Studies of Atherogenic Lipoproteins Using Mass Spectrometry–Based Lipidomics

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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).

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Cover illustration: Mass spectrum of purified VLDL-triacylglycerols

ABSTRACT

The prevalence of type 2 diabetes is increasing worldwide and is about to reach epidemic proportions. The disease is often associated with dyslipidemia, which is characterized by an atherogenic lipoprotein profile including elevated serum triacylglycerol levels, low high-density lipoprotein levels and high levels of small low-density lipoproteins. Several large clinical studies have shown that this change in serum lipoprotein profile constitutes a major cardiovascular risk factor, but the molecular mechanisms are still not completely understood. It has been proposed that intrinsic properties of the particles, such as protein and lipid composition, might be responsible for the increased risk, mediated through an increased accumulation of lipoproteins in the artery wall, leading to atherosclerosis. The primary aim of this thesis was to isolate and, with a lipidomics approach, characterize the atherogenic lipoproteins from patients with type 2 diabetes. A secondary aim was to link compositional changes to atherosclerotic processes *in vitro*.

Initially, an ultracentrifugational method was developed for the isolation of lipoproteins at physiological settings. Then, in order to make a comprehensive lipid characterization of the lipoproteins, an analytical platform for lipidomics analyses was established. This platform consists of a normal-phase HPLC system with an evaporative light scattering detector that is used in combination with a hybrid quadrupole time-of-flight instrument, equipped with a chip-based nanoelectrospray interface.

By using this platform, atherogenic lipoproteins from patients participating in two major studies were characterized. The results revealed several alterations in the lipid and protein composition of the atherogenic lipoproteins isolated from patients with type 2 diabetes. Several of these alterations could, by the use of different *in vitro* systems, be linked mechanistically to proatherogenic processes such as lipoprotein retention and tissue inflammation. We also showed that changes in lipoprotein lipid composition were mainly associated with dyslipidemia.

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

I. **Proteomics and lipids of lipoproteins isolated at low salt concentrations in D_2O/sucrose or in KBr**

Ståhlman M, Davidsson P, Kanmert I, Rosengren B, Borén J, Fagerberg B and Camejo G.

Journal of Lipid Research; 2008; 49(2): 481-490

II. **ApoCIII-enriched LDL in type 2 diabetes displays altered lipid composition, increased susceptibility for sphingomyelinase, and increased binding to biglycan**

Hiukka A*, Ståhlman M*****, Pettersson C, Levin M, Adiels M, Teneberg S, Leinonen ES, Mattson Hultén L, Wiklund O, Orešič M, Olofsson SO, Taskinen MR, Ekroos K and Borén J.

Diabetes 2009; 58(9): 2018-2026

*These authors contributed equally

III. **Lipidomics of apoB-containing lipoproteins reveal that dyslipidemia is associated with alterations in molecular lipids leading to increased proinflammatory properties**

Ståhlman M**,** Pham H, Adiels M, Mitchell TW, Blanksby SJ, Fagerberg B, Ekroos K and Borén J.

Manuscript

ABBREVIATIONS

Apo	apolipoprotein
ASCVD	atherosclerotic cardiovascular disease
BMI	body mass index
CE	cholesteryl ester
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
CID	collision induced dissociation
CM	chylomicron
CVD	cardiovascular disease
DAG	diacylglycerol
DIWA	diabetes in women and atherosclerosis
DNL	*de novo* lipogenesis
EGIR	European group for study of insulin resistance
EM	electron multiplier
eNOS	endothelial nitric oxide synthase
ESI	electrospray ionization
FC	free cholesterol
FDB	familial defective apoB100
FID	flame ionization detector
FIELD	fenofibrate intervention and event lowering in diabetes
FTICR	fourier transform ion cyclotron resonance
GC	gas chromatography
HAEC	human aortic endothelial cells
HDL	high density lipoprotein
HL	hepatic lipase
HPLC	high performance liquid chromatography
IDF	international diabetes federation
IDL	intermediate density lipoprotein
IGT	impaired glucose tolerance
KEGG	Kyoto encyclopedia of genes and genomes
LCAT	lecithin-cholesteryl acyl transferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
MALDI	matrix-assisted laser desorption ionization
MCP	multichannel plate

MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	methyl-*tert*-butyl ether
NAFLD	nonalcoholic fatty liver disease
NCEP:ATPIII	national cholesterol education program's adult treatment panel III
NHANES	national health and nutrition examination survey
OzID	ozone induced dissociation
PC	phosphatidylcholine
PCA	principal component analysis
PE	phosphatidylethanolamine
PG	proteoglycans or phosphatidylglycerol
PI	phosphatidylinositol
PIS	precursor ion scanning
PKC	protein kinase C
PL	phospholipids
PLA_2	phospholipase A_2
PS	phosphatidylserine
QqQ	triple quadrupole
QqTOF	quadrupole time-of-flight
RCT	reversed cholesterol transport
sdLDL	small dense LDL
SIMS	secondary ion mass spectrometry
SMase	sphingomyelinase
SRM	selected reaction monitoring
T2D	type 2 diabetes
TAG	triacylglycerol
TOF	time-of-flight
UPLC	ultra performance liquid chromatography
VACAM-1	vascular cell adhesion molecule 1
WHO	world health organization

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“If we knew what it was we were doing,

it would not be called research, would it?”

Albert Einstein

BIOLOGICAL BACKGROUND

Cardiovascular disease

Cardiovascular disease (CVD) is a group of disorders of the heart and blood vessels and includes coronary heart disease (CHD) (e.g. myocardial infarction and angina pectoris), cerebrovascular disease (e.g. stroke) and peripheral artery disease. CVD is the number one killer in the world, representing 29% of all global deaths [1]. The most important behavioral risk factors, responsible for about 80% of the coronary heart disease and cerebrovascular disease, are unhealthy diets, inactivity and tobacco use [1]. The effects of unhealthy diets and inactivity are manifested as obesity and type 2 diabetes (T2D), which are also strong predictors for cardiovascular disease. These will be discussed below.

Obesity

"Let me have men around me that are fat…"

In Shakespeare's *Julius Caesar*, the Roman emperor suggests that a high body mass correlates with a well-balanced mental disposition. In those days, the prevalence of obesity was low and the state not considered a medical risk factor. Today obesity is a growing problem about to reach epidemic proportions [2]. In a recently published report, WHO estimates that about 400 million adults (+15 years) are obese (BMI≥30) with an additional 1.6 billion people being overweight (25<BMI>30) [3]. The increased prevalence of obesity and overweight, which to a great extent can be explained by the change to a more sedentary lifestyle and the constant access to palatable, energy-dense foods, is associated with several negative consequences. Not only do obese subjects have an elevated risk of mental illness such as depression and anxiety [4], they also have an increased risk of premature death [5-7]. Much of this increased risk can be explained by the wide range of comorbidities associated with obesity including dyslipidemia, T2D, hypertension and stroke [8].

Type 2 diabetes and insulin resistance

In parallel to the rise in obesity, the prevalence of T2D is increasing alarmingly. According to estimations by the international diabetes federation (IDF), 285 million people are suffering from the disease, which is thought to increase to almost 440 million by the year 2030. This increase will affect all global regions, with projected increases ranging from 21% in Europe to 111% in Africa [9].

Diabetes is a chronic disease that can be divided into two subclasses where type 1 diabetes is primarily due to autoimmune mediated destruction of the pancreatic β-cells, resulting in insulin deficiency. T2D, which constitutes about 90% of all cases [10], develops over several years and is initiated by an inability of the peripheral tissue to pick up glucose from the circulation as a response to the insulin signal (insulin resistance). This leads to a compensatory increased production of insulin by the pancreatic β-cells and increased levels in the circulation. This results in normal, or near-normal, plasma glucose levels, which can disguise the pathological condition for years. The period, between the appearance of insulin resistance and the onset of type 2 diabetes, is today often termed pre-diabetes and is characterized by an impaired glucose tolerance (IGT). IGT, which is defined as hyperglycemia (with values intermediate between normal and diabetes) following a glucose load, is in itself associated with cardiovascular risk and it is estimated that 40% of these subjects will develop T2D over 5-10 years [10]. The state of diabetes occurs, often as a result of β-cells dysfunction, when the pancreas can't produce sufficient levels of insulin to maintain homeostasis. This will lead to a reduced glucose clearance by peripheral tissue and increased glucose production by the liver, causing the blood levels to rise [11].

The medical and socioeconomic burden of T2D, which is caused by the associated complications, imposes enormous strains on health-care systems. The complications are both macro- and microvascular diseases as a consequence of accelerated atherogenesis. T2D is the fifth leading cause of death in the US [12] and cardiovascular morbidity in patients with T2D is two to four times higher than in non-diabetic patients [10].

Metabolic Syndrome

The metabolic syndrome, also known as "syndrome X" [13], "the insulin resistance syndrome" [14] and the "deadly quartet" [15], is a clustering of factors associated with an increased risk of CVD and T2D [16-21]. The development of the metabolic syndrome is driven by obesity and/or insulin resistance and results in atherogenic dyslipidemia, elevated blood pressure, elevated plasma glucose, a

pro-thrombotic and a pro-inflammatory state, which are all considered core risk factors (figure 1). The rational for using the metabolic syndrome as a tool in practice is that the accumulated risk for a person displaying several risk factors is higher than that of its individual components [17]. However, questions have been raised to the clinical utility of putting efforts into establishing diagnostic criteria for the metabolic syndrome [22-23].

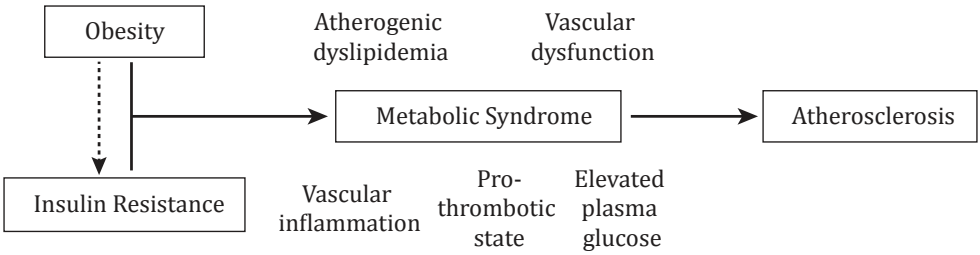


Figure 1. Obesity and T2D are the driving forces behind the development of the metabolic syndrome, which is associated with several risk factors leading to atherosclerosis and premature death.

Several definitions of the metabolic syndrome are available. The most commonly used are from WHO [24], the European Group for Study of Insulin Resistance (EGIR)[25] and the National Cholesterol Education Program's Adult Treatment Panel III (NCEP:ATPIII) [26]. To be classified as having the metabolic syndrome according to the WHO criteria, which is the definition mostly used in Sweden, the patient must have either T2D, impaired glucose tolerance (IGT) or insulin resistance and also two (or more) additional risk factors (figure 2). In the US, the definition from the NCEP:ATPIII is often used. This definition is similar to the one proposed by WHO but doesn't require the presence of T2D, insulin resistance or IGT, thereby making it less "glucose-centric".

Despite attempts in recent years to reach an agreement on the definition of the metabolic syndrome, it remains difficult to compare the prevalence published for different populations. However, a highly consistent finding is that the prevalence is strongly age-dependant [27]. It has been shown from studies performed in several countries, involving different ethnicities, that the prevalence is usually lower than 10% for both men and women below 30 years of age [28-30]. However, for people in the 60-69 age group, the prevalence might be as high as 67% [30].

In a study performed in Gothenburg Sweden, the prevalence of the metabolic syndrome in 778 elderly (70-year-old) individuals were estimated to be 26% for men and 19% for women using the NCTP:ATPIII definition [31]. Another study performed in the same region, with a cohort consisting of 104 middle aged men, showed similar prevalence with, depending on the definition used, 22% or 28% of the individuals having the metabolic syndrome [32].

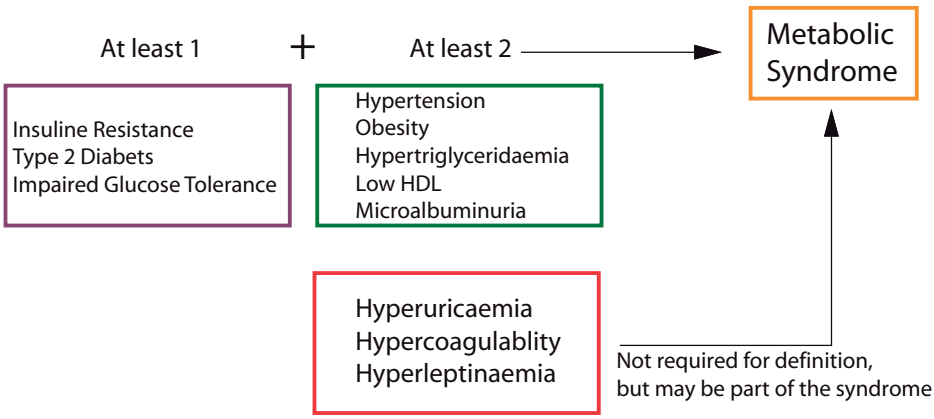


Figure 2. Inclusion criteria for defining the metabolic syndrome according to the WHO [24]. Adapted from Zimmet et al.[10]

Atherosclerosis

Atherosclerosis is a consequence of the metabolic syndrome and the underlying cause of most cardiovascular disease including coronary artery disease, ischemic gangrene, aortic aneurysms and many cases of heart failure and stroke. It has been estimated that atherosclerotic cardiovascular disease (ASCVD) (CVD caused by atherosclerosis) is responsible for 50% all deaths in Europe [33-34]. Parts of the very complex pathogenesis of atherosclerosis will be described in the following sections.

