ESTABLISHMENT OF NEW POTENTIAL BIOMARKERS FOR CARDIOMETABOLIC DISEASES

Karin Littmann

Stockholm 2020
Establishment of new potential biomarkers for cardiometabolic diseases
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Karin Littmann

Principal Supervisor:
Professor Paolo Parini
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Chemistry and
Department of Medicine
Metabolism Unit

Co-supervisors:
Ann-Charlotte Bergman, PhD
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Chemistry

Jonas Brinck, PhD, MD
Karolinska Institutet
Department of Medicine
Metabolism Unit

Opponent:
Professor Kjetil Retterstøl
University of Oslo
Department of Nutrition
Division of Clinical Nutrition

Examination Board:
Associate Professor Linda Mellbin
Karolinska Institutet
Department of Medicine
Division of Cardiology

Associate Professor Mats Gåfvels
Uppsala University
Department of Medical Science
Division of Clinical Chemistry

Associate Professor Per Svensson
Karolinska Institutet
Department of Clinical Science and Education,
Södersjukhuset
Division of Cardiology

The thesis will be defended at
4U Solen, ANA Futura, Karolinska Institutet,
Alfred Nobels Allé 8, Huddinge
Friday 15th of May 2020 at 9.30 a.m.
"Först kaffe, sen förändrar vi världen"

Herregud & Co

Printed with permission from Royne Mercurio
Till Viktor och Filip, ni är bäst!
Jag älskar er! ♥
ABSTRACT

Cardiometabolic diseases is a growing health issue worldwide. New knowledge about the pathophysiological processes have been gained and several research discoveries have contributed to better management of the diseases. Even so, the prognosis, diagnosis, and treatment of cardiometabolic diseases needs to be further improved. For this, the discovery and implementation of additional biomarkers is extremely relevant. New advancing technologies have increased the opportunities to perform discovery studies and to identify new potential biomarkers. However, for several reasons, only few biomarkers survive the long journey from discovery into clinical implementation and there is a need to make this process more efficient.

The objective of the thesis was to evaluate new potential biomarkers to improve the diagnosis and management of cardiometabolic diseases. An additional aim was to establish an efficient process for rapid transfer of new potential biomarkers, identified in discovery studies into evaluation in the routine care setting. Four studies in different patient cohorts, characterized by separate designs, and reflecting diverse phases in the implementation of biomarkers for cardiometabolic diseases are presented.

Lipoprotein (a) \([\text{Lp}(a)]\) is a modified low-density lipoprotein (LDL) particle and its concentration in plasma is mainly genetically determined. High levels of \(\text{Lp}(a)\) is associated with an increased risk for cardiovascular diseases (CVD). Despite the fact that it is not yet clear whether reduction of plasma \(\text{Lp}(a)\) levels translate into a reduced CVD risk, more knowledge about its role as a risk factor in different cohorts and diagnoses is needed to better understand how patients with high \(\text{Lp}(a)\) levels should be managed.

In Paper I we investigated the distribution of plasma \(\text{Lp}(a)\) levels and its association with CVD in a large cohort of patients who had their plasma levels of \(\text{Lp}(a)\) determined in routine care. Laboratory data from 23,398 patients was linked to data retrieved from National Board of Health and Welfare registers and National Quality registers. \(\text{Lp}(a)\) levels had a skewed distribution, increased with age, and was higher in females. Patients with \(\text{Lp}(a)\) levels in the 4th quartile had a 1.36-fold (95% Confidence Interval (CI) 1.14-1.61, \(p=0.001\)) increased risk for ischemic heart disease compared to patients belonging to the 1st quartile. The risk was independent of age, previous CVD, diabetes, and LDL-cholesterol levels. Hence, \(\text{Lp}(a)\) is an important risk factor for ischemic heart disease also in patients referred from hospitals, outpatient clinics, and general practitioners in the Region Stockholm.

Very little is known about the role of \(\text{Lp}(a)\) as a risk factor for CVD in patients with type 1 diabetes. Therefore, in Paper II we investigated the association of \(\text{Lp}(a)\) with cardiovascular complications and metabolic control in 1860 subjects with type 1 diabetes. \(\text{Lp}(a)\) levels had a skewed distribution, increased with age, and was not influenced by sex. Patients with poor metabolic control (HbA1c >52 mmol/mol) had higher \(\text{Lp}(a)\) levels compared to patients with good metabolic control. Patients with high \(\text{Lp}(a)\) levels (>120 nmol/L) had a 1.51-fold (95% CI 1.01-2.28, \(p=0.048\)) increased risk for any macrovascular diseases, a 1.68-fold (95% CI
1.12-2.50, \( p=0.01 \)) increased risk for albuminuria, and a 2.03-fold (95% CI 1.02-4.01, \( p=0.043 \)) increased risk for calcified aortic valve disease compared to patients with very low levels (<10 nmol/L). In summary, Lp(a) is a relevant risk factor also in patients with type 1 diabetes.

In Paper III we aimed to establish an efficient process for transfer of newly discovered potential biomarkers into evaluation in the routine care setting. The prototype was based on the evaluation of chemokine ligand 16 (CCL-16), previously identified as interesting biomarker for acute coronary syndrome (ACS) in a discovery project called Vinncardio, initiated by the Science for Life laboratory, Royal Institute of Technology (KTH), Stockholm, Sweden. Patients eligible for inclusion were identified when their plasma was analyzed for high sensitive Troponin T at the Karolinska University Laboratory, Stockholm, Sweden. The plasma samples were temporarily stored and meanwhile the patients received a letter of invitation to participate in the study. A positive response was retrieved from ~40 % and 1631 patients were included. No significant differences in CCL-16 were observed between patients with ACS and other diagnosis and CCL-16 do not appear to be a valid biomarker for ACS. Despite this negative result, we manage to establish a process for early evaluation of new potential biomarkers in routine care settings and to rapidly create a biobank and include patients referred to the hospital with an acute medical condition.

Clinical randomized trials have shown that addition of ezetimibe to simvastatin treatment further improve the reduction of CVD events, especially in patients with type 2 diabetes where elevation of remnant-cholesterol is characteristic. Remnant-cholesterol is a new and interesting biomarker and mendelian randomization studies have identified it as an independent risk factor for CVD, also promoting and sustaining low grade inflammation. In Paper IV we aimed to in detail study how the lipoprotein metabolism is affected by simvastatin and ezetimibe treatment, alone or in combination, to gain further understanding of the molecular effects of these two widely used lipid lowering drugs. Forty patients eligible for cholecystectomy were randomized to four-week treatment before surgery to placebo, simvastatin (80 mg daily), ezetimibe (10 mg daily), or to combination of both. The combination of simvastatin and ezetimibe resulted in further reduction of cholesterol and cholesteryl esters in remnant- and LDL-particles, as well as reduction of apolipoprotein B (apoB) containing particles, and reduced apoB-containing lipoprotein affinity for arterial proteoglycans compared to simvastatin. These additional positive effects on atherogenic lipoproteins and especially remnant-particles can possibly explain the further reduction of CVD events previously observed, and the combination of ezetimibe and simvastatin seems to be the optimal treatment in conditions with elevated remnant-cholesterol.

In conclusion, these four studies have provided further knowledge about the different biomarkers investigated. Also, they can contribute to an improved management of patients with cardiometabolic diseases and indicate the way to a rapid recruitment of patients in clinical studies. Hence, this thesis adds to a deeper understanding of the complexity in the process to validate and implement new biomarkers.
LIST OF SCIENTIFIC PAPERS

I. **Littmann K**, Hagström E, Häbel H, Bottai M, Eriksson M, Parini P*, Brinck J*. Distribution of plasma Lipoprotein (a) levels assessed in a Swedish clinical routine laboratory and association to cardiovascular disease. 
   *Manuscript*
   *These authors contributed equally to this work.*


   *Manuscript*
   *These authors contributed equally to this work.*

   *These authors contributed equally to this work.*
## CONTENTS

1 Background.......................................................................................................................... 1
   1.1 Biomarkers..................................................................................................................... 1
      1.1.1 Definition and characteristics of biomarkers...................................................... 1
      1.1.2 Biomarker research ............................................................................................... 1
   1.2 Cardiometabolic disease .............................................................................................. 2
   1.3 Cardiovascular diseases (CVD) ................................................................................... 2
      1.3.1 Estimating the CVD risk ....................................................................................... 3
      1.3.2 Biomarkers for CVD ............................................................................................ 4
   1.4 Lipoproteins and CVD risk .......................................................................................... 4
      1.4.1 Brief overview of lipoproteins ............................................................................. 4
      1.4.2 Assessment of atherogenic lipoproteins for CVD risk estimation ....................... 6
      1.4.3 Lipid lowering treatment with statins and ezetimibe ........................................... 8
   1.5 Lipoprotein (a) [Lp(a)] .................................................................................................. 9
      1.5.1 Metabolism of Lp(a) ............................................................................................. 9
      1.5.2 Genetics and phenotypes ..................................................................................... 9
      1.5.3 Role of Lp(a) in pathophysiological mechanisms ............................................ 10
      1.5.4 Laboratory assessment of Lp(a) ......................................................................... 10
      1.5.5 Lp(a) and CVD .................................................................................................. 11
      1.5.6 Lp(a) lowering therapies ................................................................................. 11
   1.6 Acute Coronary Syndrome (ACS) ............................................................................. 12
      1.6.1 Definition and diagnosis of ACS ....................................................................... 12
      1.6.2 Biomarkers for ACS ........................................................................................... 13

2 Objective ................................................................................................................................. 15
   2.1 Objective of the thesis ............................................................................................... 15
   2.2 Specific objectives in the different Papers ............................................................... 16

3 Clinical relevance ..................................................................................................................... 17

4 Methodology ................................................................................................................................ 19
   4.1 Description of the different cohorts .......................................................................... 19
   4.2 Laboratory assays ........................................................................................................ 20
      4.2.1 Routine laboratory parameters ........................................................................... 20
      4.2.2 Lp(a) .................................................................................................................. 20
      4.2.3 CCL-16 ............................................................................................................... 21
      4.2.4 Lipoproteins and apolipoproteins ....................................................................... 21
      4.2.5 Proteoglycan binding ........................................................................................ 21
      4.2.6 Messenger ribonucleic acid (mRNA) expression in liver biopsies ..................... 22
   4.3 Statistical analysis ......................................................................................................... 22

5 Ethical considerations ............................................................................................................. 24
   5.1 General ethical considerations within research projects ......................................... 24
   5.2 Specific ethical consideration in the different Papers ............................................... 25

6 Results and discussion ........................................................................................................... 28
   6.1 Paper I ........................................................................................................................ 28
6.1.1 Distribution of plasma Lp(a) levels .................................................................28
6.1.2 Lp(a) levels and association with mortality and CVD ..........................30
6.2 Paper II .................................................................................................................30
   6.2.1 Distribution of plasma Lp(a) levels ............................................................30
   6.2.2 Lp(a) levels and vascular complications ..................................................30
   6.2.3 Lp(a) levels in relation to metabolic control .............................................32
6.3 Paper III ...............................................................................................................32
6.4 Paper IV ...............................................................................................................33
   6.4.1 Treatment effect on lipoproteins and lipoprotein subclasses ..................33
   6.4.2 Treatment effect on plasma proteoglycan binding .....................................34
   6.4.3 Treatment effect on key genes regulating hepatic cholesterol and lipoprotein metabolism .................................................................34
7 Conclusions and future perspectives .................................................................36
8 Concluding remarks ..............................................................................................39
9 Svensk sammanfattning .......................................................................................40
10 Acknowledgments .................................................................................................42
11 References ............................................................................................................45
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAT</td>
<td>Acetyl-coenzyme A acetyltransferase</td>
</tr>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>AMI</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Apo(a)</td>
<td>Apolipoprotein (a)</td>
</tr>
<tr>
<td>ApoA1</td>
<td>Apolipoprotein A1</td>
</tr>
<tr>
<td>ApoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass graft</td>
</tr>
<tr>
<td>CAVD</td>
<td>Calcified aortic valve disease</td>
</tr>
<tr>
<td>CCL-16</td>
<td>Chemokine ligand 16</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl esters</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CKMB</td>
<td>MB isoform of creatine kinase</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicron</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>cTroponin</td>
<td>Cardiac specific Troponin</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>EAS</td>
<td>European Atherosclerosis Society</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Emergency room</td>
</tr>
<tr>
<td>ESC</td>
<td>European Society of Cardiology</td>
</tr>
<tr>
<td>GDPR</td>
<td>General data protection regulation</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>β-Hydroxy β-methylglutaryl-CoA</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>hs-Troponin T</td>
<td>High-sensitive Troponin T</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>IFCC</td>
<td>International Federation of Clinical Chemistry</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>IMPROVE-IT</td>
<td>Improved reduction of outcomes: Vytorin efficacy international trial</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter quartile range</td>
</tr>
<tr>
<td>K2-EDTA</td>
<td>Potassium- ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>KIV-2</td>
<td>Kringle IV type 2</td>
</tr>
<tr>
<td>KUL</td>
<td>Karolinska University Laboratory</td>
</tr>
</tbody>
</table>
LCAT       Lecithin–cholesterol acyltransferase
LDL       Low density lipoprotein
Li-Heparin Lithium Heparin
Lp(a)     Lipoprotein (a)
LPL       Lipoprotein lipase
MI       Myocardial infarction
mRNA     Messenger-Ribonucleic acid
NEFA     Non esterified fatty acids
NMR      Nuclear magnetic resonance
NPC1L1    Niemann-Pick C1-like 1
NSTEMI    Non-ST-elevation myocardial infarction
MTTP      Microsomal triglyceride transfer protein
PAD       Peripheral artery disease
PCI       Percutaneous coronary intervention
PCSK9     Proprotein convertase subtilisin/kexin type 9
PG        Proteoglycan
RRR       Relative risk ratio
SCORE     Systematic coronary risk estimation
SD        Standard deviation
SHARP     Study of heart and renal protection
SR-B1     Scavenger receptor type B class I
SREBP2    Sterol regulatory element-binding protein 2
SREBP2F2  Sterol regulatory element binding transcription factor 2
STEMI     ST-elevation myocardial infarction
TG        Triglyceride
UA        Unstable angina
URL       Upper reference limit
VLDL      Very-low density lipoprotein
VTE       Venous thromboembolism
1 BACKGROUND

1.1 BIOMARKERS

Why are only approximately 100 different biomarkers currently used in the clinic, though >150,000 potential new biomarkers have been suggested from discovery studies (1)? Is there no need for more biomarkers? Are those identified biomarkers false? Is the process to establish new biomarkers too challenging or is their clinical implementation too complex?

