Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
Department of Circulation and Medical Imaging

Sigri Bakken Sperstad

Biomarkers of Subclinical Atherosclerosis

Master's thesis in Molecular Medicine
Supervisor: Anja Bye
May 2021

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CERG
Cardiac Exercise Research Group

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Abstract

Background: Traditional biomarkers used to measure risk of myocardial infarction (MI) only explain a modest proportion of the incidence. With an increasing prevalence, there is a need for new biomarkers to improve prediction and prevention of the disease.

Aims: The primary aim was to explore the potential of lipoprotein subfractions and two proteins as biomarkers of subclinical atherosclerosis. Secondary aims were to 1) identify gender-specific risk markers, 2) identify biomarkers of imminent MI, 2 years after baseline and 3) establish if samples used for nuclear magnetic resonance (NMR) lipidomics can be reused to quantify proteins.

Methods: A retrospective observation case-control (1:2) study with a 5-year observation period was performed. Apparently healthy participants that later experienced a MI (n=50) were compared to matched controls (n=100). NMR lipidomics quantified 112 lipoprotein subfractions in serum. Protein quantification of matrix metalloproteinase-9 (MMP-9) and aldehyde dehydrogenase 4 family member A1 (ALDH4A1) was performed by enzyme-linked immunosorbent assay (ELISA). Analyses stratified by gender and subgroup analysis with a 2-year observation period were performed. The potential of reusing samples from NMR lipidomics for ELISA were tested (n=49).

Results: Apolipoprotein A1 concentration in small high-density lipoprotein (HDL) was significantly higher in cases ($p < 0.05$). In men, decreased levels of large HDL subfractions and increased levels of small HDL subfractions were associated with future MI. Subgroup analysis with a 2-year observation period had an increased concentration of triglycerides in low-density lipoprotein (LDL) in cases ($p < 0.05$). Protein levels of MMP-9 and ALDH4A1 did not differ between cases and controls. Protein concentration of fresh serum and serum used for NMR analysis correlated ($p < 0.01$).

Conclusion: Several lipoprotein subfractions seemed to be associated with future MI, potentially reflecting subclinical atherosclerosis. In men, there might be additional potential for risk prediction by exploring HDL subfractions. Promising results indicated that samples used for NMR lipidomics can be reused for protein quantification.

Samandrag

Bakgrunn: Tradisjonelle risikomarkørar nytta for å estimere risiko for hjarteinfarkt forklarar berre ein del av dei faktiske tilfella. Med ein auka førekomst av tilfelle er det behov for nye risikomarkørar som kan forbetre predikasjon og førebygging av sjukdommen.

Formål: Hovudformålet var å studere potensialet for lipoprotein subfraksjonar og to protein som mogelege risikomarkørar for subklinisk aterosklerose. Delmåla var å 1) identifisere kjønns-spesifikke risikomarkørar, 2) identifisere risikomarkørar for hjarteinfarkt berre to år etter baseline og 3) undersøkje om blodprøver som fyrst har vorte nytta til analyse av lipoprotein subfraksjonar kan gjenbrukas til proteinanalyse.

Metode: Dette er ein retrospektiv case-kontroll (1:2) studie med observasjonsperiode på fem år. Tilsynelatande friske deltakare (n=50) som seinare fekk hjarteinfarkt vart samanlikna med matcha kontrollar (n=100). 112 Lipoprotein subfraksjonar vart måla i serum med nuclear magnetic resonance (NMR) lipidomics. Konsentrasjonen av proteina matrix metalloproteinase-9 (MMP-9) og aldehyde dehydrogenase 4 family member A1 (ALDH4A1) vart estimert gjennom enzyme-linked immunosorbent assay (ELISA). Det vart utført subanalysar basert på kjønn og med to-års observasjonstid. Serumprøvar brukt til NMR analyse vart gjenbrukt til ELISA-analyse.

Resultat: Konsentrasjonen av apolipoprotein A1 i små high-density lipoprotein (HDL) var signifikant høgare hjå casane ($p < 0.05$). Subanalysar av menn viste at store HDL subfraksjonar har høgare konsentrasjon hjå kontrollane, medan små HDL subfraksjonar har høgare konsentrasjon hjå casane. I subanalysa med to-års observasjonsperiode hadde casane høgare konsentrasjon av triglyserid i LDL. Konsentrasjonen av dei to proteina, MMP-9 og ALDH4A1, var ikkje forskjellig mellom caser og kontrollar. Proteinkonsentrasjonen til serumprøvane som fyrst vert brukt til NMR analyse korrelerte med friske prøvar ($p < 0.01$).

Konklusjon: Fleire lipoprotein subfraksjonar verkar å vere assosiert med framtidig hjarteinfarkt, som potensielt kan indikere subklinisk aterosklerose. HDL subfraksjonar skil seg ut blant menn, og kan potensielt bidra til forbetring av kjønns-spesifikk predikasjon av hjarteinfarkt. Lovande resultat tydar på at det mogeleg å gjenbruke prøvar frå NMR lipidomics til proteinanalyse.

Acknowledgement

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Table of contents

Abbreviations

ALDH4A1	Aldehyde Dehydrogenase 4 family member A1
ApoA1	Apolipoprotein A1
ApoA2	Apolipoprotein A2
ApoB	Apolipoprotein B
BMI	Body Mass Index
CAD	Coronary Artery Disease
CVD	Cardiovascular Disease
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
ESC	European Society of Cardiology
HDL	High Density Lipoprotein
HDL-C	HDL-Cholesterol
sdHDL	Small, dense HDL (HDL-4)
ICD	International Classification of Diseases
IDL	Intermediate Density Lipoprotein
LDL	Low-Density Lipoprotein
LDL-C	LDL-Cholesterol
LDL-TG	LDL-Triglyceride
MI	Myocardial Infarction
MMP	Matrix Metalloproteinases
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analyses
PLSDA	Partial Least Squared Discriminant Analysis
RCT	Reverse Cholesterol Transport
SD	Standard Deviation
SEM	Standard Error of Mean
VLDL	Very Low-Density Lipoprotein

1. Introduction

1.1 Trends in epidemiology

Cardiovascular diseases (CVD) are noncommunicable diseases, defined as disorders involving the heart and blood vessels [1]. The 2019 report from the European Society of Cardiology (ESC) Atlas states that CVD remains the most common cause of death [2]. In 2016, the World Health Organization indicated that CVD represented 31% of all deaths worldwide. Figure 1 illustrates the causes of deaths in Norway in 2019, indicating that 25% are due to CVD [3]. There is a declining trend of CVD morbidity in ESC countries, including Norway [2, 3]. Despite lower mortality rates, there is still a considerable amount of people that are affected. The CVD register in Norway reported that 349 009 patients were registered with CVD in 2019 [4]. In the years to come, it is estimated that the number of people at risk will increase, due to obesity, diabetes 2, inactivity and ageing [2, 5]. This reflects the need for better diagnostic methods of people at high cardiovascular risk, making it possible to achieve preventative measures [1].

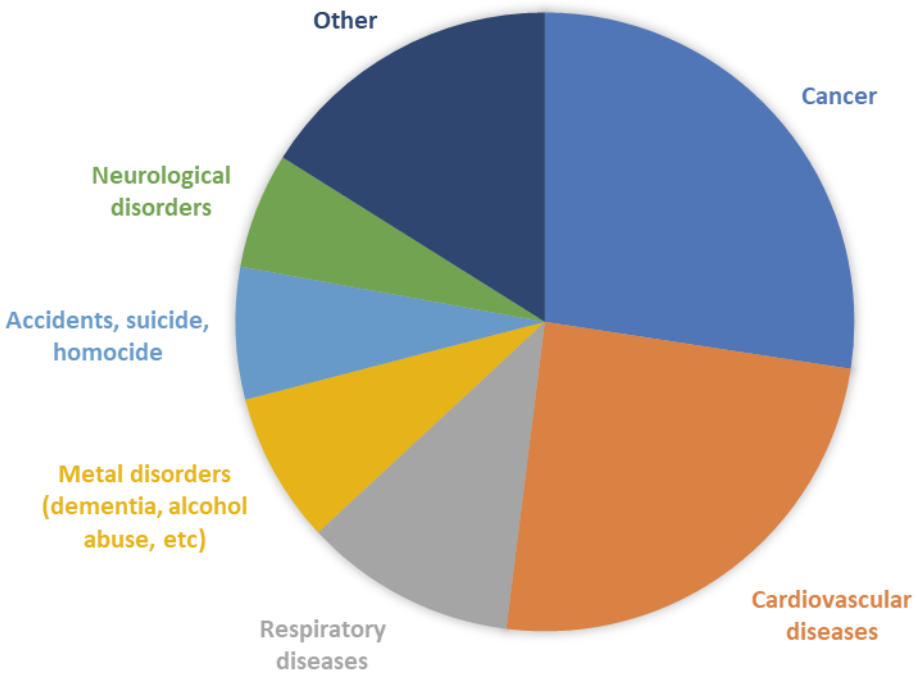

Figure 1: *Causes of death in Norway 2019.* Pie chart is based on statistics from Folkehelseinstituttet [3]

1.2 Coronary artery disease

CVDs involving the blood vessels supplying the heart are termed coronary artery disease (CAD)[1]. CAD is the leading cause of mortality and morbidity in industrialized countries [6-9]. There is also observed an increasing prevalence in developing countries, implying that this will continue to be a socioeconomic burden worldwide in the years to come [7]. CAD resulting in death is often caused by arterial blockage due to atherosclerotic plaque in the walls of large and medium-sized arteries [10]. Atherosclerosis is therefore the most prevalent underlying cause of CAD [11-13]. CAD includes myocardial infarction (MI), which is cessation of blood supply to the heart, leading to myocardial injury [14].

Atherosclerosis is a multifactorial disease involving both modifiable and non-modifiable risk factors [14]. The 2019 report from ESC presents modifiable risk factors for MI, including cigarette smoking, obesity, physical inactivity, hypertension, hypercholesterolemia, diabetes mellitus and high alcohol consumption [2]. Non-modifiable risk factors include age, male gender, and genetic predisposition [7, 9].

1.2.1 Pathophysiology of atherosclerosis

The progression of atherosclerosis represents the main underlying cause of MI and is usually asymptomatic. Atherosclerosis can be defined as a gradual thickening of the walls of the arteries through formation of atherosclerotic plaques [14]. It is characterized as a chronic immunoinflammatory and fibroproliferative disease of the arteries [7, 15]. Atherosclerosis is a complex and progressive disease where the pathological events begin in early childhood and proceeds throughout life. The process involves a series of subclinical cellular events leading to complex atherosclerotic lesions [9, 16]. Figure 2 illustrates the progression of atherosclerosis, eventually leading to a MI. Specific sites of the arteries are more prone to atherogenesis because of altered hemodynamics. Arterial branch points and curvatures experience disturbed blood flow, resulting in low shear stress. Shear stress is the force generated by the flowing blood acting on the vascular endothelium. Changes in shear stress affects the vascular function, whereas low shear stress predisposes the endothelium for early-stage atherosclerotic lesions [16-19].