The atherosclerotic plaque

Atherosclerotic lesions, termed atheromas or atherosclerotic plaques, are typically present as asymmetric focal thickenings of the inner-most layer of the artery, the intima. The atheroma is preceded chronologically by so-called fatty streaks. These can be present already in early life, even in the arteries of small children [35-36]. Atherosclerosis is a silent and slowly progressing disease and the transformation of the fatty streaks into advanced atheromas often takes many years [37]. The advanced atheroma is structurally more complex than the fatty streak and has a core rich in macrophage foam cells, dead cells, and extracellular lipid droplets. The core is often surrounded by a cap of smooth muscle cells and collagen-rich matrix [34]. During the progression of atherosclerosis, inflammation plays a major role with inflammatory cells such as T-cells, dendritic cells and mast cell present during all stages of atherogenesis [38].

As the atheroma continues to grow, the narrowing of the arteries will eventually lead to a clinical event. Partial occlusion of an artery leads to ischemia of the supported organ and might lead to angina pectoris (heart) or intermittent claudication of the legs. Furthermore, some plaques that are highly inflamed and have a thin fibrous cap might rupture. This might cause total occlusion of the blood flow leading to myocardial infarction or stroke [39-40].

Lipoproteins

Lipoproteins are spherical protein-coated lipid particles that play important roles in both the initiation and progression of atherosclerosis. Their main function is to enable the transport of hydrophobic lipids, such as cholesterol esters and triglycerides (and also vitamins), throughout the circulatory system. The hydrophobic lipids are kept in the centre of the particle and are surrounded by a monolayer of amphipathic phospholipids with their hydrophobic regions (fatty acids) turned inwards and their polar head groups facing the aqueous environment (figure 3). The composition and size of the lipoprotein particle can vary and the lipoproteins are often classified into subgroups depending on their density (table 1), which will be determined by the lipid/protein ratio. Since the size of the particle is correlated to the amount of lipid, this means that larger particles will have a higher lipid/protein ratio and thus lower density. Chylomicrons (CMs) are large triacylglycerol-rich particles secreted into the intestine and function as carriers of dietary lipids. In the circulation, the metabolism of chylomicrons occurs mainly through the action of lipoprotein lipase (LPL) (figure 4). This enzyme hydrolyzes the triacylglycerols into fatty acids and glycerol and produces smaller chylomicron remnants, which eventually

Table 1. Characteristics of different lipoprotein classes.

	CM	VLDL	LDL	HDL
Density (g/ml)	<0.95	0.95-1.019	1.019 -1.063	1.063-1.210
Diameter (nm)	80-100	25-80	20-25	8-13
Total lipid (wt %)	98-99	90-92	75-80	40-48
Lipid composition ***(wt % of total lipids)***				
CE	2-4	15-23	47-51	24-45
TAG	81-89	50-58	7-11	6-7
FC	1-3	4-9	10-12	6-8
PL	7-9	19-21	28-30	42-51

CE, cholesteryl esters; TAG, triacylglycerols; PL, phospholipids; FC, free cholesterol. Adapted from [41] and [42]. In the table above, IDL is included in the VLDL class.

will be removed from the circulation by the liver. Very-low density lipoprotein (VLDL) consists of large triacylglycerol-rich particles secreted by the liver. As VLDL is metabolized in the circulation, mainly through the action of LPL and hepatic lipase (HL), the particles are gradually deprived of their lipid constituents and reduced in size. This results in the generation of intermediate-density lipoprotein (IDL) and then to even smaller low-density lipoprotein (LDL). This process of delivery of lipids to peripheral tissue is often referred to as "forward lipid transport".

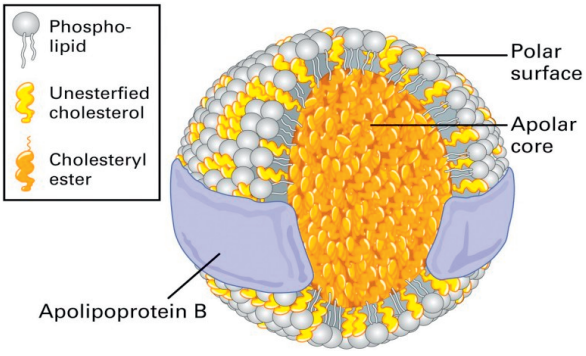


Figure 3. Structure of a lipoprotein particle with a core of hydrophobic lipids surrounded by an outer monolayer of amphipathic lipids, with their hydrophobic tails turned inward and the polar heads outward to the aqueous environment.

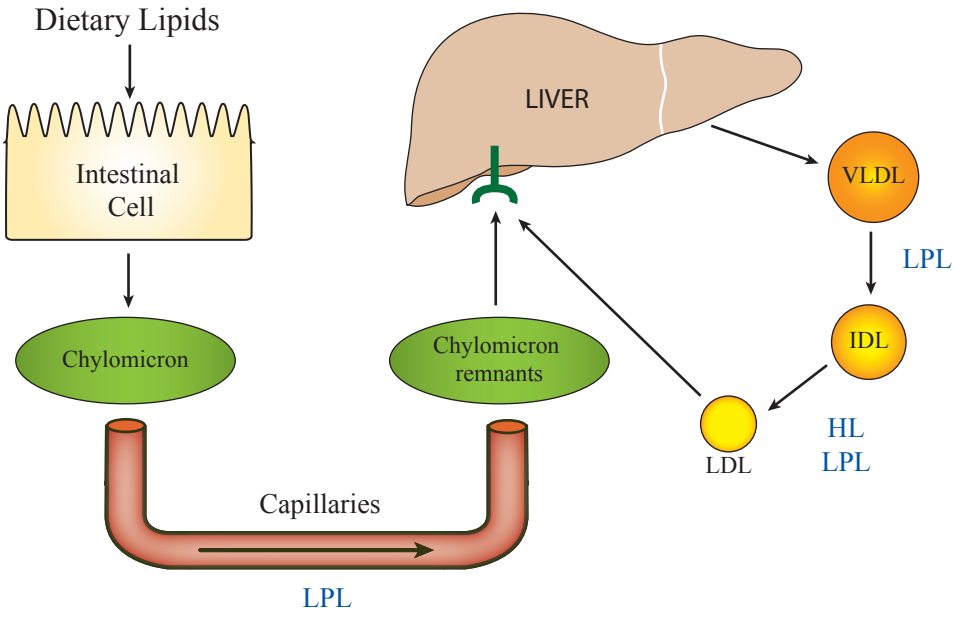


Figure 4. Both chylomicrons and VLDL are metabolized by lipases in the circulation resulting in smaller lipid deprived particles that are removed from the circulation by the liver through receptor-, and non-receptor-mediated uptake.

High density lipoprotein (HDL) is produced by the liver, but can also be formed from other sources such as the intestine [43] and macrophages [44]. It might also be formed in the circulation as a remnant particle during the lipolysis of other lipoproteins [45]. HDL is considered beneficial and atheroprotective because of its ability to remove cholesterol from peripheral tissue and return it to the liver, a process known as reversed cholesterol transport (RCT). Although RCT is considered to be the most important property of HDL in terms of its atheroprotective capacity, HDL is also associated with anti-inflammatory [46-47], anti-oxidative [48-49] and anti-apoptotic [50-52] properties, which are all thought to be contributing to its beneficial effects.

Apolipoproteins

On the surface of the lipoprotein particles a number of different proteins are attached. These are called apolipoproteins (apos) and are classified into different groups such as apoAs, apoB and apoCs. The size of the apolipoproteins varies between ~512 kDa for the apoB molecule down to less than 10 kDa for the

smaller apoCs. Apolipoproteins play several important roles in the metabolism of lipoproteins as they are crucial for the interaction of the lipoprotein particles with enzymes, transfer proteins and cell surface receptors.

Apolipoprotein B (apoB) comes in two variants. ApoB100, which is one of the largest human proteins, consisting of 4536 amino acids, is the main protein on the VLDL particle. A truncated version consisting of the amino-terminal end of the apoB100 molecule is secreted attached to the chylomicron particle in the intestine. This molecule is called apoB48 since the size of the protein is 48% of the apoB100 molecule. The apoB molecule, which always exists in one copy per particle, is a non-exchanging lipoprotein. This means that it will stay attached to the lipoprotein during its time in the circulation. The apoB molecule is important for the uptake and removal of lipoproteins via the LDL-receptor. If the sequence involved in the receptor recognition is altered, as is the case in familial defective apoB100 (FDB), this will result in a reduced clearance of these particles, with hypercholesterolemia and premature atherosclerosis as a consequence [53].

Apolipoprotein E was first described as a lipoprotein constituent of VLDL [54] but is now recognized as a major component of several classes of plasma lipoproteins [55-57]. The majority of apoE found in plasma is produced by the liver, with 20-40% being derived from extra-hepatic sources such as brain, muscle and also macrophages [58-59]. ApoE plays important roles in the circulation by facilitating the uptake and degradation of lipoproteins through its interaction with the LDL-receptor [60-61].

Apolipoprotein CIII is a small protein (8.8 kDa) produced mainly by the liver and to a lesser extent by the intestine [62]. It is present in three isoforms that are termed apo$CIII_0$, apo$CIII_1$ and apo$CIII_2$ depending on the number of sialic acid molecules (0-2) terminating the oligosaccharidic portion of the protein [63]. ApoCIII has been shown to inhibit lipolysis of lipoproteins by LPL and also delay the removal of apoB-containing lipoproteins by the hepatic LDL-receptor [64-65]. Both of these mechanisms leads to hypertriglyceridemia and the role of apoCIII in the development of dyslipidemia and atherosclerosis will be discussed separately.

Apolipoprotein AI (apoAI) is the most abundant protein on HDL. ApoAI is a 28 kDa protein that can be present in several copies on the HDL surface. ApoAI is an exchangeable apolipoprotein. This means that it is more loosely bound to the lipoprotein surface and can therefore be transferred between lipoproteins . It is also capability of acquiring lipids while in the circulation. In this aspect, apoAI is crucial, both in allowing the transfer of cholesterol and phospholipids from peripheral tissues to nascent, discoidal HDL, and also for activation of lecithin-cholesteryl acyl transferase (LCAT). This enzyme is responsible for cholesterol esterification and maturation of HDL [66-68].

Atherogenic dyslipidemia

The state of dyslipidemia often observed in subjects with T2D and the metabolic syndrome is characterized by the so called lipid triad [69]. This involves high levels of plasma triacylglycerols, low levels of HDL and the appearance of small dense LDL (sdLDL). This state, which often precedes T2D by several years, is associated with an increased risk of developing atherosclerosis and some of the mechanisms behind will be described below.

The lipid triad

An important driving force, generating all the features of atherogenic dyslipidemia described above, is the increased secretion of triacylglycerol-rich VLDL particles from the liver (figure 5). The secretion is dependent on the availability of triacylglycerol for VLDL particle formation. There are three major sources affecting the level of triacylglycerol in the liver: (i) fatty acids retrieved from the circulation, (ii) uptake of chylomicron and VLDL remnants through hepatic receptors, and (iii) *de novo* lipogenesis (DNL) [70]. All of these processe are increased in subjects with insulin resistance and T2D [70]. The resulting increase in VLDL secretion is mainly due to increased production of a subgroup of VLDL called $VLDL_1$ [71-72]. These particles are larger and contain more triacylglycerols than the smaller $VLDL_2$ particles. Furthermore, the $VLDL_1$ particle also displays altered kinetic properties. For instance, increased secretion of $VLDL_1$ leads to a reduction in the catabolic rate of apoB-containing lipoproteins, in particular LDL and IDL, resulting in an increased plasma concentration of these particles [73-75].

The formation of sdLDL is closely linked to hypertriglyceridemia [76] and it has been shown that the $VLDL_1$ level is a major predictor of LDL size in individuals with and without T2D [77-78]. The mechanisms involved are thought to be related to the reduced clearance and prolonged residence time of apoB-containing lipoproteins in the circulation [79]. This allows the exchange of triacylglycerols and cholesterol esters between VLDL and LDL, mediated by cholesterol ester transfer protein (CETP). In this process LDL becomes depleted in cholesterol esters and enriched in triacylglycerols, which makes it an excellent substrate for hepatic lipase. Hepatic lipase is commonly increased in patients with the metabolic syndrome and consequently, the prevailing condition favors the formation of sdLDL [80-82]. A number of observations witness that the abundance of sdLDL is linked to an increased risk of CVD [83-86]. The small particle size has been proposed to be the major reason for the increased atherogenicity, since this would favor penetration into the vessel wall [87]. Furthermore, sdLDL particles have a higher affinity for proteoglycans than larger LDL [88-89] and also show increased susceptibility to oxidation [90-91].

