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LIPOPROTEOMICS

A New Approach to the Identification and Characterization
of Proteins in LDL and HDL

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*To my parents Inger and Åke:
If you hadn't been there
taking care of my sweet boys,
this would never have happened.*

*To my dearest boys Tom and Piero:
I hope there will be a day
when you say you are happy it did.*

Abstract

A proteomic approach was applied to examine the protein composition of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in humans. LDL and HDL were isolated by density gradient ultracentrifugation, and proteins were separated with two-dimensional gel electrophoresis (2-DE) and identified with peptide mass fingerprinting, using matrix-assisted laser desorption/ionization-time of flight mass spectrometry, and with amino acid sequencing using electrospray ionization tandem mass spectrometry. To improve the identification of low abundant proteins in silver stained 2-DE gels, 2,5-dihydroxybenzoic acid was used instead of α -cyano-4-hydroxycinnamic acid as matrix in the peptide mass fingerprinting procedure; this was demonstrated to give more matching peptide peaks, higher sequence coverage, and higher signal to noise ratio. Altogether 18 different proteins were demonstrated in LDL and/or HDL: three of these (calgranulin A, lysozyme C and transthyretin) have not been identified in LDL before. Apo C-II, apo C-III, apo E, apo A-I, apo A-IV, apo J, apo M, serum amyloid A-IV and α_1 -antitrypsin were found in both LDL and HDL, while apo B-100 (clone), calgranulin A, lysozyme C and transthyretin were found only in LDL, and apo A-II, apo C-I, and serum amyloid A only in HDL. Salivary α -amylase was identified only in HDL₂, and apo L and glycosylated apo A-II only in HDL₃. Many of the proteins occurred in a number of isoforms: in all, 47 different isoform identities were demonstrated. A 2-DE mobility shift assay and deglycosylation experiments were used to demonstrate, for the first time, that apo M in LDL and HDL occurs in five isoforms; three that are both *N*-glycosylated and sialylated, one that is *N*-glycosylated but not sialylated and one that is neither *N*-glycosylated nor sialylated. LDL from obese subjects was found to contain more apo J, apo C-II, apo M, α_1 -antitrypsin and serum amyloid A-IV than LDL from controls, and also more of an acidic isoform (pI/M_r ; 5.2 / 23 100) of apo A-I. In addition, the new LDL-associated protein transthyretin, was found to be significantly more abundant in LDL from obese subjects. On the other hand, the amounts of apo A-IV and the major isoform of apo A-I (pI/M_r ; 5.3 / 23 100) were significantly less. Altogether, these findings (i) illustrate the power of 2-DE and mass spectrometry for detailed mapping of the proteins and their isoforms in human lipoproteins; (ii) demonstrate the presence of a number of new proteins in LDL (calgranulin A, lysozyme C and transthyretin); (iii) give precise biochemical clues to the polymorphism of apo M in LDL and HDL, and (iv) indicate that obesity is associated with

significant changes in the protein profile of LDL. It is concluded that new information on lipoproteins can easily be obtained through a proteomic approach, thus facilitating the development of a new proteomic field: lipoproteomics. Much further investigation in this field is warranted, particularly because newly discovered LDL and HDL proteins may play hitherto unknown role(s) in inflammatory reactions of the arterial wall and evolve as useful biomarkers in cardiovascular disease.

Preface

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

- I Karlsson H, Leanderson P, Tagesson C and Lindahl M. Lipoproteomics I: Mapping of proteins in low-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics*, 2005, 5; 551-565.
- II Karlsson H, Leanderson P, Tagesson C and Lindahl M. Lipoproteomics II: Mapping of proteins in high-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics*, 2005, 5; 1431-1445.
- III Ghafouri B, Karlsson H, Lewander A, Tagesson C, and Lindahl M. Peptide mass fingerprint data from silver stained proteins can be improved by using 2,5-dihydroxybenzoic acid instead of α -cyano-4-hydroxycinnamic acid as matrix in MALDI-TOF MS. 2007, *Submitted*.
- IV Karlsson H, Lindqvist H, Tagesson C, and Lindahl M. Characterization of apolipoprotein M isoforms in low-density lipoprotein. *J. Proteome Res.* 2006, 10; 2685-2690.
- V Karlsson H, Lindqvist H, Tagesson C, and Lindahl M. Comparative proteomics of low-density lipoprotein from normal weight and obese adults. 2007, *Manuscript*.

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1. Abbreviations

ACAT	acyl CoA: cholesterol acyltransferase
ACN	acetonitrile
BMI	body mass index
CETP	cholesterol ester transfer protein
CHCA	α -cyano-4-hydroxy-cinnamic acid
CHD	coronary heart disease
CID	collision induced dissociation
CVD	cardiovascular disease
DC	direct current
1-DE	one-dimensional gel electrophoresis
2-DE	two-dimensional gel electrophoresis
DHB	dihydroxybenzoic acid
ESI MS	electrospray ionization mass spectrometry
HDL	high-density lipoprotein
HL	hepatic lipase
HMG CoA	3-hydroxy-3-methylglutaryl-Coenzyme A
HUPO	Human Proteome Organisation (2001)
IDL	intermediate density lipoprotein
LCAT	lecitin: cholesterol acyl transferase
LDL	low-density lipoprotein
LPL	lipoprotein lipase
MALDI TOF MS	matrix assisted laser desorption/ionization time of flight mass spectrometry
PBS	phosphate buffer saline
PAF-AH	platelet activating factor acetyl hydrolase
PLA₂	phospholipase A ₂
PAGE	polyacrylamide gel electrophoresis
PG	proteoglycan
PMF	peptide mass fingerprinting
PON-1	paraoxonase-1
PTM	post-translational modification

RF	radio frequency
sdLDL	small dense low-density lipoprotein
SDS	sodium dodecyl sulphate
TBE	tris-borate EDTA buffer
TFA	trifluoroacetic acid
UC	ultracentrifugation
VLDL	very low density lipoprotein

2. Terms and definitions

Alternative splicing is the process that occurs in eukaryotes in which the splicing process of a pre-mRNA transcribed from one gene can lead to different mature mRNA molecules and therefore to different proteins.

Amphoteric: A substance that can react as either an acid or base. Amino acids and thereby proteins are amphoteric.

Atherogenesis: Formation of atheromas on the walls of the arteries as in atherosclerosis.

Bioinformatics: Involves the use of techniques including applied mathematics, statistics, informatics, computer science, artificial intelligence and biochemistry to solve a biological problem, usually on the molecular level.

Biomarker: A substance used as an indicator of a biologic state.

Cardiovascular disease (CVD): Disease affecting the heart or blood vessels. Cardiovascular diseases include arteriosclerosis, coronary artery disease, heart valve disease, arrhythmia, heart failure, hypertension, orthostatic hypotension, shock, endocarditis, diseases of the aorta and its branches, disorders of the peripheral vascular system, and congenital heart disease.

2-dimensional polyacrylamide gel electrophoresis (2-D PAGE): This technique is used to separate mixtures of proteins, and is particularly useful for comparing related samples such as healthy and diseased tissue. Proteins are separated according to charge (pI) by isoelectric focusing (IEF) in the first dimension and according to their relative molecular mass (M_r) in the second dimension.

Dalton: The unified atomic mass unit (u) or Dalton (Da) is a small unit of mass used to express atomic and molecular masses. It is defined to be 1/12 of the mass of an unbound atom of the carbon-12 nuclide, at rest or ground state.

Dyslipidemia: A disruption in the amount of lipids in the blood

Electrospray ionization (ESI): A method of forming gas ions from a solution of molecules. The solution is sprayed into a vacuum through a sharp needle to which a high voltage is applied prior to its introduction into the mass spectrometer.

Genome: The genome of an organism is its whole hereditary information and is encoded in the DNA (or for some viruses RNA).

Immune response: The immune system is a set of mechanisms that protect an organism from infection by identifying and killing pathogens.

Immobilized pH gradient (IPG): Refers to a plastic-backed isoelectric focusing strip with an immobilized pH gradient. Separate proteins according to their isoelectric point in gel electrophoresis.

Mass spectrometry: An analytical technique used to measure the mass to charge ratio of ions. It is most generally used to find the composition of a physical sample by generating a mass spectrum representing the masses of sample components.

Mass to charge ratio (m/z): The three-character symbol m/z is used to denote the dimensionless quantity formed by dividing the mass of an ion in unified atomic mass units (Dalton) by its charge number.

Matrix assisted laser desorption ionization (MALDI): An ionization source that generates ions by desorbing them from solid matrix material with a pulsed laser beam.

Mass analyser: Separates mixtures of ions by the mass to charge ratios. Examples are quadrupole or time of flight (TOF).

MS/MS: A technique that can be used for analyses of protein sequences. Two mass analyzers are linked in tandem so that the first mass spectrometer is used to select ions of a particular m/z value which then pass into the collision chamber and the second mass spectrometer determines the masses of the fragments.

Metabolic syndrome: A combination of medical disorders such as impaired glucose tolerance, high fasting glucose, insulin resistance, high blood pressure, dyslipidemia and central obesity that increase the risk for cardiovascular disease and diabetes.

Proteome: The term was coined by Mark Wilkins in 1995 and is used to describe the entire complement of proteins in a given biological organism or system at a given time, i.e. the protein products of the genome.

3. Introduction

3.1 Proteomics

Proteomics can be described as the qualitative and quantitative comparison of proteomes under different conditions to investigate biological processes. The proteome refers to all the proteins produced by an organism. The word is derived from PROTEins and genOME, since proteins are expressed by the genome. There are fewer protein-coding genes in the human genome than there are proteins in the human proteome (~20,000 to 25,000 genes vs. ~1,000,000 proteins). This is explained by post-translational modifications [Mann 2003] of many proteins or that RNA can be alternatively spliced [Abelson1998]. The proteome (in contrast to the genome) differs from cell to cell and is constantly changing during the life time through its interactions with the genome and the environment. To be able to learn more about these mechanisms, the search for biomarkers (proteins used as indicators of a biologic state) has become a challenging goal for scientists. An international collaboration is being coordinated by the Human Proteome Organisation (HUPO) with the purpose of achieving maps of all human proteins and revealing their functions and interactions.

Proteins can be studied in various perspectives and the choice of technique in proteomic research is dependent on the aim. Specialized methods, such as phosphoproteomics and glycoproteomics, have been developed to study proteins with post-translational modifications. For protein separation of complex mixtures, 2-D gel electrophoresis is highly recommended. 2-DE separates proteins in two dimensions according to their isoelectric point and molecular weight. Protein spots in a gel can be visualized using chemical stains or fluorescent markers and they can also be quantified by the intensity of their stain, which is an advantage in comparative studies. For identification, peptides can be recovered from in-gel digested proteins [Shevchenko 2002] and identified by mass spectrometry. Protein mixtures can also be analyzed without prior separation. If the sample is extremely complex or contains large (>250 kDa) or hydrophobic proteins it may be suitable to use liquid chromatography. This procedure begins with proteolytic digestion of the proteins. The resulting peptides are then often injected onto a high-pressure liquid chromatography column (HPLC), which separates peptides based on hydrophobicity, coupled directly to a mass spectrometer. Furthermore, if the aim is to capture proteins that interact with one another or a specific surface, suitable techniques could be protein immunoaffinity chromatography or protein microarray. Surface-

enhanced laser desorption and ionization (SELDI) is a technique that combines array separation with mass spectrometry. Mass spectrometers are important instruments in most chemistry laboratories. In earlier times, only small molecules could be identified but in 2002, Koichi Tanaka (Japan) and John Fenn (USA) received the Nobel Prize for the development of soft desorption ionization methods for mass spectrometric analyses of biological macromolecules to identify and reveal the structures of such molecules. The method developed by Tanaka, and during the same time by Karas and Hillenkamp, is known as matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry; a laser beam induces transfer of proteins or peptides from a solid phase into the gas phase before entering the TOF section. The method by Fenn is known as electrospray ionization (ESI) mass spectrometry; proteins or peptides are transferred into gas phase from a liquid phase through a high-voltage spray. The data received from the mass spectrometers are then interpreted with the help of bioinformatic tools available in proteomic databases such as the UniProt Knowledgebase <http://expasy.org/sprot/>. The term “lipoproteomics“ is hereby used to describe how these different techniques can be used as a new approach to characterize lipoprotein particles.