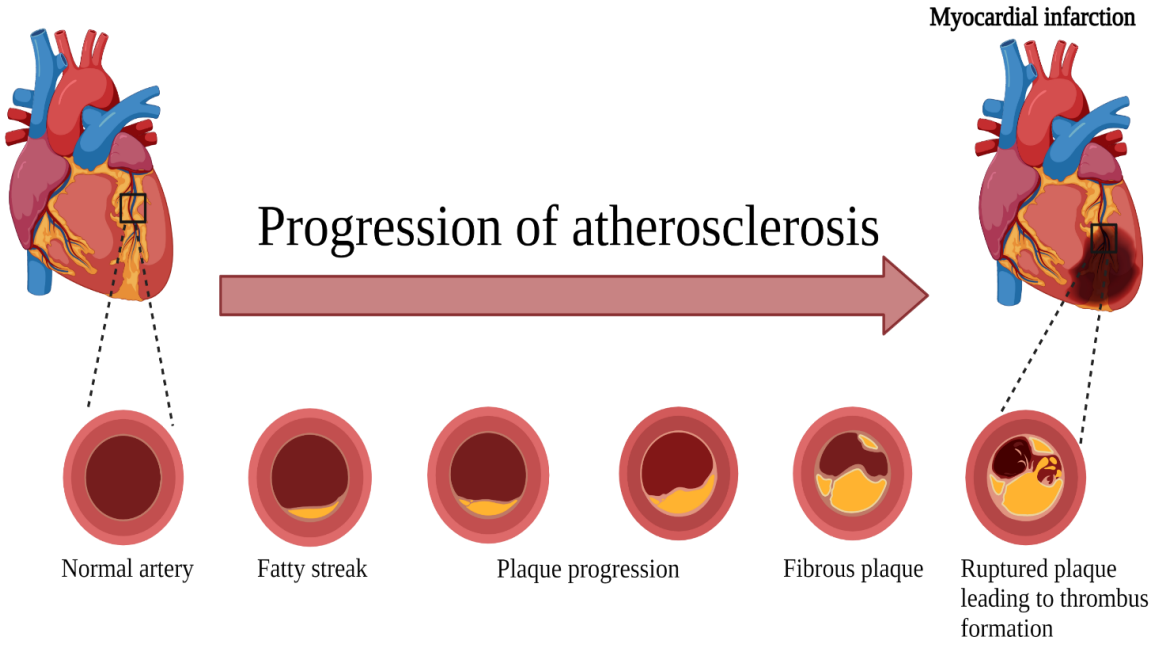

Figure 2: *Progression of atherosclerosis in the coronary artery.* Illustration of the gradual development and thickening of atherosclerotic plaque from a healthy artery, plaque formation and thrombosis, leading to a myocardial infarction. The figure is created with BioRender.com.

The vascular endothelium forms an effective barrier between the blood and vessel wall, as it regulates vasoconstriction and vasodilation by secreting chemicals acting on smooth muscle cells [14]. Injury to the endothelium caused by physical or chemical irritants leads to endothelial dysfunction, which represents the first step in atherosclerosis. Oxidative stress affects the endothelial and vascular function and is also associated with impaired bioavailability of nitric oxide, which is one of the main causes of endothelial dysfunction. Damage to the endothelium disrupts the effective barrier, leading to increased endothelial permeability, generation of proinflammatory cytokines, platelet aggregation, leukocyte recruitment, adhesion and subendothelial transmigration. Activated platelets interacting with a damaged endothelium contribute to the proatherogenic effect [9, 14, 20]. Increased permeability results in an accumulation of apolipoprotein B100 (ApoB) containing lipoproteins, especially low-density lipoprotein (LDL), in the subendothelial space. The lipoproteins are retained there through binding to matrix proteoglycans [14, 21]. LDLs undergo oxidative modifications, altering the particles to promote inflammation through activating endothelial cells, smooth muscle cells and monocytes. Infiltrated monocytes travel from the blood to the intima where they differentiate into macrophages and internalize oxidized LDL via scavenger receptors. The formation of lipid-loaded macrophages cells, foam cells, is the earliest visible atherosclerotic lesion. This is also called the fatty streak [6, 8, 15, 20], which is illustrated in Figure 2.

Foam cells will go through apoptosis. Accumulation of apoptotic cells will form a lipid-rich necrotic core within the plaque. Foam cells stimulate a release of proinflammatory cytokines that promote atherosclerotic plaque progression [14, 17, 22]. The activated endothelium will also release chemokines and growth factors. Together, foam cells and the epithelium stimulate proliferation and migration of smooth muscle cells from the arterial media into the intima. There, smooth muscle cells synthesize the extracellular matrix (ECM), building up the fibrous cap surrounding the necrotic core [17, 23]. This progressive process of atherosclerotic plaque formation causes luminal narrowing of the artery, called stenosis [23, 24]. Plaque rupture, erosion or a calcified nodule might lead to arterial thrombosis. A fully occluded coronary artery causes a restriction of blood supply, preventing the heart from receiving enough oxygen. Eventually, this leads to acute ischemia and MI (Figure 2). The thrombus-mediated acute coronary events depend on both the composition and vulnerability of the plaque, as rupture-prone plaques are associated with a thin fibrous cap and abundance of inflammatory cells [21, 23]. A key factor for plaque rupture is the production of matrix metalloproteinases (MMP) as they stimulate thinning of the fibrous cap [25]. Stable plaques, containing a thick fibrous cap progress to stenosis. Stenosis reduces blood flow but is usually not as fatal as thrombosis. However, stable plaques can be transformed into rupture-prone plaques [23, 24].

1.3 Lipoproteins

Accumulation of lipoproteins and lipids plays a crucial role for the development of atherosclerosis [20, 26]. Lipids are a diverse group of hydrophobic compounds having several biological functions, acting as structural components of cell membranes, energy storage source and intermediates in signaling pathways [27]. Due to their hydrophobicity, plasma lipids as cholesterol and triglycerides are packaged with apolipoproteins to be soluble in plasma. These spheroidal macromolecules, called lipoproteins, transport lipids through the blood. Phospholipids, apolipoprotein, and cholesterol make up the hydrophilic coat, whereas the hydrophobic core consist of triglycerides and free cholesterol, illustrated in Figure 3.

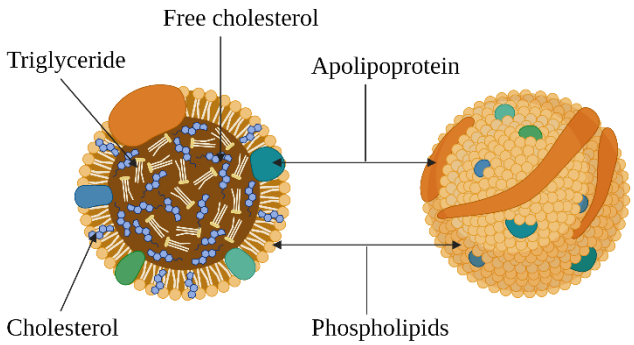

Figure 3. *General composition of a lipoprotein.* The figure is created with BioRender.com

In order of increasing density, lipoproteins are classified into chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoproteins (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Figure 4). They are distinguished by their composition, size, density and function [20, 28]. The composition of triglycerides, cholesterol and main apolipoprotein are also indicated in Figure 4. Lipoproteins continuously transport cholesterol and triglycerides within the blood. Figure 5 illustrates a simplified overview of lipoprotein metabolism. Dietary fat is absorbed in the small intestine and becomes incorporated into chylomicrons, which transport lipids for storage or energy use. Chylomicron remnants are removed by the liver. Cholesterol and triglycerides are packaged into VLDL in the liver. VLDL transport the lipids through the bloodstream to adipose- and muscle tissue. Within the blood, extraction of triglycerides gradually converts VLDL to LDL through IDL. LDL transport cholesterol from the blood to peripheral tissues and the liver, while HDL transport cholesterol from peripheral tissues to the liver, which is called reverse cholesterol transport (RCT) [14, 20].

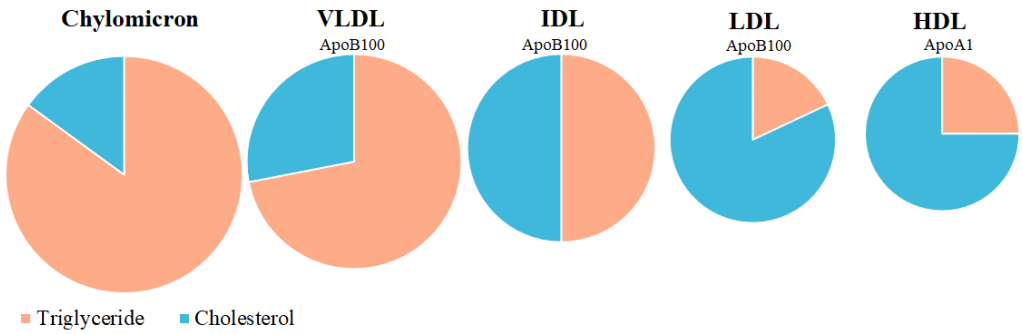

Figure 4. *Lipoprotein composition*. Distribution of triglyceride and cholesterol in chylomicron, VLDL, IDL, LDL and HDL. Size of the lipoproteins reflect their density Their major apolipoprotein component is also illustrated. VLDL: Very low-density lipoprotein, IDL: Intermediate density lipoprotein, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, ApoB: Apolipoprotein B100, A1: Apolipoprotein A1

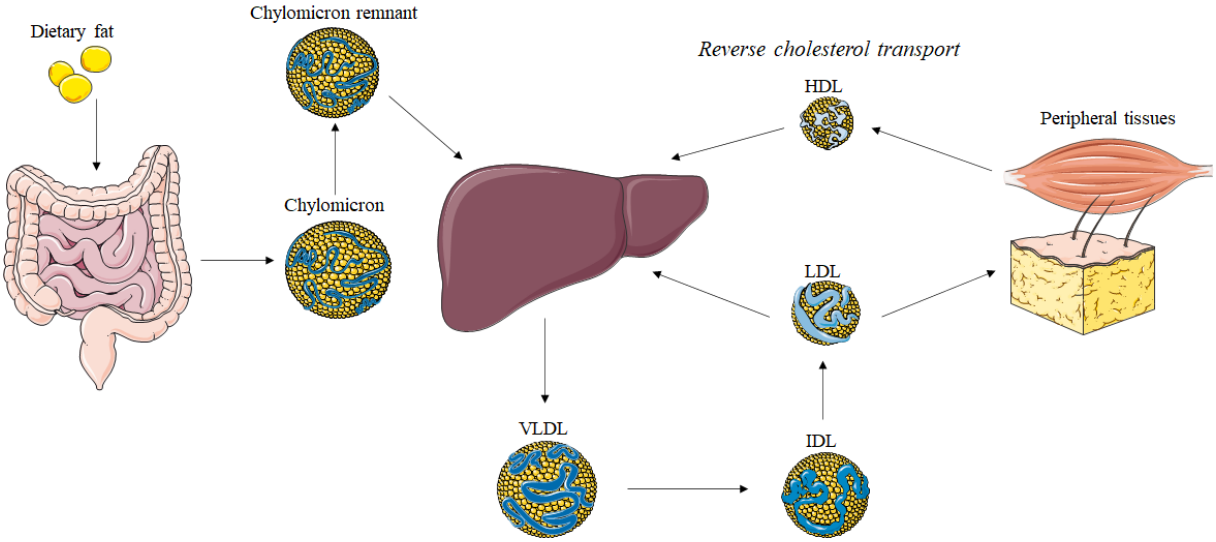

Figure 5. *Simplification of lipoprotein metabolism, showing transportation of chylomicrons, LDL, IDL, VLDL, LDL and HDL through the bloodstream to tissues*. VLDL: Very low-density lipoprotein, IDL: Intermediate density lipoprotein, LDL: Low-density lipoprotein, HDL: High-density lipoprotein.