HDL is considered beneficial and atheroprotective mainly due to its involvement in RCT. In a dyslipidemic state the increased triacylglycerol concentration and secretion of large $VLDL_1$ particles will lead to a reduction in the amount of HDL particles [92-93]. Again, CETP is involved, enriching the HDL particles with triacylglycerols. This enrichment has been shown to increase the clearance of HDL through an increased activity of hepatic lipases [94-95]. In association with this metabolic alteration, the HDL particles progressively lose normal biological activities. Such altered HDL particles have been called "functionally defective" [96] and show signs of reduced cholesterol efflux capacity [97-98] as well as reduced anti-oxidative [49, 99-100] and anti-inflammatory properties [101-102]

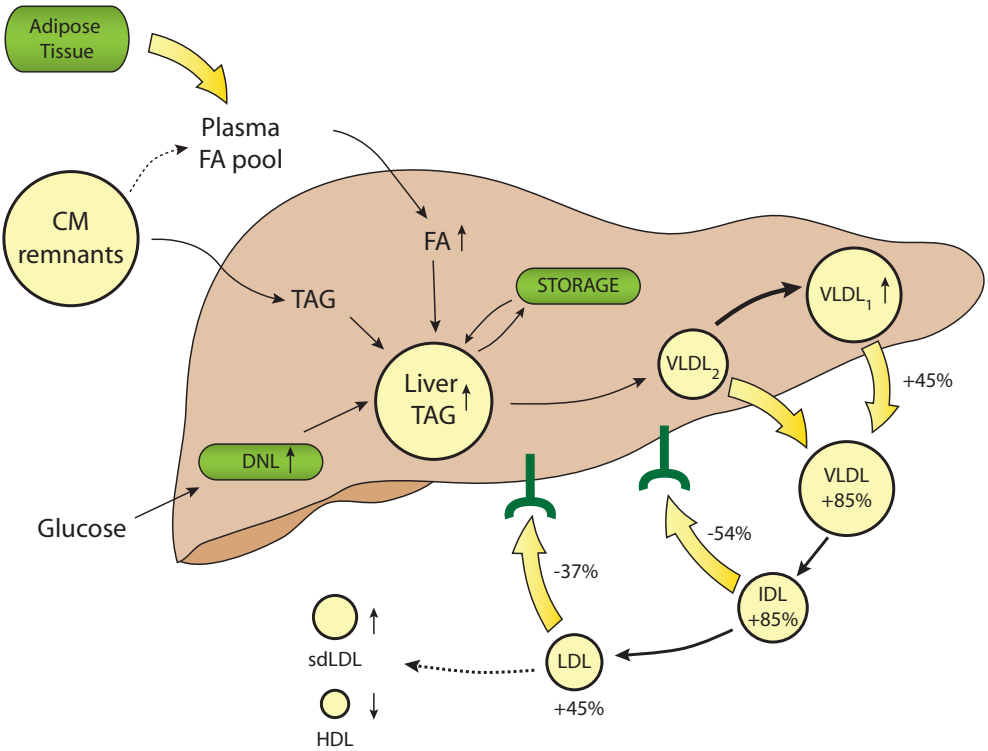


Figure 5. The metabolic syndrome is associated with an intrahepatic lipid accumulation. This accumulation, which is the result of increased de novo lipogenesis (DNL) and increased utilization of the plasma fatty acid (FA) pool, results in increased production of large triglyceride-rich $VLDL_1$. These particles have reduced clearance and the prolonged plasma residence time are associated with the formation of small dense LDL (sdLDL) and reduced levels of HDL, which are both proatherogenic events.

Dyslipidemia and liver fat

Nonalcoholic fatty liver disease (NAFLD) is a disease characterized by increased hepatic triacylglycerol levels. It ranges in severity from simple fatty liver (hepatic steatosis), with benign prognosis, to a potentially progressive form, nonalcoholic steatohepatitis, which might lead to liver fibrosis and cirrhosis. The disease is associated with obesity, T2D, hypertension and hyperlipidemia [103-104], and has therefore been recognized as the hepatic component of the metabolic syndrome [105]. The mechanisms leading to excessive accumulation of triacylglycerols in the liver are mainly linked to an increased delivery of fatty acids from peripheral adipose tissue, and enhanced *de novo* lipogenesis (DNL) in the liver itself [106]. By using a multiple-stable-isotope approach, Donnelly et al. estimated the contribution of DNL to hepatic triacylglycerol content in insulin resistant NAFLD subjects to be almost 30% [107]. This can be compared to a DNL contribution of 5% under non-pathological conditions [108]. This might be important in driving the dyslipidemia since a strong correlation exists between DNL and secretion of VLDL particles [106]. This secretion, which is mainly the result of increased number of $VLDL_1$ particles, is under normal conditions suppressed by the action of insulin. However, in subjects with fatty liver, this suppression is reduced [109].

The connection between liver fat and insulin resistance is more uncertain. Emerging data suggests a causal role of intrahepatic lipid accumulation for the development of hepatic insulin resistance [110-112]. Even though it has been shown in several rodent models that reducing the intrahepatic triacylglycerol depots will improve insulin sensitivity [113-115], this is most likely not a consequence of reduced triacylglycerol levels *per se*. Instead there is much evidence pointing to the importance of lipid intermediates such as diacylglycerols and ceramides in the development of insulin resistance [116-117].

Dyslipidemia and apoCIII

The plasma level of apoCIII is increased in patients with dyslipidemia and correlates well with the levels of triacylglycerols [118]. Furthermore, in a number of studies with angiographic or clinical end points, plasma concentrations of apoCIII, or apoCIII-containing apoB particles, have been shown to be strong independent risk factors for cardiovascular disease [119-123]. Genetic studies have also shown the importance of apoCIII levels for predicting cardiovascular disease. In a study published recently by Pollin and coworkers, it was shown that carriers of a heterozygous null mutation in the gene encoding for apoCIII had lower LDL and triacylglycerol levels as well as an increase in HDL levels. Furthermore, subclinical atherosclerosis was also lower in these subjects indicating a cardioprotective effect of this lifelong deficiency [124].

The mechanisms for the increased risk of cardiovascular disease in patients with high apoCIII levels can, to some extent, be explained by the inhibitory effect of apoCIII on LPL, the enzyme responsible for lipolysis and catabolism of triacylglycerol-rich lipoproteins in the circulation. Furthermore, recent publications also suggest that apoCIII inhibits hepatic uptake of VLDL and LDL overriding the opposite influence of apoE when both are present [125-126]. Both of these mechanisms result in a decreased catabolic rate and prolonged plasma residence time for apoB-containing lipoproteins. It was also reported that apoCIII facilitates the conversion of LDL into sdLDL [125], another proatherogenic event.

In addition to the mechanisms described above, convincing evidence now indicates that apoCIII is a multifunctional protein with effects beyond metabolic regulation. In a number of elegant articles from Kawakami and coworkers, apoCIII has been shown to be directly involved in inflammatory events in the vascular wall. For example, apoCIII, alone or bound to apoB-containing particles, was shown to increase the adhesion of circulating monocytes to vascular epithelial cells, which is an important event in the inflammatory aspect of atherosclerosis [127]. The mechanisms behind this were: (i) activation of monocytes and (ii) activation of vascular endothelial cells by protein kinase C (PKC) β, which results in an increased expression of vascular cell adhesion molecule 1 (VCAM-1) [128]. The activation of PKCβ was further shown to inhibit insulin-mediated activation of the endothelial nitric oxide synthase (eNOS) pathway, reducing the amount of the vasodilatory and atheroprotective nitric oxide. In conclusion, these findings suggest novel mechanisms for apoCIII in the pathogenesis of atherosclerosis and further link dyslipidemia to endothelial dysfunction [129].

The pathogenesis of atherosclerosis

A primary initiating event in atherosclerosis is the subendothelial accumulation of apoB-containing lipoproteins in susceptible areas of the artery wall [130]. Even though LDL is considered the lipoprotein that is most prone for penetration into the subendothelium, remnant particles originating from partial lipolysis of triacylglycerol-rich CM and VLDL might also be retained [131-132]. The retention is mainly mediated through the interaction of the apoB-containing particles with the artery wall proteoglycans (PGs) [133-135]. There are two factors that influence the retention of apoB-particles. The first one is the serum concentration. It can easily be appreciated that an increased amount of particles will increase the chance for penetration and subsequent retention. Accordingly, LDL levels have in several large clinical studies been recognized as one of the most important risk factors for cardiovascular disease [93, 136]. The other is the propensity of the particle, once inside the endothelium, to become trapped. This might be due to either intrinsic properties of the particle such as size, charge, protein and lipid composition, or properties of the surrounding such as endothelial permeability or PG characteristics.

Once trapped inside the intima, the lipoproteins are exposed to enzymatic and non-enzymatic modifications. Oxidation plays an important role due to the generation of oxidized phospholipids. These are known to be mediators of inflammatory cell recruitment and endothelial activation [137-138]. Oxidation might be the result of enzymatic attack by myeloperoxidase and lipoxygenase, or by non-enzymatic attack by reactive oxygen species [139-140]. Alternatively, secretory phospholipase A2 (sPLA2) might be responsible for producing lyso-phosphatidylcholine, which is known to be a proinflammatory lipid involved in the atherogenic process [141-143]. Another modification thought to be important is caused by secretory sphingomyelinase (SMase), which is responsible for mediating the conversion of sphingomyelin to ceramide. This increase in ceramide promotes lipoprotein aggregation, which facilitates its uptake by arterial wall macrophages, leading to foam cell formation [144]. These mechanisms of lipoprotein modifications are accompanied by an inflammatory response and together they accentuated the essence of the complex pathogenesis of atherosclerosis.

The progression of the disease results in an expansion of the atheroma and the development of the lipid core. It is thought that much of the non-cellular material located in the basal intima originates from the death of foam cells and smooth muscle cells. The core is also filled with lipid debris originating from dead cells as well as from free lipoproteins [145].

STUDY POPULATIONS

Subjects from two major study cohorts have been used in the work included in this thesis. These studies are described shortly below

Fenofibrate Intervention and Event Lowering in Diabetes

In paper II the study subjects were recruited from the cohort originally included in the "Fenofibrate Intervention and Event Lowering in Diabetes" (FIELD) trial [146]. FIELD was a double-blind, placebo-controlled trial investigating the benefits of using fenofibrates for the prevention of coronary heart disease in people with T2D. The main eligibility criteria were: T2D with onset after 35, men and women between 50 and 75 years of age, average total cholesterol between 3.0-6.5 mM and triacylglycerol/HDL ratio > 4.0 or triacylglycerols > 1.0 mM. The trial, which was performed between 1998 and 2000, was performed in Australia, New Zeeland and Finland. In Finland Marja-Riitta Taskinen was one of the principal investigators. From the 9795 individuals that participated, 270 were recruited from the Helsinki Center. Of these, 101 volunteered for the study performed in paper II. The control group was recruited according to supplemental material in paper II.

Diabetes in Women and Atherosclerosis

In paper III the subjects were chosen from the community-based study "Diabetes in women and atherosclerosis" (DIWA). This was performed during 2001-2003, with Björn Fagerberg as principal investigator, and involved all the 64-year-old women living in Gothenburg, Sweden. Of the 4586 women invited, 2595 participated and went through a screening examination, which included a glucose tolerance test. In addition, the examination also included anthropometric measurements together with a questionnaire regarding previous disease, current medication, smoking habits and heredity for diabetes. Subjects from this cohort were selected with the inclusion criteria stated in paper III.

LIPIDS

Once considered passive bystanders, with the only functions as providers of energy and a basic structure of membranes, lipids are now considered multifunctional and it is difficult to find an area of cell biology where lipids do not play important roles as signaling or regulatory molecules. The importance of understanding lipid biology, for academic but also for clinical chemistry and pharmaceutical industry, is essential since alterations in lipid metabolism is involved in the pathogenesis of several common diseases such as T2D, Alzheimer's disease, schizophrenia, cancer and atherosclerosis.

Definition and classification

Even though most lipid chemists and biochemists have a pretty clear picture of what is meant by the term lipid, there is no satisfactory and widely accepted definition of the term. In many textbooks, lipids are often described as naturally occurring substances that are hydrophobic in nature and soluble in organic solvents [147]. Even though this is true in most cases, there are substances that we now see as lipids (saccharolipids for example) that are more soluble in water than in chloroform and, vice versa, several peptides and very hydrophobic proteins are actually soluble in chloroform [148]. There are other definitions that use a more biological approach. William Christie defines lipids as "fatty acids and their derivates and substances related biosynthetically or functionally to these compounds" [149].

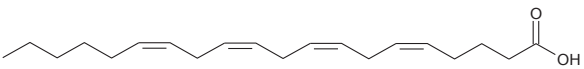
In an effort to reach a consensus, several of the world leading lipid researchers recently proposed a lipid classification system [150]. Here lipids are defines as "hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensation of thioesters or isoprene units". They also make a classification of lipids into 8 groups based on chemical and structural similarities (table 2) and propose schemes for nomenclature. This effort, which is made with the intention of facilitating the systematization and cataloging of lipids, will be very useful when creating lipid databases for the use in lipidomics and system biology. Below will follow a short description of the lipid classes relevant for the work included in this thesis.

Table 2. Lipid classification as suggested by Fahy et al. [150]

Lipid class	Abbreviation	Example
Fatty acyls	FA	Fatty acid, eicosaniod
Glycerolipids	GL	Triacylglycerol
Glycerophospholipids	GP	Phosphatidylcholine
Sphingolipids	SP	Ceramide, Sphingomyelin
Sterol lipids	ST	Cholesterol, Cholesterol ester
Prenol lipids	PR	Retinol
Saccharolipids	SL	Lipid X
Polyketides	PK	Tetracycline

Structure and function

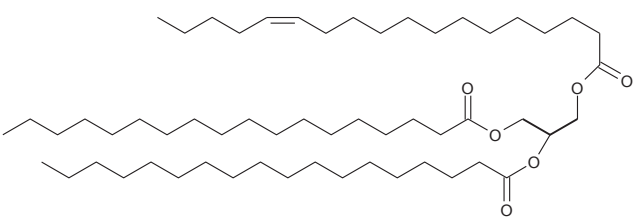
Fatty acyls



(Arachidonic acid)

This group includes the fatty acids, which are synthesized in a fashion that leads to ethylene (C_2H_4)-spaced products of different chain lengths. Double bonds are included at various positions and at various stages in the biosynthetic and metabolic processes resulting in a vast amount of different molecules. Besides being used as building blocks for several other lipid classes, the fatty acids, and especially arachidonic acid, are also important precursors for biologically active lipids such as prostaglandins, leukotriens and thromboxanes.

Glycerolipids

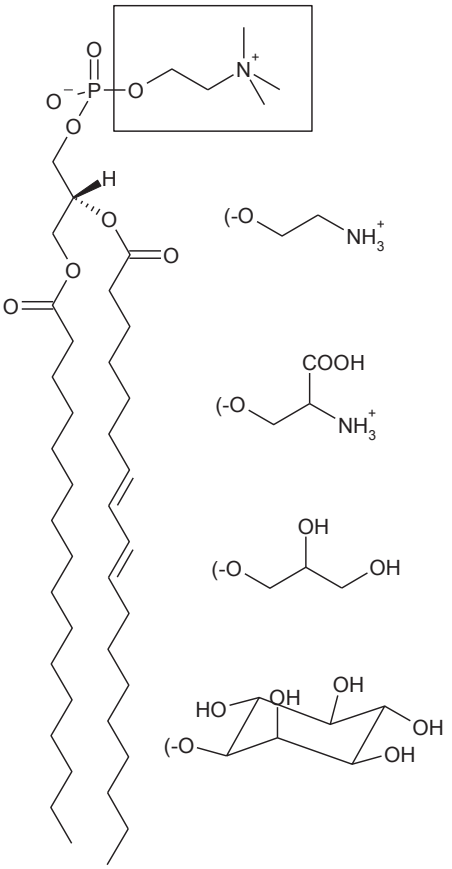


(Triacylglycerols)

The glycerolipids include all the lipids that contain a glycerol backbone, except for the glycerophospholipids, which constitutes a separate category. The glycerolipids are dominated by the mono-, di-, and triacylglycerols, which all

have fatty acids esterified to the glycerol backbone. Triacylglycerols are the main source of energy in the body while the diacylglycerols functions as membrane constituents but also, under certain conditions, as intracellular messengers [151]. Increased intra-cellular levels of diacylglycerols have also been suggested to be induce insulin resistance [152].