3.2 Lipoprotein particles

A wide variety of body tissues makes demands on the plasma lipid pool; triglycerides are an important energy source for cardiac and skeletal muscle, phospholipids are important components of the cell membrane and cholesterol is used as precursor to hormones and bile acids. Since lipids are poorly soluble in water their extracellular transport is performed as lipoprotein particles, which are mainly synthesized in the intestine and the liver. The structure of a lipoprotein particle resembles that of a cell membrane but consists of a monolayer instead of a bilayer. The hydrophilic part of the phospholipids, the hydroxyl group of the free cholesterol and the proteins are orientated at the surface of the particle while the hydrophobic components, the cholesteryl esters and the triglycerides are present in the middle (Figure 1).

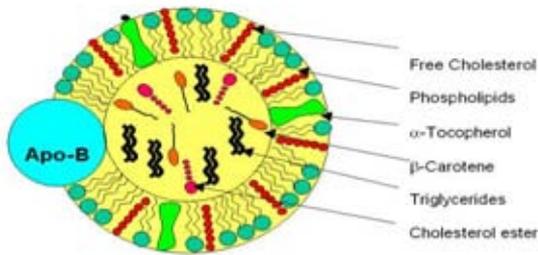


Figure 1. Schematic view of LDL (Sattler W. -94)

The lipoprotein transport system exists to deliver these hydrophobic compounds through the aqueous medium of blood plasma to cells in a directed and regulated manner [Packard 1999]. Most cells have only limited capacity to store cholesterol and triglycerides and take steps, like down regulation of specific receptors, to limit the intake when having sufficient stores. The body does not possess feedback mechanisms to inhibit intestinal absorption however, and excessive fat intake leads to the accumulation of lipids in the circulation and may thereby lead to pathological consequences, with cholesterol in particular being deposited in the blood-vessel walls. Lipoprotein particles are divided into several different classes according to density, electrophoretic mobility and apolipoprotein composition (Table 1).

Table 1. Characteristics of lipoprotein particles (Pownall, H.J and Gotto, J.R -99).

	Chylomicrons	VLDL	IDL	LDL	HDL₂	HDL₃
Electrophoretic						
mobility	α_2	pre- β	slow-pre β	β	α_1	α_1
Density (g/ml)	> 0,93	0,95-1,006	1,006-1,019	1,019-1,063	1,063-1.125	1.125-1.212
Diameter (Å)	> 800	300-800	250-350	216	100	75
Composition (% of dry mass)						
Proteins	2	8	19	22	40	55
Phospholipids	7	18	19	22	33	25
Triglycerides	86	55	23	6	5	3
Cholesterol esters	3	12	29	42	17	13
Free cholesterol	2	7	9	8	5	4

3.2.1 Chylomicrons

Chylomicrons are described as triglyceride-rich lipoproteins that contain apolipoprotein B-48. Within the enterocyte, triglycerides are regenerated from free fatty acids and partial glycerides and most of the cholesterol is esterified by the action of membrane-bound acyl coenzyme A; cholesterol acyltransferase (ACAT) [Packard 1999]. Apo B-48 is synthesised in the enterocyte and its presence is essential for the intracellular assembly of the particle [Hussain 1996]. The chylomicrons are secreted by the enterocytes and reach the circulation via the lymph system. In the circulation other apolipoproteins such as apo C-I, C-II, C-III and E are added to the chylomicron surface. These proteins are freely exchangeable and in competition for equivalent binding sites on the particle surface, associating through hydrophobic interactions with the lipid droplet as a consequence of their amphipatic properties. After entering the blood the chylomicrons rapidly becomes the target of the enzyme lipoprotein lipase (LPL), acting at the endothelial surface of capillaries in sites such as adipose and muscle tissue. Together with its cofactor apo C-II, they are the most important factors for chylomicron triglyceride clearance. At the same time, as the triglycerides are hydrolyzed, the

chylomicrons release phospholipids, apoproteins and free cholesterol, resulting in a chylomicron “remnant” particle containing apo E and B-48, proteins that are recognized by the chylomicron remnant receptor in the liver [Redgrave1999].

3.2.2 *Very Low-Density Lipoprotein (VLDL)*

Apo B-100 is required for the initial assembly of VLDL in the hepatocytes. Besides B-100, the particle also consists of E and C apolipoproteins [Ginsberg 1999]. VLDL is the main transport form for triglycerides and cholesteryl esters from the liver to the tissue. Apo C-II activates LPL and thereby the hydrolysis of triglycerides in VLDL. Large consumers of triglycerides are cardiac, adipose and muscle tissue. After the hydrolyzing step the VLDL particle changes in density and composition and now contains fewer triglycerides, one apo B-100 and multiple copies of apo E [Pownall 1999]. This particle is defined as intermediate-density lipoprotein.

3.2.3 *Intermediate-Density Lipoprotein (IDL)*

Relatively little is known about IDL. The density of IDL is between the density of VLDL and LDL and it contains apo B-100 and apo E. IDL can either be recognized by the LDL receptor or the LDL-related protein receptor (LRP) for cellular uptake. Alternatively, it can be further hydrolyzed by hepatic lipase, which hydrolyzes both triglycerides and phospholipids, and the particle then reaches the density interval of low-density lipoprotein.

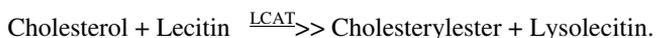
3.2.4 *Low-Density Lipoprotein (LDL)*

LDL, the product of IDL hydrolysis, is the main distributor of cholesteryl esters from the liver to the tissue and also the main lipoprotein fraction involved in atherogenesis (Figure 2). LDL synthesis is influenced by diet, drugs and genetic variation but the catabolic steps follow regulated mechanisms, which include the LDL receptor mediated uptake and the macrophage scavenger activity [Packard 1999]. Most cells have LDL receptors, but the majority is located in the liver and excess cholesterol goes back to the liver for storage or bile production. The ligand for the LDL receptor and the dominating protein in LDL is apo B-100, even if other minor components such as apo E [Sattler 1994], apo M [Duan 2001], sPLA₂ and PAF-AH [Flood 2004, Chait 2005] have been described. Cellular uptake is performed by endocytosis of the lipoprotein-receptor complex and in lysosomes the cholesteryl esters and the proteins

are degraded into free cholesterol and amino acids. The expression of the LDL receptors is regulated by the intracellular cholesterol levels, which means that when a cell has sufficient sterol for membrane synthesis, sex hormone or bile production, the transcription of the LDL receptors are down-regulated. Within the cell, the cholesterol biosynthesis includes the formation of mevalonate from 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA). This reaction is the rate-limiting step and is catalyzed by the enzyme HMG CoA reductase. Hypolipidaemic drugs such as statins inhibit the action of HMG CoA-reductase [Bernini 1999] and thereby inhibit the cholesterol biosynthesis and the down-regulation of LDL receptors.

3.2.5 High-Density Lipoprotein (HDL)

Unlike other plasma lipoproteins, HDL is not made as a spherical, mature lipoprotein. The initial HDL, the precursor HDL, is a discoidal structure made of phospholipid bilayer and has at least two sources: nascent discoidal structures secreted from the liver and the intestine and surface remnants generated during lipolysis of triglyceride-rich lipoproteins [Eisenberg 1999]. Based on density, different subclasses of HDL have been described. Most abundant in plasma are the less dense HDL₂ and the denser HDL₃. Apo A-I is the dominating protein in HDL but several other apolipoproteins such as apo A-II, A-IV, apo D apo E, apo C-I, apo C-II, apo C-III [Eisenberg 1999], apo J [Calero 2000], apo M [Duan 2001] and acute phase reactants [Ducret 1996, Artl 2000] have also been described. In the circulation, HDL is exposed to the action of enzymes and lipid transfer proteins during the interaction with cells in the vascular bed and other lipoproteins. HDL is referred to as the “good” cholesterol by its involvement in the reversed cholesterol transport (Figure 2). This term is used to describe the transport of cholesterol from peripheral tissues transferred via lipoproteins to the liver for either recycling or excretion from the body as bile acid [Barter 1999]. In the short term, apo A-I and apo A-IV interact with the cell surface and promote cholesterol efflux and lecithin cholesterol acyltransferase (LCAT) activation. LCAT catalyses the reaction:



Then, lysolecitin associates to albumin while the cholesteryl ester is stored in the inner hydrophobic part of the HDL particle. Cholesterol ester transfer protein (CETP), another transfer protein in HDL, mediates the transfer of cholesteryl esters from HDL to the VLDL/LDL fractions for LDL receptor-mediated uptake [Barter 1999] in exchange for

triglycerides. Another protein of interest is paraoxonase: this protein contributes to the anti-atherogenic role of HDL by decreasing the levels of lipid peroxides generated within LDL during oxidation and hence catalyzing their removal [Suckling 1999]. The strong inverse correlation between plasma HDL cholesterol levels and coronary artery disease [Eisenberg 1999, Franceschini 2001] is largely explained by the role of HDL in the reversed cholesterol transport, but the cardio-protective properties have also been attributed to anti-inflammatory effects of the lipoprotein particles [Viles-Gonzalez 2003, Fan 2003].

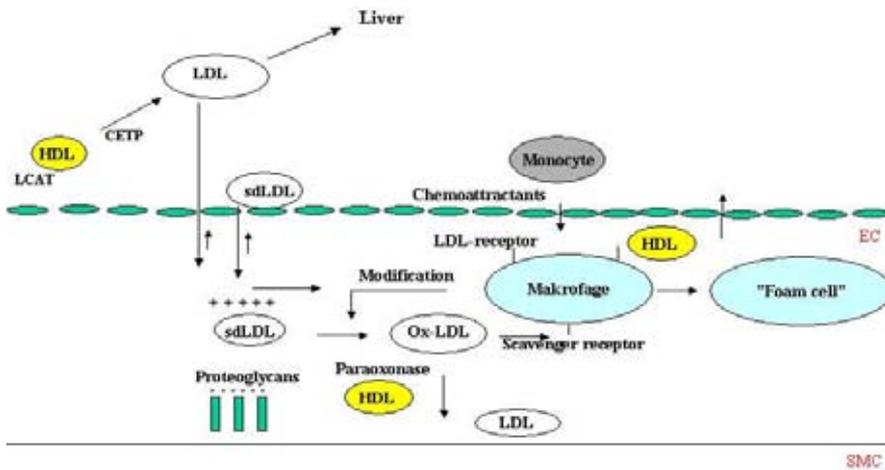


Figure 2. Suggested atherosclerotic mechanisms in the arterial wall.

3.3 Atherosclerosis

Atherosclerosis is a cardiovascular disease (CVD), which is considered to be the most common cause of death in Sweden and other developed countries [The National Board 2006, Mathers 2001]. According to the National Heart Lung and Blood Institute atherosclerosis is a slow, progressive disease that may start in childhood and includes the hardening and narrowing of the arteries. The initiation of atherosclerosis has been debated for many years. One of the earliest hypotheses was the response to injury hypothesis when Ross and colleagues hypothesized that atherosclerosis occurs in response to localized injury to the

lining of the artery wall which may cause a proliferation of smooth muscles. Triggering agents could be high cholesterol levels, smoking, diabetes, or oxidized fatty acids [Ross 1977]. This hypothesis was followed by the response to oxidation hypothesis, reported by Hessler and colleagues in 1979, suggesting that oxidation of LDL generates products such as cholesterol hydroperoxides that are injurious to the artery wall. In 1995, based on previous results according to the interaction between lipoproteins and the arterial wall by among others, Camejo and colleagues in 1985, the response to retention hypothesis was reinforced by Williams *et al.* This hypothesis supports an important role for modified lipoproteins in early atherosclerosis by subendothelial retention. Once retained, these lipoproteins provoke responses that lead to disease in a previously unaffected artery.

