-oxidant and anti-inflammatory. HDL particles are believed to protect against atherosclerosis by mediating the return of excess tissue cholesterol to the liver for secretion in bile.[35-37] However, HDL has more roles than the ones in plasma cholesterol transport. It is also known that HDL inhibits the formation of oxidized LDL, promotes cholesterol from arterial macrophages and inhibits the expression of adhesion proteins, e.g. vascular cell adhesion molecule-1 (VCAM-1) and E-selectin.[8,38-40] The molecular basis for these functions is still not fully understood, let alone less mapped to the different components of the HDL particle.[41,42] Myocardial infarction (MI) occurs when the atheromatous process prevents blood flow through the coronary artery.[31,34] The infarction is mostly caused by the formation of an occluding thrombus on the surface of the plaque. The thrombosis in turn is caused by plaque rupture and endothelial erosion.[30,31,43]

APOLIPOPROTEINS

Apolipoproteins, the protein components of lipoproteins, have three main functions. 1) They help stabilize apolar lipids in plasma. 2) They bind to cell surface receptors and thereby determine the sites of cellular uptake and degradation of lipoproteins. 3) They regulate the activity of enzymes involved in the lipid metabolism.[44,45] The main apolipoproteins are of the subclasses A, B, C, D or E. They all have different metabolic functions.[46] More apolipoprotein have been identified (apoF – apoO), but little is known about their functions.

ApoA-I and **apoA-II** are the major HDL proteins (Table 2).[47] In addition to binding lipids, apoA-I enhances the activity of LCAT. The main function of apoA-II is not known.[48] The plasma concentration of apoA-I in humans is around 1 mg/ml, making it one of the most abundant proteins in human plasma.[8] Although apoA-I is often used to estimate the HDL levels, apoA-I is exchanged between lipoproteins and the number of apoA-I molecules varies between particles. ApoA-II is the second most abundant apolipoprotein of HDL.[48] It binds to phospholipids with higher affinity than does apoA-I and is found mainly in the spherical HDL particles.[8,47]

Apolipoprotein B (apoB) is a large amphipathic protein existing in two forms, apoB-100 and apoB-48. It is essential for the intracellular assembly of VLDL and chylomicrons in the secretory pathway of the cells.[49,50] ApoB-48 corresponds exactly to the N-terminal 48% of apoB-100.[49-51] ApoB-100 is found on the surface of VLDL, IDL and LDL, whereas apoB-48 is only present

on chylomicrons.[49,50] Structural aspects of apoB-100 are difficult to study because of its huge size (512 kDa) and insoluble nature once it is separated from its lipid environment.[51] One apoB-100 molecule is found on each particle making it possible to use apoB as a measurement of the number of LDL or VLDL particles present in plasma.[29] Normally, more than 90% of plasma apoB-100 is bound to LDL, and the remainder to VLDL.

Apolipoproteins C (apoC) exists in three different isoforms in humans, C-I, C-II and C-III (Table 2).[52-54] They are all present in chylomicrons, VLDL and HDL.[53] Several functions of apoC-I have been documented. ApoC-I inhibits the uptake of triglyceride rich lipoproteins via hepatic receptors, particularly by LRP.[53,55,56] ApoC-I is also involved in HDL remodeling by activation of LCAT and inhibition of CETP.[55,56] C-II is the key cofactor for LPL.[57,58] The sequence for the activation of LPL reside in the C-terminal domain of the apoC-II peptide.[59] ApoC-III is the most abundant of the C-apolipoproteins in humans (Table 2). ApoC-III inhibits LPL and LDLr-mediated endocytosis of lipoprotein particles.[46,52] The attenuated clearance of triglyceride rich lipoproteins is associated with increased coronary risk and the induction of myocardial ischemia.[58,60]

Apolipoprotein D (apoD) occurs mainly in HDL although it is present also in LDL and VLDL.[48,61] ApoD has long been predicted to be a member of the lipocalin protein family.[61-64] In 2007, Eichinger *et al* were able to solve the crystal structure for free apoD, and its complex with progesterone, confirming the lipocalin fold.[65] *In vitro* studies have shown that apoD is able to bind cholesterol, progesterone, bilirubin and arachidonic acid, although, as for many lipocalins, the physiological ligand is unknown.[61,65-68] The function of apoD is not known but it has been suggested to be a multi-ligand, multi-functional transporter.[61]

ApoE is a polymorphic 34.2 kDa protein (Table 2).[70] It is a crucial ligand for receptor-mediated uptake of chylomicron remnants and IDL, binding the lipoproteins to several receptors including the LDLr, LRP and the VLDL-receptor.[44,45,47,71] ApoE has two structural domains: a 22 kDa N-terminal domain

Table 2. Properties of the major plasma apolipoproteins[1,8,49,61,69]

Protein	Lipoprotein	Function	Concentration	Site of expression
A-I	HDL, chylomicrons	Activate LCAT	1-2 g/l	Liver, intestine
A-II	HDL	Not known	300-400 mg/l	Liver
B-48	chylomicrons	Secretion of chylomicrons and uptake of chylomicron remnants in the liver	–	Intestine
B-100	VLDL, IDL, LDL	VLDL secretion, uptake of LDL	0.5-1.2 g/l	Liver
C-I	HDL, VLDL, chylomicrons	Inhibits uptake of lipoproteins by hepatic receptors. Activates LCAT and inhibits CETP.	~60 mg/l	Liver
C-II	HDL, VLDL, chylomicrons	Activates LPL	~40 mg/l	Liver
C-III	HDL, VLDL, chylomicrons	Inhibits LPL	~120 mg/l	Liver
D	HDL	Not known	~120 mg/l	Adrenal glands, pancreas, kidneys, placenta, spleen, lungs, ovaries, testes, brain, peripheral nerves and cerebrospinal fluid
E	IDL, chylomicron remnants	Uptake of lipoproteins in cells	30-70 mg/l	Liver

containing the LDLr binding region and a 10 kDa C-terminal domain that contains the major lipid- or lipoprotein binding elements.[44,70] There are three common isoforms of apoE: E2, E3 and E4.[72,73] ApoE3, the most abundant isoform, is considered the "parent" protein.[44] ApoE4 has been identified as a major risk factor for Alzheimer's disease.[70,74,75] Despite intense research, the

molecular mechanisms underlying the association of apoE4 with Alzheimer's disease is not clear.[75]

Apolipoproteins in cardiovascular diseases

Lately there have been a number of studies regarding which is more important for predicting CVD – the cholesterol that HDL and LDL carry, or the actual particle count, measured as apoA-I and apoB.[76-81] Several studies have suggested that apoA-I and apoB are better predictors for coronary heart disease (CHD) and MI than lipoprotein levels.[76-78,80,81] In a Danish population study, based on the Copenhagen City Heart study (CCHS), we showed that apoA-I and apoB were just as good at predicting the development of CHD as HDL and LDL.[82]