Glycerophospholipids



Choline
(Phosphatidylcholine)

Ethanolamine
(Phosphatidylethanolamine)

Serine
(Phosphatidylserine)

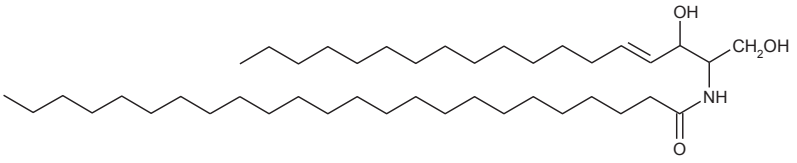
Glycerol
(Phosphatidylglycerol)

Inositol
(Phosphatidylinositol)

Even though containing a glycerol backbone, the glycerophospholipids are considered as a separate group because of their high abundance and importance as membrane constituents. The glycerophospholipids are ubiquitous in nature and can be subdivided into distinct classes depending on the polar head group attached to the *sn*-3 position on the glycerol backbone. The phosphatidylcholines and phosphatidylethanolamines are the most common sub-classes and may constitute as much as 55 and 35 %, respectively, of the total lipids in certain tissues [149]. Other sub-classes include phosphatidylserine, phosphatidylglycerol

and phosphatidylinositol. Hydrolysis of phospholipids can occur by the action of phospholipases, which will lead to the production of lyso-phospholipids and free fatty acids. Both lyso-phosphatidylcholine and lyso-phosphatidic acid are potent signaling molecules and have been implicated in several disease states [153]. Furthermore, in response to various cell stimuli, the action of phospholipase C on phosphatidylinositol 4,5-bisphosphate leads to the production of diacylglycerol and inositol 1,4,5-triphosphate, which are both secondary messengers in what can now be considered to be a classical pathway [154-155].

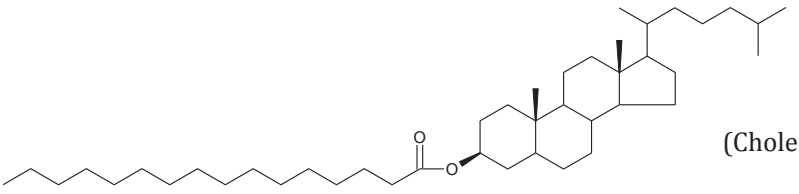
Sphingolipids



(Ceramide)

The sphingolipids are a complex family of compounds that share a sphingoid base backbone as a common structural feature. The sphingoid base is synthesized from a serine and a long-chain fatty acyl-CoA and further metabolism leads to the generation of several other sub-groups including ceramides, sphingomyelins, and glycosphingolipids (such as glucosylceramides and gangliosides). Ceramides, which are an important sub-group, have a fatty acid of varying length (mostly between 16-26 carbons long) attached to the sphingoid base backbone. They are bioactive molecules that have been implicated in a variety of physiological functions including apoptosis, cell growth arrest, differentiation, cell senescence, cell migration and adhesion [156]. They are also being considered as regulators of insulin signaling and action [157].

Sterol lipids



(Cholesteryl ester)

The sterols are widely studied in mammalian systems. Cholesterol is an important constituent of biological membranes and also the precursor for many steroids (hormones) such as estrogen, testosterone and cortisol. The storage

form of cholesterol, the cholesteryl ester, is highly abundant in many cells and also in the circulation as a major part of the lipoprotein particles.

Lipid diversity

Analysis of mammalian tissue suggest that the number normally-abundant fatty acids is typically in the order of 30-60 [158]. By combining these with different lipid backbones and head groups, a large number of different molecular lipids can be formed. For example, the theoretical number of triacylglycerols that could be generated from 50 fatty acids would be more then 100 000. Furthermore, more than 300 different oligosaccharide side chains and 60 different sphingoid bases have been characterized so far in the sphingolipid class [159]. By combining these with 50 fatty acids the number of theoretical combinations reaches almost 1 million. Fortunately, from an analytical point of view, many of these doesn't exist, or exist at very low abundance, due to non-random distribution of fatty acids and head groups. For example, phospholipids often have saturated fatty acids at *sn*-1 position while the *sn*-2 position is occupied by an unsaturated fatty acid [158]. Another example is ceramides, which usually consists of long saturated fatty acids and sphingosine as the sphingoid base [160]. Nonetheless, the number of molecular lipids is huge and therefore a comprehensive investigation of the lipidome relies on powerful analytical techniques, such as mass spectrometry (MS).

Lipid abbreviations

Throughout this thesis, and also in the articles included, molecular lipids are denoted with the abbreviation of the lipid class followed by the number of carbons and double bonds included in the fatty acids attached to the core structure. For example, PC 16:0/18:1 means phosphatidylcholine with a fatty acid comprising of 16 carbons with no double bonds (palmitic acid) on the *sn*-1 position, and a fatty acid comprising 18 carbons with 1 double bond on the *sn*-2 position on the glycerol backbone. If the positions of the fatty acids are unknown it is annotated PC 16:0-18:1. Furthermore, the fatty acid denoted 18:1 in the example above might be either oleic or vaccenic acid depending on the position of the double bond. If it was oleic acid the structure would be written PC 16:0/18:1 n-9 with the "n-9" informing us that the double bond is positioned between carbon number 9 and 10 counting from the methyl end. (These are sometimes called omega-9 fatty acids). If the fatty acid was vaccenic acid, the lipid would have been written PC 16:0/18:1 n-7.

LIPIDOMICS PLATFORM

"*…through chemistry one could understand the nature of evils in the shape of disease, and the means of curing or mitigating them*".

J. L. W. Thudichum 1876

Lipidomics

Since the term genomics was introduced in the middle of the 80's (supposedly by the scientist Thomas Roderick [161]) a number of different *-omics* have now emerged in the field of biology. One of them is metabolomics, which has the ambitious task of characterizing all the metabolites of a biological system (such as a cell or organism) at a given time and under certain conditions. Metabolomics lies further down in the *-omics* cascade and, in some aspects, therefore more accurately reflects the physiological state of the cell, tissue or organism of interest. This makes metabolomics a highly interesting alternative when searching for new diagnostic biomarkers or for evaluating therapeutic interventions. Furthermore, by combining data from metabolomics-, proteomics-, and genomics studies, inter-related information is attained that can be used to understand and predict the behavior of a system as a whole. This discipline is sometimes called systems biology.

Lipidomics is categorized as a subgroup of metabolomics, and has been defined as "the system-level analysis and characterization of lipids and their interacting moieties" [158]. Other definitions imply that lipidomics is more than just the characterization of lipids and involves also the comprehensive understanding of the influence of all lipids on a biological system [162]. The emergence of this new discipline, which was first mentioned in a peer-reviewed scientific paper in 2003 [163], is built on the foundation of three major inter-related factors. First, the importance of lipids as a part of the etiology of diseases such as T2D, atherosclerosis, obesity and Alzheimer's disease has been recognized. Secondly, researchers have realized that the metabolism of several molecular lipids and lipid classes are interwoven. Therefore, the study of specific pathways requires the analysis of multiple lipids. Finally, and perhaps most importantly, the field has expanded because of important developments in the arsenal of analytical

tools available. Of major importance is MS, which allows even low abundant lipids to be analyzed in a high-throughput manner.

The analysis of lipids usually consists of three major parts: (i) lipid extraction, (ii) sample analysis (often by HPLC and MS), and (iii) data analysis and interpretation. These parts will be discussed below.

Lipid extraction

The first step in the analysis of lipids (unless imaging techniques are employed) is the extraction of the lipids and removal of non-lipid components from the tissue or biofluid of interest. This is in most cases achieved by extraction with organic solvents. A number of different organic solvents can be used, and the extractions can be tailored for high recoveries of specific lipid classes [149]. Two of the most common methods for isolating a broad spectrum of lipid classes are the methods described by Folch, Lees and Sloane [164], and by Bligh and Dyer [165]. The basic principle of these methods is that initially chloroform and methanol are added to the sample creating a mono-phase system that extracts the lipids from the sample matrix. Then an aqueous buffer is added, which cleans the lipid extract from non-lipid components.

In the work included in this thesis, all extractions were made according to Folch et al. and the procedure has been automated by the use of a robot [166]. The robot uses the 96-well format and the extraction, which is completed in about one hour, is performed in 1.3 ml glass inserts placed in a custom-made alumina block. Validation of the method revealed similar extraction recoveries and quantification accuracy compared to a manually performed extraction. In addition, the method resulted in increased reproducibility.

Even though the Folch procedure is highly used and excellent for extracting lipids within a wide range of polarity, the routine and automated use of this method suffers from certain drawbacks, all related to the use of chloroform. Despite being an excellent solvent for lipids, chloroform is a known carcinogen with several negative effects on the environment and human health [167]. Furthermore, the low solubility of water in chloroform reduces the sample to solvent ratio used in the initial mono-phase system of the Folch procedure to 1:20. This means that when using 1.3 ml glass vials only 30 µl of sample can be extracted. Despite the increased sensitivity of modern mass spectrometers, there still are occations when the analysis of low abundant lipids in certain matrices (such as cerebrospinal fluid or isolated cellular compartments) would benefit from using a higher initial volume of sample matrix. Finally, chloroform is a dense liquid

ending up at the bottom at the tube when adding the aqueous buffer to the extraction mixture. This means that the buffer and inter-phase have to be penetrated for recovery of the lipid-containing fraction. For some samples the water phase, and especially the inter-phase, can be filled with precipitate from proteins and other matrix components, which might disturb accurate recovery of a pure lipid fraction. Alternatives to the Folch procedure are therefore required. The Dole procedure is based on a ternary system of isopropanol, hexane and 16 mM H_2SO_4 [168]. This system has an initial sample to solvent ratio of 1:5 and the lipids end up in the upper organic phase. However, this method is only suitable for non-polar lipids and show low recoveries for more polar lipids such as phospholipids and sphingolipids. Recently a method was described using methyl-*tert*-butyl ether (MTBE), methanol and water (10/3/2.9; v/v/v) for the use in automated lipidomics applications [169]. Although this method displays high recoveries for a broad range of lipids, it still suffers from low sample to solvent ratio in the first mono-phase system. An alternative method (manuscript in preparation) has been developed that is based on butanol, methanol, ethyl acetate, heptane and 1mM acetic acid. This method has a high sample to solvent ratio (1:4) and has an upper organic phase, which makes it ideal for automation.

Chromatography of lipids

Basics

Chromatographic methods allow the separation of closely related analytes of complex mixtures. In all chromatographic methods the analytes are transported in a mobile phase that can be a gas, liquid or supercritical fluid. The mobile phase is forced through a stationary phase that is fixed inside a column or on a plate. Due to the differences in distribution of the analytes between the two phases, they will be separated from each other. The analytes that favors the mobile phase will travel rapidly through the stationary phase. In contrast, the analytes that are highly retained by the stationary phase will move slowly with the flow of the mobile phase.

In **gas chromatography** (GC) the sample is vaporized and injected into a chromatographic column. The analytes are carried by a gas and allowed to interact with the stationary phase, which is normally a liquid (gas-liquid chromatography). A limitation of GC is the requirement of the analytes to be easily vaporized. This often requires time-consuming derivatization procedures to reduce the boiling point of the analytes. Another limitation of the GC methodology is the inability to analyze thermally unstable compounds. In the field of lipid analysis GC has been used extensively and even though it has been

replaced by HPLC in many applications, it is still the preferred technique for analyzing free fatty acids.

The use of **high-performance liquid chromatography** (HPLC) (or high-pressure liquid chromatography as it was originally called) has accelerated and is now the most widely used analytical separation method. One important reason for this is the applicability to substances of major interest for industry, many fields of science and the public. In HPLC, the stationary phase is contained inside a column with the dimensions normally ranging from 50-250 mm in length and 1-5 mm in diameter. The stationary phase often consists of small beads (3-5μm) coated with different material depending on the separation required or, in other words, on the analytes of interest. In early work, HPLC was based on highly polar stationary phases such as water or triethyleneglycol supported on silica or alumina particles. Non-polar mobile phases, such as hexane and ethers, were commonly used. For historical reason, this type of chromatography is referred to as normal-phase and is today used for separations of analytes with relatively hydrophobic properties such as lipids. Even though normal-phase HPLC is used in several applications, more common today is reversed-phase HPLC. Here the stationary phase is nonpolar alkyl chains (e.g. C18 or C8) covalently bound to the support surface while the mobile phase is based on more polar solvents such as water, methanol and acetonitrile. The name reversed-phase originates from the fact that in this kind of HPLC, the elution order of the analytes is reversed compared to normal-phase. In normal-phase HPLC the least polar analyte is eluted first, while in reversed-phase it is eluted last. Besides the C18 and C8 stationary phases mentioned above, several other exist such as cyano- and phenyl-bonded phases that give the column slightly different retention properties that might be useful for specific applications. Due to the robustness and applicability of reversed-phase HPLC it can be found in a number of different fields. Even though the method is usually used for more polar substances, it is also used extensively in the field of lipid analysis.

