Dept. of Biomedical Engineering and Computational Science

Computational models and methods for lipoprotein research

Linda Kumpula

Aalto University

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Linda Kumpula

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Abstract

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Abstract

Lipoproteins are self-assembled nanoparticles for water-insoluble lipid transportation in the circulation. Lipoprotein particles form a key metabolic system in a variety of normal physiological processes but also play an essential role in many pathological conditions. In particular, certain lipoprotein abnormalities are associated with the development of atherosclerosis, a disease state of arteries, common in cardiovascular disease.

Computational modelling is a potential but so far rarely used method to study lipoprotein particles. This thesis contributes to lipoprotein research by various computational approaches where experimentally isolated and biochemically characterised lipoprotein particles serve as a starting point. This thesis deals with estimating the number of lipid molecules within lipoprotein particles, i.e., composition information, and approximating the molecular structure of lipoprotein particles in each subclass. It also proceed the ultracentrifugal particle isolation by a kind of in silico sub-classification resulting from utilisation of the self-organising map (SOM) method. This, when applied to experimental data, with lipoprotein lipid concentration and composition information combined, shows that there is variability in the compositional/metabolic relations between individuals, i.e., distinct lipoprotein phenotypes. Furthermore, this thesis introduces a method to estimate lipoprotein particle concentrations in each subclass, which also provides a reference particle library for NMR-based lipoprotein particle concentration estimation.

Applications of the models to experimental data show that triglyceride and cholesterol ester molecules, which are conventionally held as core lipids, may also locate in significant amounts in the surface. The lipoprotein phenotype analysis shows that per particle compositions, which appear as a fundamental issue in metabolic and clinical corollaries, can not be deduced solely from the regularly measured plasma lipid concentrations nor from the particle concentration estimates.

Keywords lipoprotein, ultracentrifugation, self-organizing map, lipoprotein composition, lipoprotein concentration

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Laskennalliset mallit ja menetelmät lipoproteiinitutkimuksessa

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Tiivistelmä

Lipoproteiinit ovat nanokokoisia hiukkasia, jotka kuljettavat veteen liukenemattomia rasvoja verenkierrossa. Lipoproteiinit ovat osa normaalia aineenvaihduntaa, mutta ne voivat osallistuvat myös monien tautitilojen syntyyn. Tietyt lipoproteiinipoikkeavuudet ovat tärkeitä sydän- ja verisuonitaudeissa yleisesti esiintyvän ateroskleroosin eli valtimonkovettumistaudin riskitekijöitä.

Lipoproteiinien laskennallinen mallintaminen on lupaava mutta toistaiseksi vähän käytetty menetelmä lipoproteiinien tutkimisessa. Tässä väitöskirjassa sovelletaan lipoproteiinitutkimukseen useita erilaisia laskennallisia malleja käyttäen lähtökohtana kokeellisesti saatua tietoa. Väitöskirjan keskeisiä aiheita ovat lipoproteiinien sisältämien rasvamolekyylien lukumäärien arviointi yhtä lipoproteiinipartikkelia kohti sekä lipoproteiinien molekyylirakenteen estimointi. Väitöskirja tarjoaa myös laskennallisen tavan parantaa ultrasentrifugilla eristettyjen lipoproteiinipartikkeleiden erottelua. Kokeelliseen dataan sovellettuna tämä menetelmä osoittaa, että ihmisten välillä on eroavaisuuksia lipoproteiinien koostumuksissa ja aineenvaihdunnallisissa ominaisuuksissa, ts. lipoproteiinifenotyypeissä. Väitöskirja esittelee myös tavan arvioida lipoproteiinipartikkeleiden konsentraatioita verenkierrossa. Tämä mahdollistaa myös vertailutietokannan rakentamisen partikkelikonsentraatioiden estimoimiseksi NMR-lähtöisellä tavalla.

Väitöskirjan mallien soveltaminen kokeelliseen dataan osoittaa, että triglyseridit ja kolesteroliesterit, joita tavanomaisesti pidetään lipoproteiinien ytimissä sijaitsevina rasvamolekyyleinä, voivat sijaita huomattavina määrinä myös hiukkasten pinnan tuntumassa. Lipoproteiinifenotyyppien analysointi osoittaa, että lipoproteiinihiukkasten koostumusta, jolla näyttää olevan merkitystä ihmisen aineenvaihduntaan ja kliiniseen tilaan, ei voida päätellä pelkästään plasman rasvakonsentraatioista tai lipoproteiinipartikkelien konsentraatioista.

Avainsanat lipoproteiini, ultrasentrifugi, itseorganisoituva verkko, lipoproteiinien koostumus, lipoproteiinien konsentraatio

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Preface

I have been exceptionally lucky since I found a research subject which I felt important and interesting and which challenged me daily. Thus these years have been intriguing time for me. A part of the research I contributed is collected to this thesis for the degree of Doctor of Science in Technology. The work was performed mostly during the years 2006-2010 in the Department of Biomedical Engineering and Computational Science (former Laboratory of Computational Science) at the Aalto University School of Science (former Helsinki University of Technology). My work has been funded by Academy of Finland Centre of Excellence program, Vilho, Yrjö and Kalle Väisälä Fund, Jenny and Antti Wihuri Fund, Finnish Concordia Fund and Emil Aaltonen Foundation.

I would like to express my gratitude to several people who have influenced me and my work during these years. I am grateful to my supervisor Prof. Kimmo Kaski for many things such as introducing me this research subject in the first place, giving me the opportunity to work in the lab, believing in me during the difficult times and, finally, guiding me during the preparation of this manuscript. I am also thankful to Prof. Mika Ala-Korpela for guiding me through various academic challenges and for the fortune to be a member of Computational Medicine Research Group. I also wish to thank the other present and former members of the group, DSc (Tech) Ville-Petteri Mäkinen, Taru Tukiainen, Pasi Jylänki, Jaakko Niemi, Tomi Peltola, Antti Kangas, Janne Ojanen, Johanna Hokkanen, Aino Salminen and Niko Lankinen. You have given me company I will never forget. I will particularly miss the Friday afternoons full of laugh, some science and the funny aphorisms of the days. My thanks go also to several other co-workers in the lab at the time, namely, Iina Aaltonen, Jenni Hulkkonen and DSc (Tech) Riitta Toivonen. It was always nice to make the world a bit better with you. I want also thank my collaborators Prof. Marja-Riitta Taskinen from Division of Cardiology, University of Helsinki; Docent Matti Jauhiainen from National Institute for Health and Welfare, Department of Molecular Medicine and Prof. Markku Savolainen, PhD Minna Hannuksela and Sanna Kuusisto from Institute of Clinical Medicine & Department of Internal Medicine, Clinical Research Center & Biocenter Oulu, University of Oulu and Oulu University Hospital.

Beyond the work, I am grateful to my parents Pirjo and Eero for providing me safe, inspirational and encouraging environment to grow up. I am sure it was sometimes difficult to find interesting things to do to a child like me and my mom truly made her best. My dad told me thousands of times about his work in hard metal industry which probably influenced my choices of schooling and profession. I am also grateful of your time particularly during these two years when I prepared this manuscript. I want also thank my parents-in-law Helena and Kari, sister-in-law Johanna, brother-in-law Tuomas and my best friend Eija for being interested in my things including work and being part of my life.

Finally, my deepest gratitude belongs to my husband Jussi and our daughters Oona and Iina, the joys of my life. Jussi, I enjoy the discussions we have about life, science, sports, your work and mine. Your support and advices have been valuable especially in times I have struggled with my studies or work. You have also helped me to 'have it all', to combine work and family, by taking high responsibility of our everyday wellbeing. Oona and Iina, I am also fortune to be your mom and be able to view your growth and interests in the small and large things in this world.

Eurajoki, March 2011

Linda Kumpula

"Never forget that only dead fish swim with the stream."
-Malcolm Muggeridge-

Contents

Author's contribution

This thesis is a monograph and completely written by the author, Linda Kumpula. The research reported in this thesis is, however, a result of collaboration between Kumpula and several other authors. Kumpula has had a distinct and independent role in each of the studies included in this thesis; the author had the main responsibility of the theoretical analysis and implementation of the computer programs or pre-processing scripts in the studies. The author has also had a significant role in conceiving and designing the studies and the analysis of the numerical results. Two of the three studies reported in here have also been published in refereed journals, see[424, 467]. The author of this thesis had also a substantial role in preparing these manuscripts.

List of abbreviations

ABCA1	ATP-binding cassette transporter A1, a cholesterol efflux regulatory protein
ABCG1/4	ATP-binding cassette transporter G1 and G4, cholesterol efflux regulatory proteins
ATP I	Adult Treatment Panel I, first guidelines from the National Cholesterol Education Program to evaluate the risk of atherosclerosis
ASCOT-LLA	Anglo-Scandinavian Cardiac Outcomes Trial – Lipid lowering arm, a clinical study
apo	apolipoprotein, a lipid-binding protein in lipoprotein particles
BMI	body mass index, a measure of body weight based on a person's weight and height
C, Chol	cholesterol, includes both free, unesterified cholesterol and cholesterol ester molecules.
CAD	coronary artery disease, a disease state of the arteries in the heart
CARE	Cholesterol and Recurrent Events Trial, a clinical study
CE	cholesterol ester, a lipid
CETP	cholesteryl ester transfer protein, a transfer protein
CM	chylomicrons, lipoproteins
CPPT	Coronary Primary Prevention Trial, a clinical study
FC	free or unesterified cholesterol, a lipid
GGE	gradient gel electrophoresis, a lipoprotein characterisation method
HDL	high-density lipoproteins
HDL-E	high-density lipoprotein particles with apoE
HL	hepatic lipase, an enzyme
HMG-CoA	3-hydroxy-3-methylglutaryl-Coenzyme A

HPGC	high-performance gel-filtration chromatography, a lipoprotein isolation and characterisation method
HPLC	high-performance liquid chromatography, a lipoprotein isolation and characterisation method
hs-CRP	high-sensitive C-reactive protein, an inflammation marker in blood
IDL	intermediate-density lipoproteins
LCAT	lecithin-cholesterol acyltransferase, an enzyme
LDL	low-density lipoproteins
LDL-P	concentration of an LDL particle
LDL-R	LDL-receptor, a receptor internalising lipoprotein particles
LPL	lipoprotein lipase, an enzyme
Lp(a)	lipoprotein (a), LDL particle with a specific additional protein
LRP	LDL-receptor-related protein, a receptor internalising lipoproteins
NCEP	National Cholesterol Education Program, a program to reduce illness and death from coronary artery disease in the United States
NIH	National Institutes of Health, a medical research centre in the United States. Now known as the National Heart, Lung, and Blood Institute (NHLBI)
NMR	nuclear magnetic resonance, NMR spectroscopy is a technique exploiting the magnetic properties of certain nuclei
ORP	oxysterol binding protein-related proteins, proteins acting as sterol sensors or transporters
PC	phosphatidylcholine, a phospholipid
PL	phospholipid
PLTP	phospholipid transfer protein

PROT	protein
sdLDL	small, dense LDL, subclass of LDL particles
Sf	Svedberg flotation unit, a measure of flotation in ultracentrifugation
SM	sphingomyelin, a phospholipid
SOM	self-organising map, a type of artificial neural network in machine learning
SR-BI	scavenger receptor BI, a receptor internalising lipoproteins
TG	triglyceride (or triacylglycerol), a lipid
UCF	ultracentrifugation, a lipoprotein isolation method
VHDL	very high-density lipoprotein
VLDL	very low-density lipoproteins
WHO	World Health Organization, a specialized agency of the United Nations that acts as a coordinating authority on international public health
4S	Scandinavian Simvastatin Survival Study, a clinical study

List of symbols

α	the relative portion of a lipid in the core of a lipoprotein particle
aa	amino acid
c	concentration of a lipid [mol/l]
f,s	lipoprotein fraction, such as VLDL, or subclass, such as VLDL1
i	individual
l	lipid
M	molecular mass of a lipid or protein [g/mol]
N	number of lipid molecules
P	concentration of a lipoprotein particle [mol/l]
ρ	concentration of total proteins [mg/dl]
v	molecular volume of a lipid or an amino acid [nm^3]
$\tilde{v}_l$	averaged molecular volume of lipids [nm^3]
d	diameter of a lipoprotein particle [nm]
V	volume of a lipoprotein particle [nm^3]
V_{core}	volume of the core [nm^3]
V_p	volume of total proteins within a lipoprotein particle [nm^3]
W	weight percentage [%]

1. Introduction

Lipoprotein particles are lipid transporters in the circulation of all vertebrates and are thus key players also in human physiology. One of the physiological processes is transport of triglycerides for cells' energy metabolism. Lipoprotein particles are also important in transporting cholesterol molecules which are used in cellular plasma membranes and subcellular organelles as structural elements and for production of certain important molecules such as vitamin D, bile acids and several hormones including steroid hormones. Although cholesterol is an essential molecule for proper cell functioning, it is harmful to the cells in large amounts and thus lipoprotein particles, as the main cholesterol transporters, have also key roles in human pathology. Interest in lipoprotein particles has arisen particularly due to their association with cardiovascular disease, which is the major cause of death in the Western World[1]. The main cause of cardiovascular disease is atherosclerosis, which is a disease state of arterial walls. The etiology of atherosclerosis is not, however, yet totally explained although much has been learned over decades about this complex disease. Nevertheless, the role of lipoprotein particles in the development of atherosclerosis is currently widely acknowledged. This introductory chapter reviews the common history of atherosclerosis and lipoprotein particle research and gives the framework to the following chapters of lipoprotein particles.

1.1 The history of atherosclerosis and lipoprotein research

In the 19th century, atherosclerosis was recognised as an inevitable accompaniment of ageing about which nothing much could be done. Nevertheless, the century provided grounds to the development in the following century: a lipid molecule, which became also called *the molecule of the 20th century*, was discovered. It was found in the early 19th century by a French chemist Michael E. Chevreul who was the first to isolate and purify the lipid from gallstones[2]. Chevreul also named the molecule *cholesterol* from Greek words *chol* for bile and *stereos* for solid. By the end of the 19th century, the presence of cholesterol in human blood was also confirmed, cholesterol was observed in athero-

sclerotic lesions, and, interestingly, blood lipid accumulation was suggested to cause atherosclerosis as reviewed elsewhere[3].

First suggestions about the association between high blood cholesterol and the development of atherosclerosis

The current era of atherosclerosis research began in 1913 when a young experimental pathologist Nikolai Anitschkow showed that simply feeding rabbits purified cholesterol dissolved in sunflower oil induced vascular lesions while the control rabbits fed only the sunflower oil showed no lesions[4, 5]. In addition, the cholesterol-induced lesions resembled those of human atherosclerosis both grossly and microscopically. Over the ensuing years, Anitschkow and colleagues established sharp-eyed overview of essential parts of the development of atherosclerosis known today[6]. These included infiltration of leukocytes into the arterial wall, their enrichment with cholesterol, development into early and further into advanced lesions over time. They also conducted that the cholesterol came from the blood, the degree and the duration of increased blood cholesterol effected on the extend of the lesions, and that the early lesions were also reversible.

The seminal work by Anitschkow was, however, rejected or at least not followed up and the serious role of cholesterol in human atherosclerosis did not really become appreciated until years later. Some laboratories actually tried to confirm Anitschkow's finding, but, instead of using rabbits as laboratory animals, most used rats and dogs with which they were more familiar with[7]. Those animals are, however, very efficient in converting cholesterol to bile acids and thus, even on very high cholesterol intake, the diet does not increase their blood cholesterol levels appreciably to enhance the development of atherogenic lesions. As a result, these new cholesterol feeding studies failed and the investigators concluded that Anitschkow's results must reflect some peculiarity of the rabbit. Thus, unfortunately, the rabbit model was dismissed as irrelevant for human disease. In retrospect, the misfortune was not directed only on the choice of the laboratory animals, but the researchers should have recognised an important causal relation relevant for atherosclerosis: feeding of the cholesterol followed by increase in blood cholesterol levels.

Another reason why Anitschkow's work was not taken seriously was that his rabbits had extraordinary high blood cholesterol levels ranging from 500-1000 mg/dl (13-26 mmol/l) or even higher. In humans, cholesterol levels that high are rarely measured and extrapolation was felt unwarranted. Soon after the first study, Anitschkow showed that more modest increases of cholesterol levels in rabbits were sufficient to induce lesions although it took longer[7]. The general prejudice was, however, strong among the *cholesterol disbelievers*:

how cholesterol feeding within a few months can cause the illness which was generally accepted to be an inevitable accompaniment of ageing.

Interestingly, the rarely occurring individuals with extraordinary high plasma cholesterol levels (up to 1000 mg/dl, 26 mmol/l) could have proved the Anitschkow's case. As early as in 1889, Lehzen and Knauss[8] had reported case studies of children with xanthomas, large deposits of lipids just beneath the skin or attached to tendon sheaths on the backs of the hands or at the ankles. At that time, it was noted that a number of these children developed serious heart problems at a startlingly early age and the disease had high familial expression. These cases were, however, very rare, and xanthomas were much more commonly associated with liver disease or diabetes, or most commonly, found in healthy older human subjects. About twenty years later, and a few years before Anitschkow's results, Pinkus and Pick had found that the individuals with xanthomas have high plasma cholesterol levels and the lipids in xanthomas were cholesterol esters[9]. They also had suggested that the cholesterol in the deposits originated from the blood and attached to tendons and vascular walls. It is impossible to say if Anitschkow was aware of these studies, and whether he had realised the value of these early studies to his results.

Anyhow, the next interesting observations of xanthomas came from Carl Müller, a Norwegian professor of internal medicine, who in 1939 reviewed the concurrent familial expression of xanthomas, hypercholesterolemia and heart disease. He was also able to add observations of almost a hundred cases from almost 20 Norwegian families[10]. At the time, consanguineous marriages were still fairly common in some isolated communities in Norway increasing the prevalence of the rarely occurring individuals with high plasma cholesterol levels with the classical xanthomas of skin and tendons. Professor Müller concluded in his now-classical paper[10] that:

> *"The reports I have presented confirm the previous observations on xanthomatosis as a cause of hereditary heart disease. They reveal further that the syndrome of cutaneous xanthomatosis, hypercholesterolemia and angina pectoris presents itself as a well defined clinical entity. There can be hardly any doubt but that xanthomatous deposits in the coronary artery and consecutive myocardial ischemia are the cause of the angina pectoris."*

Thus Müller stated that a familially occurring high plasma cholesterol level, or familial hypercholesterolemia, was the cause of heart diseases. Over the next twenty-five years, Müller's characterisation of familial hypercholesterolemia borne out a series of extensive studies which established familial hypercholesterolemia as a monogenic defect implying that the arterial disease was secondary to the elevated blood cholesterol analogous to the pathogenes-

is of Anitschkow's rabbits[11-13]. Thus the linkage of hypercholesterolemia and cardiovascular disease was shown in human beings.

Discovery of lipoprotein particles – a competitor to the blood cholesterol

During the two first decades of 20th century, a number of researchers began to think that the considerable amount of cholesterol molecules should be present in plasma either within some sort of emulsion particles or associated with proteins. The first definitive studies of these lipid transporters, which were named lipoproteins, were reported by Macheboeuf and colleagues in 1929[14]. Over the next 10 to 20 years, they succeeded in purifying and characterising a lipoprotein from horse serum. During World War II, Cohn and coworkers[15] and Oncley, Scatchard and Brown[16] at Harvard developed large-scale methods to fractionate human serum to provide materials useful in treating wounded. The method turned out to be useful also in lipoprotein research since the developers noticed that human serum contained not one but two major lipoprotein fractions named α- and β-lipoproteins which were later on called high-density lipoproteins (HDL) and low-density lipoproteins (LDL), respectively. At the time, nothing was known about the origin, the fate, or the biological significance of lipoprotein particles but they were soon associated with the development of atherosclerosis. In 1951, Russ, Eder and Barr[17] used the methods developed in the Cohn/Oncley laboratory and found that women before menopause had consistently higher blood levels of the α-lipoproteins than men. The authors speculated that the difference may explain the lower incidence rate of coronary artery disease (CAD) in pre-menopausal women. In retrospect, this view is still currently supported but even more importantly, Russ, Eder and Barr brought new perspective on atherosclerosis research by suggesting that different lipoprotein fractions might have distinct biological functions and that there may be a linkage between specific lipoprotein patterns and the risk of coronary artery disease.

The results of Russ, Eder and Barr induced questions about the total number of different lipoprotein particles and their function in human beings. The man who was to answer several of these questions was John W. Gofman. Gofman's background was appropriate to enhance the lipoprotein and atherosclerosis research; he had roots both in medicine and physics, and he was thus able to apply methods and ideas from one field to another. Gofman was also familiar with Anitschkow's work which he, unlike most others at the time, took very seriously. Anitschkow's work, together with the limited genetic, biochemical and epidemiological evidence available, had convinced Gofman that blood cholesterol was centrally important in the development of

atherosclerosis.

Gofman was also familiar with a technique called analytical ultracentrifugation which was developed by Theodor H. E. Svedberg in Sweden. The method proved to be invaluable for characterising proteins and measuring their molecular sizes and relative concentrations in mixtures. Gofman wanted to see if this methodology could be used to separate lipoprotein particles and purchased an ultracentrifuge, which was the second of its kind, in his laboratory in California. It turned out that the analytical centrifuge was indeed useful in characterising lipoproteins[18]. After a while, Gofman with his colleagues Frank T. Lindgren and Harold Elliot managed to devise an accurate and reproducible way to separate lipoproteins in plasma into fractions and to reliably measure their amounts in mass units. This was a breakthrough in lipoprotein research and enabled Gofman and his colleagues to study the relationship between coronary artery disease and not just total cholesterol but also the amounts of the lipoprotein particles that carried that cholesterol. Gofman and his colleagues believed that it mattered a great deal in which lipoprotein fractions the blood cholesterol was carried, and in 1950 they presented preliminary data on a limited number of patients suggesting that the Sf 10-20 fraction was particularly pro-atherogenic[19]. This fraction corresponds closely to the so-called intermediate density lipoprotein (IDL) particles which are also today realised to be strongly pro-atherogenic[20-23].

A few years later, Gofman and his colleagues published a formula, a *lipoprotein index*[24], to weight the different lipoprotein classes according to their atherogenicity. Gofman and his colleagues suggested that the different classes of lipoproteins should be weighted differently in estimating the risk of coronary artery disease using this atherogenic index. However, the number of samples on which the conclusions were based was quite small, and thus a large prospective study was needed to make the case. There were, however, various problems on the way: large prospective studies would require hundreds of blood samples and there were only two analytical ultracentrifuges in the country, ultracentrifuges were extraordinary expensive and technically difficult to operate and National Institutes of Health (NIH) had turned down Gofman's grant application. The study[25] was, however, done in collaboration with three other laboratories supported by a large grant from NIH. Unfortunately, the attempt fell into technical problems with the analytical ultracentrifuge; after the study had already begun, Gofman noticed a technical glitch in his methodology but could correct it. However, by that time, a large number of samples had already been used up or there was not enough left to reanalyse them. As a result, the final report from three of the four participating laboratories had to be based on the original method only, whereas the results from

Donner laboratory, where Gofman was working, were corrected and reported in both the original and the revised, more accurate form. The final report[25], published in 1956, contained two formal Discussion sections, one for Donner-'s laboratory and the another for the three other laboratories dividing the authors into two parties. The original report suggested that the total plasma cholesterol level and lipoprotein particle amounts were equally good predictors of coronary artery disease risk while the researchers in Donner laboratory favoured the lipoprotein measures.

The study was epochal in many ways. Before the study, the battle had been between researches who either believed or disbelieved that cholesterol molecules have a key role in the development of atherosclerosis. Now, the believers were also divided into two parties, the ones who had faith in plasma cholesterol levels and the ones who became interested in lipoprotein particles as predictors of the risk of coronary artery disease. Thus among the *lipoprotein believers*, the work of Gofman and his colleagues induced a huge increase in research on the plasma lipoprotein particles and their relationship to atherosclerosis.

High blood cholesterol strengthens its role in the development of atherosclerosis

At the same time as Gofman was working with lipoproteins, the National Heart Institute was carrying out a study in the small town of Framingham, Massachusetts, and the study became known as *The Framingham Heart Study*[26-28], the most impacted single epidemiological study in atherosclerosis research. At the time, a large majority of the 28 000 residents of Framingham agreed to participate in the study which was designed to gather large-scale information on most of the potentially relevant factors of coronary artery disease known at the time. In later years, additional measurements were added as more was learned about atherosclerosis and the study is actually continuing actively to this day studying the offsprings of the original cohort[29].

The first article of the Framingham project was published in 1957[28] after four years of follow-up, and papers have been published on the project after that almost annually. The Framingham Heart Study was the first to provide solid and unarguable evidence that individuals with higher blood cholesterol levels are more likely to experience a heart attack. However, the Framingham study was also the first to show that several other factors than high plasma cholesterol, such as high blood pressure and smoking, may increase the risk of atherosclerotic heart disease at least in additive manner.

The cholesterol believers were not, however, completely pleased with the results. Daniel Steinberg described the feelings after the first results from the

Framingham project is his historical review[30] as:

> *"What was needed was an intervention trial, a controlled experiment that would show that lowering cholesterol levels as a single variable could reduce coronary risk. However, safe and effective drug treatment for hypercholesterolemia was still some way down the road, and the effectiveness of manipulating dietary fat was only becoming clear in the late 1950s."*

The era of large-scale clinical dietary intervention trials was beginning at the time encouraged by the results of the Framingham Heart Study.

Diet-heart hypothesis and dietary studies

When coming to the 1960s, American Heart Association, whose goal is to prevent and reduce the incidence of cardiovascular disease in the United States, was convinced about a causal relationship of increased blood cholesterol and atherosclerotic heart disease[31]. The American Heart Association also recommended, based on limited, small sized dietary trials at the time, that people at high risk should be advised to modify their diet to avert heart attacks. Interestingly, however, the modifications concerned the amount and, even more interestingly, the quality of fatty acids rather than the amount of cholesterol in diet. Some years later these recommendations were extended to include the general public. These recommendations became known among researches as a so-called *diet-heart hypothesis*; eating less saturated fats lowers serum cholesterol levels, and the lower serum cholesterol leads to a reduced risk of heart attacks.

Some investigators took the chance to test the effect of the diet-heart hypothesis. Obviously, the freshly started studies would take years before publicising and thus Ancel Keys, a pioneer in nutritional research, who had been convinced much earlier that blood cholesterol level was determined significantly by the amount and the nature of the fat in the diet[32], hurried to publish his results first. The study, *The Seven Countries Study*[33], was published in 1966.

In the late 1940s, Keys, with his colleagues, had decided to launch an ambiguous study to test the hypothesis that populations with high fat-diets should have higher blood cholesterol levels leading to higher heart attack rates than other populations. Keys and his colleagues selected seven countries which spanned the full range of blood cholesterol levels. These countries included Japan, Yugoslavia, Italy, the Netherlands, Finland, Greece and the United States. In each country, blood samples were drawn for cholesterol measurements, the nature of diet was determined by a questionnaire and the coronary artery disease death rate (per 1000 men over 10-year period) was

then correlated with these two variables. It turned out that when coronary artery death rate was plotted against the blood cholesterol levels for all the seven countries, the data points fell roughly on the same straight line, strongly suggesting that the population risk was roughly proportional to the blood cholesterol level over the range of values studied[33]. Similarly, the blood cholesterol concentrations plotted against the contribution of saturated fats to the daily calorie intake fell roughly along a straight line[33]. Thus, taken together, the data showed that the population risk of fatal heart attacks was proportional to the blood cholesterol level, which was, in turn, proportional to the dietary intake of saturated fats.

The relationship between saturated fats and blood cholesterol level was, however, only correlational, although strong, and thus it could not establish causality. Conceivably, genetic differences or other differences in living habits might be the true explanation for the correlation. Keys and his colleagues were aware of this limitation in their study design. At the same time, a group of researchers in Hawaii investigated Japanese immigrants in Hawaii and San Francisco and compared them with native Japanese living in Japan[34].

Hawaii and San Francisco have a very large population of Japanese immigrants who have lived in their new home country only a few generations and thus their gene pools could not have changed significantly. The investigators determined the blood cholesterol levels and heart attack rates of these Japanese immigrants and compared them with the same measures in native Japanese in Japan. The result was striking: The Japanese who had moved to Hawaii had higher cholesterol levels and heart attack rates than the Japanese in Japan[34]. The difference was even more striking in those who had settled in San Francisco. Since these results could not be explained by genetic factors, the rise in blood cholesterol levels and the accompanying increase in heart attack rates following the migration must have been due to environmental factors, most likely changes in dietary habits[34].

The results of several dietary intervention trials, which were started after the first results of the Framingham Heart Study, became available a few years later. Three studies in particular – the Leren Oslo Study[35], the Wadsworth Veterans Administration Study[36] and the Finnish Mental Hospital Study[37, 38] – confirmed the results from The Seven Countries Study that saturated fats tend to increase the serum cholesterol levels but also showed that diets rich in polyunsaturated fat could significantly lower the serum cholesterol levels. Thus it became clear that the quality of dietary fats has a marked effect on blood cholesterol levels.

Associations between lipid and lipoprotein variables – lipoprotein phenotypes

Work on the association of lipoproteins with atherosclerosis was also proceeding; Donald S. Fredrickson, working at NIH, was convinced of the correctness of Gofman's view that patterns of lipoproteins might contain valuable information beyond the measurements of the component lipids only, namely plasma or serum levels of cholesterol and triglyceride molecules. Fredrickson was, however, also aware that Gofman's method, analytical ultracentrifugation, was just too complicated and expensive for clinical use. Fredrickson felt that it was important to bring easier methods to the clinics and got soon familiar with a technique called paper electrophoresis which was introduced to him by his young colleague. Fredrickson immediately saw the enormous clinical potential of the method; Paper electrophoresis could separate the lipoprotein classes, based on their mobility, to four different bands[39]. In the following years, Fredrickson and this colleagues studied and classified lipoprotein patterns of hundreds of patients referred to the Clinical Centre at NIH and found that most of them could be classified into one of five or six lipoprotein types, or phenotypes[40-43], as shown in Table 1.1.

Table 1.1 Fredrickson's phenotypes

Phenotype	Elevation	Plasma C	Plasma TG	Atherogenicity	Prevalence
I	CM	↔ to ↑	↑↑↑↑	−	< 1 %
IIa	LDL	↑↑	↔	+++	10 %
IIb	VLDL and LDL	↑↑	↑↑	+++	40 %
III	IDL	↑↑	↑↑↑	+++	< 1 %
IV	VLDL	↔ to ↑	↑↑	+	45 %
V	VLDL and CM	↑ to ↑↑	↑↑↑↑	+	5 %

C, cholesterol; TG, triglyceride; CM, chylomicrons; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins; IDL, intermediate-density lipoproteins.

At the time Fredrickson was formulating the lipoprotein phenotype classification with his colleagues, it was known that also triglyceride molecules, in addition to cholesterol molecules, are located within lipoprotein particles and that there are two classes of lipoproteins which are specialised in transporting these triglycerides, namely chylomicrons and VLDL particles. Thus, Fredrickson's classification of hyperlipidemias related both plasma cholesterol and triglyceride levels to increased relative numbers of specific lipoprotein fractions and could also distinguish several new familial syndromes with high plasma triglyceride levels. The phenotypes which were classified as the most

atherogenic were the ones containing elevated IDL or LDL particles in accordance with the results of Gofman and his coworkers some years earlier.

Fredrickson's phenotyping of lipoproteins was warmly welcomed in clinical practice in the United States and also abroad. In addition, World Health Organization (WHO) recommended the lipoprotein phenotyping system as a worldwide standard in 1970. It was, however, obvious from the beginning that the phenotypes were neither homogeneous entities nor true genotypes but were frequently secondary to other, although unknown, conditions. The phenotyping system had, though, an important role in lipoprotein research since it introduced lipoprotein particles to physicians. The designation of individuals into the Fredrickson's phenotypes was also the first step toward better diagnosis and management of hyperlipidemia; there were unique clinical features in each phenotype, different responses to both diet and drug regimens as well as important differences in the complications of the hyperlipidemia and in familial expression.

When entering into 1970s, Fredrickson and his colleagues continued to make the lipoprotein phenotyping system more practical. At the time, the electrophoretic measurements were performed in local or regional laboratories and the diagnoses of the printouts, which were sent to the practitioners, were regularly poor in identifying Fredrickson phenotype III with high IDL. The phenotype, although rare, brought a lot of attention once recognised and treated since the condition was highly atherogenetic with visually observable xanthomatous skin deposits which melted away within a few weeks of proper treatment. The practitioners were becoming, however, annoyed by the situation which led Fredrickson and his colleagues to search of algorithms and other tools for the use of practitioners with minimum extra laboratory analysis.

Friedewald's formula – a transform from lipoproteins to lipid variables

At the same time, various laboratories were publishing average compositions of VLDL particles (see, for example,[44] and references therein). Using these compositions and observations made from hundreds of patients with high plasma triglyceride levels, Fredrickson and his colleagues were searching a way to estimate LDL particle concentrations[45]. The main idea of Fredrickson and his colleagues was that the ratio of triglyceride to cholesterol molecules in VLDL particles was constant. Thus the amount of cholesterol molecules in VLDL particles could be estimated by their triglyceride content. Fredrickson and his colleagues also observed that in the absence of chylomicrons most of the triglycerides in plasma were in VLDL particles. Thus the amount of VLDL

triglycerides in plasma could be approximated by the plasma triglyceride concentration.

These estimations yielded the most well known formula in predicting the cholesterol content in LDL particles. The concentration of LDL cholesterol can be estimated by the difference of total cholesterol and cholesterol content in other lipoprotein particles, such as HDL and VLDL particles, namely,

$$c_{LDL-C} = c_{total\,C} - c_{HDL-C} - \frac{c_{TG}}{2.2} \quad , \qquad (1.1)$$

where c_{LDL-C} and c_{HDL-C} are the concentration of cholesterol in LDL and in HDL particles, respectively, and $c_{total\,C}$ and c_{TG} are the concentration of plasma total cholesterol and triglycerides, respectively, in mol/l[21, 46]. The last term in this equation represents the concentration of cholesterol in VLDL particles. The formula is called Friedewald's formula[46] in the honour of biometrics expert, William Friedewald, who checked the validity of the formula for Fredrickson and his colleagues. Use of the equation was restricted to individuals with less than 4.5 mmol/l of plasma triglycerides[21, 46].

This equation became popular because it utilises variables which can be easily measured in any laboratory. The equation is also pretty good in estimating ultracentrifuged LDL cholesterol although it tends to overestimate the LDL cholesterol in individuals with elevated IDL cholesterol levels[21]. Beyond the success in LDL cholesterol estimation, the purpose of the model was, however, to find a way to estimate LDL particle concentration indirectly via calculated LDL cholesterol concentration. The quality of this estimate, to the best of the author's knowledge, has not been published. The good correlation between calculated and experimental LDL cholesterol measures was incorrectly thought to prove the case. Thus, despite the work of Gofman and his colleagues as well as the improvements in lipoprotein research by Fredrickson, this estimate of particle concentrations led part of the *lipoprotein believers* from the lipoprotein values back to the lipid values. This time the term *total plasma cholesterol* was only replaced by the word *LDL cholesterol*.

Progress in atherosclerosis research

Anitschkow's work became slowly acknowledged: In the 1930s, lesions in cholesterol-fed rabbits and in human coronary arteries were noticed to be similar in most respects[47]. In the 1950s, the accumulation of cholesterol and other lipids in lesions was found to be one of the most striking morphologic

features of both human and experimental cholesterol atherosclerosis[48] and macrophages laden with lipids were observed in the vessels of cholesterol-fed rabbits[49]. In the 1960s, smooth muscle cells were detected as the predominant cell type in larger lesions and some of these smooth muscle cells were found to contain lipid droplets[50]. In the 1970s lesions in rabbits and in non-human primates were perceived to regress when hypercholesterolemic diet was discontinued[51, 52].

In the late 1960s and early 1970s, the research of the development of atherosclerosis among pathologists was directed to the role of smooth muscle cells. Two hypotheses of the initiation and the development of atherosclerosis were proposed and became widely accepted. Interestingly, the hypotheses were not based on hypercholesterolemia although high plasma cholesterol was accepted as a primary causative factor of atherosclerosis by the American Heart Association[31] just a few years earlier. These two hypotheses were proposed by Earl Philip Benditt and John M. Benditt (group 1) and Russell Ross and John Glomset (group 2) working in the same department at the University of Washington, and the hypotheses were named as *the monoclonal hypothesis*[53] and *the response-to-injury hypothesis*[54, 55], respectively, both published initially in 1973.

Benditt and Benditt proposed that the smooth muscle cell accumulation in any given localised atherosclerotic lesion originated from a single cell that had, for some reason, been triggered to grow rapidly to become a tumour of no danger to health[53]. In the same year, Ross and Glomset were studying cultured smooth muscle cells when they suddenly found the missing piece in their colleagues' study: a growth factor[56]. Ross and Glomset found that serum from blood that had been allowed to clot contained a growth factor for smooth muscle cells that was absent in serum separated from whole blood not allowed to clot. Thus, the growth factor was evidently present in platelets and was released when the platelets aggregated. Ross and Glomset combined this observation with the existing evidence that mechanical injury to the endothelium could lead to platelet aggregation and thickening of the inner layer of the vessel walls. Thus, the response-to-injury hypothesis[54, 55] stated that some still-unidentified form of *insult* occurs to the arterial endothelium, part of endothelium erodes and exposures underlying matrix to which platelets adhere and release growth factor(s). The growth factor(s) access the cells in the subendothelial space and stimulate smooth muscle cell proliferation. Repetition of such injuries over years, or chronic injuries, then led to complex lesions. The hypothesis was further developed so that the type of the injury was changed from mechanical to functional[57, 58] after the endothelium overlying the initial lesion was found unaltered[49, 59-61]. Later on, Ross reviewed the

factors which might be responsible for the injury to endothelium and mentioned hypercholesterolemia in this context with the same weight as homocysteinemia, hypertension, infection and some other potential pro-inflammatory factors[57].

The mystery of familial hypercholesterolemia and an LDL-receptor

Also the research branch of lipoprotein particles, initiated by Gofman and his colleagues and continued by various researches including Fredrickson, was proceeding further. Fredrickson's phenotyping had been strictly phenotypic and little was known about the origin and the metabolism of lipoprotein particles or their relationships to one another. The mechanisms behind Fredrickson's phenotypes remained unknown, and it was unclear to what extent the phenotypes were genetically determined. However, the situation was going to change. It was established in 1973 that the relationships of myocardial infarctions with elevated plasma triglyceride and/or plasma cholesterol concentrations were complex. In a few cases the disorders were monogenic and inherited in a Mendelian fashion. In many families hypercholesterolemia was genetically determined but involved multiple genes and, finally, there were multiple cases where elevated triglyceride levels appeared not to be genetic[62-64]. The accordance of this new classification with Fredrickson's phenotyping was poor; sometimes two or more phenotype patterns appeared from a single genetic disorder. Over the next few years, the new classification system was confirmed and revised by more sophisticated studies of lipoprotein metabolism and improved methods of genetic analysis.

The time seemed right for two forthcoming Nobel Prize winners, Joseph L. Goldstein and Michael S. Brown, to solve the mystery of familial hypercholesterolemia which had been popping out once in a while in the history of atherosclerosis research. These two men had sought the opportunity to work together for years and their collaboration got under way at Southwestern Medical School at the University of Texas Health Science Center in Dallas from the beginning of 1970s and the collaboration is actually still going on after almost forty years!

Goldstein and Brown wanted to investigate metabolic diseases and both were intrigued by the still-mysterious disorder, familial hypercholesterolemia. At the time Goldstein and Brown started their collaboration, it was known that individuals with familial hypercholesterolemia had high lipoprotein levels, particularly LDL particles[43, 65], and had a high prevalence of atherosclerosis[10]. It was also known that the disease was caused by a single gene mutation[10, 12, 13, 66] although the target gene was still unknown. Thus, Goldstein

and Brown started their search with the rate-limiting enzyme in the synthesis of cholesterol, 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase, that had recently been partially purified and characterised by Brown with his colleagues[67]. Goldstein and Brown hypothesised that the cells in individuals with familial hypercholesterolemia might be producing cholesterol at an abnormally high rate secondary to a genetic flaw in the reductase enzyme or in its regulation and planned a series of studies to confirm the hypothesis.

Goldstein and Brown decided to test their hypothesis by growing cells in culture. Since the fundamental role of the liver in lipid metabolism was already known[68], the obvious choice of cells to grow would have been liver cells. The human liver cells were not, however, available and thus Goldstein and Brown chose human skin fibroblasts which were recently successfully used to discover and characterise several gene abnormalities underlying metabolic disorders. They planned to assay the activity of the HMG-CoA reductace enzyme and take that as a measure of the rate of cholesterol synthesis.

In their very first set of experiments[69], Goldstein and Brown found that normal cells grown in the presence of serum synthesised low amounts of cholesterol. However, when the serum was removed and cells were incubated overnight in a simple, protein-free medium, the rate of cholesterol synthesis rose sharply, as much as 10-fold. In contrast, cells from individuals with familial hypercholesterolemia always showed a high rate of cholesterol synthesis, even in the presence of serum. Moreover, the addition of LDL to the medium, which reduced synthesis 10-fold in normal cells, had absolutely no inhibitory effect on cells from individuals with familial hypercholesterolemia. The cells from individuals with familial hypercholesterolemia did, however, respond similarly as normal cells if pure cholesterol dissolved in alcohol was added to the culture medium instead of LDL particles.

By 1972, it was known that the rate of cholesterol synthesis is suppressed by cholesterol in diet and that cholesterol synthesis in cultured cells is suppressed by cholesterol in the incubation medium. By using this knowledge, Goldstein and Brown deduced that they were visualising a system for regulating the synthesis of cholesterol that was faulty in individuals with familial hypercholesterolemia. In more detail, the *defect* was caused by one of the genes involved in the feedback regulation. Furthermore, the defective feedback regulation should have something to do with *internalisation of LDL particles* since cells in individuals with familial hypercholesterolemia could not take up cholesterol when it was offered as a component of LDL but could respond normally to cholesterol once it got inside the cells.

These observations by Goldstein and Brown[70-72] led to the birth of concepts

of *LDL-receptor* and *LDL-receptor pathway* for internalisation mechanism for LDL particles and the regulation of cholesterol synthesis via the LDL-receptors, respectively (see Fig. 1.1). The discovery of LDL-receptors was a major milestone in the lipoprotein field and beyond since it was the first transport receptor of many to be characterised. Eventually, the LDL-receptor pathway was characterised in detail: The amount of LDL-receptors at the cell surface is regulated by the cellular cholesterol content and thus internalisation of cholesterol decreases the amount of the receptors as well as cell's own cholesterol synthesis and increases the storage of cholesterol molecules as cholesterol esters in the cytoplasm. Defective LDL-receptors, on the contrary, do not internalise lipoprotein particles at the normal rate leading to an increase in the number of IDL and LDL particles in plasma, and secondary in the level of plasma cholesterol, and cause atherosclerosis in individuals with familial hypercholesterolemia.

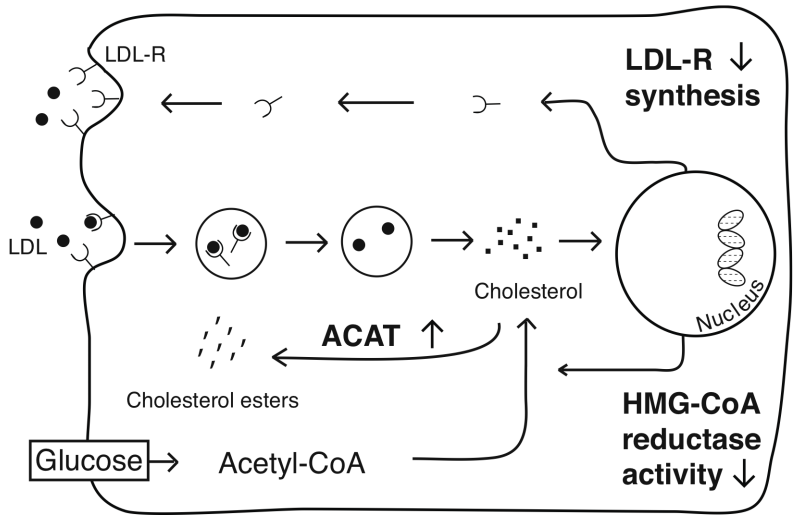

Figure 1.1 LDL particles bind to LDL-receptors (LDL-R) at the cell surface and become internalised and hydrolysed in lysosomes. The resulting increase in cellular cholesterol content decreases the LDL-receptor and cellular cholesterol synthesis and increases the cellular stores of esterified cholesterol as cholesterol esters via action of acyl-CoA cholesteryl acyl transferase (ACAT).

The discovery of Scavenger receptor A

The discovery of LDL-receptors did, however, leave an open question of how increased numbers of IDL and LDL particles in plasma could cause atherosclerosis in individuals with familial hypercholesterolemia. It was clear that these individuals had defective LDL-receptors which caused decreased internalisation of IDL and LDL particles by most cells, and particularly cells in the liver. However, evidently these individuals had cells in xanthomas and in ath-

erosclerotic lesions which were heavily loaded with cholesterol suggesting that the cells might be taking up cholesterol rapidly. Yet, since the LDL-receptors were defective in these individuals and since the accumulating particles must be IDL and LDL particles, Goldstein and Brown deduced that these particles have to be internalised by some alternative mechanism(s) into xanthomas and atherosclerotic lesions.

By the end of the 70s, the lipid-loaded cells within atherosclerotic lesions were shown to be largely derived from circulating monocytes rather than proliferated smooth muscle cells as suggested few years earlier. Goldstein and Brown tried to generate lipid-loaded cells *in vitro* by incubating mouse peritoneal macrophages or circulating monocytes with high concentration of LDL particles. The uptake of LDL particles was, however, low even with very high LDL particle concentrations[73]. Since Goldstein and Brown were convinced of the key role of LDL particles in the development of atherosclerosis in individuals with familial hypercholesterolemia, they reasoned that the circulating LDL particles must undergo some modifications and that the modified LDL particles could enter into the macrophages. The obvious question was then how the LDL particles should be modified *in vitro* to enhance their internalisation into the macrophages, and would this modification appear *in vivo*.

Goldstein and Brown explored a number of chemical and enzymatic modifications of LDL particles and found one, acetylation, which worked: acetylated LDL bound with high affinity to macrophages and was taken up rapidly by a receptor-mediated pathway leading to strikingly increased content of cellular cholesterol[73]. Acetylated LDL were not, however, generated *in vivo,* and Goldstein and Brown considered it unlikely to occur at all *in vivo*. The first step of this two-stage process was, however, proven: modified LDL particles are internalised by macrophages via receptors which were later on called *scavenger receptors A*. The second part, the mechanism which modifies LDL particles *in vivo* remained, however, unknown.

In 1979, Henriksen, Evensen and Carlander in Oslo[74] and Hessler, Robertson and Chisolm in Cleveland[75] independently made an interesting observation which ultimately led to the discovery of modified LDL present also *in vivo*. The researchers found that cells cultured in the presence of LDL and in the absence of serum underwent severe damage leading to cell death within 24 h. The cell death was, however, avoided by adding serum or high density lipoprotein (HDL) particles in the culture. In 1981, Henriksen et al.[76] found that during the incubation, the LDL particles had drastically changed becoming more dense, more electronegative and, interestingly, becoming ligands for receptor(s) on macrophages. This so-called *endothelial cell-modified LDL* was found to increase the cholesterol content in macrophages suffi-

ciently to give macrophages foamy appearance, i.e., forming foam cells. A few years later, Hessler et al.[77] showed that the changes in LDL particles induced by incubation with endothelial cells were free radical-catalysed oxidative modifications. Steinbrecher et al.[78] came independently to the same conclusion with regard to the mechanism by which endothelial cells converted LDL particles into ligands for macrophage receptor(s). These and later findings led to the development of *the oxidative modification hypothesis* of LDL where native LDL particles penetrate in the the vessel wall, become oxidised, start to attract circulating monocytes and inhibit exit of monocyte-derived macrophages. Finally, oxidised LDL is internalised by macrophages turning them into foam cells. Oxidised LDL particles were also suggested to damage endothelial cells thus further enhancing the penetration of LDL particles in the vessel wall. This hypothesis is now strongly supported by both *in vitro* and *in vivo* evidence, and more than 3000 articles have been published about the role of oxidised LDL in the development of atherosclerosis as reviewed elsewhere[79-83].

National programmes to treat individuals with high blood cholesterol

In the 1970s, many of the experts in atherosclerosis and preventive cardiology, and the American Heart Association, were convinced about the causality of elevated blood cholesterol and coronary artery disease. However, almost none of the general practitioners and very few practising internists or cardiologists were paying very much attention to their patients' high blood cholesterol levels. The National Institutes of Health was thinking about launching a national program to enhance the treatment of individuals with hypercholesterolemia. Steinberg described the situation in his recent historical review[84] as:

> *"The National Institutes of Health (NIH) realized that launching a national program to treat high blood cholesterol levels would be enormously complex and expensive. They could not justify that expense without first having iron clad proof that treatment would work. In any case, the medical community would have to be convinced before it could be expected to make serious efforts to implement any proposed treatment programs."*

Thus, the National Institutes of Health initiated in the 1970s a well-designed, large-scale, long-term, double-blinded study to demonstrate the impact of treatment on coronary artery disease. At the time of the initiation of this so-called Coronary Primary Prevention Trial (CPPT)[85, 86], several studies had briefly mentioned the effect of dietary cholesterol consumption on the serum

cholesterol level[35-37]. The majority of these studies suggested that the decreased cholesterol content of the diet decreases serum cholesterol levels, but the effect is less impressive than the effect of saturated/unsaturated fat content. In CPPT, a drug called cholestyramine, which inhibits cholesterol absorption from small intestine, was used to investigate its effect on lowering blood cholesterol and on the incidence rate of coronary artery disease. Thus CPPT was the first study to evaluate the role of absorbed cholesterol per se on plasma cholesterol concentrations and coronary artery disease without co-operative action of all dietary fats. The study population included approximately 3800 men with ages between 35 and 59 and without history of coronary artery disease or signs of current coronary artery disease. All the men had a high baseline blood cholesterol level, more or equal to 265 mg/dl (6.8 mmol/l) representing the highest 95 % of men of that age. The men received either cholestyramine or placebo. In addition, the amount of dietary cholesterol was slightly reduced in both groups. After an average of 7 years of treatment, the blood cholesterol level in the cholestyramine-group was decreased 13 % and LDL cholesterol 20 %. The number of events (definite coronary artery disease death and/or nonfatal myocardial infarction i.e., heart attack) was 19 % lower in the cholestyramine-group than in the placebo-group and the difference was, for the first time, significant ($p < 0.05$). The two groups did not, however, differ in total mortality.