1.3.1 Lipoprotein and atherosclerosis

Blood cholesterol and atherosclerosis is highly associated. HDL and LDL are the main cholesterol-carrying lipoproteins (Figure 4), having apolipoprotein A1 (ApoA1) and ApoB as the main apoprotein component, respectively. HDL and LDL are therefore of pivotal significance in the atherosclerotic process [20, 29, 30]. Measurement of LDL and HDL have primary been based on their cholesterol concentration [20, 31].The general assumption is that HDL, and its main apolipoprotein, ApoA1, protects against atherosclerosis because of its function in RCT, removing excess cholesterol from macrophages within the artery wall. This interferes with the development of atherosclerotic plaque. In addition, HDL function as an anti-inflammatory factor, protecting the vascular endothelium, and is also reported to have antithrombotic properties. However, the exact role of HDL in pathophysiology of atherosclerosis is still

controversial [24, 32, 33]. LDL is atherogenic, promoting initiation and progression of atherosclerosis. LDL stimulate cholesterol collection in macrophages and pro-inflammatory effects inside the vessel wall [7, 15, 34]. In addition, Apo B-containing lipoproteins smaller than 70 nm in diameter can freely cross the disrupted endothelium, stimulating the formation of atherosclerotic plaque. LDL constitutes more than 90% of these lipoproteins, the rest is VLDL and IDL [34]. Both LDL and oxidized LDL activate endothelial cells, smooth muscle cells and monocytes, promoting the formation and progression of atherosclerotic plaque. In addition, they facilitate thrombosis leading to acute cardiovascular events [8, 20].

1.3.2 Cholesterol as a biomarker for cardiovascular disease

The causal role of cholesterol in the pathogenesis of atherosclerosis was suggested over 100 years ago [35, 36]. Cholesterol levels are today well-established biomarkers used in the clinic for evaluation of CVD risk and CVD treatment decisions. A biomarker is a measurable indicator of a normal biological process, pathogenic process, or pharmacological response to a therapeutic intervention [37]. Total serum cholesterol estimates the total amount of cholesterol contained in all the lipoprotein particles [38]. Most cholesterol is carried in LDL particles. Thus, there is a strong correlation between cardiovascular risk and concentration of both total serum cholesterol and LDL-cholesterol (LDL-C). Hypercholesterolemia is according to the ESC defined as a total serum cholesterol above 6.2mmol/L [2]. In Norway, drug treatment is recommended for patients having a total cholesterol higher than 7.0 mmol/L. In absence of other risk factors, women that have reached menopause are excepted from this recommendation [39]. Because of the central role of atherogenic lipoproteins, maintaining optimal cholesterol levels is necessary to achieve ideal cardiovascular health [40].

High levels of LDL-C and low levels of HDL-cholesterol (HDL-C) is associated with an increased CVD risk. Elevated levels of LDL-C are related to progression of atherosclerosis and is of relevance for risk prediction [38, 41]. The main therapeutic aim for reduction of CVD risk is to reduce LDL-C, as this is one of the most important modifiable risk factors [20, 42]. Implementation of lifestyle changes, especially diet by reducing trans fatty acid content of food is effective to lower LDL-C. Alternatively, use of cholesterol lowering medication is implemented [41, 43]. LDL-C can be measured directly but is most often calculated using the Friedewald formula [42], estimating LDL-C based on levels of total cholesterol, HDL-C and triglycerides [44]. LDL-C is applied in both cardiovascular risk prediction and in deciding

treatment options [33]. Low HDL-C is a risk factor for CVD. High HDL-C levels are associated with lower risk but does not necessarily protect against disease. Thus, the established inverse association between HDL-C and CVD does not appear to be causal [33, 45, 46]. Measurement of HDL-C is included in current risk prediction, but only levels underneath a certain threshold are implemented in the model [46].

1.4 Improving cardiovascular risk prediction

1.4.1 The current risk prediction models

Accurate risk prediction is essential for clinicians to help eliminating or minimizing the impact of CVD. Mapping a patient's lipid profile is of interest as hyperlipidemia is one of the most modifiable risk factors for CVD. Despite this, risk prediction involves more than lipid profiling as atherosclerosis is a product of several risk factors [41]. Guidelines for risk prediction presented by both the American Heart Association and ESC involves a systematic approach assessing several risk factors to reflect the total CVD risk. This is represented as a prediction of 10-year CVD risk. These risk prediction models are used as a tool for clinicians to determine what interventions are needed, helping to avoid both under- and over-treatment [42, 47]. The United States utilize the Framingham Risk Score, which estimate the 10-year risk of a first fatal CVD event [42]. In 2017 the Norwegian Directorate of Health established a new model for CVD risk, NORRISK 2.The model is based on existing guidelines for prevention of CVD [46]. NORRISK 2 predicts the 10-year risk for acute myocardial infarction and cerebral stroke for men and women aged 45-79 years. The risk factors included in the model are gender, age, daily smoking, serum total cholesterol, HDL-C, systolic blood pressure, the present use of antihypertensive drugs, and if any first-grade relatives have suffered from myocardial infarction before the age of 60 [46]. Total serum triglyceride levels are not included, but is usually measured for the patient's total lipid profile. Levels above 1.7 mmol/L are associated with a potential increased cardiovascular risk [39]. Some other additional factors as rheumatoid arthritis, south Asian ethnicity, abdominal obesity and previous mental illness is also evaluated for a complete risk profile [39]. The model is based on a linkage between a large population-based study (Cohort of Norway; CONOR) and the CVD project, which is a database of CVD hospital discharge diagnoses and mortality in Norway. A precise estimate of an individual's total risk is valuable for determination of which preventative interventions that are necessary [46].

1.4.2 Identification of new biomarkers

Accurate risk prediction models are important to prevent CVD, but the traditional risk factors included in the models today fail to fully predict the individual's risk. Patients estimated at low risk suffer from MI each year [48, 49]. Around 50% of people having CAD are classified as having low or intermediate risk [49]. It is important to diagnose atherosclerosis at an early stage to prevent the progression and complications of the disease. There is a growing interest in identifying new biomarkers that could help identify individuals with subclinical atherosclerosis [13, 49, 50].

Proteins involved in inflammation, oxidation and haemostasis leading to atherosclerotic plaque have great potential as biomarkers. Research also indicates that a combination of biomarkers will estimate the CVD risk better, as one biomarker alone cannot reflect the complexity of disease [51, 52]. Two potential and relatively unexplored protein biomarker candidates are matrix metalloproteinase-9 (MMP-9) and aldehyde dehydrogenase 4 family member A1 (ALDH4A1). MMPs has a central role in the degradation of the ECM composed fibrous cap surrounding the atherosclerotic plaque. The ECM is constantly being remodeled, degradation of ECM will thus influence the stability of the plaque [53, 54]. Recent research has shown that MMPs are elevated during acute MI [54]. Tibaut et.al 2019 have previously suggested that MMP-9 can serve as a biomarker of plaque vulnerability and first time CHD events [52]. ALDH4A1 has recently been proposed as a potential biomarker of atherosclerosis [55]. ALDH4A1 is a dehydrogenase catalyzing a step of the proline degradation pathway. The recently published study aimed to investigate autoantibodies in atherosclerotic plaque based on the increasing interest between autoimmunity and atherosclerosis. ALDH4A1 was then identified as a target antigen to one of the highly expressed autoantibodies. Further analyses indicated that circulating levels of ALDH4A1 were increased in mice and humans with atherosclerosis [55].

Traditional lipid biomarkers have several limitations and fail to fully capture cardiovascular risk [56]. Research have illustrated that the described role of LDL-C in atherosclerosis is a major oversimplification [50]. Statistics show that 40% of CAD-cases have a total serum cholesterol level of less than 5.2 mmol/L, which is within the recommended range [41]. Current literature argue that conventional lipid measurements represent a simplification by not differentiating between size, density, or concentrations and compositions of lipoproteins. Investigating lipoproteins in smaller fractions might therefore reveal important information about the complexity of CAD, potentially identifying new lipid biomarkers [57].

1.5 Lipidomics

Lipidomics is a relatively recently developed research field using high-throughput profiling to produce a comprehensive analysis of cellular lipids [58, 59]. Lipidomics data provides information about the structure and function of the complete set of lipids produced in each cell [60]. In general, lipidomic analyses may provide information for early diagnosis of dyslipidemia, which is observed before clinical symptoms in several diseases [61]. Thus, it has the potential to provide information for early prevention and detection of CVD, as lipids play a pivotal role in the pathophysiology of atherosclerosis [62, 63].

Several different methods are used for lipidomic analyses, and Nuclear Magnetic Resonance (NMR) spectroscopy is one of them. The extensive profiling of lipoprotein subfractions is achieved by combining ultracentrifugation and NMR spectroscopy-based data. Different signals from the proton nuclear magnetic resonance (^{1}H-NMR) spectrum reflect the differences in lipoprotein composition, size and density. With the use of regression models combining ultracentrifugation- and NMR spectroscopy based data, information on the lipoprotein main- and subfractions are extracted [64, 65]. NMR lipidomics is a fast and inexpensive method for high throughput profiling. In addition, the analysis requires minimum sample preparation. Deviating from other lipidomics methods, NMR is non-destructive [60, 62, 66, 67]. This enables the material to possibly be reused for several analysis, representing a potential advantage of NMR lipidomics compared to other methods. NMR lipidomics quantifies density, size and particle number of different lipoprotein subfractions, in addition to lipid concentration and composition. This extended classification is described as a lipidomics profile, which might contribute to a growing understanding of the function of lipoprotein subfractions, and how they are affected by disease and treatment [68, 69].

1.5.1 Current knowledge on lipidomics in cardiovascular disease

There are promising results indicating that lipid profiling will provide new insights into the role of lipids in CVD [63, 70, 71]. Altered lipid metabolism and dyslipidemia associated with inflammation and oxidative stress are important contributors in the development of atherosclerotic lesions and plays a role in the transition from stable to unstable plaques [26, 72]. Holmes et al. performed a large-scale NMR analysis to investigate atherogenic lipoprotein subfractions. The study identified opposing associations of cholesterol and triglycerides with MI and ischemic stroke risk within HDL particles, especially in large and medium sized HDL

particles [71]. Furthermore, Wurtz et al. presented an extended model of the Framingham Risk Score, including lipoprotein subfractions quantified by NMR. The results showed an improved risk stratification for subclinical atherosclerosis in the extended model compared to the model including conventional lipids [57].

Lipidomic analyses separates lipoproteins by size, density and lipid content. Small, dense LDL particles are commonly believed to represent the most atherogenic lipoprotein subfraction [20, 59, 73-75]. These particles are proposed to be more atherogenic than large LDL particles for several reasons. First, the small size enhances their capacity to penetrate the vessel wall. Interaction with LDL-receptors clear LDL particles from the bloodstream. Small dense LDL particles have lower affinity to LDL-receptors, leading to longer circulation time in the bloodstream. This makes these particles more susceptible to chemical modifications as oxidation [26, 59, 74]. However, a recently published consensus-based recommendation from the European Atherosclerosis Society and the European Federation of Clinical Chemistry and Laboratory Medicine states that all LDL particles are atherogenic, regardless of size. Thereby concluding that treatment decisions should not emphasize on the size of LDL subfractions, the focus should be to reduce the number of LDL particles [76].

