Doctoral thesis for the Degree of Doctor of Philosophy, Faculty of Medicine

Studies on the atherogenicity of apoB-containing lipoproteins in type 2 diabetes

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2008

A Doctoral Thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted, or in manuscript).

Printed by Geson Hylte Tryck AB, Box 10323, 434 39 Kungsbacka

Göteborg, Sweden, 2008

ISBN 978-91-628-7687-6

ABSTRACT

Type 2 diabetes (T2D) and the metabolic syndrome (MetS), two conditions that are rapidly increasing in prevalence, as well as the dyslipoproteinemia and subclinical inflammation characteristic for these conditions, are associated with an increased risk for developing and dying of cardiovascular disease (CVD). The aim of this thesis was to investigate possible atherogenic properties of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) in T2D and MetS. We compared the susceptibility to lipolysis by secretory phospholipase A_2 group V (sPLA$_2$-V) of VLDL and LDL from individuals with T2D and MetS and from healthy individuals. We also characterized LDL-associated proteins, and compared the protein composition of LDL in T2D and MetS with LDL from healthy individuals. Finally, we investigated if lysozyme, one of the proteins that was increased in T2D-MetS-LDL, was elevated in serum of individuals with T2D and MetS as well.

Lipid-enriched VLDL and small, cholesterol-poor and triglyceride-rich LDL from T2D-MetS-individuals were more extensively lipolyzed by sPLA$_2$-V than control VLDL and LDL. 31 LDL-associated proteins, important for lipoprotein metabolism, complement, coagulation, oxidation, and inflammation, were identified in LDL. VLDL and LDL from T2D-MetS-individuals contained more apolipoprotein (apo) C3 per particle, and an increased LDL-apoC3 content correlated with a lower cholesterol content of LDL and a smaller LDL-size. T2D-MetS-LDL also contained less apoA1 and more apoJ and lysozyme than did control LDL, and higher abundances of apoJ and lysozyme also correlated with a lower cholesterol content in LDL. Lysozyme was also found to be elevated in serum of T2D-MetS individuals, and lysozyme levels correlated with serum creatinine and insulin levels.

The identified LDL-associated proteins might be of importance for the inflammation following LDL retention in the intima. An increased sPLA$_2$-V-mediated lipolysis of VLDL and LDL in individuals with T2D and MetS may cause increased retention of LDL and lead to high local concentrations in the intima of proinflammatory fatty acids and lysophosphatidylcholine. This might lead to an accelerated atherosclerosis development in these individuals. An increased understanding of lipoprotein alterations in diabetes may furthermore serve as a basis for the development of new treatment strategies for atherosclerosis in T2D and MetS.

Keywords: Type 2 diabetes, metabolic syndrome, atherosclerosis, VLDL, LDL, secretory phospholipase A_2 group V, inflammation, complement, apolipoprotein, lysozyme.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-III.

I. **Increased lipolysis by secretory phospholipase A_2 group V of lipoproteins in diabetic dyslipidemia**

Pettersson Camilla, Fogelstrand Linda, Rosengren Birgitta, Ståhlman Sara, Hurt-Camejo Eva, Fagerberg Björn, Wiklund Olov.

J Int Med 2008 Aug;264(2):155-65.

II. **LDL-associated proteins revealed using qualitative and quantitative proteomics – specific distribution in individuals with type 2 diabetes and the metabolic syndrome**

Pettersson Camilla, Karlsson Helen, Fagerberg Björn, Lindahl Mats, Larsson Thomas, Ståhlman Marcus, Fogelstrand Linda, Borén Jan, Wiklund Olov.

Submitted, under revision.

III. **Elevated levels of serum lysozyme in type 2 diabetes**

Pettersson Camilla, Fogelstrand Linda, Fagerberg Björn, Douhan Håkansson Lena, Borén Jan, Wiklund Olov.

Submitted.

LIST OF ABBREVITATIONS

2-DE two-dimensional gel electrophoresis

AGEs advanced glycation end products

Apo apolipoprotein

ABC ATP-binding cassette

CHD coronary heart disease

CETP cholesterol ester transfer protein

CD36 cluster of differentiation 36

CML N^{ε}-(carboxymethyl) lysine

CRP C-reactive protein

CVD cardiovascular disease

HDL high-density lipoprotein

HL hepatic lipase

IDL intermediate-density lipoprotein

IFG impaired fasting glucose

IGT impaired glucose tolerance

IL-6 interleukin-6

LDL low-density lipoprotein

LDL-R LDL-receptor

LCAT lecithin:cholesterol acyltransferase

LpL lipoprotein lipase

MMPs matrix metalloproteinases

MetS the metabolic syndrome

NCEP-ATP III National Cholesterol Education Program - Adult Treatment Panel III

NEFA non-esterified fatty acids

NGT normal glucose tolerance

oxLDL oxidized LDL

PON1 paraoxonase 1

PC phosphatidylcholine

PGs proteoglycans

PLA_2 phospholipase A_2

PAI-1 plasminogen activator inhibitor-1

RAGE receptor for AGE

SM sphingomyelin

SMCs smooth muscle cells

T2D type 2 diabetes

TNF-α tumor necrosis factor-α

VLDL very low-density lipoprotein

WHO World Health Organization

A1AT α-1-antitrypsin

LC-ESI-MS/MS liquid chromatography-electrospray ionization-tandem mass spectrometry

MALDI-TOF matrix-assisted laser desorption/ionization-time of flight

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Regarding the innate human desire to ask questions:

"There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another which states that this has already happened."

/ Douglas Adams
(1952–2001)

INTRODUCTION

Atherogenesis means the formation of atherosclerotic plaques, which is a complex process in the arterial intima, including lipid deposition, cellular proliferation, deposition of extracellular matrix, and inflammation. This thesis is focused on possibly atherogenic properties of the apoB-containing lipoproteins, very low-density lipoprotein and low-density lipoprotein, in individuals with type 2 diabetes and the metabolic syndrome, a group of individuals at high risk of developing cardiovascular disease caused by atherosclerosis.

1. Cardiovascular disease, type 2 diabetes, and the metabolic syndrome

1.1 Cardiovascular disease

Atherosclerosis is the main cause of cardiovascular disease (CVD) such as coronary heart disease (CHD) (including myocardial infarction and angina pectoris), stroke, peripheral artery disease, and ischemic heart failure. About 12% of Swedish inhabitants suffer from CVD [1]. CVD is the main killer in the world today, accounting for about 30% of all deaths globally [2], and 41% of all deaths in Sweden [3]. In the United States, as in Sweden, the single most common cause of death is acute myocardial infarction, the second is cancer, and the third is stroke. According to the National Centre for Health Statistics (USA), if all forms of major CVD were eliminated, life expectancy would increase by almost seven years [4].

During the last decades there has been a dramatic reduction of CVD in the western industrialised countries, with a 50% decrease of death from CHD [5, 6]. This is explained by improved health care and a reduction in cardiovascular risk factors in the population. However, simultaneously diabetes and obesity has caused an increase in CHD deaths by 18%, thus the achieved reduction in CHD deaths would have been even greater were it not for obesity and diabetes [6].

1.2 Type 2 diabetes

The rapidly increasing prevalence of diabetes has been compared to an epidemic. According to the International Diabetes Federation, in 1985 an estimated 30 million people worldwide

had diabetes. In 2007, that number was up to 246 million. By 2025, 380 million people are expected suffer from diabetes [7]. The increase in prevalence and incidence in especially type 2 diabetes in the U.S. can probably mostly be explained by an increased prevalence of overweight [body mass index (BMI) 25-30] and obesity (BMI $\geq$30) due to an increasingly sedentary lifestyle [8-10]. Between the time period 1976-1980 and the time period 2003-2004, the prevalence of obesity among U.S. adults (age 20-74 years) increased from 15% to 33%. The prevalence in overweight over the same time period increased from 47% to 66% [11, 12]. In Sweden in 2004, 10% of adult males and 12% of adult females were obese, and shockingly 51% of males and 42% of females were overweight [3].

Diabetes is considered a CHD risk equivalent, meaning that the risk of developing CHD if you have diabetes is similar to the risk of having recurrent CHD if you already have established CHD [13, 14]. Individuals with type 2 diabetes (T2D) run a risk of developing and dying of CVD that is several times higher than that of individuals without T2D [15, 16]. According to the European Guidelines on prevention of CVD, in the SCORE algorithm for risk calculation, the risk of dying in CHD or stroke during a 10-year period should be multiplied by a factor of three for men, and with a factor of five for women, if they have diabetes [17].

According to the American Diabetes Association, T2D accounts for about 90% of all diabetes. Type 1 diabetes is a result of destruction of the insulin-producing pancreatic β-cells, often due to autoimmunity, leading to an impaired insulin production and subsequent elevated blood glucose levels. T2D, on the other hand, is a result of prolonged insulin resistance. Insulin resistance is characterized by a cellular insensitivity to insulin signaling, leading to a decreased uptake of glucose into the cells of e.g. skeletal muscle (among other effects of decreased insulin-sensitivity, some of which are described in the section *Atherogenic mechanisms in the metabolic syndrome* below). At first, this is compensated for by increased β-cell production of insulin, but eventually insulin production can no longer compensate for the decreased insulin sensitivity, in part due to β-cell dysfunction. A decreased glucose uptake, together with a hepatic insulin resistance leading to increased hepatic glucose production, causes blood glucose levels to rise, and diabetes develops [18, 19].

In a community-based study that screened all 64-year old women living in Gothenburg for diabetes, impaired glucose tolerance (IGT), and impaired fasting glucose

(IFG), the prevalence of previously known diabetes was 4.7%. In addition, the prevalence of individuals with diabetes discovered during screening (using oral glucose tolerance tests), was found to be 4.8%, adding up to a total diabetes prevalence of 9.5%. The prevalence of IGT and IFG in the same study were 14.4% and 6.4% respectively [20].

1.3 The metabolic syndrome

In the recent INTERHEART study, a case-control study including 52 countries [21], nine risk factors could account for over 90% of the risk of having a first acute myocardial infarction. Four of these were: diabetes, obesity, dyslipoproteinemia and hypertension. These risk factors often appear together and are clustered into the metabolic syndrome (MetS). The clustering of risk factors into a syndrome facilitates the appreciation of the accumulated risk of CVD for an individual displaying multiple risk factors. The MetS-definition according to the World Health Organization (WHO) [22, 23], which can be seen in *Table 1*, is the one most commonly used in Sweden. In the United States, the definition by the National Cholesterol Education Program - Adult Treatment Panel III (NCEP-ATP III) [14] (*Table 1*) is more widely used. These definitions are similar, although not identical; both definitions include impaired glucose metabolism as a criterion, but only in the WHO-definition it is a requirement. Several of the cardiovascular risk factors associated with diabetes, such as hyperglycemia, impaired glucose metabolism, subclinical inflammation, and dyslipoproteinemia, are already manifested during the pre-diabetic state of insulin resistance described above. Therefore, in MetS, the impaired glucose metabolism-criterion is defined as *either* insulin resistance or T2D.

The prevalence of MetS was analyzed in the cohort of the American community-based Framingham Offspring Study, including 3224 individuals aged 30–79 years. Using the NCEP ATP III-definition, the prevalence of MetS was found to be 26.9% among non-diabetic white men, and 21.4% among non-diabetic white women, and using the WHO-definition, the prevalence of MetS was 31.8% for the men and 19.7% for the women [24]. An analysis of the Atherosclerosis and insulin resistance (AIR) study, including 104 middle aged men in Gothenburg, suggests that these numbers may be relevant also for Sweden. In the AIR cohort, the prevalence of MetS was found to be between 22% and 28 %, depending on definition used [25]. In the much larger Botnia study [26], including 4,483 Swedish and Finnish individuals at the age of 35–70 years, 10% of the individuals with normal glucose tolerance

(NGT) and 70% of the individuals with impaired insulin sensitivity qualified for MetS, according to the WHO-definition.

Table 1. Definitions of the metabolic syndrome

Criteria	WHO-definition	NCEP-ATP-III-definition
Required	**IGT** (FBG <6.1 mmol/L and BG 2h OGTT ≥7.8 <11.1 mmol/L) **or T2D** (FBG ≥6.1 mmol/L and/or BG 2h OGTT ≥11.1 mmol/L) **or insulin resistance** (insulin top quartile)	- -
	And/or ≥2 of:	**≥3 of:**
Glucose	- -	FBG ≥6.1 mmol/L
HDL-cholesterol	<0.9 mmol/L (men), <1.0 mmol/L (women)	<1.0 mmol (men), <1.3 mmol/L (women)
Triglycerides	≥1.7 mmol/L	≥1.7 mmol/L
Obesity	WHR <0.9 (men), >0.85 (women) or BMI ≥30 kg/m^2	Waist circumference ≥102 cm (men), ≥88 cm (women)
Hypertension	≥140/90 mm Hg	≥130/85 mm Hg
Microalbumiuria	Urinary albumin excretion ≥20 ug/min or albumin:creatinine ratio ≥30 mg/g	- -

IGT, impaired glucose tolerance; FBG, fasting blood glucose; BG, blood glucose; 2h OGTT, 2 hours after 75 g oral glucose tolerance test; HDL, high-density lipoprotein; WHR, waist:hip ratio; BMI, body mass index. Adapted from [22] and [23].

2. Atherosclerosis

2.1 The atherosclerotic plaque

Atherosclerosis is a slow process which usually takes place unnoticed over the course of a lifetime. This is partly due to vascular remodeling; initially, as the atherosclerotic plaque grows within the vascular wall, the vessel compensates by expanding outwards, keeping the lumen diameter fairly constant [27]. A cardiovascular event arises when the blood flow through the arteries supporting the target organ is restricted, causing ischemia. The occlusion may be partial, due to a plaque protruding into the lumen, causing angina pectoris or intermittent claudication of the legs, or it may be total, as in the case of thrombus formation following erosion or rupture of a plaque, causing myocardial infarction, acute ischemia of the leg, or stroke [28, 29].

Several hypotheses have been put forward regarding the mechanisms for the formation of atherosclerotic plaques. According to the response to retention hypothesis [30, 31], atherosclerosis is initiated by the retention of apoB-containing lipoproteins to proteoglycans (PGs) in the arterial intima, the innermost layer of the arterial wall. This retention, subsequent modification of lipoproteins, and the resulting formation of the atherosclerotic plaque will be described below.