LIPOCALINS

The lipocalin family is a large, and expanding, group of proteins. They are typically small (150-250 amino acid residues), extracellular proteins and their physiological role usually lies in the transport or storage of hydrophobic and/or chemically sensitive compounds (e.g. vitamins, lipids and steroids).[83-85] They share several common molecular recognition properties: the binding of small, principally hydrophobic molecules (such as retinol); binding to specific cell-surface receptors (such as megalin); and the formation of covalent and non-covalent complexes with other soluble macromolecules (such as the retinol binding protein (RBP)-transthyretin complex).[83-85] Lipocalins occur in many organisms, such as vertebrates, insects and plants, and even in bacteria.[83,86] Although they have been classified as transport proteins it is now clear that members of the lipocalin family fulfill a wide variety of different functions. Roles include retinol transport, cryptic coloration, pheromone transport, and the enzymatic synthesis of prostaglandins.[85,87-90] The lipocalins have also been implicated in the regulation of the immune response[91] and the mediation of cell regulation.[92] Their wide variety of functions are also reflected in the use of several lipocalins as biochemical markers of disease. Clinical indications relate to almost any medical field, such as inflammatory disease, cancer and lipid disorders as well as liver and kidney function.[93]

Structure

The amino acid sequences of lipocalins in general show a low percentage (below 20%) of similarity between members but they share a well-conserved three-dimensional structure.[94,95] The lipocalin fold consists of an eight-stranded antiparallel β-barrel, flanked by an α-helix (Figure 3). In cross-section, the barrel has a flattened or elliptical shape that encloses an internal ligand-binding site.[85,94,96] The eight β-strands are linked by seven loops, all typical short β-hairpins, except the first loop which is a large Ω loop which forms a lid that partially or completely closes the ligand-binding site.[84,85] The conserved folding is partly accounted for by a highly conserved gene structure with a typical seven exons/six introns arrangement. Exons 2-5, whose sizes are a typical feature of lipocalin genes, code for the entire set of β-strands.[97]

Most lipocalins, the kernel lipocalins, share three characteristic structurally conserved regions[84] while other more divergent family members, the outlier lipocalins, share only one or two.[84,98] RBP and apoD, two lipocalins of known three-dimensional structure, are kernel lipocalins (Figure 3).[65,98,99] A tryptophan at the bottom of the ligand-binding pocket is highly conserved in lipocalins. Extensive structural studies of RBP showed that lack of this tryptophan (Trp24) leads to losses in stability and decreased yields of protein when refolding *in vitro* while it was not needed for maintaining the function of the protein.[100]

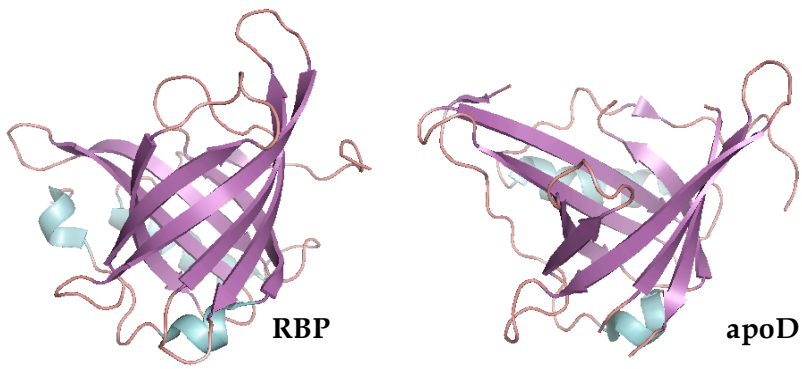

Figure 3. Ribbon representation of the crystal structures of the human lipocalins RBP and apoD.[65,99]

Ligand-binding

The large cup-shaped cavity within the β-barrel is well adapted for ligand-binding. The selectivity is determined by the amino acid composition of the pocket and the loop scaffold, as well as their overall size and conformation.[94] The binding site can form a wide, funnel-like opening, as in neutrophil gelatinase-associated lipocalin (NGAL).[101,102] Alternatively, the loops can close the cavity within the β-barrel and fully encapsulate the ligand, as for major urinary protein (MUP).[89,103] That pH-induced structural changes can affect the binding affinity has been shown for several lipocalins and their ligands.[98] Many lipocalins bind molecules with critical biological functions, e.g. retinoids, arachidonic acid and various steroids. Lipocalins with known endogenous ligands include RBP, which binds retinol,[90,104] MUP, which binds odorants in urine from male mice[89] and histamine-binding proteins, which have been shown to bind histamine.[105] However, it is important to draw a distinction between the demonstration of a binding *in vitro* and the identification of endogenous ligands. For example, the retinol binding exhibited by various lipocalins[66,94] may reflect a general affinity for a range of different small hydrophobic ligands. A broad selectivity of binding may reflect a general transport role, such as the clearance of unwanted compounds and can even give rise to pathological conditions.[94,106] Alpha 2u-N is a syndrome in male rats that are exposed to a number of environmental chemicals and pharmacological agents. The disease is caused by binding of these chemicals or their metabolites to alpha 2u, which, in turn, is believed to lead to a less digestible chemical-protein com-plexes.[106] Gutiérrez *et al.* suggested that more recently evolved lipocalins tend to show 1) show a greater rate of amino acid substitutions, 2) have a more flexible protein structure based on the number of disulfide bonds, and 3) bind smaller hydrophobic ligands to increase the efficiency of their ligand-binding contacts.[107] This can be used in drug-development where the use of lipocalins to design artificial binding proteins, anticalins, has become a new class of potential drugs for medical therapy.[96,108-110]

APOLIPOPROTEIN M

ApoM is a 25 kDa plasma protein expressed in liver and kidney.[111-113] It was first discovered in 1999 in chylomicrons but is mainly associated with HDL particles.[111] The three-dimensional structure has, until now, not been experimentally determined but apoM is identified as a lipocalin with the ability to bind small lipophilic substances.[114,115] The function of apoM is today, 10 years after its discovery, unknown but several studies have suggested that apoM has antiatherogenic and anti-inflammatory functions.[116-119]

The gene regulation of apoM

The human apoM gene is located on chromosome 6, at position p21.3 in the major histocompatibility complex (MCH) class III region.[111] The apoM gene is surrounded by *BAT4* and *NG34* on one side and *BAT3* on the other.[111] The MHC contains genes essential to both the adaptive and innate immune systems.[120] The class III region is the most gene-dense region of the human genome.[121] By the use of comparative analysis it has been shown that a cluster of MHC genes including, tumor necrosis factor *(TNF)*, lymphotoxin alpha *(LTA*) (or its putative teleost homolog (*TNF-N*), *BAT3* and *apoM* have remained together for over 450 million years, predating the divergence of mammals from fish.[120] The importance of this is unknown.