The reception by professionals and by the press was mainly positive, and the major medical journals around the world hailed the results of the trial as finally providing the rationale for treating hypercholesterolemia[87-89]. After the results were published in 1984, the National Institutes of Health held a Consensus Development Conference on lowering blood cholesterol to prevent heart disease. Fourteen experts were invited to join a panel of the conference and, after reviewing all essential parts of the research and after having presentations and general discussions, the panelists voted unanimously that the causal relationship between blood cholesterol and coronary artery disease was established and that they were convinced that reduction of blood cholesterol level could prevent coronary artery disease.

The panelists also gave recommendations to prevent coronary artery disease[90]. The recommendations included desirable upper limits for blood total cholesterol concentration for individuals of all ages as well as advice to exercise, to reduce total calories to maintain normal body weight, to reduce total calories from dietary fat to 30 % (less than 10 % from saturated fats) and reduce dietary cholesterol intake to less than 300 mg. The panellists also advised that a national program should be established to educate both physicians and the public on the importance of controlling cholesterol levels. The

program was officially launched in 1985 as the National Cholesterol Education Program (NCEP). The most important contribution of NCEP, in addition to providing education, was to propose guidelines for diagnosis and treatment: at what level of LDL cholesterol would dietary intervention be indicated and at what level would the use of drugs be warranted. In 1988, the first Adult Treatment Panel (ATP I) of NCEP published its detailed guidelines for detecting, evaluating, and treating high serum cholesterol levels in adults[91]. These guidelines identified LDL as the primary target for lowering cholesterol. The guidelines quickly became the gold standard on who to treat and how to treat. Many other countries followed the United States lead and convened expert groups to develop their own guidelines which were remarkable similar to those of NCEP.

Statins - the miracle medicines?

At the time the first adult treatment panel was preparing its statements, a new cholesterol lowering drug was cleared for use by the Food and Drug Administration in the United States[84]. The drug was called lovastatin. Interest in pharmacological approaches to lower plasma cholesterol levels had begun as early as the 1950s, years before the time plasma cholesterol level was shown to impact the incidence of atherosclerosis and coronary artery disease. However, the race of the first commercially marketed statin started in the 1980s, after introduction of the very first statin, mevastatin, in the late 1970s by a Japanese researcher Akira Endo.

The story of statins began in 1971 in the pharmaceutical company Sankyo Co. in Tokyo, where Akira Endo speculated that the broths in which fungi were being grown in hunt for new and better penicillin just might also contain natural inhibitors of cholesterol synthesis. Endo himself said later on that he hoped that some micro-organisms might “produce such compounds as a weapon in the fight against other microbes that required sterols or other isoprenoids for growth”[92]. These thoughts of Endo were clearly inspired by Fleming's discovery of penicillin in 1928[93, 94] but were not directly supported by the knowledge of the time. Endo was, however, stubborn with this and kept testing for more than 6000 broths within two years with his colleagues. It was, however, worthwhile since they finally came up with a promising compound!

In 1976, mevastatin was isolated, characterised and published[95, 96]. Interestingly, shortly after the publication, Brown and Goldstein became aware of Endo's paper and asked Endo for a sample to study regulation of cholesterol biosynthesis. Endo sent the samples and the three men met in Dallas in 1977. Endo, Goldstein and Brown found that their experiments on the compound

were concordant and published the results[97] jointly in 1978 giving grounds to what we know now about statins. Endo's compound did not, however, ever reach the commercial market since Sankyo Co. dropped its million dollar project without any public explanation.

Mevastatin belonged to a family of molecules called statins. Currently there are several different statin compounds including atorvastatin, fluvastatin, lovastatin, pitastatin, pravastatin, rosuvastatin and simvastatin. Statins inhibit cholesterol synthesis in the liver and decrease plasma cholesterol levels. Statins are also called HMG-CoA reductase inhibitors since they inhibit the action of HMG-CoA reductase, a rate-limiting enzyme in cholesterol synthesis[95-98]. The inhibition results from competition of the action of HMG-CoA reductase between structurally similar statin and HMG-CoA molecules which are precursors of cholesterol. The decreased cholesterol synthesis leads to increased synthesis or recycling of LDL-receptors in the liver[98-100] and increased uptake of LDL particles from the circulation. In addition to the quantitative effects of statins on LDL particles, they have also shown to affect the quality of LDL particles by increasing their size in individuals with preponderance of small LDL particles[101].

The effect of statins to lower LDL cholesterol was first studied in large scale in 1994[102], and it turned out that statins are much more efficient than any other earlier drug or dietary manipulation. In the study, designed as the Scandinavian Simvastatin Survival Study (4S)[102], 4444 patients with coronary artery disease and hypercholesterolemia were randomised to placebo or simvastatin (20 mg/day) and followed for five years. In the statin group, LDL cholesterol was lowered by 35 % and total cholesterol by 25 %. Total mortality, the primary end point of 4S, was reduced by 30 % and all other coronary end points by 30 to 40 % relative to the placebo group. For the first time in the history of cholesterol lowering trials, the total mortality was significantly decreased due to the actions taken to decrease plasma cholesterol levels. The results of the 4S study were quickly confirmed and extended by two other studies[103, 104] showing that patients with history of coronary artery disease benefit from the statin treatment.

Individuals without history of coronary disease also seem to benefit from statins. In the West of Scotland Coronary Prevention Study[105], 6595 men with hypercholesterolemia on pravastatin lowered their LDL cholesterol by 26 % and the primary end point, coronary death or nonfatal myocardial infarction, was reduced by 31 % relative to placebo group. In the Air Force/Texas Coronary Atherosclerosis Prevention Study[106], 6605 middle-aged individuals with average baseline total and LDL cholesterol (5.71 ± 0.54 mmol/l and 3.89 ± 0.43 mmol/l, respectively) and below-average HDL cholesterol (0.94 ± 0.14

mmol/l for men and 1.03 ± 0.14 mmol/l for women) reduced their LDL cholesterol by 25 % on lovastatin. The first acute major coronary events were reduced by 37 % in the lovastatin group compared to the placebo group. Thus, the first wave of statin trials taught us that individuals with multiple characteristics will benefit from statin therapy.

By the end of 1990s, the Cholesterol and Recurrent Events (CARE) Trial[103, 107] suggested that there might be a threshold of LDL cholesterol at about 3.2 mmol/l below which reducing cholesterol would not reduce the risk of CAD. However, the Heart Protection Study[108] showed that reducing LDL cholesterol from less than 3 mmol/l to less than 2 mmol/l reduced events as efficiently as earlier studies with higher LDL cholesterol levels. The results were confirmed by several other studies[109, 110].

Recently, efficacy and safety of statins were discussed in a meta-analysis of 14 statin trials with a total of 90 056 individuals[111]. It stated:

> *"Statin therapy can safely reduce the 5-year incidence of major coronary events, coronary revascularisation, and stroke by about one fifth per mmol/L reduction in LDL cholesterol, largely irrespective of the initial lipid profile or other presenting characteristics. The absolute benefit relates chiefly to an individual's absolute risk of such events and to the absolute reduction in LDL cholesterol achieved. These findings reinforce the need to consider prolonged statin treatment with substantial LDL cholesterol reductions in all patients at high risk of any type of major vascular event."*

In other words, LDL cholesterol lowering achieved by statins benefits all individuals in risk largely irrespective of their initial lipid profile. This view has raised arguments of overusage of statins[3] and even accusations of unscientific interpretation of cholesterol lowering trials[3, 112]. These accusations are mainly directed to the use of relativeness in reporting the incidence rates of coronary artery disease between the statin and placebo groups. For example, it is common to argue that statins have *"reduced coronary artery disease risk by 30-40 % in the 5 years statin studies"*[113]. This 30-40 percent does not mean that 30-40 percent of individuals on statins have been saved from a coronary event because of the statins. The figure means that statin trials have shown 30-40 % difference in the incidence of coronary end points between the groups. For example, in the Anglo-Scandinavian Cardiac Outcomes Trial – Lipid lowering arm (ASCOT-LLA) study[109] the rate of coronary events (either fatal CAD or non-fatal myocardial infarction) was 36 % after 3.3 years follow-up. The rate of adverse events was 1.9 % in the group of patients taking atorvastatin (10 mg/day, 5168 individuals) and 3.0 % in the placebo group (5137 individuals). The absolute risk reduction was 1.1 percentage units in the study and the relative risk reduction was 3.0 % minus 1.9 % over 3.0 % giving 36 %.

While 1.9 is 36 % less than 3.0, only 1.1 % of individuals on statin treatment actually did benefit from the treatment. In other words, for each 91 individual who took the drug, 1 did and the 90 did not benefit from statins in this study. Universally, the number of individuals who would need to be treated with statins in order for one patient to get the expected benefit, i.e., to be saved from a coronary event, is 30 to above 100 after approximately five years treatment[112]. This figure seems astonishing since statins are often presented as medicines which will save our lives[113-115]. This does not, however, necessarily mean that statins are inefficient. They may be to some extent misused due to inability to identify the individuals who would benefit most from the statins[116, 117] but maybe also due to low dosage[113], old starting age[113], early evaluation of the effects[113], and multiple variables beyond LDL cholesterol influencing the treatment effect[118]. We may simply need more research on this including more detailed characterisation of patient's lipoprotein particles.

1.2 The state of the art in atherosclerosis and lipoprotein research

Atherosclerosis is recognised as a systemic, progressive disease and its development begins at early age[119]. It is also a multi-factorial disease affected by both genetic and lifestyle factors[1]. Atherosclerosis has been characterised by a complex interplay between cholesterol accumulation and chronic inflammation leading, via a series of biological responses, to appearance of hard deposits called plaques in the arterial wall[1, 119, 120]. These plaques increase in time due to ongoing cholesterol accumulation, migration of monocytes and smooth muscle cells into the plaques, and formation of smooth muscle cell-derived fibrous elements. The increasing size of the plaques narrows down the artery lumen and may reduce flow of oxygen-rich blood in the artery. The narrowing of the artery may also be compensated by aneurysms, local enlargements of the artery, leading to thinner arterial wall and possible arterial ruptures[121, 122]. Plaques may also rupture or erode forming blood clots before becoming large and occlusive leading to acute vascular events and insufficient oxygen supply to the tissues and organs[119]. Atherosclerosis is typically asymptomatic for decades[119] but may eventually cause severe diseases, including coronary artery disease[119] and carotid artery disease[123], and may lead to subsequent clinical end-points, such as heart attacks[1] and strokes[123], respectively.

The cause of atherosclerosis has been under debate for the whole 20th century. During the time, tens of risk factors, variables which have been associated with the incidence of atherosclerosis, have been reported[1, 124-126]. Some of these have been accepted as major risk factors yielding a combination of ge-

netic susceptibility, unhealthy environment and increased lifespan[1](Table 1.2). At the time, the key role of lipoprotein particles in the development of atherosclerosis is appreciated but the concerned risk factors are usually denoted via lipid variables, such as VLDL triglycerides and LDL cholesterol, rather than lipoprotein variables such as VLDL and LDL particle concentrations, respectively.

Some of the major risk factors co-occur in an individual surprisingly often and tend to together increase the risk of atherosclerosis and type II diabetes considerably[124]. The combination of these risk factors is called the metabolic syndrome and its components are all metabolic disturbances such as abdominal body fat distribution, insulin resistance, atherogenic dyslipidemia denoting abnormal lipid and lipoprotein levels regularly present in atherosclerosis, elevated blood pressure, proinflammatory state and prothrombotic state meaning prevalence to form blood clots in the arteries. The atherogenic dyslipidemia is defined as raised plasma triglycerides, low concentration of HDL cholesterol, elevated apoB molecules as well as increased number of small, dense LDL and small HDL particles. These components of the metabolic syndrome add to each other multiplicatively and likely have reciprocal associations although their nature is not currently clear. Metabolic syndrome is, however, increasing strikingly worldwide with increasing age distribution and the global epidemic of obesity and insulin resistance[127-129].

Table 1.2 Risk factors of atherosclerosis in alphabetical order. For references, see[1, 130].

Factors with a strong genetic component		Environmental factors
Diabetes and obesity	Male gender	Alcohol consumption†
Elevated blood pressure	Metabolic syndrome	High-fat diet
Elevated levels of haemostatic factors°	Reduced levels of HDL particles	Infectious agents
Elevated levels of lipoprotein(a)	Systemic inflammation (hs-CRP)	Lack of exercise
Elevated levels of VLDL, IDL and LDL particles		Low antioxidant levels
Family history of cardiovascular diseases	**Increased lifespan**	Smoking

° *These haemostatic factors include fibrinogen, plasminogen activator inhibitor type 1 and platelet reactivity.* † *Relationship of alcohol usage and the risk of atherosclerosis is a J-curve with risk highest for heavy drinkers, lowest for light drinkers and intermediate for teetotallers. VLDL, very low density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hs-CRP, high-sensitive C-reactive protein.*

The risk of atherosclerosis is detected, evaluated, and treated by the guidelines of the third Adult Treatment Panel report (ATP III) from the National Cholesterol Education Program (NCEP)[131] or similar national program-

s[132-136]. For detection and evaluation, ATP III recommends examination of various lipid measures such as total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides, and recommends evaluation of this lipoprotein profile with other risk factors such as age, blood pressure, and smoking. For treatment, ATP III has the main focus in lowering LDL cholesterol similar to earlier reports although the optimal LDL cholesterol level for individuals at high risk is now lower, 100 mg/dl (2.6 mmol/l), than in earlier reports and more intensive pharmaceutical treatment is recommended to those individuals. In Finland, the optimal lipoprotein lipid levels are slightly different[136], as shown in Table 1.3. The Finnish recommendations emphasise also other cholesterol measures, such as the ratio of total to HDL cholesterol (total C / HDL-C) and cholesterol content in all lipoprotein particles excluding HDL particles, i.e., non-HDL-C concentration (total C – HDL-C), as risk factors.

Table 1.3 Optimal lipoprotein lipid concentrations according to ATP III and the Finnish 'Käypä hoito' - recommendations.

Individuals at low risk	**ATP III**	**Finnish recommendations**
TG (mmol/l)	< 1.7	< 2.0
total C (mmol/l)	< 5.2	< 5.0
LDL-C (mmol/l)	< 4.1*	< 3.0
HDL-C (mmol/l)	> 1.0	> 1.0
Individuals at high risk	**ATP III**	**Finnish recommendations**
TG (mmol/l)	< 1.7	< 2.0
total C (mmol/l)	< 5.2	< 4.5
LDL-C (mmol/l)	< 3.4** or < 2.6***	< 2.5
HDL-C (mmol/l)	> 1.0	> 1.0

*ATP III classifies individuals into three categories such as individuals with 0 or 1 risk factors, individuals with 2 or more risk factors and individuals with CAD or CAD risk equivalent including type II diabetes. Since Finnish recommendations classify individuals only into two categories, for practical reasons, also individuals in ATP III recommendations are regrouped. Individuals with 0 or 1 risk factors are labelled under 'individuals at low risk' and those with two or more risk factors, CAD or CAD risk equivalent under 'individuals at high risk'. Recommendation of LDL cholesterol level * for individuals with 0 or 1 risk factors, ** for individuals with 2 or more risk factors and *** for individuals with CAD or CAD risk equivalent are, however, given separately. These risk factors for classification in ATP III recommendations include smoking, hypertension (blood pressure ≥ 140/90 mmHg), low HDL-C (less than 1.0 mmol/l), family history of premature CAD and age (men ≥ 45, women ≥ 55). If the HDL-C level is more than 1.6 mmol/l, one risk factor can be subtracted. Division of individuals into low risk and high risk categories is quite similar also in the Finnish recommendations. The risk can also be estimated by risk score calculators, such as SCORE[133] and FINRISKI[137].*

The predictive role of extremely high total and LDL cholesterol concentrations is established in the development of cardiovascular disease. However, the relationship between 'normal' cholesterol levels and the risk of athero-

sclerosis is complex. This has caused arguments for even lower optimal level of LDL cholesterol at least for high risk individuals[108, 138-140].

This discrepancy has also inspired active discussion of whether we are using the best measure(s) to identify the individuals at risk of atherosclerosis and should advanced lipoprotein testing, i.e., measures beyond triglyceride, total cholesterol, LDL cholesterol and HDL cholesterol concentrations, be used. Currently, the best validated measure of CAD risk obtained from advanced lipid testing is the concentration of atherogenic particles via apolipoprotein-B (apoB) concentrations[141-146] or LDL particles determined by NMR[146-150].

In most of these studies[141-143], but not in all[144], apoB was more closely associated with CAD risk than LDL cholesterol. Similarly, CAD risk was more closely associated with NMR LDL particle concentrations than LDL cholesterol. However, in the Framingham Study[145], apoB associated with CAD risk over a 15-years follow-up of 3322 individuals with a magnitude of association comparable to total/HDL cholesterol concentration. Similarly, in the Women's Health Study[146], apoB and NMR LDL particle concentrations associated with CAD risk over an 11-years follow-up of 27 000 women with a magnitude of association comparable to total/HDL cholesterol or non-HDL cholesterol concentrations.

The poor predictive power of LDL cholesterol for risk of CAD has also emphasised the potential role of particle properties such as altered LDL particle size[20, 151]. Particularly small, dense LDL particles (sdLDL) have been associated with increased risk of CAD (for references, see[117]). The discussion has also extended to other lipoprotein particles than LDL. It has been suggested that the quality of HDL particles is altered to less atheroprotective or pro-atherogenic particles in individuals with atherosclerosis or the metabolic syndrome[152, 153]. Therefore, although lipoprotein particles have been studied for decades, there still exist unanswered questions regarding differences in the quantity and the quality of lipoprotein particles between individuals and associations of these properties with the risk of cardiovascular disease.

1.3 Aims of the study

This dissertation contributes to lipoprotein research mainly by providing new computational models and methods to characterise lipoprotein particles. It deals with the three important properties of lipoprotein particles including structure (chapter 3, Study I), composition/metabolism (chapter 4, Study II) and particle concentration (chapter 5, Study III). In more detail, this thesis aims to clarify whether hydrophobic lipoprotein lipids locate also in the surface layer of lipoprotein particles; whether computational, i.e., *in silico,* clas-

sification enhances the experimental ultracentrifugation-based isolation of lipoprotein data and whether computational approaches related to lipoprotein lipid data are useful in estimating lipoprotein particle concentrations. Summary of these models and methods are given in chapter 6. This dissertation starts, however, from the basic concepts of lipoprotein particles such as their isolation from blood plasma, chemical composition, size, structure and metabolism to give grounds to the following chapters.

2. Basic concepts

This chapter will review the main properties of lipoprotein particles beginning with their isolation and characterisation, then continuing into their general and molecular structure and, finally, into their complex metabolism in plasma. The purpose of this chapter is to give the appropriate background of lipoprotein particles from a biochemical point of view to be able to follow the subsequent chapters on computational modelling of the structure and metabolism of lipoprotein particles.

2.1 Isolation and characterisation of lipoprotein particles

Lipoproteins are highly heterogeneous particles, and they differ in various physical properties including hydrated density, surface charge, particle size and chemical composition. These different properties can be used to classify lipoproteins into several groups of particles. However, no single method can classify lipoproteins based on all of their properties at once and thus there are several isolation and characterisation methods based on the different properties. These methods include ultracentrifugation, precipitation, electrophoresis, chromatography, enzymatic methods yielding chemical compositions and nuclear magnetic resonance.

2.1.1 Isolation by hydrated density in ultracentrifugation

The hydrated density in an ultracentrifuge is the most commonly used criteria to isolate lipoprotein particles, and ultracentrifugation is the gold standard in lipoprotein research. Hydrated densities in ultracentrifugation have also defined the nomenclature of lipoprotein particles. Lipoprotein particles have relatively low densities and will float if they are ultracentrifuged in a solvent of greater density. Therefore, it is possible to isolate lipoprotein fractions with a density less than one value but greater than another by carrying out successive ultracentrifugation runs where the density of the solvent is increased

by a known amount each time. In addition, by a specific ultracentrifugation method, i.e., density gradient ultracentrifugation, several lipoprotein fractions can also be separated simultaneously using discontinuous density gradient of the solvent. Hydrated densities of lipoprotein particles form, however, a continuum, and thus the limits of five standard lipoprotein classes have been chosen discretionarily and they variate slightly between research groups.

Frequently used ranges are < 0.94 g/ml, 0.94-1.006 g/ml, 1.006-1.019 g/ml, 1.019-1.063 g/ml and 1.063-1.21 g/ml[154-156]. The particles of the first range are chylomicrons which were named already in 1924[157], about thirty years before the first ultracentrifuge. The rest of the lipoprotein particles are named according to their densities to very low-density lipoproteins (VLDL, 0.94-1.006 g/ml), intermediate-density lipoproteins (IDL, 1.006-1.019 g/ml), low-density lipoproteins (LDL, 1.019-1.063 g/ml) and high-density lipoproteins (HDL, 1.063-1.21 g/ml). Because the procedure is very flexible to isolate particles within any density range, these main lipoprotein fractions have been further partitioned into narrower density ranges to represent lipoprotein subclasses. Particularly HDL particles are further divided to lighter HDL_2(1.063-1.125 g/ml) and denser HDL_3 (1.125-1.21 g/ml) particles[158]. Also HDL particles lighter than HDL_2 (HDL_1, 1.055-1.063 g/ml)[159] and denser than HDL_3 (very high-density lipoprotein, VHDL or HDL_4, 1.210-1.250 g/ml)[160] have been isolated by ultracentrifugation although their concentrations in plasma are small. Moreover, in a density range of 1.050-1.080 g/ml[156], overlapping with the LDL and HDL ranges, exists lipoprotein (a) particles, abbreviated as Lp(a). Lp(a) is an LDL particle enriched with a specific protein, apolipoprotein(a)[161].

2.1.2 Isolation by precipitation

Lipoprotein particles can also be isolated from blood plasma or serum by various chemical precipitation techniques. In contrast to ultracentrifugation, precipitation technique disposes unwanted lipoprotein particles by forming of a solid phase in a solution during a chemical reaction. The chemical reaction is achieved by introducing certain precipitation agents which selectively and conveniently aggregate and precipitate lipoproteins with appropriate choices of reagents and conditions.

The precipitation procedures can isolate lipoprotein fractions and main HDL subclasses from blood plasma or serum by various chemical precipitation techniques but are particularly used to separate HDL particles. HDL particles can be separated by precipitation techniques using polyanions, sometimes combined with divalent cations, as these precipitation reagents selectively aggregate lipoprotein particles with a large, non-exchangeable

apoB[162, 163]. The precipitated apoB-containing particles can then be removed leaving HDL particles in the remaining solution or supernatant. Precipitation methods are, however, often based upon empirical findings and the precise physicochemical basis is not always clear.

2.1.3 Characterisation by electrophoresis

Electrophoresis can be used to characterise lipoprotein particles according to their surface charge or size. These methods have also been combined sequentially yielding two-dimensional separation of the particles[164, 165].

2.1.3.1 Agarose gel electrophoresis

Lipoprotein particles can be characterised by their surface charge as they are electrically charged due to their surface constituents and thus migrate in an electric field. Lipoprotein particles differ in their electrical charges mostly due to their protein contents and conformations but also by variations in phospholipid species. Hence the lipoproteins can be classified by their electrophoretic mobility on porous agarose gels into four main classes, namely, α-lipoproteins, pre-β-lipoproteins, β-lipoproteins and chylomicrons. The names of the classes, except chylomicrons, originate from the fact that the mobilities of lipoprotein particles are comparable to mobilities of globulins, heterogeneous families of large and less soluble serum proteins. Thus the α-lipoproteins and the β-lipoproteins have the same mobilities as the fast α-globulins and slower the β-globulins, respectively, and the mobility of pre-β-lipoproteins is a little greater than that of the β-lipoproteins. When α-, pre-β- and the β-lipoproteins are compared to those isolated by ultracentrifugation, they correspond mainly to HDL, VLDL and LDL particles, respectively[166, 167]. Chylomicron particles, if present in the sample, remain in the electrophoretic origin because their size is larger than the pore sizes of the supporting medium[166].

2.1.3.2 Non-denaturing polyacrylamide gradient gel electrophoresis

The most common procedure to characterise lipoprotein particles by size is to use non-denaturing polyacrylamide gradient gel electrophoresis (GGE) which combines electric field with a continuous molecular sieve gradient in the supporting medium. Thus, the lipoprotein particles move in electric field until they cannot migrate through the pores of decreasing size. The size of the trapped particles could then be determined by comparing them to charged reference molecules with known size or to their migration distance.

Non-denaturing polyacrylamide gradient gel electrophoresis can be used either to ultracentrifugally isolated lipoprotein fractions[168] or directly to plasma or serum samples[169] to characterise their subclass composition. The

method has found lipoprotein subclasses in each of the lipoprotein fractions although its use has been particularly directed to LDL and HDL particles; VLDL have been grouped into two subclasses with diameters larger than 35 nm and between 30 - 33 nm, respectively, and two IDL subclasses with diameters 28-30 nm and 26.8-28.4 nm in density ranges of 1.008-1.022 g/ml and 1.013-1.028 g/ml, respectively[170]. Two subclasses of IDL particles have also been observed in the density range of 1.006-1.030 g/ml yielding larger IDL-I (mean diameter 31.7 ± 0.7 nm) and smaller IDL-II particles (25.7 ± 2.4 nm), respectively[171]. Similar procedures have also identified seven LDL subclasses (I, 27.2-28.5 nm in diameter; IIa, 26.5-27.2 nm; IIb, 25.6-26.5 nm; IIIa, 24.7-25.6 nm; IIIb, 24.2-24.7 nm; IVa, 23.3-24.2 nm and IVb, 22.0-23.3 nm)[20, 172, 173] or even eight LDL subclasses (LDL-8, 17.7 ± 0.6 nm) added by an even smaller LDL particle observed in individuals with severe hypertriglyceridemia[169, 174]. HDL particles have, however, been subdivided to even more particle classes, displaying fourteen particles (from 12.46 nm to less than 7.86 in diameter)[175] although only five subclasses are usually met in any given individual. These include two HDL subclasses of HDL_2 (HDL_{2b}, ~9.7-12.9 nm in diameter; and HDL_{2a},~8.8-9.7 nm) and three of HDL_3 particles (HDL_{3a}, ~ 8.2-8.8 nm; HDL_{3b}, ~7.8-8.2 nm; HDL_{3c}, ~7.2-7.8 nm) in apparently healthy individuals[176, 177].

It is also possible to use two electrophoretic procedures sequentially as two-dimensional electrophoresis. This may be done by characterising the particles by surface charge in the first and by size in the second dimension[178-181]. The first dimension separates the HDL particles into pre-β-, α- and pre-α-migrating particles on basis of the mobilities that are slower than, similar to, or faster than albumin, respectively. Then the agarose strip is transferred on the top of a non-denaturing gradient gel and electrophoresed in the second dimension. Two-dimensional electrophoresis has then yielded five pre-β- (two pre-β1 particles around 7.10 nm in diameter and three pre-β2 particles around 17 nm), four pre-α- (between 17 and 8.2 nm) and three α-migrating particles (α1 around 11.2 nm, α 2 at 9.51 nm, and α3 at 7.12 nm). Knowledge of apolipoprotein concentrations in α-, pre-β- and the β-lipoproteins has also been increased by crossed immunoelectrophoresis combining electrophoresis in the first and electroimmunodiffusion in the second dimension[182, 183]. Electroimmunodiffusion is an immunochemical method combining electrophoretic characterisation with immunodiffusion by incorporating antibody against apolipoproteins into the support medium.

2.1.4 Isolation and characterisation by chromatography

Chromatography is a collective term for a family of laboratory techniques for

separation of mixtures, and the separation is based on differing mobilities of molecules or particles within the mixtures in a supporting medium. Chromatographic techniques have become common in lipoprotein research as they are fast and versatile.

2.1.4.1 Apolipoprotein content in immunoaffinity chromatography

Lipoprotein particles have variable apolipoprotein compositions by which they can be separated into various classes via immunoaffinity chromatography. In this procedure, antibodies, raised against individual apolipoprotein antigens, are immobilised covalently on agarose or a similar insoluble chromatography matrix and the lipoproteins including the apolipoproteins in interest can be entrapped and then collected while the others are discarded.

The specific antibodies for all apolipoprotein molecules are available and they have been used to separate lipoproteins into various classes including apoB-containing particles corresponding to chylomicron, VLDL, IDL and LDL particles and non-apoB-containing particles corresponding to HDL particles. The apoB-containing particles are further classified into a class containing a truncated form of apoB, apoB-48, corresponding to chylomicrons and full length apoB, apoB-100, corresponding to VLDL, IDL and LDL particles. Both of these classes contain also particles with or without apoC and apoE in all mixtures[184]. The main interest of separation of lipoprotein particles by immunoaffinity chromatograph is, however, directed to the non-apoB-containing particles.

The non-apoB-containing particles are largely separable into two main classes where the other contains apoA-I without apoA-II molecules, abbreviated as LpA-I, while the other contains apoA-I with apoA-II molecules, abbreviated as LpA-I:A-II[185, 186]. Positioning of these LpA-I and LpA-I:A-II particles to HDL subclasses derived by other methods has proved to be difficult and only partly converging. It has been suggested that LpA-I particles have major components in the density range of large, spherical HDL_2 particles[187] or in subclasses HDL_{2b}, HDL_{3a} and HDL_{3c}[188] while LpA-I:A-II particles are mostly in the density range of small, spherical HDL_3 particles[187] or in subclasses HDL_{2a}, HDL_{3a} and HDL_{3b}[188]. Combined use of two-dimensional non-denaturing gel electrophoresis and immunoaffinity studies have, however, provided the most sophisticated view of the positioning of the LpA-I and LpA-I:A-II particles: It seems that LpA-I:A-II particles are present only among small, spherical HDL particles (α2 and α3) while LpA-I particles are present among all HDL particles or at least in all except the ones including LpA-I:A-II particles (pre-β, α1, pre-α)[189]. Thus LpA-I particles are present in both large, spherical as well as small, discoidal, HDL particles. In addition to apoA-I containing HDL particles, some HDL particles may have apoE, apoA-

IV or even some minor apolipoprotein as their only apolipoprotein molecules but their concentration in plasma is low[190, 191].

2.1.4.2 Size exclusion chromatography

Size exclusion chromatography is a chromatographic method in which large molecules or particles in solution are separated based on their size[192, 193]. Separation of the particles is usually achieved with a column which consists of a hollow tube tightly packed with small beads with pores of different sizes. Therefore, as the mixture of particles travels down the column, smaller particles enter more often into the pores and thus have longer path and elution time in the supporting medium than larger particles. As a result, the filtered solutions are collected in constant volumes and subjected to further analysis by other techniques or are analysed on-line without actual gathering.

Size exclusion chromatography is generally considered a low resolution chromatography. One option to increase its resolution is to utilise a pump to propel the solution through the column. In this case, the column may also be shorter and denser than in the original size exclusion chromatography. The increased density arises from the decreased size of the porous beads. This form of column chromatography is usually called high-performance or high pressure liquid chromatography (HPLC).

High-performance liquid chromatography has been used for decades to isolate and characterise lipoprotein particles[194-196]. Recently, an on-line dual detection method was described for lipoprotein analysis[194, 195]. The method allows separation of twenty lipoprotein subclasses and enzymatic determination of their cholesterol and triglyceride profiles from a single injection of sample. This is achieved by using two different tandem connected gel permeation columns, two different reagents and a dual detection system. This method is used in chapter 5 of this thesis for lipoprotein isolation and characterisation.

2.1.5 Characterisation by chemical composition

Lipoprotein particles have complex chemical compositions which vary between individuals and within an individual under different physiological and pathological conditions. They contain variable amounts of three main classes of lipids including phospholipid, triglyceride and cholesterol molecules. Cholesterol molecules are further divided into unesterified or free cholesterol and cholesterol ester molecules since they behave quite differently in lipoprotein particles. Lipoproteins have also significant amounts of proteins designated apolipoproteins but they may also contain other associated protein components.

Lipoprotein particles can be characterised by determining their lipid and protein concentrations. There are numerous methods to measure the lipid and protein concentrations of which the most widely used at the time are based on enzymatic, colorimetric methods. These methods are widely used to measure the concentrations of triglyceride, (total) cholesterol, free cholesterol and phospholipid molecules. The concentration of cholesterol esters is then calculated as the difference in total cholesterol and free cholesterol molecules. The concentration of total protein is most often determined by a modified Lowry method[197, 198], a colorimetric method based on interaction of divalent copper ions with peptides. There are also several immunological methods to measure the concentration of apolipoprotein molecules in a sample[182, 183, 199, 200].

In clinical practise, lipoprotein particles are characterised less comprehensively, usually only triglyceride, total cholesterol and HDL cholesterol concentrations are measured. HDL cholesterol concentration is measured by enzymatic methods after removal of apoB-containing particles by precipitation or by so-called direct methods that do not require the physical separation of the different lipoprotein classes[201]. Similarly, LDL cholesterol concentration is measured either by the direct methods or calculated by using Friedewald's equation[46] (see equation 1.1 in page 11)[202]. The direct methods are based on different epitopes, i.e., targets of immune responses, on the HDL and LDL particles, and in 2008 there were seven different commercial direct methods available for HDL and LDL cholesterol concentration determination[203].

2.1.6 Characterisation by nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a new method among lipoprotein characterisation procedures while it has a long history in structural studies among lipoproteins and other biological aggregates. NMR provides an alternative for measuring sizes of lipoprotein particles in plasma although the sizes are held more relative than absolute. The size estimates are based on the subclass-specific frequencies of spectral signals emitted simultaneously by VLDL, LDL and HDL subclasses of different sizes and their particle weight-averaged correspondence with GGE-derived or other subclass size measures[204]. Lipoprotein particles produce multiple peaks in NMR spectrum but the composite signal of terminal methyl groups (-CH_3) is enough for lipoprotein particle quantification. This signal forms from the aggregate number of terminal methyl groups on the lipids contained within the particle and is distinguishable from the methyl signals of other subclasses by their characteristic shape and frequency. This technique has identified three VLDL sub-

classes (> 60 nm in diameter, 35-60 nm and 27-35 nm), one IDL (23-27 nm in diameter), three LDL subclasses (21.1-23 nm in diameter, 19.8-21.1 and 18-19.8) as well as three HDL subclasses (8.8-13 nm in diameter, 8.2-8.8 nm and 7.3-8.2 nm)[205, 206]. The size ranges of HDL subclasses match closely those measured by gradient gel electrophoresis while the others remain somewhat smaller[206]. Particularly LDL particles have shown to be uniformly smaller by approximately 5 to 6 nm compared with those determined by GGE[147, 206, 207]. In addition to NMR's ability to characterise lipoprotein particles by size, NMR can be used to approximate the concentrations of lipoprotein lipids based on its signal amplitudes.

To summarise, lipoprotein particles are heterogeneous in terms of hydrated density, size, surface charge, lipid composition and apolipoprotein content. Lipoprotein particles may be classified into various fractions and further into subclasses according to their different properties. Each of the isolation methods decreases the heterogeneity of the particles by the corresponding property. Lipoproteins may be further characterised by their chemical composition which forms basis for understanding the structure and the metabolism of lipoprotein particles.

2.2 General and molecular structure of lipoprotein particles

Lipoprotein particles are micellar aggregates of lipids and specific lipid binding proteins called apolipoproteins[208-211]. Mature lipoproteins are spherical particles at physiological temperatures with diameters ranging from a few nanometers to a few micrometers[212, 213]. Lipoprotein particles contain triglyceride, cholesterol, cholesterol ester and phospholipid molecules as well as variable amounts of fraction-specific apolipoproteins.

The general structure of lipoprotein particles is well known[160, 210, 211, 214-216] (see Fig. 2.1). The location of a bulk of the lipids and apolipoproteins is mainly determined by their hydrophilic and/or hydrophobic nature. Phospholipids, consisting of a head group, a phosphoryl group, glycerol or sphingosine backbone and two fatty acids, form the basis of a one-molecule thick surface layer of lipoprotein particles. Hydrophilic, polar phosphoryl-linked head groups of phospholipids are in contact with aqueous environment while their hydrophobic, unpolar fatty acids form the inner part of the surface layer. Apolipoproteins have both hydrophilic and hydrophobic regions and reside mainly at the surface of the lipoprotein particles forming a mixed film with the phospholipids. Free cholesterol molecules are abundant in the surface as structural surface lipids but are also present in the interior parts of the particles, or in the core, as they are only slightly polar due to their single hydroxyl group.

Triglycerides and cholesterol esters are hydrophobic molecules and are, thus, mainly buried in the core of the lipoprotein particles. However, this conventional view is quite restricted regarding metabolism of lipoprotein particles, and the issue of location of triglycerides and cholesterol esters is thus reconsidered in chapter 3 of this thesis.

2.2.1 The molecular organisation of lipids in lipoprotein particles

The molecular structure of lipoprotein particles is less clear than the general structure and there exists uncertainties around the details of lipid-lipid and lipid-protein interactions. Plenty of information is, however, available on the properties of lipoprotein composing lipids derived from planar layers[217, 218] as well as spherical lipoprotein model systems such as micelles and vesicles[219-224]. It could be excepted that the main interactions between, for example, phospholipids and free cholesterol molecules in these curved surface layers are similar[210] although vesicles have a phospholipid bilayer rather than a monolayer present in lipoproteins and micelles.

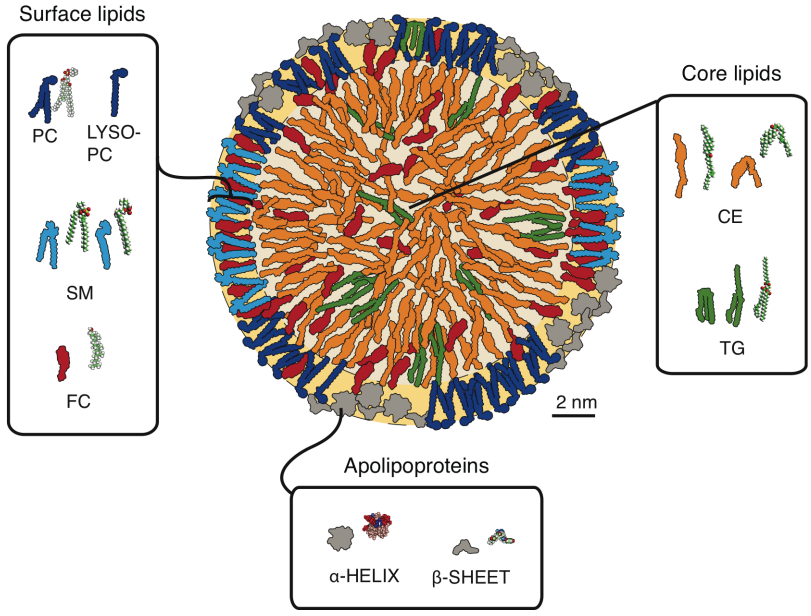

Figure 2.1 Structure of lipoprotein particles. Lipoprotein particles are spherical, micellar structures with a core and a surface layer surrounding the core. Lipoprotein particles contain lipids and proteins called apolipoproteins. The lipids in lipoprotein particles are generally divided to surface and core lipids based on their main location in lipoprotein particles. Surface lipids including phospholipids, such as phosphatidylcholine (PC), lyso-phosphatidylcholine (lyso-PC) and sphingomyelin (SM), and free cholesterol molecules, while core lipids is a common term for triglyceride (TG) and cholesterol ester (CE) molecules. Apolipoproteins reside mostly at the surface of lipoprotein particles.

It is likely that phospholipid and free cholesterol molecules are not randomly distributed at the surface of lipoprotein particles since free cholesterol molecules do not interact equally with all phospholipid molecules. In fact, free cholesterol prefers sphingosine to glycerol backbone of phospholipids, although preferences also exists between phospholipids with similar glycerol backbone but different head groups[219, 225]. It is likely that this leads to formation of two types of distinct nano-sized lipid environments, i.e., nanoenvironments[210], at the surface of lipoprotein particles, namely, one that is rich in both sphingomyelin (with sphingosine backbone) and free cholesterol molecules, and the other that is rich in phosphatidylcholine (with glycerol backbone) but poor in free cholesterol molecules[210].

Triglycerides and cholesterol esters seem not to have any specific order in the core of lipoprotein particles in physiological temperatures but occur in random orientations[210, 226]. Below the physiological temperatures, cholesterol esters of LDL particles are capable of undergoing phase transition from liquid-like state into a highly ordered smectic-like liquid crystal state with two radial layers of cholesterol esters[227-230]. The transition is reversible and occurs at around 30°C but may vary as much as 10°C in the peak temperature depending on the core lipid composition, particularly triglyceride to cholesterol ratio. Whether the phase transition could occur *in vivo*, and its possible effects on LDL particle metabolism, are, however, unknown.

The triglyceride to cholesterol ester ratio has also an important role in partitioning of free cholesterol molecules between lipoprotein core and surface and thus affects surface properties of lipoprotein particles. In emulsions of phosphatidylcholine, free cholesterol and triglyceride molecules without cholesterol esters, 80 % of cholesterol molecules were associated with phosphatidylcholines[231]. When cholesterol esters were added into the emulsion, the corresponding figure was only 50 % probably due to a favourable interaction between cholesterol rings in the core[231]. In the lipoprotein particles, which contain both triglycerides and cholesterol esters, approximately 60-70 % of free cholesterol molecules reside in contact with phospholipids[232, 233].

Similarly to ring-ring-interactions of cholesterol moieties, also fatty acids likely interact with each other. Thus it seems natural that fatty acids of triglycerides and cholesterol esters would interpenetrate towards the fatty acids of phospholipid molecules in a radial order[210] as long as they are shielded from water. In fact, several experimental studies have observed small amounts of triglycerides and cholesterol esters in the surface of large vesicles[234-240] and emulsion particles[241, 242] and recently triglycerides have also been observed in the surface of hen VLDL particles[243]. Although the triglycerides and cholesterol esters could have two different locations and multiple orientations to inter-

penetrate the phospholipid monolayer, most of these studies suggest that those triglycerides and cholesterol esters that locate in the lipoprotein-water interface have their fatty acids pointing towards the core.

Another possibility is that triglycerides and cholesterol esters interpenetrate from the core towards the surface. Interpenetration in this way is likely nanodomain-dependent since the surface nanodomains likely differ due to their different physio-chemical properties determined by their lipid constituents[210]. For example, free cholesterol molecules have a tendency to increase the order of phospholipid fatty acids and thus domains rich in sphingomyelin and free cholesterol molecules might be overly rigid to allow interpenetration of triglycerides and cholesterol esters towards the surface. The phosphatidylcholine-rich and cholesterol-poor regions, on the contrary, might favour penetration of triglycerides and cholesterol esters toward the surface[210]. This view is also partially supported by the fact that increasing amounts of free cholesterol molecules decrease the solubility of triglycerides and cholesterol esters into phospholipid bilayers[234, 235], probably because they compete for the same space between fatty acid tails of phospholipids[244].

2.2.2 Apolipoproteins determine the metabolic fates of lipoprotein particles

Lipoprotein particles contain several different apolipoproteins which were originally named with a series of letters A-E as they were separated by electrophoresis. Soon it became apparent that most of these lettered proteins could be further divided into several individual proteins and thus the apolipoproteins are now usually referred as apoA-I, apoA-II, apoA-IV, apoB, apoC-I, apoC-II, apoC-III, apoD and apoE.

The primary structure of apolipoprotein molecules has been discovered; apoA, apoC, and apoE molecules share similar amino acid sequence with 11 and 22 amino acid homogenous repeats the latter consisting of two 11-mers[245]. ApoB and apoD molecules, in contrast, bear little resemblance to the sequences of apoA, apoC, and apoE molecules or each other[245-247]. The secondary structures of apolipoproteins have also been defined; apoA, apoC, and apoE molecules are rich in α-helixes[248-250], apoB has alternating α-helixes and β-sheets[210, 248, 251], and apoD is composed primarily of β-sheets[252]. Tertiary structures are not currently that clear, particularly in the case of apoB (for more details see[210, 211, 251, 253-256]). Most if not all apolipoproteins seem, however, more or less to wrap around the lipoprotein particles and also form apolipoprotein regions at the surface of lipoprotein particles.

Apolipoprotein molecules interact with both the lipid constituents of lipoprotein particles and the surrounding aqueous environment and thus help to

solubilise the hydrophobic triglyceride and cholesterol ester molecules within the lipoprotein particles. Since apolipoprotein molecules more or less surround the lipoprotein particles, increasing amount of triglycerides and cholesterol esters, and thus increasing size of the particles, require flexibility from the apolipoproteins. In fact, the apolipoproteins have the ability to change conformation to adjust to changing lipid or protein contents,[257-260] compositions[258, 259] and metabolic states[174, 249, 261] of the lipoproteins although also the apolipoprotein content of lipoprotein particles per se may increase or decrease to adapt to these changes[209]. The change of apolipoprotein content is possible due to the capability of α-helical apoA, apoC and apoE molecules to dissociate from one particle and attach to another.

The most important apolipoprotein in HDL particles is apoA-I which is present as multiple copies in each HDL particle. The amount of apoA-I molecules increases with increasing size of HDL particles, reaching the maximum of 4-5 in the largest particles. In contrast, the most important apolipoprotein in chylomicron, VLDL, IDL and LDL particles is apoB, and each of the lipoprotein particles contains a single copy of the protein. The apoB occurs, however, in chylomicrons in a truncated form (apoB-48) with length of only 48 % of the sequence from the N-terminus of the full length apoB (apoB-100) molecule[262]. The apoB molecules are non-exchangeable apolipoproteins and thus they are bound into the same particle until particle degradation. Exchangeable apolipoproteins, apoC and apoE, transfer avidly between lipoprotein particles and participate in their metabolism[209].

Apolipoprotein molecules determine the metabolic fate of lipoprotein particles; apolipoproteins function as activators or inhibitors of plasma enzymes. These effects are direct or indirect via influencing the conformation of the activator apolipoproteins. For example, apoC-II activates lipoprotein lipase which hydrolyses triglycerides in lipoprotein particles. ApoC-III has, on the contrary, been shown to inhibit the action of lipoprotein lipase[20, 209].

Apolipoproteins function also as ligands for cell surface receptors in particle endocytosis while certain apolipoproteins may also inhibit particle endocytosis. For example, LDL-receptors, which take up VLDL, IDL and LDL particles into the cells, recognise both apoB-100 and apoE molecules as their ligands. ApoC-I and apoC-III molecules, which are usually abundant in large triglyceride-rich particles, have, however, the ability to inhibit particle endocytosis, likely by displacing or otherwise disturbing apoE, and may thus protect the newly synthesised particles from premature endocytosis[20, 209, 263].

In addition to the well-known functions of apolipoproteins in lipid binding and solubilisation, modulation of enzyme activities and receptor recognition, also other functions have been described for apolipoproteins. For example,

apoE and apoD have been suggested to play a key role in *in situ* nerve repair and regeneration[264] as well as in neuronal protection during plaque formation in Alzheimer's disease[265, 266] and apoA-IV in signalling satiety in the fed state[267, 268].

To summarise, lipoprotein particles are micellar, spherical particles of lipids and proteins in the circulation. The structure of the particles may be divided into two compartments, the core and the surface layer surrounding the core. The lipids and proteins are not randomly located in the compartments but are ordered according to their hydrophobic/hydrophilic properties as well as lipid-lipid and lipid-protein interactions. In a recent study[243], small amounts of triglycerides have also been observed at the surface of large hen lipoprotein particles.

2.3 Metabolism of lipoprotein particles

Metabolism of lipoprotein particles can be viewed as lipid transportation phenomena between the site of lipoprotein assembly and the tissues and organelles consuming the lipoprotein lipids or lipoprotein particles. The lipid transportation events are essential for proper metabolic functioning of human beings and can be characterised as apolipoprotein-driven processes. These processes are divided into four parts in this thesis and include exogenous lipid transportation, endogenous lipid transportation, forward cholesterol transportation and reverse cholesterol transportation. The exogenous and endogenous lipid transportation processes concentrate mainly on transfer of dietary and self-made triglycerides, respectively, for energy metabolism of tissues and organelles. The forward and reverse cholesterol transportation processes concentrate on transfer of cholesterol from the liver into extrahepatic tissues or in the reverse direction, respectively, to maintain cells' cholesterol homeostasis. These transportation processes occur simultaneously in the circulation and interact or even overlap with each other. The interactions are also viewed in the end of the chapter.

2.3.1 Exogenous lipid metabolism

Exogenous lipid metabolism refers to the transfer of dietary lipids from the place of absorption into the tissues and organelles consuming them for energy or storing them for later use (see Fig. 2.2). Dietary lipids contain typically 90-95 % of triglycerides with smaller amounts of phospholipid and cholesterol molecules[269]. Dietary lipids are hydrolysed in the small intestine, emulsified with bile salts and absorbed across the epithelial cell membranes.

In the epithelial cells, the dietary lipids are reassembled again and packed with newly synthesised lipids, apoB-48, apoA-I, apoA-II, and apoA-IV molecules to form chylomicron particles.

Chylomicron particles are the largest and lightest lipoproteins known. They can be separated from the density less than 0.94 g/ml[154] of blood plasma and their diameters vary in the range of 80 - 1000 nm[269]. Chylomicrons are secreted from intestinal epithelial cells into the lymphatics and then transported through thoracic duct into the bloodstream. The large, triglyceride-rich chylomicron particles begin to appear in blood shortly after oral fat intake, and these triglyceride-rich chylomicrons make the greatest contribution to human energy metabolism after meals in a so-called postprandial state[270]. While the total triglyceride content of chylomicrons is very high in the postprandial state, their particle concentration is very low, less than 5 % of VLDL particles[271] due to their rapid disposal. It is debatable whether small chylomicron particles are also secreted in the fasted state in humans as shown in rodents and rabbits[272]. In these animals, chylomicrons are secreted constantly and their size rather than number depends on the availability of lipid substrates[272].

Chylomicrons retain their initial composition while they remain in lymph but face multiple lipid and protein exchange reactions mainly with HDL particles when entered into the circulation; chylomicrons interchange some apoA for apoC and apoE molecules[273-277] as well as receive cholesterol and phospholipid molecules from HDL particles. Thus, while in the bloodstream, chylomicrons contain approximately 98 % lipids by weight and all the major apolipoproteins including A, B, C and E[154, 269, 278]. Most of the lipids are triglycerides (80-90 %), while the content of phospholipids is 10-20 % and the content of cholesterols is less than 2 %[278], see Table 2.1.