Unfortunately, this thesis will not answer all these questions, but it will present further understanding of the challenges that the search for biomarkers presents, with the hope to contribute with novel approaches for the evaluation of potential biomarkers in clinical settings.

1.1.1 Definition and characteristics of biomarkers

Biomarkers should reflect a stage in the pathophysiological process of diseases. They have a broad area of use, including screening of patients, prognosis, diagnosis, and monitoring of diseases, as well as to be surrogate end points in clinical trials. Some biomarkers are also risk factors since they are not only involved in the pathophysiological process but are also causal for the disease. However, the difference between a biomarker and a risk factor is subtle (2, 3).

The optimum features of biomarkers depend on their intended use, but some features are generally desirable. They should be specific and able to distinguish definite pathologic conditions from physiologic states or from other diseases, be measurable in accessible biological sources (e.g., blood, urine), be quantifiable with accurate assays, and defined reference levels should also be available (2). A relevant biomarker should add value to existing biomarkers, be generalizable to different populations, easy to interpret for the clinicians, and have proven cost effectiveness (3-5).

1.1.2 Biomarker research

Before starting projects aiming to discover new biomarkers, it is important to consider whether there are real needs for the healthcare that are not met by the already available biomarkers. Otherwise the outcomes of those projects cannot be implemented in clinical settings.

The strategy leading to biomarker implementation in the routine care can be divided into the following phases: discovery, qualification, verification, assay development, and evaluation. The discovery phase should be characterized by an unbiased approach and identify a definite number of possible new biomarkers that is not too excessive, otherwise this phase fails to identify relevant candidates. This indeed is a potential threat in discovery studies were genomics, proteomics, metabolomics, and pharmacogenomics are used. Careful selection of samples for screening in the discovery phase is also very important to prevent bias and to favor the identification of truly relevant candidates. Selection of the top candidates to be further
qualified should be based on literature reviewing, biological knowledge and performance in the discovery phase (2, 3, 6).

The qualification phase of top candidate biomarkers aims to assess the translatability of the discoveries. For example, if an animal model has been used to identify the candidate biomarker, it is mandatory to prove that this is detectible in human samples and differently expressed in healthy and pathological conditions. Similar needs are also present when human material stored in biobanks is used in the discovery phase. In the next phase, verification of the candidate biomarkers should be performed in a larger number of samples in order to test the sensitivity and specificity by using cases and controls (6).

Suitable technologies with acceptable analytical performance, including accuracy (bias), precision (repeatability and reproducibility), and measurement range (linearity, lower level of detection and lower level of quantification) should be used to develop an assay for measurement of the biomarkers before evaluation in clinical care settings. In addition, the optimal preanalytical conditions for the biomarkers should be determined (6-11).

The strategy for evaluation in clinical care settings depends on the conditions and the intended use of the biomarkers. However, some general important parts are the determination of reference levels in healthy individuals and the levels of the biomarkers in patients with different diseases, in order to understand the consequences of pathological values (3, 6). It is also important to test the performance of the biomarkers in term of possible prediction of the diseases and outcomes in prospective trials (3, 11). It is thus obvious that evaluation and implementation of biomarkers for routine use in healthcare can take many years, require great efforts, and that many candidates fail during the process. Hence, it is very important to have a common strategy designed in collaboration between the research laboratory, the routine laboratory, and the clinicians from the start (12, 13).

1.2 CARDIOMETABOLIC DISEASE

The definition of cardiometabolic diseases is ambiguous. In this text it is referred to a group of diseases in which metabolic disorders (e.g. metabolic syndrome, diabetes, dyslipidemia) accelerate and cause the development of cardiovascular diseases (CVD).

1.3 CARDIOVASCULAR DISEASES (CVD)

CVD comprise a variety of conditions in the heart and blood vessels including coronary heart disease, cerebrovascular disease, peripheral vascular disease, heart failure, rheumatic heart disease, cardiomyopathies, and congenital heart disease. Atherosclerosis is an important component in the development of CVD and dyslipidemia, inflammation and the immune response are all causal factors in atherogenesis (14).

CVD is the leading cause of morbidity and mortality worldwide, and the World Health Organization mention CVD as a threat to global health in 2019. In the European Union,
approximately 1.8 million die from CVD every year, accounting for more than 35% of all deaths and is considerable higher than cancer that is the second most common cause of death (15). In Sweden, CVD accounts for approximately 33% of all deaths (National Board of Health and Welfare statistics for causes of death 2018) and over 2 million people have a CVD (16).

Overall, the prevalence of CVD has declined over the last 20 years and the outcome of CVD has improved. However, there is an inequality in CVD burden and deaths across the countries, where low- and middle-income countries are more affected than the high-income countries (15-18). Better primary and secondary prevention, including treatment with statins and antihypertensive drugs, as well as lifestyle interventions and decreased smoking rates, have all contributed to the reduction of CVD and its sequelae. Unfortunately, other pathologic conditions, such as obesity and type 2 diabetes, are increasing and this explain why CVD is now a major global health issue (3, 15, 18). Early identification of individuals at high risk for CVD is essential to further improve the prevention and treatment of the diseases and biomarkers can contribute to this process.

### 1.3.1 Estimating the CVD risk

CVD risk factors are numerous. Some risk factors are modifiable (e.g. smoking, dyslipidemia, diabetes, hypertension, and unhealthy lifestyles) while others are not (e.g. sex, age, and genome). Additional circumstances, such as presence of other comorbidities, epigenetics, and psychosocial and environmental factors, may also influence the CVD risk further (4, 18, 19).

Several tools have been developed to assist in CVD risk estimation. The different tools adopted in different countries show similar performance when used in the correct populations. A commonly used risk assessment tool for European populations is the Systematic Coronary Risk Estimation (SCORE) that based on age, sex, smoking status, blood pressure, and total cholesterol estimate an individual’s 10-year risk for CVD-related mortality. Other risk assessment tools to estimate the 10-year risk for CVD events are the Framingham Risk Score based on an American population and the QRISK that are developed in the United Kingdom (18).

CVD risk estimation using a risk estimation system such as the SCORE is recommended for asymptomatic adults >40 years according to guidelines presented by the European Atherosclerosis Society (EAS) and European Society of Cardiology (ESC). The CVD risk is categorized as very-high (calculated SCORE ≥10 % for 10-year risk of fatal CVD), high (calculated SCORE ≥5 % of 10-year risk for fatal CVD), moderate (calculated SCORE ≥1 % for 10-year risk of fatal CVD), and low (calculated SCORE ≤1 % for 10-year risk of fatal CVD). Some individuals are classified to be at very-high or high CVD risk without the SCORE calculation, i.e. patients with established CVD, significant plaques visible on computer tomography scan or ultrasound, diabetes with organ damage or duration >20 years, chronic kidney disease (CKD) with estimated glomerular filtration rate (eGFR) <60 ml/min/1.73m$^2$ or
patients with familial hypercholesterolemia. Young patients with shorter duration of diabetes are classified to be at moderate risk. Other contributing factors such as remnant-cholesterol, apolipoprotein B (apoB), triglycerides (TG), high density lipoprotein (HDL) cholesterol, Lipoprotein (a) [Lp(a)], C-reactive protein (CRP), and calcium score modifies the risk and can contribute to reclassification of the patient into another risk category (19).

It is important to remember that the risk assessment is based on major risk factors from general population studies. Assessment of the individual’s CVD risk, considering additional risk modifying factors is challenging but crucial. In this context, biomarkers can help to improve the individual risk stratification and contribute to a more personalized medical care (20).

1.3.2 Biomarkers for CVD

Several different biomarkers for CVD are currently in use and many more have been suggested, including circulating biomarkers (e.g. troponins, natriuretic peptides, CRP, lipoproteins, apolipoproteins, copeptin, galectin-3, soluble suppression of tumorigenicity-2, growth derived factor 15 etc.), imaging biomarkers (e.g. calcium score, carotid intermedia thickness, and echocardiography), and anthropology measurement biomarkers (e.g. blood pressure, oxygen saturation, body mass index, and heart rate) (21). They can be categorized in several ways and usually according to the pathophysiological processes that the biomarkers are involved in. For example, biomarkers are categorized in relation to their role in myocardial injury, myocardial stress, inflammation, coagulation or metabolic disorders (e.g. dyslipidemia or glucose intolerance) (3, 18, 22).

The search for new CVD biomarkers to further improve the individual risk assessment, prevention, diagnosis, prognosis and treatment of the diseases has been extensive, and a deep understanding of the pathophysiological mechanisms of the diseases is a prerequisite in this search (3, 4). The advanced techniques used in the biomarker discovery have in addition to identifying many candidate biomarkers, contributed to further understanding of the diseases. Genome wide association studies have generated new insights from genetic biomarkers and single nucleotide polymorphisms, and the mendelian randomization approach have revealed causal relationships of several biomarkers and CVD (2, 20, 21, 23, 24).

1.4 LIPOPROTEINS AND CVD RISK

1.4.1 Brief overview of lipoproteins

In the circulation, fat molecules are transported in lipoproteins consisting of TG, free-cholesterol, cholesteryl esters (CE), phospholipids, and proteins. The most TG rich lipoproteins in the circulation are chylomicrons (CM) and very low-density lipoproteins (VLDL). CM are large TG-rich particles, only synthesized in the intestine and the major structural protein is apolipoprotein B48. In the circulation exchange of apolipoproteins, preferably with HDL-particles occurs and apolipoprotein C and apolipoprotein E are added to the CM. The enzyme
lipoprotein lipase (LPL) is present at the endothelial capillary cells and mostly expressed in adipose tissue, skeletal-, and cardiac muscle. Non esterified fatty acids (NEFA) released from the LPL-mediated hydrolysis of TG in CM are immediately absorbed by the tissues. The NEFA not absorbed are directed, bound to albumin, to the liver (so called spillover of NEFA). The muscle tissues are very effective in the uptake of NEFA, that are oxidized to produce energy. The uptake is less effective in white adipose tissue were NEFA are re-esterified to TG for storage. LPL and hydrolysis of TG are under strict hormonal regulation especially from insulin and catecholamines. Factors like apolipoprotein CIII, apolipoprotein E, and angiopoietin-like proteins also modulate LPL activity. After LPL mediated hydrolysis of TG, the remaining CM-remnants are rapidly taken up by the liver (25-30).

VLDL are synthesized by the liver and are smaller compared to CM. VLDL contains relatively less TG and more cholesterol, have a longer half-life in plasma (1-2 hours), and apolipoprotein B100 is the structural protein instead of apolipoprotein B48. After hydrolysis of TG by LPL in the circulation, VLDL became intermediate dense lipoproteins (IDL), also called VLDL-remnant. The IDL are in turn further processed, also by the action of hepatic lipase into low-density lipoprotein (LDL). LDL have a 2-3 days half-life, are the most abundant lipoproteins and transports most of the cholesterol in the circulation (27-30).

The liver has a key role in the cholesterol homeostasis. It is the main organ for de novo synthesis, but it should be remembered that most cells in the body can synthesize cholesterol. The rate-limiting enzyme in cholesterol synthesis is 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase. The liver can also acquire cholesterol from the circulation. The uptake of LDL is mediated by LDL-receptors that are mostly expressed in the liver but can be present on cell-surfaces in all cells. The dietary cholesterol absorbed in the small intestine is transported in CM from the gut into the circulation and finally to the liver as CM-remnants. HDL particles can carry cholesterol back to the liver by docking to the scavenger receptor type B class I (SR-BI). The liver can excrete cholesterol into the circulation in VLDL or into the bile directly or after conversion into bile acids. A considerable amount of the bile acids and cholesterol secreted into bile are reabsorbed in the small intestine and returned to the liver in the enterohepatic circulation (27-30).

HDL are important in the reverse cholesterol transport i.e. the transport of cholesterol from peripheral tissues back to the liver. HDL are synthesized from the intestine and the liver as nascent-HDL, consisting mainly of phospholipids, apolipoproteins and unesterified cholesterol. The characteristic apolipoprotein of HDL is apolipoprotein A1 (apoA1), a cofactor for lecithin cholesterol acetyltransferase (LCAT). Once in the circulation, HDL take up cholesterol that is esterified into CE by LCAT. The nascent HDL-particle gradually increases in CE content and turns into the mature HDL particle that exists in two subclasses with different density, HDL2 and HDL3 (31, 32). The CE in HDL can be returned to the liver in different ways. The SR-B1 removes CE from HDL in the liver while HDL remains in the circulation. In addition, CE can
be removed from HDL in exchange for TG from VLDL. This process is mediated by cholesteryl ester transfer protein (CETP), an enzyme that also exchange CE for TG between LDL and VLDL (28, 33, 34).

1.4.2 Assessment of atherogenic lipoproteins for CVD risk estimation

The evidences for LDL-cholesterol as causal factor for CVD are extensive as well as the beneficial effects of LDL-lowering therapies for both primary and secondary CVD prevention (35). However, all apoB containing lipoproteins up to ~70 nm in size, i.e. CM-remnants, VLDL, IDL, LDL, and Lp(a) can enter the endothelium and contribute to the development of atherosclerosis (36, 37).

LDL is the most abundant of the apoB-containing particles in plasma and the major contributor of cholesterol depositions in the arterial wall (38). In conditions with elevated TG-rich particles or high Lp(a) levels, the contribution of other apoB-containing lipoproteins in the atherosclerotic process is more significant. This can add to the residual CVD risk seen in some patients even after reaching desirable LDL-cholesterol levels and assessment of biomarkers beyond LDL-cholesterol can in these cases improve estimation of an individual’s CVD risk (36, 37, 39-41).