Hepatocyte nuclear factor 1-alpha (HNF-1α) is a transcription factor involved in the regulation of a large set of hepatic genes, including albumin, and α1-antitrypsin. Patients with

maturity onset of diabetes in the young (MODY) type 3 carry mutations in the *HNF-1α* gene in the heterozygous state.[122] In addition to being an important regulator of insulin secretion, HNF-1α is an essential transcriptional regulator of bile acid and HDL-c metabolism.[123] HNF-1α binds to the apoM gene promoter and HNF-1α deficient mice have no or little apoM expres-sion.[124,125] Also, as will be discussed below, an association between apoM plasma levels and MODY3 has been suggested, but the results are conflicting.[124,126,127]

The **liver receptor homolog 1** (LRH-1) has been reported to play an important role in bile acid biosynthesis[128,129] and reverse cholesterol transport.[130-132] It has also been suggested to be a negative regulator of the hepatic acute-phase response by inhibiting interleukin-6 and serum amyloid A (SAA).[133] There is a LRH-1 response element in both the human and the mouse promoter region of apoM and LRH-1 regulates apoM transcription in cultured cells.[134] Venteclef *et al.* has demonstrated that bile acids suppress apoM expression by inhibiting LRH-1 transcriptional activity.[134]

Foxa2 belongs to the Foxa superfamily of winged helix/forkhead box (Fox) transcription factors, which has three members: Foxa1, Foxa2 and Foxa3.[135] They control embryonic development and organogenesis of liver, pancreas, brain, lung, thyroid and prostate.[135-140] A Foxa2 binding site was identified in the *apoM* promoter region at position -474.[141] It was also shown that haploinsufficient Foxa2$^{+/-}$ mice have decreased apoM expression.[141] The Foxa2$^{+/-}$ mice in addition have decreased HDL and preβ-HDL levels.[141]

The **liver X receptor** (LXR) belongs to the nuclear receptor family and is a ligand–activated transcription factor involved in the regulation of lipid metabolism and inflammation.[144,145] The apoM expression was downregulated both in HepG2 cell cultures and *in vivo* in mice after addition of a LXR agonist.[146] A similar effect was seen after addition of 9-cis retinoic acid, known to affect the lipid metabolism through the retinoic X receptor.[147] The downregulation of apoM in liver caused by a LXR agonist was recently confirmed by Calayir *et al*.[148] They also

showed that apoA-I was downregulated in a similar manner. Surprisingly, an upregulation of apoM, apoA-I and ABCA1 was found in the small intestine as well as in Caco-2 cells.[148] The reason for the differential effects in liver and intestine is unknown but it is interesting that apoM behaves comparably to other main genes involved in reverse cholesterol transport in both tissues.

Leptin, a hormone secreted by adipose tissue, has been shown to influence the hepatic lipid and lipoprotein metabolism.[149,150] The apoM expression *in vivo* was increased by Leptin,[150,151] however, addition of supra-physiological leptin concentrations were shown to inhibit the apoM transcription in HepG2 cells.[152] Further investigations are needed to explain the contradictory effects of leptin *in vitro* and *in vivo*.

HepG2 cell cultures have been used extensively to study apoM expression and how it is affected by various factors. Two studies have investigated the effect of inflammatory parameters on apoM.[142,143] In the first study platelet-activating factor (PAF) significantly enhanced the apoM mRNA levels and the secretion in HepG2 cell cultures, an effect that the PAF-receptor antagonist, Lexipafant, significantly suppressed.[143] In the second study the four cytokines, transforming growth factor-alpha (TGF-α), transforming growth factor-beta (TGF-β), epidermal growth factor and hepatic growth factor were able to decrease apoM expression levels, with TGF-β being the most effective.[142]

The structure and ligand-binding abilities of apoM (Papers I-II)

Knowledge of the three-dimensional structure of a protein is of major importance in providing insights into its molecular functions, as proteins with similar folds may have similar functions. Already in 1999, apoM was proposed to be a lipocalin. This hypothesis was based on sensitive sequence searches, threading and, later on, comparative model building.[111,115] As has been discussed in a previous chapter, lipocalins share a highly conserved fold, consisting of an eight-stranded antiparallel β-barrel, flanked by a α-helix.[83,84] The lipocalin family also contains a highly conserved tryptophan

that points towards the inside of the hydrophobic pocket (Trp47 in apoM) (Fig 4). As previously shown for RBP, the highly conserved tryptophan was important for maintaining the stability of apoM and receiving high yields during the refolding process of recombinantly produced apoM.[114,153] ApoM contains, apart from the conserved tryptophan (Trp47), a second tryptophan at position 100 (Trp100) facing the hydrophobic pocket close to its opening (Fig 4). By using the fluorescence from the two tryptophans for intrinsic fluorescence quenching, the ligand binding abilities of apoM was studied.[114] Small lipophilic substances such as cholesterol, progesterone, arachidonic acid and vitamin K were tested. Binding of retinol and its two metabolites all-*trans*-retinoic acid and 9-*cis*-retinoic acid was detected with dissociation constants around 2-3 μmol/l whereas the other ligands did not bind.[114] The intrinsic fluorescence of two apoM mutants, carrying single tryptophans, was quenched by retinol and retinoic acid to the same extent as wild-type apoM, indicating that the environment of both tryptophans was affected by the binding.[114] Plasma RBP binds retinoids with ten times higher affinity than the one estimated for apoM in this study. Moreover, RBP has a higher plasma concentration (3 μmol/l) than apoM (0.9 μmol/l), questioning the physiological importance of the detected retinoid binding.