3.3.1 Risk factors

Apart from positive family history or infection [Korner 1999], there are several classical risk factors such as physical inactivity, smoking, high blood pressure, and dyslipidemia. One topical risk factor is the so-called western lifestyle, characterized by high intake of energy in combination with low physical activity. This behavior is believed to be the main cause of the increased prevalence of overweight and obesity in industrialized countries, conditions that are closely linked to the development of cardiovascular events. A cluster of risk factors called the metabolic syndrome has been defined for prediction of the development of CVD. The World Health Organization criteria (1999) require presence of diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin resistance in combination with two of the following: blood pressure $\geq 140/90$ mmHg, dyslipidaemia (triglycerides, ≥ 1.69 mmol/L and/or high-density lipoprotein cholesterol ≤ 0.9 mmol/L (male), ≤ 1.0 mmol/L (female)), central obesity waist: hip ratio > 0.90 (male), > 0.85 (female), BMI > 30 kg/m² or micro-albuminuria. In addition, according to the American Heart Association high levels of LDL-cholesterol is a risk factor: they recommend a total blood cholesterol level (LDL and HDL cholesterol) less than 200 mg/dL.

3.3.2 Hypercholesterolemia in atherogenesis

Hypercholesterolemia is defined as the presence of high levels of cholesterol in the blood. This condition can be of genetic origin caused by disturbed cholesterol metabolism but is most common in relation to over-nutrition. Longstanding hypercholesterolemia leads to

accelerated atherosclerosis, *xanthoma* (thickening of tendons due to accumulation of cholesterol) and a number of other complications in connection with the depositing of cholesterol excess. It is important to distinguish the LDL cholesterol from the HDL cholesterol. While LDL is a risk factor for atherosclerosis, HDL is not and high HDL levels might even be favorable as this reflects an effective reverse cholesterol transport. High levels of LDL cholesterol in the circulation lead to prolonged retention time of lipoprotein particles and thereby increased risk of exposure to several hydrolyzing agents such as hepatic lipase (HL), lipoprotein lipase (LPL) [Ginsberg 1999] or secretory phospholipase A₂ (sPLA₂) [Camejo 2000, Flood 2004]. This may lead to the formation of a smaller and denser LDL particle, which is considered to be more atherogenic. Small dense LDL (sdLDL) is also more likely to originate from triglyceride-rich larger VLDL particles (VLDL1) than the normal VLDL (VLDL 2). This triglyceride-rich particle is often seen in insulin-resistant subjects [Magnusson 2006]. Altered lipid content in sdLDL appears to induce changes in the conformation of apolipoprotein B-100, leading to increased exposure of proteoglycan-binding regions and these conformational changes may be the reason for the high-affinity binding of sdLDL to arterial proteoglycans (PG) that has been observed [Boren 1998]. LDL bound to PGs seems to be more susceptible to oxidation [Steinberg 2002] and it is well known that oxidized LDL is taken up avidly by macrophages and induces foam cell formation.

Several biological effects of ox-LDL have been described, such as, release of monocyte chemoattractant proteins, cytotoxic effects on endothelial cells and mitogenic effect on macrophages and smooth muscle cells [Steinberg 2002]. There are different receptors on the surface of the arterial tissue macrophage. The native LDL receptor (LDLR), which is down regulated when the levels of native LDL are high, and the ox-LDL scavenger receptor (CD36), which is not down regulated [Itabe 2003]. The uptake of modified LDL particles by the scavenger receptors, with accumulation of cholesterol, is a crucial step since the foam cells are triggers of many events seen in developing lesions. The vessel response on lesion development and plaque progression includes increased vessel wall volume, increased shear stress and thereby further luminal narrowing. These events might then progress into plaque instability, rupture and thrombosis.

3.3.3 Inflammation in atherogenesis

Hypercholesterolemia and ox-LDL are prime candidates for initiating and sustaining the pathological processes underlying atherosclerosis and much interest is focused on monocyte-driven inflammation and its role in pathological lipid accumulation in the arterial wall and the risk of thrombosis [Steinberg 2002, Mertens 2001]. But enzymatic non-oxidative degradation of LDL has also been proposed to initiate atherosclerosis. Thus, the activity of trypsin, neuraminidase and cholesterol esterase has been shown to create an atherogenic LDL particle (E-LDL), avidly taken up by macrophages and, in contrast to ox-LDL, also able to activate complement [Bhakti 1998 Torzewski 1998]. Another aspect that lately has received increased attention is the possibility that bacterial infections may cause inflammatory responses in the arterial wall that may contribute to atherosclerosis. For example, the gram-negative *Chlamydia pneumoniae* has been found in atherosclerotic plaque [Korner 1999]. This respiratory pathogen contains proteolytic enzymes that are able to degrade apo B-100 in LDL [Hashimoto 2006] and chronic dental infections caused by *Porphyromonas gingivalis* have been suggested as a potential risk factor for the development of atherosclerosis [Beck 2005].

It has been observed that lipoproteins are affected by infection /inflammation; the bile production is decreased and thereby the excretion of excess cholesterol, and the triglyceride-rich lipoprotein levels are increased so that they are able to decrease the toxicity of a variety of harmful biological and chemical agents [Hardardottir 1995]. Interestingly, lipoprotein particles have also been described to have anti-parasitic activity [Ormerod 1982]. Moreover, Esteve *et al.* suggest that lipoprotein particles may carry the LPS binding receptor CD14. LPS is a major component of the outer membrane of gram-negative bacteria and during acute infection, the binding of LPS to the lipoprotein particle is considered beneficial, preventing LPS stimulation of monocytes and macrophages, but during chronic inflammation it is not. Transport of LDL-LPS complexes into the subendothelial space might then initiate an inflammatory response and thereby promote an atherosclerotic reaction. Overall, infection and inflammation trigger an acute-phase response, with formation of a number of cytokines such as TNF- α and IL-1. At the same time, increased triglyceride levels, decreased HDL-cholesterol, impaired reverse cholesterol transport and reduced LDL protection against oxidation can be seen. This could be an evolutionary-conserved mechanism aimed at accumulating cholesterol in cells during infection for tissue repair [Esteve 2005], but at the

same time with a less beneficial effect: promoting atherosclerosis.

3.3.4 *Proteins and modifications that may influence atherogenesis*

Retention of lipoproteins in the subendothelial space is a key step in the pathogenesis of atherosclerosis. Research concerning these particles has been going on for decades, and many important conclusions have been presented. For example: particles smaller than 70 nm such as IDL and LDL, are easily taken up by the arterial wall [Nordestgaard 1995]; the size of the particle and the volume of the intima are important factors [Bjornheden 1996]; and sub-endothelial accumulation of lipoproteins correlates with both positive and negative risk factors for CVD, such as estrogen and tobacco smoke respectively [Walsh 2000, Roberts 1996]. Despite all the information available, there is an unanswered question: What about the lipoprotein-associated proteins?

Only a few years ago, it was still an established truth that LDL only contains apo B-100. However, with new proteomic techniques available it has also become possible to investigate low abundant proteins. Such studies of LDL and HDL were performed in Papers I and II. As mentioned above, conformational changes of apo B-100 might lead to exposure of PG-binding regions and this may be one reason for the high-affinity binding of sdLDL to arterial PG. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis has been studied by Skålen *et al.* But probably other proteins can be involved too. Three proteins that are known to directly interact with PG are apo E [Olin 2001], serum amyloid A [Lewis 2004] and LPL [Merkel 1998]; another protein that has been found to be able to influence the retention without direct interaction, by a mechanism that is not yet understood, is apo C-III [Olin-Lewis 2002]. These findings are important, and it is even more important to find out in what kind of lipoprotein particles they are present, if the distribution changes during different conditions and finally, if there are more unknown proteins that have similar properties. Recently a proteomic study was performed by Davidson *et al.*, studying the protein composition in LDL subclasses in patients with metabolic syndrome and type II diabetes. It was observed that sdLDL was enriched in apo C-III and depleted of apo C-I, apo A-I and apo E in both patient groups compared to healthy controls. This study demonstrates the value of defining the protein composition of lipoprotein particles under different conditions. In line with this, in Paper V a study was performed comparing the LDL protein composition in

healthy normal weight and obese adults.

Many proteins are expressed as different isoforms that may determine their activity state, localization, turnover and interactions with other molecules. Apo E has amino acid substitutions in positions 130 and 176, resulting in isoforms such as apo E2 (Cys/Cys), the most common apo E3 (Cys/Arg) and apo E4 (Arg/Arg). Apo E4 is considered to be a more atherogenic isoform and also involved in the development of Alzheimer`s disease, which may be related to a structural preference of apoE4 to remain functional in solution [Chou 2006]. Apo A- I can be found in its inactive proform and after cleavage of six N-terminal amino acids, in its active form, and isoforms caused by amino acid substitutions are known [Rall 1984]. Many isoforms are due to post-translational modifications (PTMs) and it has been estimated that 50-90% of all proteins in mammalian cells undergo PTM. There are many different PTMs such as truncation, acetylation, hydroxylation, phosphorylation and glycosylation, many of them with profound impact on the function of the protein. The most common PTM is glycosylation and this modification is known to affect the properties of lipoprotein particles [Remaley 1993, Camejo 1985]. Glycosylation of proteins involves the addition of carbohydrate residues. The three major types of enzymatic glycosylation often seen in proteins associated to lipoproteins are: *N*-glycosylation, *O*-glycosylation and sialylation. *N*-linked glycosylation is initiated in the endoplasmatic reticulum membrane (ER) and continues in the golgi apparatus in the secretory pathway. The glycans are attached to asparagine (Asn) residues by the following criteria: Asn-Xaa-Ser/Thr/Cys [Lis 1993], and Asn-Pro-Leu [Miletich 1990] (Xaa could be any amino acid). *O*-linked glycosylation occurs in the golgi apparatus and involve the attachment of glycans to the hydroxyl group of serine and threonine side chains. Glycosylation affects the conformation and stability of the protein. Sialylation involves sialic acid attachment as terminal oligosaccharide residues on *N*- and *O* glycosylated proteins. The significance of sialic acid on lipoproteins is not fully understood although it has been observed that sialic acid increases the negative net charge of the lipoprotein particle, increases lipoprotein solubility and decreases interactions with the vascular matrix. Accordingly, low levels of sialic acid in LDL are associated with CVD [Camejo 1985, Millar 1999]. In addition to enzymatic glycosylation, non-enzymatic glycosylation (glycation) is found in subjects with high levels of blood sugar. Glycations are able to form advanced glycosylated end products and results in increased interaction with PGs

and thereby retention of lipoproteins in the subendothelial space [Edwards 1999]. Altogether, this implicates the importance of determining glycosylated isoforms in lipoproteins. One apolipoprotein that has been shown to be *N*-glycosylated is the recently discovered apo M [Xu 1999, Duan 2001]. The function of apo M is not yet completely understood, but apo M has been proposed to be required for cholesterol efflux to HDL and thereby protective against atherosclerosis [Wolfrum 2005, Dahlbäck 2006]. In Papers I and II three isoforms with different isoelectric points of apo M were identified, indicating that the protein besides *N*-glycosylation also is sialylated. This possibility was pursued in Paper IV by deglycosylation experiments followed by 2-DE/MS analysis to characterize the glycosylation pattern of apo M.