A “standard” lipid profile includes measurement of total-cholesterol, HDL-cholesterol and TG. The LDL-cholesterol can either be calculated by the Friedewald’s formula (LDL-cholesterol = total-cholesterol – (0.45 x TG) – HDL-cholesterol) or directly measured. The reference method for LDL-cholesterol measurement is beta-quantification where the lipoprotein particles are partially separated by ultra-centrifugation. After removal of CM and VLDL the apoB-containing particles (mainly LDL-cholesterol) in the bottom fraction are precipitated using heparin-manganese, the remaining HDL-cholesterol in the solution is quantified and LDL-cholesterol can be calculated by subtraction of HDL-cholesterol from total cholesterol in the bottom fraction (42-44). This is a very time-consuming procedure and therefore Friedewald’s formula has been used to estimate LDL-cholesterol due to its simplicity, good precision, and cost effectiveness. Hence, most clinical studies have been or are based on calculated LDL-cholesterol. Calculation of LDL-cholesterol assumes a constant ratio between VLDL-TG and cholesterol, no presence of CM or remnant-particles, and requires a fasting state. The estimation is imprecise in hypertriglyceridemia and should not be used if TG levels are > 4.5 mmol/L. Several homogenous assays with good reproducibility and specificity for direct measurement of LDL-cholesterol is commercially available today. Due to the great heterogeneity of LDL-particles direct measurement is challenging, and diverse assays can distinguish in their ability to detect the different subfractions of LDL. Hence, the results can vary depending on the assay and especially in conditions with elevated TG levels (45, 46). Direct measurement of LDL-cholesterol usually reports 10-20 % lower values compared to calculated LDL-cholesterol levels depending on the assay and TG levels (47-50). Both calculated and directly measured LDL-
cholesterol includes the cholesterol content of Lp(a) (approximately 30 % of its mass) and can therefore be overestimated in patients with high Lp(a) levels (45, 46).

LDL-cholesterol levels are especially important for CVD risk assessment and is the primary treatment target. In the ESC/EAS 2019 guidelines, a LDL-cholesterol reduction ≥50 % and levels less than 1.4 mmol/L in patients at very-high risk or levels less than 1.8 mmol/L in patients at high risk is recommended. Even lower LDL-target levels of 1.0 mmol/L are desirable in very-high risk patients with recurrent events (19). Intensive statin treatment and adding of other lipid lowering therapies is often required to reach these levels and in this era of very low LDL-cholesterol target levels, laboratory assays with improved precision at low levels are desirable.

Elevated TG levels (41) and elevation of remnant-cholesterol (51) have both been demonstrated to be associated with increased CVD risk. The risk observed in hypertriglyceridemia seems to be due to elevated cholesterol content of atherogenic TG-rich particles rather than the TG per se (41). The cholesterol content of all atherogenic lipoproteins, i.e. the non-HDL cholesterol can be estimated by subtraction of HDL from total cholesterol and is preferable in risk estimation especially in conditions with hypertriglyceridemia and elevation of remnant-particles (i.e. diabetes, metabolic syndrome, and CKD) (46, 52). Reduction of non-HDL cholesterol reduce the CVD risk (53) and is recommended as a secondary treatment target in combined hyperlipidemia and conditions with elevated remnant-particles (19). Remnant-cholesterol is the cholesterol in non-HDL and non-LDL particles, i.e. the free cholesterol and CE carried in CM-remnants, VLDL and IDL. It can be estimated both in the fasting and non-fasting state by subtraction of LDL- and HDL-cholesterol from total cholesterol. Remnant-cholesterol is a causal risk factor for ischemic heart disease (IHD) and in contrast to LDL-cholesterol also associated with low-grade inflammation and contribute to the residual CVD risk observed after LDL-cholesterol reduction (40, 51, 52, 54-57). In addition, remnant-cholesterol was recently shown to be associated with increased risk of ischemic stroke (58).

All atherogenic lipoproteins derived from either VLDL or CM carries one apoB particle each, hence, the apoB level corresponds to their particle count in plasma. The use of apoB for CVD risk estimation has been investigated in several prospective population cohort studies (59-61) and meta-analysis (62-64). It seems to be at least equal to, but some suggests superior to LDL-cholesterol in CVD risk assessment. The advantage is especially in dyslipidemias including increased TG-rich lipoproteins or when there is a discrepancy between LDL-cholesterol and apoB levels (62). In addition, reduction of apoB with statins or other common used LDL-receptor dependent lipid lowering therapies can reduce the CVD risk (65). Therefore, measurements of apoB is recommended as an alternative CVD risk marker in combined hyperlipidemias or in patients with metabolic syndrome, diabetes or CKD, and can also be considered as a secondary treatment target. It may be preferred over non-HDL cholesterol since it is superior for estimation of atherogenic particle count in the circulation and in conditions with elevated small dense LDL-particles, this can be of special interest (19, 39). Other
advantages of apoB is the existing immunoassays with good analytical quality, that the measurement is independent of TG levels, and fasting is not required. Hence the analytical errors, especially when hypertriglyceridemia is present are less significant compared to LDL-cholesterol (39, 52).

1.4.3 Lipid lowering treatment with statins and ezetimibe

Statins are the cornerstones in the treatment of dyslipidemia today. Lovastatin was the first statin approved by the U.S. Food and Drug Administration in 1989 for human use to lower cholesterol levels. It is a natural occurring compound in food, for example red yeast rice. Since then, substantial evidence of the positive effect of statins for lipid lowering, primary and secondary prevention of CVD exists (66). A statins is the first drug of choice for the management of hypercholesterolemia and dyslipidemias with few side effects (19, 67). The most frequently appearing side effect is statin associated muscle symptoms that can limit its use. To terminate treatment, lower the dose, or change to another statin can reduce this side effect (67, 68).

Statins inhibits HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, and hence the cholesterol synthesis in the liver. Reduction of cholesterol synthesis results in increased expression of the LDL-receptor, increased uptake of LDL and other apoB-containing particles and a reduction of these atherogenic lipoproteins in the circulation (69). There is a dose dependent cholesterol reduction that varies between different types of statins. Rosuvastatin is the most potent statin and can lower the LDL-cholesterol levels up to approximately 60 % (66, 70).

Cholesterol from the diet is absorbed in the proximal jejunum in the small intestine where Niemann-Pick C1-like 1 (NPC1L1) is an important mediator. Ezetimibe is an inhibitor of NPC1L1 and reduces the intestinal absorption of cholesterol (71, 72). Thereby, ezetimibe can lower the LDL-cholesterol levels by approximately 15-20 %, and when added to statin treatment the additional reduction is 15-20 % (73, 74). The Study of Heart and Renal Protection (SHARP), a randomized double blind trial in approximately 9000 subjects with CKD, showed that the combination of simvastatin 20 mg daily and ezetimibe 10 mg daily safely reduced major atherosclerotic events by 17 % compared to placebo (75). The Vytorin Efficacy International Trial (IMPROVE-IT) showed an additional LDL-cholesterol lowering effect and improved CVD outcome by combined treatment with simvastatin 40 mg daily and ezetimibe 10 mg daily compared to only simvastatin in 18 000 patients with previous acute coronary syndrome (ACS) (76, 77). The effect was most pronounced in patients with type 2 diabetes, where the dyslipidemia is characterized by elevated TG-rich lipoproteins (i.e. remnant-particles), increased small dense LDL-particles, and low HDL-cholesterol levels (78, 79). It has also been shown that when ezetimibe is added to ongoing simvastatin treatment the remnant-cholesterol is reduced further than just doubling the statin dose (80).
1.5 LIPOPROTEIN (A) [LP(A)]

1.5.1 Metabolism of Lp(a)

Lp(a) is a LDL-cholesterol particle with an apolipoprotein(a) [apo(a)] covalently bound by disulfide bridges to apolipoprotein B100 (81). The physiological function, metabolism, and catabolism of Lp(a) has not yet been described in detail and is an ongoing research area of interest (81, 82).

Apo(a) is synthesized in the liver and binds to the LDL-particles. Exact where the binding takes place is not known, but the hepatocyte, space of Disse, or the plasma compartment are possible locations (83). The major catabolic pathway for Lp(a) is believed to be through the liver and to a smaller extent through the kidneys (81). Alterations of plasma Lp(a) levels have been observed in patients with renal disease, supporting a role of the kidney in the Lp(a) metabolism (84, 85). The receptors involved in the catabolism remains obscure and the role of the LDL-receptor is debated. Some studies have shown that Lp(a) binds to the LDL-receptor and that the interaction is important in the Lp(a) clearance but other studies have demonstrated the opposite (86-89). Additional receptors suggested to be involved in the catabolism of Lp(a) are the LDL-receptor related protein, asialoglycoprotein receptors, macrophage scavenger receptors, and megalin gp330 receptors (90). Since treatment with proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors decrease plasma levels of Lp(a), a function of PCSK9 in the Lp(a) metabolism is plausible. A major role of PCSK9 in the secretion of Lp(a) from the hepatocytes has been suggested (91), while other studies demonstrate a role of PCSK9 in the Lp(a) catabolism (88, 92), and some suggests that the Lp(a) reduction from treatment with PCSK9-inhibitor are mediated through other mechanisms than by PCSK9 (93).

1.5.2 Genetics and phenotypes

Plasma Lp(a) levels are mainly genetically determined and affected by ethnicity. Age, sex or lifestyle factors seems to be of minor importance. Apo(a) is coded from the LPA-gene on chromosome 6q2.6-q2.7. The LPA-gene share similarities with the plasminogen gene, consisting of two K-domains (IV and V) and of a protease domain, which is catalytically inactive (82, 90). The K IV-domain occurs in 10 different types, were the type 2 (KIV-2) have a large copy number variation and exist in multiple copies, from 1 to > 40. Every KIV-2 copy is 5.5 kbp, consists of two exons separated by a long 4 kbp intron and, a short intron with 1.2 kbp separate each KIV-2 copy. The exon 1 is identical in all copies while exon 2 exists in three different types that are differentiated by synonymous mutations (82). Depending on the KIV-2 copy number, the molecular weight of apo(a) varies between 200-800 kDa (94). A majority of the population (70-90 %) are heterozygous for the LPA alleles and have two different Lp(a) particles in the circulation (82, 95).

Plasma Lp(a) levels are skewedly distributed in the population, ranges within three orders of magnitude, and are inversely correlated to the copy number of KIV-2. The correlation is not linear, and the strength varies between different populations. The strongest association between
KIV-2 copy numbers and Lp(a) levels has been demonstrated in Asian and European populations and are weaker in African populations (82).

1.5.3 **Role of Lp(a) in pathophysiological mechanisms**

Elevated plasma levels of Lp(a) are associated with increased risk for CVD, i.e. IHD, stroke, and calcified aortic valve disease (CAVD) (81, 96-100). Association with peripheral artery disease (PAD) and venous thromboembolism (VTE) has also been demonstrated (101, 102).

The mechanism of Lp(a) in the pathogenesis of CVD is not fully understood, but is probably due to both atherogenic and prothrombotic properties of the particle (81). Lp(a) possess different pro-atherogenic characteristics, including interactions with endothelial cells, accumulation in the arterial wall, initiation of an pro-inflammatory response, and its carrying of a large amount of oxidized phospholipids (83, 90). The structurally similarity of apo(a) to plasminogen is important and Lp(a) is suggested to interfere with the plasminogen activity. Lp(a) can bind to similar structures as plasminogen but lacks fibrinolytic activity, resulting in an anti-fibrinolytic action (82, 90, 103). Other mechanisms for prothrombotic activity, including interference with tissue plasminogen activator, tissue factor pathway inhibitor, altered fibrin clot properties, and increased expression of plasminogen activator inhibitor-1 and tissue factor has also been suggested (90, 103).

1.5.4 **Laboratory assessment of Lp(a)**

The laboratory assessment of Lp(a) is challenging because of the great size heterogeneity of apo(a). The covalent binding between apo(a) and apoB, and the homology between apo(a) and plasminogen also contributes to the difficulty to develop specific antibodies against apo(a). Due to absence of a common reference material and harmonization of different Lp(a) assays, the consistency between laboratories has been poor. Since the reactivity of antibodies directed to the repeated segment in KIV-2 vary with the copy number, a calibrator cannot be representative for all apo(a) sizes and the assay will either overestimate or underestimate the Lp(a) concentration depending on whether the samples contains a larger or a smaller apo(a) size than the calibrator. Lp(a) has historically been measured by immunoassays that report total mass concentration of Lp(a). Today several different assays that report Lp(a) in mass or in molar concentrations are commercially available and the different assays are to a various degree dependent on the apo(a) size (104, 105).

The use of a standardized, size independent method, traceable to an international reference material is desirable for the Lp(a) measurement and the possibility to compare results from different studies (106, 107). In 1995, the International Federation of Clinical Chemistry (IFCC) initiated a project in collaboration with manufacturers to establish a reference material for standardization of Lp(a) assays. From this project a proposed reference material was suggested as a common calibrator (108, 109). To further evaluate the proposed reference material, it was
used to calibrate 22 different assays and to assign a target value of the different assay calibrators. This revealed a good inter-assay coefficient of variation (CV) of 2.8 % for the reference material and inter-assay CV of 9-15 % for quality control samples. Hence the use of the proposed reference material has been recommended for an accurate calibration and standardization of Lp(a) assays. However, in patient samples the 22 different assays displayed different results and the impact of apo(a) isoform varied greatly between the methods (104).

The IFCC standardized reference material is traceable to Lp(a) values in nmol/L. Other reference materials for mg/dL exists but these are not standardized to a common reference material and the use of a Lp(a) assay traceable in nmol/L to the IFCC reference material is preferred (106). Factors for conversion of Lp(a) between mg/dL and nmol/L have been proposed. The factors vary from 2.85 for a small apo(a) size to 1.85 for a large size and a mean factor of 2.4 have been suggested. However, factor for conversion are more or less imprecise depending on the apo(a) size and the use of conversion factors are very controversial (106, 110).