2.2. Lipoproteins

To facilitate the transport of lipids in the circulation, they are packaged into lipoproteins (*Figure 1*). Triglycerides, which are the main lipids used for energy storage, and cholesterol esters are packed inside a spherical monolayer of phospholipids. Phospholipids are amphipathic by nature, meaning that they have one hydrophobic end (facing inwards, towards the lipid core) and one hydrophilic end (facing outwards). The phospholipid monolayer also contains unesterified cholesterol and several different proteins, in general designated apolipoproteins. In general, lipoproteins are classified into five classes, based on their differences in density (*Table 2*). These classes are: chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). There are also alternative ways to classify the lipoproteins based on e.g. size, electrophoretic mobility, and dominant apolipoprotein (apo).

Below, the structure, function, and metabolism of the five general lipoprotein classes (reviewed in [32]) will be described shortly. **Chylomicrons** (*Table 2*) are large, triglyceride-rich particles that are produced by the intestine from dietary triglycerides and their main protein component, apoB48. The triglyceride content of the chylomicrons is lipolyzed into fatty acids by lipoprotein lipase (LpL), which is situated on the capillary endothelium. The fatty acids are delivered to recipient tissues and the chylomicron remnants are then taken up and further catabolized by the liver. The liver produces triglyceride rich **VLDL** from triglycerides, cholesterol, and the main protein component apoB100 (apoB). Triglycerides in VLDL are also delivered to peripheral tissues via lipolysis by LpL. As VLDL decreases in triglyceride content, and thus decreases in size and increases in density, it is first classified **IDL**, and finally **LDL**.

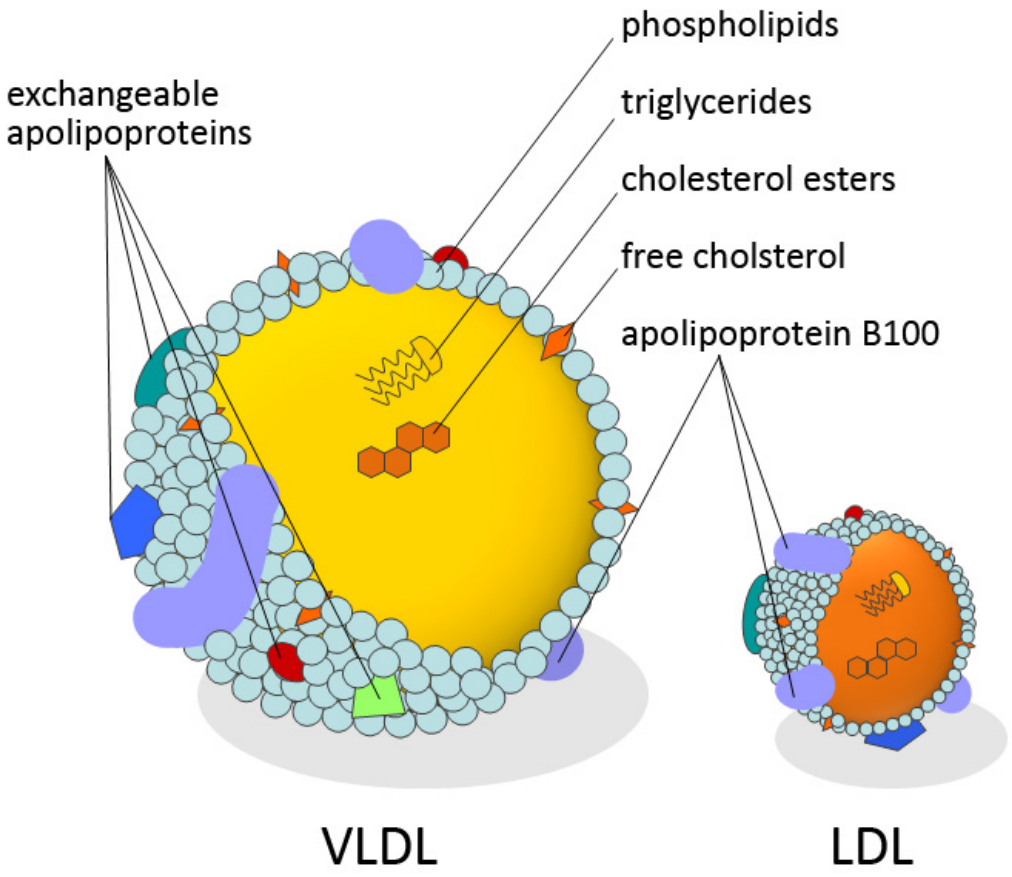

Figure 1. Lipoprotein composition. Schematic figure of the lipid- and apolipoprotein composition of lipoproteins, here exemplified by VLDL and LDL.

LDL is a cholesterol ester-rich particle in which the majority of plasma cholesterol is transported to cells through uptake of LDL via the LDL-receptor (LDL-R) [33]. The majority of LDL is however taken up by the liver, also via LDL-R, where remaining cholesterol is secreted via the bile.

HDL is produced by the liver and intestine, in a process which is still not fully elucidated, as lipid-poor phospholipid discs, containing the main apolipoprotein of HDL, apoA1 [34, 35]. These discs are transformed into spherical particles, which constitute the majority of circulating HDL, through uptake of cholesterol from peripheral cells and macrophages, mediated by cholesterol transporters, e.g. ATP-binding cassette (ABC) A1 and ABCG1, and esterification of HDL cholesterol by e.g. lecithin:cholesterol acyltransferase (LCAT). HDL promotes reverse cholesterol transport by transferring cholesterol from peripheral tissues and macrophages to the liver. This is accomplished either as direct uptake of HDL-particles by the liver or via transfer of cholesterol esters from HDL to apoB-containing lipoproteins mediated by cholesterol ester transfer protein (CETP) [35].

Table 2. Lipoprotein classes

	Density (g/ml)	Diameter (nm)	TG (%)	CE (%)	PL (%)	FC (%)	Dominant apolipoprotein
Chylomicrons	<0.95	80–100	90–95	2–4	2–6	1	apoB48
VLDL	0.95–1.006	30–80	50–65	8–14	12–16	4–7	apoB100
IDL	1.006–1.019	25–30	25–40	20–35	16–24	7–11	apoB100
LDL	1.019–1.063	20–25	4–6	34–35	22–26	6–15	apoB100
HDL	1.063–1.210	8–13	7	10–20	25	5	apoA1

TG, triglycerides; CE, cholesterol esters; PL, phospholipids; FC, free cholesterol. Adapted from [36].

2.3. Apolipoproteins

The major protein constituent of VLDL, IDL and LDL is apoB, which is necessary for the secretion of VLDL from the liver [37]. It is a large protein of 512 kDa, encircling the lipoprotein particles. ApoB is a non-exchangeable apolipoprotein that is tightly anchored to the core lipids of VLDL and LDL by β-sheets that penetrate the phospholipid monolayer [38]. ApoB contains several clusters of positively charged amino acids (mainly arginines and lysines), by which it interacts with LDL-R that mediates the clearance of LDL from the circulation [39]. **ApoA1**, which is the main apolipoprotein of HDL, usually exists on all types of HDL-particles, and also in a lipid free/lipid poor form. The major function of apoA1 is mediating cholesterol efflux, via interaction with the cellular cholesterol transporters ABCA1 and ABCG, and activation of LCAT [40]. **ApoA2** is the other major protein in HDL. It is mostly abundant in the denser HDL fraction (HDL_3), however its function is still unknown [41].

ApoE is an exchangeable apolipoprotein that associates with all lipoprotein classes [42-45]. ApoE has been suggested to have different functions in atherosclerosis and lipoprotein metabolism. These include cholesterol efflux from macrophages to HDL and interaction with LDL-R and the LDL receptor related protein (LRP) leading to uptake of apoE-containing lipoproteins [46]. The apoC-proteins are also exchangeable apolipoproteins associated with all lipoprotein classes [43, 47] and have various functions in lipoprotein metabolism [reviewed in [48]. **ApoC1** decreases the uptake of lipoproteins via LDL-R, LRP, and the VLDL-receptor, possibly through interaction with apoE, and it has also been shown to inhibit CETP. **ApoC2** is an essential activator of LpL, enabling the catabolism of triglyceride-rich lipoproteins. It may also share some of the inhibitory effects on receptor-mediated uptake with apoC1. **ApoC3** inhibits the activity of LpL and hepatic lipase (HL). It has also been shown to interfere with receptor mediated uptake of lipoproteins, and possibly also retention, through modulation of the affinity of lipoproteins for PGs [49-51].

Several of the apolipoproteins belong to, sor resemble, the super-family of lipocalins, which bind small, hydrophobic molecules in a β-barrel structure, which possibly explains their affinity for lipoproteins. **ApoM**, which is one of them, associates mainly with HDL, and to a lesser extent with VLDL and LDL [52]. The functions of apoM are not yet fully understood, but it seems to be able to protect lipoproteins against oxidation [53]. **ApoD** is also a lipocalin, and mainly associated with HDL [54]. However, as many of the less common

apolipoproteins, its effect on lipoprotein properties is not yet known. **ApoJ** is an apolipoprotein first discovered in HDL [55], and more recently in LDL [44]. In addition to many other suggested ligands, apoJ can also bind small, lipid molecules, and it has been suggested to have both anti-oxidative and cholesterol efflux-promoting properties [56, 57]. It accumulates in atherosclerotic lesions during atherosclerosis development, but has also been suggested in several other age-related diseases [58].

Some of these exchangeable apolipoproteins are probably bound to the outer lipid moieties of the lipoproteins, like apoE that even without apoA1 can form HDL-like phospholipid discs out of phospholipids [59], and apoM, which probably binds to lipoproteins via its hydrophobic signal peptide sequence [60]. ApoC3 has been shown to interact closely with the lipid monolayer of micelles, suggesting a similar interaction with lipoproteins [61].

2.4. Retention and modification of lipoproteins

The interaction of lipoproteins with arterial PGs is dependent on the clusters of positive amino acids in apoB [30]. PGs are a family of highly glycated proteins that are produced and secreted mainly by smooth muscle cells (SMCs) [62]. They are constituents of the extracellular matrix and provide elasticity and volume to the arterial wall. PGs contain negatively charged glucosaminoglycan chains of varying number and length that can interact with the positively charged amino acids in apoB.

The arterial retention of apoB-containing lipoproteins mainly includes LDL, which due to its smaller size more easily enters the subendothelial space, but also IDL and VLDL [63, 64]. However, as most studies on atherosclerotic plaque development have focused on LDL, I will hereafter refer to retained lipoproteins as LDL. After deposition in the intima, LDL is subjected to various modifications. Several different mechanisms for modifications have been suggested. Most focus has been on oxidative modification of LDL, mediated by local release of reactive oxygen species or by oxidative enzymes such as myeloperoxidase and lipoxygenases. Another suggested enzymatic modification is mediated by phospholipases.

2. 5. Secretory phospholipases

There are different families and groups within the super-family of phospholipase A_2 (PLA_2), with specific affinities for different phospholipid classes and fatty acid positions. PLA_2 lipolysis of phospholipids gives rise to the release of lysophospholipids and fatty acids (*Figure 2*). These products of phospholipid lipolysis have varying biological functions depending on the type and fatty acid composition of the phospholipid. The secretory phospholipases A_2 ($sPLA_2$) lipolyze the stereospecific numbering- (sn-) 2 fatty acyl ester bond in glycerophospholipids. A fatty acid that can occur in glycerophospholipids is arachidonic acid, a potent inflammatory activator that metabolizes further to highly bioactive inflammatory mediators, such as leukotriens and prostaglandins [65-67].

The dominant phospholipid in cell membranes, as well as in the phospholipid monolayer of lipoproteins, is the glycerophospholipid phosphatidylcholine (PC), comprising about 67% of the total phospholipid content. $sPLA_2$-lipolysis of lipoproteins in the intima would result in high local concentrations of proinflammatory fatty acids and lysophosphatidylcholine [65-67]. In addition, LDL modified by $sPLA_2$ has a higher binding capacity for arterial PGs than unmodified LDL and is also more prone to be oxidized and further modified by e.g. sphingomyelinase (SMase) [68-72].

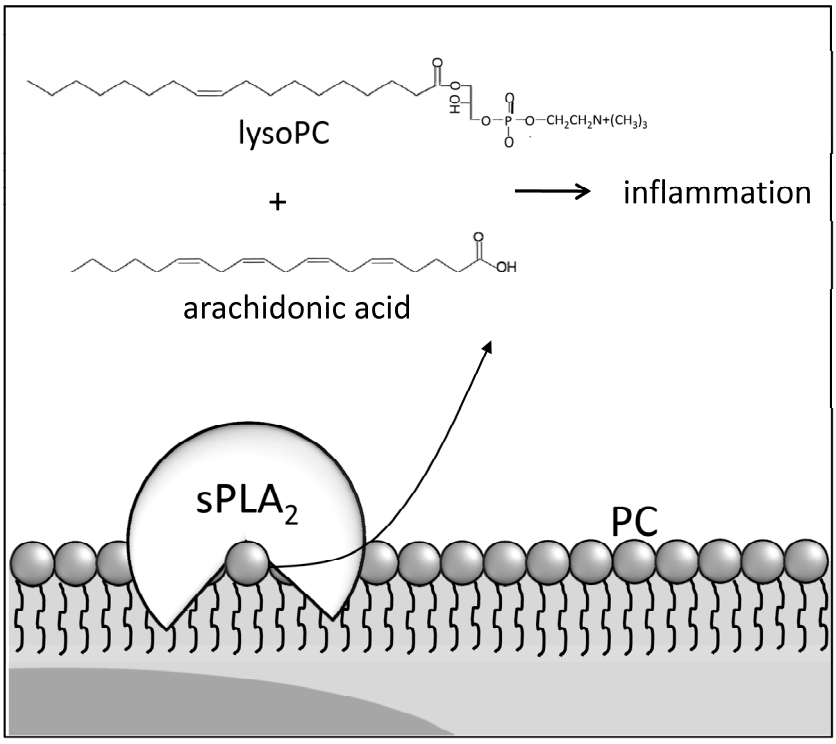

Figure 2. $sPLA_2$ in inflammation. $sPLA_2$ enzymes lipolyzes the sn-2 fatty acyl ester bond in cell membrane glycerophospholipids. This results in lyso-phospholipids and fatty acids, such as the pro-inflammatory mediators lysophosphatidylcholine (lysoPC) and arachidonic acid, which both can promote inflammation.