Considerable interest has lately been focused on the role of inflammatory proteins in atherosclerosis, many of them lipoprotein associated. They are divided into two groups, positive acute phase proteins, which increase in abundance during inflammation and negative, which decrease. Serum levels of serum amyloid A and apo J increase during inflammation while apo A-I, the major apolipoprotein in HDL, decreases. Apo J has been suggested to promote cholesterol efflux from foam cells [Gelissen 1998] while apo A-I might be reduced because of decreased apo A-I synthesis, accelerated HDL catabolism and apo A-I replacement by serum amyloid A [Artl 2000]. Studies have also indicated that HDL could be oxidatively modified by myeloperoxidase from inflammatory cells during the atherosclerotic process, and thereby limit the action of apo A-I in the reverse cholesterol transport [Chait 2005]. Based on protein content HDL particles are divided into families, e.g. the apo A-I and the apo A-I/A-II family. Within a family the density may vary but it appears that the apo A-I family is more abundant in HDL₂, and apo A-I/A-II is more abundant in HDL₃. The functional significances of these different subclasses of HDL are not clear. However, apo A-I HDL initiates cholesterol translocation while apo A-I/apo A-II HDL rather inhibits this action [Eisenberg 1999]. Additionally, a strong direct relation between a high apoB/apoA-I ratio and an increased risk of myocardial infarction has been described [Walldius 2005], a finding that underlines the importance of the protein composition of the lipoprotein particles. Also interesting is that infusion of apo A-I Milano (an isoform with a substitution Arg→Cys¹⁹⁷) in phospholipid complexes, decreased mean percent coronary atheromas, indicating that apo A-I might be a new tool in the treatment of atherosclerosis [Olsson 2004].

All these findings indicate the value of studies to determine the protein content and protein modifications in LDL and HDL (Papers I, II, III and IV). Today, the rapidly expanding use of mass spectrometry-based techniques is applicable to both mapping of complex protein mixtures [Banks 2000] as well as analysis of individual proteins [Dayal 2002] within the area of cardiovascular diseases. For instance, different isoforms of apolipoproteins have been identified by MS after isoelectric focusing [Farwig 2003]. Analysis of LDL and HDL using MALDI-and ESI-MS after 2-DE, may therefore contribute to an improved understanding for the lipoprotein-related progress of atherosclerosis.

4. Aims of this thesis

Abnormal lipoproteins are considered to be independent risk factors for CVD and there is an increasing interest in their protein composition. By using 2-DE in combination with new mass spectrometry techniques it is possible to detect both abundant and low abundant proteins in large scale.

Specific aims were:

·To create protein reference maps of proteins in the LDL, HDL₂ and HDL₃ density fractions from a pool of healthy individuals by using two-dimensional gel electrophoresis in combination with mass spectrometry.

·To improve methods for identification of low abundant proteins in the 2-DE patterns of LDL and HDL.

·To further characterize isoforms of novel proteins found in LDL and/or HDL.

·To compare the LDL-protein patterns from normal weight and obese subjects, searching for alterations that may lead to an improved understanding for the role of LDL in CVD.

5. Methodological aspects of lipoproteomics

5.1 Isolation of lipoprotein particles

The heterogeneity of lipoprotein particles has been recognized for many years and a number of techniques have been developed for detecting and characterizing the variations, including non-denaturing gradient gel electrophoresis, density-gradient ultracentrifugation, size-exclusion chromatography and immunoaffinity techniques. All methods have their advantages and drawbacks, and a method for lipoprotein isolation has not yet been developed that can be considered the perfect method. Isolation of lipoprotein particles by immunoaffinity techniques [Duverger 1993] according to their content of specific proteins is a gentle and preservative method but includes the risk of cross reactivity and low affinity. For example, LDL may be prepared by immunoaffinity against apo B-100 but this preparation will also contain IDL and VLDL as apo B-100 is present in these lipoprotein particles. Size-exclusion chromatography is also a gentle and preservative method, but includes the risk of contaminants due to complex formation and unspecific binding. On the other hand, density-gradient ultracentrifugation, the most common method for isolation of lipoprotein particles, is not very gentle and the risk of loss and exchange of proteins between the density/size ranges of different lipoprotein classes are well-known drawbacks [Sattler 1994]. There is also a risk of some overlap between the density/size ranges of different lipoprotein classes. At the same time, this overlap may not only represent a separation problem but also reflect the *in vivo* situation. After evaluating the different methods, two-step short-spin density-gradient ultracentrifugation was used to isolate LDL and HDL in these studies. Short-spin, (2h instead of 22-36h), ultracentrifugation was used with the intent to minimize the loss and exchange of proteins, and two repeated centrifugation steps, to eliminate as many contaminants as possible. Furthermore, size-exclusion chromatography was used in comparison to confirm the findings in Papers I and II.

5.1.1 Isolation of LDL and HDL using two-step short-spin density-gradient ultracentrifugation

Preparations of LDL and HDL were performed by methods described by Da Silva *et al.* and by Sattler *et al.*, respectively, including slight modifications. Blood samples in EDTA-containing tubes were pooled from healthy volunteers after an overnight fast. After 10 minutes centrifugation at 700g at room temperature plasma was obtained. EDTA and sucrose

were added to prevent LDL/HDL oxidation and aggregation, respectively. Five milliliters of EDTA-plasma was adjusted to a density of 1.22 g/mL (LDL) and 1.24 g/mL (HDL) with solid KBr. The plasma samples were then layered in the bottom of a centrifuge tube and were gently overlaid with phosphate buffer solution (PBS) with a density of 1.006 g/mL (LDL) or with KBr/phosphate buffer solution with a density of 1.063 g/mL (HDL). The first ultracentrifugation step was performed at 290 000g for 2h at 4°C (LDL) and 2h at 15°C (HDL). By the LDL isolation procedure, HDL with a density of 1.063-1.210 g/mL is located near the plasma, LDL with a density of 1.019-1.063 g/mL is located in the middle of the PBS fraction and IDL 1.006-1.019 g/mL /VLDL <1.006 g/mL are located closer to or at the top of the tube. Since LDL contains yellow carotenoids, this fraction is clearly visible (Figure 3). During the HDL isolation procedure, HDL is divided into two sub-fractions; HDL₂ with a density of 1.063-1.125 g/mL and HDL₃ with a density of 1.125-1.210g/mL. They are both located in the middle of the KBr/PBS solution while IDL, LDL and VLDL are located at the top of the tube since their densities are lower than 1.063 g/mL. In order to detect the position of the different fractions one parallel sample is needed, in which the lipoproteins have been stained by Coomassie. After centrifugation, LDL, HDL₂ and HDL₃ were collected separately by penetrating the tube with a syringe.

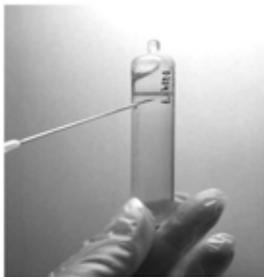


Figure 3. Collection of LDL after the first ultracentrifugation step.

To avoid contamination by serum proteins, all fractions were then further purified by a second centrifugation step performed as described previously [Da Silva 1998]. LDL was added to KBr solution with a density of 1.10 g/mL and the two HDL fractions were added to KBr

solution with a density of 1.24 g/mL separately, which resulted in that all fractions were positioned at the top of their tubes. LDL, HDL₂ and HDL₃ were then collected and desalted, the protein content was measured and the samples were lyophilized prior to further analysis.

5.1.2 Preparation of LDL and HDL using size-exclusion chromatography

To compare and verify the results from the ultracentrifugation procedure, LDL and HDL were purified from human plasma by size-exclusion chromatography. Separation of lipoproteins according to size was performed in Tris-borate buffer at a flow rate of 0.5 mL/min on a Superose™ 6 prep grade column. One ml of plasma was injected and after 15 min, 60 fractions of 0.5 mL were collected. For comparison, LDL and HDL isolated by ultracentrifugation were applied on the column. The chromatographic conditions used clearly separated the two lipoprotein-fractions and the peak fractions for LDL and HDL were eluted after 29-31 minutes and 37-39 minutes, respectively. The peak fractions collected were then pooled and desalted, the protein concentration was measured and the samples were lyophilized prior to further analysis.

5.2 Separation of proteins with two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis is an excellent method for separation of proteins from most kinds of tissues and complex mixtures of proteins (up to 10 000) can be separated [Klose 1995]. Both qualitative characterization of the protein expression, including post-translational modifications and quantitative characterization comparing the protein expression in different individuals or groups, is possible by this technique. As can be seen in Figure 4, this method includes two steps, the isoelectric focusing (IEF) step, where the proteins are separated according to their isoelectric point (pI), and the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) step, where the proteins are separated according to their molecular weight. Since it is rare that two proteins have the same isoelectric point and molecular weight, this will result in each protein migrating to its own unique position.

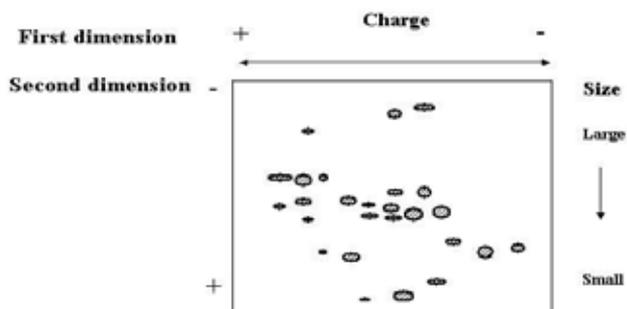


Figure 4. Schematic view of 2-DE. Denaturated proteins are separated according to charge (isoelectric point) and size (molecular weight).

5.2.1 The first dimension.

The net charge of a protein is dependent on the amino acid composition and thereby the character of the side chains of the amino acids. Amino acids are ampholytes and their charge is dependent on the pH in the surrounding. The net charge of a protein is the sum of all the charges within the protein, including the side chains and the carboxy- and amino terminal. Proteins are positively charged in pH values below their own *pI* and negatively charged in pH values above their own *pI*. The presence of an immobilizing pH gradient (IPG) is necessary for IEF. This is a polyacrylamide gel with a gradient of covalently linked acidic and basic buffering groups. The proteins are then forced to move in this gradient by an electric field. This means that a protein with positive net charge will migrate towards the cathode (-), high pH, and thereby becomes less positively charged and a protein with negative net charge will migrate towards the anode (+), low pH, and thereby becomes less negatively charged due to proton exchange with the matrix. Then the proteins will stop migrating when the net charge is zero. If a protein would start to diffuse, it would get charged again and migrate back to its own *pI* position. Here, samples containing 300-500 µg of denatured and reduced proteins were applied by in-gel rehydration for 12 h using low voltage (30V) in pH 3-10 NL IPGs. The proteins were then focused at 40 000-53 000 Vh at maximum voltage of 8000 V. IPGs were

either used immediately for the second dimension (SDS-PAGE) analysis or stored at -70°C.

5.2.2 *The second dimension.*

Prior to the second dimension, the gel with the separated proteins from the first dimension has to be equilibrated under denaturing conditions. The equilibration solution contains urea, glycerol and SDS to reduce electroosmotic effects [Görg 1988], to keep proteins denatured and to form negatively charged protein-SDS complexes. DTT is also added to reduce the denatured proteins and finally iodoacetamide to alkylate the thiol groups of the reduced proteins to prevent oxidation and cross bridging of cysteins during the second dimension. These modifications would affect the migration of the proteins.

SDS-PAGE separates denatured, reduced and alkylated proteins according to molecular weight. The migration takes place in a polyacrylamide gel containing sodium dodecyl sulphate (SDS). The gels consist of chemically co-polymerized acrylamide monomers with a cross linking agent such as N, N bisacrylamide. Thereby the pore size can be controlled and additionally, the gel can be homogenous or casted with a gradient. The gel-strip from the first dimension is placed on the polyacrylamide gel and the negatively charged proteins migrate in an electric field from the cathode towards the anode. Small proteins migrate more easily than large proteins in the polyacrylamide gel and are thereby detected closer to the anode. Here, the second dimension was performed by transferring the proteins to homogenous home cast gels (12% or 14%) on gelbond PAG film (0.5*180*245 mm) running at 20-40 mA at 10°C at 40-800 V over night.