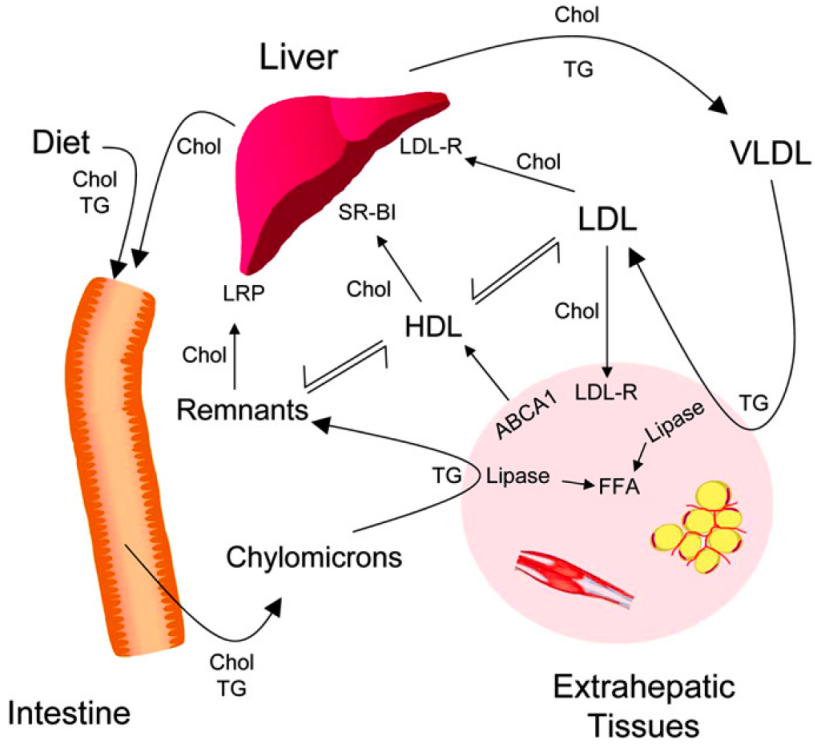

Figure 2.2 Overview of metabolism of apoB-containing lipoprotein particles. In exogenous lipid transport, chylomicrons deliver dietary triglycerides from intestine into the extrahepatic tissues (followed by lipolysis of triglycerides to free fatty acids, FFAs). Chylomicrons become chylomicron remnants which are internalised mostly by the liver via LDL-receptors and LDL-receptor related proteins. The liver uses endogenously synthesised triglyceride and cholesterol molecules as well as lipids derived from chylomicron remnants to synthesise VLDL. In this endogenous lipid transport, VLDL particles (and IDL; not shown) transport triglycerides into extrahepatic tissues in a similar way as chylomicron particles. Some of the IDL particles become LDL particles which are the main cholesterol transporters (forward cholesterol transport) in human circulation. VLDL remnants as well as IDL and LDL particles are internalised mainly by the liver via LDL-receptors and LDL-receptor related proteins. ApoB-containing particles exchange lipids, particularly triglycerides and cholesterol esters, with HDL particles. TG, triglyceride; Chol, cholesterol; SR-BI, scavenger receptor class B type I, LDL-R, LDL-receptor; LRP, LDL-receptor related protein. Reprinted from[279] with permission.

Table 2.1 Size, density, main apolipoproteins and weight percentages of the main lipoprotein fractions and HDL subclasses.

Lipoprotein	Diameter (nm)	Density (g/ml)	Main apos	W_{TG}	W_{CE}	W_{FC}	W_{PL}	W_{PROT}
CM	80 - 1000	< 0.94	apoB	86	3	2	7	2
VLDL	30 - 80	0.94 - 1.006	apoB	55	12	7	18	8
IDL	25 - 35	1.006 - 1.019	apoB	23	29	9	19	19
LDL	18 - 29	1.019 - 1.063	apoB	6	42	8	22	22
HDL2	9 - 13	1.063 - 1.125	apoA	5	17	5	33	40
HDL3	7 - 9	1.125 - 1.210	apoA	3	13	4	25	55
Lp(a)	30	1.055 - 1.085	apoB, Lp(a)	3	33	9	22	33

Apo, apolipoprotein; W, weight percentage; TG, triglyceride; CE, cholesterol ester; FC, free cholesterol; PL, phospholipid; PROT, protein. For references, see[154, 168, 169, 269, 280].

In addition to these exchange reactions, chylomicron particles promptly loose triglycerides into various tissues including skeletal muscles, myocardium and adipose tissues. At the endothelial surface of the capillaries of these tissues, each triglyceride is first hydrolysed into two fatty acids and a monoacylglycerol molecule, by apoC-II-activated action of an enzyme called lipoprotein lipase (LPL). Then, the fatty acids pass into the tissues, the monoacylglycerol is transported in the liver and, after a while, the chylomicron particles dissociate from the endothelial surface back into the bloodstream.

Transportation of triglycerides in chylomicron particles is a sequential process. These delipidation steps can be characterised by binding, desorption and rebinding at other sites leading to progressive loss of approximately 50-70 %[278, 281] of triglycerides from each chylomicron particle. The loss of triglycerides shrinks the cores of chylomicron particles and decreases the need of surface material covering the cores. This leads to massive reorganisation of molecules in chylomicron particles and shedding of phospholipid, free cholesterol and apoC molecules from the surface. This excess surface material, or surface remnants, recycles back to HDL particles. The decreased apoC-II content of chylomicrons slows the delipidation, and decreased apoC-I and apoC-III content changes the conformation of the remaining apolipoproteins. The delipidated chylomicrons, or chylomicron remnants, are then rapidly cleared from the blood plasma, mainly into the liver, through a receptor-mediated process where apoE molecules on chylomicron remnants serve as ligands. The chylomicron remnants are internalised mainly by LDL-receptors[282] or by LDL-receptor-related proteins (LRP) which show sequence homology to LDL-receptors[283, 284].

2.3.2 Endogenous lipid metabolism

Endogenous lipid metabolism refers to the delivery of self-made triglycerides

from the liver into various tissues. These triglycerides originate essentially from three sources including incompletely delipidated remnant particles internalised into the liver, fatty acids entering in the liver from adipocytes, and *de novo* synthesis in the liver[285]. The particles responsible for transporting these endogenous triglycerides in the circulation are VLDL and IDL particles. In addition to triglycerides, these particles also contain significant amounts of cholesterol esters which originate from similar sources as triglycerides including remnant particles and *de novo* synthesis but also from cholesterol recycling[285].

VLDL particles are the precursors of VLDL-IDL-LDL delipidation cascade where parallel processing pathways generate heterogeneous IDL and further LDL particles from VLDL particles[286-290] (see Fig. 2.2). The VLDL fraction consists of heterogeneous particles with diameters of 30-80 nm[280] and density of 0.94-1.006 g/ml[154]. Half of the weight of VLDL particles are triglycerides, the content of both phospholipids and cholesterol is approximately 20 % and thus the B-, C- and E-apolipoproteins account for the remaining 10 %[280] (see Table 2.1). VLDL particles are smaller in size and denser than chylomicrons and have a full length apoB-100 in contrast to the truncated apoB molecule present in chylomicrons. The apoB-100 molecule is present in VLDL particles with some apoC and a few apoE at the time of secretion, and extra apoC and apoE molecules are attached to VLDL particles in the circulation[291].

VLDL particles are secreted from the liver into the bloodstream, and both the size of the secreted particles and the rate of particle secretion seem to be regulated, mainly by triglyceride availability in the liver[20, 292-294]. The synthesised VLDL particles are structurally and metabolically different and are, therefore, discretionarily divided into the subclasses VLDL1 and VLDL2[170].

VLDL1 particles are large and triglyceride-rich[295, 296] and influence most the plasma triglyceride level in fasted state[294, 295]. VLDL1 particles are secreted into the blood plasma mainly in fasted state when insulin level[292] and the amount of chylomicron-derived lipids in the blood is low. Thus the VLDL1 particles compensate the need of triglycerides in the peripheral tissues, mostly skeletal muscles. VLDL1 particle secretion is acutely suppressed by the presence of insulin in fed state[292] but these particles may be produced if the liver is supplied with increased amounts of free fatty acids as observed in obesity[297, 298], with a diet high in simple carbohydrates[299, 300], with high alcohol consumption[301], in insulin resistance state[295], and in type 2 diabetes[295, 302].

VLDL2 particles are smaller and have lower triglyceride content and higher cholesterol ester content than VLDL1 particles[20, 303]. VLDL2 particles are, in contrast, secreted when triglyceride availability in the liver is suppressed or when cholesterol synthesis and cholesterol ester availability in the liver are in-

creased[304]. A significant portion of particles in the density range of VLDL2 particles do, however, originate from delipidated VLDL1 particles[286, 305] and are indistinguishable from the secreted VLDL2 particles by density.

VLDL particles are sequentially hydrolysed by lipoprotein lipase similar to chylomicron particles yielding VLDL remnants and IDL particles. It is currently unknown how VLDL particles are destined for VLDL remnants or IDL particles. Evidence is, however, emerging that particularly VLDL1 particles would be readily converted into VLDL remnants[305, 306], while VLDL2 particles are delipidated into IDL particles[307]. The VLDL remnants are cleared from the plasma mainly by the liver in a receptor-related manner, which is influenced by both their apolipoprotein and lipid content; VLDL remnants with a high number of apoE relative to apoC apolipoproteins are cleared to greater extent than apoE-poor particles[308] as apoE[309, 310] molecules serve as ligands for mainly LDL-receptors[311] and LDL-related receptors[284]. Increased cholesterol ester content, derived from action of other enzymes in the circulation, also seems to direct those VLDL remnants into the receptors[209, 312]. The VLDL remnants not cleared from the blood plasma persist in the circulation in the density range of VLDL and IDL particles and thus they might be incorrectly denoted as VLDL or IDL [20].

IDL are heterogeneous particles of 25-35 nm in diameter[280] and the density range of 1.006-1.019 g/ml[154] (see Table 2.1). IDL particles are transient particles between VLDL and LDL and their concentration in blood plasma of healthy individuals is low[294]. IDL particles are rich in cholesterol esters, approximately 30 % by weight, but still contain a significant portion of triglycerides, approximately 20 %, while free cholesterol constitutes 10 % and phospholipids and proteins both approximately 20 %[280]. IDL particles still contain apoB, apoC and apoE molecules similar to VLDL particles but their apoC content is reduced[313, 314].

IDL are heterogeneous particles similar to VLDL, and they can also be divided into two subclasses, namely IDL1 and IDL2[170, 171]. IDL1 particles are large, triglyceride-rich and cholesterol ester-poor while IDL2 particles are small, triglyceride-poor and enriched in cholesterol esters[20]. The larger IDL1 particles also contain twice as much apoC-II and apoC-III molecules as IDL2 particles[315]. The IDL subclasses may also differ by their origin. It has been suggested that IDL1 particles alone constitute the class of IDL particles which are delipidated from VLDL, particularly from VLDL2, since their concentration increases with increasing plasma triglycerides[20, 171]. IDL2 particles, in contrast, might by directly secreted by the liver when the triglyceride content in the liver is low[20].

A portion of IDL particles may be internalised, mainly via LDL-receptors

and LDL-related receptors using apoE as a ligand[316] (see Fig. 2.2). The remaining IDL particles are further delipidated in the bloodstream as a result of action of lipoprotein lipase and yet another enzyme called hepatic lipase (HL). Hepatic lipase has homology to lipoprotein lipase and locates at the luminal side of the liver parenchymal cells and in capillaries lining the tissues synthesising steroid hormones. Hepatic lipase hydrolyses triglycerides similar to lipoprotein lipase but has affinity also for phospholipid molecules and prefers smaller lipoprotein particles, i.e., IDL, LDL and HDL particles, than lipoprotein lipase. Thus, the delipidation pathway from VLDL particles to LDL via IDL particles is finalised by hepatic lipase and results in the loss of a majority of triglycerides, some phospholipids, and virtually all exchangeable apolipoproteins from IDL particles.[317]

2.3.3 The forward cholesterol transport

Most human cells are capable of synthesising small amounts of cholesterol. Cholesterol is also transported into the extrahepatic, peripheral cells from the liver in a so-called forward cholesterol transport process. The cholesterol in this pathway is transported within LDL particles.

LDL particles are heterogeneous in size ranging from 18 to 29 nm in diameter[168, 169] in the density range of 1.019-1.063 g/ml[154] (see Table 2.1). Each LDL particle has one apoB-100 molecule, alone forming 20 % of the weight of LDL[280], and trace amounts of other apolipoproteins, mainly apoE[174]. In contrast to chylomicrons and VLDL particles, the major lipids in LDL particles are cholesterol esters (40 %)[280]. The content of triglycerides is approximately 10 % and phospholipids 20 % by weight[280].

LDL particles are delipidated from triglyceride-rich lipoproteins secreted by the liver although newly secreted particles from the liver might also have density in the range of LDL particles when the level of plasma triglycerides is low[307] (see Fig. 2.2). However, it remains controversial whether these particles have the physiological function of LDL or whether they represent VLDL or IDL particles with extremely low triglyceride content.

As many as eight distinct subspecies of LDL particles have been identified by gradient gel electrophoresis[168, 169]. These subspecies have been grouped, based on density, into four major subclasses designated LDL1, LDL2, LDL3 and LDL4 in order of decreasing size and increasing density[168, 318, 319]. LDL1 particles comprise approximately a quarter of the LDL particles in plasma[320] and their concentration decreases with increasing insulin resistance, plasma triglycerides, obesity and activity of hepatic lipase[294]. LDL1 particles are suggested to be delipidated from IDL2 particles secreted directly from the liver[20, 171].

LDL2 particles comprise the largest group of the LDL particles in individuals with normal lipid metabolism[320]. LDL2 concentration, relative to total LDL, has, however, been shown to decrease in CETP deficiency[20, 321]. The absolute concentration of LDL2 particles has also been shown to decrease in men with a high level of plasma triglycerides[294]. LDL2 are suggested to be a product of VLDL2-IDL1 delipidation pathway[20] which, in addition to the IDL2-LDL1 pathway, is also prominent in cases where the level of plasma triglycerides is low[20, 304]. Thus both LDL1 and LDL2 subclasses are large and rich in cholesterol esters. In contrast, the concentrations of the smallest LDL particles, LDL3 and LDL4, are usually high when the level of plasma triglycerides is high[20, 294, 322, 323] and they predominate in insulin resistance state[322, 324, 325]. LDL3 and LDL4 are suggested to be delipidated from the VLDL1-VLDL remnant pathway[20, 304].

LDL particles are internalised into cells mainly via LDL-receptors[69, 70, 72]. LDL-receptors are expressed by most cell types to acquire cholesterol for their needs. The liver is, however, in whole-body terms the main site of LDL-receptor-mediated removal of LDL particles[326, 327]. The internalisation of LDL particles is highly regulated by the amount of LDL-receptors at the cell surfaces. In healthy individuals, approximately 60-80 % of LDL particles are internalised via LDL-receptors[328, 329] but several factors including age, gender, dietary alterations, hormonal disturbances, and administration of pharmacological agents affect the activity of LDL-receptors[285].

2.3.4 The reverse cholesterol transport

The reverse cholesterol transport refers to transport of cholesterol from the peripheral tissues into the liver. The reverse cholesterol transportation is an essential process in maintaining constant cholesterol content in the cells by balancing cholesterol influx and *de novo* synthesis, which both increase the cholesterol content, by efflux of cholesterol from the cells. The reverse cholesterol transport process has also been suggested to play a major role in decreasing or preventing the development of atherosclerosis particularly by decreasing the cholesterol load of vessel wall macrophages[330-334]. The reverse cholesterol transport contains two phases, cholesterol acquisition and cholesterol delivery, and the HDL particles have a key role in both phases.

HDL particles are the most numerous lipoprotein particles in human plasma, their number is approximately 10-20 times that of other lipoprotein particles together[269]. HDL particles are also the smallest lipoproteins in the circulation ranging from 7 to 13 nm in diameter[175, 176, 324], and represent the density range of 1.063-1.210 g/ml[280] (see Table 2.1). The protein content of HDL particles is approximately half of their weight and is relatively the

highest of all lipoprotein particles[280]. The apolipoprotein essential for the formation of a HDL particle is apoA-I which accounts for approximately 60-70 % of total HDL protein[335, 336]. ApoA-I occurs with or without other apolipoproteins including apoA-II, apoA-IV, apoC and apoE[335, 336]. HDL particles without apoA-I but with apoA-IV or apoE with sequence similarity to apoA-I have been identified in human plasma although their concentration is low[190, 191]. The most typical lipid in the HDL particles is phospholipid (30 %) due to the small size of HDL particles; the particles contain less than 20 % of cholesterol esters and 5 % of triglycerides[280].

HDL particles are regularly divided into subclasses by their density in ultracentrifuge yielding HDL_1 (1.055-1.063 g/ml), HDL_2 (1.063-1.125 g/ml), HDL_3 (1.125-1.210 g/ml) and HDL_4 (1.210-1.250 g/ml) subclasses[158-160]. HDL_2 and HDL_3 are the main subclasses present in human beings and can be further divided into HDL_{2b} (9.8–12.9 nm) and HDL_{2a} (8.8–9.8 nm) as well as into HDL_{3a} (8.2–8.8 nm), HDL_{3b} (7.7–8.2 nm) and HDL_{3c} (7.2–7.7 nm) according to their size in non-denaturing gradient gel electrophoresis[176, 324].

The concentration of HDL subclasses seems to alter in a reciprocal manner. Case-control and angiographic studies suggest that the risk of cardiovascular disease may increase when concentration of the largest HDL particles, HDL_{2b}, decreases relative to the smallest HDL particles, HDL_{3c} and HDL_{3b}[320, 337-339]. Relative concentration of the large particles also decreases and that of the smallest particles increases in insulin resistance and type II diabetes[324, 340, 341]. By contrast, cholesterol feeding increases the size of the HDL particles and the relative concentrations of HDL_{2b}, HDL_{2a} and HDL_{3a} particles[342]. Several other factors have also been associated with these larger HDL particles, i.e., loss of excess body weight increases HDL_{2b} and decreases HDL_{3b} concentrations relative to the total HDL particle concentration[343] and elevated levels of HDL_{2a} and HDL_{2b} concentrations have been observed in heavy alcohol users[344-346]. It is, however, unknown what regulates the subclass distribution and to what extent these subclasses have different functions.

The reverse cholesterol transport is conceived as the main metabolic function of HDL particles although they may also have other roles such as antiapoptotic, antioxidative, antiinflammatory, and vasodilatory activities[347-350]. In the reverse cholesterol transport process, the delivery of cholesterol to the liver occurs via cell surface receptors not requiring the degradation of the participating HDL particles, and thus the metabolism of HDL particles may be seen as a cycle of HDL particles between lipid-rich and lipid-poor states categorising them into various HDL particle subclasses. These lipid-rich and lipid-poor states of HDL particles result from a complex crosstalk of lipid-hydrolysing enzymes, lipid transporters and specific lipoprotein receptors af-

fecting significantly the size, composition and structure of HDL particles as well as their properties as cholesterol transporters. Due to the complexity of reverse cholesterol transport process, its details are still unclear and thus this pathway is currently under active investigation.

The site of HDL particle synthesis depends on the definition of a HDL particle; These sites include the liver, the small intestine and the circulation[250, 334, 335, 352] (see Fig. 2.3). The main structural components of HDL particles, apoA-I and apoA-II molecules, are synthesised in the liver and in the small intestine[334, 352, 353] and are transported into the circulation as lipid-free molecules[334, 335, 354], nascent HDL particles[334, 335, 352] or attached into the surface of triglyceride-rich particles[291, 312]. These apoA molecules dissociate from the surfaces either as lipid-free or lipid-poor apolipoproteins when entered into the plasma compartment. In addition to the newly secreted lipid-free and lipid-poor apoA compounds, also the plasma offers similar, likely recycled lipid-free and lipid-poor apolipoprotein molecules which are dissociated either from HDL particles during their modifications in the plasma[334, 335, 355] or from delipidated VLDL and CM particles[334, 356, 357]. Thus the maturing HDL particles appear at first in plasma as lipid-free or small lipid-poor apolipoprotein-phospholipid aggregations of which the apoA-I- phospholipid and apoA-II- phospholipid complexes are most common although also other apolipoprotein-phospholipid complexes exists[334, 358, 359].

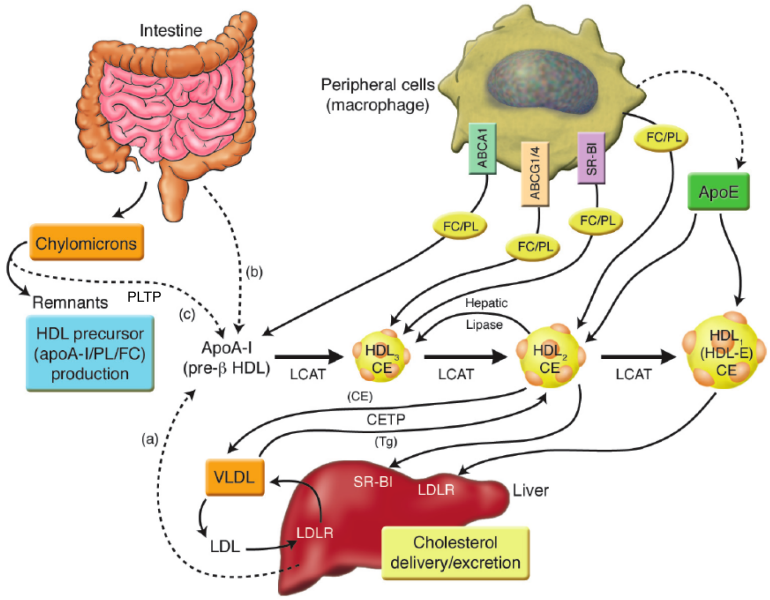

Figure 2.3 Metabolism of HDL particles. HDL particles transport cholesterol from peripheral cells to the liver. HDL precursors (pre-β HDL) are produced by the liver and intestine (apoA-I/PL/FC) or derived from surface

material from chylomicrons and VLDL particles facilitated by PLTP after lipolysis (a,b,c). Pre-β HDL, HDL_3 *and* HDL_2 *can accept cholesterol for reverse cholesterol transport. HDL particles donate cholesterol to the liver selectively, i.e., without whole-particle uptake, via scavenger receptor BI (SR-BI). Larger HDL particles with apoE molecules (*HDL_2 *or* HDL_1*) can deliver cholesterol to the liver directly via the LDL receptor. FC, free cholesterol; PL, phospholipid; CE, cholesterol ester; Tg, triglyceride, apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; pre-β HDL, HDL precursors; HDL-E, high-density lipoprotein particles with apoE; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; ABCA1, ATP-binding cassette transporter A1; ABCG1/4, ATP-binding cassette transporter G1 and G4; SR-BI, scavenger receptor class B type I; LDLR, LDL receptor. Reprinted from*[351] *with permission.*

In the course of HDL particle maturation, the lipid-poor apoA-I and apoA-II compounds acquire additional phospholipid and free cholesterol molecules from several resources via various processes. Firstly, the lipid-free and lipid-poor apolipoproteins receive phospholipids from the large lipoprotein particle surfaces in a process which is facilitated by phospholipid transfer protein (PLTP)[355, 360]. PLTP transports phospholipids down their concentration gradients, resulting in phospholipid-enriched aggregations. Secondly, lipid-free apoA molecules and lipid-poor apoA-phospholipid complexes receive avidly additional lipids, particularly excess intracellular free cholesterol molecules and phospholipids from the peripheral cell surfaces via a specific membrane transporter called ATP-binding cassette transporter A1 (ABCA1) which changes the HDL precursors into discoidal HDL particles[335, 354, 361, 362]. The flow of lipids into these discoidal HDL precursors is followed by the break-down of phosphatidylcholines into lyso-phosphatidylcholines and esterification of free cholesterol molecules into cholesterol esters by the action of lecithin-cholesterol acyltransferase enzyme (LCAT) at the surface of apoA-I-, apoA-IV-, apoC-, or apoE-containing HDL particles[359, 363, 364]. The action of LCAT increases the amount of cholesterol esters and decreases the amount of free cholesterol and phosphatidylcholine molecules in the particles and thus maintains the concentration gradient of free cholesterol and phospholipid molecules. Thirdly, the HDL precursors gain free cholesterol molecules and phospholipids via diffusion from cells and other lipoprotein particles[336, 362, 365]. The enrichment of lipids, particularly the cholesterol esters, and the attachment of additional freely exchanging apoA-I molecules gradually change the discoidal apoA-I containing HDL precursors into small, spherical HDL_3 particles[335, 366]. The HDL precursors containing only apoA-II remain, however, discoidal since LCAT is not activated by apoA-II molecules[367-369].

Small, spherical HDL_3 particles continue to receive lipids from various routes such as phospholipid and cholesterol molecules via diffusion[365], phos-

pholipids via PLTP[355, 360], cholesterol via other members of ATP-binding cassette super-families such as ATP-binding cassette transporter G1 and G4 (ABCG1 and ABCG4)[370-372], as well as via a bidirectional scavenger receptor class B type I (SR-BI) pathway[365, 373, 374], and surface lipids and apolipoproteins from chylomicrons and VLDL particles during their delipidation[334, 356, 357]. The increased free cholesterol content is again partially esterified by LCAT to cholesterol esters at the surface of apoA-I-, apoA-IV-, apoE- or apoC-containing HDL_3 particles resulting in an increased size of the HDL_3 particles. These HDL_3 particles require additional apoA molecules to stabilise their structures and the apoA molecules are received from various sources including lipid-free apolipoproteins[354], lipid-poor apolipoproteins[375, 376], discoidal HDL particles in a LCAT-mediated particle fusion[377] or fusion of two HDL_3 particles facilitated by PLTP[355]. In the LCAT-mediated fusion, a small HDL_3 particle combines with either a discoidal apoA-I- or apoA-II-containing particle resulting in a large, spherical LpA-I or LpA-I:A-II particle. In the PLTP-mediated fusion, two small apoA-I- containing HDL particles (having total of 2*3 apoA-I molecules) fuse to yield either three small HDL particles (3*2 apoA-I molecules) or a large HDL particle and two dissociated apoA-I molecules (1*4+2 apoA-I molecules). As a result, the subsequent increase in the content of lipids and apolipoproteins increases the size of the HDL_3 particles and, at some point, shifts them into HDL_2 particle range.

In the last step of the reverse cholesterol transport, cholesterol, mainly in HDL_2 particles[334, 351], is delivered into the liver by two receptor-mediated processes. The cholesterol in internalised mainly through receptors internalising apoB-containing particles[311, 328, 329] but also through SR-BI receptors[378-380].

In the SR-BI mediated process, HDL_2 binds to a cell and cholesterol, primarily in the form of cholesterol esters[374, 381, 382], is transferred efficiently via the receptor to the liver in the absence of HDL particle internalisation. The formed lipid-depleted HDL particle subsequently dissociates from the cell and re-enters into the circulation as a small HDL_3 particle and free apoA-I molecules[366, 383]. These HDL precursors are then capable of initiating the reverse cholesterol transport again or are degraded as lipid-poor apolipoprotein molecules[375, 383, 384].

HDL cholesterol molecules are also internalised via LDL-receptors and LDL-receptor related proteins. Most of the cholesterol esters internalised by this route are first transferred by cholesteryl ester transfer protein (CETP) into apoB-containing particles[385-387] although large, apoE-containing HDL particles may also be internalised directly via these receptors[351, 388-390]. CETP is a hydrophobic glycoprotein that is secreted from the liver and circulates in plasma bound mainly to HDL particles[391, 392]. It promotes the redistribution of

cholesteryl esters and triglycerides between plasma lipoproteins down their concentration gradients. Thus CETP activity results in a net mass transfer of cholesterol esters from HDL to apoB-containing lipoproteins in exchange for triglycerides from VLDL and CM particles. In apoB-containing particles, the HDL-derived cholesterol esters follow the metabolic path of the apoB-containing particles and are received mainly by the liver via LDL-receptors or LDL-receptor-related proteins[284, 311, 316, 328, 329]. The resulting HDL particles are depleted of cholesterol esters and may again receive cholesterol in the circulation.

2.3.5 Atherogenic lipoprotein phenotype

Metabolism of lipoprotein particles takes place as a complex crosstalk between the four lipid transportation systems. The lipid transportation processes are all highly connected, which is mainly caused by action of enzymes and lipid transporters. In the following, these associations are discussed from the point of view of atherogenic lipoprotein phenotype where the interactions are clearly seen.

Broadly, the atherogenic lipoprotein phenotype is a combination of lipid or lipoprotein abnormalities frequently met in individuals with atherosclerosis. Originally, the atherogenic lipoprotein phenotype was characterised by the prevalence of small, dense LDL particles, elevated plasma triglycerides and reduced HDL cholesterol[393]. Later on, when the concentrations of VLDL, LDL, and HDL particles *per se* were recognised as important variables of lipoprotein metabolism, the definition of the atherogenic lipoprotein phenotype was expanded. Therefore, currently, the atherogenic lipoprotein phenotype constitutes elevated plasma triglycerides or VLDL particles; elevated LDL particles particularly in their small, dense region; and reduced HDL cholesterol or HDL particles[1, 20, 206, 394].

Elevated plasma triglyceride level seems to be one of the most important determinants of the atherogenic lipoprotein phenotype and abnormal lipoprotein metabolism[322, 338, 395, 396]. As plasma triglyceride level is fairly easy to measure, elevated levels of plasma triglycerides have been studied extensively and linked to various conditions including several lifestyle factors such as obesity, overweight, physical inactivity, cigarette smoking, excess alcohol intake, high carbohydrate diet (> 60 % of energy intake); several diseases such as type II diabetes, chronic renal failure, nephrotic syndrome; certain drugs such as corticosteroids, estrogen, retinoids and beta-blockers as well as various genetic dyslipidemias[131]. Thus, there are multiple reasons for developing high levels of plasma triglycerides.

Increase in plasma triglyceride level results from accumulation of trigly-

ceride-rich lipoprotein particles in plasma, and the particles mainly responsible for the rise are VLDL1 particles[322, 397]. Accumulation of VLDL1 particles occurs due to alternative or combined metabolic defects in their lipid transportation processes including increased production and decreased rate of conversion or catabolism. The reason for the increased amount of VLDL1 particles has been investigated from various directions including insulin resistance[292, 325], incompetent apolipoprotein compositions[263, 398-400] as well as a saturable delipidation pathway of VLDL and CM particles[401-403].

Production of VLDL1 particles is highly controlled by the level of insulin[292]. Thus individuals with increased insulin resistance are less sensitive to the insulin-induced inhibition of VLDL1 secretion[292]. The overproduction of VLDL1 particles is also the main cause of elevated VLDL1 particles in insulin-resistant individuals while their delipidation rate of VLDL1 particles may also be decreased[325].

In apparently healthy, insulin-sensitive individuals, the elevated VLDL1 particle concentrations are mostly due to decreased delipidation rate rather than increased production of VLDL1 particles[394]. One reason for decreased delipidation of VLDL1 particles may be improper apolipoprotein composition effecting on the activity of lipoprotein lipase. For example, lipoprotein lipase is activated by apoC-II molecules and inhibited by several other apolipoproteins including apoC-III. It has been shown that apparently healthy individuals with high plasma triglyceride level have increased concentration of large VLDL particles which are rich in apoC-III compared to individuals with normal plasma triglyceride level[398-400]. ApoC-III has also been shown to inhibit hepatic clearance of triglyceride-rich lipoproteins[263, 399].

Increase in concentration of VLDL1 particles has also been observed in postprandial state. Concentration of VLDL1 particles may be increased due to the fact that chylomicrons and VLDL1 share a common, saturable delipidation pathway[401-403] since they compete for the same, active lipoprotein lipase molecules limiting the delipidation process. It seems that chylomicrons become delipidated faster than VLDL since chylomicrons bind more avidly to lipoprotein lipase than VLDL particles and since their particle concentration is at least 20-fold lower than that of VLDL[401]. In the postprandial state, the increase in VLDL1 particles has been observed in both insulin-resistant and insulin-sensitive individuals although the effect is more pronounced in individuals with insulin resistance[404].

Elevated levels of VLDL1 particles and shift of LDL particles into smaller particle sizes occur simultaneously surprisingly often[322, 396]; approximately 50 % of the variation in LDL particle size is explained by variation in plasma triglyceride levels[20, 294]. Individuals with decreased LDL size distribution are

designated as having LDL subclass B (pattern B) or individuals with prevalence of small, dense LDL particles (sdLDL). The prevalence of sdLDL particles depends highly on age and gender and is approximately 30-35 % in adult men, 5-10 % in young men (less than twenty years old), 5-10 % in premenopausal women[393, 405] and 15-25 % of post-menopausal women[406, 407]. The prevalence of sdLDL has also been linked to several gene regions important in LDL particle metabolism indicating that the prevalence of sdLDL is affected also by genetic factors[408-410]. In fact, there are multiple genes which may contribute and the responsible genetic mechanisms may vary among families[20]. Thus it seems that both genetic and non-genetic, environment factors are important determinants of LDL particle size distribution.

The mechanism by which LDL particles become small and dense in the presence of elevated plasma triglyceride concentration depends on the action of at least two lipid hydrolysing or transporting enzymes, HL and CETP[160, 411], as well as the level of plasma triglycerides. The process also requires time and thus the LDL particles should have a prolonged residence time (more than one extra day) in plasma[394]. The size reduction is similar to that seen in HDL particles as a part of their normal metabolic cycle although here the elevated plasma VLDL1 concentration has changed the weight of relative CETP-mediated lipid transport process between the lipoprotein particles[160, 322]. In the process, the amount of cholesterol esters in LDL particles becomes decreased and triglycerides increased due to the decreased cholesterol ester transfer from HDL and increased heteroexchange of LDL cholesterol esters for VLDL triglycerides as compared to the normolipidemic state. If this heteroexchange occurs to a significant degree, the triglycerides in LDL particles become hydrolysed by HL decreasing further the size of the particles. Thus, the combined action of CETP and HL in the presence of elevated level of VLDL1 particles leads to the remodelling of LDL particles into cholesterol ester-depleted small, dense LDL particles.

The concentration of total LDL particles, not only the concentration of small LDL particles relative to all LDL particles, seems to increase with increasing plasma triglycerides[322]. When plasma triglyceride concentration in a population rises from 0.5 to 1.5 mmol/l, there is a steady increase in LDL level although the increase is more marked in the intermediate-sized LDL particles and the level of small LDL remains low. Above the plasma triglycerides of 1.5 mmol/l, the total LDL concentration does not seem to increase but the level of intermediate-sized particles falls and level of small LDL rises sharply[322]. Increase in the total number of LDL particles is also observed to coincide the increased concentration of small LDL particles in many recent clinical outcome studies[147, 412-416].

Increased levels of plasma triglycerides and VLDL1 particles affect HDL particles as well[160, 395, 417]. When the level of VLDL particles is normal, CETP modulates HDL size distribution by transferring HDL cholesterol esters preferentially to LDL particles[392, 418] and thus enables, for its part, the normal cyclic metabolism of HDL particles[385-387]. However, the metabolism may become altered when the concentration of large, triglyceride-rich VLDL1 particles is increased; CETP transfers HDL cholesterol esters preferentially to VLDL1 particles for heteroexchange of VLDL triglycerides, resulting in triglyceride-enriched HDL particles[392, 418, 419]. The triglyceride-enriched HDL particles are then modified by lipid-hydrolysing lipases, for example HL, which decreases the size of the HDL particles. These *remnant HDL particles* have also shown *in vitro* to have enhanced holoparticle binding, internalisation and degradation in tissues involved in HDL catabolism thus decreasing the HDL particle concentration in plasma[417, 420-422]. Likewise, the decreased hydrolysis of VLDL1 particles may also inhibit the normal metabolism of HDL particles by yielding decreased amounts of surface lipid and apolipoprotein molecules needed to increase the size of HDL particles[357]. The shift of HDL particles to the smaller sizes may also have a feedback effect on the metabolism of VLDL1 particles since small HDL particles usually do not contain apolipoprotein other than apoA[417, 423] and thus cannot donate apoC-II and apoE molecules to VLDL and CM particles to assist their delipidation and endocytosis.

While the lipid transportation processes *per se* are complicated events, the interactions between the processes multiply the complexity resulting in highly sophisticated lipoprotein particle metabolic networks. Lipoprotein particle metabolism is also susceptible to various modifications in the function of the participating components, e.g. inefficient lipolysis of triglyceride-rich particles, although normal metabolism may to some extent be maintained via alternative routes, e.g. efflux of cholesterol via various channels in the reverse cholesterol transport. Due to the complexity of lipoprotein particle metabolism even in normal situations, the effect of each altered lipid transportation process on the cause of pathological conditions, such as atherosclerosis and cardiovascular disease, remains unclear.

To summarise, the lipoproteins are heterogeneous particles which vary on various properties including density, size, surface charge and chemical composition. Several methodologies have been developed to isolate lipoprotein particles according to their different properties into lipoprotein fractions and further lipoprotein subclasses. All lipoprotein particles have been shown to have a similar micellar structure but there still exists uncertainties about the molecular structure of lipoprotein particles. Lipoprotein particles face complex metabolism in blood plasma and their metabolism can be viewed as lipid

transportation phenomena between the site of lipoprotein assembly, degradation and in some cases cellular uptake. The lipid transportation processes are highly interconnected and regulated by various genetic and non-genetic factors. Association between lipoprotein metabolism and the development of atherosclerosis is widely studied and has led to formulation of an atherosclerotic lipoprotein phenotype which includes several lipoprotein particle components such as increased VLDL and LDL particles and decreased HDL particles as well as various lipid variables including increased VLDL triglyceride and decreased HDL cholesterol concentrations. Despite intensive investigation of lipoprotein particles for decades, the detailed contribution of lipoprotein particles to the development of atherosclerosis remains still unclear and novel methodologies and technologies are needed to increase our knowledge of various aspects of these lipid-carrying particles.

3. Computational modelling of lipoprotein particle structures

So far, we have discussed the basics of the structure and the metabolism of lipoprotein particles. From now on, we will move forward to discuss computational modelling of lipoprotein particles. The next four chapters deal with this topic and are based on the studies I have contributed to. This chapter will first introduce computational modelling of lipoprotein particle structures in general. Then, a new structural lipoprotein particle model concerning the location of hydrophobic lipids, i.e., triglycerides and cholesterol esters, within lipoproteins is presented and discussed in the light of the structure-metabolic relationship of the particles. The research discussed in the chapter has also been published in a refereed journal, see[424].

3.1 Current state of computational modelling of lipoprotein particles

The general structure of lipoprotein particles is well known. However, there still exists uncertainties about the molecular level structures[160, 210, 211, 215, 216]. While the general structures have been revealed by several physicochemical methods, including small-angle X-ray and neutron scattering[230, 425-427], NMR spectroscopy[232, 233, 428-430], and electron microscopy[212, 251, 431-434], these methods have not been successful in revealing the molecular structures. On the other hand, computational modelling is expected to be particularly useful in clarifying the molecular structure of lipoprotein particles.

Lipoprotein particles are complex systems to model. They have large molecular dimensions as even the smallest of the lipoprotein particles contain hundreds of lipid molecules and proteins constituents consisting of a large number of amino acid residues. While a few hundred molecules might not seem a particularly high number, it nevertheless means tens of thousands of atoms and even more pair-wise interactions. The composition of lipoprotein

particles is also heterogeneous as they contain triglyceride, cholesterol ester, phospholipid and free cholesterol molecules as well as various apolipoproteins. This heterogeneity increases the type of interactions present within lipoprotein particles. The lipid constituents of lipoprotein particles have also complex thermodynamic properties.

This overall complexity of lipoprotein particles presently hampers their computational modelling by techniques capable of featuring detailed lipid interactions. These include molecular and dissipative particle dynamics which are based on tracking individual atoms or group of atoms, respectively, via classical Newton's equations. Currently, these methods have been successfully used to model an isotropic system of cholesterol esters[435]; small, discoidal HDL particles[436], roughly spherical HDL particles[437] and spherical HDL particles with a half amount apoA-I molecules with somewhat limited motions[438]. While the role of molecular and dissipative particle dynamics will likely increase in computational modelling of lipoprotein particles in the future, simpler models requiring less computational power are at present the only option to gather new information on the structure of lipoprotein particles by computational means.

After the establishment of micellar structure of lipoprotein particles in 1968[208], tens of structural, computationally less demanding lipoprotein models have been constructed[174, 210, 211, 214, 215, 439-441]. As far we are aware, there are four models which seek the molecular arrangements of lipid molecules in lipoprotein particles by calculating the number of lipids in each lipoprotein particle from measured biochemical data. However, in contrast to our model introduced here, all of these older models essentially obey the conventional view that all triglyceride and cholesterol ester molecules reside in the interior of the lipoprotein particles whereas all phospholipids and polar parts of proteins locate in the surface layer of lipoprotein particles as discussed in the following paragraphs.

Shen, Scanu and Kedzy assumed that all lipoprotein particles have an equal molecular arrangement of lipids and proteins, and the arrangement can be calculated based on simple linear equations[215]. In the model, all free cholesterol molecules were located in the surface layer since their partition between the core and the surface layer was then not known. As a consequence, the model revealed that triglycerides and cholesterol esters form alone a core of a particle having a 2.05-nm-thick surface layer. Moreover, phospholipid and free cholesterol molecules were tightly packed in the core-surface layer interface preventing the interpenetration of triglycerides and cholesterol esters to the surface. Furthermore, although free cholesterol molecules resided in the surface layer, they were not in direct contact with water but were located un-

der protein molecules.

Soulages and Brenner studied the structure of high density lipophorin (insect lipoprotein) particles[439]. Lipophorins do not contain cholesterol esters but hydrocarbon and diglyceride molecules in addition of triglycerides as core lipids. Interestingly, while all triglycerides and hydrocarbons were placed in the core of the particles in this model, diglycerides were assumed to locate both in the core and in the surface layer. The amount of diglycerides in the surface layer was also assumed to be proportional to the number of phospholipid molecules in the particle. The results confirmed that there actually exists diglycerides in the surface layer of lipophorin particles but their amount was very low.

Distinct from the other models, Schnitzer and Lichtenberg published a model of LDL where apoB-100 was assumed to form a single cap on the surface of an LDL particle, or partly within the surface layer of an LDL particle, without covering the phospholipid head groups[441]. Instead, triglyceride, cholesterol ester and free cholesterol molecules could also interpenetrate under the apoB-cap. When the number of triglycerides or cholesterol esters was increased in the model, while keeping the number of other molecules fixed, the interpenetration in the surface layer underneath the protein increased although an increase in the radius of the core was significantly faster.

The most recent geometric model was published by Teerlink et al.[440]. In addition to biochemical measurements, size measurements of LDL particles by high-performance gel-filtration chromatography were also performed. The main result of their work, the non-spherical shape of LDL particles at 25 °C, was based on differences in measured and calculated radii of LDL particles. However, data on the partitioning of lipids between the core and the surface layer suggested that different lipids contribute different dimensions of non-spherical LDL particles. The larger dimensions were mostly affected by free cholesterol molecules locating at the surface: if the content of free cholesterol is low, the radius is smaller due to interaction of fatty acid chains of cholesterol ester and phospholipid molecules whereas high free cholesterol content would prevent the interaction. In contrast, triglycerides and cholesterol esters contributed the most to the smaller dimensions.

To summarise, computational modelling based on lipid and protein compositions has been found to be a feasible way to achieve information on the molecular organisation of lipids and apolipoproteins within lipoprotein particles. The current knowledge of molecular structure of lipoprotein particles is advanced greatly by these calculations although they are characterised as more straightforward and computationally less demanding than the molecular dynamic simulations.

3.2 Materials and methods: A structural lipoprotein particle model

I will introduce here a new structural lipoprotein particle model which challenges the conventional view of molecular organisation of hydrophobic lipids, i.e., triglycerides and cholesterol esters, within lipoprotein particles. The modelling is performed separately for eleven lipoprotein subclasses, including VLDL1, VLDL2, IDL, LDL1, LDL2, LDL3, HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c}, covering the whole range of different lipoprotein particles. The purpose of the modelling is to find an averaged, molecular particle structure for each of the lipoprotein subclasses.

3.2.1. Lipoprotein isolation and compositional analysis

Data for the modelling were obtained from 12 individuals. Blood samples were drawn after an overnight fast for 12 h into EDTA-containing tubes and the blood plasma was separated by low-speed centrifugation. Fasting plasma triglyceride-rich lipoproteins were separated by density gradient ultracentrifugation (a Backman L8-70 ultracentrifuge, a SW40 TI swinging-bucket rotor). Three fractions were isolated containing large VLDL (VLDL1, Svedberg flotation units Sf = 60 – 400), small VLDL (VLDL2, Sf = 20 – 60) and IDL (Sf = 12 – 20). VLDL1, VLDL2 and IDL were isolated from 2 ml of plasma the density of which was increased by adding 0.341 g dry NaCl. The plasma was then overlaid with a density gradient consisting of NaBr solutions of decreasing densities (1 ml of d = 1.0988 g/ml, 1 ml of d = 1.0860 g/ml, 2 ml of d = 1.0790 g/ml, 2 ml of d = 1.0722 g/ml, 2 ml of d = 1.0641 g/ml and 2 ml of d = 1.0588 g/ml). The VLDL1 (1 ml) was harvested after ultracentrifugation for 1 hour and 21 minutes at 39 000 rpm at 23°C. Thereafter 1 ml of d = 1.0588 g/ml saline was added and VLDL2 (0.5 ml) was isolated after centrifugation for 16 hours and 22 minutes at 18 000 rpm and IDL (0.5 ml) after 2 hours and 18 minutes at 39 000 rpm at 23°C. The centrifuge was allowed to stop with the brake off.[424, 442]

LDL and HDL samples for density gradient ultracentrifugation were isolated by preparative ultracentrifugation (a Beckman Optima TL centrifuge, a Beckman TLA 100.3 rotor). For an LDL sample, tubes containing 2.0 ml of plasma were overlaid with 1.0 ml of NaBr solution of density 1.0190 g/ml and ultra-centrifuged at 100 000 rpm for 3 h at 20 °C. After ultracentrifugation a thin, fairly yellowish layer containing VLDL and IDL was removed from the top of the tubes by aspiration. The density of the infranatant containing LDL particles was increased to 1.0900 g/ml by adding 1.5 ml of NaBr solution d = 1.100 g/ml and saved for later use. To obtain a HDL sample, the apoB-con-

taining lipoprotein particles were first removed by preparative ultracentrifugation. For this, 2 ml of plasma in 0.3 ml NaBr solution d = 1.535 g/ml was overlayered with 0.7 ml NaBr solution d = 1.0600 g/ml and ultracentrifugated at 100 000 rpm for 3 h at 15 °C. Then, the supernatant layer containing VLDL, IDL and LDL was removed by aspiration and the infranatant was saved for later use.[443]

LDL and HDL subclasses were then isolated by density gradient ultracentrifugation (a Backman L8-70 ultracentrifuge, a SW40 TI swinging-bucket rotor). For LDL subclasses, the discontinuous NaBr solution gradient was prepared by layering from bottom to top: 0.5 ml d = 1.1900 g/ml; 2.5 ml sample (the infranatant of a volume of 1.0 ml from VLDL + IDL separation in 1.5 ml NaBr d = 1.100 g/ml); 1.5 ml d = 1.060 g/ml; 1.5 ml d = 1.055 g/ml; 1.5 ml d = 1.045 g/ml; 2.0 ml d = 1.034 g/ml; 2.0 ml d = 1.023 g/ml; and 1.0 ml d = 1.019 g/ml. After ultracentrifugation at 40 000 rpm for 23 h 45 min at 23°C, the tubes were discharged from the top using Beckman Recovery System and by infusing Maxidens solvent (Nyegaard & C. A/S, Oslo, Norway) to the tubes. The protein absorbance profiles in the tubes were monitored with an absorbance meter (Pharmacia, Uppsala, Sweden). The density gradient of the tubes was controlled with a DMA 46 density meter (Anton-Paar GmbH, Graz, Austria) placed before the fraction collector. Three LDL subclasses, LDL1 (d = 1.024 – 1.031 g/ml), LDL2 (d = 1.031 – 1.040 g/ml) and LDL3 (d = 1.040 – 1.054 g/ml), were collected in a volume of 2.1 ml each.[443, 444]

For HDL subclasses, the density of the HDL sample was increased by adding 1.0 g dry NaBr. A 2-ml volume of the sample was pipetted to a tube and the discontinuous gradient was prepared by layering NaBr solutions d = 1.250 g/ml and d = 1.220 g/ml, 1.5 ml and 6.7 ml above the sample, respectively, and 2.0 ml distilled water above the salt solutions. After ultracentrifugation at 40,000 rpm for 18 h at 15 °C, five 1.3-ml fractions corresponding to HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c} were collected.[443, 445]

The concentrations of lipids, including total cholesterol, free cholesterol, triglyceride and phospholipid concentrations, were then determined from each density range with Specific Chemistry Analyser (Kone, Finland) using enzymatic colorimetric methods (kits by Boehringer Diagnostica, Mannheim GmbH, FRG). The concentration of cholesterol esters were calculated as the difference between total cholesterol and free cholesterol concentrations. The total protein contents were measured by the modified Lowry method[197, 198].

3.2.2 Molecular composition and volume calculations

The numbers of lipid molecules in the lipoprotein particles were calculated based on the experimental data. Thus, the number of triglyceride, cholesterol

ester, phospholipid and free cholesterol molecules were obtained by the equation

$$N_l^{i,s} = \frac{c_l^{i,s}}{\rho_p^{i,s}} M_p^s \quad , \tag{3.1}$$

where $c_l^{i,s}$ is the concentration of the particular lipid (*l*) in an individual (*i*) in a lipoprotein subclass (*s*) in mol/l, $\rho_p^{i,s}$ is similarly the total protein (*p*) content in the sample in g/l, and M_p^s is the total molecular mass of the proteins in each particle.

The amount of proteins per an average lipoprotein particle was estimated based on recent literature[184, 323, 446] (see Tables 3.1 and 3.2) and fine-tuned to yield appropriate weight percentages and particle sizes. Some metabolic variations take place in the apolipoprotein contents, particularly in the amount of exchangeable apolipoprotein molecules[184, 447], within lipoprotein subclasses, e.g., in VLDL1[323]. Nevertheless, we noted that even substantial variations in the apolipoprotein contents within the lipoprotein subclasses did not interfere with the key trends in the cholesterol ester and triglyceride distributions within the particles (data not shown).

This equation derived by the author is capable of characterising lipoprotein particles individually and is valid for all lipoprotein subclasses. It is thus an extension of previous equations either using average weight composition data over subjects[215, 439, 441] or only dealing with LDL particles[174, 440]. The resulting numbers of lipids in lipoprotein particles are shown in Table 3.3.