The phospholipases mostly studied in relation to CVD are the lipoprotein associated phospholipase A_2, also known as platelet-activating factor acetylhydrolase, and secretory phospholipase A_2 group IIA ($sPLA_2$-IIA). High circulating levels of both of these PLA_2 have been associated with an increased risk of CVD [73, 74]. More recently acknowledged in relation to atherosclerosis are the $sPLA_2$ group III, group V ($sPLA_2$-V) and group X, which are active on lipoproteins and have been found in human atherosclerotic plaques [68, 75-77].

2.5.1. $sPLA_2$-V

$sPLA_2$-V has been detected in mouse as well as in human atherosclerotic lesions and $sPLA_2$-V mRNA and protein have been identified in SMCs, macrophages and endothelium from human atherosclerotic plaques [68, 77]. The genes encoding $sPLA_2$-V and $sPLA_2$-IIA are both located in homologous regions on mouse chromosome 4 and human chromosome 1 [78], which has been identified as an atherosclerosis susceptibility locus in the LDL-R-deficient mouse and a candidate susceptibility locus in humans [79]. They share the same promoter, but are transcribed in reverse directions [78] and they seem to be differently regulated. In humans, $sPLA_2$-IIA is considered to be an acute phase protein and a marker of inflammation [73], and in mice, lipopolysaccharide-induced inflammation causes expression of $sPLA_2$-IIA in aorta. Aortic expression of $sPLA_2$-V is not induced by inflammation, but instead after administration of Western diet and in genetically induced hyperlipidemia [68]. In addition, $sPLA_2$-V is more active in lipolyzing lipoprotein phospholipids than is $sPLA_2$-IIA [68]. A recent study provided the first evidence that both overexpression and deficiency of bone marrow-derived $sPLA_2$-V affects the extent of atherosclerosis, measured as arterial lipid deposition, in LDL-R-deficient mice [80].

2.6. Recruitment and activation of inflammatory cells

During modification of LDL, a number of bioactive, proinflammatory molecules are generated. These molecules are proposed to activate the endothelium to produce cellular adhesion molecules and monocyte chemoattractant protein-1, enabling the recruitment of inflammatory cells, primarily monocytes but also T-cells, antigen presenting cells, and mast cells, to the intima [81]. Activated inflammatory cells secrete proinflammatory cytokines and

chemokines, like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), that may further sustain the inflammatory reaction in the intima [82-84]. In the intima, monocytes differentiate into macrophages, which subsequently increase their expression of scavenger receptors, like scavenger receptor A and cluster of differentiation 36 (CD36) [85, 86]. These receptors recognize and mediate uptake of modified LDL, causing transformation of the macrophages into lipid filled foam cells [87, 88].

2.7. The unstable plaque

The capacity of macrophages to handle the cholesterol load from modified lipoproteins is limited, leading to an extensive cell death and extracellular lipid accumulation, resulting in a necrotic core in the atherosclerotic plaque. SMCs proliferate and migrate from the media through the intima, to form a fibrous cap made out of SMCs, collagen fibers, and elastin. This cap covers the necrotic core, protecting the circulation from its highly thrombogenic content, e.g. tissue factor. However, activated macrophages and mast cells also produce proteases, such as matrix metalloproteinases, which can degrade fibrous cap components like collagen and elastin, thus weakening the fibrous cap. This can eventually lead to erosion or rupture of the cap, enabling contact between the content of the core and the coagulation system, resulting in thrombus formation. The balance between unstable properties of the plaque, such as macrophages, matrix metalloproteinases and lipids, and stabilizing properties of the plaque, such as SMCs and collagen, determines whether a plaque is stable or unstable, and thus prone to rupture [28].

3. Atherogenic mechanisms in the metabolic syndrome

Clustering separate, but associated, risk factors into MetS may, apart from highlighting this group of individuals at high risk, also provide a better basis for understanding the complex mechanisms interconnecting these metabolic abnormalities that predispose to a more rapid development of CVD. Below is a summary of some of the main proposed atherogenic properties of MetS: dyslipidemia, inflammation, oxidation, and advanced glycation end products (AGEs).

3.1. The atherogenic lipoprotein phenotype

In MetS, the lipoprotein metabolism is shifted towards a dyslipidemia which is called the atherogenic lipoprotein phenotype. The main characteristics of this phenotype are hypertriglyceridemia, the appearance of small dense LDL, and low levels of HDL [89, 90].

3.1.1. Hypertriglyceridemia

The elevated serum levels of triglycerides are due to an increased hepatic secretion of $VLDL_1$, which are larger, more triglyceride-rich VLDL-particles than the other VLDL subtype, $VLDL_2$ [91]. $VLDL_1$ secretion is increased when there is an increased hepatic availability of lipid substrate for triglyceride synthesis, due to an increased delivery of fatty acids to the liver and hepatic triglyceride content [92]. The features determining $VLDL_1$ secretion are not yet fully elucidated (reviewed in [92]) but liver fat, intra-abdominal fat, fasting insulin, and plasma glucose (which are all often increased in T2D and MetS) have been shown to predict the rate of $VLDL_1$ production [93]. In addition, hepatic insulin resistance results in failure to execute the insulin-mediated suppression of $VLDL_1$ secretion [92, 94].

An exact mechanism for the direct atherogenicity of $VLDL_1$ is not yet clear. Despite it being an apoB-containing lipoprotein, thus able to bind to arterial PGs, $VLDL_1$ is probably too large to enter the subendothelium, thus retention is probably not increased for $VLDL_1$. However, competing with chylomicrons for LpL, causing prolonged residence time in the circulation for smaller, apoB48-containing, and thus also PG-binding, chylomicron remnants may be one contributing factor. Indirectly though, $VLDL_1$ is atherogenic by promoting the formation of small dense LDL- and HDL-particles.

3.1.2. Small dense LDL

Elevated $VLDL_1$ is a strong predictor for the formation of small dense LDL, which is another characteristic of the atherogenic lipoprotein phenotype. $VLDL_1$ has a prolonged residence time in circulation. This may be due to an increased VLDL-content of apoC3, which inhibits LpL-mediated VLDL-triglyceride lipolysis [95]. The increased amount of triglycerides needed to be lipolyzed before it is cleared by the liver may also prolong the residence time of $VLDL_1$.

In addition, even though insulin is a positive regulator of LpL, studies have shown a downregulation of adipose tissue LpL in T2D [96]. The prolonged residence time of $VLDL_1$ in the circulation favors CETP-mediated exchange of triglycerides against cholesterol esters from $VLDL_1$ to LDL. This results in a more triglyceride-rich LDL, which is a better substrate for HL, the activity of which is also increased in T2D [90]. Increased lipolysis of LDL-triglycerides by HL results in small dense LDL-particles [97].

The atherogenicity of small dense LDL is believed to be a combined effect of different atherogenic properties. Small dense LDL has a lower affinity for LDL-R [98], which gives it a prolonged residence time in circulation, thus causing a higher probability of retention or modification of LDL. The smaller size allows a higher influx of LDL-particles into the vascular wall [63, 64, 99]. The conformation of apoB is different on the smaller LDL-particle [98], possibly exposing an additional PG-binding site [100], causing the observed stronger interaction between LDL and arterial PGs [101, 102], thus increasing arterial retention of small dense LDL. Small dense LDL has also been shown to be enriched in apoC3[103], which also increases the binding of LDL to arterial PGs [49]. Finally, small dense LDL has been shown to be more easily oxidized [104, 105] and glycated [106], two modifications shown to cause proinflammatory responses in vascular cells.

3.1.3. Low HDL

The third property of the atherogenic lipoprotein phenotype: low levels of HDL, is also due to the CETP-mediated exchange of triglycerides against cholesterol esters from $VLDL_1$, to HDL. The resulting triglyceride-enriched HDL is, as in the case of LDL, also a better substrate for HL. An increased HL-activity on HDL results in small dense, cholesterol-poor HDL and thus lower levels of HDL-cholesterol. The catabolism of HDL and apoA1 has also been shown to be increased for triglyceride-rich HDL and in individuals with insulin resistance and T2D [107, 108].

The atherogenic effect of decreased HDL levels is commonly thought to be largely due to a decrease in reverse cholesterol transport from macrophages, but it may also be due to the lack of other anti-atherogenic effects of HDL, including anti-oxidative, anti-inflammatory, and anti-thrombotic properties [109, 110].

3.2. Inflammation, oxidation, and advanced glycation end products

3.2.1. Inflammation

As mentioned above, obesity is a criterion for MetS. As BMI increases, so does the number and the size of the adipocytes [111], and the number of infiltrating macrophages in the adipose tissue [112]. These alterations in the adipose tissue lead to increased production of proinflammatory cytokines and adipokines, such as TNF-α, IL-6, and IL-8 [113, 114] which can contribute to the increased systemic inflammation seen in obese individuals and individuals with T2D and insulin resistance [115, 116]. This elevated systemic inflammation, seen as elevated levels of e.g. C-reactive protein (CRP), IL-6, TNF-α, and soluble cellular adhesion molecules, is associated with a higher risk of CVD [117-119].

3.2.2. Oxidation

An increased oxidative stress and elevated levels of oxidized LDL (oxLDL) are also seen in individuals with T2D and MetS [120-122]. OxLDL has been proposed to induce infiltration of inflammatory cells into the arterial wall, by increasing the expression of cellular adhesion molecules and monocyte chemoattractant protein 1 in the endothelium, and also to induce differentiation of monocytes to macrophages, macrophage foam cell formation, and secretion of proinflammatory cytokines [123]. As mentioned above, HDL has both anti-oxidative and anti-inflammatory properties, thus low levels of HDL, as in the MetS dyslipoproteinemia, may also contribute to this elevated oxidative and inflammatory state.

3.2.3. Advanced glycation end products

Hyperglycemia (elevated blood glucose levels) leads to the formation of AGEs, which have been shown to induce oxidative stress and inflammation [124-126]. AGEs are a heterogeneous group of products that form on proteins and lipids through a stepwise, enzyme-independent reaction initiated by glycation [127]. AGE-modification preferentially occurs on proteins with long residence time in circulation, e.g. LDL. Different AGEs such as N^{ε}-(carboxymethyl) lysine (CML) and hydroimidazolone have been shown to be elevated in diabetic individuals, and an association between AGE-levels and CHD has been shown [128-132]. AGE-LDL, which

also is elevated in T2D, is more susceptible to further oxidative modification and it is not cleared as efficiently via LDL-R [132, 133]. AGEs have also been shown to induce experimental atherosclerosis [134]. AGEs induce the production of reactive oxygen species, and subsequent expression of proinflammatory cytokines in vascular cells, via interaction with the membrane bound receptor for AGE (RAGE) [135]. Other receptors for AGEs seem to be able to dampen the proinflammatory effects of AGEs, including AGE-receptors 1-3, various scavenger receptors, and the soluble receptors sRAGE and lysozyme [135].

3.2.3.1. Lysozyme

Lysozyme (also known as muramidase) is a well-characterized bacteriolytic enzyme of the innate immune defense that preferentially hydrolyzes the β-1,4-glycosidic linkage between the N-acetylmuramicacid and N-acetylglucosamines in the peptidoglycan bacterial wall structure [136]. In addition, lysozyme acts a as a soluble receptor for AGEs [136], increasing both hepatic and macrophage-mediated clearance of AGEs [137]. Lysozyme has been suggested to be able to counteract the adverse effects of AGEs since it is able to increase macrophage clearance of AGEs without eliciting a proinflammatory macrophage response [137].

Lysozyme is abundantly present in body secretions such as milk, tears, saliva, and blood, and the dominating sources of circulating lysozyme are monocytes and macrophages [138]. It is upregulated during differentiation of mononuclear and polymorphonuclear blood cells, and measurement of circulating lysozyme can be used as a marker for an elevated leukocyte proliferation, as in monocytic and myelomonocytic leukemia [139]. Quite recently lysozyme was also identified in LDL for the first time [44]. In addition, overexpression of lysozyme in the apoE knock-out mouse model for atherosclerosis results in reduction of oxidative stress and reduction of atherosclerosis, manifested as a smaller plaque area with less infiltration of inflammatory cells, suggesting a protective role of lysozyme in murine atherosclerosis [140, 141].

The relationship between lysozyme and human atherosclerosis or T2D, though, has hitherto not been studied. One previous study has shown an increase in lysozyme activity in leukocytes isolated from patients with unstable angina or acute myocardial infarction, compared with healthy controls. In the same study, an elevated leukocyte lysozyme activity was shown for diabetic patients with unstable angina or acute myocardial infarction compared with non-diabetic patients with the same conditions.

AIMS OF THE THESIS

This thesis is focused on the atherogenic lipoproteins, especially LDL as LDL probably is the main initiator of atherosclerosis. I have chosen to study individuals with T2D and MetS for several reasons. As the prevalences of these conditions are increasing rapidly, bringing with them a considerable increase in risk of CVD, they will have a major effect on the burden of CVD on our society. Also, this group of high-risk patients would benefit greatly from improved treatment aimed specifically at their dyslipoproteinemia, since it is different from the high-LDL cholesterol phenotype traditionally associated with high risk of CVD [142]. Finally, since these individuals have an accelerated development of atherosclerosis and CVD, they constitute a good model for finding possible rate-limiting steps in the development of atherosclerosis.

The overall aim of this thesis was to investigate possible atherogenic properties of apoB-containing lipoproteins from individuals with T2D and MetS.

The specific aims of the individual papers were:

Paper

I. To investigate of there is a difference in susceptibility to $sPLA_2$-V-mediated lipolysis between VLDL and LDL from individuals with T2D and MetS and corresponding lipoproteins from healthy individuals.

II. **a.** To identify LDL-associated proteins.

b. To compare the protein composition of LDL from individuals with T2D and MetS with that of LDL from healthy individuals.