ApoM was identified as an outlier lipocalin.[98] For outlier lipocalins, if not for all, the sequence diversity makes crystallization and structure solution the only certain way of determining their inclusion in the lipocalin family. Recently, we managed to determine the 1.95 Å resolution crystal structure of recombinant apoM expressed in E. Coli (Figure 4).[153] ApoM displays the typical lipocalin fold that was predicted, i.e. characterized by an 8-stranded antiparallel β-barrel enclosing an internal ligand-binding pocket, flanked by a α-helix. The eight strands are linked by seven loops, the first being more extended and partially closing the internal ligand-binding pocket at one end. ApoM contains three disulfide bridges. Two of these covalently attach the extended N- and C-terminal tail segments to the lipocalin core fold. The ligand-binding pocket in apoM can be subdivided into a hydrophilic upper part, surrounded by several positively and negatively charged residues (Arg98, Arg143, Asp169 and Glu136), and a hydrophobic lower part

lined by numerous hydrophobic residues. An unexpected discovery was the binding of either myristic acid or glycerol-1-myristate in the hydrophobic pocket. Fatty acid and lipid binding studies, once again using intrinsic fluorescence quenching,

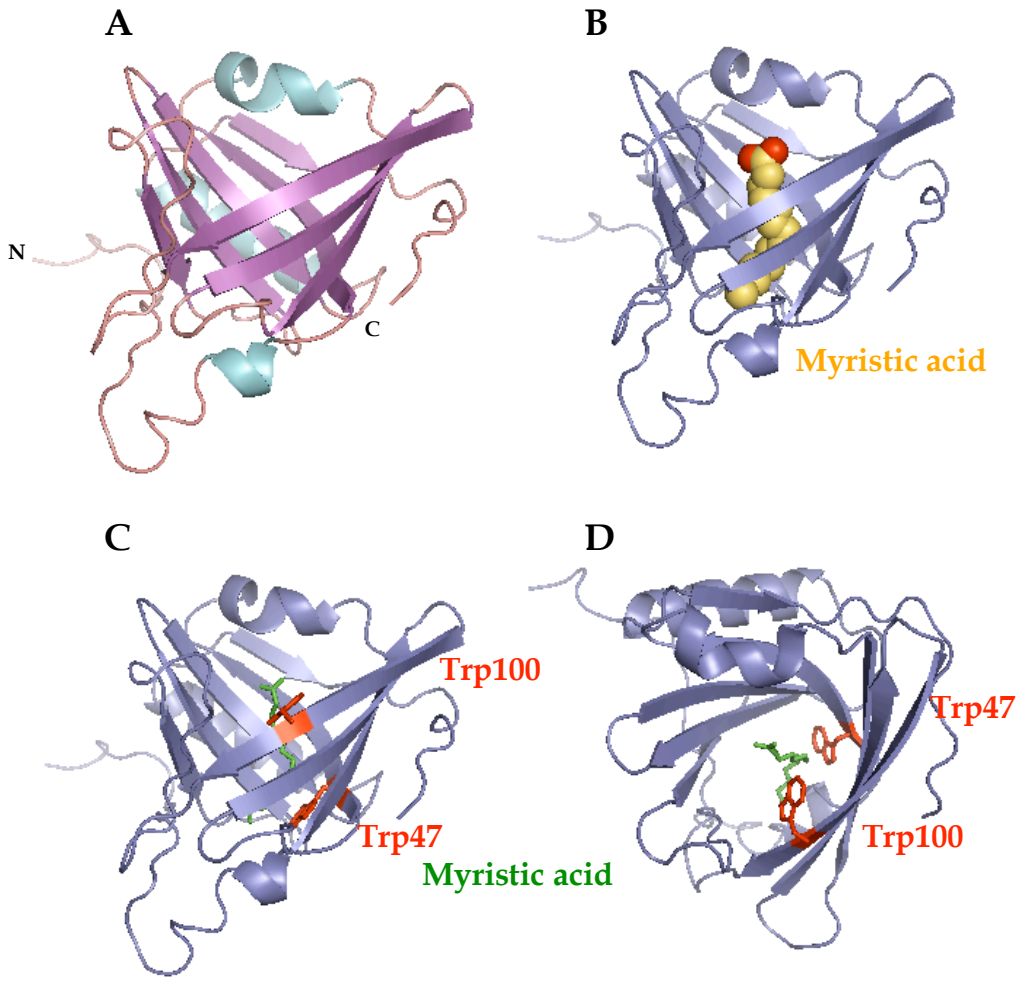

Figure 4. Overall structure of apoM. (A) Barrel-like fold in apoM showing the β strands (purple), helixes (turquoise), loops (brown) and the N and C-termini. (B) The cocrystallized myristic acid is shown as a sphere model and coloured yellow and red. (C) The tryptophans are shown in red and myristic acid in green. (D) ApoM, as pictured in C, rotated by 90° around the horizontal axis.[153]

identified D-sphingosine (IC_{50} of 0.39 μmol/l) and sphingosine-1-phosphate (IC_{50} of 0.90 μmol/l) as two possible physiological ligand candidates.[153] D-sphingosine is phosphorylated by sphingosine kinase types 1 and 2, to form Sphingosine-1-phosphate.[154]

Sphingosine-1-phosphate is a signalling sphingolipid, but is also referred to as a bioactive lipid mediator.[155] Sphingosine-1-phosphate is mainly associated to HDL but its major source is haematopoietic cells (erythrocytes, platelets and leukocytes).[156,157] It may, according to several studies, account for many of the anti-atherogenic activities of HDL, including HDL acting as an immunomodulator and having anti-oxidant functions.[155,157] By considering the lipocalin family in general, and the specificity of the ligand-binding and binding of sphingosine-1-phosphate in particular, some speculation as to the physiological function can be made. The mechanisms governing translocation of sphingo-sine-1-phosphate from cells to the HDL particle is not known, neither is it known where in the HDL particle sphingosine-1-phosphate is located.[157] ApoM could be involved in the uptake and storage of sphingosine-1-phosphate in HDL and the protective effects seen by both apoM and sphingosine-1-phosphate might be connected.

The presence of myristic acid in the binding pocket made it difficult to estimate the affinity of the binding and also questions the dissociation constants estimated for the retinol binding. Both sphingosine-1-phosphate and D-sphingosine quenched the fluorescence of Trp100 specifically. The sphingosine-1-phosphate mediated quenching could be reversed by myristic acid and D-sphingosine whereas the fluorescense of Trp47 was unaffected.[153] This suggests that the strong quenching observed by addition of D-shingosine and sphingosine-1-phosphate is caused by the exchange of myristic acid and the other ligand, and that they all bind with the hydrocarbon chain down in the pocket so that Trp47 remains unaffected.[153] Retinoic acid on the other hand quenches both tryptophans equally and does not compete with sphingosine-1-phosphate, questioning whether this substance binds into the same hydrophobic pocket.[153] However, the shifts in fluorescence we see can still be results from conformational changes of the β-barrel, making any conclusions about binding sites premature.