5.3 Staining and image analysis

5.3.1 *Staining*

Proteins separated by gel electrophoresis can be visualized by a number of methods. The different stains interact differently with the proteins and some of them are not specific for proteins. The degree of sensitivity is also different. In these studies Coomassie Brilliant Blue, SYPRO Ruby and silverstaining were used. Coomassie Brilliant Blue staining is the most common method for protein visualization on gels and also the least sensitive of the three. The detection limit is estimated to about 100 ng [Rabilloud 2000]. Coomassie Brilliant Blue is a sulphonated triphenyl methane dye that binds to proteins by electrostatic interaction. Anions

of Coomassie Brilliant Blue formed in acidic staining medium combine primarily with the protonated amino groups (Arg⁺ and Lys⁺) but also with hydrophobic sites of proteins [Silber 2000] under specific conditions. Fluorescent SYPRO Ruby staining method is a permanent stain comprised of ruthenium as part of an organic complex that interacts non-covalently with proteins. This method can detect about 5-10 ng of stained protein in an SDS gel but requires special equipment to obtain and detect the fluorescence. One advantage with SYPRO Ruby is that it has a wider linear dynamic range than silver staining [White 2004] and is therefore the staining method to recommend in comparative proteomics. Silver staining, on the other hand, is the most sensitive method; detecting gel separated low abundant proteins at 1-10 ng [Merrill 1984]. Silver ions bind proteins by electrostatic interactions with carboxyl groups of Asp and Glu [Nielsen1984] or by forming complex with the imidazole, SH, SCH₃ or NH₃ groups of His, Cys, Met and Lys respectively [Rabilloud 1990]. In the staining procedure silver ions are reduced to form an insoluble brown precipitate of metallic silver. In the method performed here, this occurs by oxidation of formaldehyde under alkaline conditions. The oxidation of formaldehyde (to formic acid) is controlled by an alkaline buffer so that silver reduction can continue until a solution is added to stop the reaction. Silver staining is a complex, multi-step process and precise timing, high-quality reagents, and cleanliness are essential for reproducible and high-quality results.

5.3.2 *Image analysis*

Processing data from stained protein gels by computers includes the gel images being digitized by an imaging system. Here, the images of the protein patterns were analyzed by a CCD (Charge-Coupled Device) camera digitizing at 1340*1040 pixel resolution in a UV-scanning illumination mode for Sypro Ruby stained gels or at 1024*1024 pixel resolution in white light mode for Coomassie and silver stained gels using a Flour-S-Multi Imager in combination with a computerized imaging 12-bit system (PDQuest 2-D gel analysis software, version 7.1.0). The unit of the signal intensity differs depending on what light source is used. The unit of the UV light source is expressed in counts while the unit of the white light source is expressed as optical density (OD). Gel images were evaluated by spot detection, spot intensities and geometric properties. Then the gels included in the comparative study were put into a match set for comparison and statistical analysis. This specialized PDQuest 2-D gel program, which also demands considerable manual interpretations, makes it possible to detect

differences in protein expression. The protein spots from all the matched gels are compared and a computer-animated master gel containing all the included protein spots from all the gels is created. It is also recommended to perform normalization of the match set; this step reduces the risk of false results due to variation in protein amounts or variations in the staining step. In the comparative study, proteins were quantified as fluorescence intensity (counts) per total protein fluorescence on the 2-D gels, expressed as percent.

5.4 Protein identification using mass spectrometry

Mass spectrometry has become the method of choice for identification and characterization of proteins. Mass spectrometric measurements are carried out in the gas phase on ionized analytes. A mass spectrometer consists of an ion source, a mass analyser that measures the mass-to charge ratio m/z of the ionized analytes and a detector that registers the number of ions at each m/z value. There are different mass spectrometry instruments that can be combined with different ionization sources. In these analyses 2-DE was used for protein separation prior to peptide mass fingerprinting and amino acid sequencing. The protein identification was performed by using matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF MS) where peptides are transferred from solid phase to gas phase and in selected cases, electrospray ionization tandem mass spectrometry (ESI MS/MS) where peptides are transferred from liquid phase to gas phase.

5.4.1 *Peptide mass fingerprinting*

The term peptide mass fingerprinting (PMF) includes that a protein is identified by its unique amino acid composition and thereby the resulting peptides. Proteins are digested by an enzyme such as trypsin (that cleaves proteins into peptides C-terminal of Lysine and Arginine), or a chemical such as cyanobromide (cleaves proteins C-terminal of methionine). Every protein has its own peptide profile and the user masses will after mass spectrometric analysis be compared to theoretical masses in different databases.

5.4.2 *MALDI-TOF MS*

Matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF MS) is a powerful tool in large-scale proteome analysis (Figure 5). In this study, peptides

dissolved in 0.1% TFA were mixed with excess matrix (α -cyano-4-hydroxycinnamic-acid (CHCA)) or 2,5-dihydroxybenzoic acid (DHB) and dried on a MALDI plate. Evaporation and ionization of the peptides in solid phase occurs via proton transfer from the matrix. A laser flash (N_2) transfers the intact peptide ion into gas phase and the ions are then accelerated in an electric field by the application of a high voltage. Then the ions are transferred into the high vacuum of the time of flight analyzer. The principle of the TOF analyzer is that if ions are accelerated with the same potential at a fixed point and time and are allowed to drift in vacuum, the ions will separate according to their mass to charge ratios. This procedure results in that lighter ions travel faster than heavier ions to the detector. The instrument is equipped with a reflector that acts as an ion mirror and compensates for the initial energy spread of ions with the same mass and, therefore, improves resolution. Since all peptides are given the charge 1^+ , the time of flight will give us an estimation of the monoisotopic mass of the peptide. Baseline correction, deisotoping and calibration of the spectrum improve the mass accuracy and the obtained mass list is then used for database searches and protein identification.

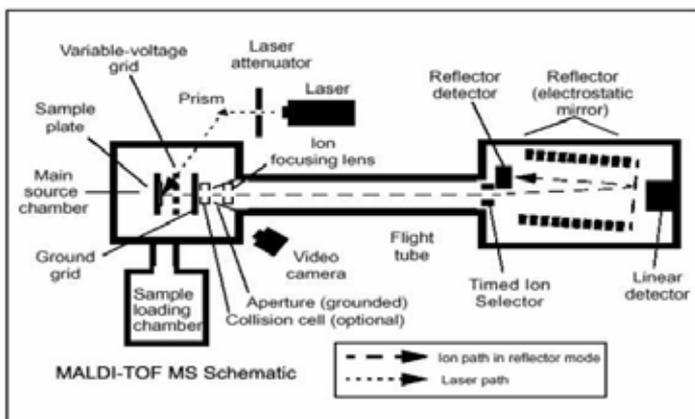


Figure 5. Schematic overview of the principles for MALDI-TOF MS.

5.4.3 Matrices

When protein identification is performed by MALDI-TOF MS, peptides are transferred from solid phase to gas phase and the presence of a matrix is a prerequisite for this process. A matrix consists of an organic UV-absorbing acid, working as a proton donor and every peptide renders one positive charge. Common matrices are sinapinic acid for intact proteins and for peptides; α -cyano-4-hydroxycinnamic-acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB). CHCA and DHB result in a completely different crystal pattern on the MALDI plate (Figure 6). It is also known that matrix clusters and metal ion adducts interfere with peptide ionization and peptide mass spectrum interpretation. This is particularly evident at low sample concentrations and therefore detection of low abundant proteins may become difficult [Xiangping 2003]. DHB has previously been shown to produce less noise than CHCA [Laugesen 2003]. Furthermore, as demonstrated in Paper III, DHB improves PMF data from silver stained proteins compared to CHCA. Therefore, DHB was used as matrix for identification of low abundant proteins in LDL and HDL.

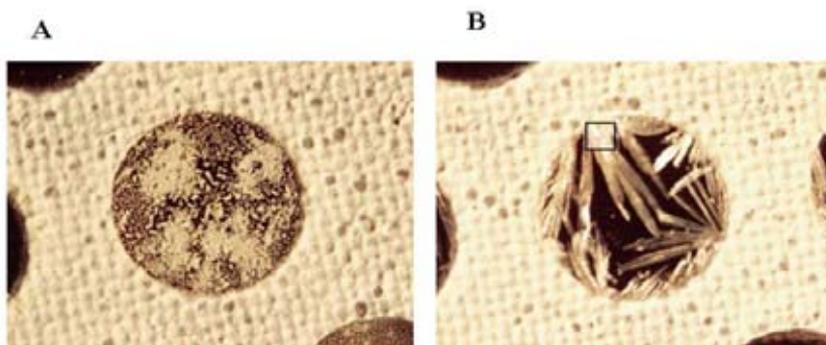


Figure 6. MALDI plates with A; α -cyano-4-hydroxycinnamic acid and B; 2,5-dihydroxybenzoic acid as matrix. The marked area indicates the position of the laser where the best signal to noise was obtained.

5.4.4 ESI MS/MS

Electrospray ionization (ESI) is a method where ions in solution are passed along a narrow inlet capillary into a strong electrostatic field (Figure 7). Charged droplets are formed at the tip of the capillary, the droplets then shrink and split into smaller droplets as the solvent (50% acetonitrile (ACN) 9/0.1% formic acid (FA)) evaporates. After solvent evaporation and droplet fission, multiply charged gas-phase ions are produced by dry gas (N_2) and the ions are then accelerated into the mass spectrometer. The quadrupole system consists of four parallel rods and the opposite electrodes are electrically connected. A potential consisting of direct current (DC) and radio frequency (RF) voltage is applied on one pair of rods and the polarity of the DC on the other pair of rods is reversed. The voltages on the pair of rods are shifted in phase at the RF. The motion of an ion is then determined by a time-dependent potential, which is a function of DC and RF. Using the quadrupole as a massfilter results in that quadrupole rods are set so that only ions in a selected range of m/z will reach the detector and the rest will collide with the rods.

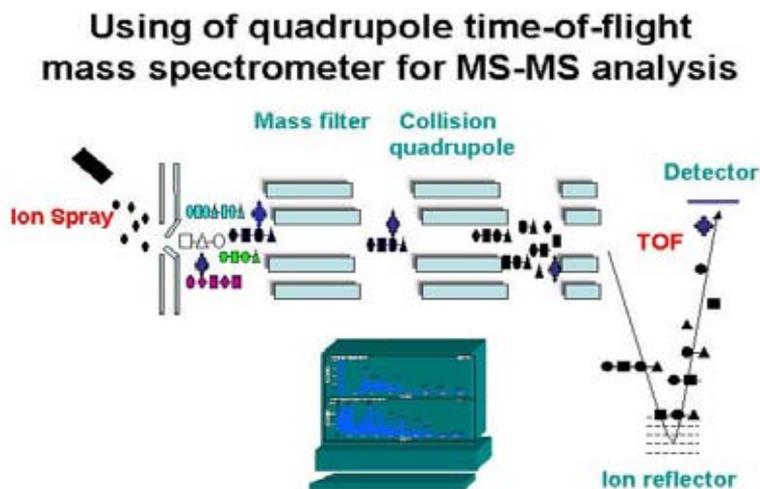


Figure 7. Schematic overview of the principles for ESI Q-TOF MS (Applied Biosystems).

In this procedure a Q-TOF spectrometer is applied which combines the front part of a triple quadrupole instrument with a TOF section for measuring the mass of the ions. Briefly described, the instrument was set in nano positive mode, an MS-scan was performed and a predicted precursor ion was selected. An MS/MS product ion scan (Figure 8) was then performed which means that only ions with chosen m/z (the first quadrupole) are sent into the collision cell containing an inert gas (the second quadrupole) for collision-induced dissociation (CID). The ions are then concentrated and sent into the TOF mass analyzer (the third quadrupole). Fragmented ions were analyzed in the TOF section and the spectrum was interpreted manually. The cleavage sites for the different ions are presented in Figure 9.