Table 3.1 A description of protein composition within the apoB-containing lipoprotein subclasses.

Lipoprotein	apoA (%)	apoB (%)	apoC (%)	apoE (%)	Total protein / apoB
VLDL1	2	50	38	10	2.01
VLDL2	2	70	23	5	1.43
IDL	–	80	8	12	1.26
LDL1	–	90	10	–	1.11
LDL2	–	93	7	–	1.08
LDL3	–	97	3	–	1.03

Lipoproteins were isolated by density gradient ultracentrifugation. VLDL1, large VLDL particle, Svedberg flotation units Sf = 60 – 400; VLDL2, small VLDL particle, Sf = 20 – 60; IDL, Sf 12 – 20; LDL-1, LDL subclass in density range of 1.024 – 1.031 g/ml; LDL2, d = 1.031 – 1.040 g/ml; LDL3, d = 1.040 – 1.054 g/ml; apoA, apolipoprotein A; apoB, apolipoprotein B; apoC, apolipoprotein C; apoE, apolipoprotein E; Total protein / apoB, the ratio of total protein concentration to apoB concentration. The amounts of apoA, apoB, apoC and apoE molecules are expressed as percent of total protein concentration.

Table 3.2 A description of protein composition within HDL lipoprotein subclasses.

Lipoprotein	apoA-I (%)	apoA-II (%)	Others (%)	$M_{protein}$ (kDa)
HDL_{2b}	70	20	10	179
HDL_{2a}	70	20	10	174
HDL_{3a}	70	25	5	144
HDL_{3b}	70	25	5	135
HDL_{3c}	70	25	5	101

HDL particles were isolated by density gradient ultracentrifugation into five fractions corresponding to HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} *and* HDL_{3c}. *ApoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; Others, other apolipoproteins than apoA-I and apoA-II; Mprotein, total molecular mass of the proteins in Daltons. The amounts of apoA-I, apoA-II and other protein molecules are expressed as percent of total protein concentration.*

The volumes of the apolipoprotein molecules were estimated based on the protein content in each lipoprotein subclass; the number of amino acids in each apolipoprotein; and the volume of an average amino acid, 0.12286 nm^3 [448]. The estimates of lipids and proteins were then used to calculate particle volumes. The total volumes of the lipoprotein particles were obtained by summing the volumes of all lipid and apolipoprotein molecules. The volumes used for the lipid molecules were: 1.572 nm^3 for triglycerides; 1.138 nm^3 for cholesterol esters; 0.656 nm^3 for free cholesterol molecules and 1.284 nm^3 for phospholipids[449].

Table 3.3 The weight percentage composition and the numbers of lipid molecules in the lipoprotein particles isolated via ultracentrifugation.

Lipoprotein	W_{TG}	W_{CE}	W_{FC}	W_{PL}	W_{PROT}	N_{TG}	N_{CE}	N_{FC}	N_{PL}
VLDL1	67 ± 9	6 ± 9	4 ± 1	15 ± 2	8 ± 1	10585 ± 1492	501 ± 278	1561 ± 292	2491 ± 213
VLDL2	49 ± 3	10 ± 3	8 ± 2	20 ± 1	13 ± 1	34915 ± 225	950 ± 362	1294 ± 314	1558 ± 115
IDL	26 ± 6	22 ± 6	12 ± 3	23 ± 1	17 ± 1	1223 ± 288	1425 ± 454	1319 ± 369	1187 ± 117
LDL1	8 ± 3	33 ± 6	15 ± 4	23 ± 1	21 ± 3	278 ± 87	1519 ± 480	1123 ± 238	884 ± 137
LDL2	6 ± 1	35 ± 5	13 ± 3	23 ± 1	23 ± 3	177 ± 60	1454 ± 370	918 ± 211	783 ± 124
LDL3	10 ± 2	30 ± 6	13 ± 4	22 ± 1	25 ± 2	258 ± 61	1063 ± 300	748 ± 229	623 ± 78
HDL_{2b}	5 ± 3	20 ± 6	9 ± 4	31 ± 5	35 ± 9	33 ± 24	174 ± 105	126 ± 72	228 ± 115
HDL_{2a}	6 ± 5	17 ± 5	7 ± 2	30 ± 2	40 ± 3	33 ± 31	114 ± 36	84 ± 30	169 ± 24
HDL_{3a}	3 ± 2	16 ± 3	5 ± 1	28 ± 2	48 ± 2	11 ± 9	77 ± 16	41 ± 11	108 ± 10
HDL_{3b}	3 ± 1	15 ± 3	4 ± 1	26 ± 2	52 ± 3	8 ± 4	61 ± 13	26 ± 8	84 ± 9
HDL_{3c}	3 ± 2	14 ± 2	3 ± 1	22 ± 1	58 ± 3	6 ± 3	38 ± 7	15 ± 5	49 ± 5

The numbers of lipids were calculated by equation 3.1 and the weight percentages were calculated using the molecular masses of 859.2 for triglycerides, 647.9 for cholesterol esters, 386.7 for free cholesterol molecules and 786.0 phospholipids. The values are mean ± S.D. from 12 individuals; W, weight percentages; N, numbers of lipid molecules; TG, triglyceride; CE, cholesterol ester; FC, free cholesterol; PL, phospholipid; PROT, total protein. Plasma cholesterol and triglyceride concentrations (mean ± S.D.) were 4.76 ± 1.32 mmol/l (range 2.63 – 6.58 mmol/l) and 1.38 ± 0.59 mmol/l (range 0.68 – 3.02 mmol/l), respectively.

3.2.3 The geometric lipoprotein particle model

In the modelling process, we assume lipoproteins as spherical particles with a core and an approximately 2 nm thick surface layer. The volumes of the cores could then be calculated from the total volumes of the particles by using these assumptions. We made also some assumptions about the molecular organisation of lipids based on the current knowledge of lipoprotein particle structures[160, 210, 211, 214, 215, 232, 233, 242]. Firstly, we assumed that all phospholipids and proteins locate in the surface layer. Secondly, we constrained the portion of free cholesterol molecules in the surface to be directly proportional to the number of phospholipid molecules in the particles. The used molar surface free cholesterol to phospholipid ratios were 0.6 for VLDL[242], 0.55 for IDL and LDL[233], and 0.22 for HDL_2 and 0.16 for HDL_3 particles[232]. The rest of the free cholesterol molecules were assumed to be in the core. Thirdly, the rest of the space within the particles was reserved for the triglycerides and the cholesterol esters.

We can now write an equation of volumes of the cores for each of the 12 individuals and for all lipoprotein subclasses separately; one side of the equation was obtained via the total volumes of the particles and the other side depended on the number of triglyceride, cholesterol ester and free cholesterol molecules in the core. Thus the equation for the volumes of the cores is

$$\begin{pmatrix} N_{TG}^{1,s} v_{TG} & N_{CE}^{1,s} v_{CE} & N_{FC}^{1,s} v_{FC} \\ N_{TG}^{2,s} v_{TG} & N_{CE}^{2,s} v_{CE} & N_{FC}^{2,s} v_{FC} \\ \vdots & \vdots & \vdots \\ N_{TG}^{n,s} v_{TG} & N_{CE}^{n,s} v_{CE} & N_{FC}^{n,s} v_{FC} \end{pmatrix} \begin{pmatrix} \alpha_{TG}^{s} \\ \alpha_{CE}^{s} \\ \alpha_{FC}^{s} \end{pmatrix} = \begin{pmatrix} V_{core}^{1,s} \\ V_{core}^{2,s} \\ \vdots \\ V_{core}^{n,s} \end{pmatrix} . \qquad (3.2)$$

Here the numbers of triglyceride ($N_{TG}^{i,s}$), cholesterol ester ($N_{CE}^{i,s}$) and free cholesterol ($N_{FC}^{i,s}$) molecules, and volumes of the cores ($V_{core}^{i,s}$), were obtained from the twelve (*n=12*) individuals as described earlier. v_{TG} , v_{CE} and v_{FC} are the average molecular volumes of triglyceride, cholesterol ester and free cholesterol molecules, respectively. The alphas (α_{TG}^{s} , α_{CE}^{s} and α_{FC}^{s}) are the relative portions of triglyceride, cholesterol ester and free cholesterol molecules in the core from which the portions of

triglycerides and cholesterol esters were to be solved while the portion of free cholesterol molecules was fixed. The portions of triglycerides and cholesterol esters in the core were also restricted between zero and one to ensure a physically relevant solution. The portions of these lipids in the surface layer could then be obtained as one minus the corresponding alpha.

The alphas were solved by optimisation since we had two unknowns and twelve equations. The optimisation was performed by minimising an error function that was built based on the equation presented above. The error function also included penalty terms taking care of the restrictions. The estimates of distributions and errors were obtained by repeating the optimisation 1000 times using mainly the Monte Carlo methods[450] and, then, calculating the averages. Finally, leave-one-out cross validation[451] was performed to estimate the errors for α_{TG}^{s} , α_{CE}^{s} , i.e., the alphas were calculated 12 times in the case of each subclass for *n-1* individuals, leaving each individual out of the analysis once. The standard deviations of these α_{TG}^{s} and α_{CE}^{s} values were taken to represent their errors. This kind of assessment provides a simple statistical estimation of the expected variations in the alphas in the case of more data.

3.3 Results

The key results of the optimisation and their comparison to existing experimental data are shown in Fig. 3.1. Panel a) shows the percentages of triglycerides and cholesterol esters in the surface of lipoprotein particles. As seen from the figure, HDL particles and apoB-100-containing particles form two separate groups: HDL particles have high percentages of triglycerides and cholesterol esters in their surface while VLDL, IDL and LDL particles have low percentages. The values for the triglyceride and cholesterol ester molecules are similar within both groups.

Panel b) of Fig. 3.1 shows the percentage of triglyceride and cholesterol ester molecules of all surface lipids in lipoprotein particles. In here, within both groups, some co-operative behaviour can be seen: In apoB-100-containing particles, the percentage of triglycerides increases and that of cholesterol esters decreases as their radius increases. On the other hand, in HDL particles, the increase in radius coincides with a decrease in the percentage of cholesterol esters but does not particularly affect the percentage of triglycerides.

In all cases, the surface percentages of triglyceride and cholesterol ester molecules can be explained by a combination of the total amount of the lipid and its percentage in the surface layer. For example, although the percentage of triglyceride in the surface layer of HDL particles is high (panel a), the percentage of triglycerides of all surface lipids is low (panel b) since the total amount of triglycerides in HDL particles is low. Correspondingly, since the total amount of triglycerides in VLDL1 particles is extremely high, the percentage of triglycerides of all surface lipids is high (panel b) although the percentage of triglycerides in the surface layer is low (panel a).

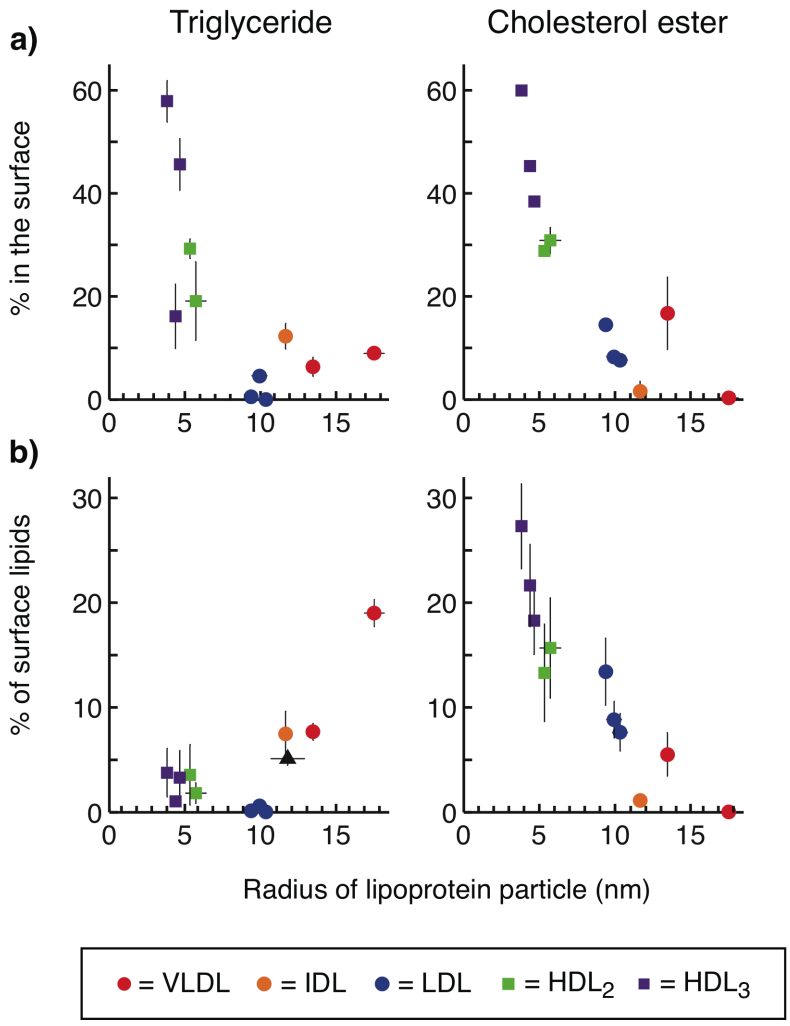

Figure 3.1 Distribution of triglyceride and cholesterol ester molecules in lipoprotein particles. Proportion of triglycerides and cholesterol esters in the surface of lipoprotein particles a) and their percentages of all surface

lipids b). All values are mean ± S.D. (non-visible S.D.-bars are within the bullets). The black triangle represents the sole experimental value for native VLDL particles isolated from hen[243]. *Modified from* [424].

3.4 Discussion

I have introduced here a structural lipoprotein model which was built based on both experimental lipid and protein concentrations in the plasma and the current knowledge of lipoprotein particle structures. The structural model is general as it is built separately for eleven lipoproteins subclasses and provides largely consistent results over the subclasses. The model indicates, in contrast to the conventional lipoprotein model, that particle size-dependent proportion of hydrophobic lipids, namely triglyceride and cholesterol ester molecules, may locate in the surface of lipoprotein particles.

The idea of triglycerides and cholesterol esters locating at the surface of lipoprotein particles might at first appear strange due to their hydrophobic nature. It should, however, be remembered that free cholesterol molecules and hydrated forms of triglycerides, diglycerides, which are only slightly polar, have been detected at the surface of lipoprotein particles[232, 233, 452]. Experimental data on the location of the hydrophobic triglycerides and cholesterol esters in the surface of lipoprotein-like particles is, however, exceptionally scarce. A recent ^{13}C NMR spectroscopy study of hen VLDL particles revealed that the amount of triglycerides of the surface lipids was 5.1 ± 0.6 mol%[243]. This value, indicated as a black triangle in Fig. 3.1 panel b, is in excellent agreement with our computed values for native human lipoproteins of similar particle size.

The structure of native human lipoprotein particles has also been widely studied by ^{13}C NMR[232, 233, 428, 429]. However, since ^{13}C NMR is incapable of operating in natural abundance of ^{13}C, the visibility of a molecule depends on its ^{13}C enrichment. Therefore, in the light of the hen lipoprotein ^{13}C NMR study mentioned above, it is possible that the surface triglycerides and cholesterol esters in human lipoprotein particles have not yet been observed due to their insufficient ^{13}C enrichment under the experimental conditions used.

There is some experimental evidence that a small portion of triglycerides and cholesterol esters locate in the surface of vesicles and emulsion particles[234-243]. The vesicle systems are not, however, ideal models for lipoprotein particles since they have a phospholipid bilayer, which is incapable of solubilising large amounts of hydrophobic lipids, and an interior aqueous compartment not present in lipoproteins (see Fig. 3.2). Furthermore, the emulsion particles have been large, 20-100 nm in diameter, and thus resembled only LDL up to VLDL particles and not HDL. The surface portions of

triglycerides and cholesterol esters from these model systems are, however, in good accordance with our optimised values. Both triglycerides and cholesterol esters mix in similar amounts into phospholipid bilayers with as high values as 10 mol% of all the surface lipids being measured for triglycerides[236, 239]. The percentages of triglyceride and cholesterol esters of all surface lipids in emulsion particles were similar as in bilayers although the results seemed to depend significantly on the actual lipid model system leading to over 10-fold differences in the observed amount of triglycerides of all surface lipids in similarly sized emulsion particles with different types of triglyceride molecules[241].

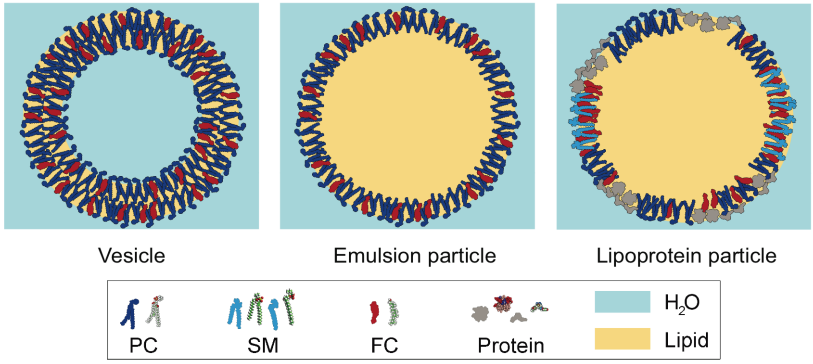

Figure 3.2 Schematic cross-sectional illustration of the overall structural characteristics of vesicles as well as emulsion and lipoprotein particles. The vesicles are biophysically distinct from the emulsion and lipoprotein particles. While the core of an emulsion and lipoprotein particle is filled with lipids, the lipid soluble area in a vesicle is limited to the surrounding bilayer. PC, phosphatidylcholine; SM, sphingomyelin; FC, free cholesterol. Modified from [424].

As the previous lipoprotein model systems have been unable to model HDL particles, we concentrated on finding studies dealing with reconstituted HDL particles to get some references for our model. Reconstituted HDL particles are frequently used to study HDL particles and they are prepared as small, micellar complexes of exchangeable apolipoproteins with lipids that mimic HDL in shape, composition and functional properties[453-456]. The one important property of reconstituted HDL particles is, among many, that they can be prepared to contain only one or few defined lipid components.

Rye et al. have investigated the role of different phospholipid molecules affecting the CETP-mediated remodelling of HDL particles[455]. Reconstituted HDL particles were prepared with one type of phosphatidylcholine, free cholesterol, cholesterol ester and apoA-I molecules, and the sizes of the particles were measured by non-denaturing gradient gel electrophoresis. By using the stoichiometry and the sizes of the reconstituted, unmodified HDL particles, we could estimate the percentages of cholesterol esters of all surface lipids

directly without optimisation as there were no triglycerides involved. The percentages increased from 2 up to 31 % the value being systematically larger for smaller particles. These values and the trend are comparable to the corresponding results for native HDL particles of similar size in our study (Fig. 3.1, panel b).

While the results of the present study on surface triglycerides and cholesterol esters are in line with the experimental outcomes from various lipoprotein model systems, the results of this study were obtained by optimisation purely based on geometric assumptions. The fact that lipid-lipid and lipid-protein interactions were not considered in the model means that the similar percentages of triglycerides and cholesterol esters in panel a of Fig. 3.1, cannot be thought of as co-operative behaviour between these lipids but rather indicate the molecular dimensions and absolute numbers of triglycerides and cholesterol esters in the lipoprotein particles.

The portions of triglycerides and cholesterol esters in the surface of lipoprotein particles might have been somewhat different if the molecular interactions would have been explicitly considered in the model. At present, the detailed influence of these molecular interactions on the optimised triglyceride and cholesterol ester distributions within the lipoprotein particles cannot be addressed. However, it is anticipated that a high triglyceride content in the core of the particles will slightly increase the proportion of cholesterol ester and free cholesterol molecules in the surface. Similarly, a high cholesterol ester content in the core would attract the alike, i.e., cholesterol ester and free cholesterol molecules, and thus slightly reduce the proportion of cholesterol ester and free cholesterol molecules and increase the proportion of triglycerides in the lipoprotein particle surface.

Fig. 3.3 illustrates the molecular structures of VLDL2, IDL, LDL2, HDL_{2b}, and HDL_{3c} particles drawn based on the existing knowledge of lipoprotein particle structures[208, 210, 211, 214, 215, 232, 233, 236, 239, 241, 243, 425, 435, 439-441, 449, 452, 455, 457, 458] and the estimates of this study, the distribution of triglycerides and cholesterol esters between the core and the surface region. As shown in Fig. 3.3, triglycerides and cholesterol esters likely penetrate into the particle surface via loosely packed phosphatidylcholine-rich, free cholesterol-poor environments (a-e)[210]. In contrast, the nanoenvironments consisting of mainly sphingomyelin and free cholesterol molecules are tightly packed and impede the penetration of triglycerides and cholesterol esters into the surface (f)[210].

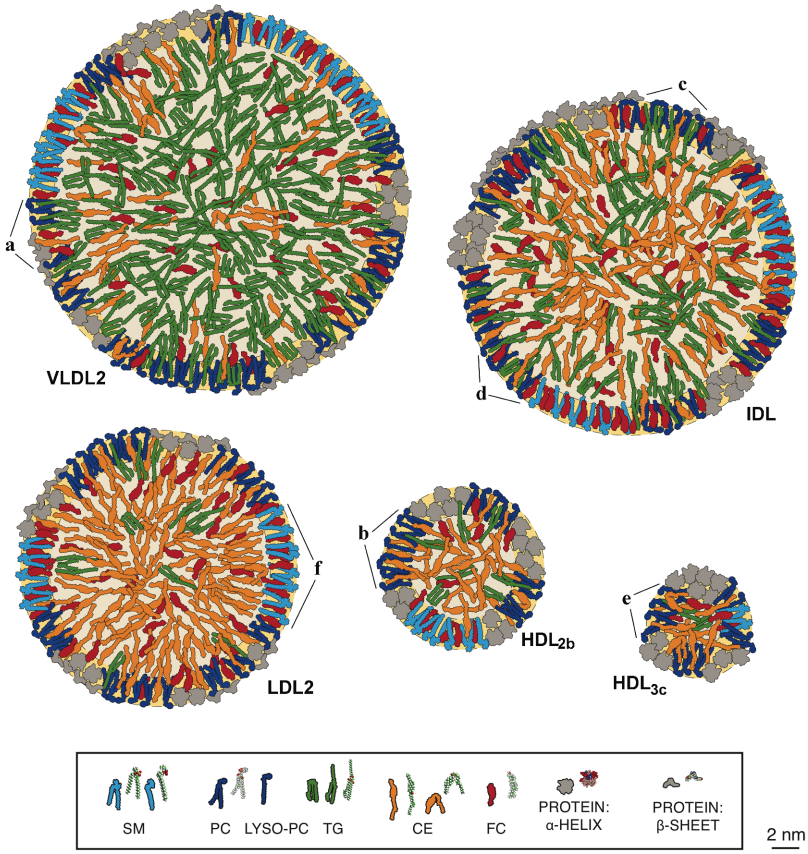

Figure 3.3 Schematic cross-sectional molecular models of VLDL2, IDL, LDL2, HDL_{2b} and HDL_{3c} particles with the optimised distribution of triglycerides and cholesterol esters between the core and the surface layer. The molecular compositions are from 12 individuals with these particles isolated using ultracentrifugation. The penetration of triglycerides and cholesterol esters into the particle surface occurs mainly via loosely packed phosphatidylcholine-rich, free cholesterol-poor domains[210] (a-e). Note also the tendency of cholesterol rings to interact (a); CE penetration (b,c,e) and triglyceride penetration with fatty acids chains ahead (a-e); a kinked triglyceride (d) and a kinked cholesterol ester molecule in the surface (e); the nanodomains consisting of mainly sphingomyelin and free cholesterol molecules are tightly packed and impede the penetration of triglycerides and cholesterol esters into the surface layer[210] (f). Modified from [424].

The presented distributions of triglycerides and cholesterol esters in the particles provide a coherent structural rationale for various molecular processes during lipoprotein metabolism. For example, the portion of triglycerides of the surface lipids decreases from VLDL to LDL particles concurrently with the action of lipoprotein and hepatic lipase closely associated with the metabolic conversion of triglyceride-rich VLDL to LDL particles. These

catalytic operations occur at the particle surfaces[459, 460] with no unambiguous evidence of enzyme intrusion into the core of lipoprotein particles[461]. These findings and the occurrence of the lipid transfer at the phospholipid-protein interface are also in line with findings for oxysterol binding protein-related proteins, ORPs[462-464] acting as sterol transporters between the subcellular membranes.

The recent structural information on cholesteryl ester transfer protein[465] bares also an important functional link to our current findings on the high-cholesterol ester and low-triglyceride proportion in the surface of HDL particles and an opposite situation, i.e., low-cholesterol ester and high-triglyceride proportion in the surface of VLDL and IDL particles (Fig. 3.1, panel b). As mentioned earlier, one of the key physiological functions of CETP is the equimolar heteroexchange of cholesterol esters from HDL particles for triglycerides from apoB-100-containing lipoprotein particles[392, 465, 466]. The recent crystal structure of CETP indicates that the transport site for the hydrophobic lipids is a tunnel of a limited capacity to discriminate between different neutral lipids; i.e., CETP has similar binding affinities for cholesterol ester and triglyceride molecules[465]. Thus, the known heteroexchange of cholesterol esters and triglycerides between HDL particles and apoB-100-containing lipoprotein particles is rational only if the acceptor and donor surfaces have different molar proportions of cholesterol esters and triglycerides. The presented structural model, using simultaneous volumetric optimisation of cholesterol ester and triglyceride distributions within lipoproteins, clearly reveals such a situation for compositionally characterised human lipoprotein particles.[424]

4. *In silico* phenotyping of lipoprotein particles

The previous chapter dealt with the computational modelling of lipoprotein particle structures and introduced a method to estimate the composition of various lipoprotein subclass particles individually. In this chapter, I will use these composition variables together with the commonly measured lipid concentrations to study the compositional/metabolic properties of lipoprotein particles. I will also discuss the heterogeneity of lipoprotein particles and the limitations in the use of lipid concentrations alone to reveal the underlying lipoprotein particle heterogeneity. The research discussed in this chapter has been published in a refereed journal, see[467].

4.1 Lipoprotein profile in describing lipoprotein metabolism

Lipoprotein metabolism plays a key role both in health and disease. The state of lipoprotein metabolism is usually examined in clinics by determining a few lipid measures from plasma of over-night fasted individuals[131, 134, 468, 469]. This lipoprotein profile, including the concentration of plasma triglycerides, plasma total cholesterol, LDL cholesterol, and HDL cholesterol, is then used among other non-lipid variables to describe the individual's overall metabolic status and potential risk for atherosclerosis and vascular disease. Lipoprotein metabolism appears, however, significantly more complex than indicated by these simple lipid measures. Evidence is accumulating that these commonly used measures of lipoprotein metabolism cannot, at least alone, properly account for the complex interactions prevailing in lipoprotein (patho)physiology[23, 470-473].

In clinics, the components of lipoprotein profile, particularly LDL cholesterol and HDL cholesterol, are measured instead of the concentration of lipoprotein particles, i.e., concentration of LDL and HDL particles, respectively. The reason is purely analytical; measuring the amounts of cholesterol or triglyceride in plasma or particular classes of lipoproteins (LDL, HDL) is straight-

forward, whereas direct assessment of lipoprotein particle number is not[205, 206]. This distinction between lipoprotein lipid levels and lipoprotein particle numbers is potentially important clinically because the two measures are not equivalent due to the heterogeneity of the lipoprotein particles[117, 205, 206]. For example, since LDL cholesterol level is a sum of cholesterol molecules over all LDL particles in a plasma sample, the LDL cholesterol concentration may be the same within two randomly selected individuals although they may differ greatly in their LDL particle numbers. The reason for the discrepancy is particle heterogeneity among individuals. It may arise either from a different size distribution of the particles affecting the total lipid content per particle and/or the relative cholesterol content of a particle (see Fig. 4.1).

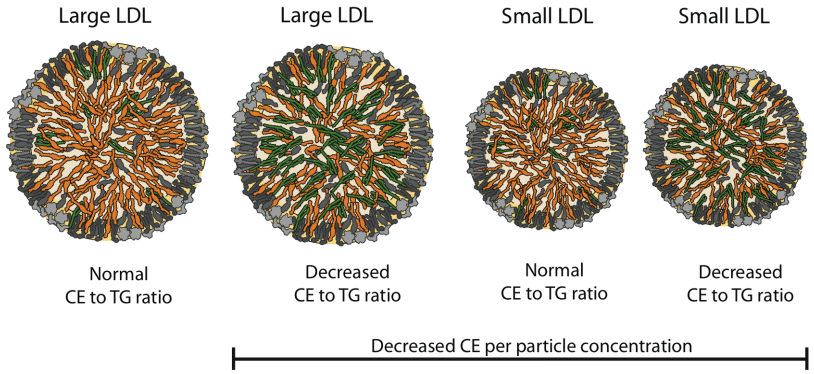

Figure 4.1 Schematic representation of the heterogeneity of LDL particle composition. The per particle cholesterol content, among the LDL particle concentration, affect substantially the LDL cholesterol concentration in plasma.

The problem of particle heterogeneity has been attempted to overcome by physical isolation of lipoprotein particles into more homogeneous lipoprotein subclasses by sequential ultracentrifugation (UCF). These procedures have proved useful in appreciating various counteracting metabolic phenomena and also in assessment of the risk for various vascular outcomes[474-476]. UCF-based isolations are, however, tedious and expensive thus restricting their use for the analysis of lipoprotein subpopulations. Detailed attention is also rarely paid on the molecular composition of the isolated particles although the structural integrity and pertinent molecular composition of lipoprotein particles are the basis for the proper functioning of lipoprotein metabolism[210, 424].

Here, we focus on an extensive set of UCF-based lipoprotein data where the apoB-containing particles are isolated to VLDL, IDL and LDL fractions and the HDL particles to HDL_2 and HDL_3 fractions[477-479]. This data set provides an experimental extreme available for clinically oriented lipoprotein studies and

can therefore be considered optimal for computational, or *in silico*, fractionation of lipoprotein data. The subclassification of physically isolated particles is performed by the use of a self-organising map (SOM) method that enables a holistic combination of various lipid and lipoprotein estimates including lipoprotein compositions, concentrations and metabolic interactions. In the analysis, we used in-house, free access software termed MeliKerion which is essentially an enhanced self-organising map method[480, 481].

4.2 Properties of the self-organising map method

The self-organising map method is a well known, unsupervised pattern recognition technique[482]. It has been developed as one of the most popular neural network methods in various disciplines[483] after the initial introduction of the SOM method for data clustering and visualisation in early 1980's[482]. The most characteristic feature of the self-organising map method is the ability to map non-linear relations in multidimensional data sets into visually more approachable, typically two dimensional planes of nodes.

4.2.1 Clustering

SOM method clusters data, termed as input data, based on all parameters of all cases according to given similarity criteria. The input data from each case *i,* i.e., plasma sample from an individual *i* in this particular application, contain a number of parameters used to form a vector $d^i = \left(d^i_1, d^i_2, \ldots, d^i_{N-1}, d^i_N \right)$ (see Fig. 4.2). The SOM algorithm[482] then transforms the input data vectors into various feature vectors (vector *j,k* $x^{j,k} = \left(x^{j,k}_1, x^{j,k}_2, \ldots, x^{j,k}_{N-1}, x^{j,k}_N \right)$) of the same size and sophisticated, averaged parameter values. Thus each feature vector represents the original *N* dimensional parameter space and is similar, although not necessarily exactly the same with a small group of original parameters. In the final state of clustering, each case *i* is attached to a feature vector with the most similar parameters, and thus similar cases end up in the same or similar feature vectors[484].

4.2.2 Visualisation

The strength of SOM analysis compared to several other clustering methods lies in its visualisation capabilities[480, 483-485]. In visualisation, each feature vector represents a node or cell (in place *j,k)* in a two-dimensional map with total

of J rows and K columns (j goes over the rows and k over the columns). The end product of visualisation is a group of two-dimensional maps called component planes representing each of the N components of the feature vectors and thus giving a general overview of the distribution and values of the original parameters. In-house developed scripts also allow visualisation of other variables (outputs) of the cases solely based on the organisation of the input variables[480, 481]. As the maps are formed based on the feature vectors in fixed places, each case i is located in the same place in each component plane enabling direct comparison of the planes.

Each of the component planes is coloured so that the colour of a node is related to parameter-specific median over cases giving reddish, white or bluish colour corresponding to high, near median or low values, respectively. The in-house scripts also enable saturation differences between the planes. The saturation of the colours is related to the significance of the clustering giving high saturation in case of high significance. The significance is also given by numeric values corresponding to the probability of the clustering to occur by chance. The use of colour coding in the component planes is particularly helpful since clearly coloured areas as well as correlated changes in the colours of different parameters are visually easy to detect. While it is difficult to exactly define clusters in the organised map, subtle changes in colours are also good in indicating potentially diffuse borderline areas between various clusters.

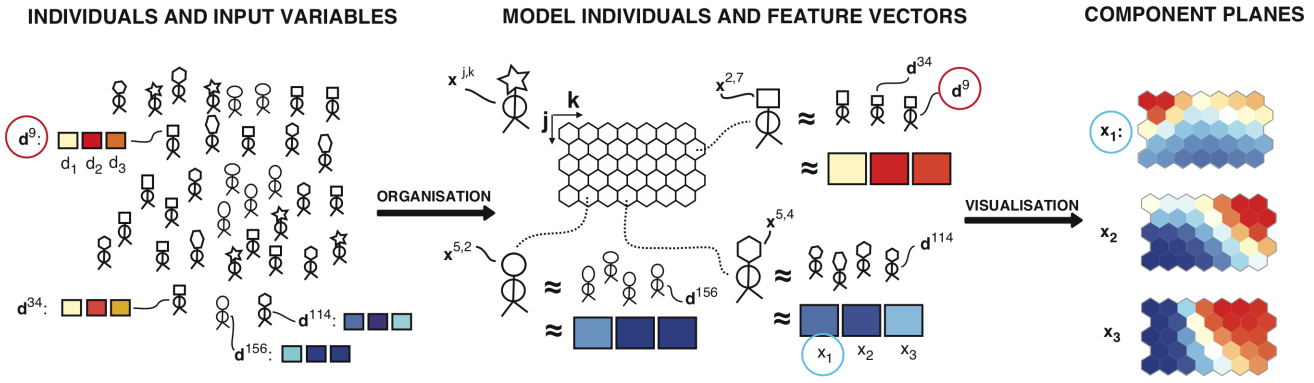

Figure 4.2 A methodological overview and illustration of the overall concept of self-organised map analysis. An individual (d^i) is described by N (here N=3) lipoprotein lipid variables (d_1,d_2,d_3) as illustrated by series of coloured rectangles for four different individuals on the left. Based on the individual inputs, the SOM algorithm results in j·k model individuals $x^{j,k}$, each with a definite location j,k in the map. For example, model individual $x^{2,7}$ in location j=2, k=7 represents a group of similar individuals including individual d9. Each of the model individual is described by a feature vector which is formed through the averaging process of the SOM algorithm. Each model individual, or feature vector, have the same amount of descriptive variables as the real individuals as illustrated by the large, coloured rectangles in the middle. The feature vectors of the neighbouring nodes in the two dimensional map are similar to each other and thereby, importantly, the individuals ending up close by in the map are also similar in the original N-dimensional space as illustrated by the shapes of the heads. The distribution of the N variables in the feature vectors is visualised by N component planes as shown in the right. Thus each of the component planes in the right illustrates also a variable used as an input for the SOM analysis. In each of the component planes, the colour of the node comes from the relative value of the corresponding component in the feature vector locating in that particular node. For example, the first component (x_1) in the feature vector $x^{5,4}$ gives the colour to the node j=5, k=4 in the upper component plane. Bluish colours refer to values lower, and reddish colours to higher, than the median value of the variable.

4.2.3 Self-organising maps applied for lipoprotein lipid data

Lipoprotein lipid data is a manifestation of a complex metabolic situation. This indicates that the data likely contain multi-parametric and non-linear relationships demanding advanced properties from the methodologies used for the analysis. The self-organising map method has been recognised as an effective and advantageous tool to handle complex data in various areas (see, for example,[481, 484-487]) and thus the applications of SOM methodology have potential to produce logical and easily interpretable results also in the context of lipoprotein lipid data.

In this particular application, the SOM analysis is able to interpret both compositional characteristics within each lipoprotein particle category (e.g., IDL) and metabolic associations between different lipoprotein categories (e.g., VLDL and HDL_2). Interestingly, the analysis also provided a kind of *in silico* subfractionation allowing to characterise various metabolic and structural details within each of the experimentally distinguished lipoprotein particle categories.

The SOM algorithm thus offers the possibility to generate a form of average representations of model individuals and identify both compositional and metabolic similarity of individuals out of multidimensional complexity of various interrelated lipoprotein lipid parameters. Comparing the component planes of two or more parameters in the two-dimensional map may provide insights into the interdependencies between the variables and their potential similarities or dissimilarities for the various clusters of model individuals.

4.3 Materials and methods: An application of SOM analysis

In our study we had at our disposal biochemical lipoprotein lipid analyses from 302 distinct plasma samples of 233 individuals. The lipid data consisted of heavy alcohol drinkers (122 samples, 40 % of the samples), hysterectomised postmenopausal women on estrogen replacement therapy (117 samples, 39 %) and apparently healthy controls (63 samples, 21 %), thereby representing a wide range of plasma lipoprotein lipid values (see Table 4.1). The numbers of samples from women and men were almost equal, 160 (53 %) and 142 (47 %), respectively. Blood samples were drawn after an overnight fast of 12 h into EDTA-containing tubes. The blood plasma was then separated by centrifugation at 1200 ^{x} g – 1500 ^{x} g for 10-15 min at 4°C. VLDL (d < 1.006 g/ml), IDL (d = 1.019-1.063 g/ml), LDL (d = 1.063-1.125 g/ml), HDL_2 (d = 1.063-1.125 g/ml) and HDL_3 (d = 1.125-1.210 g/ml) fractions were isolated from

each individual using sequential ultracentrifugation[477-479]. Lipoprotein fractions were isolated from fresh plasma samples. The concentrations of total cholesterol, free cholesterol, triglyceride, and phospholipid molecules were then determined from each density range using enzymatic colorimetric methods[477, 478]. Concentrations of cholesterol esters were calculated as difference between total cholesterol and free cholesterol concentrations. The total protein contents were measured by the modified Lowry method[197, 198]. The lipid and protein analyses commenced immediately after isolation of each fraction. The concentration variables were expressed as mmol/l in plasma for lipids and mg/dl for proteins, respectively (see Table 4.1).

*Table 4.1 The average plasma lipid and protein concentrations and weight percentage compositions (marked as *) of the lipoprotein particles isolated via sequential ultracentrifugation.*

Lipoprotein	TG	C	FC	PL	Prot.	TG*	FC*	CE*	PL*	Prot.*
VLDL	1.01 ± 1.58	0.50 ± 0.61	0.22 ± 0.32	0.32 ± 0.43	19.7 ± 20.6	53 ± 6	5 ± 2	12 ± 5	16 ± 4	14 ± 4
IDL	0.11 ± 0.07	0.25 ± 0.23	0.06 ± 0.07	0.09 ± 0.08	6.5 ± 5.0	27 ± 11	6 ± 3	31 ± 10	18 ± 6	18 ± 6
LDL	0.31 ± 0.14	3.39 ± 1.20	0.85 ± 0.33	1.06 ± 0.42	89.0 ± 32.3	7 ± 3	8 ± 2	41 ± 6	21 ± 4	23 ± 4
HDL_2	0.17 ± 0.09	1.25 ± 0.60	0.23 ± 0.16	1.00 ± 0.50	114.0 ± 44.3	6 ± 3	3 ± 1	23 ± 4	27 ± 5	41 ± 6
HDL_3	0.12 ± 0.08	0.75 ± 0.26	0.08 ± 0.06	0.65 ± 0.19	137.0 ± 45.5	4 ± 3	1 ± 1	18 ± 4	21 ± 4	56 ± 6

Values are in mmol/l for TG, C, FC and PL concentrations and mg/dl for protein concentration. Values are given as mean ± SD. TG, triglyceride; C, total cholesterol; CE, cholesterol ester; FC, free cholesterol; PL, phospholipid; PROT, total protein.

The lipoprotein lipid concentrations and protein measures were then used to build a set of input variables for the SOM analysis. We ended up in using two kinds of lipoprotein lipid measures, termed as *concentration inputs* and *composition inputs*, as both concentrations of lipids and the chemical composition of lipoprotein particles are important determinants of lipoprotein particle metabolism. The concentration inputs correspond to the concentrations of triglyceride, cholesterol ester, free cholesterol and phospholipid molecules over the particles in each physically isolated lipoprotein fraction as such while the composition inputs approximate the molecular composition of the lipoprotein particles i.e., the number of lipid molecules (~mol/g) in each averaged lipoprotein particle. The molecular composition is obtained by scaling the concentrations of lipids by the corresponding total protein measure individually in each fraction (see also equation 3.1 on page 62).

The two input variable types were deliberately used together in the SOM analysis to enable direct association of the lipid concentration and composition information. The composition inputs are not, however, intuitive variables to interpret, and therefore I will present the compositional variability between individuals by mass percentages which shared the essential distributions of the composition variables. Also other output variables, such as distri-

butions of heavy alcohol drinkers, estrogen treated women, control subjects, men, body mass index, age as well as total cholesterol, total protein and lipoprotein particle sizes in each fraction are presented as component planes. The particle sizes were estimated as described in the previous chapter. Briefly, the number of lipid molecules in a particular lipoprotein particle was calculated on the basis of the experimental data, and the known average volumes of the lipid and protein molecules were then used to calculate the average particle size.

Before the SOM analysis, some pre-processing was performed: The input data were scaled between -1 and 1 by rank transformation to prevent unjustified domination of any of the variables and then normalised to smooth the distribution of individuals into the grid. We chose a 5 x 7 map of hexagonal units resulting in 8.6 samples per unit on average and a Gaussian neighbourhood function. We also performed several runs with different map sizes leading to essentially similar results, as expected, since SOM is known to be rather insensitive to choices of its size[484]. After the positions of the individuals were computed, the component planes were coloured according to the feature vectors. A few numbers were localised on selected cells to show the local prevalence (binary variables) or mean value (continuous variables) for that particular region, and the statistical significance of the patterns was obtained by overall permutation estimations for the p-values[480, 481]. The p-value estimates were interpreted for the output variables while they are omitted for the input variables since they are no longer valid. All the analyses were performed using the in-house scripts in the MATLAB programming environment. The MeliKerion[480, 481], for SOM analyses in the MATLAB/Octave programming environment, is freely available via http://www.computationalmedicine.fi/software. After constituting the SOM, the main regions with differing metabolic features were chosen by visual examination for further analyses. Some individuals residing in the borderline areas were excluded to obtain clearer lipoprotein phenotypes. All the analyses were performed on a laptop PC with an Intel Core2 Duo, 2.0 GHz processor which trained a typical SOM and calculated the colourings in a few minutes.

4.4 Results and discussion

4.4.1 Distribution of individuals

The data contained lipid and protein measures from three distinct groups of individuals including heavy alcohol drinkers, hysterectomised postmenopausal women on estrogen replacement therapy and apparently healthy con-

trols. The numbers of men and women were almost equal. Fig. 4.3 represents the distribution of these individuals in the self-organising map constructed based on the combined concentration and composition of lipoprotein lipid variables.

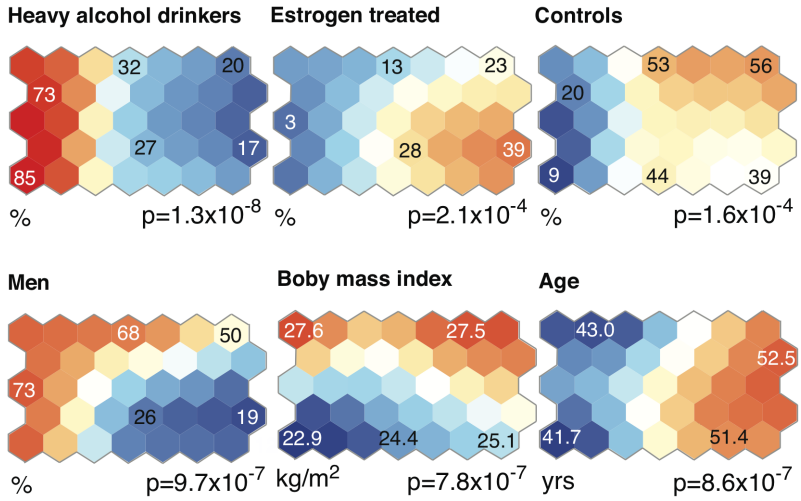

Figure 4.3 Distribution of the individuals in the SOM analysis based on the combined lipoprotein lipid concentration and composition variables. The %-values shown in the component planes referring to the groups are percentages of the total study population in that particular cell. In all of the component planes, the values are colour-coded to visualise whether they are above (reddish), at (white) or below (bluish) the median of the variable. The numbers on the selected units tell the local mean value for that particular region.

As evident from Fig. 4.3, the separate groups lie differently on the map; Heavy alcohol drinkers locate mostly in the western region of the map whereas estrogen treated women occupy the south-east region. Controls are mostly located in the east. The locations of men and women represent gender of the individuals in the three groups rather than distribution of men and women in general; most men lie in the north, west and north-west areas of the SOM since most heavy alcohol drinkers and some controls were men. The most notable difference in the distributions is that while controls and estrogen treated individuals occupy the same area, the heavy alcohol drinkers separate quite clearly from these two groups. Fig. 4.3 also shows distribution of body mass index (BMI) and age. The estrogen treated women seem to be the oldest and the heavy alcohol drinkers the youngest group of individuals in this study. Individuals with the highest BMI belong mostly to either controls or heavy alcohol drinkers. Individuals with the lowest BMI are also heavy alcohol drinkers.

4.4.2 Component planes of lipids, proteins and particle sizes

Fig. 4.4 illustrates the component planes of concentration input variables for triglyceride, cholesterol ester, free cholesterol and phospholipid molecules in VLDL, IDL, LDL, HDL_2 and HDL_3 particles. The figure contains also component planes for various output variables such as total cholesterol and total protein concentrations in each of the five fractions. Fig. 4.5 shows particle compositions as weight per cent for triglyceride, cholesterol ester, free cholesterol, phospholipid and protein molecules in VLDL, IDL, LDL, HDL_2 and HDL_3 particles as well as the estimated sizes of the lipoprotein particles in each fraction. These two figures are discussed together in the following to yield an appropriate view of lipoprotein particles from both lipoprotein lipid concentration and composition view. The two figures can also be interpreted in two dimensions, horizontally yielding compositional information within a fraction and vertically relating to a metabolic view within a lipid in several fractions. For convenience, the word *plasma* is often used to help in separating concentration variables from composition variables in the following text. For example, plasma LDL cholesterol refers to the commonly determined LDL cholesterol, i.e., the concentration of cholesterol molecules over all LDL particles. The concentration inputs are also referred from now on without a asterisk such as VLDL-TG (plasma concentration of triglycerides in VLDL particles) and the composition variables are referred with the asterisk such as VLDL-TG* (weight per cent of triglycerides in a VLDL particle).

In Fig. 4.4, the component planes of plasma concentration variables are horizontally, or within a fraction, quite similar as judged by the similar colouring of the component planes. This is particularly seen in the case of largest particles but the effect decreases towards smaller particles and is less pronounced for triglycerides in HDL particles. This parallel relatively high (reddish colour) or low (bluish colour) level of lipids within a fraction is actually quite surprising and may be due to several reasons. Firstly, if each lipoprotein particle contains some triglyceride, cholesterol ester, free cholesterol and phospholipid molecules, then a high number of particles results in high or elevated plasma concentrations of all these lipids. Secondly, if the amount of a lipid within a lipoprotein particle is high, the plasma concentration of the lipid is also high even for a normal particle concentration. In this case, the coexisting high level of all lipids may result from some particle secretion- or plasma metabolism-related phenomenon. The third possibility is that both the number of lipids within a particle and the concentration of particles are high within a fraction. Fig. 4.4 shows also concentrations of proteins within the fractions giving similar distributions as the plasma concentrations of lipids. The concentrations of proteins are particularly interesting since they es-

timate the concentrations of lipoprotein particles if the amount of proteins per particle is assumed to be conserved over individuals in each lipoprotein fraction.

Vertically in Fig. 4.4., component planes are, in general, less similar. For example, individuals with the highest concentration of VLDL lipids lie in the north-west corner and those with high LDL lipid concentrations in the north-east and eastern areas of the component planes. Furthermore, individuals with high HDL_2 lipids (reddish region in the south) may have either low LDL lipid concentrations (bluish region in south-west) or high LDL lipid concentrations (reddish region in the south-east). These associations are pronounced for phospholipid, free cholesterol, cholesterol ester and total cholesterol molecules but are somewhat different between LDL-TG and HDL_2-TG. The reason why the component planes of each lipid through the fractions are not similar is that lipoprotein particles face events in plasma which are quite fraction-specific and affect strongly both their particle composition and concentration.

This view is supported by few fractions which actually have mutual similarities also in the vertical direction: The distributions of plasma concentrations of lipids within IDL and LDL fractions seem quite similar likely because of similar metabolic routes i.e., they are both delipidation products of VLDL and compete for the same cell surface receptors. Likewise, the distribution of VLDL and HDL_2 lipid concentrations associate with each other although the relation is negative rather than positive. This agrees with the metabolism of VLDL and HDL particles since during the delipidation process of VLDL particles, extra surface material, such as cholesterol, phospholipid and protein molecules, is shedding from their surfaces and transported to HDL particles[488] thereby increasing both the size and the number of HDL_2 particles[489].

In contrast to the opposite colouring of VLDL and HDL_2 lipids in general, individuals with high VLDL triglycerides also have high HDL_2 and HDL_3 triglyceride concentrations. Since this association is apparent only for triglycerides rather than all VLDL and HDL lipid concentrations, the reason for the relation is likely the change in particle compositions rather than in particle concentrations. The association between VLDL and HDL likely occurs via action of CETP which transfers triglycerides from VLDL to HDL in exchange for cholesterol esters. This increases the concentration of HDL triglycerides which would otherwise be almost negligible.