HDL particles are highly heterogenous and increasing evidence suggest that HDL subfractions might be differentially associated with future cardiovascular events [20, 77]. Research trying to reveal the link of HDL subfractions and coronary risk has therefore received much attention. Li et al. reported that only high levels of large HDL subfractions are associated with lower CVD risk, not levels of medium or small HDL subfractions, nor levels of total HDL-C [78]. Several studies have presented similar results [29, 32]. In addition, small HDL subfractions have previously been positively associated with cardiovascular disease [29, 57, 79]. One article suggest that small, dense HDL (sdHDL) is responsible for most HDL functions, especially RCT. Furthermore, the article proposed that measuring cholesterol in sdHDL is a better marker than cholesterol in total HDL (HDL-C) [80]. However, another article proposes that medium and large sized HDL carries most cholesterol in RCT [81].

Increasing evidence suggests that the roles of HDL and LDL in atherosclerosis cannot simply be described by cholesterol levels. The current knowledge on lipidomics in CVD indicates that more refined lipid analyses may provide additional information that is missing in today's evaluation of CVD risk. Lipidomic analyses might provide new insight in the molecular mechanisms underlying atherosclerosis. This can further lead to development of new treatment strategies and better risk prediction.

1.6 Purpose and aim

Traditional risk factors for MI implemented in risk prediction models only explain a modest proportion of the actual cases [49]. This illustrates the clinical need for new biomarkers that could identify patients at risk with greater precision than today.

The primary aim of this study was to explore the potential of lipoprotein subfractions and two promising proteins, MMP-9 and ALDH4A1, as biomarkers of subclinical atherosclerosis.

Secondary aims of this current study were to 1) identify gender-specific risk markers for MI, 2) identify risk markers of imminent MI, less than 2 years after blood sampling, 3) establish if serum samples used for NMR lipidomic analysis can be reused to quantify circulating proteins.

2. Materials and methods

2.1 Study design

This is a retrospective observational case-control (1:2) study with a 5-year observation period. Lipoprotein subfractions and serum proteins were compared between apparently healthy participants that developed a MI during the follow-up (cases) to matched participants that remained healthy during follow-up (controls).

2.2 Subjects

The Nord-Trøndelag Health Study (HUNT) is a large population-based health study. All inhabitants of Nord-Trøndelag County in Norway aged 20 years or older were invited. Four health surveys have been completed so far, HUNT1 (1984-1986), HUNT2 (1995-1997), HUNT3 (2006-2008) and HUNT4 (2017-2019). The HUNT study includes questionnaire data, clinical measurements, and biological samples [82]. This large biobank provides enormous potential for identification of new biomarkers. In the present study, 150 apparently healthy participants aged 45-74 from HUNT3 were included. The exclusion criteria for cases and controls lead to a selection of participants regarded as apparently healthy at baseline (HUNT3). The selected cases (n=50) experienced a MI within 5 years after HUNT3. An observation period of 5 years was selected as these cases were believed to have advanced atherosclerosis. Cases were identified by using data from the local myocardial infarction registry (Helse Nord-Trøndelag), coded I21 by the International Classification of Diseases 10th revision (ICD-10).

Variables related to anthropometry, laboratory measures, medication, self-reported diseases, and other CVD risks were collected from the HUNT database. Exclusion criteria for cases and controls were self-reported CVD (including MI, stroke, angina pectoris, heart failure or other reported heart diseases), chronic kidney disease, diabetes, and body mass index (BMI) > 40. Furthermore, to exclude participants prone to experience a MI within the next 5 years, NORRISK 2 risk was calculated. All variables included in the NORRISK 2 model was collected from the HUNT database. Calculation of risk was performed according to the model presented by Selmer et.al [46], whereas the risk level discriminates between different age groups. Participants having medium or high 10-year risk of acute myocardial infarction or cerebral stroke were excluded. Cases that had reported use of lipid-lowering medicine at the time of HUNT3 (self-reported data from HUNT4) were excluded. Follow-up data from HUNT4

ensured that the age-and gender matched controls (n=100) remained healthy thought the next 10 years. A flowchart of the selection criteria of cases and controls in this study is shown in Figure 6.

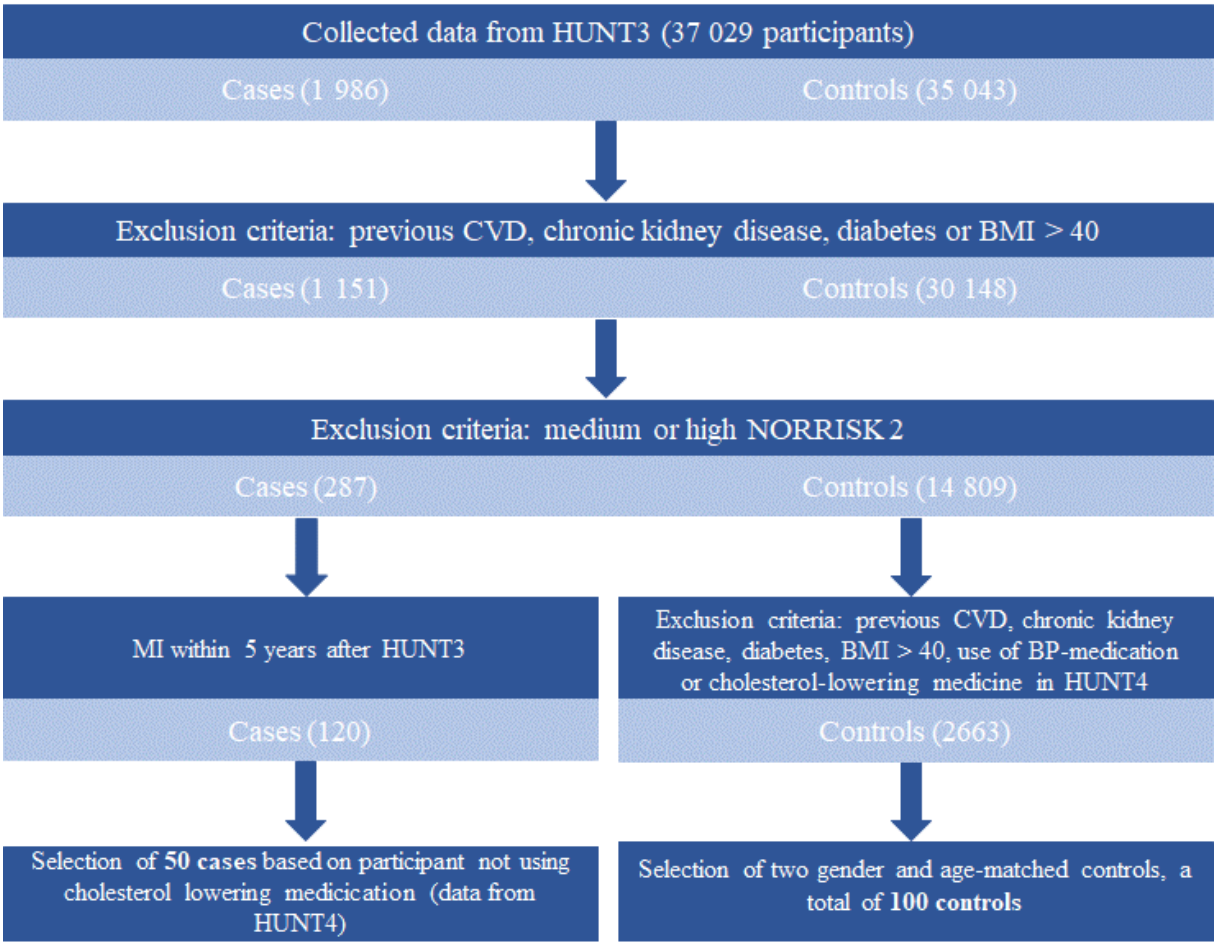

Figure 6. *Flowchart showing inclusion criteria of controls and cases in this study. HUNT: Helseundersøkelsen i Nord-Trøndelag, CVD:* Cardiovascular disease, BMI: Body mass index, MI: Myocardial infarction, BP: Blood pressure.

2.3 Clinical measurements

Clinical measurements for participants in HUNT3 was performed at the time of participation, from 2006 to 2008. Weight and height were measured on a combined scale (Model DS-102, Arctic Heating AS, Nøtterøy, Norway), and BMI was calculated as weight divided by height squared (kg/m^2). Blood pressure and resting heart rate were both measured while sitting (Critikon Dinamap 845XT, GE Medical Systems, Lille Chalfont, Buckinghamshire, United Kingdom) and followed established guidelines [82].

2.4 Blood sample collection

Blood sampling of the participants were performed non-fasting. Standard biochemical analyses were performed on fresh venous blood samples at Levanger Hospital, Norway. Nonfasting glucose (mmol/L) was analysed by Hexokinase/G-G-PDH methodology (reagent kit 3L82-20/3L82-40 Glucose, Abbot, (Clinical Chemistry, USA). HDL-cholesterol (mmol/L) was analysed by Accelerator selective detergent methodology (reagent kit 3K33-20 Ultra HDL, Abbot, Clinical Chemistry, USA). Triglycerides (mmol/L) were analysed by Glycerol Phosphate Oxidase methodology. LDL-cholesterol was calculated using the Friedewald formula. Collected serum samples were stored at -80°C. For this study, 250 µL frozen serum from each participant were collected from the HUNT biobank, aliquoted in two matrix tubes (150 µL and 100 µL). The aliquots containing 150 µL serum were used for NMR lipidomic analysis. The aliquots of 100 µL serum were used for enzyme-linked immunosorbent assay (ELISA) analyses. In addition, some of the samples used for NMR lipidomic analysis were reused for ELISA, to compare the protein concentration of reused and fresh samples.

2.5 Lipidomics

NMR lipidomics of serum samples was performed in collaboration with the MR Cancer Group (MR Core Facility, NTNU). Bruker BioSpin GmbH (Rheinstetten, Germany) was used to conduct the lipoprotein subfraction analyses, based on ^{1}H-NMR spectroscopy of serum samples, using regression analysis of ultracentrifugation results. The serum samples were diluted 1:1. 120 µL thawed serum sample was mixed with 120 µL buffer (20% D_2O with 0.075 M Na_2HPO_4, 6 mM NaN_3, 4,6 mM trimethylsilylpropanoic acid, pH 7.4) in Eppendorf tubes. Mixed serum and buffer were transferred to 3-mm NMR tubes by syringe for NMR lipidomic analyses. The analyses were performed on Bruker Avance III UltraShield Plus 600 MHz spectrometer (Bruker BioSpin) equipped with a 5mm broad band inverse (BBI) probe. Further procedures were fully automated using a SampleJet with Icon-NMR on Topspin 3.6.2 (Bruker BioSpin). 1D 1H Nuclear Overhauser effect spectroscopy (NOESY) and Carr–Purcell–Meiboom–Gill (CPMG) spectra with water presaturation were obtained at 310 K using acquisition and processing parameters similar to Dona et al. [64].