1.5.5 Lp(a) and CVD

The evidence for Lp(a) as a strong independent causal risk factor for CVD is convincing and has been extensively demonstrated in large genome wide association studies, mendelian randomization studies and several meta-analysis (97, 111-113). Lp(a) levels above the 80\textsuperscript{th} percentile in the population, corresponding to 50 mg/dL (~120 nmol/L) in the Copenhagen general population study (81) indicate an increased risk for IHD, CAVD, and PAD (97, 99, 102). The risk for myocardial infarction (MI) is elevated already at Lp(a) levels >30 mg/dL (~75 nmol/L) and increases with higher Lp(a) levels to almost 4-fold at Lp(a) levels >85 mg/L (~212 nmol/L) (114). Patients with extremely high Lp(a) levels (>180 mg/dL or >430 nmol/L) have equal lifetime risk for CVD as patients with heterozygous familial hypercholesterolemia (115). Hence, the 2019 ESC/EAS guidelines suggests that measurement of Lp(a) levels should be considered at least once in every adults life to identify those with extremely high Lp(a) levels that are at the highest risk (19).

High Lp(a) levels has been shown to be associated with ischemic stroke in several meta-analysis (94, 100, 116). The evidence is less extensive than for IHD and seems to be different depending on ethnicity (117). Since Lp(a) is suggested to possess proatherogenic properties an association with VTE could be assumed. Such association has been demonstrated in a meta-analysis (101) but the results from prospective trials are diverse where some show that Lp(a) is an independent risk factor for VTE in adults (118), but others do not (119, 120).

1.5.6 Lp(a) lowering therapies

Although, the evidence for the importance of Lp(a) as a risk factor for CVD is accumulating the major question remains whether Lp(a) lowering therapies can reduce the CVD risk. There are currently no available treatments to exclusively lower the plasma Lp(a) levels and no clinical outcome trials have investigated the effect of Lp(a) lowering on CVD risk. Therapies that lower
the plasma Lp(a) levels have a positive effect on the total lipid profile it is difficult to discern the specific impact on CVD risk due to Lp(a) reduction. However, based on data from studies with treatments that also affect the Lp(a) levels, it has been estimated that by reducing plasma Lp(a) levels to <50 mg/dL 1 of 14 cases of MI and 1 of 7 cases of CAVD could be prevented (96). In a placebo controlled trial, treatment with a PCSK9-inhibitor reduced plasma Lp(a) levels approximately 20 % which corresponded to a clinically significant reduction in CVD events independently from LDL-cholesterol (121). Other studies have demonstrated that a large Lp(a) reduction (65.7-100 mg/dL or 154-240 nmol/L) is required for a clinically significant CVD risk reduction (115, 122).

Whilst, traditional lipid-lowering therapies (e.g. stains, fibrates, and ezetimibe) have no or marginal effect on decreasing plasma Lp(a) levels, treatment with CETP-inhibitors and PCSK9-inhibitors can reduce the Lp(a) levels by 25-30 %, and lipoprotein apheresis by 25-40 % (123, 124). A meta-analysis from different statin trials revealed that statin treatment might even elevate the plasma Lp(a) levels up to 24 %, and the effect seems to be most pronounced from atorvastatin (125).

Novel therapies to exclusively lower plasma Lp(a) levels are emerging, and positive results from a hepatocyte specific apo(a) antisense nucleotide (AKCEA-APO(a)-LRx) have been demonstrated. In a randomized, double-blind, placebo-controlled trial the plasma Lp(a) levels were reduced up to 80 % in patients with established CVD after 6-12 months treatment with the AKCEA-APO(a)-LRx (126).

1.6 ACUTE CORONARY SYNDROME (ACS)

1.6.1 Definition and diagnosis of ACS

ACS is the acute manifestations of IHD, including the diagnosis of unstable angina (UA) and acute myocardial infarction (AMI). The pathophysiology behind ACS is atherosclerotic changes, atherosclerotic plaque rupture or superficial plaque erosion, and thrombosis formation leading to an acute myocardial ischemia and myocardial injury (127, 128).

ACS diagnosis is based on typical symptoms, electrocardiography (ECG) changes, and elevation of biomarkers. According to the fourth universal definition of MI (2018), the term myocardial injury should be used when there is at least one cardiac specific Troponin result, above the 99th percentile upper reference limit (URL) and is considered acute if there is rise or fall in cTroponin levels. AMI is defined when there is an acute myocardial injury with evidence for acute myocardial ischemia and i) a rise or fall in cTroponin levels and at least one value above the 99:th percentile URL and ii) at least one of; symptoms of myocardial ischemia; new ischemic ECG changes; development of pathological Q-waves; imaging evidence of ischemia or identification of coronary thrombus by angiography or autopsy. AMI is further divided into non-ST-elevation myocardial infarction (NSTEMI) and ST-elevation infarction (STEMI)
depending on the observed ECG changes. In addition, different types of MI are defined where type 1 is the traditional MI caused by ischemia in the myocardium due to plaque rupture and thrombosis formation. Type 2 MI is due to imbalance in oxygen demand and supply, for example caused by tachyarrhythmia or heavy physical effort; type 3 is sudden death caused by MI; type 4 and 5 MI are procedure related caused by percutaneous coronary intervention (PCI) (4a), stent thrombosis (4b) or by coronary artery bypass graft (CABG) (type 5) (127, 129, 130).

UA are defined as “myocardial ischemia at rest or minimal exertion in the absence of cardiomyocytes necrosis” (131). The prevalence of UA has changed during the years and especially when cTroponins replaced the previously used less specific and sensitive biomarkers. After the introduction of high sensitive cTroponins analyses the diagnosis for UA has decreased since those patients are diagnosed with NSTEMI instead (131-133).

1.6.2 Biomarkers for ACS

Previously, unspecific biomarkers like aspartate transaminase, lactate dehydrogenase, myoglobin, creatine kinase, and the MB isoform of creatine kinase (CKMB) was used in the diagnostics of ACS but today the high sensitive cTroponins are the preferred biomarkers. In order to improve the sensitivity and specificity of cTroponins and the prognosis of the disease other additional biomarkers for ACS have been suggested. Frequently discussed are for example heart-type fatty acid binding protein, natriuretic peptides, soluble suppressor of tumorigenicity 2, and growth differentiation factor 15. None of them have proven to outperform or add value to high sensitive cTroponin in the diagnosis of ACS and only CKMB and copeptin seem to be relevant so far (2, 130, 131, 134).

Troponin was first described in the 1960:s and has since then remarkably changed the diagnosis of ACS (135). Troponins are important mediators in the thin muscle filament contractions in striated and cardiac muscle tissues. Three different types of Troponins exist, Troponin T that attaches the troponin complex to actin, Troponin I that inhibits the interaction between actin and myosin when calcium is not present, and Troponin C that reduces the Troponin I inhibition of myosin after binding to calcium, leading to muscle contraction. Cardiac specific isoforms of Troponin T and Troponin I exists, but not for Troponin C. The cTroponins are mainly bound to the thin filaments, and only a small fraction (3 %) is free in the cytoplasm. After a cardiac injury, the cytoplasmic cTroponins are rapidly released, followed by a later release of the structural bound troponins (136). Peak levels are seen after 10-12 hours, the half-life of cTroponin is approximately 2 h and increased levels can be detected up to 14 days after onset of disease. The kinetics between cTroponin I and Troponin T are slightly different, where cTroponin I reaches a higher peak-level and returns faster to the normal level, probably due to faster release and degradation (130, 136-138).
A rise or fall in cTroponin levels indicates an acute myocardial injury, while a more stable elevation of cTroponin is seen in many different conditions (e.g. heart failure, pulmonary embolism, critical illness, sepsis, aortic dissection, myocarditis, kidney failure) (131, 135). To clarify a rise or fall in cTroponin levels, repeated testing is recommended in a suspected ACS. Different algorithms and cut off levels have been suggested for rule-in or rule-out ACS. A 0/3-hour and a 0/1-hour algorithm is presented in the 2015 ESC guidelines. In the 3-hour algorithm a first blood test is collected at entry into the emergency room (ER) and a second test 3 hours later. An ACS is likely if there is a change in cTroponin levels and at least one result over the upper reference limit. In the 1-hour algorithm, an initial very low level of cTroponin can rule-out ACS directly, otherwise a second cTroponin is measured after 1 hour. ACS can then be ruled-out if there is an initial low level with no change after 1 hour or ruled-in if the 1-hour value is elevated and show a change from the first measurement. The exact cut-off levels and delta changes of cTroponin are assay dependent (131). In patients with elevated cTroponin levels due to other causes, these algorithms to rule-in or rule-out of ACS cannot be used and the interpretation of cTroponin levels is more difficult.

CKMB, the MB isoform of CK is not specific for myocardium but is the most abundant isoform found in cardiomyocytes. CKMB have a faster clearance than cTroponin and returns to normal levels within 48-72 hours after an ACS. Before the introduction of cTroponins, this was the biomarker of choice for diagnosing ACS, but today it is due to the rapid clearance, most useful in the diagnosing of an early reinfarction (131).

Copeptin is derived from the c-terminal part of the pre-prohormone of vasopressin (i.e. antidiuretic hormone). It is released in proportional manner to vasopressin but is more stable in the circulation and easier to measure. Copeptin levels rises early in multiple acute conditions as an indicator of endogenous stress (139-141). Even if not specific, copeptin has been suggested as an additional biomarker in diagnosis of AMI and seems to add value to conventional cTroponin assays for the early rule-out of AMI (142-145). However, in combination with the high sensitive cTroponin assays, the additional value of copeptin is more uncertain (146-148). Hence, the use of copeptin is only recommended for early rule-out of AMI when high sensitive cTroponin assays is not available (131, 141, 149).
2 OBJECTIVE

2.1 OBJECTIVE OF THE THESIS

The objective of the thesis is to evaluate new potential biomarkers to improve the diagnosis and management of cardiometabolic diseases. An additional aim is to establish an efficient process for rapid transfer of new potential biomarkers, identified in discovery studies into evaluation in the routine care setting.

The thesis contains four studies in different patient cohorts, characterized of separate designs, reflecting diverse phases in the implementation process of biomarkers for cardiometabolic diseases (Figure 1).

Figure 1. Overview of the strategy to implement new biomarkers and how the four separate Papers contribute in the different phases

<table>
<thead>
<tr>
<th>Phases in biomarker research</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluation in the routine care</td>
<td>Evaluation in the routine care</td>
<td>Discovery Qualification and verification Assay development Evaluation in the routine care</td>
<td>Qualification and verification Evaluation in the routine care</td>
<td></td>
</tr>
<tr>
<td>Study design</td>
<td>Retrospective observational registry based cohort study</td>
<td>Observational cross-sectional registry study</td>
<td>Biomarker screening in existing biobanks Prospective cohort study</td>
<td>Randomized case-control</td>
</tr>
<tr>
<td>Data/study subjects</td>
<td>Registry data from patients with Lipoprotein (a) measurements performed in the routine care</td>
<td>Reviewing of electronical medical records from routine care patients with type 1 diabetes</td>
<td>Patients with measurement of high sensitive Troponin T in the acute care setting.</td>
<td>Patients eligible for elective cholecystectomy recruited from the clinic</td>
</tr>
<tr>
<td>Importance in biomarker development</td>
<td>Improved knowledge about Lipoprotein (a) and its role as a biomarker for cardiovascular disease in patients referred from hospitals, out-patient clinics and general practitioners to have their Lipoprotein(a) levels measured in routine care.</td>
<td>Improved knowledge about Lipoprotein (a) and its role as a biomarker for cardiovascular disease in patients with type 1 diabetes.</td>
<td>Evaluation of a new potential biomarker for acute coronary syndrome in the routine care. Define a concept for patient recruitment, sample collection, and evaluation of biomarkers in the acute care setting.</td>
<td>Further understanding of how the lipoprotein metabolism, and especially the biomarker remnant-cholesterol is affected from simvastatin and ezetimibe treatment, alone or in combination.</td>
</tr>
</tbody>
</table>
2.2 SPECIFIC OBJECTIVES IN THE DIFFERENT PAPERS

Paper I
To investigate the distribution of plasma Lp(a) levels and its association with mortality and CVD in a large cohort of patients that had their plasma levels of Lp(a) determined in routine care.

Paper II
To investigate the importance of plasma Lp(a) levels as a risk factor for CVD and its association with metabolic control in patients with type 1 diabetes.

Paper III
To establish an efficient process for rapid transfer of potential new biomarkers, identified in discovery studies into evaluation in the routine care and to define a concept for inclusion of patients and biobanking in the acute care setting. This is prototyped by the evaluation of chemokine ligand 16 (CCL-16) as a potential new biomarker for ACS.

Paper IV
To study in detail how the lipoprotein metabolism is affected by simvastatin and ezetimibe treatment, alone or in combination, in order to gain further understanding of the molecular effects and especially in conditions with elevated remnant-cholesterol.
3 CLINICAL RELEVANCE

The healthcare is facing a great challenge with an extensive burden of cardiometabolic diseases worldwide. New knowledge about the pathophysiological processes have been obtained and important research discoveries have improved management of the diseases. Despite that, cardiometabolic diseases is a growing health issue in the population and the prognosis, diagnosis, and treatments needs to be further improved. In this process, discovery and implementation of additional biomarkers is extremely relevant and can contribute to better medical care of the patients.

New advancing technologies have increased the possibilities to discover new potential biomarkers but the process from discovery into implementation in the clinic is still long and challenging. Very few biomarkers survive all the way through, and a more efficient process is desirable.

In Paper III we present our approach for an efficient transfer of a new potential biomarker, identified in a discovery project into early evaluation in the routine care setting. The study contributes with further understanding of the process and the important considerations that must be made in biomarker studies. In addition, we define a lean process for inclusion of patients and biobanking of samples in acute care settings.