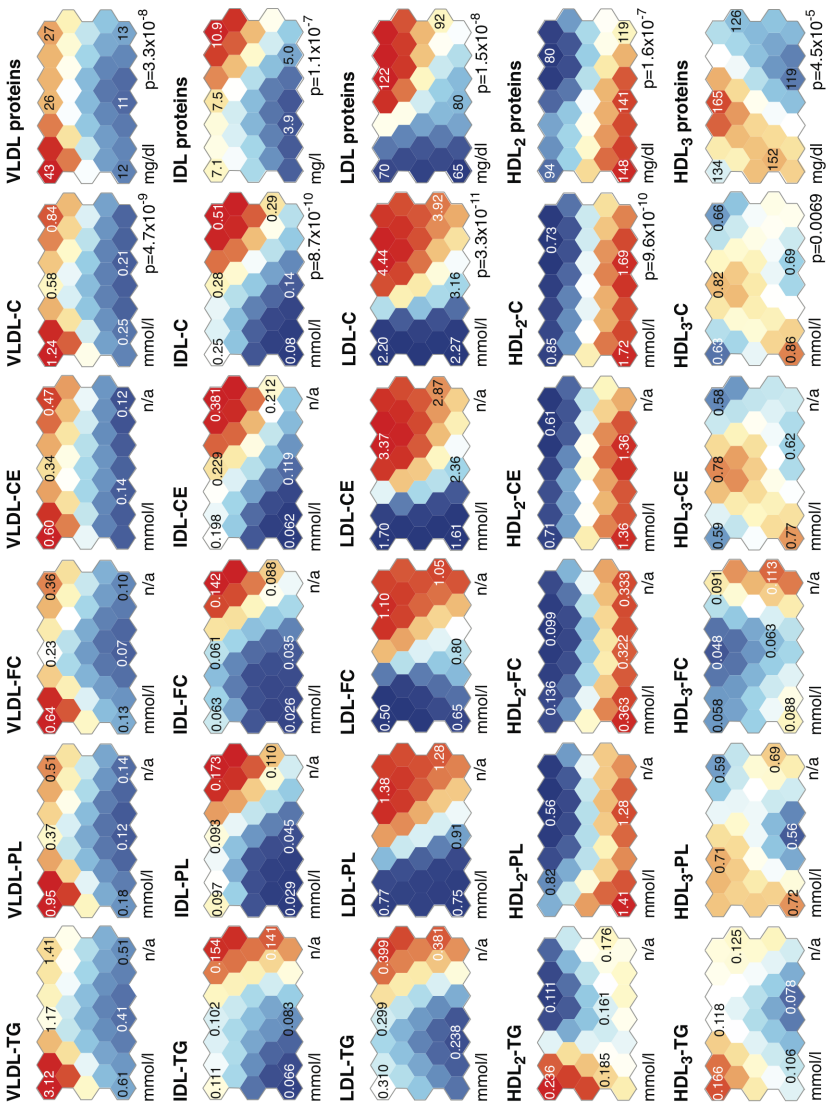

Figure 4.4 Component planes of lipoprotein lipid concentration measures in mmol/l and proteins in mg/dl in the SOM analysis of the combined lipoprotein lipid concentration and composition variables. The p-values below the plots indicate the probability of observing equivalent regional variability for random data. The p-values of input variables are replaced by marking 'n/a' as they are no longer valid. Importantly, the very same SOM analysis is the basis for all the component planes shown (holds also for Fig. 4.5) and thus each of them can be directly compared, i.e., the distribution of the individuals is the same in every component plane. TG, triglyceride; PL, phospholipid; FC, free cholesterol; CE, cholesterol ester; C, total cholesterol. Modified from [467].

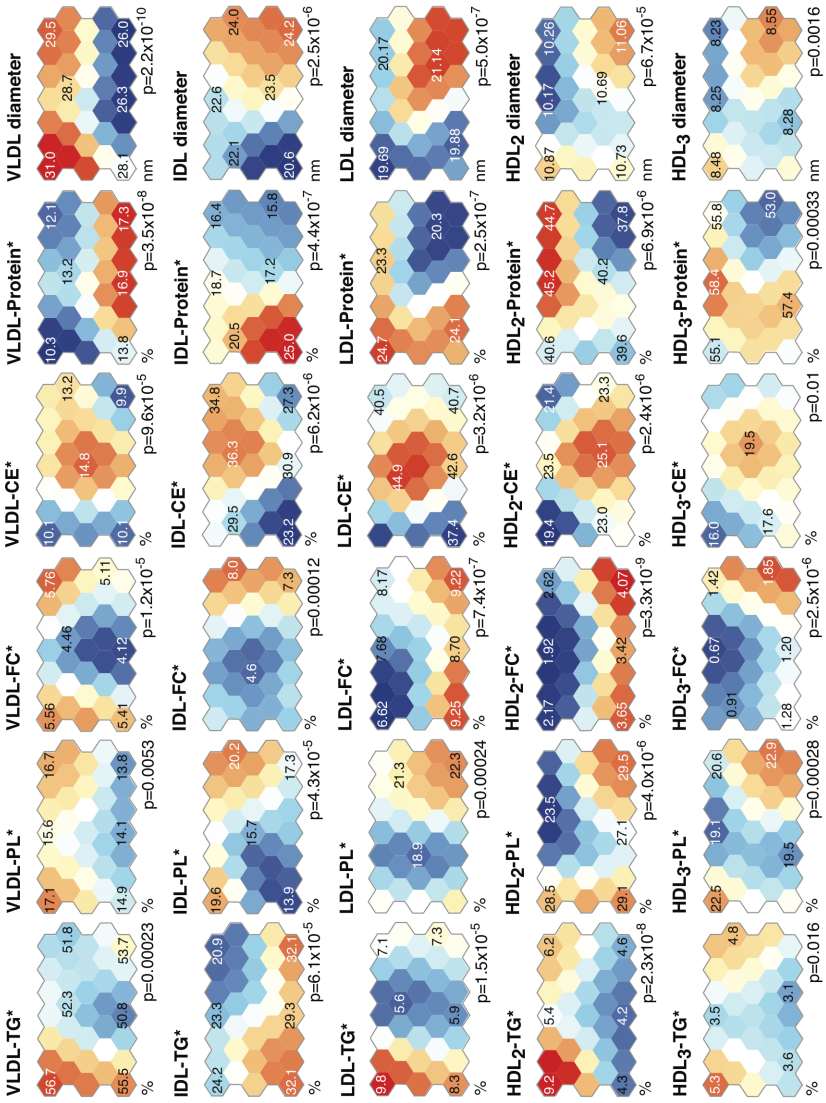

Figure 4.5 Component planes of lipoprotein composition measures and diameters in the SOM analysis of the combined lipoprotein concentration and composition variables. For each lipoprotein fraction, the values are represented as weight percentages (*) for lipids and protein or nm for the particle sizes. All other details and abbreviations are as given in the caption for Fig. 4.4. Modified form [467].

Fig. 4.5 supports the discussion above. The individuals with high VLDL triglycerides (VLDL-TG in the north-west region in Fig. 4.4) have also triglyceride-rich VLDL, LDL, HDL_2 and HDL_3 particles (-TG* in the north-west in Fig 4.5). These LDL, HDL_2 and HDL_3 particles are also cholesterol ester-poor in line with the action of CETP. The VLDL particles are, however, not particu-

larly enriched in cholesterol esters. Fig. 4.5 also completes the associations of VLDL, LDL and HDL lipid concentrations introduced above: In the south-east region, the high HDL_2 and low VLDL lipid concentrations relate to relatively high concentrations of plasma LDL lipids and large IDL, LDL, HDL_2 and HDL_3 particles together with small VLDL particles. The LDL, HDL_2 and HDL_3 particles are enriched in phospholipids (-PL*) and free cholesterol molecules (-FC*) while the VLDL and IDL particles are relatively phospholipid-poor (-PL*). In contrast, in the south-west regions of the component planes, the high HDL_2 and low VLDL lipid concentrations are associated with low concentrations of plasma lipids in LDL as well as in IDL. All these apoB-100-particles are also relatively small. In general, there are clear associations between the lipoprotein particle sizes and the phospholipid as well as protein weight percentages of the particles; high amounts of phospholipids and low amounts of proteins indicate large lipoprotein particles.

Fig 4.4 and 4.5 also show associations of plasma LDL cholesterol levels and compositional subtypes of LDL. Quite contradictory results have been published regarding the association of plasma LDL cholesterol concentrations with the composition and characteristics of the LDL particles. Individuals with prominence of small, dense LDL particles may have the same plasma LDL cholesterol concentration as individuals with large LDL particles[472, 473]. On the other hand, the individuals with large LDL particles may have higher LDL cholesterol concentrations than the individuals with small, dense LDL particles[323]. Furthermore, it has been shown that the LDL particle numbers can differ without affecting the LDL-C concentration in plasma[490]. Increased number of LDL particles may, however, result either from large or small LDL particles since the binding of LDL particles to LDL-receptors has been shown to be reduced both for small, dense and large LDL particles when compared to LDL particles with intermediate sizes[491].

Our findings indicate that the large LDL particles associate with relatively high LDL-C concentrations (the south-east region of the LDL-C planes) while the small LDL particles are mostly related to low plasma LDL cholesterol concentrations (western half of the LDL-C planes). These associations are not, however, inclusive since there are also individuals with high concentration of relatively small LDL particles leading to high plasma LDL concentrations (the north-east region). It is also worth noticing that small LDL particles associated with a high plasma LDL cholesterol concentration (LDL-C) seem to be rather triglyceride-poor (LDL-TG*) whereas small LDL particles associated with low LDL cholesterol concentrations (LDL-C) are triglyceride-enriched (LDL-TG*). Thus the chemical composition, size and particle number affect plasma LDL cholesterol concentrations and the apparently contradictory res-

ults are likely only a reflection of different characteristics in the study populations.

4.4.3 *In silico* lipoprotein phenotyping

Simultaneous analysis of figures 4.4 and 4.5 actually provides detailed subgroupings of the lipoprotein particles within each fraction, *i.e.*, a detailed *in silico* lipoprotein phenotyping beyond the experimental data. The term *lipoprotein phenotype* is used here to denote a collection of lipoprotein subtypes for VLDL, IDL, LDL, HDL_2 and HDL_3 which each have characteristic plasma lipoprotein lipid concentration profiles as well as distinct compositional and metabolic features. An overview of the lipoprotein phenotypes is given in Fig. 4.6.

Fig. 4.6 shows the distinct lipoprotein phenotypes arranged on two platforms, one for triglyceride-enriched and the other for triglyceride-poor particles due to the significant role of triglycerides particularly in apoB-containing lipoprotein metabolism. The HDL particles are also arranged accordingly into the two groups with respect to their relative triglyceride contents. Five phenotypes were discovered and they are named by alphabets from A to E corresponding to the regions in the south-west, north-west, north-east, south-east and in the middle, respectively.

Lipoprotein phenotype B (in the north-east region) is particularly worth noticing since it resembles the lipoprotein phenotype characteristic of metabolic syndrome[492]. In lipoprotein phenotype B, all lipoprotein particles are enriched in triglycerides except the IDL particles which are fairly triglyceride-poor. In addition, the concentration of HDL_2 and HDL_3 cholesterol is low. The VLDL particles are large while the IDL and LDL particles are quite small. Interestingly, while the VLDL particles are also enriched in free cholesterol and phospholipid molecules, the corresponding IDL, LDL, HDL_2 and HDL_3 particles are poor in free cholesterol molecules. The concentration of VLDL particles is high but those of LDL and HDL_2 are low and the concentration of IDL particles is also somewhat elevated.

These lipoprotein characteristics in phenotype B are in line with studies of metabolism of apoB-100-containing lipoprotein particles. Increased plasma concentrations of particularly large and TG-enriched VLDL have been shown to result in low concentrations of LDL[493] with the preponderance of small, TG-enriched LDL particles[322, 323, 494] and elevated particle concentrations in IDL density range containing VLDL remnants persisting in the plasma[293, 495]. It is also notable that the low percentage of FC in the IDL, LDL and HDL particles of phenotype B may be a structural property that enhances the oxidative susceptibility of these lipoproteins[496].

Fig. 4.6 illustrates also the concentration of the proteins within each fraction of all these five lipoprotein phenotypes. In VLDL, IDL and LDL particles, the total protein concentrations were virtually identical to the concentrations of apoB molecules (data not shown). Thus the protein concentrations in these lipoprotein fractions can be considered as estimates of lipoprotein particle concentrations. The VLDL and IDL particle concentrations are very similar in phenotypes A and E but the particles have significantly different compositions; the VLDL and IDL particles are triglyceride-enriched and cholesterol ester-poor in phenotype A, and the situation is the opposite in phenotype E. Similarly, concentration of LDL is average in phenotypes C and E with remarkable variations in the composition of LDL particles and even more pronounced differences in composition and sizes of VLDL and IDL particles between the phenotypes. Thus it seems that the plasma lipoprotein concentrations alone can give only a limited view on the overall lipoprotein metabolism. This is not unexpected considering that lipoprotein metabolism involves a complex crosstalk of various lipoprotein particles as well as enzymes and lipid transfer proteins affecting the concentrations of all lipid molecules within lipoprotein particles.

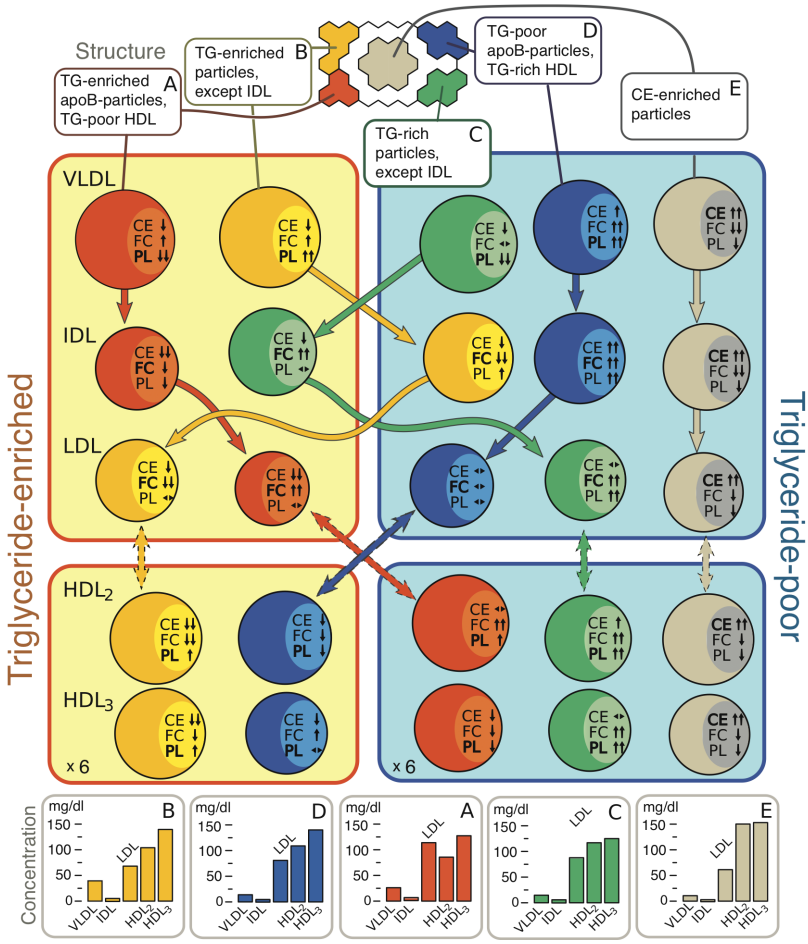

Figure 4.6 A metabolic overview of the lipoprotein phenotypes arisen from the SOM analysis illustrated in Figs. 4.4 and 4.5. The application of the SOM analysis to the combination of lipoprotein lipid concentration and composition data resulted in a novel perspective and provided also a subgrouping of the lipoprotein particles in each fraction, i.e., an in silico *lipoprotein phenotyping beyond the experimental data. The term lipoprotein phenotype refers here to a collection of lipoprotein subtypes for VLDL, IDL, LDL, HDL_2 and HDL_3 related to a particular plasma lipoprotein concentration profile and forming a metabolically connected entity. Five different phenotypes were discovered (marked as A-E and colour-coded on the SOM), all with characteristic plasma concentration profiles (as indicated in the bottom) as well as distinct compositional features (as summarised on the top). The scales in the concentration profiles indicate the total protein concentrations of the lipoprotein fractions in mg/dl. The metabolic pathways of the lipoprotein phenotypes are organised here in two platforms, one for triglyceride-enriched (on the left with an orange background) and one for triglyceride-poor particles (on the right with a bluish background) since the apoB-containing VLDL-IDL-LDL cascade relates primarily to the transport and hydrolysis of triglycerides. The HDL particles are also accordingly divided into the two platforms with respect to their relative triglyceride content. The solid, colour-coded arrows represent the metabolic pathways of the apoB-containing lipoprotein particles within each lipoprotein phenotype. The connections*

between apoB-containing and HDL particles are indicated by the bidirectional dashed arrows. The sizes of apoB-containing particles as well as HDL particles are in scale although the sizes of the HDL particles are enlarged by a factor of 6. The relative contents of the various lipids in the lipoprotein particles are indicated by the up- and downward arrows. Structurally characteristic lipids in each particle are bolded. The abbreviations are as given in the caption for Fig. 4.4. Modified from [467].

As the individuals in this study had various backgrounds, effect of the background, i.e., use of alcohol and estrogen replacement therapy, on the distribution of individuals should be considered. In general, there is a clear tendency of the individuals in the different groups to differ on the basis of their lipoprotein phenotypes; e.g., there is a preponderance of heavy alcohol drinkers in lipoprotein phenotypes A and B (see Fig. 4.2 and 4.6) while the estrogen treated hysterectomised postmenopausal women and apparently healthy controls relate mostly to the phenotypes C, D, and E. The separation is, however, unambiguous for none of the groups, and, for example, in the north-east corner of the SOM (phenotype D), the distribution is 20 % of heavy alcohol drinkers, 23 % of estrogen treated women and 56 % of apparently healthy controls. Thus the distribution of the individuals in the SOM genuinely reflects the distribution of lipoprotein lipid-related variables and less the membership in one of the three groups per se.

To summarise, the purpose of this work was to enhance the concept of lipoprotein profile which is regularly characterised by plasma concentration values of triglycerides as well as total, LDL and HDL cholesterol molecules. Originally, we had 302 plasma samples which were isolated into VLDL, IDL, LDL, HDL_2 and HDL_3 fractions by ultracentrifugation and characterised further to yield lipid concentrations of triglyceride, cholesterol ester, free cholesterol, phospholipid molecules as well as concentration of protein molecules. The samples represented a wide range of plasma lipoprotein lipid values. As the next step, we combined the ordinary although extensive set of biochemical variables in a novel way to gain information simultaneous from the concentrations of lipoprotein lipids and the compositions of the lipoprotein particles. This let us to define five distinct lipoprotein phenotypes which differed from each other on particle size, chemical composition, and lipoprotein lipid and particle concentrations. The work was performed with the help of a self-organising map method which clustered the samples based on their similarity over all used variables. Thus the results are conditional to the used variables ensuring that they represent entirety of the variables, i.e., the overall metabolism of lipoprotein particles in this case. The self-organising map method also visualises the distribution of the variables in two-dimensional component planes yielding easily interpretable and directly comparable com-

ponent planes. This is particularly useful in case of complex, multidimensional data such as lipoprotein lipid data since it allows to detect individuals with opposing course of associations between multiple variables.

Even though detailed data on lipoprotein particles would currently be preferred in cardiovascular research, the subpopulation analysis is usually based on particle size (e.g., using gradient gel electrophoresis or nuclear magnetic resonance spectroscopy) and therefore the chemical composition of the particles remains unknown[117, 177]. In analytical lipid biochemistry, sequential ultracentrifugation is the gold standard for physical lipoprotein isolation allowing for subsequent analyses of the molecular composition of the particles[477-479]. However, the UCF-based lipoprotein work is most often restricted regarding the analysed subfractions. This is because the finer the density ranges used for the isolation the more tedious and expensive the analyses become[497]. Consequently, it would generally be beneficial to computationally enhance the UCF-based lipoprotein data as illustrated in this work. In particular, deeper insight into the compositional variations in the lipoprotein particles appears a fundamental issue. The physiology and pathophysiology of lipoproteins are about transfer and exchange of various lipid molecules between the lipoprotein particles and tissues. Thus, not only the concentration but also the quality of lipoprotein particles and the form of transportation are of great importance.[467]

5. Towards lipoprotein particle concentrations

At this point, I have discussed two fundamental aspects of lipoprotein particles, the structure and the metabolism, from the point of view of computational modelling. In this chapter, I will move forward towards the third essential aspect of lipoprotein particles, the lipoprotein particle concentrations, and I will, after an introductory section, present a model to estimate lipoprotein particle concentrations on grounds of lipoprotein lipid concentrations. In this introductory section, I will also discuss lipoprotein particle sizes which are, at least at times, regarded as the most atherogenic property of lipoprotein particles. These size estimates have recently and convincingly been related to the lipoprotein particle concentrations.

5.1 Lipoprotein particle concentrations in health and disease

Lipoprotein particle concentrations, particularly LDL concentrations, are some of the most intriguing manifestations of lipoprotein particle heterogeneity. It is well established that lipoprotein particle concentrations in circulation vary substantially among individuals depending on age, gender, food intake, metabolic/hormonal state, and disease state of the individual[101, 324, 414, 498-502].

The concept of lipoprotein particle concentration dates back to late 1940s when Gofman and his colleagues managed to isolate the full spectrum of lipoprotein particles by analytical ultracentrifugation[18]. In 1950s, Gofman and his colleagues showed that differences in lipoprotein mass profiles could predict the coronary artery disease risk[24]. The time was, however, full of conflicts among the scientific community searching for the cause of atherosclerosis and cardiovascular disease, and Gofman, although having made a breakthrough in the field, lost interest in lipoprotein research. Also the analytical ultracentrifugation gave way to other, technically less difficult methods which could not, however, measure concentrations of lipoprotein particles even

roughly. The next sequence of events supporting the importance of lipoprotein particle concentrations was the discovery of LDL-receptor by Goldstein and Brown in the 1970s[69, 70, 72]. They showed that increased number of LDL and IDL particles in individuals with familial hypercholesterolemia is a sufficient cause for the development of atherosclerosis and cardiovascular disease.

By the end of the 20th century, discussion had, however, turned into the quality of lipoprotein particles, and particularly their size, rather than the particle concentrations[117, 339, 405, 406, 503-507]. At the time, the paper and agarose gel electrophoresis that Fredrickson was using in the 1980s, was refined to non-denaturing gradient gel electrophoresis that could characterise lipoprotein particles by their size[168, 169]. In the following years, it was observed that individuals with atherosclerosis had smaller LDL particles than apparently healthy individuals and thus these small, dense LDL were suggested to be the most atherogenic lipoprotein particles[504, 508, 509].

The relationship of LDL particle size and CAD risk has been investigated extensively in approximately thirty studies as summarised elsewhere[117]. Most of the studies found a significant relation between small, dense LDL particles and the risk of coronary artery disease. In a few studies, however, large LDL particles were associated with the risk of CAD. Indeed, both the small and the large LDL particles may have decreased binding affinity to LDL-receptors when compared to intermediate-sized particles[117]. Small LDL particles enter the arterial wall and remain in the wall more easily than larger ones, have enhanced oxidation potential and participate directly in the production of subendothelial macrophage foam cells[117, 510]. Per particle basis, the larger LDL particles do, however, transfer more cholesterol molecules into the wall than the smaller particles.

In the studies of LDL particle size and CAD risk, the sizes of LDL particles were measured by non-denaturing gradient gel electrophoresis which measures the relative size distribution of the particles and then the most prominent size, i.e., average lipoprotein particle size, is obtained as a point estimate of the distribution. Since the method does not directly quantify the number of small and large particles, the obtained size estimate reflects changes in total LDL particle concentration and thus, for example, a decreased LDL particle size may be caused either by elevated total LDL particles with elevated number of small particles or by fewer total LDL particles with fewer large LDL particles[511]. Therefore, when studying the relation of particle size with cardiovascular disease, the total particle concentrations should also be taken into consideration.

The association of atherogenic lipoprotein particle concentrations with the risk of CAD has also been investigated in clinical trials either via apoB con-

centrations[141-146] or NMR-derived LDL particle numbers[146-150]. In most of the studies apoB[141-143] and NMR-derived LDL particle[147-150] concentrations were more closely associated with CAD risk than LDL cholesterol. ApoB and NMR-derived LDL particle concentrations associated also with CAD risk with a magnitude of association comparable to total/HDL cholesterol ratio or non-HDL cholesterol concentration[145, 146].

While the particle concentrations were comparable but not superior to that of standard lipids in cardiovascular disease risk prediction, it should be remembered that cholesterol molecules are transported within lipoprotein particles in circulation. Thus lipoprotein particle concentrations, along with certain other properties of the particles, i.e., their lipid content and composition, are important for a proper understanding of the complex interactions prevailing in lipoprotein physiology.

Practical difficulties are, however, present in measuring lipoprotein particle concentrations. At present, no method can directly count lipoprotein particle numbers within various lipoprotein fractions. Therefore, a number of approaches have been used to approximate lipoprotein particle concentrations according to the concentration of some of their constituents. However, there are certain disadvantages in using these variables due to lipoprotein particle heterogeneity.

5.2 Surrogate variables of lipoprotein particle numbers

There are a few variables which are often used as surrogate measures for lipoprotein particle concentrations in plasma. These include the main lipid and the main apolipoprotein concentrations as well as concentrations of the total lipids or their NMR-derived resemblance. In general, the idea of lipoprotein particle estimation via these variables is quite simple. In order to estimate the lipoprotein particle concentration individually in a chosen fraction, for example in LDL, two related measures are needed: one which accounts for total amount of a constituent over the lipoprotein particles in that fraction, e.g., plasma LDL cholesterol, and another which accounts for amounts of that constituent within a single lipoprotein particle, e.g., number of cholesterol molecules per a lipoprotein particle. Then the numbers could just be divided to obtain the concentration of those lipoprotein particles. In principle, this approach is an acceptable way to estimate lipoprotein particle concentrations but it requires certain important characteristics from the constituents; within a lipoprotein fraction under question, the constituent must be present in each particle in measurable quantities, and each particle must have an equal amount of that constituent. Since measurement of the contents of a single

particle is not currently possible, this assumption of homogeneous particles within a fraction should hold also between individuals. Lipoproteins are, however, heterogeneous, and thus lipoprotein particle estimation culminates in finding the best possible constituent(s).

5.2.1 Concentrations of the main lipids

In most cases, lipoprotein particle concentration is related with the concentration of the main lipid within the lipoprotein fraction in interest. Thus LDL and HDL particle concentrations are approximated by the concentration of cholesterol in LDL and HDL particles, respectively[46, 205, 206, 348]. By analogy, VLDL particle concentrations could then be estimated by the concentration of triglycerides in VLDL particles but it is usually characterised by plasma triglyceride concentration[46, 205], which mostly reflects the triglyceride content in VLDL particles in fasted state[46, 322, 397, 403, 512]. The use of the main lipids is justified in the sense that those lipids are always present in these lipoprotein particles and their amounts are usually relatively high. The estimates are, however, based on the lipids that face significant changes during the metabolism of the particles in the circulation.

When main lipid content is used as a surrogate variable of lipoprotein particle concentration, it is assumed that the main lipid content per particle is the same, although unknown, in each individual in the particular fraction. However, it is clear that lipoprotein particles are heterogeneous in their main lipid compositions and thus the use of main lipids as lipoprotein particle estimates, particularly LDL cholesterol as a surrogate measure of LDL particles, has recently faced criticism.

A significant portion of individuals with 'normal' cholesterol values still develop atherosclerosis and cardiovascular disease. On the other hand, the 'normal' value is also quite difficult to deduce, and the desirable value has been lowered during decades particularly due to this discordance. The error made by the assumption of homogeneous particle composition seems, however, quite large. Approximately 12% change in diameter of an LDL particle (3 nm) represents approximately 40 % less volume in the core and on this basis, a person with smaller LDL particles will require 70 % more particles to carry equal amount of cholesterol as the person with larger LDL particles.

Besides the problems with LDL cholesterol concentration, it has still held its status since it is quite easy to use and for extreme cholesterol values it is sufficiently accurate. Individuals with high LDL cholesterol values (> 4.1 mmol/l) have a significant probability to develop atherosclerotic cardiovascular disease while individuals with low LDL cholesterol levels (< 2.6 mmol/l) rarely develop the disease[131, 513, 514]. In these individuals, the errors made in as-

suming a homogeneous particle composition do not significantly affect the outcome: in individuals with extremely high LDL cholesterol values, also the LDL particle concentrations must be high, and in individuals with extreme low LDL cholesterol values, also the LDL particle concentration must be low. Most of the individuals have, however, LDL cholesterol values between these two extremes. In those individuals, the elevated LDL cholesterol concentration may either implicate normal or even slightly reduces number of larger than normal LDL particles or elevated number of smaller LDL particles, but the LDL cholesterol concentration alone cannot distinguish between these possibilities.

5.2.2 Concentration of the main apolipoproteins

Concentrations of the main apolipoproteins have also been used as surrogate variables of concentrations of lipoprotein particles. Particularly, the use of plasma apoB is justifiable to estimate the concentration of apoB-containing particles, such as VLDL, IDL, and LDL particles as a whole, since the particles contain a constant number of apoB molecules, i.e., one molecule per particle, throughout their metabolism.

It has been estimated that more than 90 % of plasma apoB is associated with LDL particles[515, 516], and, consequently, plasma apoB concentration has been widely used as an estimate of LDL particle concentration. However, the portion of LDL apoB from total plasma apoB has been observed to vary almost 10 % in apparently healthy individuals and to be somewhat lower in individuals with elevated VLDL particles[515]. Therefore, plasma apoB concentration, in contrast to LDL apoB measure, is not a particularly good estimate of LDL particle concentration.

By analogy to apoB and apoB-containing lipoprotein particles, apoA-I concentration could be considered as an indicator of the concentration of HDL particles[395, 517]. The limitations in the use of plasma total apoA-I concentrations are, however, more pronounced than those with apoB and thus the association between the apoA-I and HDL particle concentration is rarely explicitly expressed.

There are two challenges with the use of apoA-I as a surrogate variable for HDL particle concentration. Firstly, apoA-I molecules are present, in addition to HDL particles, also in CM particles[154, 269]. Secondly, the number of apoA-I molecules can vary in HDL particles due to their exchangeable nature. This variability is, however, highly dependent on HDL particle size[423, 518] and thus apoA-I concentrations measured in certain HDL subclasses can be used to estimate their particle concentrations. Therefore, the challenges with apoA-I would be solved by additional lipoprotein isolation procedures in a similar

manner as for apoB-containing particles.

5.2.3 Proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy

The latest advance in lipoprotein concentration estimation has been achieved by a method which differs greatly from the conventional biochemical assays, namely, ^{1}H NMR spectroscopy. NMR spectroscopy is a holistic, specific and quantitative method mainly used in organic chemistry to identify molecules in liquids[519, 520]. NMR spectroscopy is based on nuclear magnetic resonance which exists in measurable quantities only on high magnetic fields in nuclei with non-zero total spin. Each molecule has a characteristic behaviour in the magnetic fields which determines its specific location in the resulting signal spectrum of all molecules present in the liquid. This specific location can then be used to identify the molecule, and its signal amplitude is directly related to its concentration in the liquid.

During the last decades, NMR spectroscopy has gained ground also in lipoprotein research since it is fast and can potentially replace many of the biological assays used in the characterisation of lipoprotein particles[204, 497, 521]. One particularly important advantage of NMR spectroscopy is that lipoproteins do not need to be physically isolated from the serum sample beforehand. The method does, however, require advanced computational methods when used for lipoprotein quantification. Several groups have developed computational approaches including a commercial deconvolution method by Otvos et al.[116, 206, 414, 511, 522-526] and regression models by Ala-Korpela et al.[527].

In both models, the signals used to estimate lipoprotein-related concentrations come from methyl regions, or -CH_3 groups, of all lipid molecules and thus the resulting concentrations resemble total lipid concentrations. These can then be transformed into lipoprotein fraction specific triglyceride or cholesterol concentrations by assuming an average lipid composition of the lipoprotein particles[206, 528, 529] or, interestingly, into lipoprotein particle concentrations by approximating the lipoprotein particle diameter as well as core lipid volume and mass[206, 522-526]. The deconvolution method for lipoprotein particle concentration estimates has been recently used in various clinical studies and its performance in predicting individuals at risk of cardiovascular disease has been promising[101, 116, 147-149, 412, 414, 415, 498, 530, 531].

5.3 Materials and methods: Lipoprotein particle concentrations

In the following subsections, I will introduce a method to estimate lipoprotein particle concentrations on the grounds of chemically measured lipoprotein

lipid concentrations. Since Otvos et al. have used a quite similar approach to generate model particles, or a reference particle library, for lipoprotein particle concentration estimation via NMR spectroscopy[206, 522-526], also the differences of the methods are discussed.

5.3.1 Data collection

Data for the modelling was selected to contain a group of individuals with a wide range of different lipoprotein variables, i.e., lipoprotein subclass concentrations. The blood samples from a hundred individuals were collected after an overnight fast for 12 h into EDTA-containing tubes and lipoproteins were isolation by high-performance liquid chromatography (HPLC)[194, 195]. Lipoprotein particles were separated by their size (gel permeation columns, TSK-GEL LipopropakXL; Tosoh) into twenty subclasses which included two chylomicron subclasses (< 90 nm and 75 nm in mean diameter), five VLDL subclasses (64 nm, 53.6 nm 44.5 nm, 36.8 nm and 31.3 nm), six LDL subclasses (28.6 nm, 25.5 nm, 23.0 nm, 20.7 nm, 18.6 nm and 16.7 nm) and seven HDL subclasses (15.0 nm, 13.5 nm, 12.1 nm, 10.9 nm, 9.8 nm, 8.8 nm and 7.6 nm). The concentrations of triglyceride, free cholesterol, cholesterol ester, and phospholipid molecules were also measured by enzymatic methods (see Table 5.1). Numbers of lipid molecules in the table were estimated by equation (3.1) in page 62 or by equation (5.2) below.

Table 5.1 Diameters, weight percantage compositions of lipoprotein lipids and proteins as well as estimated numbers of lipids in twenty lipoprotein subclasses isolated by high-performance liquid chromatography.

Class	Diameter	W_{TG}	W_{CE}	W_{FC}	W_{PL}	W_{PROT}	N_{TG}	N_{CE}	N_{FC}	N_{PL}
CM1	< 90.0	83 ± 7	4 ± 3	3 ± 2	9 ± 6	1 ± 0	205590 ± 16502	12940 ± 10628	17880 ± 11781	24070 ± 16023
CM2	75.0	79 ± 8	3 ± 2	4 ± 2	13 ± 6	1 ± 0	113000 ± 10769	6650 ± 4575	13460 ± 7135	20020 ± 8842
VLDL1	64.0	74 ± 8	4 ± 3	4 ± 3	17 ± 8	1 ± 0	66151 ± 6480	4960 ± 3825	8030 ± 5489	16506 ± 7679
VLDL2	53.6	67 ± 7	5 ± 3	5 ± 2	21 ± 5	2 ± 0	35627 ± 3713	3847 ± 2188	5506 ± 1980	12049 ± 3261
VLDL3	44.5	56 ± 8	10 ± 4	6 ± 1	24 ± 4	4 ± 0	17311 ± 2408	4177 ± 1815	4215 ± 777	7979 ± 1394
VLDL4	36.8	46 ± 8	14 ± 4	8 ± 1	27 ± 3	5 ± 0	8205 ± 1342	3184 ± 1027	3212 ± 520	5181 ± 655
VLDL5	31.3	29 ± 8	16 ± 5	9 ± 1	38 ± 6	7 ± 0	3268 ± 863	2443 ± 703	2309 ± 366	4605 ± 713
LDL1	28.6	16 ± 5	27 ± 4	12 ± 1	37 ± 2	8 ± 0	1370 ± 425	3110 ± 451	2286 ± 274	3452 ± 241
LDL2	25.5	12 ± 3	31 ± 3	13 ± 1	33 ± 2	11 ± 0	748 ± 210	2530 ± 254	1718 ± 158	2257 ± 115
LDL3	23.0	10 ± 3	30 ± 3	12 ± 1	34 ± 2	14 ± 0	472 ± 133	1835 ± 191	1206 ± 120	1693 ± 121
LDL4	20.7	9 ± 3	28 ± 3	11 ± 1	33 ± 3	19 ± 0	320 ± 96	1268 ± 149	809 ± 93	1254 ± 112
LDL5	18.6	8 ± 3	23 ± 3	9 ± 1	35 ± 3	25 ± 0	203 ± 80	791 ± 116	508 ± 79	981 ± 99
LDL6	16.7	8 ± 4	19 ± 3	8 ± 1	32 ± 4	33 ± 0	150 ± 70	487 ± 83	337 ± 63	678 ± 86
HDL1	15.0	6 ± 5	18 ± 6	7 ± 1	54 ± 8	16 ± 0	82 ± 64	303 ± 96	188 ± 40	761 ± 121
HDL2	13.5	5 ± 4	16 ± 6	5 ± 1	53 ± 8	21 ± 0	52 ± 42	201 ± 76	114 ± 29	558 ± 88
HDL3	12.1	5 ± 3	17 ± 4	4 ± 1	50 ± 5	24 ± 0	35 ± 22	155 ± 40	70 ± 18	385 ± 40
HDL4	10.9	5 ± 2	18 ± 3	4 ± 1	43 ± 2	30 ± 0	24 ± 12	127± 19	51 ± 6	246 ± 13
HDL5	9.8	4 ± 2	17 ± 3	4 ± 1	44 ± 3	31 ± 0	15 ± 8	88 ± 15	39 ± 4	183 ± 14
HDL6	8.8	3 ± 2	18 ± 5	4 ± 1	46 ± 5	29 ± 0	9 ± 5	64 ± 17	24 ± 4	139 ± 15
HDL7	7.6	4 ± 1	7 ± 2	5 ± 1	49 ± 3	35 ± 0	8 ± 2	16 ± 5	19 ± 4	99 ± 5

The values are mean or mean ± S.D. from 100 individuals. The diameters are in nm. CM, chylomicron; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; W, weight percentages; N, number of lipid molecules; TG, triglyceride; CE, cholesterol ester; FC, free cholesterol; PL, phospholipid; PROT, total protein.

5.3.2 Calculation of the averaged lipoprotein particle concentrations

In general, the concentration of lipoprotein particles can be thought of as a ratio of concentration of a lipid over all particles and the amount of the lipid per an averaged lipoprotein particle. This can be calculated individually and for each lipoprotein subclass separately as

$$P^{i,s} = \frac{c_l^{i,s}}{N_l^{i,s}} \quad , \tag{5.1}$$

where $c_l^{i,s}$ is the concentration of a lipid (*l*) in an individual (*i*) in a lipoprotein subclass (*s*), and $N_l^{i,s}$ is the corresponding number of the lipid molecules within an averaged lipoprotein particle. The $c_l^{i,s}$ in the equation (5.1) is an experimental quantity and the $N_l^{i,s}$ can be estimated based on experimental lipid and protein concentrations by equation (3.1) in page 62. The equation was

$$N_l^{i,s} = \frac{c_l^{i,s}}{\rho_p^{i,s}} M_p^s \quad , \tag{5.2}$$

where $\rho_p^{i,s}$ is the protein concentration and the M_p^s is the estimated molecular mass of proteins per particle. When this equation is substituted into the equation (5.1) we have

$$P^{i,s} = \frac{\rho_p^{i,s}}{M_p^s} \quad . \tag{5.3}$$

Interestingly, in this equation, the particle concentration is directly proportional to the concentration of proteins in lipoprotein subclass *s*, $\rho_p^{i,s}$. However, this time we wanted to proceed with experimental lipid rather than protein measures. Thus we continued by estimating the concentration of proteins by using the experimental lipid concentrations and some other measure connecting the amounts of lipids and proteins within a particle. Since we had size information available from each lipoprotein subclass and since lipoprotein particles constitute almost exclusively from lipids and proteins, we selec-

ted the volume of the particles as the connecting measure. The total volumes of the particles, V^s , can be calculated from the experimental sizes by

$$V^s = \frac{\pi}{6}\left(d^s\right)^3 \quad , \tag{5.4}$$

where d^s is the diameter of the particle in subclass *s,* or they can be expressed as sums of volumes of lipids and proteins equal to

$$V^s = V_p^s + \sum_l N_l^{i,s} v_l \quad . \tag{5.5}$$

Here V_p^s is the volume of the proteins in each particle in subclass *s* and $N_l^{i,s}$ is again the number of lipid molecules in individual *i* in lipoprotein subclass *s* and v_l is the averaged molecular volume of lipid *l*. The volume of proteins can be estimated, for example, by the number and the average volume of amino acids in each lipoprotein subclass *s*.

If we then substitute the equation (5.2) to (5.5) and rearrange the terms we have

$$\rho_p^{i,s} = \frac{M_p^s}{\left(V^s - V_p^s\right)} \sum_l c_l^{i,s} v_l \quad , \tag{5.6}$$

serving as an estimate for the protein concentration for each individual *i* in lipoprotein subclass *s*. When this is substituted back into equation (5.3), the concentration of lipoprotein particles can be estimated as

$$P^{i,s} = \frac{1}{\left(V^s - V_p^s\right)} \sum_l c_l^{i,s} v_l \quad . \tag{5.7}$$

Thus, the concentration of lipoprotein particles, $P^{i,s}$, in lipoprotein subclass *s* is a lipid volume weighted average of the experimental lipid concentrations within a subclass *s*.

The equation (5.7) has an interesting form when considering the earlier particle concentration estimation methods. Firstly, if concentration of any of the lipids in equation (5.7) increases substantially compared to the concentrations of other lipids, the particle concentration approaches the concentration of that particular lipid. Secondly, if the molecular volumes of lipids in equation (5.7) are substituted by an averaged lipid volume, the particle concentra-

tion becomes directly proportional to the total lipid concentration. Finally, due to the fact that the particle concentrations and the concentrations of proteins are directly proportional, also the particle concentrations calculated by equation (5.7) should correlate strongly with experimental total protein measures for each particle fraction.

5.4 Results and discussion

Lipoprotein particle concentrations calculated by equation (5.7) are shown in Fig. 5.1. In subfigures a)-c) the particle concentrations are in absolute scale (μmol/l) while subfigures d)-f) show the relative subclass particle concentrations within each corresponding lipoprotein subclass. In the following, these absolute and relative particle concentrations as well as their correlations with other particle estimates are discussed.

The absolute particle concentrations differ significantly between lipoprotein fractions; HDL particles are the most numerous lipoprotein particles and LDL particles are more common than VLDL and chylomicron particles. The concentrations of chylomicrons are very low as usual in fasted state. The absolute particle concentration values calculated in this study are in line with the current literature. VLDL subclass particles have been shown to vary around 10 nmol/l[323], LDL subclasses in the range of 100-1000 nmol/l[174] when estimated based on VLDL and LDL apoB concentrations. HDL particles have been shown to vary around 1 μmol/l when their concentrations are estimated based on the concentrations of apoA-I molecules in HDL density ranges and their amount per particle[423, 532]. The relative concentrations of the particle subclasses are also consistent with the current literature. The relative concentration of chylomicrons and large VLDL particles of all TG-rich particles is low while small VLDL particles are the most common TG-rich lipoprotein particles. This also fits with the metabolism of the particles, i.e., conversion of large TG-rich particles to smaller TG-depleted ones upon lipid hydrolysis. It has also been shown that the most prominent LDL subclass (LDL2b; 26 ± 6 %[320]) is near 26 nm in diameter[20, 169, 174] while subclasses with slightly larger or smaller size are a little less common (around 20 %)[320]. The relative concentrations of the smallest LDL particles have been shown to be minor (less than 10 %) in apparently healthy individuals as well as in subjects with elevated plasma triglyceride concentrations[188, 320]. All these trends can be seen in Fig. 5.1.

The concentration of very large HDL particles relative to intermediate and small HDL particles is also in accordance with the literature. The very large HDL particles usually require apoE rather than apoA-I as their main apolipo-

protein and are thus rare. In addition, the inter-individual variation in HDL particle concentrations is high as suggested by literature[188, 320, 533]. Interestingly, however, the small and intermediate-size HDL particles seem to have quite similar concentrations while individuals in the current literature are classified into groups with high amount of either small or large HDL particles[160, 178, 362, 395, 517, 533, 534]. While this data set is limited in size concerning further analysis of this finding, the result seems not to be only due to the fact that individuals in this data set were not divided into groups according to their plasma triglyceride level.

The other matter worth noticing is the association of the particle concentrations with the other particle concentration estimates. Fig. 5.2 shows a collection of these associations at the lipoprotein subclass level and Fig. 5.3 at the fraction level, i.e., summed up over several subclass level particle concentration estimates. Thus the panels in Fig. 5.3 can be held as estimates of total VLDL, LDL and HDL particle concentrations. The quality of the association in each subfigure is best graded by visual examination by comparing the location of dots on the line drawn based on least squares fitting. Each subfigure also profiles square of correlation coefficient (r^2) and the points' averaged deviation from the regression line (↕).

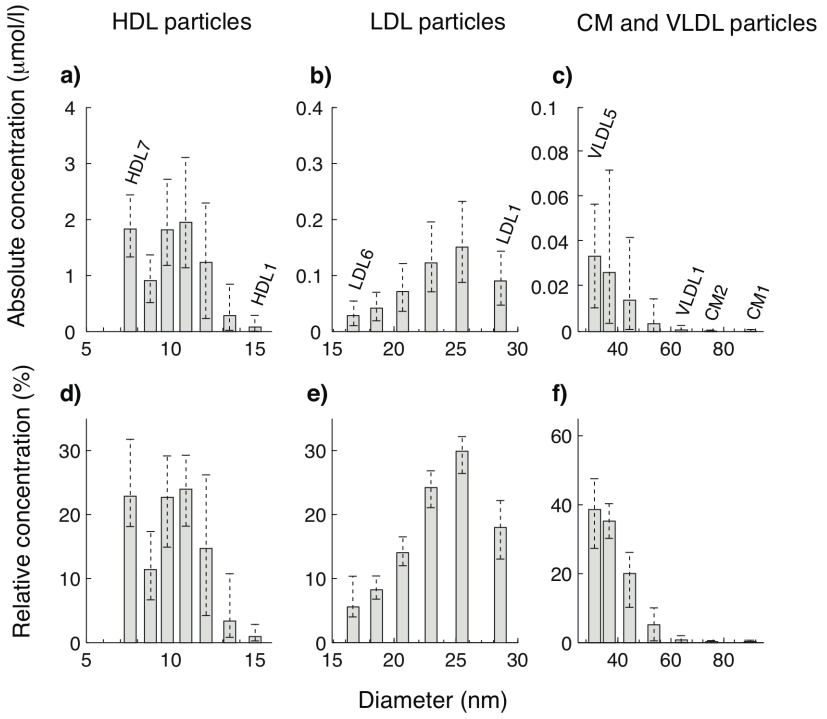

Figure 5.1 Absolute lipoprotein particle concentrations (a,b,c) and relative particle concentrations within the corresponding lipoprotein fraction (d,e,f). Samples were obtained from 100 individuals after an overnight fast. CM and VLDL particles were combined in the same subfigure. CM, chylomic-

ron; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

The panels a)-e) in Fig. 5.2 show correlations of particle concentrations with the corresponding main lipid concentrations in some of the lipoprotein subclasses. Between panels a) and b), as well as panels d) and e), there is a large difference in the strength of the correlation. It seems that particularly the subclasses with the smallest particles give the worst correlations. This is in line with the fact that as the size of the particle decreases, the concentration of the main lipid decreases in amount and, even more importantly, also relative to the concentrations of other lipids. This change in relative particle composition occurs due to differences in lipoprotein metabolism of each individual leading to significant inter-individual variations. This phenomenon is also visible in the LDL subclasses (not shown) but interestingly, it is, at least in this data set, more pronounced in VLDL and HDL subclasses. In Fig. 5.3, the amount of particles in each subclass and their subclass level association with the other particle concentration estimates affect the overall strength of the fraction level associations. Therefore, in panels a)-c), the square of the correlation coefficients is high in VLDL and LDL particles where the most abundant subclasses correlate well with the plasma triglyceride and LDL cholesterol concentrations, respectively, while in HDL particles, the smallest and the least well associated particles with HDL cholesterol are also present in high numbers giving smaller coefficients.

Panels f)-i) in Fig. 5.2 show correlations of total lipids with the particle concentrations. When comparing these figures to panels a)-e), it is obvious that when all lipid molecules are taken into account, the associations become stronger at the subclass level. For example, in VLDL5 subclass, the deviation from the regression line decreased from 12 nmol/l to less than 1 nmol/l, and in HDL7 subclass, the square of the correlation coefficient increased from 0.34 to 0.99. The increased strength of the associations with total lipid estimates stems from the fact that the total lipid measures can overcome major part of the inter-individual variation in the particle compositions. This is because the inter-individual variation at the subclass level results mainly from exchange of lipids, particularly cholesterol ester - triglyceride exchange mediated by CETP, which does not affect the total amount of lipids within the particles. On the other hand, if the total lipid measure changes, also the size of the particle changes accordingly, causing a shift to another lipoprotein subclass. The resulting subclass level correlations are all very strong and lead also to high fraction level correlations in panels d)-f) in Fig. 5.3. Therefore, based on Fig. 5.2 and 5.3, it is obvious that although the squares of correlation coefficients are high with both the main lipid and the total lipid estim-

ates, the total lipid measures are superior to main lipid estimates.