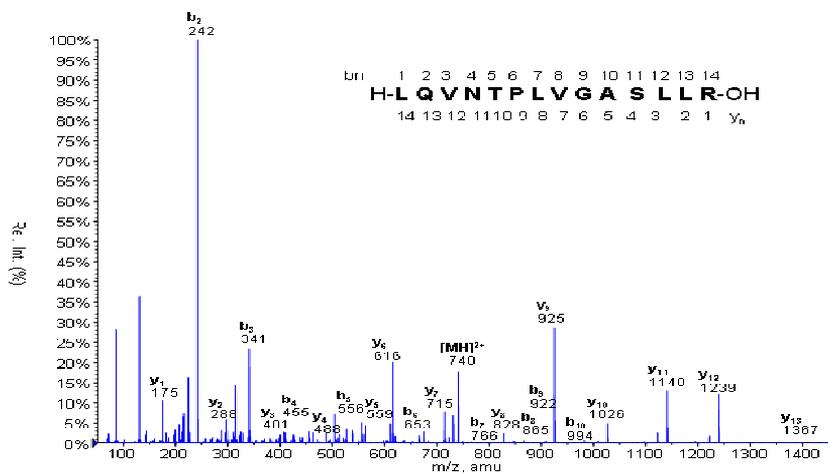


Figure 8. Example of a MS/MS scan of a $[740]^{2+}$ precursor ion with the single charged parent mass (m/z) 1480 selected in the MS-scan. b_n - and y_n -ions represent the N-terminal and C-terminal ions, respectively.

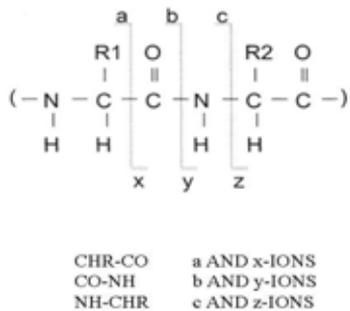


Figure 9. Definition of fragmented ions that can be obtained after collision induced dissociation (CID) using electrospray- ionization tandem mass spectrometry (ESI MS).

6. Results and Discussion

6.1 Protein identifications in LDL and HDL.

The attempt to isolate, separate and identify proteins in LDL and HDL from healthy individuals using density-gradient ultracentrifugation, 2-DE and mass spectrometry was successful. The protein maps of LDL and HDL in Papers I and II confirm the presence of proteins previously described in LDL and HDL and also reveal a number of novel members. Altogether 47 proteins were identified representing 18 different protein identities according to Papers I-V. The results are also summarized in Figure 10, Figure 11 and Table 2.

Apo B-100 and apo A-I are the well-known two most abundant proteins in LDL and HDL, respectively. In fact, apo B-100 is often regarded as the only protein in LDL. It is therefore of special interest that, although apo B-100 constitutes about 95% of the LDL protein content (Figure 1, Paper I), we have in our study identified several additional low-abundant proteins (Table 2). The protein pattern of LDL is shown in Figure 10 and it should be noted that the full size apo B-100 is not present in the 2-DE pattern, due to its large size and hydrophobic characteristics. Instead apo B-100 is represented by a specific apo B variant (clone LB25-1) that consists of the amino-terminal region [Protter 1986].

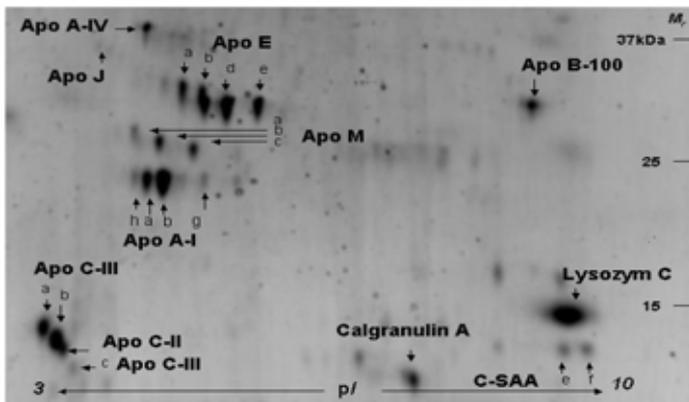


Figure 10. Proteins identified in LDL by 2-DE (modified version from Paper I)

As shown in Figure 11, apo A-I was identified as the dominating protein in HDL. The protein was separated into eight different isoforms and interestingly four of them were also present in LDL (Figure 10 a,b,g,h). One of the isoforms (g) contained a propeptide and the spot position in the 2-D pattern was the same as that of proapo A-I in human plasma [Bondarenko 2002]. Two of the isoforms (c,d) in HDL appeared with more acidic *pI*'s and higher molecular masses than predicted and the spectra data (Figure 3, Paper II) indicated that this might be due to O-glycosylation. This is a common post-translational modification of apolipoproteins [Remaley 1993], but has, to our knowledge, not been described in apo A-I before. It has, however, been shown that HDL and LCAT are less stable and less functional when apo A-I is modified by non-enzymatic glycosylation, which indicates a pathophysiological relevance of glycosylated apo A-I in atherosclerosis [Fievat 1995]. Also apo A-II, only identified in HDL, was found to be glycosylated. Interestingly, glycosylated apo A-II was only detected in HDL₃ while HDL₂ contained the non-glycosylated variant. This is illustrated in Figure 11 by their different positions in the 2-DE pattern.

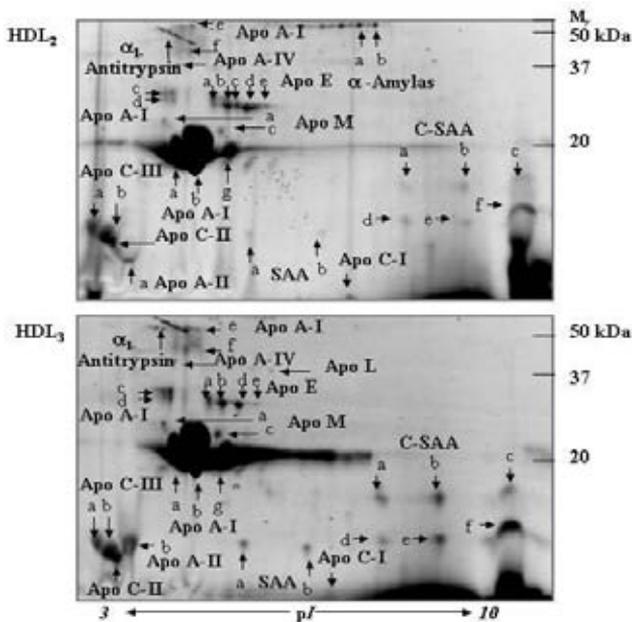


Figure 11. Proteins identified in HDL by 2 DE (modified version from Paper II)

A differential localization of glycosylated apo A-II has been indicated before [Remaley 1993] and is in line with the idea that HDL₃ is less protective against atherosclerosis than HDL₂ [Morgan 2004]. Thus it is possible that glycosylation of apo A-II could affect the capability of HDL to protect against endothelial dysfunction. Another expected apolipoprotein was apo E (Figures 10 and 11), which was separated into four isoforms according to *pI* in both LDL and HDL (a,b,d,e) and into one additional isoform in HDL₂ (c). Apo E is an important participant in the clearance of lipoprotein particles from the circulation, and one isoform, apo E4, has been associated with coronary artery disease [Chen 2003] and Alzheimer's disease [Okubo 2001]. As described in Paper I, apo E has previously been defined as three major isoforms, apo E2, E3 and E4, due to specific differences in their amino acid sequences. Since these differences influence the charges as well as the trypsin cleavage patterns, it is possible to separate and identify the isoforms by 2-DE and MS. Thus, all three isoforms were identified in our preparations and, in line with a previous study [Raffai 2001], we found that apo E4 and E5 seemed to be preferentially associated to LDL, while apo E2 and apo E3 were more abundant in HDL. However, sequence data using MS/MS are needed to fully elucidate the nature of the different apo E variants. Apo M (Figure 10,11 and 12), a protein structurally related to the lipocalin family [Duan 2001], was first identified, in Paper I, as three isoforms in LDL (a, b, c) and in Paper II, as two isoforms in HDL (a, c). As shown in Paper IV, the isoform (b) missing in the HDL 2-DE protein pattern is just "hidden" behind the dominating apo A-I. Furthermore, as demonstrated in Papers IV and V, LDL and HDL, especially in obese subjects, contains two additional isoforms of apo M (Figure 12), depending on the absence of glycosylation/sialylation. These results are discussed in section 6.2 and 6.3. The function of apo M is not clear, but recently it has been reported that apo M is required for pre β -HDL formation and cholesterol efflux, thereby inhibiting the formation of atherosclerotic lesions [Wolfrum 2005].

Lately, focus has been drawn to lipoprotein-associated inflammatory proteins [Chait 2005]. This is underlined by the identification of several possible inflammatory markers or mediators in our preparations, such as lysozyme C, calgranulin A, serum amyloid A and A-IV, apo J, α_1 -antitrypsin and salivary alpha amylase. In LDL, lysozyme C and calgranulin A (Figure 10) were identified for the first time. Lysozyme C is a well-known anti-bacterial protein towards Gram-positive bacteria [Lee 2002]. Our results clearly show that LDL has lysozyme activity

(Table 3, Paper I), but its physiological role in LDL remains to be demonstrated. However, it is possible to suggest that lysozyme C may be acting as an anti-bacterial agent in LDL. Or maybe anti-atherosclerotic agent as recently described by Liu *et al.* Calgranulin A, on the other hand, has been postulated to represent a novel means of protecting host tissue from excessive oxidative damage when high levels of hypochlorite are generated [Raftery 2001], and it is therefore tempting to speculate that calgranulin A may play a role as a natural scavenger in LDL, preventing LDL oxidation and atherogenicity. Other inflammatory mediator/marker proteins that were identified in HDL and LDL are the constitutively expressed serum amyloid A-IV, apo J and α_1 -antitrypsin, (Figure 10,11 and Table 2) while serum amyloid A was only identified in HDL.

Table 2. Summarized overview of proteins identified in LDL, HDL₂, and HDL₃ with peptide mass fingerprinting.

<u>Name</u>	<u>Gel position</u>	<u>LDL</u>	<u>HDL₂</u>	<u>HDL₃</u>	<u>Function</u>
Apo A-I	a	•	•	•	Participates in the reverse transport of cholesterol
Apo A-I	b.	•	•	•	
Apo A-I	c.		•	•	
Apo A-I	d.		•	•	
Apo A-I	e.		•	•	
Apo A-I	f.		•	•	
Apo A-I	g.	•	•	•	
Apo A-I	h.	•			
Apo A-II	a	.	•		Modulator of reverse cholesterol transport
Apo A-II	b.			•	
Apo A-IV		•	•	•	Required for activation of LPL by apo C-II
Apo B-100		•			Ligand for the LDL receptor
Apo C-I			•	•	Modulate the interaction of apoE/lipoproteins Activator of LPL
Apo C-II.		•	•	•	
Apo C-III	a.	•	•	•	Inhibitor of LPL
Apo C-III	b.	•	•	•	
Apo C-III	c.	•			
Apo E	a	•	•	•	Mediates the binding, internalisation and catabolism of lipoproteins
Apo E	b.	•	•	•	
Apo E	c.		•		
Apo E	d.	•	•	•	
Apo E	e.	•	•	•	

Apo J	a.	•	•*	•*	Promote cholesterol efflux from foam cells
Apo J	b.	•	•*	•*	
Apo J	c.	•	•*	•*	
Apo L				•	Interacts with Apo A1 in reverse cholesterol transport
Apo M	a.	•	•	•	Not yet clear, may be involved in lipid transport/ cholesterol efflux
Apo M	b.	•			
Apo M	c.	•	•	•	
Apo M	d.	•	•	•	
Apo M	e.	•	•	•	
Serum AmyloidA4	a.		•	•	Acute phase reactant, constitutively expressed
Serum AmyloidA4	b.		•	•	
Serum AmyloidA4	c.		•	•	
Serum AmyloidA4	d.		•	•	
Serum AmyloidA4	e.	•	•	•	
Serum AmyloidA4	f.	•	•	•	
Serum Amyloid A	a.		•	•	Acute phase reactant
Serum Amyloid A	b.		•	•	
α-1-antitrypsin	a.	•	•	•	Inhibitor of serine proteases, primary target; neutrophil elastase
α-1-antitrypsin	b.	•	•*	•*	
α-1-antitrypsin	c.	•	•*	•*	
α-Amylase	a.		•		Hydrolysis of oligosaccharides and polysaccharides
α-Amylase	b.		•		
Calgranulin A		•			Antioxidant?
Lysozyme C		•			Bacteriolytic function
Transthyretin		•			Retinol transport Marker of overnutrition