High-performance liquid chromatography of lipids

The use of HPLC for the separation of complex lipid mixtures has been described extensively in the literature. Due to the wide range of polarity and hydrophobicity between different lipid classes, the columns and mobile phases used also varies depending on the application. Both normal- and reversed-phase methods have been employed with some only focusing on specific lipid classes, while others use a more general approach in an attempt to separate lipids with a broader array of polarity. In the work included in this thesis lipid classes have been separated on a silica column using normal-phase HPLC according to a method published by Homan et al. [170], which is based on a prior method originally published by Christie et al [171]. Figure 6 shows a chromatogram generated from the separation of a mixture of lipid standards, using this method. In the work included in this thesis the HPLC method has been used for quantification of major lipid classes, but also as a lipid class purification system. By splitting the flow of the mobile phase post-column, selected lipids could be collected and further characterized using MS. The benefits of using this strategy, which has also been used by others [172-173], are mainly that the collected lipid class can be analyzed at lower concentrations (the sensitivity increased by a factor 3-8 depending on the lipid class) in absence of a complex sample matrix that would otherwise suppress the signal and contaminate the ion source. Another advantage is that molecular lipids originating from other lipid classes, with the same (isobaric) or very similar mass, are removed, thereby facilitating identification and quantification.

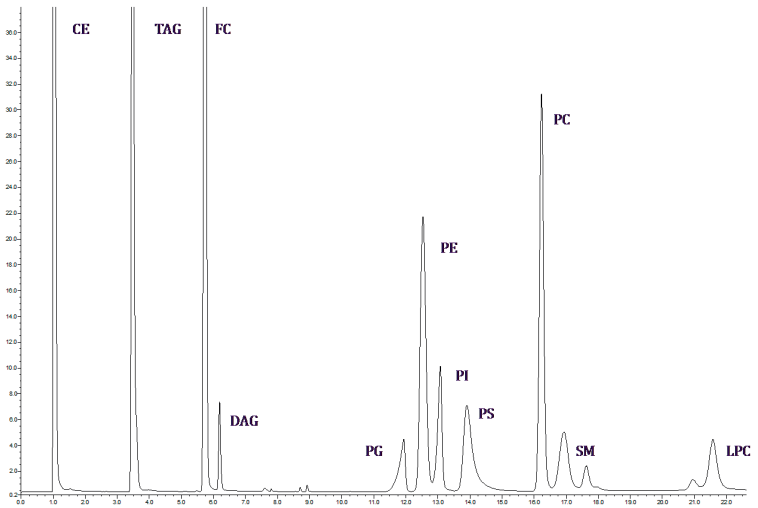


Figure 6. Chromatogram showing the separation of both non-polar and polar lipids using the method according to Homan et al. [170]

Even though the retention of lipids in a normal-phase HPLC system is based on their polar moieties, the hydrophobic parts (such as fatty acids) will also have an effect on their retention time. Thus, the composition of molecular species in the beginning of an eluting peak will be slightly different then the later part. Therefore, precautions have to taken when using HPLC for the isolation of specific lipid classes that the chromatographic system is in equilibrium and that the collection is made with sufficient margins both before and after the eluting peak. Otherwise there is a risk of generating false lipid profiles [174]. Figure 7 shows no difference in the CE profile of isolated VLDL (from the DIWA subjects in paper III) analyzed from a total lipid extract or after pre-fractionation using HPLC. Similar comparisons have been made for other lipid classes to ensure the conservation of the lipid profiles after pre-fraction.

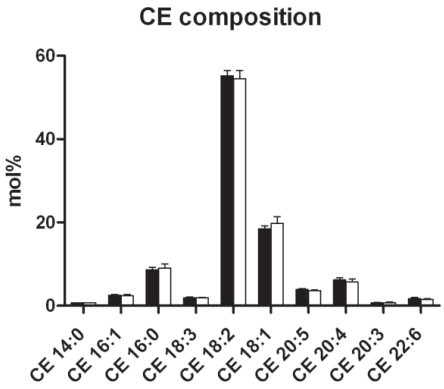


Figure 7. No significant differences could be observed in the profiles of VLDL cholesteryl esters analyzed directly as a total lipid extract (black) or after pre-fractionation using HPLC (white).

Mass spectrometry of lipids

That MS was going to play an important role in lipid research was appreciated already 50 years ago. In a review article from 1960 the local pioneers in lipid MS Ragnar Ryhage and Einar Stenhagen stated that *"...its wide applicability and power are not as yet fully appreciated"* [175]. In the following decades lipid MS, and especially GC/MS, would come to play a more important role within the field.

Since lipids are mostly nonvolatile compounds, much effort was made in the early days to get the lipids into gas-phase to be able to analyze them with GC/MS. With the advent of newer ionization techniques, especially electrospray ionization, the situation has now profoundly changed so that it is now possible to directly, without any tedious derivatization steps, produce gas-phase molecular ions for most lipids. As a consequence, GC has in many applications been replaced with HPLC or direct infusion techniques.

Basics

MS is a powerful analytical tool that can supply both qualitative and quantitative data. It has the capability to provide information about the molecular weight, elemental composition and also structural composition, which can be valuable when analyzing unknown analytes. For known analytes, it is mainly used as a selective mass filter, which means that it can sort out the mass of the analyte from interfering substances and therefore provide extremely selective data. The mass spectrometer consists of three major parts: the ion source, the mass analyzer and the detector. These will be described below.

Ion sources

Since the mass spectrometer utilizes external electric and magnetic fields to move and manipulate the analytes, one requirement is that the analytes become ionized before entering the mass analyzer. The second requirement is that the ions are in gas phase. Both of these are taken care of in the ion source. There are a number of different ion sources available and the choice depends heavily on the application. Here only the most commonly used ion source for coupling HPLC to MS will be mentioned, **electrospray ionization** (ESI). The development of ESI for mass spectrometric applications started in the middle of the eighties in the lab of John Fenn [176], who was later rewarded with the Nobel Prize in 2002. When using ESI in combination with HPLC, the HPLC effluent is emitted through a small capillary that is subjected to high voltage (1-3 kV). This voltage enables the fluid to form a Taylor cone, which is enriched with ions at the tip. From this tip a spray of charged droplets is ejected towards the mass analyzer (figure 8). The size of the droplets is decreased as a result of evaporation, which is assisted by the hot flow of nitrogen gas that is passed through the front of the ion source. In a process that has still not been fully elucidated [177] ions are subsequently formed from these charged droplets and enter the inner parts of the mass spectrometer.

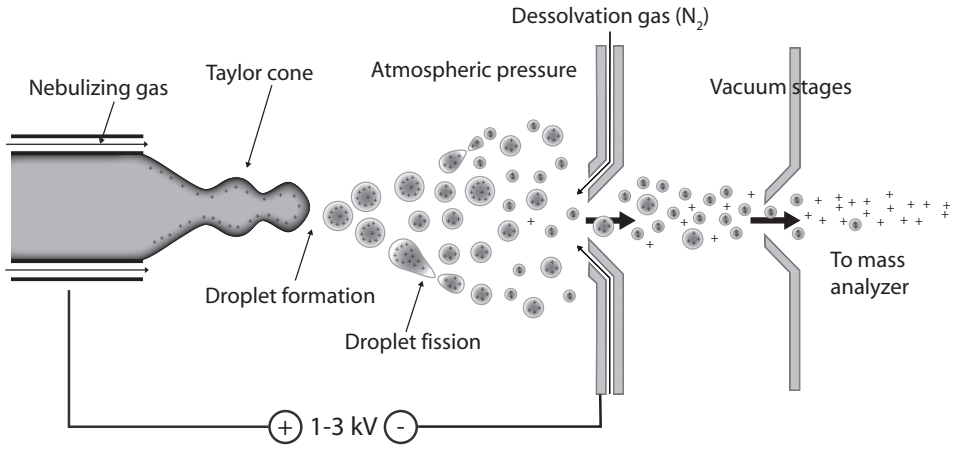


Figure 8. Electrospray ionization interface**.**

Mass analyzers

From the ion source the ions are transported through an intermediate vacuum region and further on into the high vacuum of the mass analyzer. In the mass analyzer, the ions are separated according to their mass to charge ratio (*m/z*). If the analytes are all singly charged (*z*=1), which is almost always the case for lipids, this means that the separation will only depend on the mass.

There are several types of mass analyzers available and they can be divided into different categories. Three common types are the quadrupole, the time-of-flight (TOF) and the ion trap analyzer. Since they all have their advantages and limitations, there are several factors of the mass analyzer that have to be considered when using MS. The **mass range** might be important if the analyte of interest have a high *m/z*. The **resolution**, or resolving power, is the ability of the mass analyzer to yield distinct signals for two ions with a small mass difference. The **speed** of the instrument might be important if, for example, there are multiple analytes eluting simultaneously during an HPLC run. The speed of the instrument will determine the number of data points that will be generated over the chromatographic peak, and thereby affect the robustness and power of the

analysis. If the analyte is of low abundance, which often is the case for bioactive lipids, then the **sensitivity** will be an issue. Even though the detector is responsible for signal output, the mass analyzer contributes to the sensitivity of the instrument by modulating the signal/noise ratio. Quadrupole and TOF mass analyzer are described in more detail below.

A **quadrupole** consists of four circular (or ideally hyperbolic) parallel rods. Opposite rods are electrically connected and applied with a combination of direct-current (DC) and a radiofrequency (RF) potential. The ions will travel axially through the center of the four rods where they will be affected by electrical forces. The force will depend on the combination of the DC and RF potential. This means that for a certain *m/z* only a specific combination of these potentials will enable the ion to maintain a stable trajectory through the quadrupole and reach the detector. Other ions with different *m/z* will be bent in either the x or y-axis and will be lost. In a quadrupole a spectrum is generated by stepwise scanning through the *m/z* range of interest while recording the amount of ions reaching the detector.

In a **time-of-flight** (TOF) mass analyzer the ions are accelerated by a potential often perpendicular to the ion path generated in the ion source, so called orthogonal acceleration (figure 10). Since this will give the same kinetic energy to all ions (ideally), the difference in mass between different ions will give them different velocities. The ions are then allowed to drift in a field-free vacuum region towards the detector. The difference in time between the acceleration and the pulse generated from when the ions hit the detector is the time-of-flight. This can be expressed as:

$$t_{TOF} = \frac{L}{v} = L\sqrt{\frac{m}{2qV}} \propto \sqrt{\frac{m}{z}}$$

Where L is the distance travelled, v is the velocity of the ion after the acceleration, m is the mass of the ion, q is the charge and V is the acceleration potential.

Detectors

After passing the mass analyzer, the ion beam is detected and transformed into a usable signal by the detector. Several types of detectors currently exist and they vary according to the design of the mass spectrometer and the application requirements. For all of them however, characteristics such as high sensitivity and linearity are highly desired. At present, the most commonly utilized detector is the **electron multiplier** (EM). This detector comes in a number of different variants but all work by the same principle. Ions exiting the mass analyzer strike

a conversion dynode. This creates secondary ions that generate an electron cascade multiplying the single incident with a factor of 10^6 or more. A **multichannel plate** (MCP) is an example of an EM detector that is frequently found in TOF instruments. The design of this detector allows very rapid readout and response, which is required when using a TOF mass analyzer. The drawback, at least so far, is that the MCP has a tendency to get saturated at high ion abundances, which in turn gives nonlinearities for count rates above a few thousand incident particles per second.

Tandem mass spectrometry

A big step in the analytical capability of MS was the introduction of **collision induced dissociation** or decomposition (CID) [178]. CID of preselected precursor ions often occurs in a separate part of the mass spectrometer, called the collision cell, and generates a characteristic and often unique precursor-dependant fragment pattern. By using two mass analyzers in series (tandem), separated by a collision cell, new methods could be employed (figure 9). Below different methods of performing tandem mass spectrometry (MS/MS) is illustrated with the triple quadrupole (QqQ). In this mass spectrometer, both mass analyzers are quadrupoles. However, MS/MS can also be performed in instruments with a different set-up.

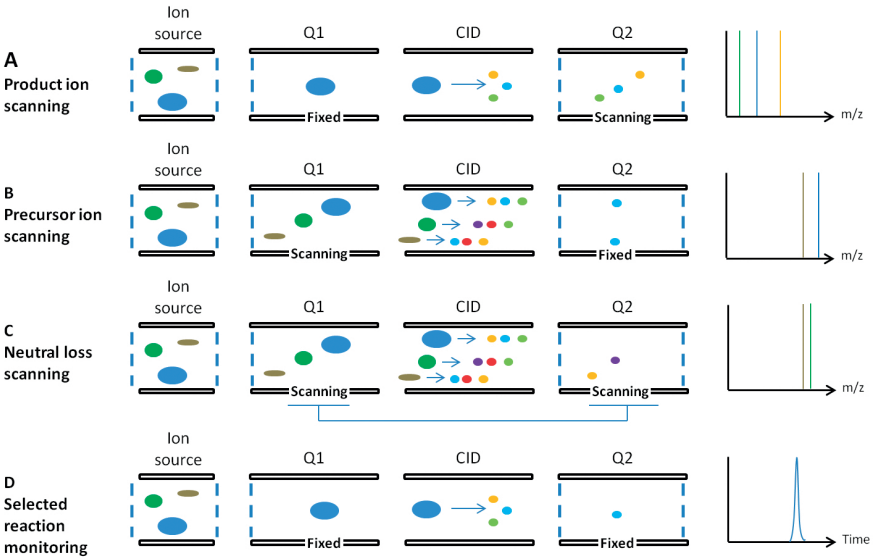


Figure 9. Mass spectrometric methods that are based on the collision induced dissociation

One way of performing MS/MS is to filter out the precursor ion of interest in the first quadrupole and expose it to CID in the collision cell. The generated fragments are then measured in the second quadrupole. This method is called **product ion scanning** and the fragmentation pattern can be used for structure elucidation of unknown compounds.