The apoM signal peptide (Papers III-IV)

Most apolipoproteins have amphipathic motifs, a feature that is important when binding to the lipoprotein particles.[158,159] Amphipathic β-sheets, located throughout the apoB-100 molecule, alternating with amphipathic α-helixes have been proposed to explain how apoB-100 is oriented in the LDL particle.[159-161] The secondary structure of apoA-I has been extensively studied, with a lot of focus on its amphipathic α-helixes and its role in lipid-binding specificity.[162,163] ApoM on the other hand does not have any amphipathic motifs and no hydrophobic patches that can explain the binding to lipoproteins. However, apoM has an unusual feature, only shared by two other plasma proteins (paraoxonase-1 (PON-1) and haptoglobin related protein), apoM is circulating with a retained signal peptide.[111,164,165] The signal peptide was hypothesized to be responsible for the association between apoM and the lipoproteins. A signal peptide is a hydrophobic, 20-30 residues long amino acid chain.[166] Normally the signal peptide is cleaved by a type I signal peptidase when it has fulfilled its purpose of guiding the growing peptide chain into the endoplasmic reticulum.[166,167] The sequence homology of signal peptides is low but they share some common features; basic at the N-terminus, apolar in the middle and small uncharged amino acid residues preceding the site of cleavage by the signal peptidase.[168,169] For the signal peptidase cleavage the amino acids on position -3 and -1 from the cleavage site are crucial.[169] By a single amino acid change from a glutamine to an alanine at position 22 (Q22A), which is position -1 from the predicted cleavage site, a cleavage site was successfully inserted in apoM.[166] By expressing both wild-type apoM and apoM^{Q22A} in human embryonic kidney 293 (HEK293) cells it could be shown that while wild-type apoM was incorporated into particles of the same size as lipoproteins, apoM^{Q22A} was secreted from the cells as free protein.[166,170] In plasma from mice injected with either human wild-type apoM or apoM^{Q22A}, wild-type apoM was recovered from the lipoprotein fraction, whereas apoM^{Q22A} was found in the lipoprotein-free fraction (Fig. 5).[171] However, apoM^{Q22A} was not detectable in plasma from transgenic mice expressing apoM^{Q22A} unless the kidney arteries were ligated

while wild-type apoM was easily detected. Thus the signal peptide is not only crucial for lipoprotein association but also for keeping apoM in circulation and preventing rapid loss by filtration in the kidneys (Figure 5).[171]

In an attempt to investigate the mechanisms governing apoM secretion, HepG2 and stably transfected HEK293 cells, expressing wild-type apoM or apoM^{Q22A}, were cultured. While endogenously expressed apoM from HepG2 cells cultured under serum-free conditions was incorporated into lipoprotein-like particles, wild-type apoM expression from HEK293 cells

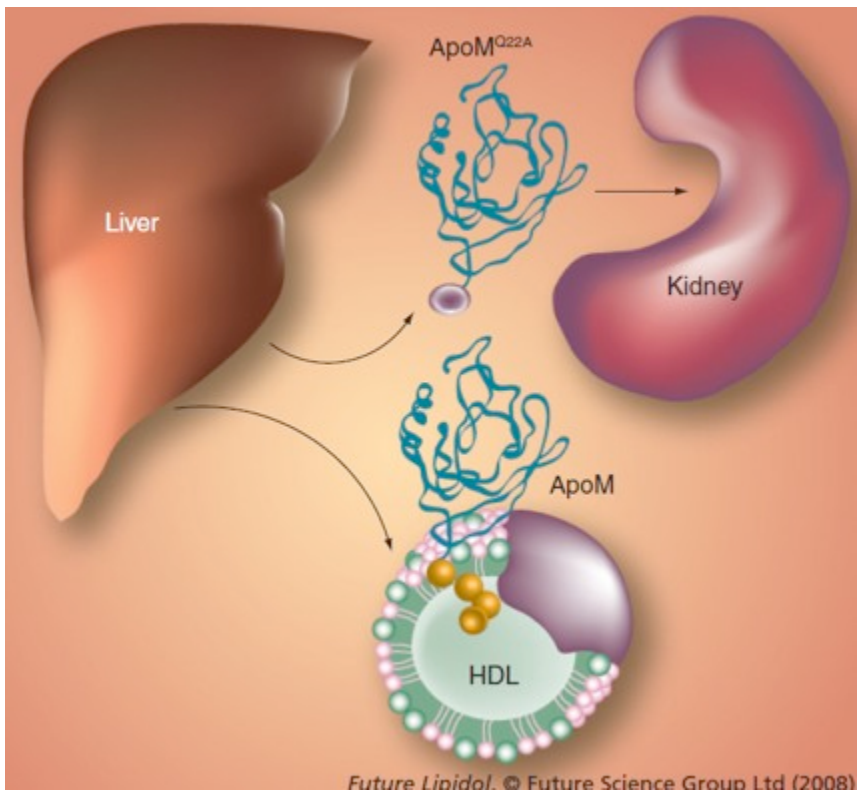

Figure 5. Wild-type apoM is synthesized in the liver and associates with HDL, but also other lipoprotein classes, in the circulation. ApoM^{Q22A} is circulating as a free protein and, due to its small size, is rapidly cleared in the kidney. Reproduced from Future Lipidology (2008) 3(5), 495-503 with permission of Future Medicine Ltd.

dropped dramatically.[170] In contrast, when cultured in mediumcontaining serum wild-type apoM was well expressed and incorporated in lipoprotein particles similar to those formed from HepG2 cells. ApoM^{Q22A} was readily expressed as a free protein from HEK293 cells both in serum-free and serum containing conditions. By measuring apoM mRNA and protein levels in cell lysates it was found that wild-type apoM accumulated in the HEK293 cells when cultured under serum-free conditions indicating that a vector in serum is needed for proper apoM secretion. Results suggested that lipoproteins might be involved in the secretion of apoM and addition of HDL, but not LDL, to the culture media completely restored wild-type apoM expression.[170] As HEK293 cells do not produce any apolipoproteins or cholesterol transporters such as ABCA-1, ABCG-1 or SR-B1 this suggests that addition of HDL to the culture media is just as efficient in stimulating apoM secretion as coexpression of lipoprotein particles.

The apoM particle

ApoM is mainly HDL associated but can still be found associated to lipoproteins of the other classes as well.[111,164,165] Christoffersen *et al.* isolated apoM-containing lipoproteins from plasma and showed that apoM is present in about 5% of the total HDL population and 2% of LDL.[117] It was also concluded that apoM appears to designate the apoM-containing population of HDL and LDL to be more antiatherogenic and less pro-atherogenic than HDL and LDL populations, respectively, as whole.[117] As described in a previous chapter, the HDL particles are very heterogeneous in size as well as in molecular weight and chemical composition. Wolfrum *et al.* detected apoM in preβ-HDL as well as in α-migrating HDL in both human and mouse serum.[119] In contrast, Christoffersen *et al.* were not able to detect any apoM in preβ-HDL while it was readily detected in 8-12 nm large particles (α-migrating).[116] Using isopycnic density gradient ultracentrifugation and liquid chromatography/electrospray mass spectrometry, apoM was found to be enriched in dense HDL_3 particles.[172,173] HDL_{3c}particles, a subgroup of HDL_3, are more potent than HDL_2

in attenuating the LDL oxidation. The potent antioxidative effect of HDL_{3c} particles was highly correlated to apoM levels as well as to SAA levels and PON-1 levels.[173] As HDL_3 also carries the highest amount of Sphingo-sine-1-phosphate (40-50 mmol Sphingosine-1-phosphate/mol HDL_3) making the binding of S1P to apoM even more intriguing.[174]