III. To investigate if lysozyme, one of the proteins revealed to be enriched on T2D-MetS-LDL in **Paper II**, is also elevated in serum of individuals with T2D and MetS.

4. METHODOLOGICAL CONSIDERATIONS

Below, some specific methodological issues are discussed. More detailed descriptions of the materials and methods used in this thesis are provided in the individual papers.

4.1. Study individuals

In all three papers, one inclusion criterion for the individuals in the case group was qualifying for MetS, however, different definitions of MetS were used in the different papers. In **Paper I** and **Paper III** the definition by WHO was used, and in **Paper II** the definition by NCEP-ATP III was used (*Table 1*). The two definitions are similar; the NCEP-ATP III-definition has marginally higher HDL-cholesterol limits than the WHO-definition and also defines obesity solely according to waist circumference ($\geq$102 cm for men and $\geq$88 cm for women), while WHO defines obesity as either WHR (>0.9 for men and >0.85 for women) or BMI ($\geq$30 kg/m^2). The major difference between the two, however, is that in the NCEP-ATP III-definition impaired glucose metabolism is not a requirement. This did not affect our selection though, as in all three papers, T2D was also an inclusion criterion for the case group.

For the case group in **Paper I** and **Paper III**, the inclusion criterion of hypertriglyceridemia (defined according to WHO as serum triglycerides $\geq$1.7mmol/L) was added to the criteria of T2D and MetS. The reasons for this was that we particularly wanted to study the atherogenic lipoprotein phenotype including small dense LDL, which is closely linked to hypertriglyceridemia. The study subjects for **Paper I** and **Paper III** were chosen from the community-based study Diabetes in Women and Atherosclerosis, or DIWA [20]. The screening for this study involved all 64-year old women living in Gothenburg, Sweden, during 2001 to 2003. Previous CVD was an exclusion criterion, and the women were recruited into three groups: NGT, IGT, and diabetes. Due to the inclusion procedure in DIWA and subsequent sampling of subjects for **Paper I** and **Paper III**, the control individuals in our study had a slightly higher BMI than the NGT-individuals in the screening for DIWA (mean BMI in **Paper I** was 27.9 vs. 26.0 kg/m^2 in the DIWA screening) [20]. This resulted in the unusually small difference in the degree of obesity between the two groups in **Paper I** and **Paper III**. In addition, there was no difference in blood pressure between the two groups (mean blood pressure in the T2D-MetS group was 136/75 vs. 143/78 in the control group).

Thus, the major difference between the T2D-MetS group and the control group in **Paper I** and **Paper III** was the presence of T2D and the atherogenic lipoprotein phenotype.

In **Paper II** we chose to study subjects from the three year-follow up of another community-based study performed in Gothenburg; Atherosclerosis and Insulin Resistance, or AIR [143], which at baseline included a random selection of the 58-year old men in Gothenburg. In **Paper II**, we matched the included individuals for BMI and WHR, to adjust for obesity. Hypertriglyceridemia was not specified as an inclusion criterion, even though the groups did differ significantly in serum triglycerides. In addition, the groups did not differ in HDL-cholesterol or ratios describing LDL-composition. Thus, in this study, the case group did not show a classic atherogenic lipoprotein phenotype.

4.2. LDL-isolation methods

Studying specific properties of LDL almost always includes extracting LDL from the *in vivo* situation. Different methods for isolating LDL have different advantages and disadvantages regarding recovery, purity, efficiency, and, probably most important but also the most difficult to assess: physiological relevance. Below, I will discuss some of the properties of the LDL-isolation methods used in this thesis. In the traditionally most used method, LDL is isolated through sequential density-based ultracentrifugation*,* using solid potassium bromide (KBr) to adjust the density. This method was not used in any of the papers in this thesis, but as it has been the golden standard for LDL-isolation, I will compare those methods used to this one.

In **Paper I**, VLDL and LDL were isolated using a method also based on sequential ultracentrifugation, but using deuterium oxide (D_2O) instead of KBr to set the density. The original reason for this modification was to avoid subsequent desalting, which requires prolonged dialysis or chromatography. Another advantage of using D_2O instead of KBr is that the lipoproteins are not subjected to high salt concentrations, which can disrupt ion-bonds between proteins and displace exchangeable apolipoproteins [144]. Proteomic analyses of LDL and VLDL isolated using the two methods mentioned above show that lipoprotein isolation using the D_2O-based method results in more proteins being associated to

LDL than using the KBr-based method, while the protein composition of VLDL is virtually the same using both methods [43].

In **Paper II**, LDL was isolated using size exclusion chromatography (*Figure 3*), which instead of densities uses the specific sizes of the lipoprotein classes to separate them from each other and from the remaining serum. In this system, the LDL-associated proteins are not exposed to any centrifugal forces and there is no need for high salt concentration solutions; instead the liquid phase is phosphate buffered saline with physiological salt concentration and pH. In **Paper II**, where we wanted to lose as little proteins as possible due to the isolation method, this was our method of choice. To achieve a good separation of the different lipoprotein classes, we used two size exclusion columns coupled in series. Also, in the more sensitive mass spectrometry analysis, the liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), we only used the central fraction of the LDL peak, to minimize the risk of contamination by proteins from other lipoprotein

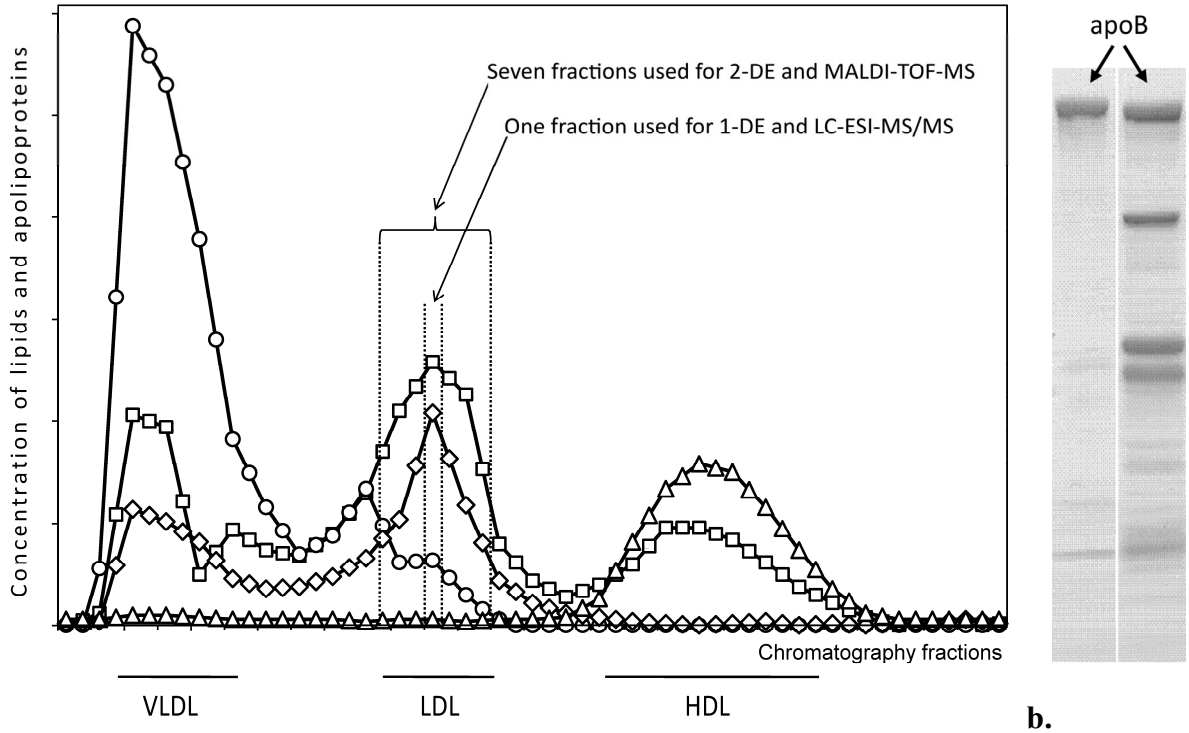

Figure 3. Size exclusion chromatography. a. Separation of lipoproteins from serum showing the concentrations of apoB (◇), apoA1 (△), cholesterol (□), and triglycerides (○) in chromatography fractions. **b.** Coomassie-stained polyacrylamide gel electrophoresis of LDL isolated using KBr-based sequential ultracentrifugation (left lane) and size exclusion chromatography (right lane), visualizing the differences in the amounts of LDL-associated proteins (arrow indicates apoB).

fractions (*Figure 3*). One drawback of all available LDL-isolation methods, however, is the fact that the lipoprotein classes overlap in density, size, and apolipoprotein content, making a complete separation of lipoprotein classes from each other impossible.

4.3. PLA2-V activity assay

In order to study the susceptibility of different lipoproteins to lipolysis by $sPLA_2$-V in **Paper I**, we developed an assay using recombinant human $sPLA_2$-V [68]. The preferred phospholipid substrate for $sPLA_2$-V in lipoproteins is PC, which constitutes 67% of the total phospholipid content of lipoproteins [145]. The measurement of non-esterified fatty acids (NEFA) provides a good estimation of lipolyzed phospholipids in VLDL and LDL, as the levels of resulting NEFA and lysophospholipids correspond very well [68]. In addition, $sPLA_2$-V does not induce changes in the levels of any other lipid classes than phospholipids, NEFA and lysophospholipids, further strengthening the use of NEFA as a measurement of phospholipid lipolysis. To keep variability as low as possible, NEFA was in **Paper I** measured using an autoanalyzer and a commercially available NEFA-kit.

The susceptibility to $sPLA_2$-V-mediated lipolysis has been shown to be highest for VLDL, followed by LDL and then HDL, when lipoproteins of the same particle concentration (measured as apoB for VLDL and LDL, and as apoA1 for HDL) are compared [68]. This is probably mostly due to the amount of phospholipids per particle, which decreases with the size of the lipoprotein. After normalization for PC concentration, there are still differences in susceptibility between lipoproteins; VLDL is the best substrate, followed by HDL and then LDL. This indicates that other properties than solely the amount of available PC in the lipoproteins determine their susceptibility to $sPLA_2$-V-lipolysis. In **Paper I**, we chose to normalize the lipoproteins isolated from the two study groups for PC concentration, to be able to see if there were any other intrinsic properties of lipoproteins that could affect their susceptibility to $sPLA_2$-V-lipolysis.

The experimental conditions of the assay in **Paper I** were optimized to achieve detectable levels of NEFA without reaching a state in the reaction where either saturation or inhibition of the enzyme might disturb the results. The final experimental PC concentrations were chosen well below the saturation limit (*Figure 4*). The reaction time of one hour was

chosen in order to minimize experimental variation and to avoid product inhibition of the enzyme. Unfortunately, the inter-assay variation proved to be too large to entitle un-paired analysis of the difference in lipolysis between the groups. Instead we chose to treat samples as paired (one patient and one control) throughout lipoprotein preparations, lipid-and apolipoprotein measurements, and enzyme reactions, and we also use paired statistics. This prevented us from performing correlation analyses between amounts of different lipoprotein subcomponents and the degree of lipolysis in order to examine what lipoprotein properties would be the most important for determining susceptibility to $sPLA_2$-V-lipolysis.

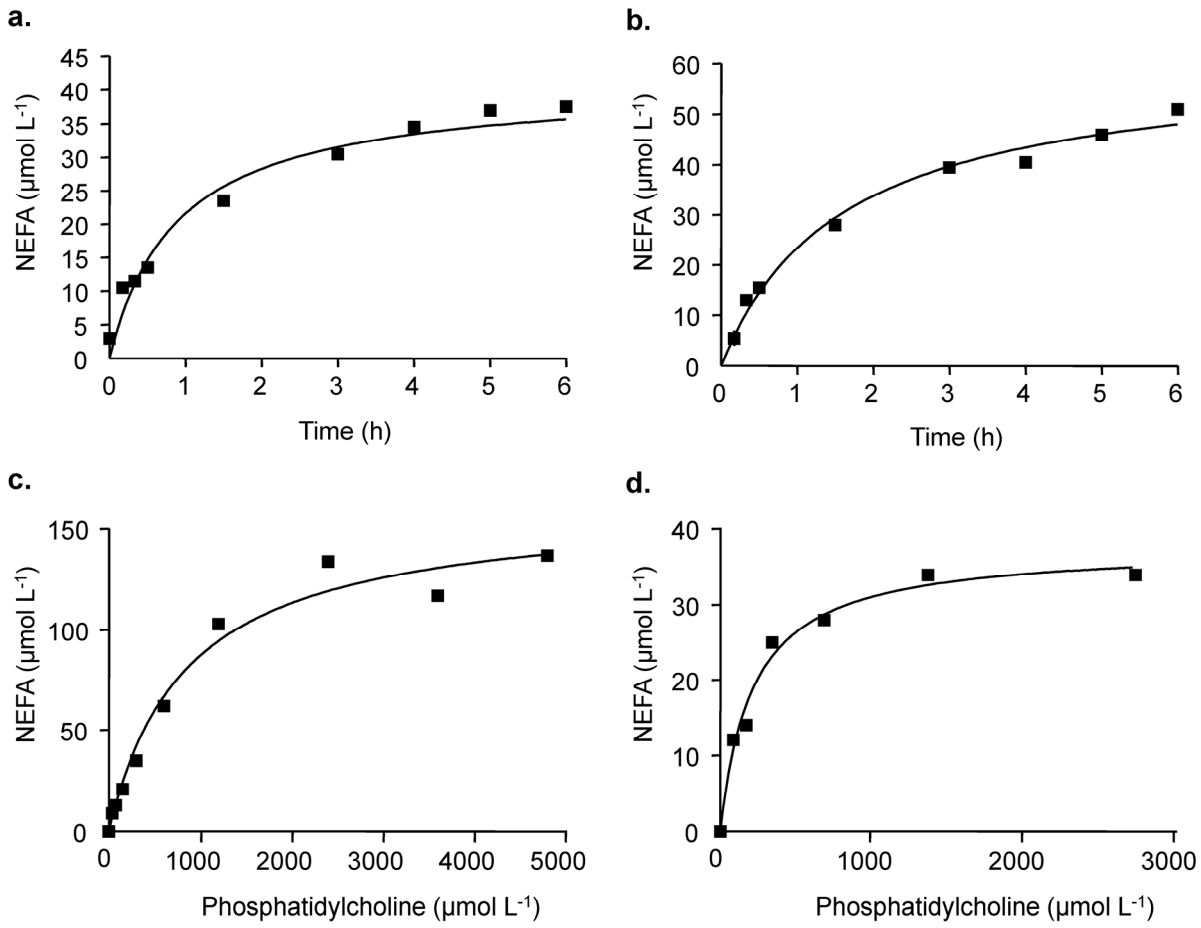

Figure 4. Lipolysis of VLDL and LDL by $sPLA_2$-V: Optimizing experimental conditions. $sPLA_2$-V-mediated lipolysis, measured as production of NEFA, are shown over time (**a, b**) and concentrations (**c, d**) of VLDL (**a, c**) and LDL (**b, d**). Concentrations used in (**a**) and (**b**) were 0.075 mmol/L PC for VLDL and 0.2 mmol/L PC for LDL. Based on (**a**) and (**b**), the final reaction time of one hour was chosen. Based on (**c**) and (**d**), where this reaction time was used, the final reaction concentrations of 0.1 mmol/L PC for VLDL and 0.2 mmol/L PC for LDL were chosen. (**Paper I**, **Figure 1**).