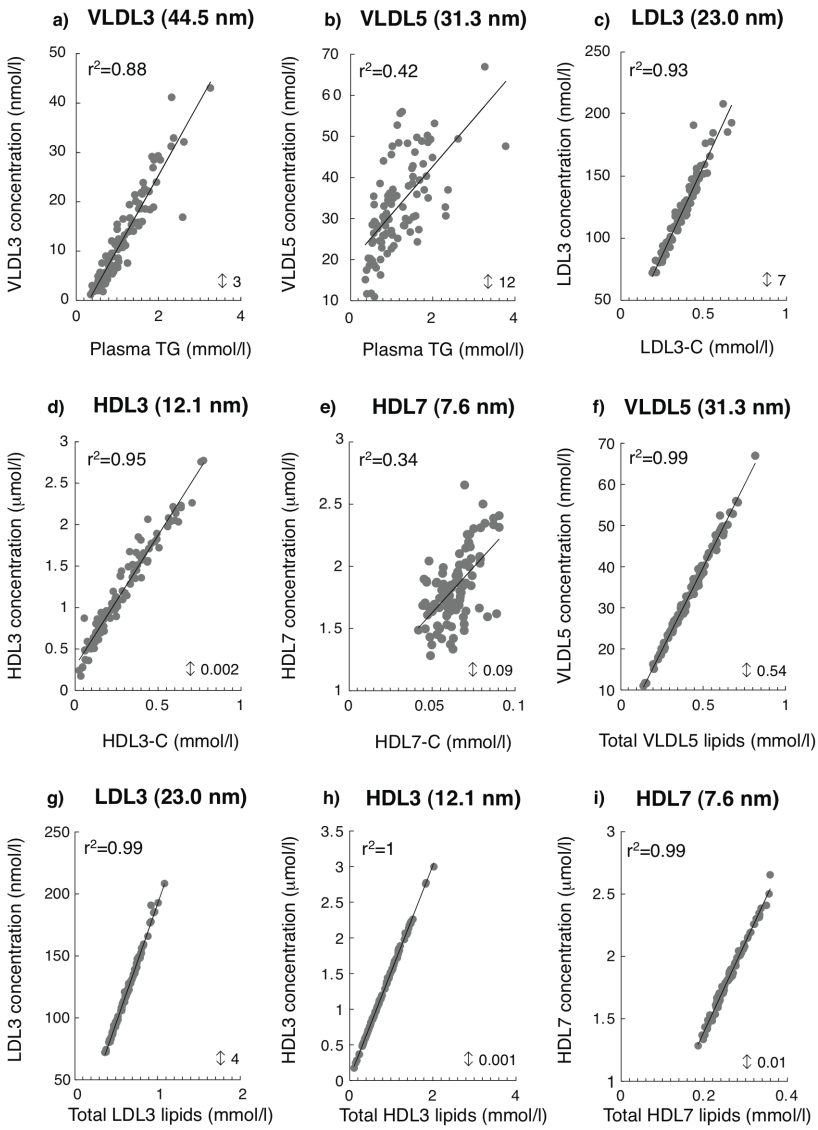

Figure 5.2 Correlation of particle concentrations of certain VLDL, LDL and HDL subclasses with their main lipid and total lipid concentrations. The subclasses were selected to show the best of and the worst of the associations (VLDL, HDL subclasses) or to show one subclass in the middle of the size range if significant variation was not present (LDL subclasses). Also some of the associations between these particle concentrations and their total lipid concentrations are shown to illustrate the strengthening of the associations. VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; C, total cholesterol.

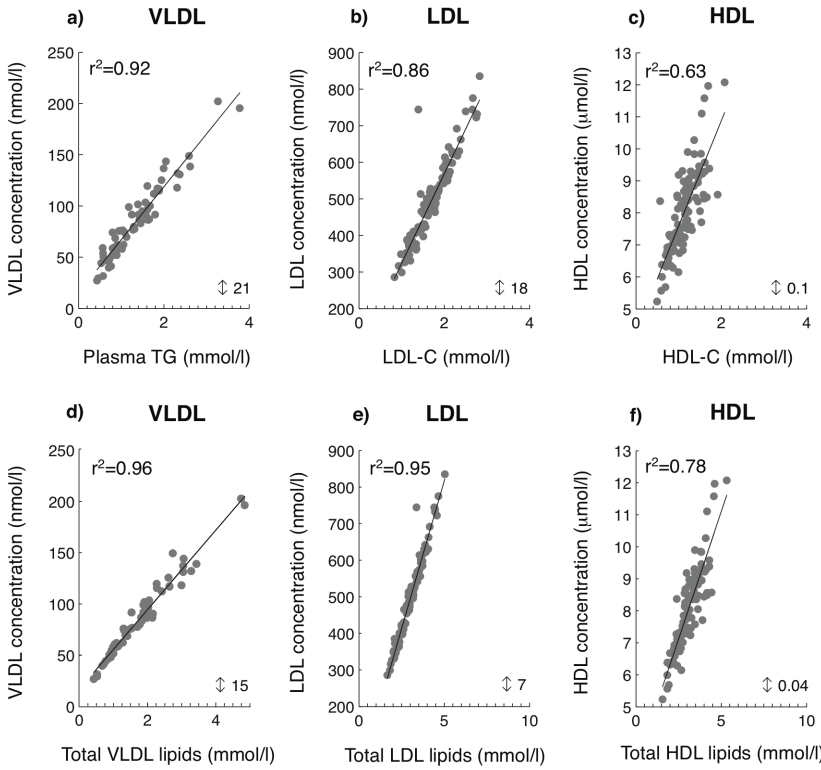

Figure 5.3 Comparison of lipoprotein particle concentrations with main and total lipid measures in VLDL, LDL and HDL fractions. The figure is constructed by summing the corresponding, estimated lipoprotein subclass particle concentrations and their lipid measures, respectively. VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglyceride; C, total cholesterol.

Due to the good performance of total lipids in particle concentration estimation, it is worth directing further interest into them. Fig. 5.4 shows the particle concentration estimates calculated by the equation (5.7) (black bars) as well as lipoprotein particle estimates built based on the total lipid concentrations (red bars). In order to compare these two estimates, we needed conversion coefficients for the total lipid concentrations. Probably the easiest way to obtain these coefficients is to use least square fitting as shown in Fig. 5.2 for some subclasses. As seen in Fig. 5.4, the differences between the methods are small for all lipoprotein particles, less than or equal to two percent in HDL, LDL, and small VLDL particles, and increase up to five percent in the largest VLDL particles. The conversion factors for total lipid concentrations can also be analytically calculated from equations (6.7) by using the same constant for all molecular volumes of lipids. For example, the use of a simple averaged lipid volume over the molecular volumes of the four lipids induced

two to three percent differences in HDL and LDL particles while the difference increased up to twenty percent in the largest triglyceride-rich particles. This larger difference is due to the fact that the use of the averaged lipid volume underestimates most the volume of triglyceride molecules as a triglyceride molecule has the largest volume of the four lipids. The difference between the particle concentration and the total lipid concentration could, however, be approximately halved by using a larger constant for the molecular lipid volumes than the average volume but then the differences in HDL and LDL particles tend to increase.

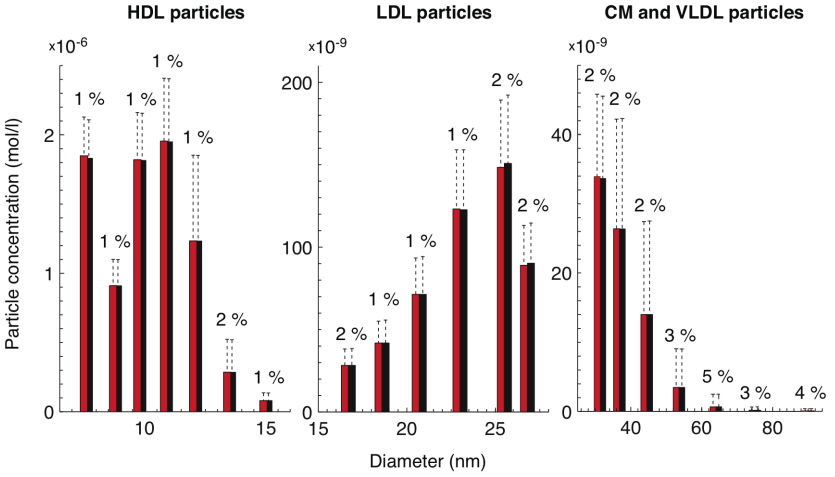

Figure 5.4 Comparison of the particle concentration estimates calculated by the equation (6.7) (black bars) with the estimates calculated based on the total lipid concentrations (red bars) in HDL, LDL, VLDL and CM subclasses. The total lipid concentrations were first converted to levels comparable with the particle concentrations by using the coefficients obtained from the least square fitting. The relative difference of the estimates in each subclass is indicated as percentage units above the bars. CM, chylomicrons; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

The reference particle library which Otvos et al. are using in their NMR studies[206, 522-526] has been generated by a method quite similar as that introduced here. However, the methods differ in the way the per particle compositions are estimated and thus likely have differences particularly in the conversion factors. Unfortunately, the conversion factors of Otvos et al., or the reference data behind the factors, have never been published, and thus direct comparison of the factors is at the moment impossible.

Nevertheless, Otvos et al. do give a biophysical description for their per particle composition estimation procedures performed separately for each lipoprotein subclass[522]; It seems that Otvos et al. estimate the amount of triglyceride and cholesterol ester molecules per particle by restricting their

relative amount per particle and by using their averaged molecular volumes and the core volume calculated from the size estimates assuming spherical particle shape and fixed thickness of the surface layer. In other words, they assume that triglyceride and cholesterol ester molecules form the cores.

The assumption Otvos et al. made on lipoprotein particle structure is rather restrictive and thus their method may suffer from certain drawbacks. Firstly, it is possible that not all triglyceride and cholesterol ester molecules reside in the cores as stated in chapter 3 in this thesis and, secondly, triglycerides and cholesterol esters are not the only molecules in the cores which contain also small amounts of free cholesterol molecules. The overall effect of these problems in the model of Otvos et al. is, however, difficult to assess since they bias the results in different directions. The first flaw tends to underestimate the total amount of triglyceride and cholesterol ester molecules per particle leading to overestimation of particle concentrations. The other flaw would, instead, tend to decrease the particle concentrations since when the free cholesterol molecules are ignored, the per particle triglyceride and cholesterol ester contents in the core increase. When the effects of these two opposite actions on the particle structures are studied by the model introduced in the chapter 3, the effect of the first drawback seems stronger leading to overestimates of particle concentrations.

Otvos et al. have made considerable attempts to verify their method. This was done either by comparing so-called *NMR-derived* lipid levels with chemically measured lipid levels or by comparing apolipoprotein levels to particle concentrations. These NMR-derived lipid levels are estimates of lipids from the methyl peak of NMR spectrum. Extraction of these features is, however, difficult since the signal cannot be split invariantly into the concentrations of various lipid molecules separately. Thus the concentrations of these lipids are estimated based on the particle concentration measures, and the conversion factors, which, at this time, transform the particle concentrations back to lipid concentrations, are again obtained from the reference particle library.

The correlations of plasma triglyceride, HDL cholesterol and LDL cholesterol concentrations with corresponding NMR-derived lipid levels drawn by Otvos et al.[206, 522] are shown in Fig. 5.5. The triglyceride and HDL cholesterol pairs have strong correlations with correlation coefficients $r=0.98$ ($r^2=0.96$) and $r=0.96$ ($r^2=0.92$), respectively, from 253 individuals. The association between NMR-derived and chemically measured LDL cholesterol values correlated less strongly with the correlation coefficient $r=0.85$ ($r^2=0.72$). These correlation coefficients are quite impressive and seem, at first, to provide considerable reliability to the model of Otvos et al. Closer examination does, however, show that the NMR-derived lipid values are not suited for judging

the fitness of the particle concentration estimation method.

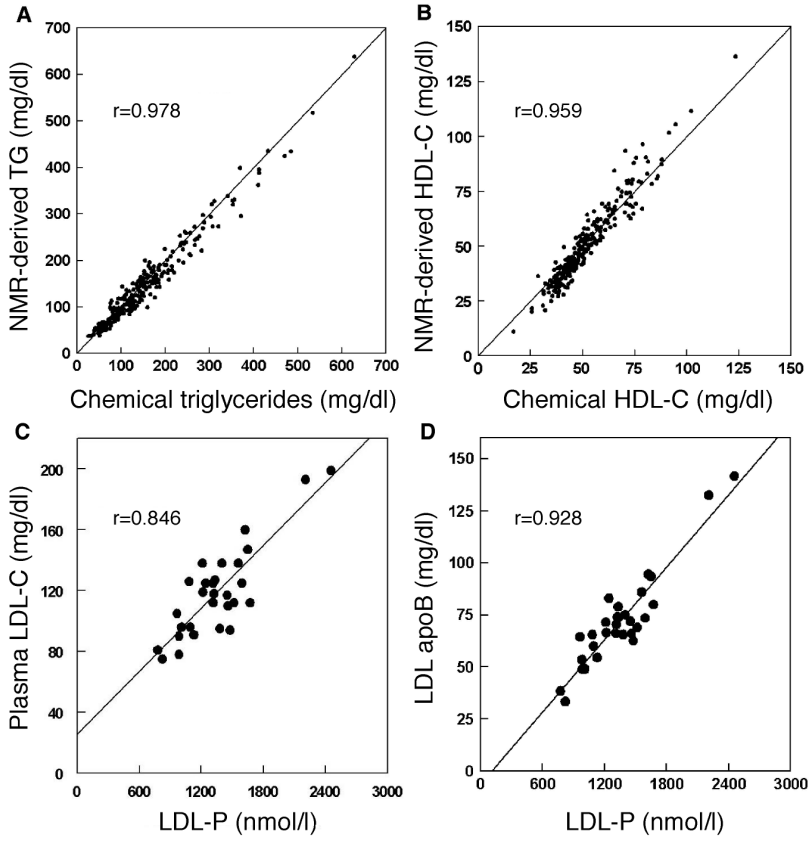

Figure 5.5 Comparison of NMR-derived and chemically measured triglyceride (A), HDL cholesterol (B), LDL cholesterol (C), and LDL apoB concentrations (D). In panels A and B, the line drawn is the identity (n=253). The regression lines and correlation coefficients are y=0.94x+2.7 and r=0.978 for triglycerides and y=1.16x-9.3 and r=0.959 for HDL cholesterol. The LDL cholesterol and apoB concentrations are obtained by first removing the VLDL fraction (density < 1.006 g/l) through ultracentrifugation and then either calculated as the difference between total plasma cholesterol and HDL cholesterol values in the bottom fraction or subjected to nephelometric apoB immunoassay giving correlation coefficients of r=0.928 and r=0.846, respectively (n=29). LDL-P, concentration of LDL particles. Panels A, B and D are reprinted from [206], Copyright (2010), with permission from Elsevier. Panel C is reprinted from [522] with permission.

The procedure from the total lipid concentration to the NMR-derived lipid concentration via particle concentration estimate utilises two conversion factors per lipoprotein subclass which are both obtained from the reference particle library. The first factor transforms the total lipid concentration to particle concentrations and is inversely proportional to the per particle lipid composition. The second factor, on the other hand, transforms the particle

concentration to the selected lipid concentration and is directly proportional to the per particle lipid composition. Therefore, when multiplying the factors together, the per particle lipid composition parameters will disappear. Thus the NMR-derived lipid concentrations do not include information of the estimated per particle composition which is the cornerstone of particle concentrations estimation and, therefore, cannot be used to validate the method. The NMR-derived lipid concentrations per se are, however, free of the errors made in per particle lipid composition estimation. They do, however, suffer from errors caused by small differences in the relative content of lipids within a subclass between an individual and the corresponding averaged reference particle library values.

Another approach that Otvos et al. have used to validate their method is to associate their estimated LDL particle concentration with chemically measured LDL apoB concentration. Since each LDL particle contains exactly one apoB molecule, this LDL apoB measure can be treated as experimental LDL particle concentration. The association of the experimental and the computational LDL particle concentration, shown in Fig. 5.5 D, is strong with a correlation coefficient r=0.93 (r^2=0.86), and thus the model of Otvos et al. seems valid for LDL. Also the biophysical consideration supports this view. As shown in Figure 3.1 in chapter 3, the conventional view of core lipids concerning triglycerides and cholesterol esters is the most valid for LDL particles as the estimated amounts of triglycerides and cholesterol esters in the surface of LDL particles were around 5 % and 10 %, respectively, and when all free cholesterol molecules are placed in the surface layer, these figures decrease to be less than a percent (data not shown).

However, as already mentioned above, the situation is different for VLDL and particularly for HDL particles. In VLDL, approximately 10 % of either triglycerides or cholesterol esters may still reside in the surface after adjustment of the effect of free cholesterol molecules. In HDL particles, the corresponding figure is approximately 20-30 %, leading to considerable errors in particle concentration estimation.

In conclusion, it has been shown within last decades that LDL cholesterol concentration is not the best lipoprotein-related measure to predict the cardiovascular disease risk of an individual[23, 470-473]. These observations have increased the interest in other lipoprotein measures, such as lipoprotein particle concentrations, in risk assessment. An NMR-based method, developed in the early 1990s by Otvos et al.[206, 522-526], has achieved wide attention in lipoprotein particle concentration estimation due to its success, particularly in the case of LDL, in several recent clinical trials[101, 116, 147-149, 412, 414, 415, 498, 530, 531]. However, the method is based on rather restrictive assumptions of lipo-

protein particle structures and may thus cause errors in the other lipoprotein particle concentration estimates. Thus there is a clear need for a method which takes into account the lipoprotein particle structures more properly. We aimed to fulfill this need and constructed such a model. The model per se requires information on the average particle size and full set of lipid concentrations in each lipoprotein subclass to estimate the particle concentrations. The method is also easily simplified to the use of total lipid concentrations leading only to small differences in VLDL, LDL and HDL particle concentrations as compared to the estimates obtained using the full set of lipid concentrations. The method may also be employed to generate a reference particle library for NMR-based lipoprotein particle estimation. The development of such an NMR-based method and demonstration of its features in clinical trials are currently under way.

6. Summary of the results

This thesis deals with various computational models and methods for lipoprotein research. The main contribution of this thesis is related to three important concepts concerning lipoprotein particles. These include structural, composition/metabolic and particle concentration issues. Experimentally isolated and biochemically characterised lipoprotein particles served as a starting point in all studies of this thesis.

The first study modelled the molecular structure, i.e., the location of triglyceride and cholesterol ester molecules, of the lipoprotein particles at subclass level. This modelling required also methods to estimate the number of lipids within a particle, i.e., composition information, as well as particle size estimates. The model was applied separately for 11 lipoprotein subclasses, i.e., VLDL1, VLDL2, IDL, LDL1, LDL2, LDL3, HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c} (very low-, intermediate-, low- and high-density lipoproteins, respectively), isolated by density gradient ultracentrifugation from 12 individuals. While triglycerides and cholesterol esters are widely considered as core lipids residing in the interior regions of lipoprotein particles, in this study, a significant portion of triglycerides was estimated to locate in the surface layer of large VLDL and, particularly, cholesterol esters in the surface layer of HDL particles. The results of the modelling gave intuitive and coherent structural rationale for the molecular interactions between the hydrophobic lipoprotein lipids and water-soluble lipolytic enzymes and lipid transporters.

The second study found several lipoprotein phenotypes with distinct, combined compositional and metabolic relationships. Data for this study included 302 blood samples representing a wide range of lipoprotein lipid values, and lipoprotein particles were isolated by ultracentrifugation into VLDL, IDL, LDL, HDL_2 and HDL_3 fractions. In the study, the self-organising map method was used for classification and visualisation of structural and metabolic relationships of lipoprotein particles, and both the plasma lipid concentrations, i.e., concentration variables, and the per particle compositions, i.e., composi-

tion variables, were used as the classification criteria. The composition variables, and also particle size estimates, were calculated according to the method introduced in the first study. When the self-organising map method was applied to the data, visible variation in the structural/metabolic relations between individuals was revealed. Accordingly, the individuals were classified into five distinct groups, i.e., lipoprotein phenotypes, with some individuals locating between the groups, thus supporting the validity of the *in silico* classification. By comparing the phenotypes, it became clear that the traditional lipid measures are not capable of distinguishing different lipoprotein phenotypes since the traditional lipid measures may be similar in many lipoprotein phenotypes although their particle compositions differ greatly. Similarly, the lipoprotein particle concentrations alone could not classify individuals properly.

The third study modelled lipoprotein particle concentrations in twenty lipoprotein subclasses. Data for the modelling included 100 blood samples from subjects selected to contain a wide range of different lipoprotein profiles, i.e., lipoprotein subclass concentrations. Lipoprotein particles were separated by high-performance liquid chromatography into two chylomicron subclasses, five VLDL subclasses, six LDL subclasses and seven HDL subclasses according to their size. For each lipoprotein subclass, lipoprotein particle concentration was estimated as the ratio of a lipid concentration in plasma and its amount in an average lipoprotein particle. The composition of averaged lipoprotein particles was again calculated accordingly to the method introduced in the first study. Contrary to the previously published model for lipoprotein particle estimates, which assumes that all triglyceride and cholesterol ester molecules reside in the core of lipoprotein particles, our model is structurally less restricted. For example, since our model is based on four (not two) experimentally measured lipoprotein lipid concentrations, our model does not require any assumption of location of the lipids within the particles. The third study provided also a reference particle library for NMR-based lipoprotein particle concentration estimation.

References

1. **Lusis, A. J.** *Atherosclerosis.* Nature, 407, 233-241*, 2000.*

2. **Vance, D. E. and Van den Bosch, H.**, *Cholesterol in the year 2000,* BBA-Molecular and Cell Biology of Lipids, 1529, 1-8*, 2000.*

3. **Hoefner, D. M.** *The cholesterol controversy continues,* MLO Med Lab Obs, 40, 24-32*, 2008.*

4. **Anitschkow, N.** *Ueber experimentelle choleserinsteatose und ihre bedeutung fur die entstehung einiger pathologischer prozesse.* Zentralbl Allg Pathol, 24, 1-9*, 1913.*

5. **Anitschkow, N.** *Ueber die veranderungen der kaninchenaorta bei experimenteller cholesterinsteatose.* Beitr Pathol Anat, 56, 379-404*, 1913.*

6. **Anitschkow, N.** *Experimental atherosclerosis in animals.*In Arteriosclerosis: A survey of the problem. (ed Cowdry, E. V.) 271-322 (Macmillan, New York, 1933).

7. **Steinberg, D.** *Thematic review series: the pathogenesis of atherosclerosis. An interpretive history of the cholesterol controversy: Part I.* J Lipid Res, 45, 1583-1593*, 2004.*

8. **Lehzen, G. and Knauss, K.**, *Ueber xanthoma multiplex planum, tuberosum, mollusciformis.* Virchows Arch A Pathol Anat Histol, 116, 85-104*, 1889.*

9. **Pinkus, F. and Pick, L.**, *Zur struktur und genese der symptomatischen xanthome,* Dtsch Med Wochenschr, 34, 1426-1430*, 1908.*

10. **Müller, C.** *Angina pectoris in hereditary xanthomatosis,* Arch Intern Med, 64, 675-700*, 1939.*

11. **Wilkinson, C. F.** *Essential familial hypercholesterolemia: cutaneous, metabolic and hereditary aspects,* Bull N Y Acad Med, 26, 670-685*, 1950.*

12. **Khachadurian, A. K.** *The inheritance of essential familial hypercholesterolemia,* Am J Med, 37, 402-407*, 1964.*

13. **Adlersberg, D., Parets, A. D. and Boas, E. P.**, *Genetics of atherosclerosis,* JAMA, 141, 246-254*, 1949.*

14. **Macheboeuf, M. A.** *Recherches sur les phosphoaminolipides et les sterides du serum et du plasma sanguins.* Bull Soc Chim Biol, 11, 268-293*, 1929.*

15. **Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. and Taylor, H. L.**, *Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids,* J Am Chem Soc, 68, 459-475*, 1946.*

16. **Oncley, J. L., Scatchard, G. and Brown, A.**, *Physical-chemical characteristics of certain of the proteins of normal human plasma.* J Phys Chem, 51, 184-198*, 1947.*

17. **Russ, E. M., Eder, H. A. and Barr, D. P.**, *Protein-lipid relationships in human plasma. I. In normal individuals,* Am J Med, 11, 468-479*, 1951.*

18. **Gofman, J. W., Lindgren, F. T. and Elliott, H.**, *Ultracentrifugal studies of lipoproteins of human serum,* J Biol Chem, 179, 973-979*, 1949*.

19. **Gofman, J. W., Lindgren, F., Elliott, H., Mantz, W., Hewitt, J., Strisower, B., Herring, V. and Lyon, T. P.**, *The role of lipids and lipoproteins in atherosclerosis,* Science, 111, 166-186*, 1950*.

20. **Berneis, K. K. and Krauss, R. M.**, *Metabolic origins and clinical significance of LDL heterogeneity,* J Lipid Res, 43, 1363-1379*, 2002*.

21. **Niemi, J., Mäkinen, V. P., Heikkonen, J., Tenkanen, L., Hiltunen, Y., Hannuksela, M. L., Jauhiainen, M., Forsblom, C., Taskinen, M. R. and Kesäniemi, Y. A.**, *Estimation of VLDL, IDL, LDL, HDL$_2$, apoA-I, and apoB from the Friedewald inputs-apoB and IDL, but not LDL, are associated with mortality in type 1 diabetes,* Ann Med, 41, 451-461*, 2009*.

22. **Williams, P. T. and Feldman, D. E.**, *Prospective study of coronary heart disease vs HDL2; HDL3; and other lipoproteins in Gofman's Livermore Cohort,* Atherosclerosis, 214, 196-202*, 2010*.

23. **Superko, H. R.** *Advanced lipoprotein testing and subfractionation are clinically useful,* Circulation, 119, 2383-2395*, 2009*.

24. **Gofman, J. W.** *Serum lipoproteins and the evaluation of atherosclerosis,* Ann N Y Acad Sci, 64, 590-595*, 1956*.

25. **Gofman, J. W., Hanig, M., Jones, H. B., Lauffer, M. A., Lawry, E. Y., Lewis, L. A. and Mann, G. V.**, *Evaluation of serum lipoprotein and cholesterol measurements as predictors of clinical complications of atherosclerosis: Report of a cooperative study of lipoproteins and atherosclerosis,* Circulation, 14, 689-741*, 1956*.

26. **Wilson, P. W., Garrison, R. J., Castelli, W. P., Feinleib, M., McNamara, P. M. and Kannel, W. B.**, *Prevalence of coronary heart disease in the Framingham Offspring Study: role of lipoprotein cholesterols,* Am J Cardiol, 46, 649-654*, 1980*.

27. **Kannel, W. B., Dawber, T. R., Kagan, A., Revotskie, N. and Stokes, J.**, *Factors of risk in the development of coronary heart disease—six year follow-up experience; the Framingham Study,* Ann Intern Med, 55, 33-50*, 1961*.

28. **Dawber, T. R., Moore, F. E. and Mann, G. V.**, *Coronary heart disease in the Framingham Study,* Am J Public Health, 47, 4-24*, 1957*.

29. **Loucks, E. B., Lynch, J. W., Pilote, L., Fuhrer, R., Almeida, N. D., Richard, H., Agha, G., Murabito, J. M. and Benjamin, E. J.**, *Life-course socioeconomic position and incidence of coronary heart disease: The Framingham Offspring Study,* Am J Epidemiol, 169, 829-836*, 2009*.

30. **Steinberg, D.** *Thematic review series: the pathogenesis of atherosclerosis. An interpretive history of the cholesterol controversy: part II: the early evidence linking hypercholesterolemia to coronary disease in humans,* J Lipid Res, 46, 179-190*, 2004*.

31. *Diet and the possible prevention of coronary atheroma; a council statement.* JAMA, 194, 1149-1150*, 1965*.

32. **Keys, A., Anderson, J. T., Fidanza, F., Keys, M. H. and Swahn, B.**, *Effects of diet on blood lipids in man particularly cholesterol and lipoproteins,* Clin Chem, 1, 34-52*, 1955*.

33. **Keys, A., Aravanis, C., Blackburn, H. W., Van Buchem, F. S., Buzina, R.,**

Djordjevic, B. D., Dontas, A. S., Fidanza, F., Karvonen, M. J., Kimura, N., Lekos, D., Monti, M., Puddu, V. and Taylor, H. L., *Epidemiological studies related to coronary heart disease: characteristics of men aged 40-59 in seven countries,* Acta Med Scand Suppl, 460, 1-392*, 1966*.

34. **Robertson, T. L., Kato, H., Rhoads, G. G., Kagan, A., Marmot, M., Syme, S. L., Gordon, T., Worth, R. M., Belsky, J. L., Dock, D. S., Miyanishi, M. and Kawamoto, S.**, *Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California. Incidence of myocardial infarction and death from coronary heart disease,* Am J Cardiol, 39, 239-243*, 1977*.

35. **Leren, P.** *The effect of plasma-cholesterol-lowering diet in male survivors of myocardial infarction. A controlled clinical trial.* Bull N Y Acad Med, 44, 1012-1020*, 1968*.

36. **Dayton, S., Pearce, M. L., Goldman, H., Harnish, A., Plotkin, D., Shickman, M., Winfield, M., Zager, A. and Dixon, W.**, *Controlled trial of a diet high in unsaturated fat for prevention of atherosclerotic complications,* Lancet, 2, 1060-1062*, 1968*.

37. **Miettinen, M., Turpeinen, O., Karvonen, M. J., Elosuo, R. and Paavilainen, E.**, *Effect of cholesterol-lowering diet on mortality from coronary heart-disease and other causes. A twelve-year clinical trial in men and women,* Lancet, 2, 835-838*, 1972*.

38. **Turpeinen, O.** *Diet and coronary events,* J Am Diet Assoc, 52, 209-213*, 1968*.

39. **Lees, R. S. and Hatch, F. T.**, *Sharper separation of lipoprotein species by paper electrophoresis in albumin-containing buffer,* J Lab Clin Med, 61, 518-528*, 1963*.

40. **Fredrickson, D. S., Levy, R. I. and Lees, R. S.**, *Fat transport in lipoproteins —an integrated approach to mechanisms and disorders,* N Engl J med, 276, 215-225*, 1967*.

41. **Fredrickson, D. S., Levy, R. I. and Lees, R. S.**, *Fat transport in lipoproteins —an integrated approach to mechanisms and disorders,* N Engl J Med, 276, 94-103*, 1967*.

42. **Fredrickson, D. S., Levy, R. I. and Lees, R. S.**, *Fat transport in lipoproteins —an integrated approach to mechanisms and disorders.* N Engl J med, 276, 34-42*, 1967*.

43. **Fredrickson, D. S. and Lees, R. S.**, *Editorial A System for Phenotyping Hyperlipoproteinemia,* Circulation, 31, 321-327*, 1965*.

44. **Griel Jr, L. C. and McCarthy, R. D.**, *Blood serum lipoproteins: A review,* J Dairy Sci, 52, 1233-1243*, 1969*.

45. **Fredrickson, D. S.** *Phenotyping. On reaching base camp (1950-1975),* Circulation, 87, III1-15*, 1993*.

46. **Friedewald, W. T., Levy, R. I. and Fredrickson, D. S.**, *Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge,* Clin Chem, 18, 499-502*, 1972*.

47. **Leary, T.** *Experimental atherosclerosis in the rabbit compared with human (coronary) atherosclerosis,* Arch Pathol, 17, 453-492*, 1934*.

48. **Duff, G. L. and McMillan, G. C.**, *Pathology of atherosclerosis,* Am J Med, 11,

92-108, *1951*.

49. **Poole, J. C. F. and Florey, H. W.**, *Changes in the endothelium of the aorta and the behaviour of macrophages in experimental atheroma of rabbits*. J Pathol Bacteriol, 75, 245-251, *1958*.

50. **Haust, M. D. and More, R. H.** *Significance of the smooth muscle cell in atherogenesis* In Evolution of the atherosclerotic plaque. (ed Jones, R. J.) 51-63 (The University of Chicago Press, Chicago, 1963).

51. **Armstrong, M. L., Warner, E. D. and Connor, W. E.**, *Regression of coronary atheromatosis in rhesus monkeys*. Circ Res, 27, 59-67, *1970*.

52. **Wissler, R. W. and Vesselinovitch, D.**, *Can atherosclerotic plaques regress? Anatomic and biochemical evidence from nonhuman animal models,* Am J Cardiol, 65, 33F-40F, *1990*.

53. **Benditt, E. P. and Benditt, J. M.**, *Evidence for a monoclonal origin of human atherosclerotic plaques,* Proc Natl Acad Sci USA, 70, 1753-1756, *1973*.

54. **Ross, R. and Glomset, J. A.**, *The pathogenesis of atherosclerosis (first of two parts)*. N Engl J Med, 295, 369-377, *1976*.

55. **Ross, R. and Glomset, J. A.**, *The pathogenesis of atherosclerosis (second of two parts),* N Engl J Med, 295, 420-425, *1976*.

56. **Ross, R., Glomset, J., Kariya, B. and Harker, L.**, *A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro*. Proc Natl Acad Sci USA, 71, 1207-1210, *1974*.

57. **Ross, R.** *Atherosclerosis-an inflammatory disease*. N Engl J med, 340, 115-126, *1999*.

58. **Ross, R.** *The pathogenesis of atherosclerosis — an update,* N Engl J Med, 314, 488-500, *1986*.

59. **Gerrity, R. G.** *The role of the monocyte in atherogenesis: II. Migration of foam cells from atherosclerotic lesions*. Am J Pathol, 103, 191-200, *1981*.

60. **Gerrity, R. G.** *The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions,* Am J Pathol, 103, 181-190, *1981*.

61. **Davies, P. F., Reidy, M. A., Goode, T. B. and Bowyer, D. E.**, *Scanning electron microscopy in the evaluation of endothelial integrity of the fatty lesion in atherosclerosis,* Atherosclerosis, 25, 125-130, *1976*.

62. **Goldstein, J. L., Hazzard, W. R., Schrott, H. G., Bierman, E. L. and Motulsky, A. G.**, *Hyperlipidemia in coronary heart disease. I. Lipid levels in 500 survivors of myocardial infarction,* J Clin Invest, 52, 1533-1543, *1973*.

63. **Goldstein, J. L., Schrott, H. G., Hazzard, W. R., Bierman, E. L. and Motulsky, A. G.**, *Hyperlipidemia in coronary heart disease II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia,* J Clin Invest, 52, 1544-1568, *1973*.

64. **Hazzard, W. R., Goldstein, J. L., Schrott, H. G., Motulsky, A. G. and Bierman, E. L.**, *Hyperlipidemia in coronary heart disease III. Evaluation of lipoprotein phenotypes of 156 genetically defined survivors of myocardial infarction,* J Clin Invest, 52, 1569-1577, *1973*.

65. **McGinley, J., Jones, H. and Gofman, J.**, *Lipoproteins and xanthomatous diseases,* J Invest Dermatol, 19, 71-82*, 1952*.

66. **Wilkinson, J., C.F., Hand, E. A. and Fliegelman, M. T.**, *Essential familial hypercholesterolemia*. Ann Intern Med, 29, 671-686*, 1948*.

67. **Brown, M. S., Dana, S. E., Dietschy, J. M. and Siperstein, M. D.**, *3-Hydroxy-3-methylglutaryl Coenzyme A Reductase. Solubilization and purification of a cold-sensitive mecrosomal enzyme*. J Biol Chem, 248, 4731-4738*, 1973*.

68. **Dietschy, J. M. and Wilson, J. D.**, *Regulation of cholesterol metabolism,* N Engl J Med, 282, 1179-1183*, 1970*.

69. **Goldstein, J. L. and Brown, M. S.**, *Familial hypercholesterolemia: identification of a defect in the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity associated with overproduction of cholesterol,* Proc Natl Acad Sci USA, 70, 2804-2808*, 1973*.

70. **Brown, M. S. and Goldstein, J. L.**, *Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity,* Proc Natl Acad Sci USA, 71, 788-792*, 1974*.

71. **Brown, M. S. and Goldstein, J. L.**, *Receptor-mediated control of cholesterol metabolism,* Science, 191, 150-154*, 1976*.

72. **Goldstein, L. J. and Brown, S. M.**, *The low-density lipoprotein pathway and its relation to atherosclerosis*. Annu Rev Biochem, 46, 897-930*, 1977*.

73. **Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S.**, *Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition*. Proc Natl Acad Sci USA, 76, 333-337*, 1979*.

74. **Henriksen, T., Evensen, S. A. and Carlander, B.**, *Injury to human endothelial cells in culture induced by low density lipoproteins*. Scand J Clin Lab Invest, 39, 361-368*, 1979*.

75. **Hessler, J. R., Robertson, A. L.,Jr and Chisolm, G. M.,3rd**, *LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture,* Atherosclerosis, 32, 213-229*, 1979*.

76. **Henriksen, T., Mahoney, E. M. and Steinberg, D.**, *Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins,* Proc Natl Acad Sci USA, 78, 6499-6503*, 1981*.

77. **Hessler, J. R., Morel, D. W., Lewis, L. J. and Chisolm, G. M.**, *Lipoprotein oxidation and lipoprotein-induced cytotoxicity,* Arterioscler Thromb Vasc Biol, 3, 215-222*, 1983*.

78. **Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L. and Steinberg, D.**, *Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids,* Proc Natl Acad Sci USA, 81, 3883-3887*, 1984*.

79. **Berliner, J. A. and Heinecke, J. W.**, *The role of oxidized lipoproteins in atherogenesis,* Free Radic Biol Med, 20, 707-727*, 1996*.

80. **Chisolm, G. M. and Steinberg, D.**, *The oxidative modification hypothesis of atherogenesis: an overview,* Free Radic Biol Med, 28, 1815-1826, *2000*.

81. **Navab, M., Berliner, J. A., Watson, A. D., Hama, S. Y., Territo, M. C., Lusis, A. J., Shih, D. M., Van Lenten, B. J., Frank, J. S. and Demer, L. L.**, *The Yin and Yang of oxidation in the development of the fatty streak a review based on the 1994 George Lyman Duff Memorial Lecture,* Arterioscler Thromb Vasc Biol, 16, 831-842, *1996*.

82. **Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L.**, *Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity,* N Engl J med, 320, 915-924, *1989*.

83. **Steinberg, D. and Witztum, J. L.**, *Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis?* Circulation, 105, 2107-2111, *2002*.

84. **Steinberg, D.** *The pathogenesis of atherosclerosis. An interpretive history of the cholesterol controversy, part IV: the 1984 coronary primary prevention trial ends it — almost,* J Lipid Res, 47, 1-14, *2006*.

85. **Lipid Research Clinics Program**. *The lipid research clinics coronary primary prevention trial results: I. Reduction in incidence of coronary heart disease,* J Am Med Assoc, 251, 351-364, *1984*.

86. **Lipid Research Clinics Program**. *The lipid research clinics coronary primary prevention trial results: II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering,* J Am Med Assoc, 251, 365-374, *1984*.

87. **Oliver, M. F.** *Hypercholesterolaemia and coronary heart disease: an answer.* Br Med J (Clin Res Ed), 288, 423-424, *1984*.

88. **Podell, R. N.** *Coronary disease prevention. Proof of the anticholesterol pudding,* Postgrad Med, 75, 193-196, *1984*.

89. **Simons, L. A.** *The lipid hypothesis is proven,* Med J Aust, 140, 316-317, *1984*.

90. **Steinberg, D., Blumenthal, S., Carleton, R. A., Chasen, N. H., Dalen, J. E., Fitzpatrick, J. T., Hulley, S. B., Mahley, R. W., O'Keefe, G. and Remington, R. D.**, *Consensus conference. Lowering blood cholesterol to prevent heart disease,* JAMA, 253, 2080-2086, *1985*.

91. **Expert Panel**. *Report of the National Cholesterol Education Program Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults,* Arch Intern Med, 148, 36-69, *1988*.

92. **Endo, A.** *The discovery and development of HMG-CoA reductase inhibitors,* J Lipid Res, 33, 1569-1582, *1992*.

93. **Fleming, A.** *Classics in infectious diseases: on the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae by Alexander Fleming, Reprinted from the British Journal of Experimental Pathology 10:226-236, 1929,* Rev Infect Dis, 2, 129-139, *1980*.

94. **Bentley, R.** *The development of penicillin,* Perspect Biol Med, 48, 444-452, *2005*.

95. **Endo, A., Kuroda, M. and Tsujita, Y.**, *ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by Penicillium citrinum,* J Antibiot, 29, 1346-1348, *1976*.

96. **Endo, A., Kuroda, M. and Tanzawa, K.**, *Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites having hypocholesterolemic activity,* FEBS Lett, 72, 323-326*, 1976*.

97. **Brown, M. S., Faust, J. R., Goldstein, J. L., Kaneko, I. and Endo, A.**, *Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase,* J Biol Chem, 253, 1121-1128*, 1978*.

98. **Ginter, E. and Simko, V.**, *Statins: The drugs for the 21st century?* Bratisl Lek Listy, 110, 664-668*, 2009*.

99. **Goldstein, J. L. and Brown, M. S.**, *The LDL receptor,* Arterioscler Thromb Vasc Biol, 29, 431-438*, 2009*.

100. **Brown, M. S. and Goldstein, J. L.**, *A receptor-mediated pathway for cholesterol homeostasis,* Science, 232, 34-47*, 1986*.

101. **Otvos, J. D., Shalaurova, I., Freedman, D. S. and Rosenson, R. S.**, *Effects of pravastatin treatment on lipoprotein subclass profiles and particle size in the PLAC-I trial.* Atherosclerosis, 160, 41-48*, 2002*.

102. **Scandinavian Simvastatin Survival Study Group**. *Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S).* Lancet, 344, 1383-1389*, 1994*.

103. **Sacks, F. M., Pfeffer, M. A., Moye, L. A., Rouleau, J. L., Rutherford, J. D., Cole, T. G., Brown, L., Warnica, J. W., Arnold, J. M. O. and Wun, C. C.**, *The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels,* N Engl J Med, 335, 1001-1009*, 1996*.

104. **Tonkin, A., Aylward, P., Colquhoun, D., Glasziou, P., Harris, P., MacMahon, S., Magnus, P., Newel, D., Nestel, P. and Sharpe, N.**, *Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels,* N Engl J Med, 339, 1349-1357*, 1998*.

105. **Shepherd, J., Cobbe, S. M., Ford, I., Isles, C. G., Lorimer, A. R., Macfarlane, P. W., McKillop, J. H. and Packard, C. J.**, *Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia,* N Engl J Med, 333, 1301-1308*, 1995*.

106. **Downs, J. R., Clearfield, M., Weis, S., Whitney, E., Shapiro, D. R., Beere, P. A., Langendorfer, A., Stein, E. A., Kruyer, W. and Gotto, J., A.M.**, *Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels results of AFCAPS/TexCAPS,* JAMA, 279, 1615-1622*, 1998*.

107. **Sacks, F. M., Tonkin, A. M., Shepherd, J., Braunwald, E., Cobbe, S., Hawkins, C. M., Keech, A., Packard, C., Simes, J. and Byington, R.**, *Effect of pravastatin on coronary disease events in subgroups defined by coronary risk factors the prospective pravastatin pooling project,* Circulation, 102, 1893-1900*, 2000*.

108. **Heart Protection Study Collaborative Group**. *MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial.* Lancet, 360, 7-22*, 2002*.

109. **Sever, P. S., Dahlöf, B., Poulter, N. R., Wedel, H., Beevers, G., Caulfield, M., Collins, R., Kjeldsen, S. E., Kristinsson, A. and McInnes, G. T.**, *Prevention of coronary and stroke events with atorvastatin in hypertensive pa-*

tients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial—Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial, 361, 1149-1158*, 2003*.

110. **Colhoun, H. M., Betteridge, D. J., Durrington, P. N., Hitman, G. A., W Neil, H. A., Livingstone, S. J., Thomason, M. J., Mackness, M. I., Charlton-Menys, V. and Fuller, J. H.**, *Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial,* 364, 685-696*, 2004*.

111. **Baigent, C., Keech, A., Kearney, P. M., Blackwell, L., Buck, G., Pollicino, C., Kirby, A., Sourjina, T., Peto, R., Collins, R. and Simes, J.**, *Efficacy and safety of cholesterol-lowering treatment: Prospective meta-analysis of data from 90056 participants in 14 randomised trials of statins,* Lancet, 366, 267-278*, 2005*.

112. **Thompson, A. and Temple, N. J.**, *The case for statins: has it really been made?* J R Soc Med, 97, 461-464*, 2004*.

113. **Steinberg, D.** *An interpretive history of the cholesterol controversy, part V: The discovery of the statins and the end of the controversy,* J Lipid Res, 47, 1339-1351*, 2006*.

114. **Vaughan, C. J., Murphy, M. B. and Buckley, B. M.**, *Statins do more than just lower cholesterol,* Lancet, 348, 1079-1082*, 1996*.

115. **Grundy, S. M.** *Statin trials and goals of cholesterol-lowering therapy,* Circulation, 97, 1436-1439*, 1998*.

116. **Freedman, D. S., Otvos, J. D., Jeyarajah, E. J., Barboriak, J. J., Anderson, A. J. and Walker, J. A.**, *Relation of lipoprotein subclasses as measured by proton nuclear magnetic resonance spectroscopy to coronary artery disease,* Arterioscler Thromb Vasc Biol, 18, 1046-1053*, 1998*.

117. **Cromwell, W. C. and Otvos, J. D.**, *Low-density lipoprotein particle number and risk for cardiovascular disease,* Curr Atheroscler Rep, 6, 381-387*, 2004*.

118. **Kwiterovich, P. O.** *Clinical relevance of the biochemical, metabolic, and genetic factors that influence low-density lipoprotein heterogeneity,* Am J Cardiol, 90, 30-47*, 2002*.

119. **Hansson, G. K.** *Inflammation, atherosclerosis, and coronary artery disease,* N Engl J Med, 352, 1685-1695*, 2005*.

120. **Libby, P., Ridker, P. M. and Maseri, A.**, *Inflammation and atherosclerosis,* Circulation, 105, 1135-1143*, 2002*.

121. **Adamson, J., Humphries, S. E., Ostergaard, J. R., Voldby, B., Richards, P. and Powell, J. T.**, *Are cerebral aneurysms atherosclerotic?* Stroke, 25, 963-966*, 1994*.

122. **Reed, D., Reed, C., Stemmermann, G. and Hayashi, T.**, *Are aortic aneurysms caused by atherosclerosis?* Circulation, 85, 205-211*, 1992*.

123. **Szabo, K., Kern, R., Gass, A., Hirsch, J. and Hennerici, M.**, *Acute stroke patterns in patients with internal carotid artery disease: a diffusion-weighted magnetic resonance imaging study,* Stroke, 32, 1323-1329*, 2001*.

124. **Alberti, K. G. M. M., Zimmet, P. and Shaw, J.**, *The metabolic syndrome—a new worldwide definition,* The Lancet, 366, 1059-1062*, 2005*.

125. **Hackam, D. G. and Anand, S. S.**, *Emerging risk factors for atherosclerotic vascular disease: a critical review of the evidence,* JAMA, 290, 932-940*, 2003*.

126. **Wilson, P. W. F., D'Agostino, R. B., Levy, D., Belanger, A. M., Silbershatz, H. and Kannel, W. B.**, *Prediction of coronary heart disease using risk factor categories,* Circulation, 97, 1837-1847*, 1998*.

127. **Park, Y. W., Zhu, S., Palaniappan, L., Heshka, S., Carnethon, M. R. and Heymsfield, S. B.**, *The metabolic syndrome prevalence and associated risk factor findings in the US population from the third National Health and Nutrition Examination Survey, 1988-1994,* Arch Intern Med, 163, 427-436*, 2003*.

128. **Cameron, A. J., Shaw, J. E. and Zimmet, P. Z.**, *The metabolic syndrome: prevalence in worldwide populations,* Endocrinol Metab Clin North Am, 33, 351-375*, 2004*.

129. **Ford, E. S., Giles, W. H. and Dietz, W. H.**, *Prevalence of the metabolic syndrome among US adults findings from the third national health and nutrition examination survey,* JAMA, 287, 356-359*, 2002*.

130. **Klatsky, A. L.** *Alcohol and cardiovascular health,* Physiol Behav, 100, 76-81*, 2010*.

131. **Antonopoulos, S.** *Third report of the National Cholesterol Education Program (NCEP) Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report.* Circulation, 106, 3143-3421*, 2002*.

132. **Jobs, C.** *JBS 2: Joint British Societies' guidelines on prevention of cardiovascular disease in clinical practice,* Heart, 91, v1-v52*, 2005*.

133. **Graham, I., Atar, D., Borch-Johnsen, K., Boysen, G., Burell, G., Cifkova, R., Dallongeville, J., De Backer, G., Ebrahim, S. and Gjelsvik, B.**, *European guidelines on cardiovascular disease prevention in clinical practice: executive summary,* Eur Heart J, 28, 2375-2414*, 2007*.

134. **McPherson, R., Frohlich, J., Fodor, G. and Genest, J.**, *Canadian Cardiovascular Society position statement–recommendations for the diagnosis and treatment of dyslipidemia and prevention of cardiovascular disease,* Can J Cardiol, 22, 913-927*, 2006*.

135. **Teramoto, T., Sasaki, J., Ueshima, H., Egusa, G., Kinoshita, M., Shimamoto, K., Daida, H., Biro, S., Hirobe, K., Funahashi, T., Yokote, K. and Yokode, M.**, *Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese,* J Atheroscler Thromb, 14, 45-50*, 2007*.

136. **Suomalaisen lääkäriseuran Duodecimin ja Suomen sisätautilääkärien yhdistys ry:n asettama työryhmä**. *Käypä hoito-suositus: Dyslipidemiat (2009).*

137. **Vartiainen, E., Laatikainen, T., Salomaa, V., Jousilahti, P., Peltonen, M. and Puska, P.**, *Sydäninfarkti- ja aivohalvausriskin arviointi FINRISKI-tutkimuksessa,* Suom Lääkäril, 48, 4507-4513*, 2007*.

138. **Cannon, C. P., Braunwald, E., McCabe, C. H., Rader, D. J., Rouleau, J. L., Belder, R., Joyal, S. V., Hill, K. A., Pfeffer, M. A. and Skene, A. M.**, *Intensive versus moderate lipid lowering with statins after acute coronary syndromes,* N Engl J Med, 350, 1495-1504*, 2004*.

139. **American Diabetes Association**. *Standards of medical care in diabetes-2006,* Diabetes Care, 29, 4-42*, 2006*.

140. **Shepherd, J., Barter, P., Carmena, R., Deedwania, P., Fruchart, J. C., Haffner, S., Hsia, J., Breazna, A., LaRosa, J. and Grundy, S.**, *Effect of lowering LDL cholesterol substantially below currently recommended levels in patients with coronary heart disease and diabetes,* Diabetes Care, 29, 1220-1226*, 2006*.

141. **Barter, P. J., Ballantyne, C. M., Carmena, R., Cabezas, M. C., Chapman, M., Couture, P., De Graaf, J., Durrington, P. N., Faergeman, O. and Frohlich, J.**, *Apo B versus cholesterol in estimating cardiovascular risk and in guiding therapy: report of the thirty-person/ten-country panel.* J Intern Med, 259, 247-258*, 2006*.

142. **Walldius, G. and Jungner, I.**, *The apoB/apoA-I ratio: a strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy-a review of the evidence*. J Intern Med, 259, 493-519*, 2006*.

143. **Thompson, A. and Danesh, J.**, *Associations between apolipoprotein B, apolipoprotein AI, the apolipoprotein B/AI ratio and coronary heart disease: a literature-based meta-analysis of prospective studies,* J Intern Med, 259, 481-492*, 2006*.