An automated Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISA^{TM}) was used to predict the lipoprotein profile [65]. The regression models estimated the concentrations of cholesterol, free cholesterol, phospholipids, and ApoA1, apolipoprotein-A2 (ApoA2) and Apo-B in total serum, as well as the concentration of these components in each of the lipoproteins (VLDL, IDL, LDL and HDL). Each lipoprotein was further divided into subfractions according to their density; VLDL into VLDL-1-5, LDL into LDL-1-6, and HDL into HDL-1-4, with increasing density (Appendix I). The concentrations of triglycerides, cholesterol, free cholesterol, phospholipids, Apo-A1, Apo-A2 and Apo-B was extracted for each lipoprotein subfraction. Particle numbers for serum, VLDL, IDL, LDL and LDL-1-6 were also extracted. In total, the NMR lipidomic analysis yield a dataset of 112 variables for each sample. Serum samples used for NMR lipidomics were transferred to tubes frozen at -80°C until they were reused for ELISA.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The candidate protein biomarkers, MMP-9 and ALDH4A1, were selected based on current literature, proposing them as promising and relatively unexplored biomarkers of subclinical atherosclerosis. Quantitative determination of serum MMP-9 and ALDH4A1 was performed to compare cases and controls. MMP-9 and adiponectin were used to investigate if samples used for NMR lipidomics could be reused for protein quantification. Adiponectin was selected in addition to MMP-9, as this was a well-established protein for ELISA analysis at our laboratory.

MMP-9 concentration was measured in 50 case-control pairs, in addition to 29 fresh and 29 diluted (1:1) NMR-samples by use of Quantikine® ELISA Human MMP-9 Immunoassay kit (R&D Systems, Minneapolis, USA). ALDH4A1 concentration was measured in 50 case-control pairs by use of Human ALDH4A1 (Delta-1- pyrroline-5-carboxylate dehydrogenase, mitochondrial) ELISA Kit (Nordic Biosite, Täby, Sweden). 20 fresh and 20 diluted (1:1) NMR-samples were used to detect adiponectin concentration by use of Tecan Adiponectin ELISA kit (IBL International GmbH, Hamburg, Germany). All kits were performed according to the manufacturer's protocol, and all samples were analysed in duplicates. A DS2 Two-plate Automated ELISA Processing System (Dynex Technologies, Chantilly, USA) was used for the fully automated immunoassays.

2.7 Statistical analyses

Statistical analyses were performed using SPSS Statistics version 27.0 (IBM SPSS, New York, USA) and MATLAB R2017a with PLS_Toolbox 8.2.1 (Eigenvector Research, Inc.) The Shapiro Wilk test of normality was conducted to check for normally distributed data. NMR lipidomic results indicated that the variables were both normally and not-normally distributed. Log transformation of the data was performed. Application of the Shapiro Wilk test showed that several variables were still not normally distributed after log transformation. Therefore, we continued with the original dataset and applied non-parametric tests. The Mann Whitney U test was used to compare clinical characteristics, lipoprotein subfractions and serum proteins between cases and controls. A significance level of $p < 0.05$ was considered statistically significant. Bonferroni correction was not performed due to the strong correlation and lack of independence between the quantified lipoprotein subfractions. Significance levels of 0.05-0.1 were discussed as trends. Continuous data are presented as mean ± standard deviation (SD) and categorical data as counts with percentages.

Analyses performed in MATLAB were auto scaled. Principal Component Analyses (PCA) plot were performed to find possible outliers in the dataset. Partial Least Squares (PLS) regression and Partial Least Squared Discriminant Analysis (PLSDA) were applied to compare differences in lipoprotein profile in cases and controls and in men and women.

2.8. Ethical considerations

The research project was approved by the Regional Committee for Medical Research Ethics (REK, 138187), HUNT (2020/18421) and by the Institute for Circulation and Medical Imaging. Data Protection Impact Assessment (DPIA) was performed and approved by the Institute for Circulation and Medical Imaging, REK, HUNT and the local myocardial infarction registry (Helse Nord-Trøndelag). All procedures were performed in line with the Declaration of Helsinki and Good Clinical Practice (GCP). All patient information was stored and handled with high levels of security according to laws and regulation.

3. Results

3.1 Characteristics of the study population

Descriptive characteristics of the study population (n=150) are presented in Table 1. All participants were apparently healthy at the time of HUNT3 (baseline), having low risk of CVD. The cases suffered from a MI within 5 years after baseline, while the controls remained healthy throughout the next 10 years (HUNT4). Cases and controls were mainly sex- and age-matched, but other CVD risk factors such as BMI and total cholesterol were also considered. Statistical analyses detected no significant differences in CVD risk factors ($p > 0.05$) between cases and controls included in Table 1, except current/previous use of medicine for hypertension ($p = 0.011$). This reduces confounding effects of CVD risk factors on the data analyses.

Mean values are presented for each variable, in addition to standard deviation. Mean blood pressure, HDL-C and triglycerides within the groups were considered within the healthy range. Mean total cholesterol levels were slightly increased compared to optimal levels, but not classified as hypercholesterolemia. Both groups of cases and controls had a BMI that is regarded as overweight (BMI > 25), 27.1 and 27, respectively.

Table 1: Baseline characteristics of the study participants.

	Cases (n=50)	**Controls (n=100)**
Sex (♀/♂)	20/30	40/60
Age (years)	56 ± 6	56 ± 5
BMI (kg/m²)	27.1 ± 3.8	27.0 ± 3.5
SBP (mmHg)	126 ± 14	126 ± 13
DBP (mmHg)	75 ± 10	74 ± 9
Total cholesterol (mmol/L)	6.1 ± 0.9	5.9 ± 0.7
HDL C (mmol/L)	1.3 ± 0.4	1.4 ± 0.3
Triglycerides (mmol/L)	1.6 ± 0.7	1.6 ± 0.8
Not-fasting glucose (mmol/L)	5.8 ± 1.1	5.5 ± 0.9
Fasting time (h)	3 ± 2	2 ± 2
Current/previous medicine for hypertension (yes)	7 (16%)	3 (3%)
Smoker (yes)	5 (10%)	5 (5%)
First grade relative suffering from MI before the age of 60 years (yes)	12 (32%)	14 (16%)

Data is shown as mean ± SD or as number (%) for categorical variables. SD: Standard deviation, BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HDL-C: High-density lipoprotein cholesterol.

3.2 Lipoprotein subfractions

The NMR lipidomic analysis of serum quantified 112 different lipoprotein subfraction for each participant. An overview including a description of the 112 lipoprotein subfractions is illustrated in Appendix II. Figure 7 illustrates the classification of lipoproteins conducted from NMR lipidomics. The subfractions of lipoprotein are differentiated based on size and density. HDL particles for example, are divided into four subfractions; HDL-1, HDL-2, HDL-3 and HDL-4, where HDL-1 is largest, and HDL-4 is small and dense.

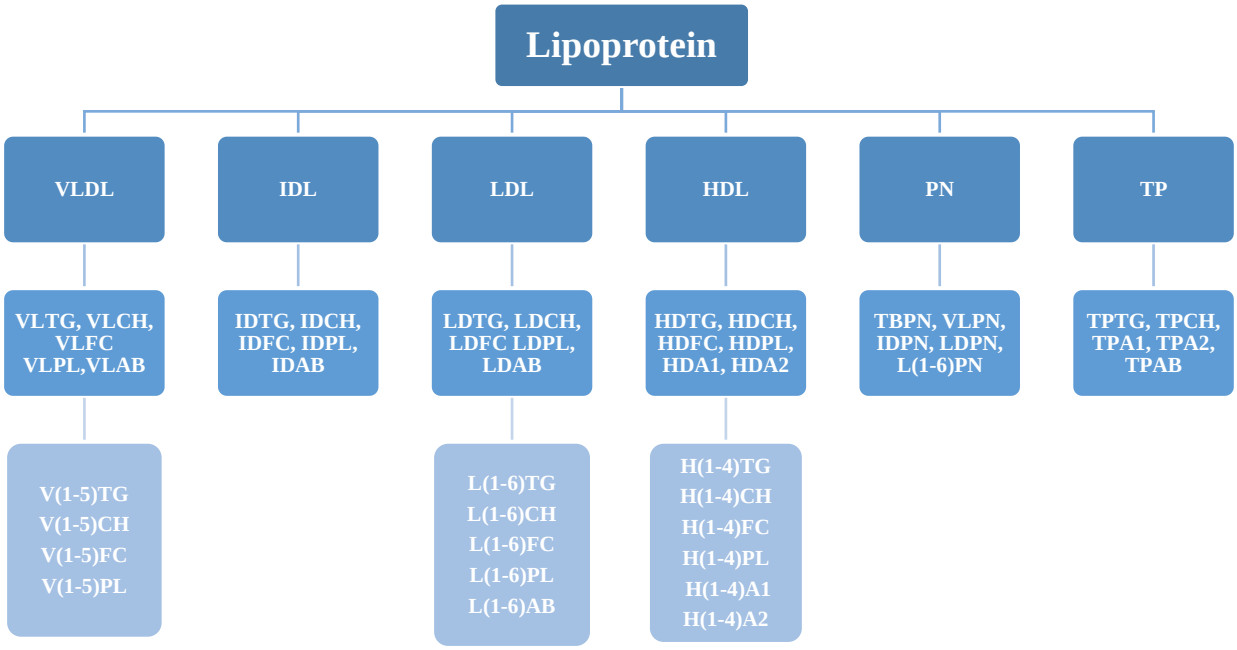

Figure 7: *Overview of lipoproteins and lipoprotein subfractions, quantified by NMR lipidomics.* PN: Particle number, TP: Total plasma, VLDL: Very low-density lipoprotein, IDL: Intermediate-density lipoprotein, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, TG: Triglycerides, CH: Cholesterol, FC: Free cholesterol, PL: Phospholipids, AB: Apolipoprotein B100, A1: Apolipoprotein A1, A2: Apolipoprotein A2.

3.2.1 Quality control of NMR lipidomic results

Standard laboratory measurements of serum HDL-C and triglycerides in HUNT were compared to the same variables when quantified by NMR lipidomic analysis. Correlation plots for HDL-C and triglycerides indicated that the concentrations quantified by NMR were consistent with the concentrations obtained from standard laboratory measurements (Appendix III). The plots have R-values of 0.91 and 0.98, respectively. The PCA-plot in Appendix IV illustrates that no multivariate outliers were detected in the dataset.

3.2.2 Lipoprotein subfractions in cases and controls

The cases (n=50) in the present study were registered with a MI within 5 years after baseline. To detect lipoprotein subfractions differentiating between these two groups, Mann Whitney U test was performed. The test showed that, among all lipoprotein subfractions measured, only serum levels of H4A1 differed between the groups ($p < 0.05$). This lipoprotein subfraction reflects the concentration of ApoA1 in HDL-4, which is the smallest and most dense HDL fraction. In our study, the cases had higher serum levels of H4A1 compared to controls (Figure 8). Of particular interest, there seemed to be an opposite direction in cases and controls for H4A1 levels as compared to ApoA1 in the other subfractions. For HDL-1, HDL-2 and HDL-3, and in total HDL (HDA1) the concentration of ApoA1 was lower in cases, although not significant. According to the figure, the concentration of ApoA1 was considerably higher in HDL-4 particles compared to the other subfractions. H4A1 levels therefore seemed to contribute substantially to total HDA1 levels. Density of the four HDL subfractions is shown in Table I, Appendix I.