4.4. Proteomic analyses

In **Paper II**, the first aim was to identify LDL-associated proteins. To accomplish this we chose to identify proteins in LDL (isolated by the size exclusion chromatography method described above), using mass spectrometric methods, which allowed us a broad and explorative identification, compared to directed protein identification methods, such as western blot or ELISA. We chose to combine a qualitative approach, using LC-ESI-MS/MS, to analyze LDL from five of the 19 individuals in the study, with a quantitative approach, using two dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) to quantify and identify LDL-associated proteins from all 19 individuals.

The mass spectrometric method chosen for the qualitative approach in **Paper II**, LC-ESI-MS/MS, is highly sensitive and has a high resolution. Thus one advantage of this method is its ability to identify even minor protein components in a complex biological sample. A disadvantage of this high sensitivity is a higher probability of identifying contaminating proteins. In order to avoid this, we used strict protein identification criteria: at least one unique peptide at the ≥99.9% level of confidence and a supporting peptide at the ≥95% level of confidence were required for positive protein identification. We also chose to present only those proteins that were identified in all analyzed LDL-samples. It is difficult to draw conclusions about the general population on the basis of five analyzed individuals, therefore the results from **Paper II** will have to be validated, perhaps using more directed methods, in a larger study sample. But for an explorative approach, set out to identify unexpected proteins in LDL, our sample size is comparable to what has previously been used in the literature [109].

By separating and staining the LDL-associated proteins on 2-D gels, protein images can be obtained and processed using specialized software, and relative intensities for each protein can be calculated. The detection limits of the 2-DE analysis are determined by the sensitivity and linearity of the protein stain. According to the manufacturer, the SYPRO Ruby protein stain (Molecular Probes, Eugene, OR) used in **Paper II** is linear down to the nanogram level, but this is still not nearly as sensitive as the LC-ESI-MS/MS analysis, which is sensitive down to at least a low femtomole level. As a result, using this approach we only identified 14 of the 31 proteins identified in the LC-ESI-MS/MS approach.

In the quantitative approach in **Paper II**, the normalization method used quantifies the relative intensity of each protein, expressed as a percentage of the total intensity of included proteins on the gel. Thus, with a large span in protein spot intensity, there is a risk that relatively minor changes in the strong spots create false fluctuations in the relative intensities of the weaker spots. This was a concern for our 2-DE analyses, as in a preliminary quantification, fibrinogen α, β, and γ constituted 48%, haptoglobin constituted 23%, and the immunoglobulins together constituted 17% of the total protein intensity on the gels (apoB does not enter the gel, thus it is not included in the total protein intensity). Therefore, after ensuring that there were no significant differences between the groups in the intensity of fibrinogen, haptoglobin, and immunoglobulins, we excluded them from further quantification. The variations of the remaining proteins (measured as standard deviations of the intensities of each protein in the whole study material) were less after these proteins were excluded, supporting our original concern. Thus, we chose to present the relative protein intensities from the second quantification. Whether the presence of these major plasma proteins represent large aggregates co-migrating with LDL in the size exclusion chromatography or actual LDL-binding proteins will have to be further explored using other LDL isolation methods.

4.5. Lysozyme measurements

A common way to measure lysozyme is by using an activity assay [146]. This assay is based on the ability of lysozyme to hydrolyze the β-1,4 linkages between N-acetylmuramic acid and N-acetylglucosamine, which constitute the polysaccharide backbone of peptidoglycans in the cell wall structure of bacteria. In this assay, a suspension of the gram-positive bacteria *Micrococcus lysodeikticus* is exposed to lysozyme, which degrades the bacterial walls, and the subsequent decrease in turbidity of the solution is monitored spectrophotometrically at 450 nm. In **Paper III**, we chose not to use this assay, as we wanted to measure the total content of lysozyme in serum, not just active lysozyme. It has been shown that the bactericidal activity of lysozyme is inhibited by binding of AGEs to lysozyme, due to an overlap between the AGE-binding site and the bactericidal site [136]. In **Paper II**, we showed an increased amount of lysozyme associated with LDL from individuals with T2D. Individuals with T2D have been shown to have increased levels of AGE-modified LDL, and one possible way for lysozyme to associate with LDL in T2D could be via the AGE-moiety of AGE-

modified LDL. Such an association would presumably inhibit the bactericidal activity of lysozyme. Therefore, to ensure that we in our measurements included also lysozyme bound to proteins and/or lipoproteins, via AGE-moieties or in other ways, we chose to use an ELISA based on a polyclonal antibody directed against human lysozyme.

5. RESULTS AND DISCUSSION

The results of this thesis are described in detail in the individual papers, and referred to as e.g. (**Paper I**, **Table 1**). Below, results are discussed in the context of the possible atherogenicity of lipoproteins and lipoprotein-associated proteins in T2D and MetS.

5.1. The atherogenic lipoprotein phenotype

Using T2D, MetS, and hypertriglyceridemia as inclusion criteria in **Paper I** and **Paper III** led to a case group (T2D-MetS group) with the typical MetS-dyslipidemia with elevated serum triglycerides and low HDL-cholesterol (*Figure 5* and **Paper I**, **Table 1**). The elevation of serum triglycerides was probably partly due to a specific increase in the production of $VLDL_1$, as the analyses of lipid content in VLDL showed a doubling of the amount of triglycerides and cholesterol per VLDL-particle in the T2D-MetS group (**Paper I**, **Table 3**). This is consistent with previous findings describing the diabetic dyslipidemia [91].

The case group also showed a shift in LDL-subclass phenotype towards small dense LDL, seen in **Paper I** and **Paper III** as a smaller LDL-peak particle diameter in the T2D-MetS group (25.9 nm vs. 27.0 nm). A common definition of small dense LDL is an LDL-particle diameter <25.5 nm, when measured by gradient gel electrophoresis [147]. (Thus, the "normal" diameter range presented in *Table 1* may have been derived using other methods, e.g. NMR, which gives a smaller measurement of LDL-particle diameter than gradient electrophoresis [148, 149].) In **Paper I**, LDL-peak particle diameter was <25.5 nm in 39% of the T2D-MetS-individuals and in none of the control individuals. However, most individuals have a broad range of LDL-particle diameters [149], meaning that small dense LDL-particles probably are present in more than 39% of the T2D-MetS-individuals.

In addition to measurements of LDL size in **Paper I** we performed an extensive characterization of the lipid constituents of LDL, extending previous knowledge of the specific composition of LDL in T2D and MetS. Small T2D-MetS-LDL contained less cholesterol and more triglycerides than larger LDL from control individuals (*Figure 5* and **Paper I**, **Table 3**), and correlation analyses showed that a decreased LDL-peak particle diameter was associated with a decrease in cholesterol content and an increase in triglyceride content of LDL. These results suggest that T2D-MetS-LDL is not just cholesterol-

poor, as was expected, but also triglyceride-rich compared with normal LDL. However, with the density ranges chosen for VLDL- and LDL-isolation in **Paper I**, IDL was within the density range of the LDL-isolation (**Paper I** and *Table 2*)**.** Thus if any IDL was present, despite overnight fasting before blood sampling, it may have affected lipid composition ratios presented for LDL. If so, this may have affected the T2D-MetS group more than the control group, as these blood samples theoretically may have contained more IDL than blood samples from controls. Thus the increase in triglyceride content of T2D-MetS-LDL might be slightly overestimated.

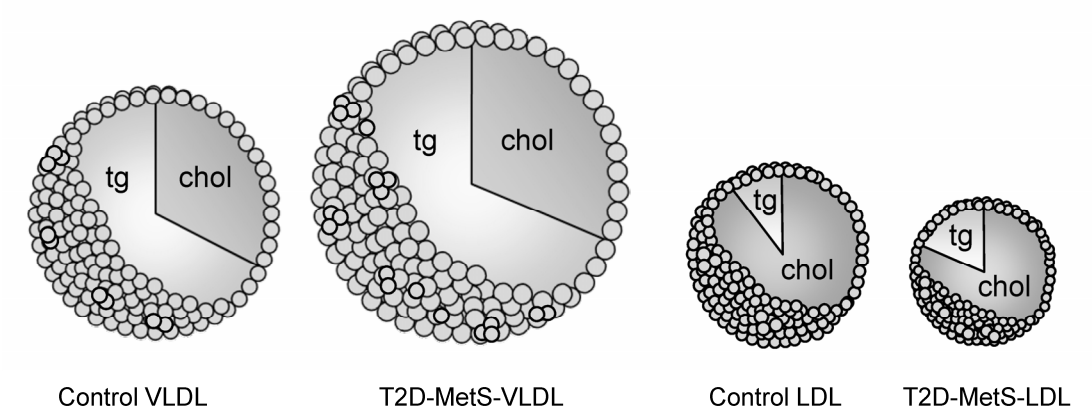

Figure 5. Altered composition of triglycerides (tg) and cholesterol (chol) in VLDL and LDL from individuals with T2D and MetS compared with VLDL and LDL from healthy (control) individuals.

The presence of small dense LDL in the T2D-Mets group in **Paper I** and **Paper III** was also reflected in lower serum LDL-cholesterol in the T2D-MetS group (**Paper I**, **Table 1** and **Paper III**, **Table 1**). It is not uncommon for this type of individuals to display normal or even low LDL-cholesterol levels, which in the presence of hypertriglyceridemia usually does not reflect a decrease in the amount of LDL-particles, but rather a lower cholesterol content in the LDL-particles [150]. A crude estimation of the relative contribution of LDL-particles to the total serum apoB concentration in each group in **Paper I** suggests that this was the case also in this study [i]. The same estimation also indicates an increase in the number of VLDL-particles in the T2D-MetS group, which is typically found in this type of individuals [91]. Elevated VLDL

[i] An estimation of the relative contribution of LDL-particles to the total serum apoB-concentration was made using the LDL ratio of cholesterol:apoB and the serum LDL-cholesterol and apoB values. The estimated apoB concentration representing LDL would according to this estimation be 0.47 g/L apoB in the T2D-MetS group and 0.46 g/L apoB in the control group. Assuming this, the remaining apoB would then be in VLDL, which would be represented by 0.83 g/L apoB in the T2D-MetS group and 0.66 g/L apoB in the control group.

confers an increased risk for CVD that often is overlooked if only the LDL-cholesterol is taken into consideration [142]. A measurement of apoB instead of LDL-cholesterol would better reflect an increase in all the atherogenic lipoproteins (VLDL, IDL, and LDL). A possibly even better measurement is the apoB:apoA1 ratio, which also takes the anti-atherogenic lipoprotein HDL into consideration. Recently, the American Diabetes Association and the American College of Cardiology suggested the additional use of apoB, in addition to LDL-cholesterol and non-HDL-cholesterol, to assess the effect of LDL-lowering treatment [151]. The benefit of using the apoB:apoA1 ratio instead of e.g. mentioned cholesterol measurements to predict risk of myocardial infarction has been shown in several studies [152, 153]. When using a ratio, e.g. the apoB:apoA1 ratio, it is however important to evaluate both individual parts of the ratio as well as the ratio itself, not to lose any valuable information.

5.2. Increased lipolysis by sPLA$_2$-V of lipoproteins in diabetic dyslipidemia

In **Paper I** we examined the susceptibility of atherogenic lipoproteins from individuals with T2D and hypertriglyceridemia to sPLA$_2$-V-mediated lipolysis. Both lipid-rich VLDL and small, cholesterol-poor LDL from these individuals showed an increased susceptibility to sPLA$_2$-V-mediated lipolysis compared with VLDL and LDL from the control group (**Paper I**, **Table 2** and **Figure 2**). On average, T2D-MetS-VLDL was lipolyzed 23% more efficiently than control VLDL and T2D-MetS-LDL was lipolyzed 17% more efficiently than control LDL. Any possible presence of IDL in the LDL fraction might have influenced the LDL results, but not the VLDL results. Considering the measured amount of VLDL- and LDL-phospholipids in the two study groups (**Paper I**, **Table 3**), together with the estimated serum concentrations of LDL and VLDL[i], the 23% increase in susceptibility of T2D-MetS-VLDL would mean an estimated 2.8-fold increase in NEFA and lysoPC produced from the VLDL-pool, and a 12% increase in NEFA and lysoPC produced from the LDL-pool, in the T2D-MetS-individuals, assuming the same amount of sPLA$_2$-V.