144. **Sharrett, A. R., Ballantyne, C. M., Coady, S. A., Heiss, G., Sorlie, P. D., Catellier, D. and Patsch, W.**, *Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein (a), apolipoproteins A-I and B, and HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study,* Circulation, 104, 1108-1113*, 2001*.

145. **Ingelsson, E., Schaefer, E. J., Contois, J. H., McNamara, J. R., Sullivan, L., Keyes, M. J., Pencina, M. J., Schoonmaker, C., Wilson, P. W. F. and D'Agostino, R. B.**, *Clinical utility of different lipid measures for prediction of coronary heart disease in men and women,* JAMA, 298, 776-785*, 2007*.

146. **Mora, S., Otvos, J. D., Rifai, N., Rosenson, R. S., Buring, J. E. and Ridker, P. M.**, *Lipoprotein particle profiles by nuclear magnetic resonance compared with standard lipids and apolipoproteins in predicting incident cardiovascular disease in women,* Circulation, 119, 931-939*, 2009*.

147. **Blake, G. J., Otvos, J. D., Rifai, N. and Ridker, P. M.**, *LDL particle concentration and size as determined by NMR spectroscopy as predictors of cardiovascular disease in women,* Circulation, 106, 1930-1937*, 2002*.

148. **Mora, S., Szklo, M., Otvos, J. D., Greenland, P., Psaty, B. M., Goff, D. C., O'Leary, D. H., Saad, M. F., Tsai, M. Y. and Sharrett, A. R.**, *LDL particle subclasses, LDL particle size, and carotid atherosclerosis in the Multi-Ethnic Study of Atherosclerosis (MESA),* Atherosclerosis, 192, 211-217*, 2007*.

149. **El Harchaoui, K., van der Steeg, W. A., Stroes, E. S. G., Kuivenhoven, J. A., Otvos, J. D., Wareham, N. J., Hutten, B. A., Kastelein, J. J. P., Khaw, K. T. and Boekholdt, S. M.**, *Value of low-density lipoprotein particle number and size as predictors of coronary artery disease in apparently healthy men and women: The EPIC-Norfolk Prospective Population Study,* J Am Coll Cardiol, 49, 547-553*, 2007*.

150. **Cromwell, W. C., Otvos, J. D., Keyes, M. J., Pencina, M. J., Sullivan, L., Vasan, R. S., Wilson, P. W. F. and D'Agostino, R. B.**, *LDL particle number and risk of future cardiovascular disease in the Framingham Offspring Study—Implications for LDL management,* J Clin Lipidol, 1, 583-592*, 2007*.

151. **Rizzo, M. and Berneis, K.**, *Low-density lipoprotein size and cardiovascular risk assessment,* QJM, 99, 1-14*, 2006*.

152. **Navab, M., Anantharamaiah, G. M., Reddy, S. T., Van Lenten, B. J., Ansell, B. J. and Fogelman, A. M.**, *Mechanisms of disease: proatherogenic HDL—an evolving field,* Nat Rev Endocrinol, 2, 504-511*, 2006*.

153. **Hansel, B., Giral, P., Nobecourt, E., Chantepie, S., Bruckert, E., Chapman, M. J. and Kontush, A.**, *Metabolic syndrome is associated with elevated oxidative stress and dysfunctional dense high-density lipoprotein particles displaying impaired antioxidative activity,* J Clin Endocrinol Metab, 89, 4963-4971*, 2004*.

154. **Jonas, A.** *Lipoprotein structure* In Biochemistry of lipids, lipoproteins and membranes, 4th edition. (eds Vance, D. E. and Vance, J. E.) 483-504 (Elsevier, The Netherlands, 2002).

155. **Herbert, P. N., Gotto Jr, A. M. and Fredrickson, D. S.** *Familial lipoprotein deficiency* In The metabolic basis of inherited disease. (eds Stanbury, J. B., Wyngaarden, J. B. and Fredrickson, D. S.) 544-588 (McGraw-Hill, New York, 1978).

156. **Harris, N., Neufeld, E. J., Newburger, J. W., Ticho, B., Baker, A., Ginsburg, G. S., Rimm, E. and Rifai, N.**, *Analytical performance and clinical utility of a direct LDL-cholesterol assay in a hyperlipidemic pediatric population,* Clin Chem, 42, 1182-1188*, 1996*.

157. **Gage, S. H. and Fish, P. A.**, *Fat digestion, absorption, and assimilation in man and animals as determined by the dark-field microscope, and a fat-soluble dye.* Am J Anat, 34, 1-85*, 1924*.

158. **Eisenberg, S.** *High-density lipoprotein metabolism* In Lipoproteins in health and disease. 71-85 (Arnold, London, 1999).

159. **Graham, J. M., Griffin, B. A., Davies, I. G. and Higgins, J. A.** *Fractionation of lipoprotein subclasses in self-generated gradients of iodixanol* In Atherosclerosis. (ed Drew, A. F.) 51-59 (Springer, 2001).

160. **Barter, P., Kastelein, J., Nunn, A. and Hobbs, R.**, *High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions,* Atherosclerosis, 168, 195-211*, 2003*.

161. **Vance, J. E.** *Assembly and secretion of lipoproteins* In Biochemistry of lipids, lipoproteins and membranes, 4th edition. (eds Vance, D. E. and Vance, J. E.) 505-526 (Elsevier, The Netherlands, 2002).

162. **Warnick, G. R., Benderson, J. and Albers, J. J.**, *Dextran sulfate-Mg^{2+} precipitation procedure for quantitation of high-density-lipoprotein cholesterol,* Clin Chem, 28, 1379-1388*, 1982*.

163. **Warnick, G. R. and Albers, J. J.**, *A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol,* J Lipid Res, 19, 65-76*, 1978*.

164. **Asztalos, B. F., Sloop, C. H., Wong, L. and Roheim, P. S.**, *Two-dimensional electrophoresis of plasma lipoproteins: recognition of new apo A-I-containing subpopulations,* Biochim Biophys Acta, 1169, 291-300*, 1993*.

165. **Asztalos, B. F. and Roheim, P. S.**, *Presence and formation of free apolipoprotein AI–like particles in human plasma,* Arterioscler Thromb Vasc Biol, 15, 1419-1423*, 1995*.

166. **Noble, R. P.** *Electrophoretic separation of plasma lipoproteins in agarose gel,* 9, 693-700*, 1968*.

167. **Sparks, D. L. and Phillips, M. C.**, *Quantitative measurement of lipoprotein surface charge by agarose gel electrophoresis,* J Lipid Res, 33, 123-130*, 1992*.

168. **Krauss, R. M. and Burke, D. J.**, *Identification of multiple subclasses of plasma low density lipoproteins in normal humans,* J Lipid Res, 23, 97-104*, 1982*.

169. **McNamara, J. R., Campos, H., Ordovas, J. M., Peterson, J., Wilson, P. W. and Schaefer, E. J.**, *Effect of gender, age, and lipid status on low density lipoprotein subfraction distribution. Results from the Framingham Offspring Study,* Arterioscler Thromb Vasc Biol, 7, 483-490*, 1987*.

170. **Musliner, T. A., Giotas, C. and Krauss, R. M.**, *Presence of multiple subpopulations of lipoproteins of intermediate density in normal subjects,* Arterioscler Thromb Vasc Biol, 6, 79-87*, 1986*.

171. **Meyer, B. J., Caslake, M. J., McConnell, M. M. and Packard, C. J.**, *Two subpopulations of intermediate density lipoprotein and their relationship to plasma triglyceride and cholesterol levels,* Atherosclerosis, 153, 355-362*, 2000*.

172. **Krauss, R. M. and Burke, D. J.**, *Identification of multiple subclasses of plasma low density lipoproteins in normal humans.* J Lipid Res, 23, 97-104*, 1982*.

173. **Nichols, A. V., Krauss, R. M. and Musliner, T. A.**, *Nondenaturing polyacrylamide gradient gel electrophoresis,* Methods Enzymol, 128, 417-431*, 1986*.

174. **McNamara, J. R., Small, D. M., Li, Z. and Schaefer, E. J.**, *Differences in LDL subspecies involve alterations in lipid composition and conformational changes in apolipoprotein B.* J Lipid Res, 37, 1924-1935*, 1996*.

175. **Li, Z., McNamara, J. R., Ordovas, J. M. and Schaefer, E. J.**, *Analysis of high density lipoproteins by a modified gradient gel electrophoresis method.* J Lipid Res, 35, 1698-1711*, 1994*.

176. **Blanche, P. J., Gong, E. L., Forte, T. M. and Nichols, A. V.**, *Characterization of human high-density lipoproteins by gradient gel electrophoresis,* Biochim Biophys Acta, 665, 408-419*, 1981*.

177. **Warnick, G. R., McNamara, J. R., Boggess, C. N., Clendenen, F., Williams, P. T. and Landolt, C. C.**, *Polyacrylamide gradient gel electrophoresis of lipoprotein subclasses,* Clin Lab Med, 26, 803-846*, 2006*.

178. **Asztalos, B. F., Collins, D., Cupples, L. A., Demissie, S., Horvath, K. V., Bloomfield, H. E., Robins, S. J. and Schaefer, E. J.**, *Value of high-density lipoprotein (HDL) subpopulations in predicting recurrent cardiovascular events in the Veterans Affairs HDL Intervention Trial,* Arterioscler Thromb Vasc Biol, 25, 2185-2191*, 2005*.

179. **Asztalos, B. F., Horvath, K. V., Kajinami, K., Nartsupha, C., Cox, C. E., Batista, M., Schaefer, E. J., Inazu, A. and Mabuchi, H.**, *Apolipoprotein composition of HDL in cholesteryl ester transfer protein deficiency,* J Lipid Res, 45, 448-455*, 2003*.

180. **Asztalos, B. F., Horvath, K. V., McNamara, J. R., Roheim, P. S., Rubinstein, J. J. and Schaefer, E. J.**, *Comparing the effects of five different statins on the HDL subpopulation profiles of coronary heart disease patients,* Atherosclerosis, 164, 361-369*, 2002*.

181. **Asztalos, B. F., Horvath, K. V., McNamara, J. R., Roheim, P. S., Rubinstein, J. J. and Schaefer, E. J.**, *Effects of atorvastatin on the HDL subpopulation*

profile of coronary heart disease patients, J Lipid Res, 43, 1701-1707*, 2002*.

182. **Nanjee, M. N., Doran, J. E., Lerch, P. G. and Miller, N. E.**, *Acute effects of intravenous infusion of ApoA1/phosphatidylcholine discs on plasma lipoproteins in humans,* Arterioscler Thromb Vasc Biol, 19, 979-989*, 1999*.

183. **Nanjee, M. N., Cooke, C. J., Olszewski, W. L. and Miller, N. E.**, *Concentrations of electrophoretic and size subclasses of apolipoprotein A-I-containing particles in human peripheral lymph,* Arterioscler Thromb Vasc Biol, 20, 2148-2155*, 2000*.

184. **Alaupovic, P.** *The concept of apolipoprotein-defined lipoprotein families and its clinical significance,* Curr Atheroscler Rep, 5, 459-467*, 2003*.

185. **Cheung, M. C. and Albers, J. J.**, *Characterization of lipoprotein particles isolated by immunoaffinity chromatography. Particles containing A-I and A-II and particles containing A-I but no A-II,* J Biol Chem, 259, 12201-12209*, 1984*.

186. **Nichols, A. V., Blanche, P. J., Shore, V. G. and Gong, E. L.**, *Conversion of apolipoprotein-specific high-density lipoprotein populations during incubation of human plasma,* Biochim Biophys Acta, 1001, 325-337*, 1989*.

187. **Cheung, M. C. and Albers, J. J.**, *Distribution of high density lipoprotein particles with different apoprotein composition: particles with AI and A-II and particles with AI but no A-II,* J Lipid Res, 23, 747-753*, 1982*.

188. **Warnick, G. R., McNamara, J. R., Boggess, C. N., Clendenen, F., Williams, P. T. and Landolt, C. C.**, *Polyacrylamide gradient gel electrophoresis of lipoprotein subclasses,* Clin Lab Med, 26, 803-846*, 2006*.

189. **Asztalos, B. F., Lefevre, M., Foster, T. A., Tulley, R., Windhauser, M., Wong, L. and Roheim, P. S.**, *Normolipidemic subjects with low HDL cholesterol levels have altered HDL subpopulations,* Arterioscler Thromb Vasc Biol, 17, 1885-1893*, 1997*.

190. **Huang, Y., von Eckardstein, A., Wu, S., Maeda, N. and Assmann, G.**, *A plasma lipoprotein containing only apolipoprotein E and with gamma mobility on electrophoresis releases cholesterol from cells,* Proc Natl Acad Sci USA, 91, 1834-1838*, 1994*.

191. **von Eckardstein, A., Huang, Y., Wu, S., Sarmadi, A. S., Schwarz, S., Steinmetz, A. and Assmann, G.**, *Lipoproteins containing apolipoprotein A-IV but not apolipoprotein AI take up and esterify cell-derived cholesterol in plasma,* Arterioscler Thromb Vasc Biol, 15, 1755-1763*, 1995*.

192. **Barth, H. G., Boyes, B. E. and Jackson, C.**, *Size exclusion chromatography,* Anal Chem, 68, 445-466*, 1996*.

193. **Mori, S.and Barth, H. G.** In *Size exclusion chromatography* (Springer Verlag, Berlin, 1999).

194. **Usui, S., Hara, Y., Hosaki, S. and Okazaki, M.**, *A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC,* J Lipid Res, 43, 805-814*, 2002*.

195. **Okazaki, M., Usui, S., Ishigami, M., Sakai, N., Nakamura, T., Matsuzawa, Y. and Yamashita, S.**, *Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in high-performance liquid chromatography,* Arterioscler Thromb Vasc Biol, 25, 578-584*, 2005*.

196. **Busbee, D. L., Payne, D. M., Jasheway, D. W., Carlisle, S. and Lacko, A. G.**, *Separation and detection of lipoproteins in human serum by use of size-exclusion liquid chromatography: a preliminary report,* Clin Chem, 27, 2052-2058*, 1981*.

197. **Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.**, *Protein measurement with the folin phenol reagent,* J Biol Chem, 193, 265-275*, 1951*.

198. **Markwell, M. A. K., Haas, S. M., Bieber, L. L. and Tolbert, N. E.**, *A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples,* Anal Biochem, 87, 206-210*, 1978*.

199. **Postiglione, L., Spanò, A., Varricchio, P., Larizza, G., Oriente, A., Gattozzi, D. and Oriente, P.**, *Serum A1 and B apolipoprotein determination: comparison of an immunoturbidimetric method with a monoclonal-antibody-based radial immunodiffusion assay,* Int J Clin Lab Res, 21, 318-320*, 1992*.

200. **Ikeda, T., Shibuya, Y., Senba, U., Sugiuchi, H., Araki, S., Uji, Y. and Okabe, H.**, *Automated immunoturbidimetric analysis of six plasma apolipoproteins: correlation with radial immunodiffusion assays,* J Clin Lab Anal, 5, 90-95*, 1991*.

201. **Warnick, G. R., Nauck, M. and Rifai, N.**, *Evolution of methods for measurement of HDL-cholesterol: from ultracentrifugation to homogeneous assays,* Clin Chem, 47, 1579-1596*, 2001*.

202. **Miller, W. G., Waymack, P. P., Anderson, F. P., Ethridge, S. F. and Jayne, E. C.**, *Performance of four homogeneous direct methods for LDL-cholesterol,* Clin Chem, 48, 489-498*, 2002*.

203. **Miller, W. G., Myers, G. L., Sakurabayashi, I., Bachmann, L. M., Caudill, S. P., Dziekonski, A., Edwards, S., Kimberly, M. M., Korzun, W. J. and Leary, E. T.**, *Seven direct methods for measuring HDL and LDL cholesterol compared with ultracentrifugation reference measurement procedures,* Clin Chem, 56, 977-986*, 2010*.

204. **Ala-Korpela, M.** *^{1}H NMR spectroscopy of human blood plasma,* Prog Nucl Magn Reson Spectrosc, 27, 475-554*, 1995*.

205. **Otvos, J. D., Jeyarajah, E. J. and Cromwell, W. C.**, *Measurement issues related to lipoprotein heterogeneity,* Am J Cardiol, 90, 22i-29i*, 2002*.

206. **Jeyarajah, E. J., Cromwell, W. C. and Otvos, J. D.**, *Lipoprotein particle analysis by nuclear magnetic resonance spectroscopy,* Clin Lab Med, 26, 847-870*, 2006*.

207. **Witte, D. R., Taskinen, M. R., Perttunen-Nio, H., Van Tol, A., Livingstone, S. and Colhoun, H. M.**, *Study of agreement between LDL size as measured by nuclear magnetic resonance and gradient gel electrophoresis,* J Lipid Res, 45, 1069-1076*, 2004*.

208. **Steim, J. M., Edner, O. J. and Bargoot, F. G.**, *Structure of human serum lipoproteins: nuclear magnetic resonance supports a micellar model,* Science, 162, 909-911*, 1968*.

209. **Ginsberg, H. N.** *Lipoprotein physiology,* Endocrinol Metab Clin North Am, 27, 503-519*, 1998*.

210. **Hevonoja, T., Pentikäinen, M. O., Hyvönen, M. T., Kovanen, P. T. and Ala-Korpela, M.**, *Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL,* Biochim Biophys Acta, 1488, 189-

210, *2000*.

211. **Segrest, J. P., Jones, M. K., De Loof, H. and Dashti, N.**, *Structure of apolipoprotein B-100 in low density lipoproteins,* J Lipid Res, 42, 1346-1367, *2001*.

212. **Sherman, M. B., Orlova, E. V., Decker, G. L., Chiu, W. and Pownall, H. J.**, *Structure of triglyceride-rich human low-density lipoproteins according to cryo-electron microscopy,* Biochemistry, 42, 14988-14993, *2003*.

213. **Forte, T. and Nichols, A. V.**, *Application of electron microscopy to the study of plasma lipoprotein structure,* Adv Lipid Res, 10, 1-41, *1972*.

214. **Morrisett, J. D., Jackson, R. L. and Gotto, A. M.,Jr**, *Lipid-protein interactions in the plasma lipoproteins,* Biochim Biophys Acta, 472, 93-133, *1977*.

215. **Shen, B. W., Scanu, A. M. and Kezdy, F. J.**, *Structure of human serum lipoproteins inferred from compositional analysis,* Proc Natl Acad Sci USA, 74, 837-841, *1977*.

216. **Prassl, R. and Laggner, P.**, *Molecular structure of low density lipoprotein: current status and future challenges,* Eur Biophys J, 38, 145-158, *2009*.

217. **Ohvo-Rekilä, H., Ramstedt, B., Leppimäki, P. and Peter Slotte, J.**, *Cholesterol interactions with phospholipids in membranes,* Prog Lipid Res, 41, 66-97, *2002*.

218. **Aittoniemi, J., Niemelä, P. S., Hyvönen, M. T., Karttunen, M. and Vattulainen, I.**, *Insight into the putative specific interactions between cholesterol, sphingomyelin, and palmitoyl-oleoyl phosphatidylcholine,* Biophys J, 92, 1125-1137, *2007*.

219. **Yeagle, P. L. and Young, J. E.**, *Factors contributing to the distribution of cholesterol among phospholipid vesicles,* J Biol Chem, 261, 8175-8181, *1986*.

220. **Yeagle, P. L., Hutton, W. C., Huang, C. H. and Martin, R. B.**, *Headgroup conformation and lipid-cholesterol association in phosphatidylcholine vesicles: a ^{31}P (^{1}H) nuclear Overhauser effect study,* Proc. Natl. Acad. Sci. USA, 72, 3477-3481, *1975*.

221. **Yeagle, P. L., Hutton, W. C., Huang, C. H. and Martin, R. B.**, *Phospholipid head-group conformations; intermolecular interactions and cholesterol effects,* Biochemistry (N. Y.), 16, 4344-4349, *1977*.

222. **Wu, W. E. N. G., Leu, S. W. E. N., Hsieh, C. H. and Chi, L. M.**, *The order of phosphatidylcholine headgroup in sonicated cholesterol/phospholipid vesicles as revealed by ^{1}H-NMR,* Chem. Phys. Lipids, 58, 241-248, *1991*.

223. **Brown, M. F. and Seelig, J.**, *Influence of cholesterol on the polar region of phosphatidylcholine and phosphatidylethanolamine bilayers,* Biochemistry (N. Y.), 17, 381-384, *1978*.

224. **Mattjus, P. and Slotte, J. P.**, *Does cholesterol discriminate between sphingomyelin and phosphatidylcholine in mixed monolayers containing both phospholipids?* Chem. Phys. Lipids, 81, 69-80, *1996*.

225. **Yeagle, P. L.** *Cholesterol and the cell membrane,* Biochim Biophys Acta, 822, 267-287, *1985*.

226. **Pregetter, M., Prassl, R., Schuster, B., Kriechbaum, M., Nigon, F., Chapman, J. and Laggner, P.**, *Microphase separation in low density lipoproteins.*

Evidence for a fluid triglyceride core below the lipid melting transition, J Biol Chem, 274, 1334-1341*, 1999*.

227. **Deckelbaum, R. J., Shipley, G. G., Small, D. M., Lees, R. S. and George, P. K.**, *Thermal transitions in human plasma low density lipoproteins,* Science, 190, 392-394*, 1975*.

228. **Atkinson, D., Deckelbaum, R. J., Small, D. M. and Shipley, G. G.**, *Structure of human plasma low-density lipoproteins: molecular organization of the central core,* Proc Natl Acad Sci USA, 74, 1042-1046*, 1977*.

229. **Laggner, P., Degovics, G., Müller, K. W., Glatter, O., Kratky, O., Kostner, G. and Holasek, A.**, *Molecular packing and fluidity of lipids in human serum low density lipoproteins,* Hoppe Seylers Z Physiol Chem, 358, 771-778*, 1977*.

230. **Müller, K., Laggner, P., Glatter, O. and Kostner, G. M.**, *The structure of human-plasma low-density lipoprotein B,* Eur J Biochem, 82, 73-90*, 1978*.

231. **Saito, H., Minamida, T., Arimoto, I., Handa, T. and Miyajima, K.**, *Physical states of surface and core lipids in lipid emulsions and apolipoprotein binding to the emulsion surface,* J Biol Chem, 271, 15515-15520*, 1996*.

232. **Lund-Katz, S. and Phillips, M. C.**, *Packing of cholesterol molecules in human high-density lipoproteins,* Biochemistry, 23, 1130-1138*, 1984*.

233. **Lund-Katz, S. and Phillips, M. C.**, *Packing of cholesterol molecules in human low-density lipoprotein,* Biochemistry, 25, 1562-1568*, 1986*.

234. **Spooner, P. J. R. and Small, D. M.**, *Effect of free cholesterol on incorporation of triolein in phospholipid bilayers,* Biochemistry, 26, 5820-5825*, 1987*.

235. **Spooner, P. J., Hamilton, J. A., Gantz, D. L. and Small, D. M.**, *The effect of free cholesterol on the solubilization of cholesteryl oleate in phosphatidylcholine bilayers: a 13C-NMR study,* Biochim Biophys Acta, 860, 345-353*, 1986*.

236. **Deckelbaum, R. J., Hamilton, J. A., Moser, A., Bengtsson-Olivecrona, G., Butbul, E., Carpentier, Y. A., Gutman, A. and Olivecrona, T.**, *Medium-chain versus long-chain triacylglycerol emulsion hydrolysis by lipoprotein lipase and hepatic lipase: implications for the mechanisms of lipase action,* Biochemistry, 29, 1136-1142*, 1990*.

237. **Hamilton, J. A. and Small, D. M.**, *Solubilization and localization of triolein in phosphatidylcholine bilayers: A ^{13}C NMR Study,* Proc Natl Acad Sci USA, 78, 6878-6882*, 1981*.

238. **Hamilton, J. A., Miller, K. W. and Small, D. M.**, *Solubilization of triolein and cholesteryl oleate in egg phosphatidylcholine vesicles,* J Biol Chem, 258, 12821-12826*, 1983*.

239. **Hamilton, J. A., Vural, J. M., Carpentier, Y. A. and Deckelbaum, R. J.**, *Incorporation of medium chain triacylglycerols into phospholipid bilayers: effect of long chain triacylglycerols, cholesterol, and cholesteryl esters,* J Lipid Res, 37, 773-782*, 1996*.

240. **Li, R., Schmidt, W., Rankin, S., Walzem, R. L. and Boyle-Roden, E.**, *Solubilization of acyl heterogeneous triacylglycerol in phosphatidylcholine vesicles,* J Agric Food Chem, 51, 477-482*, 2003*.

241. **Boyle-Roden, E. and Khan, M. A.**, *Quantitative analysis of surface-located*

triacylglycerol in intact emulsion particles, J Agric Food Chem, 49, 2014-2021, *2001*.

242. **Miller, K. W. and Small, D. M.**, *Surface-to-core and interparticle equilibrium distributions of triglyceride-rich lipoprotein lipids,* J Biol Chem, 258, 13772-13784, *1983*.

243. **Boyle-Roden, E. and Walzem, R. L.**, *Integral apolipoproteins increase surface-located triacylglycerol in intact native apoB-100-containing lipoproteins,* J Lipid Res, 46, 1624-1632, *2005*.

244. **Li, Q. T. and Sawyer, W. H.**, *Effect of unesterified cholesterol on the compartmentation of a fluorescent cholesteryl ester in a lipoprotein-like lipid microemulsion,* J Lipid Res, 33, 503-512, *1992*.

245. **Luo, C. C., Li, W. H., Moore, M. N. and Chan, L.**, *Structure and evolution of the apolipoprotein multigene family,* J Mol Biol, 187, 325-340, *1986*.

246. **Yang, C. Y., Chen, S. H., Gianturco, S. H., Bradley, W. A., Sparrow, J. T., Tanimura, M., Li, W. H., Sparrow, D. A., DeLoof, H. and Rosseneu, M.**, *Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100,* Nature, 323, 738-742, *1986*.

247. **Rassart, E., Bedirian, A., Do Carmo, S., Guinard, O., Sirois, J., Terrisse, L. and Milne, R.**, *Apolipoprotein D,* Biochim Biophys Acta, 1482, 185-198, *2000*.

248. **Segrest, J. P., Jackson, R. L., Morrisett, J. D. and Gotto, A. M.,Jr**, *A molecular theory of lipid-protein interactions in the plasma lipoproteins,* FEBS Lett, 38, 247-258, *1974*.

249. **Brouillette, C. G., Anantharamaiah, G. M., Engler, J. A. and Borhani, D. W.**, *Structural models of human apolipoprotein AI: a critical analysis and review,* Biochim Biophys Acta, 1531, 4-46, *2001*.

250. **Brouillette, C. G. and Anantharamaiah, G. M.**, *Structural models of human apolipoprotein AI,* Biochim Biophys Acta, 1256, 103-129, *1995*.

251. **Ren, G., Rudenko, G., Ludtke, S. J., Deisenhofer, J., Chiu, W. and Pownall, H. J.**, *Model of human low-density lipoprotein and bound receptor based on CryoEM,* 107, 1059-1064, *2010*.

252. **Drayna, D., Fielding, C., McLean, J., Baer, B., Castro, G., Chen, E., Comstock, L., Henzel, W., Kohr, W. and Rhee, L.**, *Cloning and expression of human apolipoprotein D cDNA,* J Biol Chem, 261, 16535-16539, *1986*.

253. **Wilson, C., Wardell, M. R., Weisgraber, K. H., Mahley, R. W. and Agard, D. A.**, *Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E,* Science, 252, 1817-1822, *1991*.

254. **Borhani, D. W., Rogers, D. P., Engler, J. A. and Brouillette, C. G.**, *Crystal structure of truncated human apolipoprotein AI suggests a lipid-bound conformation,* Proc Natl Acad Sci USA, 94, 12291-12296, *1997*.

255. **Eichinger, A., Nasreen, A., Kim, H. J. and Skerra, A.**, *Structural insight into the dual ligand specificity and mode of high density lipoprotein association of apolipoprotein D,* J Biol Chem, 282, 31068-31075, *2007*.

256. **Gangabadage, C. S., Zdunek, J., Tessari, M., Nilsson, S., Olivecrona, G. and Wijmenga, S. S.**, *Structure and dynamics of human apolipoprotein CIII,* J Biol

Chem, 283, 17416-17427, *2008*.

257. **Wang, Y. T., von Zychlinski, A. and McCormick, S.**, *Dimyristoylphosphotidylcholine induces conformational changes in apoB that lowers lipoprotein (a),* J Lipid Res, 50, 846-853, *2009*.

258. **McKeone, B. J., Patsch, J. R. and Pownall, H. J.**, *Plasma triglycerides determine low density lipoprotein composition, physical properties, and cell-specific binding in cultured cells.* J Clin Invest, 91, 1926-1933, *1993*.

259. **Curtiss, L. K., Bonnet, D. J. and Rye, K. A.**, *The conformation of apolipoprotein A-I in high-density lipoproteins is influenced by core lipid composition and particle size: A surface plasmon resonance study,* Biochemistry, 39, 5712-5721, *2000*.

260. **Rye, K. A., Wee, K., Curtiss, L. K., Bonnet, D. J. and Barter, P. J.**, *Apolipoprotein A-II inhibits high density lipoprotein remodeling and lipid-poor apolipoprotein A-I formation,* J Biol Chem, 278, 22530-22536, *2003*.

261. **Wang, X., Pease, R., Bertinato, J. and Milne, R. W.**, *Well-defined regions of apolipoprotein B-100 undergo conformational change during its intravascular metabolism,* Arterioscler Thromb Vasc Biol, 20, 1301-1308, *2000*.

262. **Chan, L.** *Apolipoprotein B, the major protein component of triglyceride-rich and low density lipoproteins,* J Biol Chem, 267, 25621-25624, *1992*.

263. **Windler, E. and Havel, R. J.**, *Inhibitory effects of C apolipoproteins from rats and humans on the uptake of triglyceride-rich lipoproteins and their remnants by the perfused rat liver,* J Lipid Res, 26, 556-565, *1985*.

264. **Boyles, J. K., Notterpek, L. M. and Anderson, L. J.**, *Accumulation of apolipoproteins in the regenerating and remyelinating mammalian peripheral nerve. Identification of apolipoprotein D, apolipoprotein A-IV, apolipoprotein E, and apolipoprotein AI,* J Biol Chem, 265, 17805-17815, *1990*.

265. **Terrisse, L., Poirier, J., Bertrand, P., Merched, A., Visvikis, S., Siest, G., Milne, R. and Rassart, E.**, *Increased levels of apolipoprotein D in cerebrospinal fluid and hippocampus of Alzheimer's patients,* J Neurochem, 71, 1643-1650, *1998*.

266. **Mahley, R. W. and Huang, Y.**, *Apolipoprotein E: from atherosclerosis to Alzheimer's disease and beyond,* Curr Opin Lipidol, 10, 207-217, *1999*.

267. **Fujimoto, K., Cardelli, J. A. and Tso, P.**, *Increased apolipoprotein A-IV in rat mesenteric lymph after lipid meal acts as a physiological signal for satiation,* Am J Physiol, 262, 1002-1006, *1992*.

268. **Fujimoto, K., Fukagawa, K., Sakata, T. and Tso, P.**, *Suppression of food intake by apolipoprotein A-IV is mediated through the central nervous system in rats.* J Clin Invest, 91, 1830-1833, *1993*.

269. **Gurr, M. I., Harwood, J. L. and Frayn, K. N.** In *Lipid biochemistry* (Blackwell Science, Oxford, 2002).

270. **Cohn, J. S., Johnson, E. J., Millar, J. S., Cohn, S. D., Milne, R. W., Marcel, Y. L., Russell, R. M. and Schaefer, E. J.**, *Contribution of apoB-48 and apoB-100 triglyceride-rich lipoproteins (TRL) to postprandial increases in the plasma concentration of TRL triglycerides and retinyl esters,* J. Lipid. Res., 34, 2033-2040, *1993*.

271. **Karpe, F., de Faire, U., Mercuri, M., Gene Bond, M., Hellénius, M. L. and Hamsten, A.**, *Magnitude of alimentary lipemia is related to intima-media*

thickness of the common carotid artery in middle-aged men, Atherosclerosis, 141, 307-314*, 1998*.

272. **Martins, I. J., Sainsbury, A. J., Mamo, J. C. L. and Redgrave, T. G.**, *Lipid and apolipoprotein B48 transport in mesenteric lymph and the effect of hyperphagia on the clearance of chylomicron-like emulsions in insulin-deficient rats,* Diabetologia, 37, 238-246*, 1994*.

273. **Havel, R. J., Kane, J. P. and Kashyap, M. L.**, *Interchange of apolipoproteins between chylomicrons and high density lipoproteins during alimentary lipemia in man,* J Clin Invest, 52, 32-38*, 1973*.

274. **Dolphin, P. J., Forsyth, S. J. and Krul, E. S.**, *Post-secretory acquisition of apolipoprotein E by nascent rat hepatic very-low-density lipoproteins in the absence of cholesteryl ester transfer,* Biochim Biophys Acta, 875, 21-30*, 1986*.

275. **Eisenberg, S., Patsch, J. R., Sparrow, J. T., Gotto, A. M. and Olivecrona, T.**, *Very low density lipoprotein. Removal of Apolipoproteins C-II and C-III-1 during lipolysis in vitro,* J Biol Chem, 254, 12603-12608*, 1979*.

276. **Goldberg, I. J., Scheraldi, C. A., Yacoub, L. K., Saxena, U. and Bisgaier, C. L.**, *Lipoprotein ApoC-II activation of lipoprotein lipase. Modulation by apolipoprotein A-IV,* J Biol Chem, 265, 4266-4272*, 1990*.

277. **Rubinstein, A., Gibson, J. C., Paterniti Jr, J. R. and Kakis, G.**, *Effect of heparin-induced lipolysis on the distribution of apolipoprotein E among lipoprotein subclasses,* J Clin Invest, 75, 710-721*, 1985*.

278. **Redgrave, T. G.** *Chylomicrons* In Lipoproteins in health and disease. 31-54 (Arnold, London, 1999).

279. **Hoofnagle, A. N. and Heinecke, J. W.**, *Lipoproteomics: using mass spectrometry-based proteomics to explore the assembly, structure, and function of lipoproteins,* J Lipid Res, 50, 1967-1975*, 2009*.

280. **Pownall, H. J. and Gotto Jr, A. M.** *Structure and dynamics of human plasma lipoproteins* In Lipoproteins in health and disease. 3-15 (Arnold, London, 1999).

281. **Hultin, M., Savonen, R. and Olivecrona, T.**, *Chylomicron metabolism in rats: lipolysis, recirculation of triglyceride-derived fatty acids in plasma FFA, and fate of core lipids as analyzed by compartmental modelling,* J Lipid Res, 37, 1022-1036*, 1996*.

282. **Yu, K. C. and Cooper, A. D.**, *Postprandial lipoproteins and atherosclerosis,* Front Biosci, 6, D332-D354*, 2001*.

283. **Redgrave, T.** *Chylomicron metabolism,* Biochem Soc Trans, 32, 79-82*, 2004*.

284. **Hussain, M. M., Maxfield, F. R., Mas-Oliva, J., Tabas, I., Ji, Z. S., Innerarity, T. L. and Mahley, R. W.**, *Clearance of chylomicron remnants by the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor,* J Biol Chem, 266, 13936-13940*, 1991*.

285. **Packard, C. J. and Shepherd, J.** *Physiology of the lipoprotein transport system: an overview of lipoprotein metabolism* In Lipoproteins in health and disease. 17-30 (Arnold, London, 1999).

286. **Packard, C. J., Gaw, A., Demant, T. and Shepherd, J.**, *Development and*

application of a multicompartmental model to study very low density lipoprotein subfraction metabolism, J Lipid Res, 36, 172-187*, 1995.*

287. **Ehnholm, C., Mahley, R. W., Chappell, D. A., Weisgraber, K. H., Ludwig, E. and Witztum, J. L.**, *Role of apolipoprotein E in the lipolytic conversion of ß-very low density lipoproteins to low density lipoproteins in type III hyperlipoproteinemia,* Proc Natl Acad Sci USA, 81, 5566-5570*, 1984.*

288. **Gaw, A., Packard, C. J., Murray, E. F., Lindsay, G. M., Griffin, B. A., Caslake, M. J., Vallance, B. D., Lorimer, A. R. and Shepherd, J.**, *Effects of simvastatin on apoB metabolism and LDL subfraction distribution,* Arterioscler Thromb Vasc Biol, 13, 170-189*, 1993.*

289. **Gaw, A., Packard, G. J., Caslake, M. J., Griffin, B. A., Lindsay, G. M., Thomson, J., Vallance, B. D., Wosornu, D. and Shepherd, J.**, *Effects of ciprofibrate on LDL metabolism in man,* Atherosclerosis, 108, 137-148*, 1994.*

290. **Caslake, M. J., Packard, C. J., Gaw, A., Murray, E., Griffin, B. A., Vallance, B. D. and Shepherd, J.**, *Fenofibrate and LDL metabolic heterogeneity in hypercholesterolemia,* Arterioscler Thromb Vasc Biol, 13, 702-711*, 1993.*

291. **Fielding, P. E. and Fielding, C. J.** *Dynamics of lipoprotein transport in the human circulatory system* In Biochemistry of lipis, lipoproteins and membranes. (eds Vance, D. E. and Vance, J. E.) 527-552 (Elsevier, Netherlands, 2002).

292. **Malmström, R., Packard, C. J., Watson, T. D. G., Rannikko, S., Caslake, M., Bedford, D., Stewart, P., Yki-Järvinen, H., Shepherd, J. and Taskinen, M. R.**, *Metabolic basis of hypotriglyceridemic effects of insulin in normal men,* Arterioscler Thromb Vasc Biol, 17, 1454-1464*, 1997.*

293. **Adiels, M., Olofsson, S. O., Taskinen, M. R. and Boren, J.**, *Overproduction of very low-density lipoproteins is the hallmark of the dyslipidemia in the metabolic syndrome,* Arterioscler Thromb Vasc Biol, 28, 1225-1236*, 2008.*

294. **Tan, C. E., Foster, L., Caslake, M. J., Bedford, D., Watson, T. D., McConnell, M., Packard, C. J. and Shepherd, J.**, *Relations between plasma lipids and postheparin plasma lipases and VLDL and LDL subfraction patterns in normolipemic men and women,* Arterioscler Thromb Vasc Biol, 15, 1839-1848*, 1995.*

295. **Adiels, M., Packard, C., Caslake, M. J., Stewart, P., Soro, A., Westerbacka, J., Wennberg, B., Olofsson, S. O., Taskinen, M. R. and Boren, J.**, *A new combined multicompartmental model for apolipoprotein B-100 and triglyceride metabolism in VLDL subfractions,* J Lipid Res, 46, 58-67*, 2005.*

296. **Griffin, B. A. and Packard, C. J.**, *Metabolism of VLDL and LDL subclasses,* Curr Opin Lipidol, 5, 200-206*, 1994.*

297. **Egusa, G., Beltz, W. F., Grundy, S. M. and Howard, B. V.**, *Influence of obesity on the metabolism of apolipoprotein B in humans.* J Clin Invest, 76, 596-603*, 1985.*

298. **Cohn, J. S., Patterson, B. W., Uffelman, K. D., Davignon, J. and Steiner, G.**, *Rate of production of plasma and very-low-density lipoprotein (VLDL) apolipoprotein C-III is strongly related to the concentration and level of production of VLDL triglyceride in male subjects with different body weights and levels of insulin sensitivity,* J Clin Endocrinol Metab, 89, 3949-3955*, 2004.*

299. **Melish, J., Le, N. A., Ginsberg, H., Steinberg, D. and Brown, W. V.**, *Dissociation of apoprotein B and triglyceride production in very-low-density lipopro-*

teins, Am J Physiol, 239, E354-362*, 1980*.

300. **Ginsberg, H. N., Le, N. A., Melish, J., Steinberg, D. and Brown, W. V.**, *Effect of a high carbohydrate diet on apoprotein-B catabolism in man,* Metabolism, 30, 347-353*, 1981*.

301. **Taskinen, M. R., Nikkilä, E. A., Välimäki, M., Sane, T., Kuusi, T., Kesäniemi, Y. A. and Ylikahri, R.**, *Alcohol-induced changes in serum lipoproteins and in their metabolism.* Am Heart J, 113, 458-463*, 1987*.

302. **Howard, B. V.** *Lipoprotein metabolism in diabetes mellitus,* J Lipid Res, 28, 613-628*, 1987*.

303. **Bittolo Bon, G., Cazzolato, G., Zago, S. and Avogaro, P.**, *Concentration, composition and apolipoprotein B species of very low density lipoprotein subfractions from normolipidemic and hypertriglyceridemic humans,* Ric Clin Lab, 15, 233-240*, 1985*.

304. **Pease, R. J. and Leiper, J. M.**, *Regulation of hepatic apolipoprotein-B-containing lipoprotein secretion.* Curr Opin Lipidol, 7, 132-138*, 1996*.

305. **Stalenhoef, A. F. H., Malloy, M. J., Kane, J. P. and Havel, R. J.**, *Metabolism of apolipoproteins B-48 and B-100 of triglyceride-rich lipoproteins in normal and lipoprotein lipase-deficient humans,* Proc Natl Acad Sci USA, 81, 1839-1843*, 1984*.

306. **Packard, C. J., Munro, A., Lorimer, A. R., Gotto, A. M. and Shepherd, J.**, *Metabolism of apolipoprotein B in large triglyceride-rich very low density lipoproteins of normal and hypertriglyceridemic subjects.* J Clin Invest, 74, 2178-2192*, 1984*.

307. **Gaw, A., Packard, C. J., Lindsay, G. M., Griffin, B. A., Caslake, M. J., Lorimer, A. R. and Shepherd, J.**, *Overproduction of small very low density lipoproteins (Sf 20-60) in moderate hypercholesterolemia: relationships between apolipoprotein B kinetics and plasma lipoproteins,* J Lipid Res, 36, 158-171*, 1995*.

308. **Aalto-Setälä, K., Fisher, E. A., Chen, X., Chajek-Shaul, T., Hayek, T., Zechner, R., Walsh, A., Ramakrishnan, R., Ginsberg, H. N. and Breslow, J. L.**, *Mechanism of hypertriglyceridemia in human apolipoprotein (apo) CIII transgenic mice. Diminished very low density lipoprotein fractional catabolic rate associated with increased apo CIII and reduced apo E on the particles.* J Clin Invest, 90, 1889-1900*, 1992*.

309. **Mann, W. A., Meyer, N., Weber, W., Meyer, S., Greten, H. and Beisiegel, U.**, *Apolipoprotein E isoforms and rare mutations: parallel reduction in binding to cells and to heparin reflects severity of associated type III hyperlipoproteinemia,* J Lipid Res, 36, 517-525*, 1995*.

310. **Ji, Z. S., Fazio, S. and Mahley, R. W.**, *Variable heparan sulfate proteoglycan binding of apolipoprotein E variants may modulate the expression of type III hyperlipoproteinemia,* J Biol Chem, 269, 13421-13428*, 1994*.

311. **Chappell, D. A., Fry, G. L., Waknitz, M. A., Muhonen, L. E. and Pladet, M. W.**, *Low density lipoprotein receptors bind and mediate cellular catabolism of normal very low density lipoproteins in vitro,* J Biol Chem, 268, 25487-25493*, 1993*.

312. **Tall, A. R.** *Plasma high density lipoproteins. Metabolism and relationship to atherogenesis.* J Clin Invest, 86, 379-384*, 1990*.

313. **Attman, P., Knight-Gibson, C., Tavella, M., Samuelsson, O. and**

Alaupovic, P., *The compositional abnormalities of lipoproteins in diabetic renal failure,* Nephrol Dial Transplant, 13, 2833-2841*, 1998*.

314. **Attman, P. O., Alaupovic, P., Tavella, M. and Knight-Gibson, C.**, *Abnormal lipid and apolipoprotein composition of major lipoprotein density classes in patients with chronic renal failure,* Nephrol Dial Transplant, 11, 63-69*, 1996*.

315. **Meyer, B. J., Duvillard, L., Owen, A., Packard, C. J. and Caslake, M. J.**, *Fractionation of cholesteryl ester rich intermediate density lipoprotein subpopulations by chondroitin sulphate,* Atherosclerosis, 195, 28-34*, 2007*.

316. **Mahley, R. W., Innerarity, T. L., Rall, S. C. and Weisgraber, K. H.**, *Plasma lipoproteins: apolipoprotein structure and function,* J Lipid Res, 25, 1277-1294*, 1984*.

317. **Zambon, A., Bertocco, S., Vitturi, N., Polentarutti, V., Vianello, D. and Crepaldi, G.**, *Relevance of hepatic lipase to the metabolism of triacylglycerol-rich lipoproteins,* Biochem Soc Trans, 31, 1070-1074*, 2003*.

318. Krauss, R. M. *Physical heterogeneity of apolipoprotein B-containing lipoproteins* (Proceedings of the workshop on lipoprotein heterogeneity., US Govt. Printing Office, Washington, DC, 1987).

319. **Krauss, R. M.** *Relationship of intermediate and low-density lipoprotein subspecies to risk of coronary artery disease,* Am Heart J, 113, 578-582*, 1987*.

320. **Williams, P. T., Superko, H. R., Haskell, W. L., Alderman, E. L., Blanche, P. J., Holl, L. G. and Krauss, R. M.**, *Smallest LDL particles are most strongly related to coronary disease progression in men,* Arterioscler Thromb Vasc Biol, 23, 314-321*, 2003*.

321. **Hirano, K., Matsuzawa, Y., Sakai, N., Hiraoka, H., Nozaki, S., Funahashi, T., Yamashita, S., Kubo, M. and Tarui, S.**, *Polydisperse low-density lipoproteins in hyperalphalipoproteinemic chronic alcohol drinkers in association with marked reduction of cholesteryl ester transfer protein activity,* Metabolism, 41, 1313-1318*, 1992*.

322. **Packard, C. J.** *Triacylglycerol-rich lipoproteins and the generation of small, dense low-density lipoprotein,* Biochem Soc Trans, 31, 1066-1069*, 2003*.

323. **Georgieva, A. M., van Greevenbroek, M. M. J., Krauss, R. M., Brouwers, M., Vermeulen, V. M. M. J., Robertus-Teunissen, M. G., van der Kallen, C. J. H. and de Bruin, T. W. A.**, *Subclasses of low-density lipoprotein and very low-density lipoprotein in familial combined hyperlipidemia: relationship to multiple lipoprotein phenotype,* Arterioscler Thromb Vasc Biol, 24, 744-749*, 2004*.

324. **Krauss, R. M.** *Lipids and lipoproteins in patients with type 2 diabetes,* Diabetes Care, 27, 1496-1504*, 2004*.

325. **Verges, B.** *New insight into the pathophysiology of lipid abnormalities in type 2 diabetes,* Diabetes Metab, 31, 429-439*, 2005*.

326. **Carew, T. E., Pittman, R. C. and Steinberg, D.**, *Tissue sites of degradation of native and reductively methylated [14C] sucrose-labeled low density lipoprotein in rats. Contribution of receptor-dependent and receptor-independent pathways,* J Biol Chem, 257, 8001-8008*, 1982*.

327. **Bhattacharya, S., Balasubramaniam, S. and Simons, L. A.**, *Role of the liver in low-density-lipoprotein catabolism in the rat*. Biochem J, 220, 333-336*, 1984*.

328. **Kesäniemi, Y. A., Witztum, J. L. and Steinbrecher, U. P.**, *Receptor-mediated catabolism of low density lipoprotein in man. Quantitation using glucosylated low density lipoprotein,* J Clin Invest, 71, 950-959*, 1983*.

329. **Shepherd, J., Packard, C. J., Bicker, S., Lawrie, T. D. and Morgan, H. G.**, *Cholestyramine promotes receptor-mediated low-density-lipoprotein catabolism,* N Engl J Med, 302, 1219-1222*, 1980*.

330. **Cuchel, M. and Rader, D. J.**, *Macrophage reverse cholesterol transport: key to the regression of atherosclerosis?* Circulation, 113, 2548-2555*, 2006*.

331. **Rader, D. J.** *Regulation of reverse cholesterol transport and clinical implications,* Am J Cardiol, 92, 42-49*, 2003*.

332. **Miller, G. J. and Miller, N. E.**, *Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease,* Lancet, 1, 16-19*, 1975*.

333. **Ross, R. and Glomset, J. A.**, *Atherosclerosis and the arterial muscle cell: proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis,* Science, 180, 1332-1339*, 1973*.

334. **von Eckardstein, A., Nofer, J. R. and Assmann, G.**, *High density lipoproteins and arteriosclerosis: role of cholesterol efflux and reverse cholesterol transport,* Arterioscler Thromb Vasc Biol, 21, 13-27*, 2001*.

335. **Barter, P. J.** *Hugh sinclair lecture: the regulation and remodelling of HDL by plasma factors,* Atherosclerosis, 3, 39-47*, 2002*.

336. **Asztalos, B. F. and Schaefer, E. J.**, *High-density lipoprotein subpopulations in pathologic conditions,* Am J Cardiol, 91, 12-17*, 2003*.

337. **Wilson, H. M., Patel, J. C. and Skinner, E. R.**, *The distribution of high-density lipoprotein subfractions in coronary survivors,* Biochem Soc Trans, 18, 1175-1176*, 1990*.

338. **Johansson, J., Carlson, L. A., Landou, C. and Hamsten, A.**, *High density lipoproteins and coronary atherosclerosis. A strong inverse relation with the largest particles is confined to normotriglyceridemic patients,* Arterioscler Thromb Vasc Biol, 11, 174-182*, 1991*.

339. **Cheung, M. C., Brown, B. G., Wolf, A. C. and Albers, J. J.**, *Altered particle size distribution of apolipoprotein AI-containing lipoproteins in subjects with coronary artery disease,* J Lipid Res, 32, 383-394*, 1991*.

340. **Syvänne, M., Ahola, M., Lahdenperä, S., Kahri, J., Kuusi, T., Virtanen, K. S. and Taskinen, M. R.**, *High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease,* J Lipid Res, 36, 573-582*, 1995*.

341. **Williams, P. T., Haskell, W. L., Vranizan, K. M. and Krauss, R. M.**, *The associations of high-density lipoprotein subclasses with insulin and glucose levels, physical activity, resting heart rate, and regional adiposity in men with coronary artery disease: The stanford coronary risk intervention project baseline survey,* Metab Clin Exp, 44, 106-114*, 1995*.

342. **Romano, G., Tilly-Kiesi, M. K., Patti, L., Taskinen, M. R., Pacioni, D., Cassader, M., Riccardi, G. and Rivellese, A. A.**, *Effects of dietary cholesterol on plasma lipoproteins and their subclasses in IDDM patients,* Diabetologia, 41, 193-200*, 1998*.