The use of high sensitive cTroponin assays has greatly improved the diagnostics of ACS but additional biomarkers to further increase sensitivity and specificity for early diagnosis are still desirable. Furthermore, in patients with suspected UA and no elevation of cTroponin or with raised levels for other reasons, additional biomarkers could help to distinguish UA from other conditions. In Paper III the transfer of a biomarker from discovery into evaluation in the routine care setting is prototyped by the evaluation of CCL-16. This chemokine was suggested as an interesting biomarker for ACS in a discovery project called Vinncardio, initiated by the Science for Life Laboratory, Royal Institute of Technology (KTH), Stockholm, Sweden. In the discovery project, high-throughput affinity plasma proteomic technologies were used to screen existing biobanks for new potential biomarkers for CVD and we evaluated whether CCL-16 could add value to hs-Troponin T in the diagnosis of ACS in the routine care setting.

High Lp(a) levels is a risk factor for CVD but it is yet not known whether the elevated risk can be reduced by Lp(a) lowering therapies. Despite that, further knowledge of the role of Lp(a) and its association with CVD in different populations and diagnoses are needed to better understand when Lp(a) should be measured and how patients with elevated Lp(a) levels should be managed. In Paper I and Paper II, we examine the role of Lp(a) as a risk factor for CVD in a large cohort of patients that had their plasma Lp(a) levels determined in the routine care and in
patients with type 1 diabetes to further understand the importance of high Lp(a) levels in different patient groups.

The management of dyslipidemias has improved over the years for several reasons. The treatment options have increased and new therapies e.g. ezetimibe and PCSK9-inhibitors have been added to the selection of treatments. The diagnosis of dyslipidemias has been developed due to additional biomarkers and the introduction of enhanced laboratory assays with better performance. Despite that, there is still a need for improved individual risk assessment and optimization of treatment in patients with dyslipidemias. A deeper understanding of the different biomarkers, their contribution to the overall CVD risk, and how they can be used to better characterize the dyslipidemia can contribute to this.

Previously the SHARP and IMPROVE-IT trials have shown additional reduction of cardiovascular events when ezetimibe was added to simvastatin treatment. The effect was most pronounced in patients with type 2 diabetes where the dyslipidemia is characterized by elevated remnant-particles, increased small dense LDL-cholesterol particles, and low HDL-cholesterol levels. Remnant-cholesterol is an independent risk factor for IHD, also promoting and sustaining low grade inflammation, and is an interesting biomarker for CVD risk assessment. In **Paper IV** we study in detail how treatment with simvastatin and ezetimibe, alone or in combination, affect the lipoprotein metabolism to further understand the molecular effects behind the additional atheroprotective effects observed in the SHARP and IMPROVE-IT trails, and especially in conditions with elevated remnant-cholesterol.
4 METHODOLOGY

This section provides an overview of the different patient cohorts and methodologies used in the separate studies. A more detailed description is found in the respective papers.

4.1 DESCRIPTION OF THE DIFFERENT COHORTS

Paper I is a retrospective observational registry based cohort study where the distribution of plasma Lp(a) levels and its association with mortality and CVD were investigated in 23,398 patients (11,238 males and 12,160 females, mean±standard deviation (SD) age 55.5±17.2 years) that had their Lp(a) levels measured in the routine clinical chemistry laboratory at Karolinska University Laboratory (KUL) between 2003-2017. The Lp(a) results were retrieved from the laboratory database together with other laboratory test results of interest. The laboratory data was linked to register data retrieved from the National Board of Health and Welfare registers (causes of death, prescribed drugs, National patient register) and National Quality registers (Swedeheart, The Swedish Stroke-register).

The patients were referred from totally 1,442 different units and almost 30 % were from the specialties of cardiology, vascular surgery and endocrinology. The indication for Lp(a) measurement is not known and are probably diverse depending on the referring unit. However, all measurements were for some reason performed in patients in the routine care and in many cases probably as part of CVD risk estimation. Hence, it is tempting to speculate that the overall CVD risk may be higher in this cohort compared to the general population. Within the cohort, 19.1 % of the patients had a previous CVD and this is similar to what is expected in the Swedish general population where approximately 2 million (~20 %) people have CVD (16). However, our definition of previous CVD is more limited and with a more general definition the prevalence might be even higher. Two major risk factors for CVD, i.e. hypertension and diabetes were present in 41.1 % and 18.5 % of the patients respectively. That is higher than the estimated prevalence of hypertension (27 %) and diabetes (5 %) in the general Swedish population (statistics from (150) and Swedish National Diabetes Register, March 2020). Of the patients, 33.5 % were treated with a lipid lowering drug and 24.2 % had a diagnosis of hyperlipidemia in the National patient register, but the prevalence is probably slightly higher due to missing registrations of the diagnosis.

Paper II is an observational cross-sectional registry study where the importance of plasma Lp(a) levels as a risk factor for CVD and association to metabolic control were investigated in 1,860 patients (mean±SD age 48±16 years, diabetes duration 27±15 years and HbA1c 62±14 mmol/mol) with type 1 diabetes. All included patients received regular care at the metabolism unit at Karolinska University Hospital and had their Lp(a) levels measured in the clinical routine at least once. Laboratory test results were retrieved from the laboratory database at KUL. Information about treatment, other diagnosis, and complications were obtained by manually reviewing the electronic medical records at Karolinska University Hospital.
In Paper III, patients eligible for inclusion were identified when their plasma samples were analyzed for hs-Troponin T at KUL. The automatic laboratory system was for 9 months programmed to save the remaining plasma from all samples referred to KUL from the ER, acute care unit or the cardiac intensive care unit for measurement of hs-Troponin T. The samples were temporarily stored for 2 months and meanwhile, a letter was sent to 4924 patients with information about the study and an invitation to participate. A signed informed consent form was returned within two months from 1986 (40.3 %) of the patients. For all others the temporarily stored plasma samples were discarded and all their information permanently deleted from the study. Including 291 patients recruited directly at the ER, a total of 2277 patients were possible to include. Unfortunately, 646 patients were excluded due to missing samples and/or information, insufficient sample volume or laboratory errors (including extensive hemolysis in the plasma samples) and finally 1631 patients (956 males and 671 females, mean±SD age 63.1±16.5 years) were included. The diagnosis from the hospital admission were retrieved by manual reviewing the electronic medical records at Karolinska University Hospital.

Repeated hospital admission occurred for 146 patients during the study inclusion time, and plasma sampled from 1777 hospital admissions with the diagnosis distributed between angina (n=28, 1.6 %), UA (n=18, 1.0 %), NSTEMI (n=52, 2.9 %), AMI (n=47, 2.6 %), and any other diagnosis (n=1632, 91.8 %) were included. It is estimated that 5 % of all patients appearing with chest pain in the ER suffer from ACS (151). The slightly higher frequency of ACS (6.6 %) in our cohort can be due to the fact that patients from the cardiac intensive care unit were also included or that patients with ACS were more motivated to participate in the study.

Paper IV is a randomized case-control study where forty patients with uncomplicated gallstone disease, eligible for elective cholecystectomy at the Department of Surgery, Danderyd Hospital, Sweden were included. The patients (14 males, 13 fertile females and 13 postmenopausal females) were randomized to four-week treatment before surgery to placebo, simvastatin (80 mg daily), ezetimibe (10 mg daily), or to the combination of both simvastatin and ezetimibe. Fasting blood samples were collected before treatment start and at the end of study. During the surgical intervention, liver biopsies and bile were collected.

4.2 LABORATORY ASSAYS

4.2.1 Routine laboratory parameters

Routine laboratory parameters were measured with certified routine assays at KUL. For hs-Troponin T, the Elecsys hsTroponin T assay from Roche (Roche Diagnostics, GmbH, Mannheim, Germany) was used. The eGFR was calculated from the revised Lund-Malmö formula (152). LDL-cholesterol was calculated from the Friedewald’s formula if TG was < 4.5 mmol/L.
4.2.2 Lp(a)

In Paper I, Lp(a) was measured at KUL by two different particle enhanced immunoassays. Before 2014 an assay that reported Lp(a) in mass concentration (Tina-quant Lipoprotein(a), Roche Diagnostics) was used. From 2014 an assay that reported Lp(a) in molar concentration (Tina-quant Lipoprotein(a) Gen 2, Roche diagnostics), standardized to the IFCC reference material (SRM 2B) was used. In Paper II all Lp(a) results were analyzed with the assay reporting in molar concentration, except from 5% of the patients that had their Lp(a) levels measured in other laboratories, using other certified Lp(a) assays (Lp(a) Ultra Sentinel Diagnostics, Beckman Coulter or Lp(a) Advia XPT, Siemens Heathcare) that report values in mass concentration.

Since two different assays for Lp(a) measurement were used in Paper I, the different results in mg/dL or nmol/L were analyzed separately or divided into quartiles to be able to compare outcomes between quartile levels of Lp(a) for mg/dL and nmol/L together. As discussed previously, the conversion of Lp(a) values between nmol/L and mg/dL are controversial since the assays are not comparable and the conversion is more or less imprecise depending on apo(a) isoform size. Hence, we consider that a conversion may have an impact on the results from this study and decided not to use that approach. However, the results measured with an alternative assay in Paper II were converted into nmol/L before statistical analysis by the conversion factor [Lp(a) nmol/L = 2.4 x (Lp(a) mg/dL)] suggested by Roche Diagnostics and Brown et. al. (110) since we considered that these values were too few to have a major impact on the final results.

4.2.3 CCL-16

CCL16 was measured with either an enzyme linked immunosorbent assay (ELISA) assay (CCL16 (human) ELISA kit, catalog number KA1724, Abnova, Taipei, Taiwan) performed with an automatic system (Freedom EVO 200, Tecan Trading AG, Switzerland) or on a Luminex MAGPIX® instrument with an antibody suspension bead array as described (153).

4.2.4 Lipoproteins and apolipoproteins

Lipoproteins were separated by size-exclusion chromatography and the cholesterol and TG content in the VLDL, LDL and HDL fractions were determined as described earlier (154). Nuclear Magnetic Resonance spectroscopy (NMR) were used for a detailed analysis of lipoprotein subclasses, ApoA1 and ApoB at Nightingale Health Ltd. Laboratory, Vantaa, Finland (155).

4.2.5 Proteoglycan binding

The binding affinity of apoB-containing lipoproteins to human arterial proteoglycans (PG) was assessed by a solid phase procedure that has been described earlier (156, 157).
4.2.6 Messenger ribonucleic acid (mRNA) expression in liver biopsies

Total ribonucleic acid (RNA) was extracted and transcribed into complementary deoxyribonucleic acid (cDNA) using Omniscript (Qiagen, Sollentuna, Sweden). For the quantification of mRNA levels, SYBRGreen mastermix and specific primers were used, arbitrary units were calculated by linearization of the cycle threshold, i.e. CT values and normalized to glyceraldehyde-3-phosphate-dehydrogenase.

4.3 STATISTICAL ANALYSIS

The statistical analysis was performed as described in detail in the separate papers and are only briefly explained here.

Binary variables are presented as positive counts and percentages. Numerical variables are presented as mean±SD if normally distributed or median (inter quartile range (IQR)) for variables with skewed distribution (Paper I, II and III). In Paper IV data are expressed as mean±standard error of the mean unless otherwise stated.

Differences in means were compared by two-sided t-tests (Paper I-III) or paired t-test (Paper III), Wilcoxon-Rank sum-test for differences in medians (Paper II), and Pearson´s chi-squared test for proportions (Paper II). In general, a p-value < 0.05 was considered significant (Paper I-IV).

The patients in Paper I were divided into quartiles of Lp(a) levels in mg/dL and nmol/L respectively. All-cause mortality, CVD related mortality, and time to CVD events were analyzed across the Lp(a) quartiles using Cox proportional hazard regression models adjusted for age, sex, smoking, diabetes, hypertension, LDL-cholesterol, statin treatment, and previous CVD.

In a subgroup analysis on time to IHD, patients with Lp(a) levels in the 4th quartile were compared to the 1st quartile using Cox proportional hazard models for each defined baseline characteristic or category and fitting adjusted models including an interaction term between the two Lp(a) quartiles and the respective binary variable. A p-value < 0.01 was considered significant for interaction according to the Bonferroni correction.

The cohort were also divided into seven age groups and the median, 80th, and 90th percentile levels of Lp(a) were compared between the groups using quantile regression.

The reason why the median, 80th, and 90th percentile Lp(a) levels are compared in Paper I and Paper II is that i) Lp(a) has a skewed distribution, hence the median is preferred in front of the mean, ii) Lp(a) levels above the 80th percentile in the population have been discussed as an indicator for increased risk (98) and therefore the 80th percentile levels in this cohort were investigated, iii)) patients with extremely high Lp(a) levels (> 180 mg/dL or > 430 nmol/L) are at highest CVD risk and consequently the extreme Lp(a) levels in this cohort were examined (19).
In **Paper II** the patients were divided into four groups according to their Lp(a) levels in nmol/L: very low <10, low 10-30, intermediate 30-120, and high >120. Crude and adjusted relative risk ratios (RRR) for the different complications were calculated using multinomial logistic regression models. The categorical variable of Lp(a) group was the dependent variable and the binary variable of the complication was the independent variable. In the adjusted models age and smoking status were covariates.

The median, 80th, and 90th percentile levels of Lp(a) were estimated in the different quartiles of age and in three groups of HbA1c levels by quantile regression models. Differences between the age quartiles or HbA1c groups were explored by Wald test.

In **Paper III**, differences in CCL-16 levels between the diagnosis at hospital admission was compared by one-way analysis of variance (ANOVA). Possible correlations between CCL-16 and hs-Troponin T or eGFR were explored by simple linear regression.

Significances in **Paper IV** were tested by multi-way ANOVA and post hoc comparison were performed according to the Fisher’s least significant difference test.

Statistical analyses were performed with Stata version 15 (StataCorp, College Station, TX, USA) (**Paper I-II**), IBM SPSS Statistics 26 (IBM Corporation) (**Paper III**) or Statistica version 12.0 (StatSoft, Tulsa, OK, Data S1) (**Paper IV**). Graphical presentations in **Paper I-III** were performed with GraphPad Prism 8 (Graph Pad Software Inc.).
5 ETHICAL CONSIDERATIONS

All studies were approved by the Swedish Ethical Review Authority (Paper I: 2017/871-31/4 and 2018/526-32; Paper II: 2017/872-31/4; Paper III: 2015/1570-31/4 and 2016/372-32; Paper IV: 2006/1204-31/1). Paper IV was also approved by the Swedish Medical Agency and registered in the EU clinical trial register (EudraCT number: 2006-004839-30).