Whether relevant amounts of sPLA$_2$-V are present in circulation, though, has not yet been possible to determine, since no satisfying assay for measurement of sPLA$_2$-V mass has been developed to date. Nevertheless, the effect of sPLA$_2$-V-mediated lipolysis of lipoproteins is probably most potent in a relatively close environment, like in the atherosclerotic plaque, since sPLA$_2$-V there might generate quite high local concentrations of

NEFA and lysoPC. For atherosclerosis, the increased susceptibility of LDL to $sPLA_2$-V may thus be more important than that of VLDL, as large VLDL probably does not enter the subendothelium as readily as LDL does. $sPLA_2$-V can bind to arterial PGs [68, 154] and has been shown in atherosclerotic plaques associated with SMCs and macrophages, and with the endothelium [68, 76, 77](*Figure 6*). $sPLA_2$-modification of LDL results in a smaller LDL-particle [155], presumably increasing its influx into the arterial intima (*Figure 6.a*). $sPLA_2$-modified LDL form aggregates [156], with higher affinity for arterial PGs [157] (*Figure 6.b*), and with an increased capacity to turn macrophages into foam cells [158, 159] (*Figure 6.c*). These effects may lead to an increased intimal retention of $sPLA_2$-V-modified LDL compared with native LDL. An increased production of NEFA and lysoPC in the plaque could result in an increased PG synthesis by SMCs [66] (*Figure 6.d*), enhanced chemokine release from endothelial cells [65], increased adhesion of monocytes to endothelial cells [67] *(Figure 6.d*), and an induced production of proinflammatory cytokines by macrophages [160] *(Figure 6.f*). All of these effects can induce intimal inflammation, which can promote plaque progression *(Figure 6.g*).

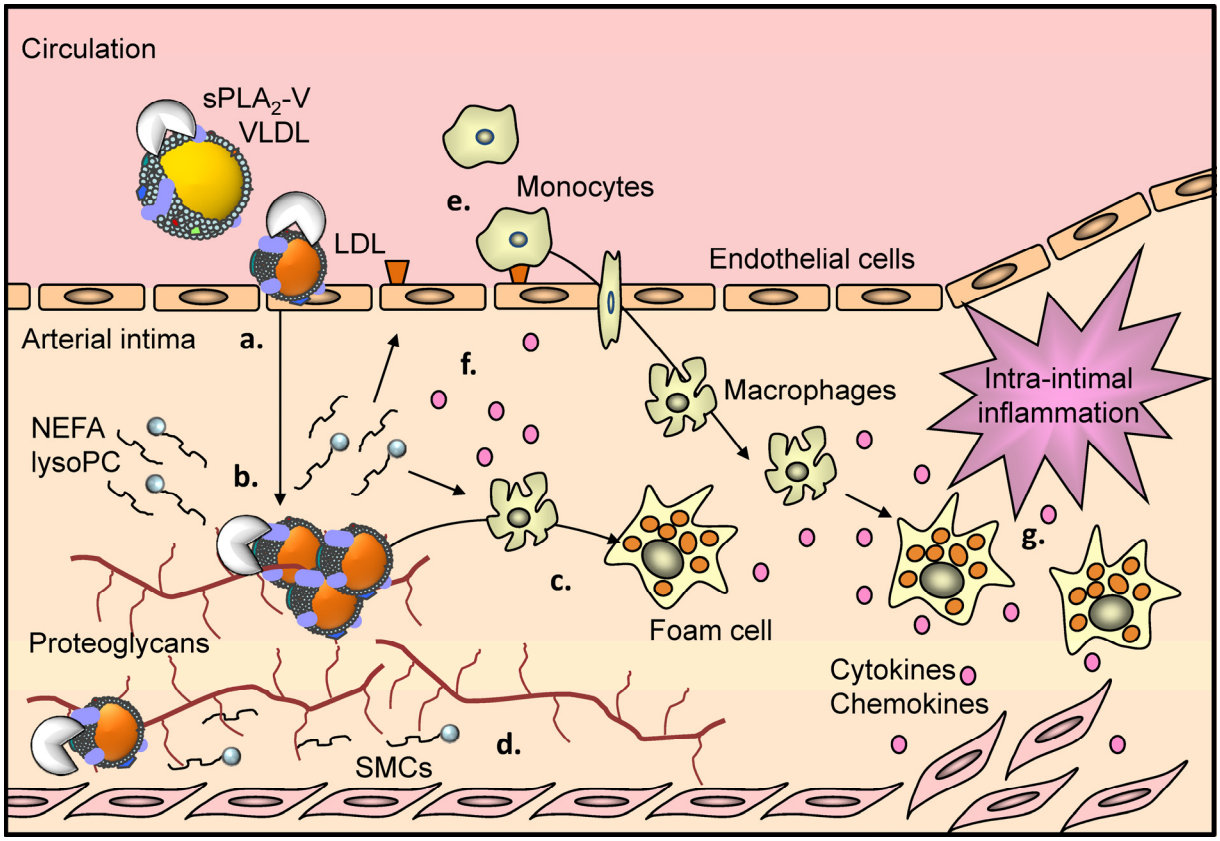

Figure 6. Suggested effects of sPLA2-V-mediated lipolysis of lipoproteins on atherosclerosis.
(***a-g**; see paragraph above*)

Together with the results in **Paper I**, this suggests that $sPLA_2$-V-mediated lipolysis of lipoproteins may lead to an increased atherosclerosis in individuals with T2D and MetS. Our results may also help to explain the elevated levels of lysoPC shown in LDL from individuals with T2D and hypertriglyceridemia [161].

The reason for varying susceptibility of different lipoproteins to $sPLA_2$-V-mediated lipolysis is not known. The total amount of PC in the lipoprotein probably affects the susceptibility, but it is not the only determining factor [68]. Below, I will discuss three possible properties of VLDL and LDL from the T2D-MetS-individuals that could possibly explain the increased susceptibility to $sPLA_2$-V-mediated lipolysis of these lipoproteins.

An increased surface fluidity of a lipoprotein, which shows an inverse correlation with the surface protein+cholesterol:phospholipids ratio, has been shown to increase the susceptibility of lipoproteins to $sPLA_2$-V-mediated lipolysis [162]. T2D-MetS-LDL contains more apoB per phospholipids and more apoC3 per phospholipids than control LDL (**Paper I**, **Table 3**), suggesting a higher surface fluidity of T2D-MetS-LDL. This would argue against an effect of the surface fluidity on $sPLA_2$-V-mediated lipolysis of T2D-MetS-LDL, as T2D-MetS-LDL was more readily lipolyzed than control LDL. The increased lipolysis of T2D-MetS-VLDL, on the other hand, might be due to an increased surface fluidity, based on the lower apoB per phospholipid and apoC3 per phospholipid ratios compared to control VLDL. This is highly speculative, and cannot be concluded from our data, as we did not measure total protein content or free cholesterol, as opposed to total cholesterol. However, surface fluidity might be one factor affecting the degree of lipolysis of lipoproteins in T2D and MetS that needs to be further investigated.

One of the characteristics of the T2D-MetS-lipoproteins in this study was an increased apoC3 content (**Paper I**, **Table 3**), which has been reported in several other studies [47, 49, 95, 103, 163]. Enrichment of phospholipid liposomes with apoC3 has been shown to increase the activity of snake venom $sPLA_2$ against the liposomal phospholipids [162, 164], however this has still to be shown for $sPLA_2$-V. Nevertheless, considering the microenvironment of $sPLA_2$ enzymes and their substrate: the PC in the phospholipid surface, it is possible that the amount of apoC3 per "surface unit" (per phospholipid) rather that per particle (per apoB) is important for the $sPLA_2$-activity, especially if a direct interaction between apoC3 and $sPLA_2$ is required. T2D-MetS-VLDL contained more apoC3 per particle, but less apoC3 per phospholipid than

control VLDL (**Paper I**, **Table 3**), which is in accordance with a previous study on similar study subjects [163]. LDL from T2D-MetS-individuals, however, contained more apoC3 both per particle and per phospholipid than control LDL did. Thus this may provide a possible explanation for the increased lipolysis of T2D-MetS-LDL, but not of T2D-MetS-VLDL.

A third factor shown to affect $sPLA_2$-V-lipolysis, which may be indirectly affected by the lipoprotein content of apoC3, is the sphingomyelin (SM) content of the lipoproteins. ApoC3 has been shown to stimulate SMase to lipolyze SM, resulting in the products ceramide and phosphorylcholine [165], and the ratio of SM to ceramide have been shown to affect $sPLA_2$-V-activity [166]. $sPLA_2$-V does not hydrolyze SM per se, but a decreased lipoprotein content of SM, as well as an increased lipoprotein content of ceramide have been shown to increase $sPLA_2$-mediated lipolysis of lipoprotein PC. In **Paper I**, we did not measure SM; however previous studies have shown that both VLDL and LDL from T2D-individuals have reduced ratios of SM per PC [167, 168]. Furthermore, in a detailed lipid analysis in another subsample of the DIWA study, lower SM content is shown for both LDL and VLDL from individuals with T2D and hypertriglyceridemia (*M. Ståhlman, personal communication*). Thus, a decreased SM content, possibly due to an increased apoC3 content (**Paper I**, **Table 3**), might provide an explanation for the increased lipolysis of VLDL and LDL from the DIWA individuals with T2D and hypertriglyceridemia in **Paper I**.

5.3. LDL-associated proteins

In **Paper II** we identified 31 LDL-associated proteins in LDL isolated through size exclusion chromatography, using a qualitative approach based on LC-ESI-MS/MS (**Paper II**, **Table 1**). The identified proteins belonged to functional groups such as apolipoproteins, inflammation-regulating proteins, complement, and protease inhibitors (*Figure 7*).

Previous proteomic studies on LDL-associated proteins have reported fewer proteins associated with LDL, probably due to the use of other LDL-isolation methods (KBr- or D_2O-based sequential ultracentrifugation) and less sensitive and/or more restrictive proteomic methods (2-DE and MALDI- or SELDI-TOF) [43, 44, 103]. Indeed, a proteomic study of HDL-associated proteins also using LC-ESI-MS/MS identified 48 proteins in HDL [109], which is more than has been identified using 2-DE and MALDI- or SELDI-TOF [43, 45].

We were also able to quantify 14 of the identified LDL-associated proteins, as well as various isoforms of several of those proteins (**Paper II**, **Table 2**), using a quantitative approach based on 2-DE and MALDI-TOF.

5.3.1. LDL-associated apolipoproteins

The largest group among the LDL-associated proteins (**Paper II**, **Table 1** and *Figure 7*) was, as expected, the apolipoproteins. Among them were the most common LDL-associated apolipoproteins; apoB, apoC3, and apoE. The other apolipoproteins identified were apoA1, apoC2, apoD, apoJ, and apoM, all previously identified in LDL [43, 44, 103, 144, 169], in addition to apoL1, which to our knowledge has not been identified in LDL before. ApoL1 has previously been shown associated with HDL and, to a very small extent, with VLDL. The fact that it was not identified in the 2-DE-based approach indicates quite small amounts of apoL1 also in LDL. ApoL1 has been suggested to be involved in lipid metabolism, based on a putative sterol response element in its promoter and its association with hypertriglyceridemia [170-172], but its functions have not yet been elucidated.

We were not able to identify apoC1 and apoA4, which previously have been identified in LDL. ApoC1 could only be identified with enough certainty in one of the five analyzed LDL and did thus not qualify as an LDL-associated protein in **Paper II**. We could not identify apoA4 using LC-ESI-MS/MS, but we did find a positive identification of apoA4 in one spot on the 2D-gels. However, it was mixed with several other proteins, making it impossible to quantify, and sequence analyses suggested that it was only a fragment.

Of the exchangeable apolipoproteins, apoE and apoA1 were the most abundant, followed by apoM, apoJ, apoC3, and apoC2 (**Paper II**, **Table 2**). The amount of apoD, as apoL1, was probably below the detection limit of the 2-DE-quantification. Even though all apolipoproteins presented in **Paper II** were present in all analyzed LDL, the intensity of some of the apolipoproteins on the 2-D-gels varied with a factor of several thousand. This illustrates the heterogeneity of the LDL lipoprotein class, with wide ranges of the contents of different exchangeable apolipoproteins.

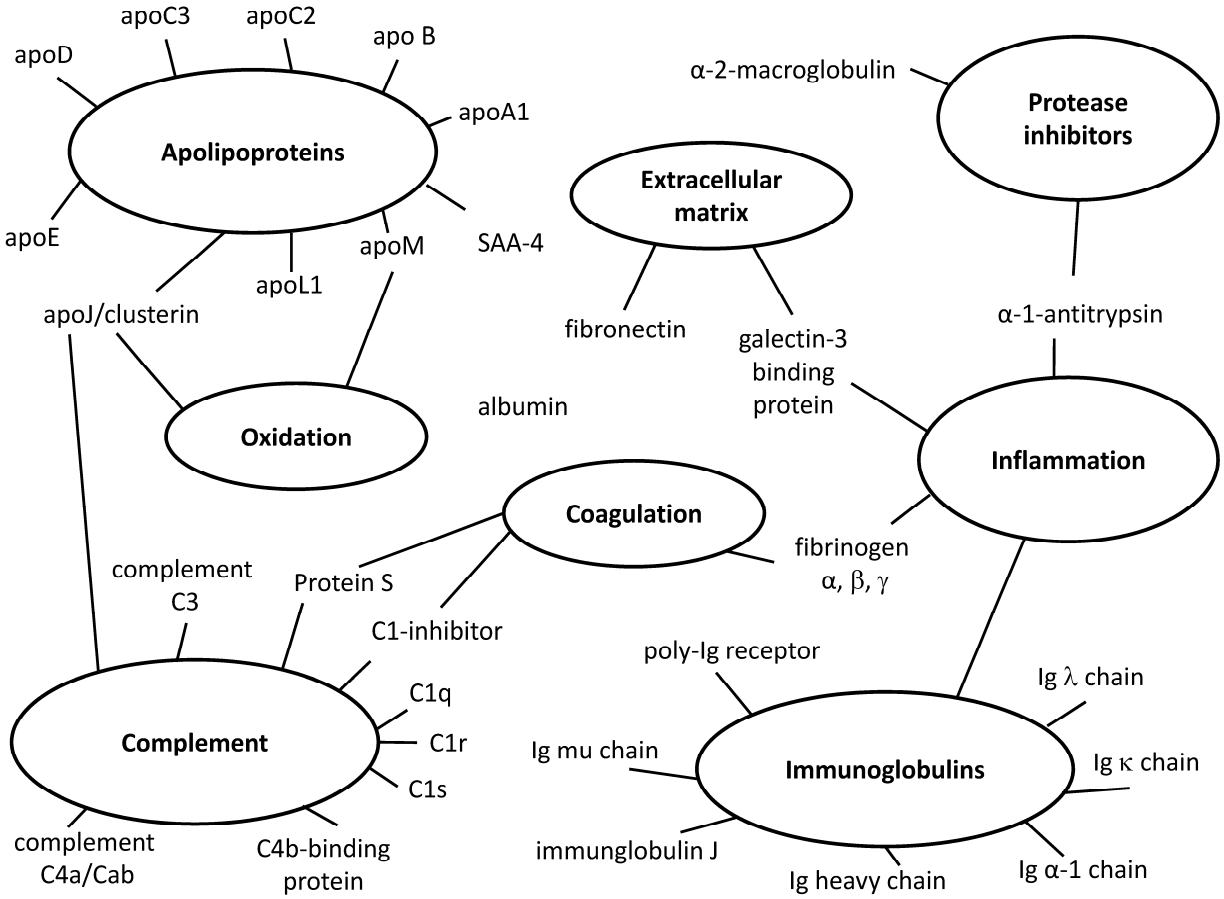

Figure 7. LDL-associated proteins revealed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) in LDL isolated using size exclusion chromatography. All proteins presented here were identified in five different LDL-preparations, isolated from five individuals with varying lipid status, BMI, and WHR.