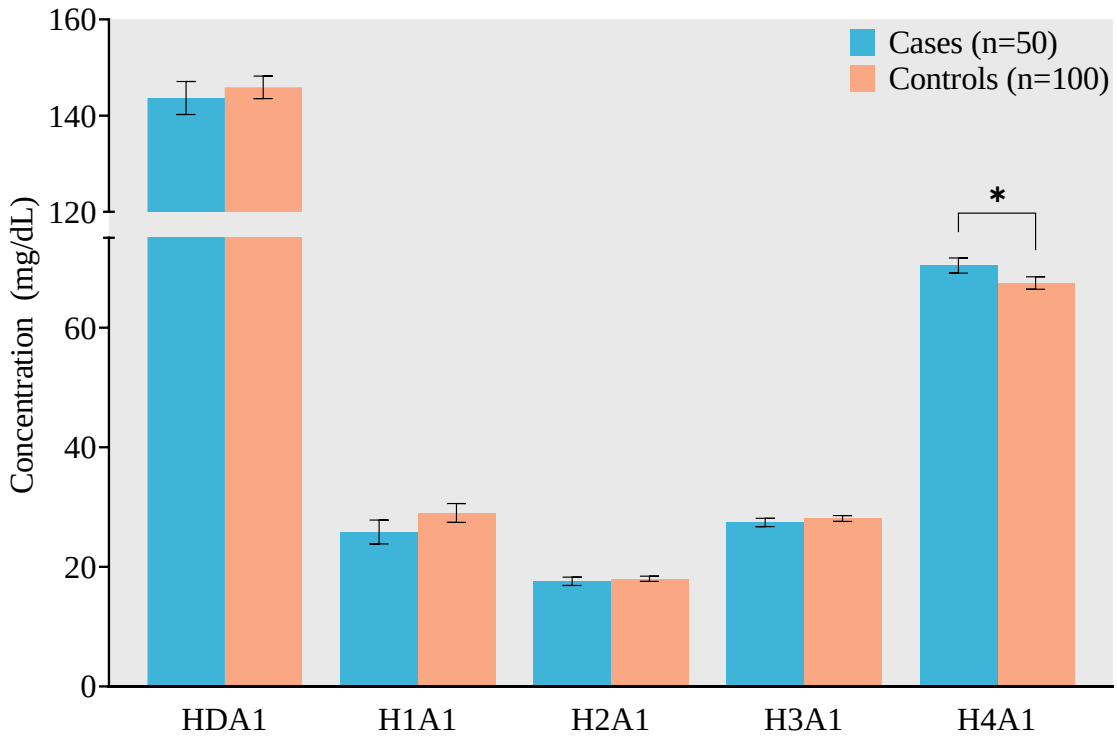

Figure 8*: Concentration of ApoA1 in total serum HDL and HDL subfractions in cases experiencing a MI within 5 years and matched controls*. HDA1 reflects the concentration of ApoA1 in total HDL, while H1-H4 reflect the concentration in the four subfractions of HDL. Group differences are calculated using Mann Whitney U test. Data are shown as mean and SEM, and the number of participants is displayed in the figure. ApoA1/A1: Apolipoprotein A1, HDL: High-density lipoprotein, MI: Myocardial infarction SEM: Standard error of mean. *$p < 0.05$.

3.2.3 Subgroup analysis: Men

PLS-DA plot B in Appendix V indicated that the lipidomics profile was significantly different ($p < 0.001$) between men and women. This verified the importance of performing gender-stratified analysis. Subgroup analysis of women (n=60) indicated that there were no significant differences in any of the lipoprotein subfractions between cases and controls. However, analysis of men (n=90) showed interesting results within the HDL subfractions. This supported the results from the PLS-DA plot, as Mann Whitney U test showed different results for men and women.

A total of five variables were significantly different ($p < 0.05$) between cases and healthy controls in men, all involving HDL subfractions. In addition, potential differences between cases and controls were found for an additional eight HDL subfractions. However, these findings were not statistically significant, but regarded as trends ($p < 0.1$). All HDL subfractions in cases and controls are displayed in Figure 9, showing mean concentration of the different lipid components in HDL. The lipid components are ApoA2, ApoA1, phospholipids, free cholesterol, cholesterol and triglycerides, respectively. H1TG, H1A2, H2PL and H2A2 reflects the concentration of triglycerides and Apo-A2 in large HDL particles, HDL-1 and HDL-2, respectively. The serum levels of all these subfractions characterized as large, were significantly lower in cases ($p < 0.05$). Subfractions involving small and dense HDL (HDL-4) deviates from the other HDL subfractions, as all of them had higher mean concentration in cases than controls. H4A1 was significantly higher in cases ($p < 0.05$), while H4A2 and H4CH represented trends ($p < 0.1$). For HDL-C, the same opposite association between cholesterol concentration in HDL subfractions (HDL-2 and HDL-4) and future MI (trend, $p < 0.1$) was detected (Figure 9). Small and large HDL particles were thus identified to have adverse association with future MI.

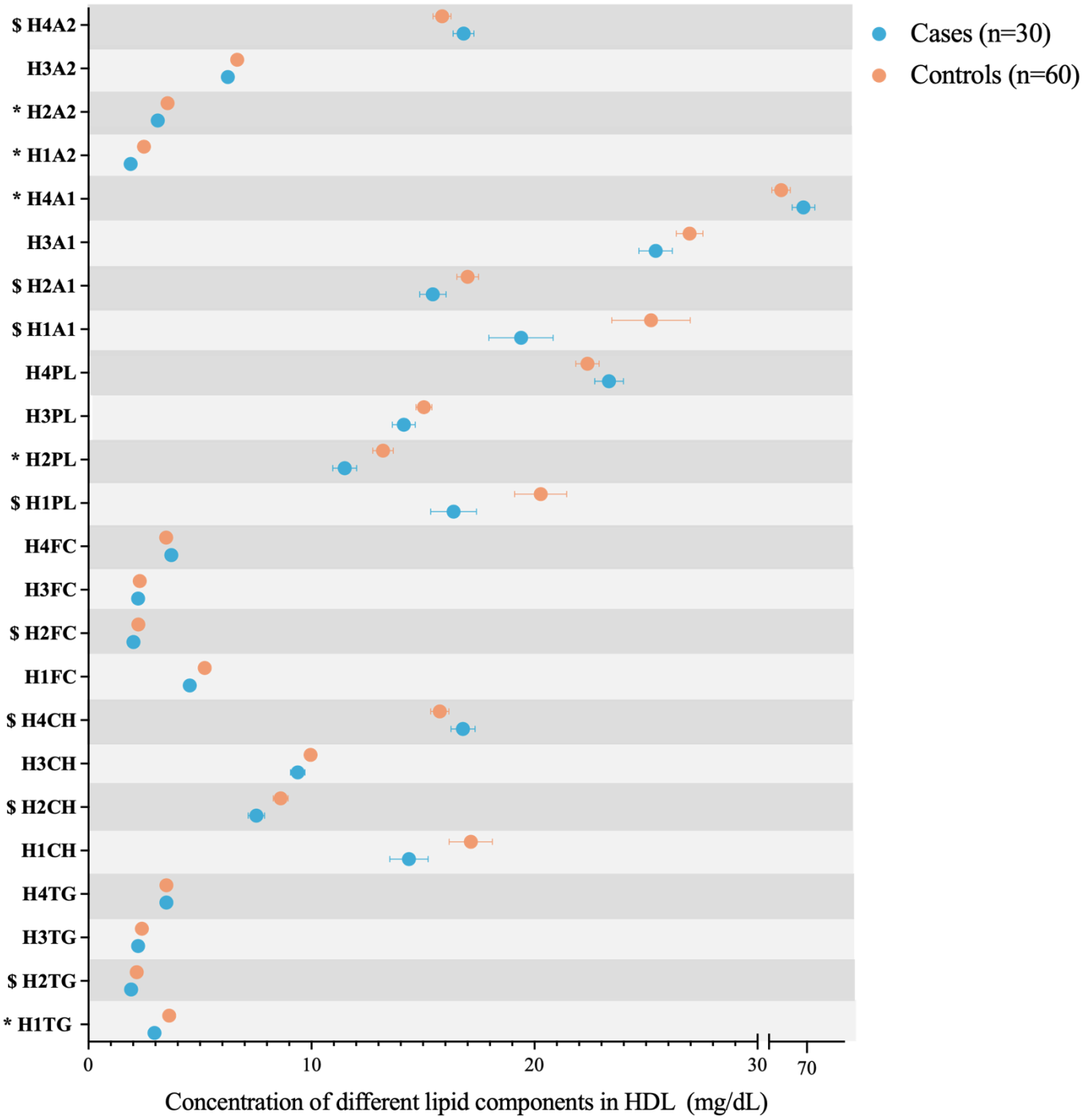

Figure 9. *Subgroup analysis of men: The concentration of different lipid components (Apolipoprotein A2, Apolipoprotein A1, phospholipid, free cholesterol, cholesterol, and triglycerides) in HDL subfractions (HDL-1 to HDL-4) in cases experiencing a MI within 5 years and matched controls*. Group differences are calculated using Mann Whitney U test. Data are shown as mean and SEM. The number of participants is displayed in the figure. HDL: High-density lipoprotein, A2: Apolipoprotein A2, A1: Apolipoprotein A1, PL: Phospholipid, FC: Free cholesterol, CH: Cholesterol, TG: Triglyceride, MI: Myocardial infarction, SEM: Standard error of mean. *$p < 0.05$. \$$p < 0.1$.

3.2.4 Subgroup analysis: 2-year observation period

All cases had a low NORRISK 2 at baseline, but still they experienced a MI within a relatively short time-period. We were particularly interested in those participants experiencing a MI within the first 2 years after baseline, as these were assumed to have a more pronounced risk profile. Therefore, a subgroup analysis was performed on the 19 participants that experienced a MI within 2 years after baseline and their matched controls, yielding with a total of 57 subjects. The results showed that one set of lipoprotein subfractions were prominent, the concentration of triglycerides in LDL (LDL-TG). There was a significantly ($p < 0.05$) higher concentration of LDL-TG in cases compared to controls (Figure 10). Additionally, the results did not discriminate between large- and small LDL subfractions. LDL-TG levels were also higher in cases than controls for the whole study population (5-year observation period), although not significantly ($p = 0,11$). Measurements of circulating triglyceride in the clinic includes the total concentration of triglyceride in VLDL, LDL, IDL and HDL. Figure 10 illustrate that neither triglyceride levels in VLDL, IDL, HDL or in total serum differed between cases and controls in this subgroup analysis.