We have had comprehensive ethical and juridical considerations in the different projects and extensively discussed juridical aspects of the study designs with the relevant authorities at Karolinska Institutet and Karolinska University Hospital. This has provided a deeper understanding of the complexity in these issues and therefore the ethical considerations deserve an extended section.

5.1 GENERAL ETHICAL CONSIDERATIONS WITHIN RESEARCH PROJECTS

The basic ethical principles, i.e. the principle of respect for autonomy and integrity, the principle of beneficence and do not harm, and the principle of justice are fundamental for ethical considerations in research projects and will be discussed in general first.

The principle of respect for autonomy and integrity means that all individuals are autonomous with the right to decide for their own life. In research, this refers to that the individual must be fully informed about the project and potential risks, and able to autonomously decide about participation. It is always voluntary to participate and discontinuation in the study is allowed at any time without explanation or disadvantage in the future care. The study subjects give the researcher a confidence to use their personal data, medical information, samples or bodies for research, and the researcher should manage this with respect for the individual’s integrity. Unnecessary handling of personal data should be avoided, the data should be handled safely, and protected from unauthorized access.

According to the principle of beneficence and do not harm, the research should not be performed on expenditure of others, all possible risks must be minimized, the research should be of high quality, and beneficial for everyone. Before initiating a research project, it must be considered whether the possible gains can motivate the research and interventions needed, and if the benefits outweigh the potential risks. During the study, the patients should be monitored safely, the investigations must be of high quality, and biological samples should be properly stored to keep the material intact and give true results. A study design of good quality favors correct results and avoidance of biased data.

Finally, the principle of justice means that research must be fair in design, selection of study participants, and performance. Everyone must be equally treated in all ethical aspects independent of age, gender, socioeconomic status etc.

Except from these basic ethical principles, there are many more aspects, including both general human rights, moral- and ethical principles and laws. Several guidelines, consensus documents, and recommendations exist. One very important is the Declaration of Helsinki, a
statement of ethical principles for medical research involving human subjects adopted by the World Medical Association. Another is the Good Clinical Practice guidelines for performing clinical trials in human subjects from the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use. The Swedish research council has also written a document about “Good research practice” that discusses guidelines, laws and important considerations in ethics.

In addition, all research in Sweden involving humans must be approved by the Swedish Ethical Review Authority. This is regulated by the Act concerning ethical review of research involving humans (SFS 2003:460). Several other laws are also important, i.e. the Patient data act (Patientdatalagen, SFS 2008:355), the Act of biobanking (SFS 2002:297) and the Health data act (SFS 1998:543). From the 25:th of May 2018 the General Data Protection Regulation (GDPR, EU 2016/679) replaced the personal data ordinance. The GDPR is a European Union regulation concerning the protection of natural persons and the handling, processing, and movement of personal data.

5.2 SPECIFIC ETHICAL CONSIDERATION IN THE DIFFERENT PAPERS

**Paper I** is a register-based study, where more than 23 000 patients were identified from a database at KUL. Only register based data are handled in this study and the individual subjects are not informed and asked for participation. According to Art. 89 in GDPR there is an exception for handling of registry based personal data for scientific research purposes, meaning that all participating subjects do not have to be informed if not feasible, provided that the data are handled safely, and according to the principle of data minimization, including pseudonymization if possible. This makes it feasible to perform register-based studies involving many thousands of subjects and studies where some of the subjects are deceased.

All personal data from KUL and the different registers were merged by the National Board of Health and Welfare by matching of the Swedish personal identification number. The data was then coded into a study ID before it was presented to the investigators and exclusively pseudonymized data are handled in the study. The key for this code is kept by the National Board of Health and Welfare and only the responsible investigators can by special request access this key, that will be permanently destroyed after three years. Pseudonymized data should be handled as carefully as personal data and there is a great responsibility for the investigators to make sure of this.

In a future second part of this project, patients with high Lp(a) levels will be investigated in more detail, additional data will be requested from the patients, and collected from their electronic medical records. For this purpose, during Spring 2018, a letter with information about the study and an invitation to participate in the second part of the project was sent to more than 4000 patients from our cohort with Lp(a) levels >125 nmol/L. Of these, 42 % accepted to participate and returned a signed informed consent form. This approach to recruit patients poses a dilemma since the patients receive a letter from the investigators without
their permission to be contacted, and this may be perceived as a violation to their privacy. Before initiation of the study we had a comprehensive discussion with the lawyers at Karolinska Institutet about the study design and in fact, one of the respondents felt offended and questioned our right to contact them. We performed the study before the current GDPR law was introduced in May 2018. According to the GDPR, this approach would not be possible unless specific enquiry to the data protection authorities whether the unique study objectives allow the investigating doctors to directly contact patients, bypassing their treating doctors.

In Paper II, all participants were patients at the Department of Endocrinology, Karolinska University Hospital Sweden and the study only include existing information from the electronic medical records. No additional data were requested, and no interventions were performed. Therefore, the patients were not asked individually for participation in the study.

Potential privacy concerns were minimalized due to careful data management, ensuring that no unauthorized persons could access it. The data were collected from the electronic medical records by the investigators, all physicians with professional secrecy working at the Karolinska University Hospital. The data were thereafter coded into a given study ID and no one except from the investigators had access to the securely stored key. After finishing the study, the key was discarded and only the anonymous data was stored. The data are presented at group level so that no individuals can be identified.

In Paper III subjects eligible for inclusion were identified when their plasma samples were analyzed for hs-Troponin T at KUL. The plasma samples were temporarily stored for a longer time than usual and a letter with an invitation to participate in the study was sent to the patients’ home, without their permission to be contacted. To minimize the risk of interfering with privacy, a letter was only sent once without any reminders. If no reply was received within the time limit, all information about the patient were permanently deleted from the study. The plasma samples were securely stored temporarily in the routine laboratory and discarded without any additional analyzing if the patient did not participate in the study.

It is a challenge to recruit patients with an acute medical condition. On the other hand, it can be unethical to not perform research aiming to improve the management of patients with acute diseases due to the difficulty. It is important to make sure that the patient understands that participation is voluntary and that their future care will not be affected if the patient disagrees to participation. This can be difficult in the acute care and a patient with an acute medical condition may not be capable to give an informed consent for participation or the inclusion procedure might delay the routine care. Therefore, our approach to use the routine blood samples and contact the patients and ask for participation afterwards is appealing. Also here, with the new GDPR legislation an enquiry about the feasibility of this kind of study should be a priory discussed with the data protection authorities, as described above for Paper I.

In Paper IV, the patients received written and oral information and were invited to participate in the study at a screening visit by the doctor or a nurse. A personal meeting is
positive to make sure that the patient has fully understood the information and are able to ask questions. On the other hand, it can be more difficult to refuse participation and a time for consideration is important to offer.

Any additional intervention in a study, beyond the routine care may involve an increased risk. In Paper IV the patients were treated with simvastatin and ezetimibe, additional blood sampling as well as liver biopsies and collection of bile during laparoscopic cholecystectomy were performed. Whether the possible benefits from the study outweighed the potential risks were carefully considered. To minimize the risks the patients were closely monitored, and the surgery was performed by experienced surgeons. In addition, the patients were fully informed about the study and the risks and were free to end their participation at any time without any explanation and without any consequence on their future care.
6 RESULTS AND DISCUSSION

6.1 PAPER I

6.1.1 Distribution of plasma Lp(a) levels

Plasma Lp(a) levels had a skewed distribution with median 15.9 mg/dL or 18.2 nmol/L, 80\textsuperscript{th} percentile at 55.6 mg/dL or 113.5 nmol/L, and 90\textsuperscript{th} percentile at 89.2 mg/dL 179.5 nmol/L (Figure 2).

Figure 2. Distribution of plasma Lp (a) levels
Lp(a) levels increased with increasing age and up to ~1.4 times higher mg/dL and ~1.7 times higher nmol/L median, 80th, and 90th percentile levels were observed in all age groups above 51 years compared to the reference age group 21-35 years. In the age group 36-50 years the Lp(a) nmol/L median, 80th, and 90th percentile levels were also significantly increased. (Data shown in Table 2, Paper I.)

The median Lp(a) levels were 11-12% higher in females in the total cohort, and when compared in different age groups, significantly higher Lp(a) levels were generally observed in females in the age groups above 51 years, but not in the younger. (Data shown in Figure 3 and supplementary Table S4, Paper I.) The median and 95% Confidence Interval (CI) Lp(a) mg/dL and nmol/L levels for males and females in different age groups are shown in Figure 3.

Figure 3. Lp(a) median (95% CI indicated by grey) levels in males and females in different age groups

Postmenopausal females seem to have higher Lp(a) levels than premenopausal (158, 159) and this could explain why age and sex influenced Lp(a) especially after 51 years of age. Hence, we compared the levels in females >51 years (i.e. mean age for menopause in Sweden) or with hormone replacement therapy (HRT) to females ≤51 years and no HRT and the two former had significantly higher Lp(a) levels (p<0.01 for all). (Data shown in Table 3, Paper I.) This reinforces the assumption that menopausal status affect Lp(a) in this cohort and indicate a hormonal influence on Lp(a) levels. In line with this, a meta-analysis showed
reduction of plasma Lp(a) levels in females receiving HRT and oral administration was more effective than transdermal (160). In two other studies, Lp(a) levels in males decreased by 30% after oral administration of estrogen (161), but was unchanged after parenteral administration (162), and the effect from HRT seems to be dependent on the administration. Whether this hormonal influence on plasma Lp(a) levels is clinically relevant and can have an impact on the Lp(a) associated CVD risk needs to be further investigated in other studies.

6.1.2 Lp(a) levels and association with mortality and CVD

Patients with Lp(a) levels in the 4th quartile had increased Hazard Ratio (HR) of 1.18 (95% CI 1.04-1.35, \( p=0.012 \)) for CVD (i.e. IHD and stroke), and 1.36 (95% CI 1.14-1.61, \( p=0.001 \)) for IHD compared to patients with Lp(a) levels in the 1st quartile. No association between Lp(a) levels and stroke was observed and the increased CVD risk seems to be mainly due to IHD. (Data shown in Figure 4, Paper I.)

Age, previous CVD, diabetes, and LDL-cholesterol did not interact with the IHD risk in a subgroup analysis comparing patients with Lp(a) levels in the 4th to the 1st quartile in each defined subgroup category. However, sex had a significant interaction (\( p=0.005 \)). (Data shown in Figure 5, Paper I.) There was no difference in age between males and females with Lp(a) levels in the 4th quartile, but the number of females were higher and the extremely high Lp(a) levels (i.e. indicating the highest CVD risk) were more frequent in females. Coherent with this, higher plasma Lp(a) levels in females with CVD have been reported in other studies (163, 164) and it has been suggested that Lp(a) is a stronger risk factor for CVD in females (163-165). However, the influence of sex on the Lp(a) associated CVD risk needs to be reviewed further.

In summary, the distribution of plasma Lp(a) levels and the increased risk for IHD in this cohort of patients referred from hospitals, out-patient clinics or general practitioners in the Region Stockholm is comparable to what has been reported in general population studies (81, 94, 97), demonstrating that elevated Lp(a) levels is also a risk factor of importance in this cohort.

6.2 PAPER II

6.2.1 Distribution of plasma Lp(a) levels

The plasma Lp(a) levels had a skewed distribution with median, 80th, and 90th percentile levels of 19, 98, and 176 nmol/L, and no difference between males and females. Lp(a) levels increased with age, and patients in the 3rd and 4th quartile of age (49-59 and 60-90 years, respectively) had approximately 1.5 times higher median, 80th, and 90th percentile levels compared to patients in the 1st age quartile (18-35 years).

6.2.2 Lp(a) levels and vascular complications

Patients with type 1 diabetes and high Lp(a) levels (>120 nmol/L) had, adjusted for age and smoking, a RRR of 1.51 (95% CI 1.01-2.28, \( p=0.048 \)) for any macrovascular complication
(i.e. composite CVD of coronary heart disease (CHD), cerebrovascular disease, and diabetic foot ulceration), a RRR of 1.68 (95% CI 1.12-2.50, \( p=0.01 \)) for albuminuria, and a RRR of 2.03 (95% CI 1.02-4.01, \( p=0.043 \)) for CAVD compared to patients with very low levels (<10 nmol/L). The unadjusted RRR for CVD was 2.2 (95% CI 1.37-3.52, \( p=0.001 \)) and for CHD 2.42 (95% CI 1.41-4.15, \( p=0.001 \)), but this was no longer significant after adjustment (Figure 4). The association between Lp(a) levels and all vascular complications investigated are showed in Table 2, Paper II.

Figure 4. RRR for vascular complications in patients with type 1 diabetes and high (>120 nmol/L) compared to very low (<10 nmol/L) Lp(a) levels

![Relative Risk Ratio](image)

*\( p<0.05 \), **\( p<0.01 \). Adjusted for age and smoking status. CVD: cardiovascular disease, CHD: coronary heart disease. Composite CVD: CHD, cerebrovascular disease and diabetic foot ulceration, CAVD: calcified aortic valve disease.