Precursor ion scanning (PIS) and **neutral loss** (NL) scanning are both powerful tools when working with lipids since they take advantage of the structural similarities of the molecular lipids within a certain lipid class. In PIS using a QqQ, all molecular ions (or molecular ion adducts) in a certain predefined mass range are exposed to CID and in the second mass analyzer the characteristic fragment ion(s) is/are monitored. In the cases where CID generates a neutral characteristic fragment, the two mass analyzers are scanned at a constant mass difference corresponding to the mass of the lost, neutral fragment. This is called neutral loss scanning.

An often used method for quantification is **selected (or single) reaction monitoring** (SRM). This is preferably used in combination with HPLC and takes advantage of the specific fragmentation pattern of an analyte of interest. By fixing both the first and the second mass analyzer on masses that are highly specific for the analyte, a very selective, and thus sensitive, method can be created for quantification. If several compounds are monitored simultaneously, the method is called **multiple reaction monitoring** (MRM).

Quadrupole time-of-flight mass spectrometry

Hybrid mass spectrometers combining both the quadrupole and time-of-flight technologies emerged in the mid 90s [179]. As shown in figure 10, the QqTOF mass spectrometer contains a resolving quadrupole (Q1), a collision cell, and an orthogonally angled TOF analyzer. Normally QqTOF instruments are more sensitive than triple quadrupoles working in scanning mode because it has the benefit of being able to record all ions simultaneously in the TOF analyzer. But when working with MS/MS methods such as PIS or MRM, the sensitivity of the triple quadrupole is usually superior. This is mainly due to the low duty cycle (5-30%) of the TOF mass analyzer. A duty cycle can in this case be defined as the proportion of the ions entering the accelerator area that are actually accelerated towards the detector. However, this limitation was overcome when bunching and trapping technologies were developed [180]. By temporarily trapping fragment ions within a certain mass range in the collision cell before releasing them into the TOF analyzer, the duty cycle could reach close to a 100% for ions in the low mass range [180]. This has increased the sensitivity of the QqTOF instruments, which are now in the same order of magnitude as the triple quadrupoles. Other important features of the QqTOF are the inherited characteristics of **high resolution and**

mass accuracy. This allows the detection of ions within a small mass range (typically 0.1 Da), which minimizes the chance of detecting a false positive and biased quantification. Furthermore the TOF analyzer allows the **simultaneous recording** of a theoretically unlimited number of ions. This makes it an attractive alternative for performing multiple PIS.

Most mass spectrometric analyses performed in the work included in this thesis utilizes this type of instrument, and are based on methods developed by Ekroos and Ejsing [181-183].

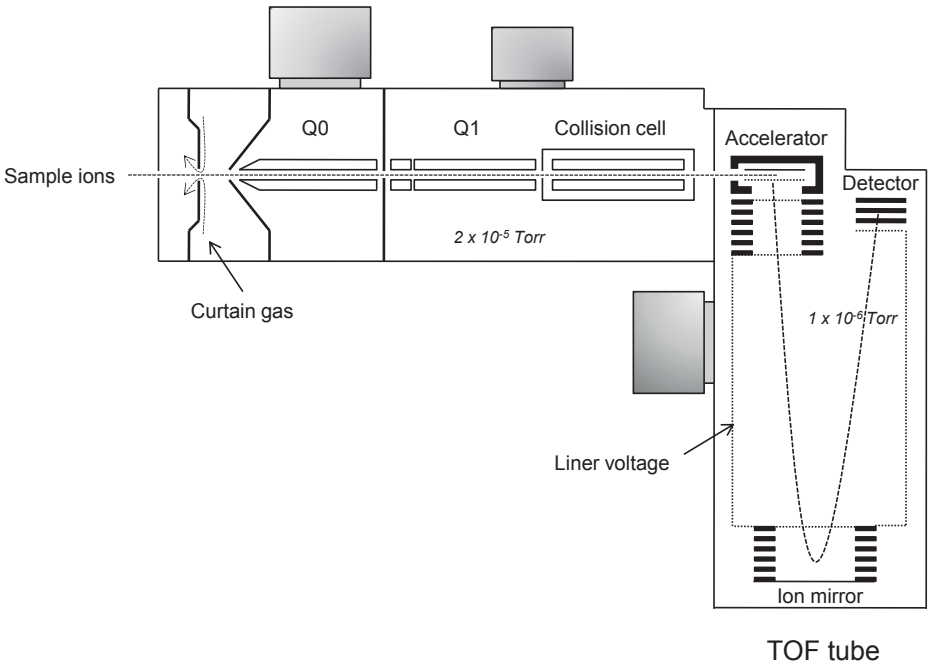


Figure 10. Overview of the QqTOF mass spectrometer.

Nano-ESI interface

For ordinary ESI sources the flow rates used for introducing the sample into the mass spectrometer are in the µl/min range. Efforts to reduce these flow rates have in recent years resulted in the development of several types of **nano-ESI** sources (nl/min range). Ideally, these ion sources benefit from increased sensitivity, primarily as a result of reduced droplet size in the ionization process. Reduced droplet size leads to an increased surface/volume ratio, which makes a large portion of the analytes available for desorption and subsequent ionization [184]. Another benefit of using nano-ESI is a reduced ion suppression, which can seriously hinder ordinary ESI [185]. Despite these advantages, the nano-ESI sources have not yet enjoyed the same success as their higher flow rate analogues [186], at least in the field of lipidomics. One reason for this is the lack of robustness of these sources, which are highly dependent on the quality and nature of the spray emitter for producing a stable spray. Other problems are that methods have been difficult to automate and often suffer from cross-contamination.

For the work included in this thesis, a static, chip-based nano-ESI interface (Nanomate; Advion BioSciences, Ithaca, NY), has been employed. This system, which is compatible with the 96-well system, uses conducing tips to aspirate the sample. The tip is then turned 90 degrees and docked with a 5µm nozzle in an ESI chip, positioned directly in front of the orifice of the mass spectrometer. With the aid of a gas pressure and high voltage, a fine spray is formed on the back of the chip. Using this interface, 5µl of sample can be infused during 30 minutes with a flow rate of approximately 150 nl/min. The low flow rate reduces ion suppression and increases the sensitivity of the analysis.

Approaches used for lipid characterization

As a consequence of the great diversity in structure, physicochemical properties, and relative abundance of different lipids, a number of methods and approaches for performing lipid-based MS are currently available. Furthermore, the field is expanding rapidly and several attempts have been made to create schemes for classification of the different approaches. However, this can often be confusing since a similar approach can be named differently by different research groups. Below, and in figure 11, I have outlined what I see as the most common approaches. In the following sections I will discuss their strengths, differences and usefulness for the analysis of different lipid classes.

Global vs. targeted lipidomics

Depending on the hypothesis and information known beforehand, the lipidomics approach can be either global (non-targeted), or targeted. This means that if there is much information known and the hypothesis addresses a specific aspect of the lipidome, a more precise and specific approach can be used. On the other hand, if not much is known in the context of expected lipidome, it may be reasonable to apply a global approach. Global lipidomics means unselectively mapping of the entire spectrum of lipids, including all molecular species, in a biological system [187]. This often starts with a Folch extraction to recover lipids with a wide range of polarity. Then analysis of the total lipid extract is made using HPLC (or UPLC) in combination with high accuracy mass spectrometers, such as TOF, Orbitrap or FTICR instruments [188-190]. The possible advantage of using this approach is that it allows the detection of previously unknown or poorly characterized lipids. These can then be identified in a complementary analysis, using a more targeted approach. The global approach generates substantial amounts of data and much effort has to be made in terms of data processing, such as peak detection, peak alignment and normalization. However, absolute quantification is a challenge due to the complexity of the analysis and lack of adequate standards.

In contrast, a targeted approach focuses on specific lipids or lipids classes. This means that selective MS methods, such as precursor ion and neutral loss scanning, can be used, which increases the sensitivity of the analysis. Using this definition, a targeted approach means everything from a simple SRM method quantifying shingosine-1-phosphate to profiling of several hundreds of triacylglycerols using neutral loss scanning. Using this approach, which is more common than a global approach, quantitative data for hundreds or lipids can be attained with high accuracy and precision. A targeted approach can be based on either HPLC/MS or shotgun methods, which are discussed below. In the work included in this thesis, all analyses are made with a targeted approach.

Shotgun- vs. HPLC-based lipidomics

While HPLC/MS-based methods uses chromatographic separation in combination with the MS analysis (on-line), shotgun techniques infuses the lipid mixture directly. The term shotgun lipidomics was coined by Han and Gross and first appeared in a peer-review paper in 2005 [191]. Since then several articles have been published describing important innovations in this type of lipidomics such as intrasource separation of lipids [192-196]. The strategy behind intrasource separation of lipids is to create an environment in the ion source that results in maximal ionization of the lipids of interest, while reducing this ionization of other lipids. Examples of parameters that can be adjusted, in order to attain

optimal ionization, are polarity of the electrospray, adduct formation, pH and declustering potentials.

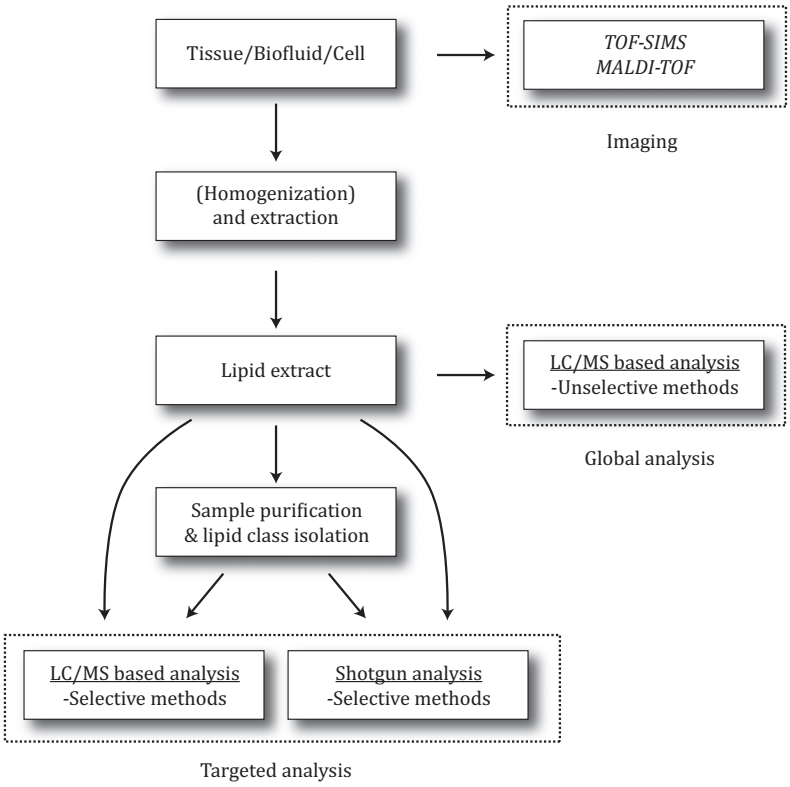


Figure 11. Workflow showing different strategies that can be used for performing lipidomics.

Although excellent for medium to high abundant lipid classes, shotgun lipidomics is rarely used directly for low abundant analytes such as ceramides and arachidonic acid derivates. Analyses of these substances are often performed by HPLC/MS-based methods. Alternatively, a purification step such as solid phase extraction, off-line HPLC or thin layer chromatography might be performed prior to the shotgun analysis. This has previously been shown for triacylglycerols [197], diacylglycerols [198], gangliosides [199] and ceramides [200]. In the work included in this thesis, diacylglycerols, ceramides and triacylglycerols (for LDL) were purified

using off-line HPLC prior to mass spectrometric analysis. Diacylglycerols and triacylglycerols were quantified using shotgun analysis while the ceramides were quantified using HPLC/MS/MS. Cholesteryl esters, free cholesterol, phosphatidylcholines, sphingomyelins and lyso-phosphatidylcholines were analyzed directly from the total lipid extract using a shotgun approach.

Imaging

During a traditional lipid analysis of tissue, no information is obtained about lipid localization. For this purpose, powerful techniques are now emerging capable of generating a two dimensional (and also three-dimensional) picture of the lipid distribution in both animal and plant tissue. The techniques most commonly used are matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [201] and time-of-flight secondary ion MS (TOF-SIMS) [202].

Ion suppression

Due to the complexity and relatively high concentration of lipids in the sample mixture the shotgun approach will be associated with some degree of ion suppression (charge competition in the ion source). For the critics of the shotgun approach, this is the major argument since it is suggested to hamper accurate quantification [203]. However, using the shotgun approach, the ion suppression is expected to be more constant during the analysis of each sample and furthermore, the ion suppression is lipid class dependant. This means that these effects will be corrected for by the use of one, or several, lipid class specific internal standards, which should be endogenously non-existing [166]. For a HPLC/MS-based analysis on the other hand, the ion suppression will vary during the chromatographic run due to the difference in the eluting mobile phase composition (for gradient HPLC/MS methods) and sample matrix. In other words, the signal intensity of different molecular lipids from the same lipid class will not only depend on the inherent physicochemical properties of the molecule, but also on the retention time. To ensure correct quantification, isotopically labeled internal standards have to be used for each molecular lipid, which in many applications is unrealistic. To conclude, both of these approaches suffer from ion suppression and, even though more substantial, ion suppression during shotgun lipidomics might be easier to control.

Methods used for lipid class profiling

In the papers included in this thesis the profiling of lipid classes (except for ceramides) have been performed using a shotgun approach on a hybrid QqTOF

instrument as described above. Furthermore, all analyses performed on the QqTOF instrument have utilized (multiple-) PIS. A short description of the settings and conditions used for the different lipid classes is presented below and in table 3.

The non-polar nature of **cholesteryl esters** make them hard to ionize using the ESI interface. However, in the presence of ammonium acetate (5 mM) they will form $[M+NH_4]^+$ adducts, which will generate a protonated cholestadiene fragment when exposed to CID [204-205]. This fragment was used for PIS according to earlier publications [205]. The deuterated cholesteryl-d_6-octadecanoate was used as internal standard. The high abundance of cholesteryl esters in both the VLDL and LDL fraction made it possible for analysis by the shotgun approach.