Glycosylations are common post-translational modifications of proteins, known to affect their properties and functions. Human apoM is partially glycosylated (~80%) in the circulation and Asn135 has been identified as the glycosylation site.[111,115,164,175-177] In total five different isoforms of apoM in LDL and HDL have been identified; three are both N-glycosylated and sialylated, one is N-glycosylated but not sialylated and one is neither glycosylated nor sialylated. The individual physiological roles of these isoforms, if any, have not been elucidated.[177]

ApoM in the kidney

The two membrane receptors megalin and cubulin are highly expressed in the endocytic pathway of the renal proximal tubule.[178,179] Both receptors are important for normal reabsorption of proteins in the proximal tubule.[179] Megalin has been shown to bind lipocalins (RBP and alpha-1-microglobulin) apolipoprotein H, albumin, transthyretin amongst others, whereas identified ligands for cubulin include transferrin, immunoglobulin light chains and apoA-I.[178,180-182] However, one of the most important functions of these receptors is to rescue protein-bound components, such as microelements and vitamins i.e. vitamin A, B_{12} and D.[181] ApoM is expressed in liver and in tubular epithelial cells in the kidney.[111,113] There are several hypotheses regarding the function of apoM expressed in the liver but the function of kidney derived apoM is far from obvious. The high apoM expression in the proximal compared to the distal tubules, and it is absence in Henle's loop, may suggest a specific function.[113] Megalin was shown to be a receptor for apoM and mediated its uptake in the kidney tubule cells.[183] ApoM was secreted in the urine of megalin deficient mice, while apoM was detected neither in wild-type mouse nor in human urine.[183] These results suggest that the megalin-

mediated endocytosis in kidney proximal tubules prevents apoM excretion in the urine.

ApoM in mice

The amino acid sequences of human and mouse apoM are 79% identical.[120,125] As in humans, mouse apoM is expressed in hepatocytes and in kidney proximal tubule cells.[125] Mouse apoM is also secreted with a retained signal peptide, but unlike human apoM it is not glycosylated.[125] ApoM levels in mouse plasma are decreased in apoA-I deficient mice, which suggests a connection between apoM and apoA-I metabolism.[125] Several studies have suggested that apoM is important for preβ-HDL formation and cholesterol efflux *in vivo* in mice and thereby inhibits formation of atherosclerotic lesions.[116,119] Christoffersen *et al.* showed that apoM increased the LCAT-independent generation of preβ-HDL in plasma.[116] Preβ-HDL particles are considered the initial extracellular acceptors of cellular cholesterol upon efflux from peripheral tissues. According to one study preβ-HDL formation was impaired in apoM deficient mice resulting in cholesterol accumulation in large HDL particles (HDL_1). This dramatic effect was observed in both HNF-1α deficient mice and in wild-type mice treated with siRNA directed against apoM, but was not seen in another study of apoM$^{-/-}$ mice.[116,119] However, in both studies mice overexpressing apoM developed smaller atherosclerotic lesions compared to controls.[116,119] The connection between apoM and preβ-HDL formation was later confirmed in humans where apoM plasma levels were positively associated with both preβ-HDL levels and preβ-HDL formation.[184]

The affect of acute phase response on apoM has also been studied in mice. ApoM mRNA levels were shown to be decreased in liver and kidney of mice treated with lipopolysaccharide (LPS), zymosan or terpentine. Moreover, treatment with TNF-α or interleukin-1 decreased apoM expression in Hep3B cell cultures.[118] In a mouse model of hepatic ischemia-reperfusion injury (IRI) apoM mRNA levels were decreased during the first 3 hours, indicating that they were significantly influenced by the acute-phase of IRI.[185]

ApoM in humans

Several interesting findings on apoM *in vitro* and in mice have been found. Unfortunately, not many of these have so far been able to be translated into the situation in humans. To be able to study apoM plasma levels in humans and how these are affected by different diseases we developed an apoM ELISA.[186] The mean apoM plasma level in a population of healthy volunteers was around 0.9 μmol/l corresponding to 23 mg/l, i.e. a lot less than the protein most abundant in HDL, apoA-I.[186] Using this ELISA we could establish reference values for healthy individuals; 0.58-1.18 μmol/l for women 18-49 years old and 0.61-1.30 μmol/l for women 50+ years and for men.[186] A positive correlation was observed for apoM levels and age among women.[186] The reason for the correlation is unknown but speculations around hormonal effects on apoM expression or elimination is tempting. Somewhat unexpectedly, a strong correlation between apoM and total cholesterol levels was revealed in the same healthy individuals (r=0.52) as well as HDL and LDL cholesterol levels.[186] This relationship and since been found in other populations.[82,187] One group has reported increased plasma apoM levels in hepatocellular carcinoma, liver cirrhosis and chronic hepatitis.[188] The method used to measure apoM levels was semi-quantitative and the study population was small so it may be too early to draw firm conclusions about the apoM levels in these liver diseases.

ApoM in diabetes

There have been many studies in both mice and humans suggesting that apoM regulation may be linked to glucose and lipid metabolism. Decreased plasma apoM levels have been found in several mouse models of type 2 diabetes.[151,189] In contrast to the results seen in mice, apoM gene expression in cultured liver cells was downregulated by insulin,[190,191] and in a recently published paper, apoM plasma levels were ~9% lower in patients with type 2 diabetes compared to controls.[184] In 2007, a study of three single-nucleotide polymorphisms (SNPs) in apoM, C-1065A, T-855C and T-778C, found that the SNP T-778C was associated with increased levels of plasma total cholesterol

and fasting plasma glucose and conferred the risk of development of type 2 diabetes.[192]

MODY is a group of six different monogenic forms of diabetes characterized by autosomal dominant inheritance, onset before 25 years of age, and pancreatic β-cell dysfunction.[122,193] MODY3 is caused by mutations in the gene encoding HNF-1α.[122] It is characterized by a severe insulin secretion defect in response to glucose.[122] HNF-1α regulates apoM gene expression in mice, as discussed in a previous chapter.[124] In 2003, Richter *et al.* found that MODY3 patients had a ~36% reduction in apoM levels compared to control subjects, whereas MODY1 patients had normal plasma apoM levels.[124] The authors suggested that apoM levels might be a useful serum marker for the identification of MODY3 patients. More recently however, apoM levels were assessed in two other populations. In none of these studies any difference in the plasma apoM levels were detected between either MODY3 patients and controls or between MODY3 patients and patients with type 2 diabetes.[126,127]