As far as we know, this is the largest study investigating the association between Lp(a) and vascular complications in patients with type 1 diabetes. Nevertheless, it is limited in size, CVD events were only present in 9 % and this constrains the ability to thoroughly investigate possible associations between Lp(a) levels and subcategories of CVD (i.e. CHD or cerebrovascular disease). However, consistent with our results, patients with type 1 diabetes had an increased risk for CVD at Lp(a) levels >30 mg/dL (~75 nmol/L) in a small prospective study (166), and a trend for positive correlation between Lp(a) and albuminuria were demonstrated in children with type 1 diabetes (167). High Lp(a) levels has been identified as a risk factor for CAVD across multiple ethnicities (99), but the relevance as a risk factor in patients with type 1 diabetes has not been investigated before, and this is a novel finding. In conclusion, high Lp(a) levels was a significant risk factor for cardiovascular complications in this cohort of patients with type 1 diabetes, and assessment of Lp(a) levels can improve the risk estimation also in this group of patients.
6.2.3 Lp(a) levels in relation to metabolic control

Plasma levels of Lp(a) were associated with metabolic control. Patients with intermediate metabolic control (HbA1c 52-70 mmol/mol) had higher 80th and 90th percentile Lp(a) levels ($p<0.05$ and $p<0.001$) and patients with poor metabolic control (HbA1c >70 mmol/mol) had higher median and 90th percentile Lp(a) levels ($p<0.01$ and $p<0.001$) compared to patients with good metabolic control. The risk to have high (>120 nmol/L) compared to very low (<10 nmol/L) Lp(a) levels were ~1.8-fold increased (RRR 1.78, 95% CI 1.15-2.76, $p=0.01$) for patients with poor metabolic control and ~1.5-fold increased (RRR 1.49, 95% CI 1.01-2.18, $p=0.043$) for patients with intermediate metabolic control compared to good metabolic control. (Data shown in Figure 2A-B, Paper II.)

Influence of metabolic control on Lp(a) levels has been observed before (168), but not in all studies (169, 170). In an investigation of patients with type 2 diabetes, the insulin levels were inversely correlated to the Lp(a) levels not regulated by genetic trait (171). Since Lp(a) levels probably are controlled mainly by its synthesis rate (172), this finding in patients with type 2 diabetes together with our results might suggest that insulin has an influence on the hepatic Lp(a) metabolism. Future studies are needed to reveal such a possible mechanism further.

6.3 PAPER III

No significant differences in CCL-16 were observed between the different diagnosis of angina, UA, NSTEMI, AMI or any other diagnosis, neither between the composite of any ACS (UA, NSTEMI and AMI) and all other diagnosis. (Data shown in Figure 7, Paper III.) CCL-16 was identified as an interesting biomarker for ACS in the discovery study, but it had no additional value to hs-Troponin T when evaluated in a routine care setting. Several factors can contribute to the contradictive results as discussed in Paper III.

In the discovery project, plasma samples collected in tubes containing potassium and the anticoagulant ethylenediaminetetraacetic acid (K2-EDTA) was used. For the evaluation of CCL-16 in the routine care, the plasma samples collected in tubes containing the anticoagulant Lithium (Li)-Heparin for measurement of hs-Troponin T was used. Therefore, measurement of CCL-16 in plasma samples collected in either of the two sample tubes was compared. The analytical performance with two different antibodies (HPA042909 and HPA051577) was lower in samples collected in tubes containing Li-Heparin compared to K2-EDTA (HPA042909 CV 39 % and 25 % respectively; HPA051577 CV 44 % and 29 %, respectively). In addition, the discrepancies between CCL-16 measured in plasma from Li-Heparin and K2-EDTA tubes were significantly different between the two antibodies ($p<0.01$). (Data shown in Figure 2-3, Paper III.) Hence, the anticoagulants in the sample tubes influenced the measurement of CCL-16 differently, depending on the antibody used and possible also due to a matrix effect. Despite this, we estimated that this would not affect our experiments and decided to use plasma collected in Li-Heparin tubes for CCL-16 measurements. Considering the contradictive outcomes from the discovery and the evaluation in the routine care setting, the matrix effect and the diverse performance of CCL-16
measurements between the sample types may still influence on the results. Therefore, it would be interesting to investigate CCL-16 differences between patients with ACS and other diagnosis in plasma collected in both types of tubes. The relative discrepancies between the diagnosis might be different depending on sample type and have an impact on the findings.

A stability test revealed that CCL-16 was susceptible to the storing conditions. After 72 hours storage a 41 \% mean increase in CCL-16 levels was observed in samples kept in room temperature. Samples stored in the fridge had a 10 \% mean decrease and a 11 \% mean increase was found in samples stored at – 20 °C. (Data shown in Figure 4, Paper III.) The plasma samples from the Carlskrona Heart Attack Prognosis Study that were used in the discovery project were collected in the routine care according to the recommended procedures at the Department of Clinical Chemistry at Blekinge Hospital at that time (173, 174). The samples had been stored for more than 20 years in the freezer and thawed at least once in 2012. We do not know how the preanalytical handling or the long storage conditions might have affected the CCL-16 levels and the results from the biomarker discovery. In our evaluation of CCL-16, the plasma samples were collected and handled in the routine care setting and exposed to common possible preanalytical errors. However, we had full control of the time and storage conditions from sample collection to biobanking. Previously it has been shown that different cytokines behave differently depending on the anticoagulant (175), but in general measurement of cytokines are more reliable and stable in plasma collected in K2-EDTA (176-178). Therefore, in retrospect it would be interesting to also perform a stability test in plasma samples collected in K2-EDTA tubes.

6.4 PAPER IV

6.4.1 Treatment effect on lipoproteins and lipoprotein subclasses

After four weeks of treatment, remnant-cholesterol and LDL-cholesterol were decreased by simvastatin 80 mg daily (-51 \% and -52 \%, \textit{p}<0.001), ezetimibe 10 mg/daily (-18 \%, \textit{p}<0.001 and -14 \%, \textit{p}<0.05), and combined therapy (-65 \% and -64 \%, \textit{p}<0.001). CE in remnant-particles were also decreased by the different treatments (-53 \%, -20 \%, and -68 \% respectively, \textit{p}<0.001), and the addition of ezetimibe to simvastatin treatment resulted in a -15 \% additional reduction (\textit{p}<0.01) compared to simvastatin alone. Simvastatin and combined treatment reduced TG in remnant-particles and LDL-particles, but this was not observed in treatment with ezetimibe. The combined treatment also caused an -10\% additional reduction of apoB-containing particles compared to simvastatin. (Data shown in Figure 2 and Figure 5 in Paper IV.)

When changes in the lipoprotein subclasses were studied in detail by NMR, simvastatin and combined therapy reduced particle number, TG, and CE in all seven subclasses of remnant-particles (i.e. non-HDL, non-LDL-particles). Ezetimibe treatment reduced CE in all subclasses except from XL-VLDL, particle number only in three subclasses (XXL-VLDL, XS-VLDL and IDL) and TG only in the XXL-VLDL subclass. In all three LDL-subclasses, simvastatin and combined therapy reduced particle number, TG, and CE. Treatment with
ezetimibe reduced CE only in L-LDL, particle number in L-LDL and TG in S-LDL. (Data shown in Figure 3-4, Paper IV.)

In summary, combined therapy with simvastatin and ezetimibe had compared to simvastatin treatment an additional effect on further reduction of cholesterol and CE in both remnant- and LDL-particles and further reduction of apoB-containing particles. Treatment with ezetimibe only reduced CE in remnant-particles, but not in LDL-particles. Intestinal and hepatic acetyl-CoA acetyltransferase 2 (ACAT2) determines the secretion of CE into CM or nascent VLDL-particles (179). Additional CE is added into VLDL-particles by LCAT (32, 180) or by CETP mediated exchange of TG and CE between VLDL- and HDL-particles (33, 34). High-dose statin treatment reduces the activity of hepatic ACAT2 (181) and CETP and increase the LCAT activity (182) while treatment with ezetimibe reduces the cholesterol available for intestinal ACAT2 and seems to reduce both CETP and LCAT activity in plasma (183). Due to this, both an additional and synergistic effect from the combination of the two therapies can be expected. This is demonstrated in our results where the combined therapy caused additional positive effects on the atherogenic lipoproteins, and especially on the remnant-particles.

6.4.2 Treatment effect on plasma proteoglycan binding

Plasma apoB-containing lipoproteins binding to arterial PG was reduced by simvastatin (-53 %; \( p<0.001 \)), ezetimibe (-17 %; \( p<0.01 \)), and the combined treatment (-57 %; \( p<0.001 \)) compared to placebo. After correction for apoB, the corresponding reduction was -27 % \( (p<0.01) \), -11 % \( (p<0.05) \), and -21 % \( (p<0.01) \), respectively. When the PG binding was corrected for total cholesterol the effect of ezetimibe was lost, but simvastatin and combined therapy still showed reduction (-40 %; \( p<0.001 \) and -28 %; \( p<0.01 \), respectively). (Data shown in Figure 6, Paper IV.)

Plasma apoB-containing lipoprotein binding to arterial PG is an indicator for lipoprotein deposition in the arterial wall, i.e. the initial step in atherosclerosis (157). The positive changes on the atherogenic lipoproteins described above translated into reduced PG binding, where simvastatin was most effective and combined therapy had no additional effect. The reduced PG binding was due to the reduction of apoB-containing particles and total cholesterol. In contrast to ezetimibe treatment, the effect remained after correction for total cholesterol or apoB in simvastatin treatment. This suggests that simvastatin not only reduces the total cholesterol and particle numbers, but further modifies the particle characteristics.

6.4.3 Treatment effect on key genes regulating hepatic cholesterol and lipoprotein metabolism

Investigation of mRNA levels of some key genes involved in hepatic cholesterol metabolism provided a further understanding of the modifications of lipid content in remnant-particles observed from combined therapy.
Transcription of the rate-limiting enzyme in cholesterol metabolism, HMG-CoA reductase is regulated by the membrane-bound transcription factors called sterol regulatory element binding proteins (SREBP) (184). HMG-CoA reductase is the target for statins and as expected, simvastatin treatment induced hepatic mRNA genes under control of sterol regulatory element binding factor 2 (SREBF2), i.e. 3-hydroxy-methylglutaryl-CoA reductase (HMGCR), 3-hydroxy-methylglutaryl-CoA synthase 1 (HMGCS1), LDL-receptor (LDLR), PCSK9 and NPC1L1 (184). No additional effect was observed from combined therapy and simvastatin seemed to fully activate the SREBP system. However, the addition of ezetimibe to simvastatin treatment reduced expression of CETP mRNA levels compared to simvastatin alone and reduced the mRNA expression of microsomal triglyceride transfer protein (MTTP) that was induced from simvastatin treatment. (Data shown in Figure 8, Paper IV.) CETP mediates exchange of CE and TG between VLDL, LDL, and HDL (33). MTTP is an essential protein for the assembly and secretion of VLDL from hepatocytes and CM in enterocytes, acting as a chaperone in the apoB folding and incorporating lipids into the particles (185). These additional changes from combined therapy on mRNA levels of CETP and MTTP might partly explain the additional modifications of lipoprotein constitution observed from combined therapy.
7 CONCLUSIONS AND FUTURE PERSPECTIVES

In Paper I and Paper II we studied the association between plasma Lp(a) levels and CVD in two different cohorts and found that Lp(a) was a significant risk factor in both. In Paper I, the patients referred from hospitals, out-patient clinics, and general practitioners in Region Stockholm to have their Lp(a) levels determined in the routine care had similar skewed distribution of plasma Lp(a) levels and Lp(a) associated increased risk for IHD as have been reported in the general population. In Paper II, high plasma Lp(a) levels was a significant risk factor for macrovascular complications, albuminuria, and CAVD in patients with type 1 diabetes. Together these findings endorse the role of high Lp(a) levels as an important riskfactor for CVD in separate cohorts and implies that measurement of plasma Lp(a) levels can improve the CVD risk estimation in different populations and diagnoses. At the time of writing, there is no evidence that therapeutic decrease of high plasma Lp(a) levels leads to reduction of CVD. Novel therapies to exclusively lower plasma Lp(a) levels are emerging and in the next coming years we will probably have an answer to this. Meanwhile, intensified conventional treatment of other risk factors should be considered in individuals with high plasma Lp(a) levels to decrease the CVD risk.

In the future, we would like to examine the association of plasma Lp(a) levels with PAD and CAVD in the cohort described in Paper I. In addition, we are planning the second part of the project where individuals with high plasma Lp(a) levels will be studied in more detail to further understand how additional factors (e.g. sex, menopausal status, heredity, and other CVD risk factors) modifies the Lp(a) associated CVD risk.

In Paper I and Paper II the plasma Lp(a) levels increased with increasing age. In Paper I females had significantly higher plasma Lp(a) levels, but in Paper II no differences were observed between the sexes. The patients in Paper II were younger, only 44 % were females, and a larger sample size is probably needed to reveal a possible difference between males and females. We suggest that menopausal status might have an influence on plasma Lp(a) levels and contribute to the findings observed in Paper I, suggesting an impact of hormonal regulation on plasma Lp(a) levels. However, the influence of age, sex, hormonal regulation, and menopausal status on Lp(a) levels needs to be investigated further, as well as whether such influence might translate into a significant effect on the CVD risk related to plasma Lp(a) levels.

Since we observed higher Lp(a) levels with increasing age in Paper I and Paper II, it can be assumed that the individual Lp(a) levels increases with age. In Paper I, almost 4000 individuals had repeated Lp(a) measurements with the same assay for a time period up to 10 years and we investigate whether the Lp(a) levels changed over time in the same individual. Preliminary analysis of data suggests that the individual variation of Lp(a) levels in plasma is larger than expected. Further, some individuals tended to increase while others tended to decrease their Lp(a) levels over time. Hence, it is too early to state that ageing per se has an effect and we will investigate this further to understand what parameters (e.g. menopausal
status, lipid lowering therapies or other diseases) may influence the effects of age in different ways.

In **Paper II** patients with poorer metabolic control had higher plasma Lp(a) levels compared to patients with good metabolic control. The Lp(a) levels seems to be mainly determined by its rate of synthesis in the liver and our finding might suggest that the metabolic control has an influence on this, as have been suggested by others before. In some minor studies the effect on Lp(a) levels after improved metabolic control have been investigated, showing contradictive results. In the future, it would be interesting to in more detail study the effect of improved metabolic control on plasma Lp(a) levels in a prospective trial to further understand how the metabolic control can affect the Lp(a) metabolism and whether such influence have a significant impact on the Lp(a) associated CVD risk.