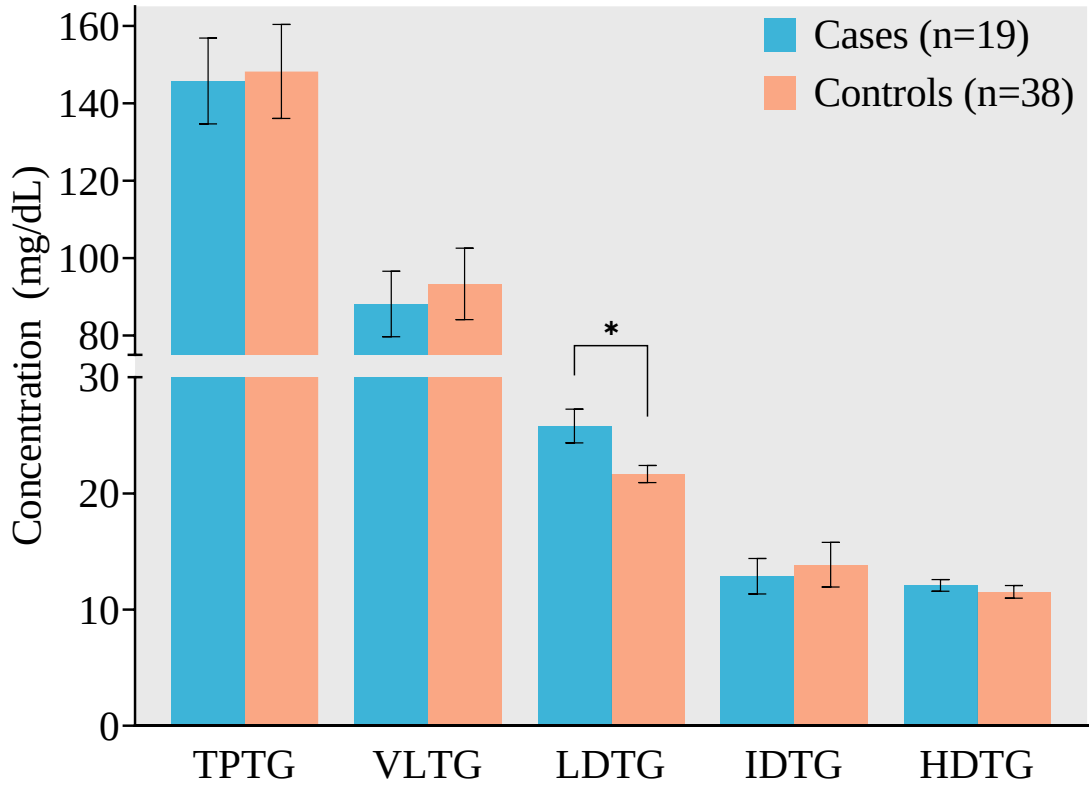

Figure 10: *Triglyceride concentration in total serum, VLDL, LDL, IDL and HDL in cases experiencing a MI within 2 years and matched controls.* Group differences were calculated using Mann Whitney U test. Data are shown as mean and SEM. The number of participants is displayed in the figure. MI: Myocardial infarction, TPTG: Total plasma triglyceride, VLTG: Triglycerides in very low-density lipoprotein, LDTG: Triglycerides in low-density lipoprotein, IDTG: Triglyceride in intermediate density lipoprotein, HDTG: Triglyceride in high-density lipoprotein, SEM: Standard error of mean. *$p < 0.05$.

3.2.5 Summary of findings from NMR lipidomics

The findings from NMR lipidomics analyses are summarized graphically in Figure 11.

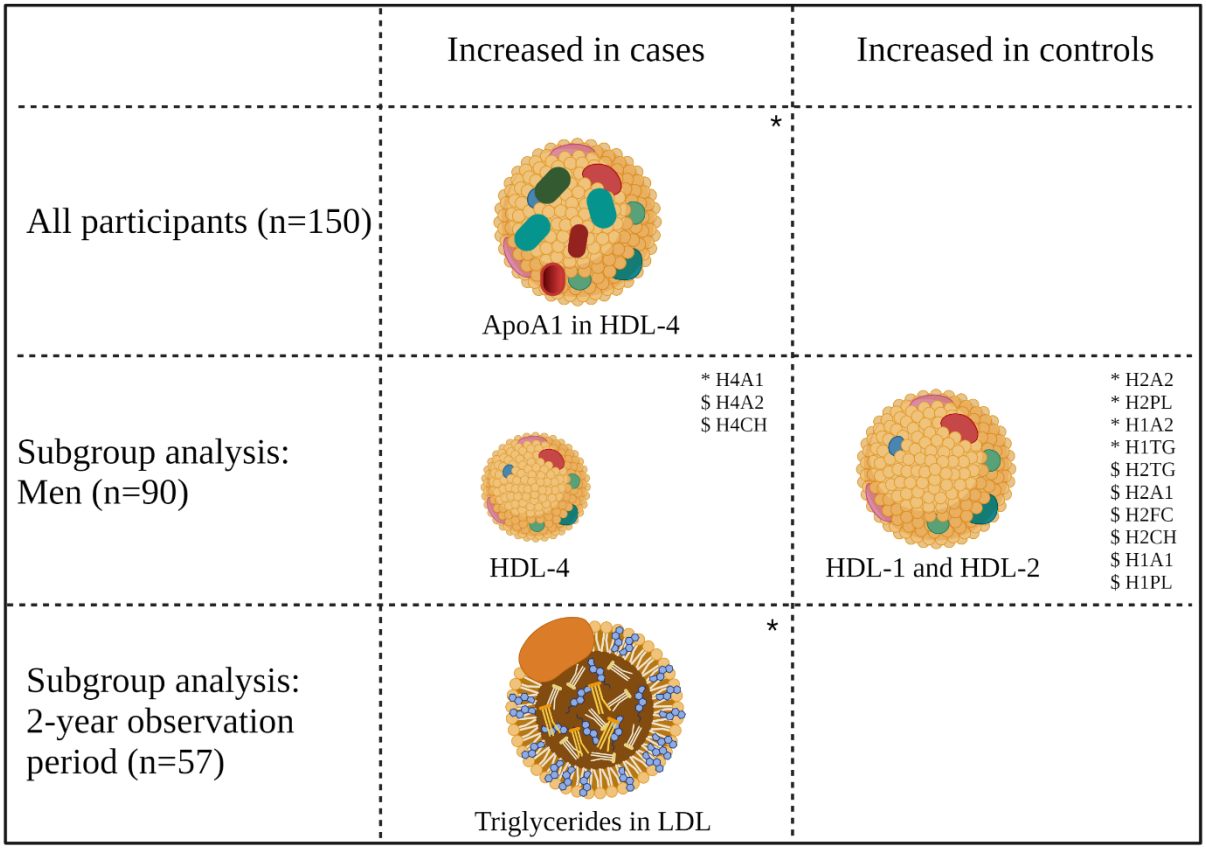

Figure 11. *Summary of the most important findings from the NMR lipidomics analysis.* The figure illustrates that concentration of ApoA1 in HDL-4 was higher in cases experiencing a MI than controls when investigating all participants 5 years after baseline. Subgroup analysis of men showed a trend where concentration of different components in small and dense HDL (HDL-4) where increased in cases, while concentration of different components in larger HDL (HDL-1 and HDL-2) where increased in controls. Analysis of the subgroup with a 2-year observation period showed an increased concentration of triglycerides in LDL. The number of participants in each analysis is displayed in the figure. NMR: Nuclear magnetic resonance, ApoA1: Apolipoprotein A1, HDL: High-density lipoprotein, LDL: Low-density lipoprotein. *p < 0.05. $p < 0.1. The figure is created with BioRender.com.

3.3 Potential protein biomarkers

3.3.1 MMP-9

Concentration of MMP-9 was measured by ELISA, whereas 50 case-control pairs were compared. Mann Whitney U test of the non-normally distributed data indicated that there were no significant differences between cases and controls (Table 2). Analyses in subgroups based on gender and MI within 2 years after HUNT3 indicated no significant difference in serum MMP-9 concentration between cases and control.

3.3.2 ALDH4A1

ALDH4A1 concentration was measured in 50 case-control pairs by ELISA. Mann Whitney U test of the non-normally distributed data indicated that there were no significant differences between cases and controls (Table 2). Analyses in subgroups based on gender and MI within 2 years after HUNT3 indicated no significant difference in serum ALDH4A1 concentration between cases and control.

Table 2: Serum concentration (ng/mL) of MMP-9 and ALDH4A1 in cases and controls. Group differences are calculated using Mann Whitney U test and p-values for the differences are shown.

	Cases (n=50)	**Controls (n=50)**	**p-value**
MMP-9	490.18 ± 238.18	495.70 ± 198.77	0.95
ALDH4A1	5.59 ± 3.33	5.44 ± 3.37	0.95

Data is shown as mean ± SD. MMP-9: Matrix metalloproteinase-9. ALDH4A1: Aldehyde dehydrogenase 4 family member A1.

3.4 Reuse of NMR serum samples

Serum from a total of 49 subjects were used to determine whether samples used for NMR lipidomics could be reused for protein quantification by ELISA. Protein concentration in serum was therefore quantified and compared in fresh serum samples and in samples already used for NMR lipidomics. MMP-9 concentration was measured in samples from 30 subjects and adiponectin concentration in samples from 19 subjects. Figure 12A illustrate the concentration of MMP-9 in both fresh and used samples, while Figure 12B illustrate adiponectin concentration. Each participant was indicated with a number on the x-axis, ranging from highest to lowest concentration with increasing number. The Pearson correlation coefficient revealed a significant ($p < 0.01$) positive correlation between fresh and used samples. Correlation plots are illustrated for both proteins in Appendix VI. For MMP-9, there was a tendency towards slightly lower concentrations of MMP-9 in fresh serum samples at high concentrations of MMP-9. In contrast, a potential trend toward slightly lower concentrations of adiponectin were found in reused serum samples at high concentrations of adiponectin. Both these findings may potentially be associated with technical challenges associated with the upper range of the standard curve used in the ELISAs.