Metabolic syndrome is a heterogeneous disease that associates abdominal obesity and several metabolic factors with elevated blood pressure levels.[194,195] It is associated with increased risk of CVD and diabetes. The main physiological processes underlying metabolic syndrome are insulin resistance and inflammation.[194-196] Dullaart *el al*. showed that plasma apoM was on average 15% lower in subjects with metabolic syndrome compared to controls in a Dutch population. Furthermore, apoM correlated inversely to body mass index (BMI) and waist circumference. However, apoM was not correlated to any of the diabetic parameters such as glucose, insulin, leptin or the intermedia thickness.[197] The lack of correlation between apoM levels and leptin is particularly interesting as it is conflicting with previously published studies where a positive association between apoM and leptin levels was found.[150,151,198]

ApoM in cardiovascular diseases (Paper V)

CHD is the leading cause of death in all ages.[20] CHD is a narrowing of the coronary arteries caused by atherosclerosis resulting in stable angina pectoris or MI. As described in the

section about apoM and diabetes three SNPs in the apoM gene have been described.[192] A case-control study showed that SNP T-778C and T-855C alleles were associated with CHD.[199,200] Since apoM correlates strongly to total cholesterol levels and there is a well-established relationship between total plasma cholesterol and CHD and MI[23,201,202] we investigated whether there is a relationship between serum apoM levels and the risk for CHD.[82] We measured the apoM levels in two separate populations, based on FINRISK '92[203,204] and CCHS.[205,206] There was no signifi-cant difference in mean apoM levels between cases developing CHD during follow up (10 years in FINRISK '92 and 23 years for CCHS) and controls. Also, in conditional logistic regression analyses, apoM was not a predictor for CHD events.[82] Recently another study was published, reporting a slight increase in plasma apoM levels in CHD patients.[207] The difference between this study and the previous ones is that they measured apoM levels in present disease, whereas the other two described the apoM levels at baseline before the disease was developed. Since the apoM levels in the latter study was measured by a method that is at best semi-quantitative (semi-quantitative dot blot technique) it is still too early to draw firm conclusions about apoM levels in established CHD.[207]

FUTURE PERSPECTIVES

The long-term goal is to elucidate the physiological function of apoM and its involvement in disease. In order to get there we have a lot of loose ends to start working with.

One important tool that we have available now that we did not have before is the 3D structure of apoM. Thus, we know for certain that apoM is a lipocalin and the search for ligands with physiological functions does now seem even more relevant. As apoM was crystallized in complex with myristic acid we know that apoM binds fatty acids. Albumin, the major transporter of fatty acids,[208] is present at almost 1000-times higher concentration in plasma than apoM which makes it is difficult to envision a biological role for apoM as a fatty acid binding protein. The binding of D-sphingosine and sphingosine-1-phosphate is more intriguing, especially as apoM and sphingosine-1-phosphate are present in the same HDL subpopulation. Since both apoM and sphingosine-1-phosphate have been suggested to be anti-atherogenic it is of course of interest to see how these effects are connected.

Another interesting question for the future is the details on apoM secretion. As the hydrophobic signal peptide is retained, the phospholipids-bound apoM needs to be transferred from the phospholipids layer of the cell membrane to the phospholipids on the lipoprotein. A docking process during which an undefined acceptor complex becomes transiently associated with the cell membrane has been hypothesized to be involved in the release of PON-1.[209] Whether a similar mechanism is conceivable for apoM or not would be interesting to elucidate.

A strong association between apoM and cholesterol has been shown in several studies, but the mechanism behind this association remains unknown. Various groups are currently trying to unravel whether the answer lies at the gene regulation level, caused by transcription factors, or at the protein level in the lipid metabolism. The involvement of apoM in inflammation and acute-phase response has also been discussed, but more extensive studies are needed. SAA, an acute phase reactant, has been shown to displace apoA-I from HDL.[210-212] Also, acute-phase HDL is more rapidly cleared from the circulation during inflammation, myocardial infarction or ischemia than what is observed for normal HDL that has apoA-I as the major apolipoprotein moiety.[211] What the functional connections between inflammation and apoM levels are is not known, but since apoM levels were unaffected in subjects that later developed CHD it seems likely that the disease in itself causes the decrease in apoM levels. However, to fully understand the role of apoM in atherosclerosis and other diseases it could be of interest to use the apoM ELISA to measure apoM levels in a variety of inflammatory diseases. Also, the availability of genetically modified mice, both apoM-knockout mice and apoM-transgenic mice is an important tool when investigating the molecular mechanisms of its involvement in disease processes.

POPULAR SCIENTIFIC SUMMARY

Fat is one of the most important energy sources for humans, but to be able to use the energy it has to be transported to the organs where it's needed. To do this our body forms particles that are called lipoproteins. Lipoproteins are round particles with water-soluble molecules on the surface surrounding a core of fat. The particles mainly contain fat, cholesterol and proteins (apolipoporoteins). It's the apolipoproteins that decide where the lipoproteins will go. The particles are divided into different groups either dependent on their density, electric charge or dependent on which proteins they contain. LDL (low-density lipoproteins) and HDL (high-density lipoproteins) are the most common classes. They are often called "bad" and "good" cholesterol, respectively, because research has shown that much LDL increases a person's risk of getting cardiovascular diseases (diseases in the heart and the blood vessels) while HDL has the opposite effect. Some of the apolipoproteins are limited to only one or a few of the lipoprotein classes while other are present in all. Apolipoprotein A-I (apoA-I) is the main protein in HDL while apolipoprotein B (apoB) is the main one in LDL. Newly discovered apolipoproteins are typically simply named with the next letter of the alphabet.

Apolipoprotein M (apoM) is mainly present in HDL, but it can be found in all other lipoprotein classes as well. The liver and kidneys produce apoM and then let it out in the blood. What apoM is good for is not known today. We know which chromosome that contains the apoM gene, and it has been

possible to follow apoM far back in evolution. Many different species have apoM, including most mammals and fish. Using different data base searches and modelling apoM was predicted to be a lipocalin, even though it was not known exactly what the protein looked like.

The lipocalins are a large family of proteins, which all look fairly similar. They form a pocket where small, fat-soluble molecules can bind. Many lipocalins transport molecules such as vitamins, cholesterol and fat. Retinol binding protein is an example of a lipocalin that is a known transporter, it transports vitamin A in the blood. However, there are a lot of other lipocalins where we don't know their function.