5.3.2. LDL-associated inflammation-regulating proteins

The second largest group of the LDL-associated proteins identified by LC-ESI-MS/MS was, somewhat unexpected, proteins related to the complement system (**Paper II**, **Table 1** and *Figure 7*). Several complement proteins were recently identified in HDL using LC-ESI-MS/MS [109], but to our knowledge this is the first study describing complement proteins in LDL. One of the identified complement proteins, C1q, has previously been shown to bind to LDL *in vitro* [173]. The complement proteins C1q, C3, C4, C1-inhibitor, C4b-binding protein, protein S, and clusterin have previously been found to be activated and/or present in human

atherosclerotic lesions, often at higher levels than in normal artery wall [174-179]. There are several possible activators of complement in atherosclerotic plaques, such as enzymatically modified LDL, CRP, immunoglobulins, cholesterol crystals, and apoptotic cells [180]. An accumulation and subsequent activation of complement could promote an inflammatory reaction in the arterial wall, possibly promoting atherosclerosis. The results of this study suggest that LDL might act as a vehicle for complement proteins into the arterial wall.

The most abundant of the non-classical apolipoproteins in LDL identified in **Paper II** (after excluding the highly abundant fibrinogen, haptoglobin and immunoglobulins; see *Methodological considerations*) was the serine protease inhibitor α-1-antitrypsin (A1AT), which has previously been identified in LDL [43, 181]. A1AT elicits inflammatory responses and lipid accumulation in macrophages *in vitro* [182] and A1AT, alone and in complex with LDL, has been found in atherosclerotic lesions [181, 182]. In addition to A1AT, several proteins able to affect immune functions, such as fibrinogen, immunoglobulins, galectin-3 binding protein, and serum amyloid A 4, were identified as LDL-associated (*Figure 7*). These results suggest that LDL might be a pro-inflammatory lipoprotein, carrying several proteins that may be able to promote a proinflammatory response after retention of LDL in the intima.

5.3.3. LDL-associated proteins in type 2 diabetes

To increase the understanding of the increased atherogenicity seen for LDL in T2D and MetS, we applied both the qualitative and the quantitative approach to LDL from the two study groups in **Paper II**. Our data suggest a different protein composition of LDL isolated from individuals with T2D and MetS compared with LDL isolated from healthy control individuals. Eight proteins were found to be present only in T2D-MetS-LDL and not in control LDL using LC-ESI-MS/MS, all of them able to affect oxidation and/or inflammation, and three of them possibly affecting complement. These eight proteins will be discussed in the following three paragraphs.

Carboxypeptidase N inhibits the proinflammatory anaphylatoxins C3a and C5a in the complement cascade, thus has an anti-inflammatory function. **PAI-1** has two functions that affect complement; it inhibits the formation of plasmin, which apart from degrading fibrin also can cleave and activate C3, and it also binds vitronectin, which is a late

downregulator of the complement cascade. Elevated plasma levels of PAI-1 are associated with CVD, insulin resistance, and hyperinsulinemia, which may explain the association of PAI-1 only with T2D-MetS-LDL and not with control LDL. The third protein with complement binding properties was **histidine-rich glycoprotein**. Histidine-rich glycoprotein has been shown to be involved in angiogenesis, cell adhesion and migration, fibrinolysis and coagulation, immune complex clearance, and phagocytosis of apoptotic cells. Among a wide range of other ligands, it also binds several of the complement components, like C1q, C8, C9, factor D and S-protein [183, 184].

Paraoxonase 1 (PON1) is mostly associated with HDL, but it has also previously been shown to be associated with chylomicrons and VLDL [185]. PON-1 conveys part of the antioxidative effect of HDL, and in mouse models of atherosclerosis it is atheroprotective [186, 187]. **Orosmucoid 2**, or alpha-1-acid glycoprotein 2, is an acute phase protein which is regulated by e.g. IL-6 and is elevated upon inflammation. This might explain the exclusive distribution in T2D-MetS-LDL, as this group of individuals often have an elevated subclinical inflammation, seen as elevated levels of inflammatory markers such as IL-6 and CRP [115, 188, 189]. Orosomucoid 2 sorts under the lipocalin family [190], as apoD and apoM, providing a possible explanation for its LDL-association. **AMBP-protein**, also known as protein HC or α-1-microglobulin, is an immunoregulatory plasma protein. It forms complexes with other plasma proteins like monomeric IgA, fibronectin, and α -2-macroglobulin, and is also a member of the lipocalin family [191].

The last two proteins found exclusively in T2D-MetS-LDL using LC-ESI-MS/MS, were the innate host defense proteins **lactoferrin** and **lysozyme**. These proteins bind each other [192] and they share many features: they are present in granules of polymorphonuclear leukocytes, have antibacterial activities, and have been shown to reduce the production of reactive oxygen species and cytokines [136, 192, 193].

Lysozyme was identified as an LDL-associated protein for the first time in LDL from pooled sera using 2-DE and MALDI-TOF [44]. Out of all the proteins found exclusively in T2D-MetS-LDL using LC-ESI-MS/MS, lysozyme was the only one abundant enough to be identified also in the quantitative approach in **Paper II**. In the LC-ESI-MS/MS analyses, where three case and two control individuals were analyzed, lysozyme was not found in any of the control individuals, but in the 2-DE analyses, where 9 case individuals and 10 control

individuals were analyzed, lysozyme was present also in some control individuals. However, in the 2-DE quantification, lysozyme was found to be significantly increased in T2D-MetS-LDL compared with control LDL (*Figure 8*). The higher presence of lysozyme in LDL from individuals with T2D might be explained by the fact that lysozyme binds to AGE-moieties [136]. AGEs are formed on proteins and lipids during hyperglycemia, and both apoB and the minor phospholipid moieties of LDL (phosphatidylinositol and phosphatidylserine) can be glycated [132]. It was recently shown that small dense LDL is more easily glycated than larger subfractions of LDL [106], and the levels of AGE-LDL are elevated in individuals with diabetes compared with non-diabetic individuals [132, 135]. Thus, one might speculate that the increased lysozyme in LDL in T2D and MetS may be due to an increased AGE-modification of this LDL. The increased content of lysozyme in T2D-MetS-LDL may however also be due to a process not affected by AGE-modification, as this type of individuals also have elevated serum levels of lysozyme (**Paper III**, **Figure I**).

Also **apoJ** (basic isoform) was found to be increased in T2D-MetS-LDL (*Figure 8*). Its main function is still unclear, though it has been shown to be a negative regulator of the terminal membrane attack complex of the complement cascade [194, 195]. ApoJ has also been shown to have anti-oxidative [57] and atheroprotective [196] effects, possibly due to its lipid-binding properties. ApoJ has been suggested to be able to sequester oxidized lipids, e.g. from LDL, thereby decreasing adverse effects of these, such as migration and proliferation of vascular cells [57, 197, 198]. It has also been shown to mediate cholesterol efflux from foam cells [56]. ApoJ is upregulated in normal and stress-induced ageing [199], and serum levels of apoJ are elevated in T2D and in patients suffering from CHD [58]. ApoJ has previously been shown to be associated with both HDL [55, 200] and LDL [43, 44], and it accumulates in the arterial wall during the development of atherosclerosis [201-203]. Thus, LDL might be one possible transporter of apoJ into the subendothelium.

ApoA1 (basic isoform) was found to be decreased in T2DM-MetS-LDL (*Figure 8*). ApoA1 has traditionally been regarded as an apolipoprotein exclusive for HDL, but several proteomic studies, using different LDL-isolation methods, have identified apoA1 also in LDL [44, 103]. This could be due to an overlap in sizes between HDL and LDL, resulting in an HDL-contamination in isolated LDL. In **Paper II**, however, several of the typical and abundant HDL proteins, such as apoA2 and acute phase serum amyloid A were not identified in LDL, even using the highly sensitive LC-ESI-MS/MS analysis, which contradicts this

explanation. Also, the finding that apoA1 was decreased in T2D-MetS-LDL is in agreement with a previous study showing that small dense LDL from individuals with T2D and MetS contain less apoA1 than small dense LDL from healthy controls [103].

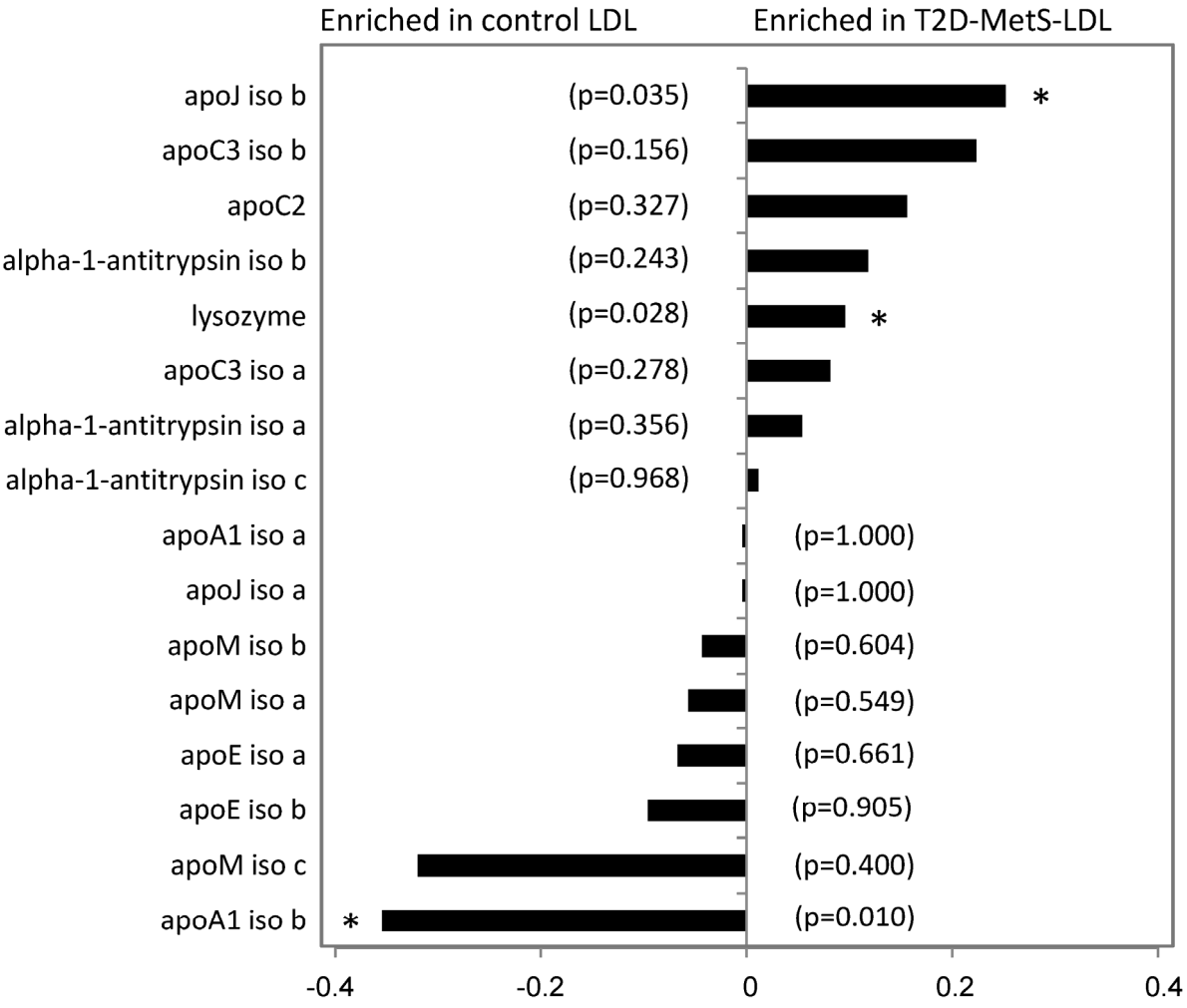

Figure 8. Quantitative differences in LDL-associated proteins between the T2D-MetS group and the control group. For each protein, the mean relative amount (%) in the control group was subtracted from the mean relative amount (%) in the T2D-MetS-group. Positive values indicate that a protein, or isoform, is enriched in T2D-MetS-LDL compared with control LDL and negative values indicate that a protein, or isoform, is enriched in control LDL.

To see if any of the quantified LDL-associated proteins were related to lipid alterations characteristic for small dense LDL, we performed correlation analyses between lipid- and

protein components of LDL. Combining the results from **Paper I** and **Paper II** [ii] we found that small, cholesterol-poor LDL contained increased amounts of apoC3, apoJ, and lysozyme. A very recent study, comparing the protein composition of LDL from obese individuals with combined dyslipidemia, including elevated serum triglycerides, low HDL and elevated LDL-cholesterol, with LDL from lean, healthy controls, provides an interesting basis for comparison of the results in **Paper II**. LDL from the obese, hypertriglyceridemic, but non-diabetic, individuals also contained less apoA1, more apoJ, and more apoC3, but similar amounts of lysozyme, compared with LDL from healthy, lean controls [204] (*H. Karlsson, personal communication*). The fact that the obese individuals in the mentioned study were hypertriglyceridemic suggests that they had small dense LDL, thus the results of this study supports our suggestion that small dense LDL is enriched with apoJ and apoC3 and depleted of apoA1. The fact that Karlsson *et al*. did not find an increased amount of lysozyme in LDL from the obese, hypertriglyceridemic, but not diabetic, group, supports our speculation that lysozyme might associate more with AGE-modified LDL.