Ionization of **triacylglycerols** are also dependant on adduct formation. Ammonium or sodium adducts are common [206-208] but also lithium adducts have been employed successfully for the characterization of triacylglycerol isomers [209-210]. Due to the huge number of unique species that can be formed from common, naturally-occurring fatty acids, the complete characterization of triacylglycerols using MS is a challenge. Strategies used include both UPLC/MS methods [211-212]] and shotgun methods [197, 207, 209-210]. In the work presented in this thesis, triacylglycerols were characterized using multiple PIS by selecting 40 of the most common diacylglycerol fragments generated during CID. While direct analysis of VLDL triacylglycerols was feasible, triacylglycerols from LDL first required purification by HPLC.

Two articles have been published recently dealing with the shotgun analysis of **diacylglycerols** [198, 213]. Due to the low abundance of this lipid class, a purification step is necessary to reach the sensitivity required. In the work included in this thesis, diacylglycerols were purified using off-line HPLC. Similar to the triacylglycerols, specific fragments formed due to the loss of a fatty acid, are generated during CID of ammoniated diacylglycerols. These monoacyl fragments were used for PIS. For triacylglycerols and diacylglycerols, internal standards containing heptadecanoic (C17:0) fatty acids are used.

Lipoproteins have a very high relative abundance of **phosphatidylcholine**, which together with **sphingomyelin** constitutes the major part of the monolayer surrounding the hydrophobic core of the lipoproteins. Both of these lipid classes are easily ionized in positive mode, primarily due to their quaternary nitrogen situated in the choline headgroup [182, 214-215]. Upon CID, both phosphatidylcholine (and also **lyso-phosphatidylcholine**) and sphingomyelin generates a fragment due to the loss of the choline head group. Therefore these three lipid classes were profiled together in positive mode using PIS of *m/z* 184.1.

When performing PIS of phosphatidylcholines in positive mode, only the brutto composition is attained. This gives only limited information about the fatty acid composition. For example, the two isobaric phosphatidylcholine species PC 16:0/18:1 and PC 16:1/18:0 both give rise to a peak at *m/z* 760.5 using PIS in positive mode. Additional information of phosphatidylcholine lipid species was therefore attained using multiple PIS in negative mode [181-183]. In negative mode phosphatidylcholines are readably detected as acetate adducts. When exposed to CID these adduct ions generate several fragments, including individual fatty acid carboxylate ions. By taking advantage of the strengths of the QqTOF instrument, about 30 different fatty acid fragments were monitored simultaneously, allowing the identification of molecular lipids.

Due to the low abundance of **ceramides** in lipoprotein fractions they were not measured by shotgun analysis. Instead a HPLC/MS/MS method was employed. During CID of sphingosine-based ceramides, a characteristic fragment at m/z 264 is generated [216]. Using this product ion, an MRM method was developed for the quantification of the most common ceramides

Table 3. Summary of the analytical methods employed for the work included in my thesis

Lipid class	Analysis	Reference
Cholesteryl esters	+PIS *m/z* 369.3	204-205
Triacylglycerols	+MPIS [a]	166
Diacylglycerols	+MPIS [b]	166
Ceramides	+ MRM *m/z* 264.3	216
Phosphatidylcholines	+PIS *m/z* 184.1 -MPIS [c]	181-183
Sphingomyelins	+PIS *m/z* 184.1	182
Lyso-phosphatidylcholines	+PIS *m/z* 184.1	182

[a] Fragments released during CID of TAG-ammonium adducts selected for MPIS
[b] Fragments released during CID of DAG-ammonium adducts selected for MPIS
[c] Fatty acid fragments released from PC-acetate adducts selected for MPIS

Elucidation of double bond positions

As described above, information about the distribution of molecular lipids within a specific lipid class can readably be attained by shotgun lipidomics in combination with methods such as precursor ion- and neutral loss scanning. However, in most cases, conventional CID of lipids does not provide information about the position of existing double bonds. Therefore, when using this approach molecular lipids that differ only in the position of a double bond are indistinguishable [217-218]. Several mass spectrometric methods for elucidation of double bond positions have been demonstrated. However, many of them require derivatization [219], sufficient volatility of the analyte [220], isolated lipids or simple mixtures [218]. To overcome these limitations a new method based on the shotgun approach has been developed, whereby lipids are exposed to ozone vapor within an ion-trap mass spectrometer [221]. This induces ozonolysis at the position of the double bond, producing fragment ions with mass-to-charge ratios specific to the position of the double bond. Using this technique, termed ozone induced dissociation (OzID), in combination with normal CID, information about both fatty acid positions and double bond position can be attained. This technique was used in paper III for the characterization of double bond position in specific triacylglycerol molecular species.

Bioinformatics

When analyzing biological material with a lipidomics approach, it is not unusual to obtain data for several hundred, or even thousands, of molecular lipids. As a consequence, tools for handling and interpreting this data become a necessity to be able to take full advantage of the wealth of new information [222]. This field of bioinformatics plays important roles in several steps of the post-analytical evaluation of the data. The first step is to create lipid databases and methods for automated data processing. Or in other words, to convert raw-data files from the instrument into final lipid datasets. There are several software packages available for this. LipidView™ is registered by Applied Biosystems for use on data generated by their mass spectrometers. This software is an extension of the prototype LipidProfiler™, for which the details were published a couple of years ago [181]. There is other dedicated lipid softwares [223-224], and several of the common metabolomics data processing tools may also be used in lipidomics. Particularly MZmine [225] and XCMS [226], which are both open-source softwares, have been broadly applied.

Following the raw data processing and the generation of lipid datasets, additional tools are required for the statistical analysis and interpretation. Commonly used are relatively simple univariate tools such as t-tests, non-parametric Wilcoxon test and ANOVAs. These methods consider only one lipid at a time, with the

potential drawback of missing important information that might exists in complex correlation structures of lipidomics datasets. For these purposes, multivariate tools can be used, which include principal component analysis (PCA) and hierarchic clustering [222, 227].

In addition to the statistical tools used for indentifying specific differences between groups and finding possible biomarkers, it might be interesting to put the observed changes in relation to a biochemical or physiological context. The KEGG (http://www.genome.jp/kegg) database is a resource commonly used for these purposes and includes a collection of manually drawn pathway maps representing current knowledge about molecular interaction and reaction networks [228].

AIMS

The overall aim of this thesis was:

To isolate, and with a lipidomics approach, study atherogenic lipoproteins from type 2 diabetic patients in search for disease specific biomarkers that would increase our understanding of the disease.

The specific aims of the different studies were:

I. To develop a method for the isolation of VLDL, LDL and HDL using ultracentrifugation at physiological pH and ion strengths.

II. To identify proatherogenic properties of apoCIII containing LDL isolated from subjects with T2D.

III. To investigate whether dyslipidemia is required on top of insulin resistance to induce the compositional changes in lipoprotein lipids often associated with T2D.

RESULTS AND DISCUSSION

Paper I

In this paper a new method for isolating lipoproteins, using sequential ultracentrifugation, is described.

Preparation of lipoproteins using ultracentrifugation dates back to the mid 50s [229-230], and is still one of the most commonly applied methods. Traditionally potassium bromide (KBr) is used for density-adjustments during the ultracentrifugation procedure. However, the use of high amounts of KBr results in a supraphysiological ion strength, which might disrupt the binding of exchangeable apolipoproteins attached to surface of the lipoprotein particles [231-232]. Therefore, when studying apolipoproteins such as apoCIII and apoE, which are suggested to be important in the pathogenesis of cardiovascular disease, it is possible that ultracentrifugation using solutions with high ion strength introduce artifacts that might attenuate the importance of these proteins in the etiology of the disease. Therefore, the method developed uses a combination of D_2O/sucrose to acquire the densities necessary for isolation of VLDL, LDL and HDL, which occurs at physiological pH and ion strength. This method is an extension of a previous method for isolation of LDL using D_2O [233].

The purity of the lipoprotein fractions was tested using a combination of native and SDS gel electrophoresis. No contaminations of LDL or albumin were detected in the HDL fractions isolated with the two methods (**figure 2 and 3, paper I**).

Next the proteins were analyzed using 2D gel electrophoresis. The results revealed no significant differences between the VLDL fractions isolated with the two methods. For LDL and HDL on the other hand, the 2D gels containing D_2O/sucrose-isolated proteins showed more intense spots for several of the proteins. MALDI-TOF MS revealed that many of them were isoforms of α-1 anti-trypsin, apoE, apoCII and apoCIII (**figure 4 and 5, paper I**).

Results from the SELDI-TOF analysis further strengthen these results as the spectra from the D_2O/sucrose-isolated LDL and HDL show additional and more intense peaks both in the LDL and HDL fractions. Also in this case, the amounts of apoCII and apoCIII isoforms ($m/z \sim 9000$) were significantly different between the two methods (**figure 6 and 7, paper I**).

The major lipid classes (cholesteryl esters, triacylglycerols, free cholesterol and phosphatidylcholine) in the isolated lipoprotein fractions were quantified using normal-phase HPLC with ELS detection. No differences were observed between the two methods (**figure 8, paper I**). This confirmed that the two methods separate very similar density ranges of the lipoprotein spectrum. We did not investigate the effect of the method on chemically fragile lipids. One has to consider the possibility that the use of 30 degrees for the isolation of HDL, which is necessary due to the high viscosity of the solutions, might induce auto-oxidative and enzyme-mediated modifications of lipids containing poly-unsaturated fatty acids (e.g. linoleic- and arachidonic acid).

When using KBr for density adjustments, it is required that the isolated lipoprotein fractions are dialyzed prior to proteomics or cell experiments. An advantage of the described method is that the isolated lipoprotein fractions might be used directly without desalting. Furthermore, sucrose improves cryopreservation of the biological and physic-chemical integrity of the lipoproteins [234]. A limitation of the method is that only the major lipoprotein fractions are isolated. No isolation of LDL and HDL subclasses is achieved.

Paper II

In this paper we wanted to identify molecular mechanisms explaining the pro-atherogenic role of apoCIII in T2D subjects.

A mechanism possibly involved in the increased atherogenicity of apoCIII-containing LDL is an increased binding to artery wall proteoglycans [235]. To investigate this, we incubated apoCIII-rich and apoCIII-poor LDL from T2D subjects, with biglycan. Biglycan is considered to be one of the most important proteoglycans involved in the retention of lipoproteins in human arteries [36, 134-135]. The results showed that apoCIII-rich LDL had a higher affinity for the biglycan. However, complementary experiments with LDL, enriched with apoCIII *in vitro,* showed that this increase could only partly be explained by the apoCIII *per se* (**figure 1A, paper II**). These complementary experiments also showed that the proteoglycan binding was not linearly correlated to the amount of apoCIII molecules on the LDL particle. Whereas moderate levels increased the binding, supraphysiological levels of LDL-apoCIII actually inhibited biglycan binding (**figure 1B, paper II**). By performing the same experiment using recombinant LDL isolated from apoB transgenic mice, we could also show that the binding was mediated by the site A (residues 3148-3158) in the apoB molecule.

During the binding experiments described above, we concluded that although apoCIII *per se* did induce a small increase in biglycan binding, other intrinsic

properties have to be involved to fully explain the increased binding of LDL with an endogenously high apoCIII/apoB ratio. Therefore we performed a detailed lipid characterization of LDL isolated from T2D and control subjects. The results revealed that T2D subjects had reduced amounts of the membrane lipids free cholesterol, sphingomyelin, ceramide and the ganglioside GM1, compared to the control subjects (**figure 2, paper II**). We also showed that, only in the T2D subjects, where the amounts of these lipids negatively correlated to the apoCIII/apoB molar ratio. Furthermore, since the level of the most abundant membrane lipid, phosphatidylcholine, was unchanged, these results suggest that LDL isolated from T2D subjects has an altered lipid composition.

Free cholesterol, sphingomyelin and ceramide have all been shown to increase membrane rigidity [236-237]. Therefore, we suggest that the relative reduction in these lipids compared to phosphatidylcholine will result in an increased membrane fluidity, which will allow apoB to acquire a conformation that is more favorable for proteoglycan binding. In addition, reduced sphingomyelin levels might also increase the susceptibility of the LDL particle to hydrolysis by group IIA and group V $sPLA_2$ [238], two enzymes known to promote atherosclerosis [141, 239].

It is not known whether the high apoCIII levels modulate the membrane composition, or if the increased fluidity of diabetic LDL simply allows more apoCIII to bind. However, it has been suggested that apoCIII can activate sphingomyelinase [240], which is responsible for the hydrolytic conversion of sphingomyelin into ceramide. Increased ceramide content has been shown to induce particle aggregation, which is an important event in the pathogenesis of atherosclerosis [241]. We therefore analyzed the kinetics of sphingomyelinase-induced hydrolysis of LDL and the kinetics of particle aggregation. We showed that for LDL isolated from T2D subjects, enrichment with apoCIII in vitro increased the susceptibility of hydrolysis and aggregation by SMase (**figure 5, paper II**). Furthermore, it has been shown that the ganglioside GM1 is an inhibitor of SMase [242]. Since our results show that LDL isolated from T2D subjects has reduced levels of GM1, this could make them even more susceptible for SMase hydrolysis.

We then investigated the relative abundance of the different apoCIII isoforms. ApoCIII exists in three isoforms ($apoCIII_0$, $apoCIII_1$ and $apoCIII_2$) differing in the number of sialic acids bound to the protein [63]. We analyzed the distribution of different isoforms on LDL isolated from control subjects and subjects with T2D. For both groups, the results showed a positive correlation between the apoCIII/apoB ratio and the degree of sialylation. Furthermore, for all apoCIII/apoB ratios the T2D subjects had higher levels of the more sialylated form (**figure 6, paper II**).

Finally, in a cell experiment using human aortic endothelial cells (HAECs), we showed that increased apoCIII sialylation results in increased secretion of the proinflammatory mediators Il-6, Il-8, TNF-α and ICAM-1 (**figure 7, paper II**).