*Unpublished results

Serum amyloid A and apo J might be useful predictors of cardiovascular events since their concentration in blood increases during acute inflammation [Chait 2005, Trougakos 2002]. Apo J, also called clusterin, has previously been found in amyloid plaques and cerebrovascular deposits [Calero 2000]. The function of apo J is not yet clear but current knowledge suggests a protective role in Alzheimers disease by maintaining solubility and preventing toxicity of the amyloid β particles [Calero 2000] and lately it has been suggested that apo J

promotes cholesterol efflux from foam cells [Gelissen 1998]. Sialylation of apo J was confirmed by neuraminidase treatment of the LDL fraction prior to 2-DE. The possibility to determine the sialylated isoforms of apo J will be important to elucidate the biological role(s) of this multifunctional protein. Another possible inflammatory mediator/marker is α_1 -antitrypsin. This serine protease inhibitor was first identified in HDL (Paper II), but was then also found in LDL (Paper V). Since neutrophil elastase, which has been found in human atherosclerotic plaque [Dollery 2003], is able to modify apolipoproteins resulting in HDL destruction [Pirillo 2000], the possibility is raised that the presence of α_1 -antitrypsin in lipoprotein particles is of importance to regulate the inflammatory process during atherosclerosis. Moreover, the identification of salivary alpha amylase for the first time in HDL₂ has to be further studied. This protein has previously been found in plasma and its level has been increased after cardiac surgery [Ihaya 2001]. It has also been proposed that salivary alpha-amylase could have an antibacterial function [Geerling 2000]. However, its function in lipoprotein particles, if any, is unknown. Finally, a novel finding was transthyretin (TTR) in LDL from obese subjects (Paper V and section 6.3). TTR is a well-known thyroxine and retinol transporter. But interestingly, it has also been suggested to be a marker for over-nutrition [Youshida 2006]. Therefore, more studies to elucidate the function of TTR in LDL are warranted.

One important question which is challenging but which must remain unanswered for many of the identified proteins is whether they have a function in LDL /HDL or whether they are just associated to the lipoprotein particles and reflect the general conditions in the blood at a given time point. Important aspects are also to determine individual variations in protein expression and to investigate possible changes associated with cardiovascular disease processes. Results from the comparative study of LDL from normal weight and obese subjects (Paper V), discussed in section 6.3, demonstrate that the 2-DE/MS-approach may be used for such studies. And in line with this, preliminary results comparing LDL from individuals with acute cardiac infarction with healthy controls show increased abundance of for example: lysozyme C, transthyretin and for the first time α -cardiac actin in the subjects with a cardiac event. This further underlines the importance of such studies.

6.2 Improvement of peptide mass fingerprint data and characterization of apo M isoforms

During characterization of apo M isoforms, two matrices, 2,5-dihydroxybenzoic acid (DHB) and α -cyano-4-hydroxycinnamic acid (CHCA) were compared in the peptide mass fingerprinting procedure (PMF), for the purpose of improving the identification of low-abundant proteins in silver stained 2-D gels (Paper III). Significantly more peptides and higher sequence coverage were found with DHB than with CHCA. Additionally, to avoid sources of error the proteins were collected from three different 2-D gels and analyzed by three different individuals. In two cases, the gels contained proteins from nasal fluid (containing typically more hydrophilic proteins) and in one case LDL (containing typically more hydrophobic proteins). As illustrated in Table 1/Paper III, the results were the same irrespective of sample or user. The most profound effect with DHB compared to CHCA was the less noisy spectra, previously noted by Laugesen *et al.* This may be explained by the fact that matrix cluster and metal ion adducts is able to interfere with peptide ionization and peptide mass spectrum interpretation and that this is particularly evident at low sample concentrations [Xiangping 2003]. The reduced noise levels resulted in a significantly higher signal to noise ratio with DHB than CHCA. A higher signal to noise ratio results in more small but significant peaks being detected and included in the peak list submitted for database search. Since the number of matching peaks is one of the most important parameters when identifying proteins with peptide mass fingerprinting, this improves the reliability of the results. Furthermore, the increased sequence coverage enhances the amount of information about the protein. The chemistry behind the interactions between matrices and proteins is complex and not well understood [Lewis 2000]. One difference between the two matrices is that the crystallization for CHCA is very fast compared to DHB. It is possible that the slow crystallization of DHB favours the incorporation of peptides instead of other contaminating and competing ions and that this becomes more pronounced when analysing low abundant proteins, especially in combination with the complex silver chemistry. This finding is consistent with previous observations demonstrating that the discrimination of peptides is influenced by the rate of matrix crystallization [Cohen 1996]. In addition, as shown in Figure 6, CHCA and DHB have very different crystal structures. CHCA is usually homo-geneously distributed on the target plate while the crystals of DHB are needle shaped. It was also observed that, with DHB, the best signal to noise was obtained if the laser was directed towards the base of these structures, i.e. towards the outside of the crystals on the target plate.

Thus, improved PMF results were obtained with MALDI-TOF MS by using DHB as matrix together with silver stained proteins after 2-DE. An increased number of peptide peaks was detected, probably as a result of higher signal to noise ratios. The improved MALDI procedure helped us to identify transthyretin in LDL from obese subjects (Papers III and V) and DHB was also used during the characterization of low abundant apo M isoforms as described below.

The recently discovered apo M, with a predicted molecular mass of 21.3 k, has previously been investigated using 1-DE, showing a larger molecular mass (26.0k) than predicted. According to Xu *et al*, this difference between apparent molecular mass and the predicted value is caused by *N*-glycosylation. In Paper I, three forms of apo M, detected at pI/M_r; 5.0/27.6k, 5.2 /27.0k, and 5.4/26.3k respectively, was identified (No. 1-3 in figure 12). This illustrates that not only the mass but also the isoelectric points of the identified apo M isoforms differ from the predicted value (5.7). To investigate *N*-glycosylation of apo M, LDL was treated with PNGase F prior to 2-DE analysis. PNGase F releases asparagine-linked oligosaccharides from glycoproteins and glycopeptides by hydrolyzing the amide of the asparagine side chain. The result of the PNGase treatment clearly indicated that apo M is *N*-glycosylated. Thus, the three apo M forms in the untreated sample almost disappeared and instead two additional forms (Figure 2/Paper IV) at positions pI/M_r; 5.4/22.0k and 5.6/22.0k, appeared after treatment with PNGase F. Moreover, a finding that further confirm the results was that MS analysis demonstrated the appearance of the peptide 2549 [MH]⁺ (Figure 2B, Paper IV), suggesting an N → D (+1) modification of peptide 2548 [MH]⁺, typical for N-linked deglycosylation. This peptide contains a proposed glycosylation site, Asn¹³⁵, and corresponds to the amino acid position 121-143 [Duan 2001].

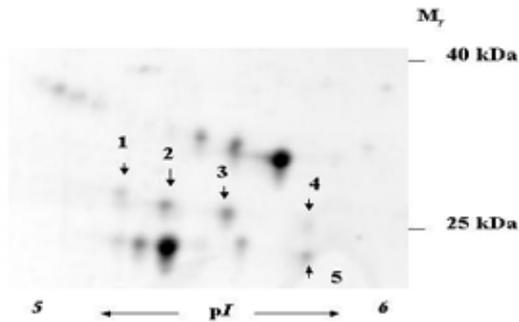


Figure 12. Apo M isoforms separated by 2-DE.

Besides the mass differences, the shift in pI between the different isoforms of apo M was further investigated. This type of pattern could depend on the presence of terminal sialyl groups on the *N*-linked saccarides. Sialylation is a common modification of apolipoproteins [Millar 2001] that renders the proteins a more negative charge state. To examine this possibility, desialylation of LDL was performed using neuraminidase. This enzyme is highly purified from *Arthrobacter ureafaciens* and releases linked sialic acids. As shown in Figure 3/Paper IV, the neuraminidase treatment induced a mobility shift toward more basic pI in apo M. Thus, there was a profound decrease in intensity of the 5.0/27.6k (No.1) and 5.2/27.0k (No.2) isoforms, accompanied by increased intensity of the 5.4/26.3k (No.3) and a new 5.6/26.3k (No.4) isoform, verified as apo M by MS analysis (Table 1, Paper IV). The relatively unchanged mass of the proteins and the mobility shifts toward more basic pI indicated that apo M, like many other apolipoproteins such as apo E, apo J, apo C-III and apo B-100 is sialylated.

In addition to the deglycosylation experiments, the expression of apo M in LDL was examined in 20 healthy subjects and apo M was detected in 17 out of these 20 individuals. However, the levels varied considerably between different individuals, from not detectable to

almost 1% of the total protein content. Apo M also appeared to be more abundant in subjects with high BMI (further studied in Paper V, and discussed in section 6.3). Interestingly, not only the three originally described isoforms were found but also the new unglycosylated and unsialylated variants were detected (No. 4 and 5, Figure 12). The importance of the different apo M isoforms is unclear. Maybe glycosylation of apo M contributes to increased solubility, correct folding or confers stability to the protein. But it may also play a role in the immune system, by oligosaccharide attachment to both host and pathogenic proteins [Rudd 2004]. Possibly, the apo M isoforms with different degree of glycosylation do have different functions. Recently, the kinetics and the respective role of the apo C-III isoforms in modulating intravascular lipid/lipoprotein metabolism were described by Mauger *et al*, implicating that further studies are warranted to elucidate the importance of apo M glycosylation.

6.3 Comparative proteomics of LDL from normal weight and obese adults

After successful identifications of new proteins and their isoforms in LDL (Papers I and IV) and HDL (Paper II), it was a natural step to compare the distribution of the proteins between different groups of individuals. Ten healthy obese adults (BMI>30) and nine normal weight adults (BMI<25) participated voluntarily in this study (Paper V). LDL was isolated using density-gradient ultracentrifugation, proteins were separated with 2-DE and identified using mass spectrometry. Proteins in the two groups were then quantified and compared using the specialized PDQuest 2-D gel program described in section 5.3.2.

It is well known that there are many health implications of obesity. Risks include hypertension, insulin resistance, diabetes mellitus and cardiovascular disease, including altered lipoprotein profile such as low HDL-C, high TG and high LDL-C [Pi-Sunyer 1991]. In line with this, the obese subjects in this study showed significantly higher levels of LDL-C and TG while the HDL-C levels were significantly lower than in the controls (Table 1, Paper V). In addition, the obese subjects possessed a clearly changed LDL-protein profile. As illustrated in Figure 1/Paper V, there were several proteins that were either decreased or increased in LDL from obese subjects compared to controls. These changes are summarized in Table 3.

Table 3. Protein alterations in LDL from obese subjects, summarized from paper V.

Protein	Increase/decrease	Comment
α_1 -antitrypsin	↑	
Transthyretin	↑	
Apo A-I	↑	Acidic isoform
Apo A-I	↓	Major isoform
Apo A-IV	↓	
Apo C-II	↑	
Apo C-III	↑	Male subjects
Apo E	↓	Male subjects
Apo J	↑	
Apo M	↑	
SAA-IV	↑	Non-glycosylated isoform

Apolipoproteins previously described in Paper I, such as apo A-I, apo A-IV, apo C-III and apo E, apo B (clone 25), apo C-II, apo J, apo M, and serum amyloid A-IV were found in almost all LDL preparations analyzed. By contrast, α_1 -antitrypsin was found only in four out of the nine controls, and lysozyme C only in two of the controls. Notably, α_1 -antitrypsin was found in eight out of the ten obese subjects. Alpha-1-antitrypsin, previously identified in HDL (Paper II) is known to be a potent regulator of the effects of activated neutrophils [Spencer 2004] and neutrophil elastase has been demonstrated in human atherosclerotic plaque [Dollery 2003]. It is therefore possible that, α_1 -antitrypsin plays a decisive role in LDL as a protective agent. Interestingly, a novel protein was detected in the 2-D gels from seven out of ten obese subjects but only in one of the controls. This protein was identified as transthyretin (TTR). In relation to lipoproteins, TTR has been found in HDL and has been suggested to interact with apo A-I and to possess proteolytic activity [Sousa 2000, Liz 2004]. In general, TTR is known as a thyroxin transporter but is also able to form complex with the retinol binding protein (RBP) for retinol transport. Interestingly, TTR has recently been shown as a biomarker for myocardial infarction [Kiernan 2006] and both TTR and RBP have been suggested to be new markers for over-nutrition and possibly also metabolic syndrome [Youshida 2006]. Like in HDL, the functional role of transthyretin in LDL has to be further investigated.