The process from biomarker discovery studies into implementation of novel biomarkers is challenging and we experienced several difficulties along the way in **Paper III**. However, we managed to establish an efficient process for patient inclusion and sample collection in the acute care setting at a low cost. For nine months we succeed to recruit almost 2000 patients, i.e. ~40 % of those surveyed by letter, and save their samples collected in the routine care for biobanking. (Interestingly, a similar response rate was observed in the second part that we are planning for in the study presented in **Paper I**, where 42 % of those surveyed by letter were included as described in section 5.2). The routine clinical chemistry laboratory was a central part to make this process work. The attempt to include patients directly in the ER was less effective and perfectly illustrates the challenge with a traditional protocol for patient inclusion in acute medical conditions.

CCL-16 was rapidly transferred from the discover study into evaluation in a routine care setting with similar conditions as its potential future use. Therefore, we could early in the implementation process, without too much effort and costs, conclude that CCL-16 did not seem to be a suitable biomarker for diagnosis of ACS.

From our experiences we identified several issues that needs to be carefully considered in future biomarker discovery studies. To be successful, the project must be carefully planned from the beginning and a close collaboration between the research laboratory, the routine laboratory, and the clinic is required from the start. The samples and technologies used throughout the project should be chosen with consideration, a proper verification of the biomarkers identified in the discovery is important, and the characteristics of the biomarker in different sample types needs to be investigated. The cohort and samples used for discovery should be representative for the indented future use of the biomarker. Correct preanalytical handling and storing of samples are essential for good quality, and unfortunately not all biobanks are suitable to use in biomarker discovery projects due to insufficient sample quality.

We have in this project established a cohort of patients with suspected ACS and a biobank where we have full knowledge about the preanalytical storage conditions of the samples. This
biobank will be used in future evaluations of biomarkers that we are planning for. Based on the experiences gained in this project we will also perform a screening for potential new biomarkers in a selection of the samples from our biobank to investigate whether the results from the initial biomarker discovery can be repeated or if other additional biomarkers of interest can be identified.

In Paper IV we found an additional positive effect on atherogenic lipoproteins when ezetimibe was added on top of statin treatment. This effect was most pronounced on remnant-particles, translated into a reduced PG binding, and is probably due to both an additional and additive effect of the two treatments on the lipoprotein metabolism. These observations might explain the positive findings in the SHARP and IMPROVE-IT trials from combined therapy, especially in patients with type 2 diabetes where elevated remnant-cholesterol is common. In conditions with elevated remnant-cholesterol the combination of ezetimibe and statins seems to be the optimal treatment. Furthermore, estimation of remnant-cholesterol can lead to better characterization of the dyslipidemia and contribute to optimization of treatment.
8 CONCLUDING REMARKS

The aim of this thesis was to investigate new potential biomarkers to improve the diagnosis and treatment of cardiometabolic diseases. In the four separate papers we have in diverse cohorts and with different approaches investigated the importance of various biomarkers in cardiometabolic disease.

In **Paper I** and **Paper II**, we investigated the association between plasma Lp(a) levels and CVD in two different cohorts and concluded that Lp(a) was a relevant biomarker in both and that measurement of Lp(a) can help to improve the CVD risk assessment.

In **Paper III** we established an efficient approach for early transfer of potential new biomarkers, identified in discovery studies into evaluation in the routine care setting and defined a concept for patient recruitment and biobanking of samples in the acute care setting. Although CCL-16 was found not to be a good biomarker for ACS, we achieved valuable experiences for future biomarker studies.

In **Paper IV**, several additional atheroprotective changes on the lipoprotein constitution were found when ezetimibe was added to simvastatin treatment. This was especially observed in remnant-particles and we concluded that estimation of remnant-cholesterol can contribute to further characterization of dyslipidemias, and in conditions with elevated levels the combined treatment with ezetimibe and simvastatin seems to be optimal.

I think that due to better understanding of the biomarkers, their contribution in the disease process, and how they are affected from different treatments, additional biomarkers will be used to a greater extent to further characterize dyslipidemias in the future. My belief is that Lp(a) will be part of the “standard” lipid profile and together with total-cholesterol, HDL-cholesterol, LDL-cholesterol, TG, and other additional biomarkers such as remnant-cholesterol be important in the CVD risk estimation. This will contribute to a better individual risk assessment, optimization of treatment, and hopefully improved outcome of the diseases.

Together, these four papers have added further knowledge about the different biomarkers and can contribute to an improved management of patients with cardiometabolic diseases. In addition, a way to transfer new biomarkers from the research laboratory into evaluation in the routine care setting has been suggested. Thus, this thesis has provided a deeper understanding of the complexity in the process to validate and implement new potential biomarkers.

Den tekniska utvecklingen av laboratoriemetoder har bidragit till att vi lättare kan identifiera nya möjliga biomarkörer. Att sedan överföra dessa nya biomarkörer från forskningen till användning inom rutinsjukvården är en lång och resurskrävande process som kan ta många år. Mer än 150 000 nya biomarkörer har föreslagits, men endast ett hundratal används idag inom sjukvården, vilket illustrerar denna svårighet.

Detta doktorandprojekt syftade till att utvärdera nya biomarkörer för att förbättra diagnostik och behandling av kardiometabola sjukdomar. Dessutom har vi försökt att etablera en effektiv process för att överföra nya möjliga biomarkörer som identifieras i forskningslaboratoriet till utvärdering i den kliniska rutinsjukvården. Detta för att snabbt kunna avgöra om biomarkörerna verkar vara användbara och kan tillföra någon nytta för patienterna. I fyra separata studier har vi på olika sätt och i olika patientgrupper utvärderat nytan av olika biomarkörer för kardiometabola sjukdomar.

Lipoprotein (a) är en typ av blodfett. Nivåerna av Lp(a) i blodet påverkas framför allt av äftlighet och inte så mycket av kost- och livsstilsfaktorer. Hög nivåer av Lipoprotein (a) innebär en ökad risk för att drabbas av hjärt- och kärlsjukdom. Även om mycket tyder på det, så vet vi inte säkert om risken för hjärt- och kärlsjukdomar kan minsas genom att sänka Lipoprotein (a) nivåerna med hjälp av läkemedel och det behöver undersökas vidare. Tyvärr så finns det idag inte några lättillgängliga behandlingar för att sänka Lipoprotein (a) nivåerna, men nya behandlingar är på väg. Oavsett det så behöver vi öka vår kunskap om betydelsen av Lipoprotein (a) som riskfaktor för hjärt- och kärlsjukdom hos olika patientgrupper för att bättre förstå hur patienter med höga Lipoprotein (a) nivåer ska behandlas för att minska deras risk.

I studie I undersökte vi sambandet mellan Lipoprotein (a) och kardiovaskulära sjukdom hos mer än 23 000 patienter som alla hade mätt sitt Lipoprotein (a) värde inom rutinsjukvården. Patienterna med de 25 % högsta Lipoprotein (a) värdena hade en nästan 1,4-gånget ökad risk för drabbas av hjärtinfarkt eller kärlkramp jämfört med de med lägre nivåer. I studie II undersökte vi sambandet mellan Lipoprotein (a) och olika kärlsjukdomar hos patienter med typ 1 diabetes. Där hade patienter med höga Lp(a) värden (≥120 nmol/L) en 1,5-gånget ökad risk för att drabbas av hjärt- och kärlsjukdom, 1,7-gånget ökad risk att få äggvita i urinen som tecken på tidig njurskada och en 2-gånget ökad risk att drabas av hjärtklaffsjukdomen aortastenos. Vår slutsats från dessa två studier är att Lipoprotein (a) är en viktig riskfaktor för hjärt- och kärlsjukdom även i dessa patientgrupper. Därför bör Lipoprotein (a) mätas och vid
förhöjda värden bör det övervägas om risken för hjärt- och kärlsjukdom kan minskas genom att förbättra behandling av övriga riskfaktorer.

**I studie III** etablerade vi en process för att överföra nya möjliga biomarker från forskningslaboratoriet till utvärdering inom rutinsjukvården och för att effektivt rekrytera patienter med akuta sjukdomar till forskningsstudier. I en tidigare studie identifierades CCL-16 som en möjlig biomarker för att förbättra diagnostiken av hjärtinfarkt och vi utvärderade därför nyttan av att mäta CCL-16 hos patienter med misstänkt hjärtinfarkt i rutinsjukvården. Det kan vara svårt att rekrytera patienter med akuta sjukdomar och att samla in deras prover till forskningsstudier eftersom den akuta behandlingen alltid går först. Vi valde därför att identifiera möjliga studipatienter när deras prover analyserades även CCL-16 och information om diagnos hämtades från patientjournalen. Tyvärr så tillförde inte CCL-16 något i diagnostiken av hjärtinfarkt i denna studie och verkar därmed inte vara en lämplig biomarker för detta. Däremot har en biobank med prover som kan användas i framtida utvärderingar av möjliga biomarker upprättats och vi har fått ökad kunskap om vad som är viktigt att tänka på i denna typ av forskningsprojekt.

Tidigare studier har visat på en ytterligare minskning av hjärt- och kärlsjukdom hos patienter som behandlas med kombinationen av de två vanliga blodfettsänkande läkemedlen simvastatin och ezetimibe jämfört med de som bara behandlas med simvastatin. Effekten var mest uttalad hos patienter med typ 2 diabetes. För att förstå varför så undersökte vi i **studie IV** hur behandling med simvastatin och ezetimibe, var för sig eller tillsammans påverkar blodfetternas sammansättning. Fyrtio patienter behandlades i 4 veckor med antingen placebo, simvastatin, ezetimibe eller en kombination av båda läkemedlen och blodprover togs innan och efter behandling. Patienterna som fick behandling med både simvastatin och ezetimibe hade en ytterligare positiv effekt på blodfetternas sammansättning jämfört med de som bara fick ett av läkemedlen. Effekten var mest uttalad för de partiklar som innehåller det resterande kolesterol (rest-kolesterol) utöver det ”onda”- (LDL-kolesterol) och ”goda”- (HDL-kolesterol) kolesterol. Vid vissa tillstånd så som diabetes typ 2 är rest-kolesterol förrört och bidrar till en ökad risk för hjärt- och kärlsjukdomar. Våra resultat visar att behandling med kombinationen av simvastatin och ezetimibe är att föredra i dessa fall.

Tillsamman har dessa fyra studier bidragit med ökad kunskap om de olika biomarkererna som kan leda till förbättrad diagnostik och behandling av patienter med kardiometabola sjukdomar. Dessutom har en strategi för att snabbt kunna utvärdera nya eventuella biomarker i rutinsjukvården föreslagits. Detta har gett en bättre förståelse för komplexiteten och utmaningarna med att införa nya biomarker
10 ACKNOWLEDGMENTS

To my main supervisor Paolo Parini. You are a fantastic, intelligent and social person and I am very lucky to have the opportunity to work with you. You believed in me from the beginning and you have helped me to start believing in myself. I have always felt confident as a PhD-student because I know that when I really need it, you are there, guiding me and fighting for me. You have not only helped me to grow as a researcher and to become a scientist but also to grow as a person. We have experienced successes and though times together and you have showed me that every failure or mistake is a chance to learn and to become better. We have discussed both science, carriers and life during the years, and I am convinced that we will continue to do so. Thank you Paolo!

To my co-supervisor Ann-Charlotte Bergman for your support and contribution with great scientific knowledge. You always add an intelligent perspective on things. You supported me in all the practical arrangements in the laboratory and I would not have managed that without you.

To my co-supervisor Jonas Brinck for your contribution with great scientific knowledge and clinical experiences. Thanks for your encouragement and energy to push things forward and for all the scientific discussions and coffee breaks.

To Mats Eriksson, for supervising me in the clinic and for your endless positivity and friendliness. You always contribute with your great scientific knowledge in combination with great clinical skills in our discussions.

To all members in the lipo-group research constellation, Lise-Lotte Vedin, Matteo Pedrelli, Camilla Pramfalk, Mirko Minniti, Thomas Jacobsson, Veronika Tillander for generating a welcoming and positive research atmosphere.

To Osman Ahmed for a good collaboration in the Stockholm study. You are a very good researcher and you inspire me.

To Lilian Larsson and Maria Olin for always being helpful and for your guidance and teaching in the laboratory.

To Maura Heverin for proof reading. I really appreciate it!

To Magnus Hansson, for being the best supervisor in clinical chemistry and the perfect roommate and colleague. For contributing with all your knowledge, all your clever thoughts and for always involving me in your exciting projects.

To Gösta Eggertsen, for always contributing with your great knowledge and opinions about clinical chemistry, lipoproteins and everything else in the world. For your help and your caring about me.
To Maria Farm, for being my role model, colleague and friend. We have experienced the time as PhD-students, residents, and newly being specialists in clinical chemistry together and I have learned from you. You are a fantastic person and you spread so much joy around you.

To Anna Sjöström, for your kindness, your ability to make everyone feel valuable, for never missing a birthday and for being a great colleague and friend.

To Mats Estonius, my former manager, for a good collaboration in the biochemistry section.

To all former colleagues at the Clinical Chemistry Laboratory at Karolinska University Hospital, Charlotte Hansson, Frida Duell, Sara Karlsson, Daniel Ekholm, Henrik von Horn, Finn Thormark Fröst, Robin Zenlander, Niklas Bark, Sven Gustavsson, Inga Bartusevicie, Jovan Antovic, Britta Landin, Magnus Axelsson and Margareta Steen-Linder.

To Anders Kallner, for sharing your great experiences in clinical chemistry and statistics and for assisting me in the writing of my first manuscript ever.

To all collaborators in the projects, Henrike Häbel, Matteo Bottai, Jacob Odeberg, Maria Jesus Iglesias, Jochen Schwenk, Philip Smith, Michael Alvarsson, Emil Hagström, Tigist Wodaije.

To Simon Sjuls and Rasmus Svensson for your contributions in the projects.

At last, I would like to thank all my family and friends. I would not have made this without you!

My parents for your endless support.
My “syster yster” Emma for always being so much wiser than me and help me find the right way in life.
My brother Olle for inspiring me with all your projects and your enthusiasm in life. I learn from you.

Malin Randeniye for being my very best friend forever.

Micke, for being there by my side and looking after me.

Viktor and Filip for all the joy and love you bring into my life.
11 REFERENCES


27. Sig-Britt W. Endokrinologi: Liber AB; 2015.