5.4. Elevated lysozyme in type 2 diabetes

As mentioned above, lysozyme was identified in LDL for the first time not long ago [44]. An increased activity of lysozyme was recently reported in leukocytes isolated from patients with acute myocardial infarction compared with healthy controls [205]. In the same study, the authors found higher leukocyte lysozyme activity in diabetic individuals with acute CVD (myocardial infarction or acute angina) than in non-diabetic patients with the same conditions. In **Paper II** and **Paper III**, we extended those findings by showing that individuals with T2D also have increased serum levels (**Paper III**, **Figure 1**) and LDL content of lysozyme (**Paper II**, **Figure 3**).

The findings in **Paper II** and **Paper III**, that lysozyme is elevated in T2D and MetS, seems to contradict the finding that overexpression of lysozyme can reduce

[ii] In **Paper I**, an increased content of apoC3 in LDL correlated with several features of small dense LDL, including decreased cholesterol content and reduced LDL peak particle diameter (LDL ppd). The LDL content of lysozyme and apoJ correlated inversely with the cholesterol:PC ratio in **Paper II**. In **Paper I**, the cholesterol:PC ratio in LDL correlated with LDL ppd (LDL cholesterol:PC vs. LDL ppd, R_s=0.252, p=0.035, n=70). Thus, assuming the same relationships between LDL cholesterol:PC ratio and LDL ppd in **Paper I** and **Paper II**, apoC3 and apoJ, and lysozyme were all elevated in small, cholesterol-poor LDL.

atherosclerosis development in apoE knock-out mice [136, 141]. The suggested atheroprotective mechanism of lysozyme is through scavenging of AGEs, leading to an increased AGE-clearance, and a decrease in both AGE-mediated and non-AGE-mediated systemic oxidative stress and subsequent vascular cell inflammatory response and proliferation [137, 140, 141].

Lysozyme is mainly cleared via the kidneys, and elevated serum lysozyme has been seen in conditions with impaired renal function [206, 207]. In agreement with previous studies, there was a correlation between lysozyme and serum creatinine in **Paper III** (**Table 2**). When creatinine clearance was calculated using the Cockcroft-Gault formula [208] however, the correlation was reduced to a non-significant trend (data not shown). Nor were there any differences in either serum creatinine (**Paper III**, **Table 1**) or creatinine clearance (data not shown) between the groups, arguing that kidney function alone could not explain the difference in serum lysozyme between the groups in **Paper III**.

In **Paper III**, we performed correlation analyses between lysozyme and available markers of inflammation and markers of glycemic and metabolic control. Significant correlations were found between lysozyme and several of the features of T2D and the MetS, including small dense LDL as discussed above. Of these features, insulin showed the strongest correlation with lysozyme (**Paper III**, **Table 2**). The relationship between insulin and lysozyme could also be seen as a difference in lysozyme levels between insulin-treated and non insulin-treated individuals. The insulin treated group had lower levels of c-peptide, suggesting that circulating insulin levels, regardless of exogenous or endogenous origin are linked to lysozyme levels. The direct effect of insulin on lysozyme production or release will have to be further addressed.

The results from **Paper III** suggest that the situation in humans might be more complex than in mice. This raises the question whether merely an increase in serum concentration of lysozyme is sufficient for the proposed atheroprotective effect, also in human T2D. Further studies on mechanisms of lysozyme in atherosclerosis and its possible regulators are warranted.

The rapidly increasing prevalences of obesity and T2D, conditions which bring about increased risk of developing CVD, presently presents a major challenge for the medical field. To be able to improve treatment and prevention of CVD, a more detailed understanding of the atherosclerosis process, especially in T2D and the associated MetS dyslipoproteinemia, is of great importance. The focus of this thesis was to investigate previously unknown properties of the atherogenic lipoproteins, in particular LDL, from individuals with T2D and MetS that may be important for atherosclerosis development. The results show several ways in which VLDL (**Paper I**) and LDL (**Paper I** and **Paper II**) from individuals with T2D and MetS are different from VLDL and LDL from healthy individuals that may be important for atherosclerosis.

An increased $sPLA_2$-V-mediated lipolysis of VLDL and LDL in individuals with T2D and MetS (**Paper I**) may cause increased LDL retention [155-157] and lead to high local concentrations of proinflammatory NEFA and lysoPC in the arterial intima, which might lead to an increased vascular inflammation and promote atherosclerosis [65, 158, 160]. The reason for the increased susceptibility of T2D-MetS-lipoproteins to $sPLA_2$-V-mediated lipolysis will have to be further addressed using *in vitro* set-ups where the individual effects of the lipoprotein properties proposed (**paper I**) to influence $sPLA_2$-V-lipolysis can be studied.

It has been suggested that LDL subpopulations containing different exchangeable apolipoproteins confer different atherogenic risk and have different metabolic properties [209-211], such as affinity for clearance receptors [46, 51], capacity to bind to PGs [49], and susceptibility to lipolysis [48]. In **Paper II**, we describe 31 LDL-associated proteins, many of them with functions in lipid metabolism, but also several with functions important for complement, inflammation, oxidation, and coagulation. In contrast to HDL, the role of which has been extended from mere lipid transporting to also include anti-oxidative and anti-inflammatory properties [109, 110], LDL seems to be a pro-inflammatory lipoprotein, carrying several proteins with the potential to affect the inflammatory events subsequent to intimal LDL retention. The results suggest that LDL is a very heterogeneous lipoprotein class, displaying wide ranges of LDL-associated lipoproteins, possibly providing LDL with a wide variety of functions.

LDL from individuals with T2D and MetS was associated with an increased amount of apoC3, apoJ, and lysozyme, and a decreased amount of apoA1 (**Paper II**). In addition, some minor protein components, with functions affecting complement,

inflammation, oxidation, and coagulation, were only found in T2D-MetS-LDL (**Paper II**). These results suggest an altered LDL-associated protein profile for LDL in T2D and MetS, which will have to be confirmed using other LDL-isolation methods and more directed quantification methods. These results should also serve as a basis for studies on the effects of these protein alterations on e.g. vascular cell response to LDL.

The finding that lysozyme, which has been suggested to be atheroprotective in mice, is elevated in LDL and serum from individuals with T2D and MetS, and also linked to insulin levels (**Paper III**), calls for further studies on the regulation and effect of lysozyme on atherosclerosis in humans.

An increased understanding of functional effects of alterations in diabetic lipoproteins may serve as a basis for the development of new treatment strategies for atherosclerosis in T2D and MetS. Also the protein composition of LDL from other groups at high risk of CVD should be examined, with the aim to find general candidate proteins of possible importance for atherosclerosis development.

ACKNOWLEDGEMENTS

Jag vill tacka alla som på något sätt är delaktiga i att denna avhandling blev skriven och börjar med min första riktiga labb-vistelse, nämligen på Klinisk Kemi på SU:

Tack **Anders Elmgren** och **Göran Larsson**, för att ni väckte mitt intresse för medicinsk forskning redan under mitt första sommarlov på molekylärbiologiutbildningen.
Tack till **Marcus Krantz**, min handledare på min första praktiska tillämpningskurs, i mikrobiologi på Lundberglab, som hade överseende med att jag satte eld på mig själv och att jag hällde ut radioaktivitet och ändå övertygade mig om att jag skulle söka till forskarskolan.
Tack **Håkan Billig** och biomedicinska forskarskolan, **GGSB 2000**, som lät mig pröva på olika labb på Medicinska fakulteten, senare Sahlgrenska Akademin. Och tack för ryggsäcken!
Tack till **Henrik Semb,** och **Joakim Håkansson** min chef och min handledare på exjobbet, och hela resten av gamla **Medkem**, inklusive **Ölklubben**, för ett grymt kul halvår!
Tack också till **Levent Akyürek**, som pekade mig i riktning mot Wallenberglaboratoriet.

Och då är vi framme vid nutid.

Tack **Olov Wiklund**, min handledare och vägledare, för att du har litat på mig och låtit mig prova så många olika projekt - jag har lärt mig otroligt mycket under vägen - och för att du hämtar in mig när jag svävar ut för långt. Tack för all kunskap om den kliniska biten, **Olle** och **Linda Fogelstrand**, min biträdande handledare och mentor - din entusiasm var ett stort skäl till varför jag fastnade för Wlab och den har hjälpt mig upp många gånger under åren. Tack för oändliga möten, på alla möjliga ställen - inte minst på msn. Du får mig att vilja bli bättre.
Jan Borén, du har ibland varit som en extra handledare för mig under åren. Tack för alla givande råd, tips och diskussioner och för intresset för LDL.
Tack **Björn Fagerberg**, för allt studiematerial, för många bra inlägg i diskussionen runt projekten och för att du också tycker att det är intressant att göra korrelationsanalyser.
Och tack till er alla för chansen att få kombinera min molekylärbiologi med kliniska studier.

Tack till alla på Wlab som har hjälpt till i mina projekt under åren och särskilt tack till:
Kicki Norén, **Kristina Skålén** och **Elin Björk**, för er hjälp med alla miljontals utkörningar av LDL i CHARLI-projektet.
Thomas Larsson, tack för all hjälp med masspektrometrin, databehandlingen och funderingar runt resultaten i CHARLI-projektet.

ACKNOWLEDGEMENTS

Marcus Ståhlman och **Martin Adiels**, tack för delat intresse för lipoproteinkomposition.
Karin Ekström, **Caroline Améen**, **Eva Brunhage** och **Sara Ståhlman** för all hjälp med sPLA2-V-projektets optimeringskrångligheter. **Caroline**, jag saknar vårt Poplabb – låt oss återuppliva det nån gång. **Sara**, jag saknar dig på labb … knäppt, men jättekul och helt som vi tänkte en gång, att vi skulle jobba ihop till slut.
Lillemor Mattson Hultén och **Christina Ullström**, samt tidigare **Anita Lund** och **Aira Lidell** – tack för fantastisk service och undervisning på Lab 16.
Fysiologgruppen, tack för allt jobb ni gör och har gjort med patienterna i AIR och DIWA och särskilt till **Marie-Louise Ekholm**, som alltid hittar plats i schemat för mina plötsliga infall.
Tack till **alla som har donerat blod** till mina studier och därmed fått stå över frukost – ni vet vilka ni är. Och till de som ställde upp på att sticka: **Bente Grüner Sveälv**, **Ulrica Prahl Abrahamsson**, **Kristina Skålén**, **Mikael Rutberg**, **Annie Janssen**, **Margareta Scharin Täng**, **Josefin Kjelldal**.

Jag vill också tacka alla samarbetspartners utanför Wlab:
Eva Hurt-Camejo och **Birgitta Rosengren**, för ett mycket roligt projekt om $sPLA_2$-V.
Lena Douhan Håkansson, tack för lysozym-intresset och bra input om lysozym och LDL.
Helen Karlsson och **Mats Lindahl** för inspirerande intresse för LDL-associerade proteiner och hjälp i CHARLI-projektet – **Helen**, tack för en underbar helg hos dig på landet. Jag har aldrig antecknat så mycket under en disputation, tidigare eller senare.
Anna Bergstrand och **Emil Gustafsson**, tack för att ni gjorde ett gott försök med att styra upp bindningsassay:erna – tyvärr(?) verkar LDL-retention vara en mycket långsam process…

Tack också till alla som har fått allt annat att flyta på Wlab:
Agneta Ladström, för att du har ett oändligt tålamod med ekonomi-ignoranta doktorander.
Ewa Landegren, för att du agerar extramamma åt hela vår grupp och för att du alltid ser till att vi har kul.
Rosie Perkins, för excellent editorial assistance, delat språkintresse och alltid snabba svar.
Mujtaba Siddiqui och sedemera **Magnus Gustafsson**, för pålitlig markservice för förvirrade forskare.
Merja Meuronen Österholm, **Claudia Abid** och **Christina Pettersen** för att ni tar hand om oss på Wlab.

ACKNOWLEDGEMENTS

Tack till resten av festkommittén, särskilt **Ulrica Prahl Abrahamsson**, för ett gäng grymma fester under åren.

Hanna Åberg, **Cissi Malmberg** och **Eleonor Brandin**, för kul sällskap den senaste tiden.

Stort tack till **alla andra på Wlab**, för en massa kul och för att ni utgör en bra plattform för forskningssamarbeten, med en väl utvald sammansättning av briljanta och roliga människor.

Tack också till **alla mina vänner** (de som fortfarande finns kvar efter en mycket ovanligt osocial höst och en nästan helt frånvarande vinter), för att ni förstår och i perioder av mycket jobb låter mig vara den sura milla som inte svarar på sms och inte kan planera mer än max en timme framåt. Särskilt mycket tack till några som hjälpt mig extra mycket just nu:

Calle Jacobson, min nattetid alltid tillgängliga vän på c-lab, som ger mig kaffe och låter mig avreagera mig, utan att klaga – du har fått tåla mycket.

Tack till **Stina Caesar**, som försökte ta ner min stress med mantrat: ”The book that nobody reads”. Jag hoppas att du har fel :)

Tack **Calle**, **Stina**, **Tor Skoglund** och **Mattias Kettil**, för korrekturläsning av boken (även om din kommentar **Tor**: ”Du måste skriva om ditt aim”, klockan 23.14 dagen innan inlämning till tryck, var lite hårdsmält...)

Tack **Erik Gustafsson**, för mat, massage och kärlek. Jag vill inte ens tänka på hur jobbig den här vintern kunde ha varit utan dig. Med dig har den varit alldeles, alldeles underbar…

Och till sist, ett jättestort tack till min familj, **Andreas**, **mamma** och **pappa** – för allt. Allt allt allt. Men just nu för en underbart lugn jul, för badkar, hämtning, väckningssamtal, uppmuntran, omtanke och alltid kravlös kärlek.

Nu är boken färdig och jag är snart doktor. Ett stort steg för.. och så vidare. Tadaaa…!

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