The quantitative results obtained in the different groups are summarized in Table 3. Thus,

LDL from the obese subjects was found to contain more apo J, apo C-II, α_1 -antitrypsin, transthyretin, an acidic isoform of apo A-I and SAA-IV than LDL from controls. Also, more of apo M was found in LDL from obese subjects compared to controls; two of the five isoforms were significantly more abundant in obese subjects (No. 4 and 5, Figure 12). Apo M is a novel apolipoprotein mainly associated to HDL, but also found in LDL. Recent results indicate that HDL and LDL containing apo M are more anti-atherogenic and less pro-atherogenic than HDL and LDL, respectively, without apo M [Christoffersen 2006]. Apo M has also been postulated to be involved in the immune response [Deakin 2006] and it is possible that apo M, like apo J, may have a protective role in LDL by transporting modified lipids or other unknown components in its hydrophobic pocket. Additionally, the suggestion that apo M may have anti-inflammatory properties, due to its link to leptin and platelet activating factor [Huang 2007], is interesting. As discussed in section 6.2, apo M is expressed as five isoforms in LDL and HDL, depending on differences in glycosylation (Paper IV). In this study only two of the isoforms were significantly increased in the obese group. However, as the results of all isoforms pointed in the same direction it is reasonable to suggest that total apo M is increased in obese subjects.

Apo J found in increased abundance in the obese subjects, has previously been described to be upregulated during several physiological disturbances, including accumulation of LDL in the arterial wall during the development of atherosclerosis and in diabetes type II [Trouwakos 2002]. Also, through interaction with HDL-paraonase, apo J has been postulated a protective role in atherosclerosis, either by involvement in cholesterol transport or by elimination of excess lipid by-products [Burkey 1992, Bailey 2001]. Another interesting protein raised in the obese subjects was SAA-IV. It is an acute phase protein that has been detected in LDL before [Paper I, Yamada 1996] and, in line with our findings, has been proposed to be a possible nutritional marker [Yamada 2001]. SAA-IV in LDL was, as in HDL, expressed as six isoforms; three with an apparent molecular mass of 18k and three with an apparent mass about 13k. Interestingly, preferentially the 13k forms appeared to be increased in the obese group. As discussed in section 6.2, LDL protein patterns were examined after deglycosylation experiments (Paper IV). The analysis showed a reduction of the SAA-IV 18k isoforms while the 13k isoforms were unaffected by deglycosylation. This indicates that the 13k isoforms, increased in obese subjects, are unglycosylated variants of SAA-IV.

One apolipoprotein whose production rate has been strongly correlated to plasma TG levels is apo C-II [Gerber 2002] and, in line with this, LDL-apo C-II was significantly increased in the obese subjects in this study. According to previous reports, serum apo C-II levels have been shown to be increased in patients with cardiovascular events [Gerber 2002] but not to be an independent discriminator of CHD [Gerber 2003]. As an initiator of triglyceride hydrolysis, raised LDL-apo C-II levels may just reflect an increased need of lipoprotein lipase (LPL) activity. Finally, the most acidic isoform of apo A-I was more abundant in LDL from obese subjects. This isoform may be caused by deamidation, a post-translational modification that is not uncommon and previously observed in apo A-I [Zannis 1980], but to our knowledge with unknown properties. On the other hand, the amounts of apo A-IV and the major isoform of apo A-I were significantly less in LDL from the obese subjects. Both proteins are considered anti-atherogenic apolipoproteins in plasma [Eckardstein 2005, Ostos 2001]. A decreased distribution of such proteins in obese subjects may therefore contribute to an increased risk of CVD. In line with our results, Davidson and coworkers observed that sdLDL from patients with the metabolic syndrome and type II diabetes were depleted of apo A-I. In addition, investigations have suggested that apo A-IV are affected by high-fat diet in a way that is not fully understood. The apo A-IV synthesis seems to be stimulated by fat diet but also auto-regulated in response to prolonged consumption of fat [Tso 2004].

According to gender, even though the results are based on few individuals, some interesting differences were observed. Lower amounts apo E was found in LDL from obese men than control men, while there was no difference in LDL apo E content between obese and control women (Table 3, Paper V). Conversely, unglycosylated apo C-III was more abundant in LDL from obese men than control men, but there was no such difference among women. Individuals with high apo C-III content in LDL are considered to be at higher risk of cardiovascular events [Gerber 2003] but in a recent study of apo C-III isoforms in plasma from men with overweight, no correlation was observed between the proportion of each isoform and BMI [Mauger 2006]. The most acidic isoform of apo A-I was more abundant in LDL from women than men, but the increased amounts found in LDL from obese subjects as compared to controls appeared both in men and women. Enrichment of apo C-III as well as depletion of apo E, apo C-I and apo A-I has recently been demonstrated in sdLDL from

patients, notably only men, with the metabolic syndrome and type II diabetes [Davidsson 2005]. In view of the results in Paper V, it is therefore tempting to suggest that the decreased abundance of apo A-I and apo E in obese subjects compared to controls, may reflect increased amounts of sdLDL particles. This subgroup of LDL is known to appear in individuals with hypertriglyceridemia in combination with low concentration of HDL-cholesterol [Packard 2003] and could therefore be expected in obese subjects. In addition, the distinct changes in LDL protein composition also suggest a remodeling of the LDL particle and remodeling has previously been observed in HDL with depletion of Apo A-I in exchange for SAA [Cabana 1999].

In conclusion, the study in Paper V implicates the importance of analysing isoform patterns of apolipoproteins in LDL. It has also shown distinct alterations of lipoprotein composition in obese subjects compared to controls. By using 2-DE in combination with MS, new interesting LDL proteins such as transthyretin and α_1 -antitrypsin have been found. Furthermore, changes such as decreased distribution of apo E in the obese subjects appeared to be related to gender. Although more studies are needed, it is possible to speculate that differences in LDL protein profiles may reflect an increased risk of atherosclerosis and CVD.

7. Conclusions and future perspectives

After isolation, separation and identification of proteins in LDL and HDL, it is possible to conclude that there are several more proteins present within the density ranges of LDL and HDL after ultracentrifugation than previously thought. The attempt to improve the identification of low abundant proteins in silver stained 2-D gels using 2,5-dihydroxybenzoic acid instead of α -cyano-4-hydroxycinnamic acid was successful. Results showed more matching peptide peaks, higher sequence coverage and higher signal to noise with 2,5-dihydroxybenzoic acid than with α -cyano-4-hydroxycinnamic acid (Paper III). Altogether 47 proteins representing 18 different protein identities were detected in LDL and HDL (Papers I, II, IV and V). We can also conclude that enzymatic treatment of lipoproteins followed by 2-DE is an excellent tool to investigate post-translational protein modifications, such as glycosylation. Thus, five isoforms of apo M depending on glycosylation/sialylation were characterized by their shift in molecular mass and isoelectric point (Paper IV). Among the identified proteins several related to infection/inflammation, such as serum amyloid A in HDL, lysozyme C, transthyretin and calgranulin A in LDL and apo J, apo M, α_1 -antitrypsin and SAA-IV in both LDL and HDL. It is possible that lipoprotein-associated proteins that change in concentration during acute and/or chronic inflammation may serve as markers of CVD. Furthermore, altered LDL-protein pattern was found in obese subjects (Paper V), with significantly higher abundance of proteins such as, apo J, apo M, α_1 -antitrypsin, SAA-IV and transthyretin compared to controls. Altogether these results suggest that further investigations of the protein composition of lipoproteins in relation to cardiovascular events are warranted.

It is well known that the choice of isolation method is a crucial step in the characterization of lipoproteins. During these studies, using ultracentrifugation to isolate LDL and HDL, we have reflected that there might be subgroups of lipoprotein particles within the density range that we are not able to separate from each other or that proteins are lost during ultracentrifugation. It has already been reported that there are “only-serum amyloid A” particles present in the HDL density range during acute phase response in mice, with completely different properties than native HDL [Cabana 1999]. Moreover, studies of apo M containing particles isolated by immuno-affinity chromatography, suggest that they designate subpopulations of LDL and HDL that protect against oxidation and stimulate cholesterol efflux more efficiently than LDL and HDL without apoM, respectively [Christoffersen 2006]. It would therefore be interesting

to isolate the lipoprotein particles by immunoaffinity procedures towards specific proteins such as α_1 -antitrypsin, transthyretin, apo J, lysozyme C or serum amyloid A-IV. This alternative could preferentially be used in combination with ultracentrifugation or a more gentle method like size-exclusion chromatography, in purpose to further characterize the protein composition of different lipoprotein particles.

2-DE in combination with MS is a highly suitable tool for detailed studies of the proteins in the lipoprotein particles. As previously mentioned, by 2-DE it is possible to separate different isoforms of the proteins, e.g. caused by mutations, splice variants or post-translational modifications such as glycosylations, phosphorylations and truncations. It is then possible to characterize these different modifications by MS, or preferentially MS/MS, analysis. Other LDL and HDL modifications of great potential for future studies of atherosclerosis, using MS-based techniques, are nitration, chlorination and other oxidative modifications of proteins. Today mounting evidence suggests the importance of such modifications in the atherosclerotic process. One example is the recent study in which HDL isolated from human atherosclerotic lesions was found to contain elevated levels of 3-nitrotyrosine and 3-chlorotyrosine, indicating that myeloperoxidase from neutrophils oxidizes and impairs HDL *in vivo* [Chao 2005].

Also interesting for future studies is the recently reported link between environmental factors, such as air pollution and cardiovascular disease. It has been observed that increased concentration of fine particulate air pollution is associated with increased risk of cardiovascular events [Bai 2006, Miller 07]. The mechanisms behind this effect are unclear but it is believed to involve systemic inflammation [Bai 2006]. It has been observed that environmental tobacco smoke leads to an increased accumulation of LDL in the arterial wall, which was mediated primarily by interactions of the smoke constituents with LDL rather than with the artery wall [Roberts 1996]. This suggests that biochemical modifications of LDL caused by reactive oxidants or particulate matter in the smoke are of importance. It is therefore not unlikely that exposure to fine particulate air pollution could cause similar effects. It is also possible that these effects are mediated through the activation of inflammatory cells. Indeed, as shown by Lindbom *et al*, airborne particles, collected from busy urban streets, activate macrophages to release pro-inflammatory cytokines. Thus, oxidants, proteolytic enzymes and other mediators from inflammatory cells may contribute to the modification of lipoproteins. Altogether, the value of MS-based studies in order to detect

and identify such modifications in LDL and HDL from individuals exposed to air pollutions is implicated.

To summarize, the availability of powerful tools such as 2-DE, MS and MS/MS in combination with large numbers of databases has given us the opportunity to identify 47 proteins in LDL and HDL and in addition, the possibility of investigating their modifications. Now we have a challenging task in front of us, trying to understand the physiological roles of these proteins and how they might be involved in CVD.

8. Acknowledgements

I have hard time finding words when I try to express how grateful I am that I have had the opportunity to do something as exciting as proteomic research. Many people have contributed to this thesis in different ways:

Since I was considered too old (or perhaps it was because I didn't play an instrument) for the Research School in Biomedicine, I would like to give a heartfelt thanks to **Docent Mats Lindahl** and **Prof. Christer Tagesson** for giving me the opportunity to complete this thesis. You believed in me, and despite financial problems you gave me the chance to do what I really had been dreaming of. This I will never forget.

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