Taken together, the results from **paper II** highlights several important properties of apoCIII-containing LDL that adds to our understanding of its role in the pathogenesis of atherosclerosis.

Paper III

In this paper we wanted to investigate whether dyslipidemia is required on top of insulin resistance to induce the compositional changes in lipoprotein lipids often associated with T2D.

Dyslipidemia, a state of high triacylglycerols and low HDL, is often associated with T2D. This makes it hard to determine whether the changes in lipoprotein lipid composition often observed in T2D subjects are the result of the dyslipidemia or the insulin resistance *per se*. For example, in **paper II** we saw several changes in LDL isolated from T2D subjects. However, many of these subjects also had dyslipidemia (**table S3, paper II**). In an attempt to clarify the role of dyslipidemia versus insulin resistance for lipoprotein modification, VLDL and LDL from T2D subjects, with and without dyslipidemia, were characterized.

The lipid class composition of VLDL and LDL isolated from normolipidemic T2D subjects was very similar to control subjects (**figure 1, paper III**). The only difference was a slight decrease in lyso-phosphatidylcholine in LDL. The dyslipidemic T2D subjects, on the other hand, showed more pronounced differences with increased triacyl- and diacylglycerol together with reduced sphingomyelin in VLDL. In LDL there was an increase in diacylglycerol, while cholesteryl ester, ceramides, sphingomyelin and free cholesterol were reduced (**figure 1, paper III**). Phosphatidylcholine were not reduced resulting in an altered membrane composition similar to what we saw in paper II.

A closer investigation of the lipid species within each lipid class revealed that both T2D groups contained increased amount of PC 16:0-20:3 and PC 18:0-20:3 (**figure 2, paper III**). In the dyslipidemic group, PC 16:0-16:1 was also increased together with increased amount (both absolute and relative) of CE 20:3 and CE 16:1. Furthermore, the amount of C16:1 and C20:3 correlated extremely well between the two lipid classes, indicating that these two lipid classes are metabolically linked through the lecithin-cholesterol acyltransferase (LCAT). In the circulation, this enzyme is responsible for transesterification of cholesterol with the fatty acid from the *sn*-2 position of phospholipids [243]. It has been shown

previously that these fatty acids (C16:1 and C20:3), at least when hydrolyzed to phosphatidylcholines and cholesteryl esters, are related to T2D [244-245]. A possible explanation for this might be that the hyperinsulinemia in these subjects induces the expression of SREBP1c [246-247]. This in turn leads to the activation of Δ9 and Δ6 desaturases, which might result in an increased amount of C16:1 and C20:3 in both cholesteryl ester and phosphatidylcholine lipid classes [245, 248-249].

Since it has been shown that dyslipidemia is linked to an increased VLDL secretion and *de novo* lipogenesis, we hypothesized that this would induce an altered fatty acid composition of the VLDL triacylglycerols from the dyslipidemic subjects. Initially the fatty acid composition was measured as methyl esters with GC/flame ionization detector (FID). This showed an increased absolute and relative amount of palmitic acid in the dyslipidemic group, suggesting increased lipogenesis [250]. Further characterization of both the triacylglycerols and the diacylglycerols using multiple PIS identified several, specific lipid species containing palmitic acid that were elevated in the group with dyslipidemia (**figure 4, paper III**). The most prominent were TAG 16:0/16:0/16:0, TAG 16:0-16:0-18:1 and TAG 16:0-16:0-16:1. Similar patterns were observed in the diacylglycerols with DAG 16:0-16:0 and DAG 16:0-16:1 being significantly increased. From the raw data acquired during PIS, a fatty acid profile very similar to the one obtained from the GC/FID could be created (figure 12).

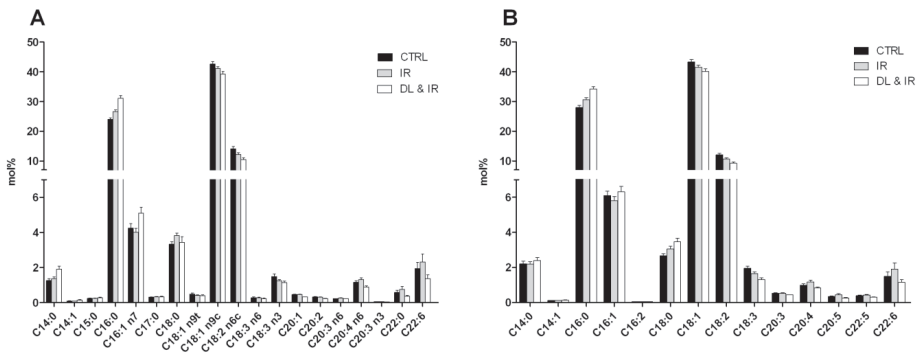


Figure 12. Fatty acid profile of VLDL triacylglycerols attained by (A) GC/FID or (B) QqTOF MS. For abbreviations and group characteristics, see methods in paper III.

Next we tested whether the significantly altered lipid species correlated with important clinical parameters such as BMI or HOMA index (**table 2 and 3, paper III**). Several of the cholesteryl ester and phosphatidylcholine species correlated well with many of the measured parameters, with PC 18:0-20:3 showing the strongest correlation. Kotronen et al. have recently shown that serum triacylglycerols, containing saturated fatty acids, are better markers of insulin resistance than total serum triacylglycerol concentration [251]. Our results show that several of the palmitate-containing triacylglycerols, especially TAG 16:0-16:0-18:1, correlate positively with insulin resistance. However, all correlations were weaker than correlations with total plasma triacylglycerols.

An increase in de novo lipogenesis would result in an increased production of palmitic acid. This will in turn activate desaturases perhaps in an attempt to transform the palmitic acid into the less toxic palmitoleic acid [252]. An increase in palmitoleic acids was seen both in the phosphatidylcholine and cholesteryl ester lipid class in the subjects with dyslipidemia. Furthermore, data from the GC/FID analysis showed that it was also increased in the VLDL triacylglycerols with borderline significance (p=0.05). Further metabolism of the palmitoleic acid into vaccenic acid (C18:1 n-7) is possible by *Elovl5*-mediated elongation [253]. However, analysis using the QqTOF instrument does not allow the determination of double bond position. Furthermore, vaccenic acid was not included in the standard mixture used for GC/FID analysis. To elucidate whether the increased abundance of palmitic acid results in increased amounts of vaccenic acid we used the novel OzID methodology [218, 221]. This allows the assignment of both the position (*sn*-1(3) or *sn*-2) of the fatty acids on the glycerol backbone and the double bond position of each fatty acid. By utilizing this methodology, the TAG 50:1 (50 carbons and 1 double bond in total on the three fatty acids) was characterized. The results revealed several findings. First, the TAG 50:1 almost exclusively consisted of the TAG 16:0-16:0-18:1. Secondly, the isomer TAG 16:0/18:1/16:0 was increased by about 10% in the two groups with T2D subjects (**figure 5, paper III**). Finally, in the dyslipidemic group, there was an increase in the abundance of vaccenic acid incorporated at the *sn*-2 position (**figure 6, paper III**).

It has previously been shown that VLDL, and especially VLDL from hypertriglyceridemic subjects, induces a pro-inflammatory response in cultured endothelial cells [254-255]. Even though it has been suggested that the composition of the particle is responsible for the induced response, the actual mechanisms are poorly understood. We wanted to test the hypothesis that the increased response, of VLDL from hypertriglyceridemic subjects, could be a result of increased palmitate in the VLDL triacylglycerols. We therefore incubated smooth muscle cells and endothelial cells with isolated VLDL containing high and low triacylglycerol palmitate (38±0.2% and 26±0.9% respectively). The result showed a highly significant increase in the secretion of several cytokines from the cells incubated with VLDL with high levels of palmitate. We cannot from these

results exclude the possibility that other intrinsic properties, such as protein composition or state of oxidation, could induce the observed response. However, there were no significant differences in any of the other measured lipid classes between the two groups.

In conclusion, VLDL and LDL from normolipidemic T2D subjects display some but rather modest changes in lipid composition compared to VLDL and LDL isolated from control subjects. In contrast, both lipoproteins display significant changes in dyslipidemic T2D subjects. Some of these are associated with inflammatory properties.

CONCLUDING REMARKS

In theory, the solution to the world epidemic of obesity and T2D is rather simple. It is all about reducing the energy intake and increasing the energy expenditure. In other words: eat less, exercise more. However, it is naive to believe that these recommendations would change the behavior of millions of people now accustomed to a sedentary lifestyle and quick access to palatable energy-dense food. Instead we are relying on the pharmaceutical and nutritional industries to provide alternative ways of relieving the burden. So in order to improve prevention and treatment of cardiovascular disease, which is often the cause of death in patients with obesity and T2D, a detailed understanding of the pathogenesis of atherosclerosis is needed. In the work included in this thesis the focus has been on dyslipidemia, which is a cardinal feature of both obesity and T2D. More specifically we wanted to link compositional changes in VLDL and LDL to the increased atherogenicity of these lipoproteins in patients with T2D. In order to do this, a lipidomics approach was used.

From our results we can conclude that both the protein and lipid composition of the particles are important for the increased atherogenicity. In **paper II** we see that apoCIII, which is a protein that has recently been recognized as a cardiovascular risk factor, is increased on LDL particles isolated from T2D subjects. In the paper we show that this is associated with increased binding of LDL to artery wall proteoglycans and increased susceptibility of the particle to SMase that drives the production of ceramides. Both are important events in the atherosclerotic process. We could also show that the sialylation of apoCIII is important for mediating an inflammatory response in cultured HAEC cells. However, the metabolism of apoCIII is complex and additional studies are needed to further clarify the role of apoCIII in atherosclerosis. Possible strategies would be to use tracer methods, possibly combined with immunoaffinity chromatography [125-126].

In both **paper II** and **paper III** we showed that VLDL and LDL isolated from T2D subjects with dyslipidemia had an altered lipid composition compared to the control subjects. We believe that this alteration is affecting the conformation of the apoB molecule making the particle more susceptible for proteoglycan interaction. However, the exact mechanism for the alteration in lipid composition remains to be determined.

The lipidomics analysis of VLDL and LDL, performed in **paper III,** revealed several interesting alterations occurring at the molecular level in the subjects with dyslipidemia. The amount of C16:1 and C20:3 were increased in several lipid classes, indicating alterations in desaturase activity. Furthermore, VLDL triacylglycerols were enriched in palmitate that induced a highly proinflammatory response when incubated with endothelial and smooth muscle cells.

By using the novel OzID-technique we took a step further towards the full characterization of lipoprotein lipids and showed that alterations might occur even at the level of double bond position. This highlights the importance of having powerful tools for indentifying potential biomarkers and to fully elucidate the mechanisms that are important in generating the atherogenic lipoproteins observed in patients with T2D.

LIPIDOMICS PERSPECTIVE

Although still in its infancy, lipidomics has already positioned itself as an important tool in biological research. The development of advanced mass spectrometric equipment in combination with selective fragmentation techniques, such as OzID, now enables us to reach far into the lipidome and acquire information about low abundant and structurally specific lipids. These tools will become crucial for further our understanding of how the molecular composition of the cellular lipidome affects important functions and maintains cell homeostasis. An example highlighting the importance of determining the molecular lipids was shown a few years back when Shinzawa-Itoh et al. showed that a specific phosphatidylglycerol molecule containing palmitic acid (on the *sn*-1 position) and vaccenic acid (on the *sn*-2 position) was needed for normal function of cytochrome c oxidase [256]. Another example has been shown for the survival of *C. elegans* exposed to anoxia. Specific molecular sphingolipids containing C24 and C26 carbons were associated with death, while molecular lipids with shorter chains were involved in survival [257]. In fact, researchers in the field of sphingolipids are probably one of the groups that will benefit the most from the advances in MS. So far, a comprehensive characterization of this group of highly interesting and multifaceted group of lipids has been limited due to the structural complexity of the lipid class and the lack of analytical power [156, 258]. Hence, as the field of lipidomics progresses we can expect many interesting discoveries in this field in the years to come

Despite predictions of future success, one has to remember that lipidomics is not just chemistry alone. It's becoming increasingly clear that just measuring hundreds, or even thousands, of lipids within a cell might not be that informative, unless we can localize the lipids to the subcellular compartments [258-259]. This means that either we find efficient ways of isolating the sub-cellular compartments or we develop imaging techniques with subcellular resolution. At the moment TOF-SIMS can reach nanometer-scale resolution but suffers from problems with relatively low sensitivity and extensive fragmentation during the ionization process. However, if new instruments can be developed where these issues have been resolved, this technique has great potential of becoming an important complement in the analytical toolbox within the field of lipidomics.

Another aspect that needs to be considered, when charting the abundance of hundreds of molecular lipids, is that the concentration of certain lipids is highly dynamic, changing rapidly within several orders of magnitude during certain stimuli. However, what is often the result of an experiment is a snapshot of the biological tissue, cell or biofluid that is being investigated. Therefore, important information about kinetic parameters and fluxes within the biological system will be overlooked. An interesting way to unravel these questions might be to use tracer lipidomics, which is a branch of lipidomics in where labeled lipids (often with stable isotopes) are followed during time.

Even though it is clear that lipidomics can add another dimension to our understanding of common diseases, it remains to be seen whether it will take the step into a more clinical setting. If it did, it would probably open up new sources of information as lipidomics screens of patients are likely to give a wider understanding how specific diseases will cause "ripple effects" through the lipidome [260]. Also in the pharmaceutical industry lipidomics might have a place both in preclinical and clinical settings. To this end, lipidomics has been shown to provide information about efficacy profiles of different compounds within a specific drug class [261-262] in addition to functioning as a tool for identifying adverse drug effects, such as muscle and liver toxicity [261, 263]. It is also tempting to believe that in the era of personalized medicine, it could provide useful information during clinical trials about responders and non-responders.

To conclude, since lipids play important roles in a vast number of cellular processes and the fact that dysregulation of lipid metabolism is associated with many different and severe diseases, it is not bold to predict that lipidomics will continue to be a major resource in many scientific and medical fields in the years to come.

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