343. **Williams, P. T., Krauss, R. M., Vranizan, K. M., Albers, J. J. and Wood, P. D.**, *Effects of weight-loss by exercise and by diet on apolipoproteins A-I and A-II and the particle-size distribution of high-density lipoproteins in men,* Metabolism, 41, 441-449*, 1992*.

344. **Schafer, C., Parlesak, A., Eckoldt, J., Bode, C., Bode, J. C., Marz, W. and Winkler, K.**, *Beyond HDL-cholesterol increase: phospholipid enrichment and shift from HDL3 to HDL2 in alcohol consumers,* J Lipid Res, 48, 1550-1558*, 2007*.

345. **Williams, P. T., Vranizan, K. M., Austin, M. A. and Krauss, R. M.**, *Associations of age, adiposity, alcohol intake, menstrual status, and estrogen therapy with high-density lipoprotein subclasses,* Arterioscler Thromb Vasc Biol, 13, 1654-1661*, 1993*.

346. **Mäkelä, S., Jauhiainen, M., Ala-Korpela, M., Metso, J., Lehto, T., Savolainen, M. and Hannuksela, M.**, *HDL_2 of heavy alcohol drinkers enhances cholesterol efflux from raw macrophages via phospholipid-rich HDL_{2b} particles,* Alcohol Clin Exp Res, 32, 991-1000*, 2008*.

347. **Dodani, S., Grice, D. G. and Joshi, S.**, *Is HDL function as important as HDL quantity in the coronary artery disease risk assessment?* J Clin Lipidol, 3, 70-77*, 2009*.

348. **Sviridov, D., Mukhamedova, N., Remaley, A. T., Chin-Dusting, J. and Nestel, P.**, *Antiatherogenic functionality of high density lipoprotein: how much versus how good,* J Atheroscler Thromb., 15, 52-62*, 2008*.

349. **Kontush, A. and Chapman, M. J.**, *Antiatherogenic small, dense HDL-guardian angel of the arterial wall,* Nat Clin Pract Cardiovasc Med, 3, 144-153*, 2006*.

350. **Navab, M., Anantharamaiah, G. M. and Fogelman, A. M.**, *The role of high-density lipoprotein in inflammation,* Trends Cardiovasc Med, 15, 158-161*, 2005*.

351. **Mahley, R. W., Huang, Y. and Weisgraber, K. H.**, *Putting cholesterol in its place: apoE and reverse cholesterol transport,* J Clin Invest, 116, 1226-1229*, 2006*.

352. **Eggerman, T. L., Hoeg, J. M., Meng, M. S., Tombragel, A., Bojanovski, D. and Brewer Jr, H. B.**, *Differential tissue-specific expression of human apoA-I and apoA-II,* J Lipid Res, 32, 821-828*, 1991*.

353. **Eisenberg, S.** *High density lipoprotein metabolism,* J Lipid Res, 25, 1017-1058*, 1984*.

354. **Lee, J. Y. and Parks, J. S.**, *ATP-binding cassette transporter AI and its role in HDL formation,* Curr Opin Lipidol, 16, 19-26*, 2005*.

355. **Settasatian, N., Duong, M., Curtiss, L. K., Ehnholm, C., Jauhiainen, M., Huuskonen, J. and Rye, K. A.**, *The mechanism of the remodeling of high density lipoproteins by phospholipid transfer protein,* J Biol Chem, 276, 26898-26905*, 2001*.

356. **Musliner, T. A., Long, M. D., Forte, T. M., Nichols, A. V., Gong, E. L., Blanche, P. J. and Krauss, R. M.**, *Dissociation of high density lipoprotein precursors from apolipoprotein B-containing lipoproteins in the presence of unesterified fatty acids and a source of apolipoprotein AI,* J Lipid Res, 32, 917-933*, 1991*.

357. **Patsch, J. R., Gotto, A. M., Olivercrona, T. and Eisenberg, S.**, *Formation of high density lipoprotein2-like particles during lipolysis of very low density lipoproteins in vitro,* Proc Natl Acad Sci USA, 75, 4519-4523*, 1978*.

358. **Nofer, J. R., Kehrel, B., Fobker, M., Levkau, B., Assmann, G. and Eckardstein, A.**, *HDL and arteriosclerosis: beyond reverse cholesterol transport,* Atherosclerosis, 161, 1-16, *2002*.

359. **Fielding, C. J. and Fielding, P. E.**, *Molecular physiology of reverse cholesterol transport,* J Lipid Res, 36, 211-228, *1995*.

360. **Huuskonen, J., Olkkonen, V. M., Jauhiainen, M. and Ehnholm, C.**, *The impact of phospholipid transfer protein (PLTP) on HDL metabolism,* Atherosclerosis, 155, 269-281, *2001*.

361. **Wang, N. and Tall, A. R.**, *Regulation and mechanisms of ATP-binding cassette transporter A1-mediated cellular cholesterol efflux,* Arterioscler Thromb Vasc Biol, 23, 1178-1184, *2003*.

362. **Barter, P. J. and Rye, K. A.**, *Relationship between the concentration and antiatherogenic activity of high-density lipoproteins,* Curr Opin Lipidol, 17, 399-403, *2006*.

363. **Kostner, G. M., Knipping, G., Groener, J. E., Zechner, R. and Dieplinger, H.**, *The role of LCAT and cholesteryl ester transfer proteins for the HDL and LDL structure and metabolism,* Adv Exp Med Biol, 210, 79-86, *1987*.

364. **Glomset, J. A.** *The plasma lecithin: cholesterol acyltransferase reaction,* J Lipid Res, 9, 155-167, *1968*.

365. **Yancey, P. G., Bortnick, A. E., Kellner-Weibel, G., de la Llera-Moya, M., Phillips, M. C. and Rothblat, G. H.**, *Importance of different pathways of cellular cholesterol efflux,* Arterioscler Thromb Vasc Biol, 23, 712-719, *2003*.

366. **Rye, K. A., Clay, M. A. and Barter, P. J.**, *Remodelling of high density lipoproteins by plasma factors,* Atherosclerosis, 145, 227-238, *1999*.

367. **Forte, T. M., Bielicki, J. K., Goth-Goldstein, R., Selmek, J. and McCall, M. R.**, *Recruitment of cell phospholipids and cholesterol by apolipoproteins A-II and AI: formation of nascent apolipoprotein-specific HDL that differ in size, phospholipid composition, and reactivity with LCAT,* J Lipid Res, 36, 148-157, *1995*.

368. **Durbin, D. M. and Jonas, A.**, *Lipid-free apolipoproteins AI and A-II promote remodeling of reconstituted high density lipoproteins and alter their reactivity with lecithin: cholesterol acyltransferase,* J Lipid Res, 40, 2293-2302, *1999*.

369. **Asztalos, B. F., Schaefer, E. J., Horvath, K. V., Yamashita, S., Miller, M., Franceschini, G. and Calabresi, L.**, *Role of LCAT in HDL remodeling: investigation of LCAT deficiency states,* J Lipid Res, 48, 592-599, *2007*.

370. **Wang, N., Lan, D., Chen, W., Matsuura, F. and Tall, A. R.**, *ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins,* Proc Natl Acad Sci USA, 101, 9774-9779, *2004*.

371. **Nakamura, K., Kennedy, M. A., Baldan, A., Bojanic, D. D., Lyons, K. and Edwards, P. A.**, *Expression and regulation of multiple murine ATP-binding cassette transporter G1 mRNAs/isoforms that stimulate cellular cholesterol efflux to high density lipoprotein,* J Biol Chem, 279, 45980-45989, *2004*.

372. **Kennedy, M. A., Barrera, G. C., Nakamura, K., Baldán, Á., Tarr, P., Fishbein, M. C., Frank, J., Francone, O. L. and Edwards, P. A.**, *ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation,* Cell Metab, 1, 121-131, *2005*.

373. **Yancey, P. G., de la Llera-Moya, M., Swarnakar, S., Monzo, P., Klein, S. M., Connelly, M. A., Johnson, W. J., Williams, D. L. and Rothblat, G. H.**, *HDL phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor-BI (SR-BI),* J Biol Chem, 275, 36596-36604, *2000*.

374. **Ji, Y., Jian, B., Wang, N., Sun, Y., Moya, M. L., Phillips, M. C., Rothblat, G. H., Swaney, J. B. and Tall, A. R.**, *Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux,* J Biol Chem, 272, 20982-20985, *1997*.

375. **Liang, H. Q., Rye, K. A. and Barter, P. J.**, *Cycling of apolipoprotein AI between lipid-associated and lipid-free pools,* Biochim Biophys Acta, 1257, 31-37, *1995*.

376. **Rye, K. A. and Barter, P. J.**, *Formation and metabolism of prebeta-migrating, lipid-poor apolipoprotein A-I,* Arterioscler Thromb Vasc Biol, 24, 421-428, *2004*.

377. **Clay, M. A., Pyle, D. H., Rye, K. A. and Barter, P. J.**, *Formation of spherical, reconstituted high density lipoproteins containing both apolipoproteins A-I and A-II is mediated by lecithin:cholesterol acyltransferase,* J Biol Chem, 275, 9019-9025, *2000*.

378. **Thuahnai, S. T., Lund-Katz, S., Williams, D. L. and Phillips, M. C.**, *Scavenger receptor class B, type I-mediated uptake of various lipids into cells,* J Biol Chem, 276, 43801-43808, *2001*.

379. **Krieger, M. and Kozarsky, K.**, *Influence of the HDL receptor SR-BI on atherosclerosis,* Curr Opin Lipidol, 10, 491-497, *1999*.

380. **Trigatti, B., Rigotti, A. and Krieger, M.**, *The role of the high-density lipoprotein receptor SR-BI in cholesterol metabolism,* Curr Opin Lipidol, 11, 123-131, *2000*.

381. **Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H. and Krieger, M.**, *Identification of scavenger receptor SR-BI as a high density lipoprotein receptor,* Science, 271, 518-520, *1996*.

382. **Wang, N., Arai, T., Ji, Y., Rinninger, F. and Tall, A. R.**, *Liver-specific overexpression of scavenger receptor BI decreases levels of very low density lipoprotein apoB, low density lipoprotein apoB, and high density lipoprotein in transgenic mice,* J Biol Chem, 273, 32920-32926, *1998*.

383. **Hill, S. A. and McQueen, M. J.**, *Reverse cholesterol transport—a review of the process and its clinical implications,* Clin Biochem, 30, 517-525, *1997*.

384. **Horowitz, B. S., Goldberg, I. J., Merab, J., Vanni, T. M., Ramakrishnan, R. and Ginsberg, H. N.**, *Increased plasma and renal clearance of an exchangeable pool of apolipoprotein AI in subjects with low levels of high density lipoprotein cholesterol.* J Clin Invest, 91, 1743-1752, *1993*.

385. **Tall, A. R., Jiang, X., Luo, Y. and Silver, D.**, *1999 George Lyman Duff memorial lecture: lipid transfer proteins, HDL metabolism, and atherogenesis,* Arterioscler Thromb Vasc Biol, 20, 1185-1188, *2000*.

386. **Morton, R. E.** *Cholesteryl ester transfer protein and its plasma regulator: lipid transfer inhibitor protein,* Curr Opin Lipidol, 10, 321-327, *1999*.

387. **Schwartz, C. C., VandenBroek, J. M. and Cooper, P. S.**, *Lipoprotein cholesteryl ester production, transfer, and output in vivo in humans,* J Lipid Res, 45, 1594-1607, *2004*.

388. **Mahley, R. W.** *Apolipoprotein E: cholesterol transport protein with expanding role in cell biology,* Science, 240, 622-630*, 1988*.

389. **Curtiss, L. K. and Boisvert, W. A.**, *Apolipoprotein E and atherosclerosis,* Curr Opin Lipidol, 11, 243-251*, 2000*.

390. **Matsuura, F., Wang, N., Chen, W., Jiang, X. C. and Tall, A. R.**, *HDL from CETP-deficient subjects shows enhanced ability to promote cholesterol efflux from macrophages in an apoE-and ABCG1-dependent pathway,* J Clin Invest, 116, 1435-1442*, 2006*.

391. **Tall, A. R.** *Plasma cholesteryl ester transfer protein,* J Lipid Res, 34, 1255-1274*, 1993*.

392. **Barter, P. J., Brewer Jr, H. B., Chapman, M. J., Hennekens, C. H., Rader, D. J. and Tall, A. R.**, *Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis,* Arterioscler Thromb Vasc Biol, 23, 160-167*, 2003*.

393. **Austin, M. A., King, M. C., Vranizan, K. M. and Krauss, R. M.**, *Atherogenic lipoprotein phenotype. A proposed genetic marker for coronary heart disease risk,* Circulation, 82, 495-506*, 1990*.

394. **Packard, C. J., Demant, T., Stewart, J. P., Bedford, D., Caslake, M. J., Schwertfeger, G., Bedynek, A., Shepherd, J. and Seidel, D.**, *Apolipoprotein B metabolism and the distribution of VLDL and LDL subfractions,* J Lipid Res, 41, 305-318*, 2000*.

395. **Yang, Y., Yan, B., Fu, M., Xu, Y. and Tian, Y.**, *Relationship between plasma lipid concentrations and HDL subclasses,* Clin Chim Acta, 354, 49-58*, 2005*.

396. **Griffin, B. A., Freeman, D. J., Tait, G. W., Thomson, J., Caslake, M. J., Packard, C. J. and Shepherd, J.**, *Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk,* Atherosclerosis, 106, 241-253*, 1994*.

397. **Gofman, J. W., Delalla, O., Glazier, F., Freeman, N. K., Lindgren, D., Nichols, A. V., Strisower, B. and Tramplin, A. R.**, *The serum lipoprotein transport system in health, metabolic disorders, atherosclerosis and coronary heart disease,* Plasma, 2, 413-484*, 1954*.

398. **Le, N. A., Gibson, J. C. and Ginsberg, H. N.**, *Independent regulation of plasma apolipoprotein C-II and C-III concentrations in very low density and high density lipoproteins: implications for the regulation of the catabolism of these lipoproteins,* J Lipid Res, 29, 669-677*, 1988*.

399. **Ginsberg, H. N., Le, N. A., Goldberg, I. J., Gibson, J. C., Rubinstein, A., Wang-Iverson, P., Norum, R. and Brown, W. V.**, *Apolipoprotein B metabolism in subjects with deficiency of apolipoproteins CIII and AI. Evidence that apolipoprotein CIII inhibits catabolism of triglyceride-rich lipoproteins by lipoprotein lipase in vivo.* J Clin Invest, 78, 1287-1295*, 1986*.

400. **Maeda, N., Li, H., Lee, D., Oliver, P., Quarfordt, S. H. and Osada, J.**, *Targeted disruption of the apolipoprotein C-III gene in mice results in hypotriglyceridemia and protection from postprandial hypertriglyceridemia,* J Biol Chem, 269, 23610-23616*, 1994*.

401. **Xiang, S. Q., Cianflone, K., Kalant, D. and Sniderman, A. D.**, *Differential binding of triglyceride-rich lipoproteins to lipoprotein lipase,* J Lipid Res, 40, 1655-

1663, *1999*.

402. **Brunzell, J. D.** *Evidence for a common, saturable, triglyceride removal mechanism for chylomicrons and very low density lipoproteins in man,* J Clin Invest, 52, 1578-1585, *1973*.

403. **Karpe, F.** *Postprandial lipid metabolism in relation to coronary heart disease.* Proc Nutr Soc, 56, 671-678, *1997*.

404. **Boquist, S., Hamsten, A., Karpe, F. and Ruotolo, G.**, *Insulin and non-esterified fatty acid relations to alimentary lipaemia and plasma concentrations of postprandial triglyceride-rich lipoproteins in healthy middle-aged men,* Diabetologia, 43, 185-193, *2000*.

405. **Campos, H., Genest Jr, J. J., Blijlevens, E., McNamara, J. R., Jenner, J. L., Ordovas, J. M., Wilson, P. W. and Schaefer, E. J.**, *Low density lipoprotein particle size and coronary artery disease,* Arterioscler Thromb Vasc Biol, 12, 187-195, *1992*.

406. **Campos, H., Blijlevens, E., McNamara, J. R., Ordovas, J. M., Posner, B. M., Wilson, P. W., Castelli, W. P. and Schaefer, E. J.**, *LDL particle size distribution. Results from the Framingham Offspring Study,* Arterioscler Thromb Vasc Biol, 12, 1410-1419, *1992*.

407. **Selby, J. V., Austin, M. A., Newman, B., Zhang, D., Quesenberry Jr, C. P., Mayer, E. J. and Krauss, R. M.**, *LDL subclass phenotypes and the insulin resistance syndrome in women,* Circulation, 88, 381-387, *1993*.

408. **Austin, M. A., Newman, B., Selby, J. V., Edwards, K., Mayer, E. J. and Krauss, R. M.**, *Genetics of LDL subclass phenotypes in women twins. Concordance, heritability, and commingling analysis,* Arterioscler Thromb Vasc Biol, 13, 687-695, *1993*.

409. **Bu, X., Krauss, R. M., Puppione, D., Gray, R. and Rotter, J. I.**, *Major gene control of atherogenic lipoprotein phenotype (ALP): A quantitative segregation analysis in 20 coronary artery disease (CAD) pedigrees,* Am J Hum Genet, 51, A336, *1992*.

410. **Austin, M. A., Jarvik, G. P., Hokanson, J. E. and Edwards, K.**, *Complex segregation analysis of LDL peak particle diameter,* Genet Epidemiol, 10, 599-604, *1993*.

411. **Chung, B. H., Segrest, J. P. and Franklin, F.**, *In vitro production of β-very low density lipoproteins and small, dense low density lipoproteins in mildly hypertriglyceridemic plasma: role of activities of lecithin: cholester acyltransferase, cholesterylester transfer proteins and lipoprotein lipase,* Atherosclerosis, 141, 209-225, *1998*.

412. **Kuller, L., Arnold, A., Tracy, R., Otvos, J., Burke, G., Psaty, B., Siscovick, D., Freedman, D. S. and Kronmal, R.**, *Nuclear magnetic resonance spectroscopy of lipoproteins and risk of coronary heart disease in the cardiovascular health study,* Arterioscler Thromb Vasc Biol, 22, 1175-1180, *2002*.

413. **Mackey, R. H., Kuller, L. H., Sutton-Tyrell, K., Evans, R. W., Holubkov, R. and Matthews, K. A.**, *Lipoprotein subclasses and coronary artery calcification in postmenopausal women from the Healthy Women Study.* Am J Cardiol, 90, 71i-76i, *2002*.

414. **Otvos, J. D., Collins, D., Freedman, D. S., Shalaurova, I., Schaefer, E.**

J., McNamara, J. R., Bloomfield, H. E. and Robins, S. J., *Low-density lipoprotein and high-density lipoprotein particle subclasses predict coronary events and are favorably changed by gemfibrozil therapy in the Veterans Affairs High-Density Lipoprotein Intervention Trial,* Circulation, 113, 1556-1563*, 2006*.

415. **Rosenson, R. S., Freedman, D. S. and Otvos, J. D.**, *Relations of lipoprotein subclass levels and LDL size to progression of coronary artery disease in the PLAC I trial,* Am J Cardiol, 90, 89-94*, 2002*.

416. **Schaefer, E., Parise, H., Otvos, J., McNamara, J., D'Agostino, R. and Wilson, P.**, *LDL particle number, size, and subspecies in assessing cardiovascular risk: results from the Framingham Offspring Study,* Circulation, 110, III-777*, 2004*.

417. **Xiao, C., Watanabe, T., Zhang, Y., Trigatti, B., Szeto, L., Connelly, P. W., Marcovina, S., Vaisar, T., Heinecke, J. W. and Lewis, G. F.**, *Enhanced cellular uptake of remnant high-density lipoprotein particles. A mechanism for high-density lipoprotein lowering in insulin resistance and hypertriglyceridemia,* Circ Res, 103, 159-166*, 2008*.

418. **Guérin, M., Le Goff, W., Lassel, T. S., Van Tol, A., Steiner, G. and Chapman, M. J.**, *Atherogenic role of elevated CE transfer from HDL to VLDL(1) and dense LDL in type 2 diabetes : impact of the degree of triglyceridemia*. Arterioscler Thromb Vasc Biol, 21, 282-288*, 2001*.

419. **Guerin, M., Egger, P., Soudant, C., Le Goff, W., van Tol, A., Dupuis, R. and Chapman, M. J.**, *Cholesteryl ester flux from HDL to VLDL-1 is preferentially enhanced in type IIB hyperlipidemia in the postprandial state,* J Lipid Res, 43, 1652-1660*, 2002*.

420. **Rashid, S., Barrett, P. H. R., Uffelman, K. D., Watanabe, T., Adeli, K. and Lewis, G. F.**, *Lipolytically modified triglyceride-enriched HDLs are rapidly cleared from the circulation,* Arterioscler Thromb Vasc Biol, 22, 483-487*, 2002*.

421. **Rashid, S., Trinh, D. K., Uffelman, K. D., Cohn, J. S., Rader, D. J. and Lewis, G. F.**, *Expression of human hepatic lipase in the rabbit model preferentially enhances the clearance of triglyceride-enriched versus native high-density lipoprotein apolipoprotein AI,* Circulation, 107, 3066-3072*, 2003*.

422. **Lamarche, B., Uffelman, K. D., Carpentier, A., Cohn, J. S., Steiner, G., Barrett, P. H. and Lewis, G. F.**, *Triglyceride enrichment of HDL enhances in vivo metabolic clearance of HDL apo A-I in healthy men,* J Clin Invest, 103, 1191-1199*, 1999*.

423. **Kontush, A. and Chapman, M. J.**, *Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis,* Pharmacol Rev, 58, 342-374*, 2006*.

424. **Kumpula, L. S., Kumpula, J. M., Taskinen, M. R., Jauhiainen, M., Kaski, K. and Ala-Korpela, M.**, *Reconsideration of hydrophobic lipid distributions in lipoprotein particles,* Chem Phys Lipids, 155, 57-62*, 2008*.

425. **Baumstark, M. W., Kreutz, W., Berg, A., Frey, I. and Keul, J.**, *Structure of human low-density lipoprotein subfractions, determined by X-ray small-angle scattering,* Biochim Biophys Acta, 1037, 48-57*, 1990*.

426. **Deckelbaum, R. J., Shipley, G. G. and Small, D. M.**, *Structure and interactions of lipids in human plasma low density lipoproteins,* J Biol Chem, 252, 744-754*, 1977*.

427. **Laggner, P., Kostner, G. M., Degovics, G. and Worcester, D. L.**, *Structure of the cholesteryl ester core of human plasma low density lipoproteins: Selective deuteration and neutron small-angle scattering,* Proc Natl Acad Sci USA, 81, 4389-4393, *1984*.

428. **Hamilton, J. A. and Morrisett, J. D.**, *Nuclear magnetic resonance studies of lipoproteins,* Methods Enzymol, 128, 472-515, *1986*.

429. **Sears, B., Deckelbaum, R. J., Janiak, M. J., Shipley, G. G. and Small, D. M.**, *Temperature-dependent ^{13}C nuclear magnetic resonance studies of human serum low density lipoproteins,* Biochemistry, 15, 4151-4157, *1976*.

430. **Kroon, P. A.** *The order-disorder transition of the core cholesteryl esters of human plasma low density lipoprotein,* J Biol Chem, 11, 5332-5339, *1981*.

431. **Orlova, E. V., Sherman, M. B., Chiu, W., Mowri, H., Smith, L. C. and Gotto Jr, A. M.**, *Three-dimensional structure of low density lipoproteins by electron cryomicroscopy,* Proc Natl Acad Sci U S A, 96, 8420-8425, *1999*.

432. **Spin, J. M. and Atkinson, D.**, *Cryoelectron microscopy of low density lipoprotein in vitreous ice,* Biophys J, 68, 2115-2123, *1995*.

433. **Van Antwerpen, R. and Gilkey, J. C.**, *Cryo-electron microscopy reveals human low density lipoprotein substructure,* J Lipid Res, 35, 2223-2231, *1994*.

434. **Coronado-Gray, A. and van Antwerpen, R.**, *Lipid composition influences the shape of human low density lipoprotein in vitreous ice,* Lipids, 40, 495-500, *2005*.

435. **Heikelä, M., Vattulainen, I. and Hyvönen, M. T.**, *Atomistic simulation studies of cholesteryl oleates: model for the core of lipoprotein particles,* Biophys J, 90, 2247-2257, *2006*.

436. **Catte, A., Patterson, J. C., Jones, M. K., Jerome, W. G., Bashtovyy, D., Su, Z., Gu, F., Chen, J., Aliste, M. P. and Harvey, S. C.**, *Novel changes in discoidal high density lipoprotein morphology: a molecular dynamics study,* Biophys J, 90, 4345-4360, *2006*.

437. **Catte, A., Patterson, J. C., Bashtovyy, D., Jones, M. K., Gu, F., Li, L., Rampioni, A., Sengupta, D., Vuorela, T., Niemelä, P., Karttunen, M., Siewert, J. M., Vattulainen, I. and Segrest, J. P.**, *Structure of spheroidal HDL particles revealed by combined atomistic and coarse-grained simulations,* Biophys J, 94, 2306-2319, *2008*.

438. **Yetukuri, L., Söderlund, S., Koivuniemi, A., Seppänen-Laakso, T., Niemelä, P. S., Hyvönen, M., Taskinen, M. R., Vattulainen, I., Jauhiainen, M. and Orešič, M.**, *Composition and lipid spatial distribution of HDL particles in subjects with low and high HDL-cholesterol,* J Lipid Res, 51, 2341-2351, *2010*.

439. **Soulages, J. L. and Brenner, R. R.**, *Study on the composition-structure relationship of lipophorins,* J Lipid Res, 32, 407-415, *1991*.

440. **Teerlink, T., Scheffer, P. G., Bakker, S. J. and Heine, R. J.**, *Combined data from LDL composition and size measurement are compatible with a discoid particle shape,* J Lipid Res, 45, 954-966, *2004*.

441. **Schnitzer, E. and Lichtenberg, D.**, *Re-evaluation of the structure of low density lipoproteins,* Chem Phys Lipids, 70, 63-74, *1994*.

442. **Taskinen, M. R., Packard, C. J. and Shepherd, J.**, *Effect of insulin therapy*

on metabolic fate of apolipoprotein B-containing lipoproteins in NIDDM, Diabetes, 39, 1017-1027*, 1990*.

443. **Tilly-Kiesi, M., Knudsen, P., Groop, L. and Taskinen, M. R.**, *Hyperinsulinemia and insulin resistance are associated with multiple abnormalities of lipoprotein subclasses in glucose-tolerant relatives of NIDDM patients. Botnia Study Group,* J Lipid Res, 37, 1569-1578*, 1996*.

444. **Griffin, B. A., Caslake, M. J., Yip, B., Tait, G. W., Packard, C. J. and Shepherd, J.**, *Rapid isolation of low density lipoprotein (LDL) subfractions from plasma by density gradient ultracentrifugation,* Atherosclerosis, 83, 59-67*, 1990*.

445. **Groot, P. H., Scheek, L. M., Havekes, L., Van Noort, W. L. and Van't Hooft, F. M.**, *A one-step separation of human serum high density lipoproteins 2 and 3 by rate-zonal density gradient ultracentrifugation in a swinging bucket rotor,* J Lipid Res, 23, 1342-1353*, 1982*.

446. **Hannuksela, M. L., Brousseau, M. E., Meyn, S. M., Nazih, H., Bader, G., Shamburek, R. D., Alaupovic, P. and Brewer, H. B.**, *In vivo metabolism of apolipoprotein E within the HDL subpopulations LpE, LpE: AI, LpE: A-II and LpE: AI: A-II,* Atherosclerosis, 165, 205-220*, 2002*.

447. **Rosseneu, M.** In *Structure and function of apolipoproteins* (CRC Press, 1992).

448. **Edelstein, C., Kezdy, F. J., Scanu, A. M. and Shen, B. W.**, *Apolipoproteins and the structural organization of plasma lipoproteins: human plasma high density lipoprotein-3,* J Lipid Res, 20, 143-153*, 1979*.

449. **Schumaker, V. N., Phillips, M. L. and Chatterton, J. E.**, *Apolipoprotein B and low-density lipoprotein structure: implications for biosynthesis of triglyceride-rich lipoproteins,* Adv Protein Chem, 45, 205-248*, 1994*.

450. **Rubinstein, R. Y.and Kroese, D. P.** In *Simulation and the Monte Carlo method* (Wiley-Interscience, New Jersey, 2008).

451. **Hastie, T., Tibshirani, R. and Friedman, J.** In *The elements of statistical learning: data mining, inference and prediction* (Springer, New York, 2009).

452. **Soulages, J. L., van Antwerpen, R. and Wells, M. A.**, *Role of diacylglycerol and apolipophorin-III in regulation of physiochemical properties of the lipophorin surface: metabolic implications,* Biochemistry, 35, 5191-5198*, 1996*.

453. **Favari, E., Calabresi, L., Adorni, M. P., Jessup, W., Simonelli, S., Franceschini, G. and Bernini, F.**, *Small discoidal pre-beta1 HDL particles are efficient acceptors of cell cholesterol via ABCA1 and ABCG1,* Biochemistry, 48, 11067-11074*, 2009*.

454. **Cho, K. H.** *Synthesis of reconstituted high density lipoprotein (rHDL) containing apoA-I and apoC-III: the functional role of apoC-III in rHDL,* Mol Cells, 27, 291-297*, 2009*.

455. **Rye, K., Duong, M., Psaltis, M. K., Curtiss, L. K., Bonnet, D. J., Stocker, R. and Barter, P. J.**, *Evidence that phospholipids play a key role in Pre-β apoA-I formation and high-density lipoprotein remodeling,* Biochemistry, 41, 12538-12545*, 2002*.

456. **Patel, S., Drew, B. G., Nakhla, S., Duffy, S. J., Murphy, A. J., Barter, P. J., Rye, K. A., Chin-Dusting, J., Hoang, A. and Sviridov, D.**, *Reconstituted high-density lipoprotein increases plasma high-density lipoprotein anti-inflammat-*

ory properties and cholesterol efflux capacity in patients with type 2 diabetes, J Am Coll Cardiol, 53, 962-971*, 2009*.

457. **Small, D. M., Clarke, S. B., Tercyak, A., Steiner, J., Gantz, D. and Derksen, A.**, *The lipid surface of triglyceride-rich particles can modulate (apo) protein binding and tissue uptake.* Adv Exp Med Biol, 285, 281-288*, 1991*.

458. **Hamilton, J. A. and Small, D. M.**, *Solubilization and localization of cholesteryl oleate in egg phosphatidylcholine vesicles. A carbon 13 NMR study,* J Biol Chem, 257, 7318-7321*, 1982*.

459. **Mead, J. R., Irvine, S. A. and Ramji, D. P.**, *Lipoprotein lipase: structure, function, regulation, and role in disease,* J Mol Med, 80, 753-769*, 2002*.

460. **Connelly, P. W.** *The role of hepatic lipase in lipoprotein metabolism,* Clin Chim Acta, 286, 243-255*, 1999*.

461. **Winkler, F. K., d'Arcy, A. and Hunziker, W.**, *Structure of human pancreatic lipase,* Nature, 343, 771-774*, 1990*.

462. **Im, Y. J., Raychaudhuri, S., Prinz, W. A. and Hurley, J. H.**, *Structural mechanism for sterol sensing and transport by OSBP-related proteins,* Nature, 437, 154-158*, 2005*.

463. **Ngo, M., Colbourne, T. and Ridgway, N.**, *Functional implications of sterol transport by the oxysterol-binding protein gene family,* Biochem J, 429, 13-24*, 2010*.

464. **Yan, D. and Olkkonen, V. M.**, *Characteristics of oxysterol binding proteins,* Int Rev Cytol, 265, 253-285*, 2008*.

465. **Qiu, X., Mistry, A., Ammirati, M. J., Chrunyk, B. A., Clark, R. W., Cong, Y., Culp, J. S., Danley, D. E., Freeman, T. B., Geoghegan, K. F., Griffor, M. C., Hawrylik, S. J., Hayward, C. M., Hensley, P., Hoth, L. R., Karam, G. A., Lira, M. E., Lloyd, D. B., McGrath, K. M., Stutzman-Engwall, K. J., Subashi, A. K., Subashi, T. A., Thompson, J. F., Wang, I. K., Zhao, H. and Seddon, A. P.**, *Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules,* Nat Struct Mol Biol, 14, 106-113*, 2007*.

466. **Jiang, X. C. and Zhou, H. W.**, *Plasma lipid transfer proteins.* Curr Opin Lipidol, 17, 302-308*, 2006*.

467. **Kumpula, L. S., Mäkelä, S. M., Mäkinen, V. P., Karjalainen, A., Liinamaa, J. M., Kaski, K., Savolainen, M. J., Hannuksela, M. L. and Ala-Korpela, M.**, *Characterization of metabolic interrelationships and in silico phenotyping of lipoprotein particles using self-organizing maps.* J Lipid Res, 51, 431-439*, 2010*.

468. **De Backer, G., Ambrosioni, E., Borch-Johnsen, K., Brotons, C., Cifkova, R., Dallongeville, J., Ebrahim, S., Faergeman, O., Graham, I. and Mancia, G.**, *European guidelines on cardiovascular disease prevention in clinical practice. Third Joint Task Force of European and other Societies on Cardiovascular Disease Prevention in Clinical Practice.* Eur Heart J, 24, 1601-1610*, 2003*.

469. **Pearson, T. A., Blair, S. N., Daniels, S. R., Eckel, R. H., Fair, J. M., Fortmann, S. P., Franklin, B. A., Goldstein, L. B., Greenland, P. and Grundy, S. M.**, *AHA guidelines for primary prevention of cardiovascular disease and stroke: 2002 update: consensus panel guide to comprehensive risk reduction for adult patients without coronary or other atherosclerotic vascular diseases,* Circulation, 106, 388-391*, 2002*.

470. **Superko, H. R. and King III, S.**, *Lipid management to reduce cardiovascular risk: a new strategy is required,* Circulation, 117, 560-568*, 2008*.

471. **Sniderman, A. D., Wolfson, C., Teng, B., Franklin, F. A., Bachorik, P. S. and Kwiterovich Jr, P. O.**, *Association of hyperapobetalipoproteinemia with endogenous hypertriglyceridemia and atherosclerosis,* Ann Intern Med, 97, 833-839*, 1982*.

472. **Katzel, L. I., Krauss, R. M. and Goldberg, A. P.**, *Relations of plasma TG and HDL-C concentrations to body composition and plasma insulin levels are altered in men with small LDL particles,* Arterioscler Thromb, 14, 1121-1128*, 1994*.

473. **Kang, H. S., Gutin, B., Barbeau, P., Litaker, M. S., Allison, J. and Le, N. A.**, *Low-density lipoprotein particle size, central obesity, cardiovascular fitness, and insulin resistance syndrome markers in obese youths,* Int J Obes Relat Metab Disord, 26, 1030-1035*, 2002*.

474. **Arsenault, B. J., Lemieux, I., Despres, J. P., Wareham, N. J., Luben, R., Kastelein, J. J. P., Khaw, K. T. and Boekholdt, S. M.**, *Cholesterol levels in small LDL particles predict the risk of coronary heart disease in the EPIC-Norfolk prospective population study,* Eur Heart J, 28, 2770-2777*, 2007*.

475. **Stein, J. H. and McBride, P. E.**, *Should advanced lipoprotein testing be used in clinical practice?* Nat Clin Pract Cardiovasc Med, 3, 640-641*, 2006*.

476. **Vekic, J., Topic, A., Zeljkovic, A., Jelic-Ivanovic, Z. and Spasojevic-Kalimanovska, V.**, *LDL and HDL subclasses and their relationship with Framingham risk score in middle-aged Serbian population,* Clin Biochem, 40, 310-316*, 2007*.

477. **Liinamaa, M. J., Hannuksela, M. L., Kesäniemi, Y. A. and Savolainen, M. J.**, *Altered transfer of cholesteryl esters and phospholipids in plasma from alcohol abusers,* Arterioscler Thromb Vasc Biol, 17, 2940-2947*, 1997*.

478. **Liinamaa, M. J., Kervinen, K., Hannuksela, M. L., Kesäniemi, Y. A. and Savolainen, M. J.**, *Effect of apolipoprotein E phenotype on plasma lipids and lipoproteins in alcohol abusers,* Alcohol Clin Exp Res, 21, 606-612*, 1997*.

479. **Karjalainen, A., Heikkinen, J., Savolainen, M. J., Bäckström, A. C. and Kesäniemi, Y. A.**, *Mechanisms regulating LDL metabolism in subjects on peroral and transdermal estrogen replacement therapy,* Arterioscler Thromb Vasc Biol, 20, 1101-1106*, 2000*.

480. **Mäkinen, V. P., Forsblom, C., Thorn, L. M., Wadén, J., Gordin, D., Heikkilä, O., Hietala, K., Kyllönen, L., Kytö, J., Rosengård-Bärlund , M., Saraheimo, M., Tolonen, N., Parkkonen, M., Kaski, K., Ala-Korpela, M. and Groop on behalf of the FinnDiane Study Group, P.H.**, *Metabolic phenotypes, vascular complications and premature deaths in a population of 4,197 patients with type 1 diabetes.* 57, 2480-2487*, 2008*.

481. **Mäkinen, V. P., Soininen, P., Forsblom, C., Parkkonen, M., Ingman, P., Kaski, K., Groop, P. H. and Ala-Korpela, M.**, *^{1}H NMR metabonomics approach to the disease continuum of diabetic complications and premature death.* Mol Syst Biol, 4, 1-12*, 2008*.

482. **Kohonen, T.** In *Self-organizing maps* (Springer, Berlin, 2001).

483. **Suna, T., Salminen, A., Soininen, P., Laatikainen, R., Ingman, P., Mäkelä, S., Savolainen, M. J., Hannuksela, M. L., Jauhiainen, M., Taskinen, M. R., Kaski, K. and Ala-Korpela, M.**, *^{1}H NMR metabonomics of plasma lipoprotein subclasses: elucidation of metabolic clustering by self-organising maps,*

NMR Biomed, 20, 658-672*, 2007*.

484. **Nikkilä, J., Törönen, P., Kaski, S., Venna, J., Castren, E. and Wong, G.**, *Analysis and visualization of gene expression data using self-organizing maps,* Neural Netw, 15, 953-966*, 2002*.

485. **Valkonen, V. P., Kolehmainen, M., Lakka, H. M. and Salonen, J. T.**, *Insulin resistance syndrome revisited: application of self-organizing maps,* Int J Epidemiol, 31, 864-871*, 2002*.

486. **Suna, T., Hardey, M., Huhtinen, J., Hiltunen, Y., Kaski, K., Heikkonen, J. and Ala-Korpela, A.**, *Self-organising map approach to individual profiles:age, sex and culture in internet dating,* Sociol res online, 11, 31.03.2006*, 2006*.

487. **Hyvönen, M. T., Hiltunen, Y., El-Deredy, W., Ojala, T., Vaara, J., Kovanen, P. T. and Ala-Korpela, M.**, *Application of self-organizing maps in conformational analysis of lipids,* J Am Chem Soc, 123, 810-816*, 2001*.

488. **Tall, A. R., Krumholz, S., Olivecrona, T. and Deckelbaum, R. J.**, *Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low density lipoproteins and high density lipoproteins during lipolysis,* J Lipid Res, 26, 842-851*, 1985*.

489. **Levy, E., Deckelbaum, R. J., Thibault, R. L., Seidman, E., Olivecrona, T. and Roy, C. C.**, *In vitro remodelling of plasma lipoproteins in whole plasma by lipoprotein lipase in primary and secondary hypertriglyceridaemia,* Eur J Clin Invest, 20, 422-431*, 1990*.

490. **Superko, H. R. and Gadesam, R. R.**, *Is it LDL particle size or number that correlates with risk for cardiovascular disease?* Curr Atheroscler Rep, 10, 377-385*, 2008*.

491. **Nigon, F., Lesnik, P., Rouis, M. and Chapman, M. J.**, *Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor,* J Lipid Res, 32, 1741-1753*, 1991*.

492. **Chapman, M. J.** *Metabolic syndrome and type 2 diabetes: lipid and physiological consequences,* Diab Vasc Dis Res, 4 Suppl 3, S5-8*, 2007*.

493. **Packard, C. J., Munro, A., Lorimer, A. R., Gotto, A. M. and Shepherd, J.**, *Metabolism of apolipoprotein B in large triglyceride-rich very low density lipoproteins of normal and hypertriglyceridemic subjects.* J Clin Invest, 74, 2178-2192*, 1984*.

494. **Magkos, F., Mohammed, B. S. and Mittendorfer, B.**, *Effect of obesity on the plasma lipoprotein subclass profile in normoglycemic and normolipidemic men and women,* Int J Obes, 32, 1655-1664*, 2008*.

495. **Chan, D. C., Watts, G. F., Barrett, P. H., O'Neill, F. H. and Thompson, G. R.**, *Plasma markers of cholesterol homeostasis and apolipoprotein B-100 kinetics in the metabolic syndrome,* Obes Res, 11, 591-596*, 2003*.

496. **Tribble, D. L., Holl, L. G., Wood, P. D. and Krauss, R. M.**, *Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size,* 93, 189-199*, 1992*.

497. **Ala-Korpela, M., Lankinen, N., Salminen, A., Suna, T., Soininen, P., Laatikainen, R., Ingman, P., Jauhiainen, M., Taskinen, M. R. and Héberger, K.**, *The inherent accuracy of ^{1}H NMR spectroscopy to quantify plasma lipopro-*

teins is subclass dependent, Atherosclerosis, 190, 352-358*, 2007.*

498. **Festa, A., Williams, K., Hanley, A. J. G., Otvos, J. D., Goff, D. C., Wagenknecht, L. E. and Haffner, S. M.**, *Nuclear magnetic resonance lipoprotein abnormalities in prediabetic subjects in the Insulin Resistance Atherosclerosis Study,* Circulation, 111, 3465-3472*, 2005.*

499. **Goff, D. C., D'Agostino, R. B., Haffner, S. M. and Otvos, J. D.**, *Insulin resistance and adiposity influence lipoprotein size and subclass concentrations. Results from the Insulin Resistance Atherosclerosis Study,* Metab Clin Exp, 54, 264-270*, 2005.*

500. **Freedman, D. S., Otvos, J. D., Jeyarajah, E. J., Shalaurova, I., Cupples, L. A., Parise, H., D'Agostino, R. B., Wilson, P. W. F. and Schaefer, E. J.**, *Sex and age differences in lipoprotein subclasses measured by nuclear magnetic resonance spectroscopy: The Framingham Study,* Clin Chem, 50, 1189-1200*, 2004.*

501. **Hsia, J., Otvos, J. D., Rossouw, J. E., Wu, L. L., Wassertheil-Smoller, S., Hendrix, S. L., Robinson, J. G., Lund, B. and Kuller, L. H.**, *Lipoprotein particle concentrations may explain the absence of coronary protection in the women's health initiative hormone trials,* Arterioscler Thromb Vasc Biol, 28, 1666-1671*, 2008.*

502. **Krauss, R. M.** *Dietary and genetic effects on low-density lipoprotein heterogeneity,* Annu Rev Nutr, 21, 283-295*, 2001.*

503. **Crouse, J. R., Parks, J. S., Schey, H. M. and Kahl, F. R.**, *Studies of low density lipoprotein molecular weight in human beings with coronary artery disease,* J Lipid Res, 26, 566-574*, 1985.*

504. **Austin, M. A., Breslow, J. L., Hennekens, C. H., Buring, J. E., Willett, W. C. and Krauss, R. M.**, *Low-density lipoprotein subclass patterns and risk of myocardial infarction,* JAMA, 260, 1917-1921*, 1988.*

505. **McNamara, J. R., Jenner, J. L., Li, Z., Wilson, P. W. and Schaefer, E. J.**, *Change in LDL particle size is associated with change in plasma triglyceride concentration,* Arterioscler Thromb Vasc Biol, 12, 1284-1290*, 1992.*

506. **Swinkels, D. W., Demacker, P. N., Hendriks, J. C. and van 't Laar, A.**, *Low density lipoprotein subfractions and relationship to other risk factors for coronary artery disease in healthy individuals,* Arteriosclerosis, 9, 604-613*, 1989.*

507. **De Graaf, J., Swinkels, D. W., de Haan, A. F., Demacker, P. N. and Stalenhoef, A. F.**, *Both inherited susceptibility and environmental exposure determine the low-density lipoprotein-subfraction pattern distribution in healthy Dutch families.* Am J Hum Genet, 51, 1295-1310*, 1992.*

508. **Lamarche, B., Tchernof, A., Moorjani, S., Cantin, B., Dagenais, G. R., Lupien, P. J. and Despres, J. P.**, *Small, dense low-density lipoprotein particles as a predictor of the risk of ischemic heart disease in men: prospective results from the Quebec Cardiovascular Study,* Circulation, 95, 69-75*, 1997.*

509. **Gardner, C. D., Fortmann, S. P. and Krauss, R. M.**, *Association of small low-density lipoprotein particles with the incidence of coronary artery disease in men and women,* JAMA, 276, 875-881*, 1996.*

510. **Krauss, R. M.** *Heterogeneity of plasma low-density lipoproteins and atherosclerosis risk,* Curr Opin Lipidol, 5, 339-349*, 1994.*

511. **Mora, S.** *Advanced lipoprotein testing and subfractionation are not (yet) ready for routine clinical use,* Circulation, 119, 2396-2404*, 2009*.

512. **Karpe, F., Bickerton, A. S., Hodson, L., Fielding, B. A., Tan, G. D. and Frayn, K. N.**, *Removal of triacylglycerols from chylomicrons and VLDL by capillary beds: the basis of lipoprotein remnant formation,* Biochem Soc Trans, 35, 472-476*, 2007*.

513. **Law, M. R.** *Lowering heart disease risk with cholesterol reduction: evidence from observational studies and clinical trials,* Eur Heart J, 1, 3-8*, 1999*.

514. **Law, M. R., Wald, N. J. and Thompson, S. G.**, *By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease?* Br Med J, 308, 367-372*, 1994*.

515. **Durrington, P. N., Bolton, C. H. and Hartog, M.**, *Serum and lipoprotein apolipoprotein B levels in normal subjects and patients with hyperlipoproteinaemia,* Clin Chim Acta, 82, 151-160*, 1978*.

516. **Sniderman, A., Vu, H. and Cianflone, K.**, *Effect of moderate hypertriglyceridemia on the relation of plasma total and LDL apo B levels,* Atherosclerosis, 89, 109-116*, 1991*.

517. **Xu, Y. and Fu, M.**, *Alterations of HDL subclasses in hyperlipidemia,* Clin Chim Acta, 332, 95-102*, 2003*.

518. **Kontush, A., Chantepie, S. and Chapman, M. J.**, *Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress,* Arterioscler Thromb Vasc Biol, 23, 1881-1888*, 2003*.

519. **Lambert, J. B.and Mazzola, E. P.** In *Nuclear magnetic resonance spectroscopy: an introduction to principles, applications and experimental methods* (Prentice Hall, 2004).

520. **Pochapsky, T. C.and Pochapsky, S. S.** In *NMR for physical and biological scientists* (Taylor & Francis, 2007).

521. **Cushley, R. J. and Okon, M.**, *NMR studies of lipoprotein structures,* Annu Rev Biophys Biomol Struct, 31, 177-206*, 2002*.

522. **Jeyarajah, E. J.** *Development and validation of a ^{1}H NMR method for lipoprotein quantification and coronary heart disease risk assessment.* (2004).

523. http://www.liposcience.com/ (10.11.2010).

524. **Otvos, J. D., Jeyarajah, E. J. and Bennett, D. W.**, *Quantification of plasma lipoproteins by proton nuclear magnetic resonance spectroscopy,* Clin Chem, 37, 377-386*, 1991*.

525. **Otvos, J. D., Jeyarajah, E. J., Bennett, D. W. and Krauss, R. M.**, *Development of a proton nuclear magnetic resonance spectroscopic method for determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement,* Clin Chem, 38, 1632-1638*, 1992*.

526. **Otvos, J., Jeyarajah, E. and Bennett, D.**, *A spectroscopic approach to lipoprotein subclass analysis,* J Clin Ligand Assay, 19, 184-189*, 1996*.

527. **Soininen, P., Kangas, A. J., Würtz, P., Tukiainen, T., Tynkkynen, T., Laatikainen, R., Järvelin, M. R., Kähönen, M., Lehtimäki, T. and Viikari, J.**,

High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism, Analyst, 134, 1781-1785*, 2009*.

528. **Ala-Korpela, M.** *Critical evaluation of 1H NMR metabonomics of serum as a methodology for disease risk assessment and diagnostics.* Clin Chem Lab Med, 46, 27-42*, 2008*.

529. **Ala-Korpela, M., Korhonen, A., Keisala, J., Hörkkö, S., Korpi, P., Ingman, L. P., Jokisaari, J., Savolainen, M. J. and Kesäniemi, Y. A.**, *^{1}H NMR-based absolute quantitation of human lipoproteins and their lipid contents directly from plasma,* J Lipid Res, 35, 2292-2304*, 1994*.

530. **Rosenson, R. S., Otvos, J. D. and Hsia, J.**, *Effects of rosuvastatin and atorvastatin on LDL and HDL particle concentrations in patients with metabolic syndrome.* Diabetes Care, 32, 1087*, 2009*.

531. **Kathiresan, S., Otvos, J. D., Sullivan, L. M., Keyes, M. J., Schaefer, E. J., Wilson, P. W. F., D'Agostino, R. B., Vasan, R. S. and Robins, S. J.**, *Increased small low-density lipoprotein particle number. A prominent feature of the metabolic syndrome in the Framingham Heart Study,* Circulation, 113, 20-29*, 2006*.

532. **Jia, L., Fu, M., Tian, Y., Xu, Y., Gou, L., Tian, H. and Tian, L.**, *Alterations of high-density lipoprotein subclasses in hypercholesterolemia and combined hyperlipidemia,* Int J Cardiol, 120, 331-337*, 2007*.

533. **El Harchaoui, K., Arsenault, B. J., Franssen, R., Despres, J. P., Hovingh, G. K., Stroes, E. S. G., Otvos, J. D., Wareham, N. J., Kastelein, J. J. P. and Khaw, K. T.**, *High-density lipoprotein particle size and concentration and coronary risk,* Ann Intern Med, 150, 84-93*, 2009*.

534. **Miller, N. E.** *Associations of high-density lipoprotein subclasses and apolipoproteins with ischemic heart disease and coronary atherosclerosis,* Am Heart J, 113, 589-597*, 1987*.

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