Through binding experiments we managed to show that apoM binds vitamin A (paper I). We don't know if this binding has any physiological function or not, but it shows that apoM binds small fat-soluble molecules and by that behaves like a lipocalin. We have recently also been able to determine the 3D structure of apoM, i.e. determine exactly what it looks like and can thereby show that it is indeed a lipocalin (paper II). It might not sound very exciting, but by knowing what the protein looks like we can get important clues that can help us figure out what it does. When we determined the structure we could also see that a particular fatty acid, called myristic acid, was bound in the pocket of apoM. We have also shown that apoM binds two molecules that are believed to add to the good effects of HDL (D-sphingosine and sphingosine-1-phosphate).

All proteins are produced inside cells. When they are produced they have a short stretch of amino-acids (the building blocks of proteins) that works much like an address-tag, which guides the protein to the right places inside the cell. For proteins that are exported to the blood, this address-tag is normally cut off at an early stage. In apoM it remains part of the finished protein, something that is very uncommon among proteins. We have been able to show that this tag is necessary for apoM to be able to bind to the lipoprotein particles (paper III). Without it, apoM would be circulating as a free protein and be so small that it would be filtered through the kidneys and be lost in the urine. However, the amino acid-tail also makes it a lot trickier for apoM to get out from the cells and reach the blood. We managed to show that some kind of "acceptor" is needed for

apoM to get out from the cells and that HDL, in contrast to LDL, may be that acceptor (paper IV).

Work in mice has shown that apoM protects against an inflammation of the arteries with build up of fat that can lead to heart attack, stroke or angina etc. One of the causes of these diseases is high cholesterol level in the blood. We have seen in humans that if you have high levels of apoM in blood you often have high levels of cholesterol and vice verse. This made it interesting to see if the apoM level in a blood sample, taken many years earlier, could predict who would later get heart attack and not. We compared a group of people who later had a heart attack with a similar group who didn't in both a Danish and a Finnish study (paper V). The apoM levels were the same. Another group of scientists have shown that people who had already had a heart attack had slightly more apoM in their blood than healthy volunteers. Perhaps the amount of apoM in the blood is affected by the disease but doesn't have anything to do with getting the disease. More research is needed to answer this question.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Fett är en av människans viktigaste energikällor. För att kunna utnyttja energin måste den kunna transporteras i blodet dit där den behövs. För att kunna göra detta bildar vår kropp partiklar som kallas för lipoproteiner. Dessa är runda partiklar som har en yta uppbyggd av vattenlösliga molekyler som omsluter en fet kärna. Partiklarna innehåller framför allt fett, kolesterol och proteiner (apolipoproteiner). Det är apolipoproteinerna som håller ihop lipoproteinerna och som styr vart de tar vägen. Partiklarna delas upp i olika grupper, antingen beroende på densitet, elektrisk laddning eller beroende på vilka proteiner de innehåller. LDL (low-density lipoproteins) och HDL (high-density lipoproteins) är de vanligaste typerna och kallas ofta för det onda respektive det goda kolesterolet. Detta beror på att höga koncentrationer av LDL i blodet ökar risken för hjärt-kärlsjukdomar, medan HDL minskar risken för de samma. Vissa apolipoproteiner finns bara i en eller några lipoproteintyper medan andra finns i samtliga. Apolipoprotein A-I (apoA-I) är det protein som det finns mest av i HDL, medan apolipoprotein B (apoB) är vanligast i LDL.

Apolipoprotein M (apoM) är ett apolipoprotein som framför allt finns i HDL, men det finns också i mindre mängder i de andra lipoproteintyperna. ApoM tillverkas i levern och i njurarna. Man vet vilken kromosom apoM genen finns i och man har kunnat följa apoM långt bak i evolutionen. Många djurarter har apoM, både däggdjur och fiskar, men exakt vad apoM är till för vet man inte. Man har med hjälp av att söka i

olika databaser och med datormodelleringar sett att apoM förmodligen är en lipokalin.

Lipokaliner är en stor proteinfamilj som alla ser ungefär likadana ut. De ser ut som en ficka som små, fettlösliga molekyler kan binda, såsom vitaminer, kolesterol och fett. Retinolbindande protein är ett expempel på en lipokalin som är en känd transportör, vilken transporterar vitamin A i blodet. Det är dock inte många lipokaliner som man vet vad de är till för.

Vi lyckades med hjälp av bindningsexperiment visa att apoM binder vitamin A (artikel I). Om denna bindning har någon fysiologisk betydelse vet vi inte, men det visar att apoM, precis som de flesta lipokaliner, kan binda små fettlösliga molekyler. Vi har nu också lyckats fastställa hur apoM ser ut genom att göra kristaller av proteinet och faktiskt visa att det är ett lipokalin (artikel II). Det kanske inte låter så intressant, men när man vet hur ett protein ser ut kan man få reda på ganska mycket om dess funktion. När vi fastställde hur apoM ser ut såg vi att en fettsyra som heter myristinsyra redan fanns bunden till apoM. Förutom att binda fettsyror har vi också sett att apoM kan binda D-sfingosin och sfingosin-1-fosfat. Dessa två molekyler tror man bidrar till den skyddande effekt som man ser hos HDL mot hjärt-kärlsjukdomar.

Alla proteiner tillverkas inuti cellerna. När proteiner produceras har de en liten svans av aminosyror, en signalpeptid, som fungerar som en adresslapp som guidar proteinet till rätt platser i cellen. Denna klipps normalt sett av innan proteinet kommer ut i blodet. Det gör den dock inte i apoM och vi har lyckats visa att signalpeptiden är nödvändig för att apoM ska kunna binda till lipoproteiner (artikel III). Om denna hade klippts av hade apoM cirkulerat fritt i blodet och varit så pass litet att det filtrerats bort i njuren och försvunnit ut i urinen. Signalpeptiden gör det svårt för apoM att komma ut ur cellen till blodet. ApoM behöver en partikel som tar emot apoM på utsidan av cellen och vi har visat att HDL kan fungera som en sådan medan LDL inte gör det (artikel IV).

I möss har man sett att apoM skyddar mot åderförkalkning, förstadiet till hjärtinfarkt, stroke, kärlkramp etc. Vi har också sett att om människor har mycket apoM i blodet har de ofta höga kolesterolnivåer och vice versa. Eftersom det är känt att höga kolesterolnivåer ökar risken för hjärtinfarkt ville vi se om apoM

nivåerna säger något om vilka som senare får hjärtinfarkt och inte (artikel V). ApoM nivåerna visade sig vara desamma hos dem som senare fick hjärtinfarkt och dem som förblev friska. Det är dock en annan grupp som har sett en liten ökning i apoM nivån i blodet hos patienter med hjärt-kärlsjukdom jämfört med friska frivilliga. Det kan vara så att apoM nivån påverkas av själva sjukdomen men inte har något med själva sjukdoms-utvecklingen att göra. Detta är dock något som behöver utredas vidare.

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