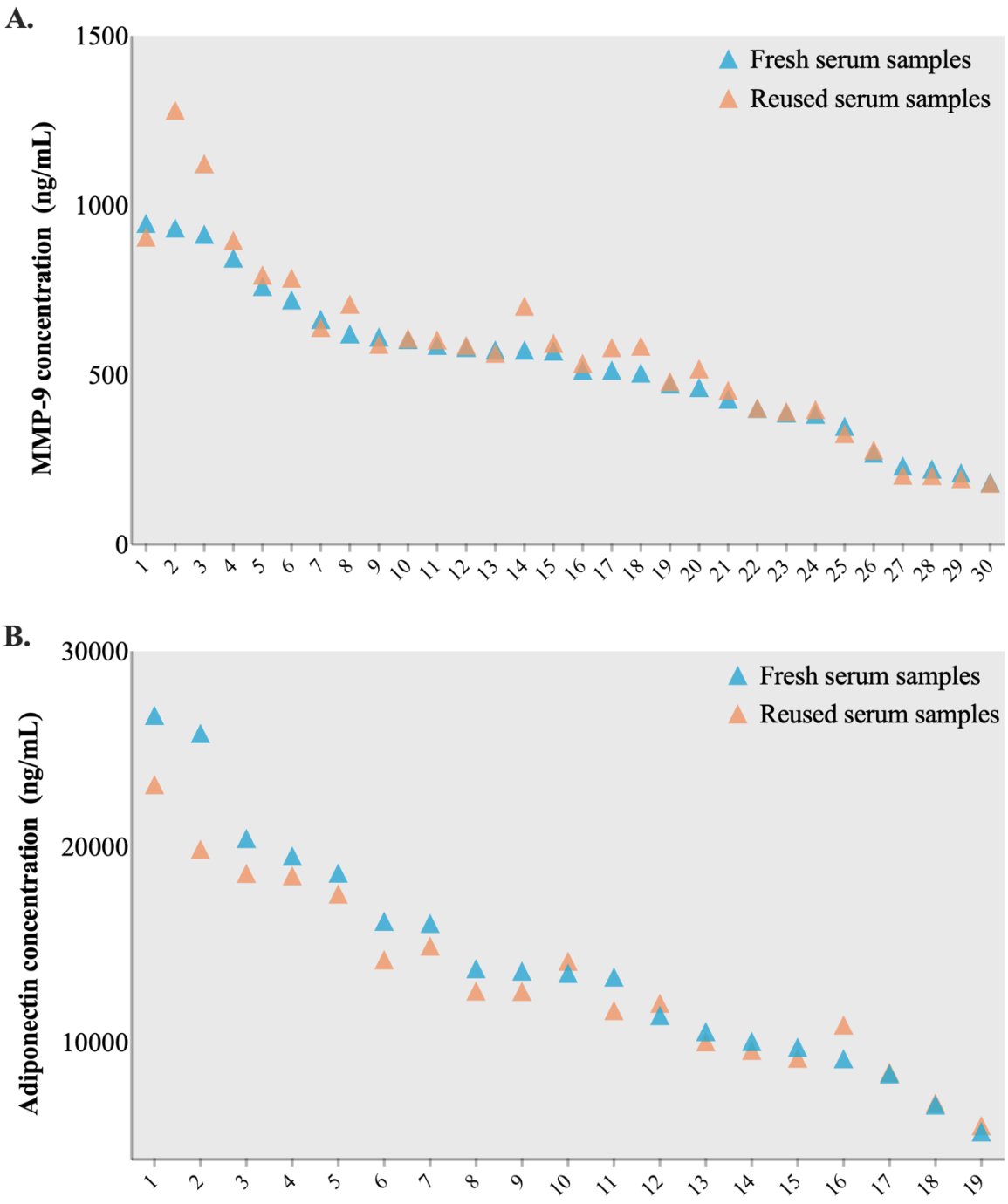

Figure 12: *Comparison of protein concentration measured by ELISA in fresh and reused serum samples. Reused samples have been used for NMR analysis*. **A.** MMP-9 concentration in fresh and reused serum, samples from 30 subjects were compared. **B.** Adiponectin concentration in fresh and reused serum, samples from 19 subjects were compared. NMR: Nuclear magnetic resonance, ELISA: Enzyme-linked immunosorbent assay, MMP-9: Matrix metalloproteinase-9.

4. Discussion

This retrospective observational study investigated potential serum biomarkers for sub-clinical atherosclerosis. The selected cases were apparently healthy at baseline. Despite their low cardiovascular risk, they experienced a MI within 5 years. Current risk prediction models failed to characterize them as high-risk individuals. A short follow-up period, only 5 years, differentiates this study from other similar studies. The gradual build-up of atherosclerotic plaque, leading to a MI, takes many years to develop. Participants experiencing a cardiac event only 5 years after baseline were thus expected to have advanced atherosclerosis, resulting in a more pronounced risk profile.

One lipoprotein subfraction; ApoA1 in HDL-4, was found to differ in concentration between cases and controls ($p < 0.05$) when investigating all participants 5 years after baseline. Interestingly, the concentration of this subfraction was higher in cases compared to controls, despite the presumed cardioprotective role of ApoA1 and HDL. In men, the serum concentration of HDL subfractions seemed to be related to differences in risk profiles. The small HDL-4 subfractions showed opposite trends between cases and controls compared to the larger HDL subfractions (HDL-1 and HDL-2). For the subgroup analysis with a 2-year observation period, the concentration of LDL-TG was significantly higher in cases, although total serum triglycerides levels were equal. Figure 11 summarize these results, and the potential function of these lipoprotein subfractions related to future MI will be further discussed in chapter 4.1. Concentration of MMP-9 and ALDH4A1 did not differentiate between cases and controls in this study, thereby not supporting their potential as biomarkers for subclinical atherosclerosis (chapter 4.2). Data from this study indicated that serum samples used for NMR lipidomics can be reused for protein quantification by ELISA, these promising results will be further discussed in chapter 4.3.

4.1 Lipoprotein subfractions

When comparing cases and controls at baseline, there were no differences in lipid risk markers used to evaluate CVD risk in the clinic today. Hence, circulating levels of total cholesterol, HDL-C and triglycerides did not differ between cases and controls (Table 1). The present study illustrated the complex composition of circulating lipoproteins in serum, assessed by NMR lipidomics. NMR lipidomics allows for a more detailed overview of lipoproteins, which may identify differences in risk profile that are overseen by traditional methods [66, 67].

4.1.1 ApoA1 in HDL-4: 5-year observation period

ApoA1, the main apolipoprotein component of HDL, has several cardioprotective functions [20]. Surprisingly, NMR lipidomic analysis showed that the concentration of ApoA1 in HDL-4 was significantly higher in cases compared to controls. This contradict the overall assumption that HDL and ApoA1 are atheroprotective, indicating that high ApoA1 concentration is not necessarily an advantage for cardiovascular health. ApoA1 has an important role in RCT [32]. Being the main apoprotein component of HDL, ApoA1 is involved in stimulating the ATP-binding cassette transported, ABCA1. ABCA1 mediates efflux of cholesterol and phospholipids from cells. Together ApoA1 and ABCA1 generate HDL particles [81, 83]. In addition, ApoA1 has anti-oxidative properties [20]. High concentration of ApoA1 is therefore associated with lower CVD risk. Quantification of circulating ApoA1 can be performed in the clinical setting but is currently not a part of standard CVD risk evaluation [39]. In this present study, we expected that ApoA1 concentration would be higher in controls, or that no significant difference was found. No significant difference was observed for total ApoA1 in HDL. However, the sdHDL-4 subfraction revealed a positive association with future MI. According to these results, it might seem like ApoA1 in HDL-4 has a potential proatherogenic effect that is concealed when measuring total ApoA1. However, it is relevant to mention that concentration of ApoA1 in HDL-4 contributes substantially to the total ApoA1 concentration. According to Figure 8, almost half of the ApoA1 concentration in total HDL is carried in HDL-4 particles. To summarize, these results showed that more comprehensive analysis of HDL has potential to reveal information regarding ApoA1s contribution to MI risk. In specific, the results indicated that ApoA1 in HDL-4 might be a potential biomarker of subclinical atherosclerosis. However, this should be verified by larger studies.

4.1.2 Gender-specific differences

Results from the multivariate analysis PLSDA showed a significant difference in lipidomics profile for men and women (Appendix V, Figure III B). HDL-C threshold levels used in NORRISK 2 are different for men and women [39], and it was therefore hypothesized to detect differences in lipoprotein subfractions as well. Analyses stratified by gender revealed several differences in lipidomics profile between cases and controls in men. No significant differences between cases and controls were detected in women. Freedman et al. detected the same trend, suggesting that women have a less atherogenic lipoprotein profile than men [84]. Women have lower cardiovascular risk than men in general, usually explained by genetic and hormonal differences. One possible mechanism that explains the difference is the involvement of estrogen in liver lipid metabolism and levels of serum lipoprotein in women [85]. The data presented in this study are in line with previous studies detecting gender-specific differences in lipidomic risk profile [84, 86]. These results strengthen the possibility that lipoprotein subfractions can contribute to generating more gender-specific cardiovascular risk prediction.

4.1.3 HDL subfractions: Men

Interestingly, there seems to be an additional potential for risk prediction in men by exploring the subfractions of HDL. Within the HDL subfractions, five subfractions were significantly different ($p < 0.05$) between the cases and controls. In addition, there were trends towards differences in eight other HDL subfractions ($p < 0.1$). Trends not reaching the statistical cut-off value of 0.05 should be interpreted with caution. To our knowledge, the concentration of free cholesterol, phospholipid, or ApoA2 in lipoproteins have not been investigated in association with CVD risk. Previous studies have debated whether ApoA2 is an anti- or pro-atherogenic protein, emphasizing that its role in lipoprotein metabolism is still uncertain [87]. Phospholipids and free cholesterol are believed to influence the anti-oxidative properties of HDL, but the mechanism is not known [20]. One previous study that investigated triglyceride concentration in HDL related to MI risk by NMR lipidomics, concluded that triglycerides in all HDL subfractions were positively associated with MI risk [71]. This deviated from our results, as a possible negative association between triglycerides in large HDL (HDL-1 and HDL-2) and future MI was detected. The potential role of these components in HDL regarding atherosclerosis is still unknown. For that reason, their concentration in specific HDL subfractions did not receive much attention in this study. Instead, the focus was to investigate the adverse trends for small and large HDL subfractions in relation to future MI.

According to our results, small and large HDL particles possess adverse associations with risk of MI in men; high levels of sdHDL and low levels of large HDL are associated with future MI. These results are consistent with previous studies [57, 88]. The positive and negative associations of small and large HDL particles will counteract each other when investigating HDL by traditional methods. Interestingly, there is a possibility that potential proatherogenic sdHDL particles mask the protective role of larger HDL particles, and vice versa. Hence, it becomes apparent that interpretation of HDL alone may not reflect the true role of the lipoprotein. Our results are in line with previous statements suggesting that HDL size affects function and could be clinically valuable for cardiovascular risk prediction [29, 89]. However, there are conflicting data on whether small or large HDL are positively associated with CAD [77, 81]. A drawback in this current study is that data for HDL particle number was not available in the NMR analysis. It would have been interesting to investigate if the same trends were observed when comparing the particle number of each size, as particle number have received much attention the last years [31, 90].

The exact role of HDL subfractions in relation to cardiovascular risk is still debated. For HDL subfractions in men, concentration of sdHDL fractions was positively associated with future MI. Three of these HDL-4 subfractions were categorized as different between cases and controls based on significance level; ApoA1, ApoA2 and cholesterol. As previously discussed for ApoA1 in HDL-4, a positive association between HDL-4 level and MI challenges the perception of HDL being atheroprotective**.** A previous study investigating HDL subfractions in CAD patients identified the same trend [79]. There was a significant positive association between concentration of sdHDL subfraction and presence of CAD. Other previous reports are in line with these results, proposing that sdHDL particles possess proatherogenic properties [91]. However, other studies have indicated that small HDL are not associated with CAD [71, 81].

Consistent with previous studies [79, 81, 89, 92], larger HDL subfractions were negatively associated with cardiovascular risk. In total, ten large HDL subfractions, belonging to HDL-1 and HDL-2, where characterized as significant or trends. These subfractions had lower concentration in cases compared to controls. This might indicate two things, 1) larger particles are mainly responsible for the cardioprotective effects of HDL, 2) low concentration of large HDL particles is related to future MI. Supporting these results, a previous study suggested that the cardioprotective functions of HDL are performed by large HDL particles [90]. Current risk prediction in Norway only encounters low levels of HDL-C in the prediction model. Reason being that high levels of HDL-C are not necessarily protective, whereas low levels are

associated with increased risk [39]. The overall lower concentration of large HDL subfractions observed in MI cases in our study might indicate that levels of large subfractions are related to future MI. Further research is necessary to confirm the adverse association between small and large HDL, and future MI. Thus, the focus should be to identifying the exact relation between HDL size and MI risk.

In the clinical setting, the HDL fraction used for CVD risk prediction is HDL-C. However, the association between HDL-C and cardiovascular risk is still controversial [31, 78]. In this present study, no differences were found in HDL-C between cases and controls at baseline. HDLs are highly heterogenous particles. This heterogeneity arises partly during RCT, as there is a constantly remodeling of lipoproteins [29] Clinical measurement of HDL-C does not reflect this heterogeneity in composition, size and biological function. This present study indicated that increased cholesterol levels in the relatively large HDL-2 particle might be associated with a lower risk of MI (trend, $p < 0.1$). The borderline significance is probably due to the small study population, as a similar NMR study identified an inverse association of cholesterol concentration in medium and large HDL particles with risk of MI [71]. The previously suggested protective role of large HDL is consistent with several other studies focusing on cholesterol concentration. It is proposed that high levels of large HDL-C are more protective than high levels of sdHDL-C. [78, 80]. In this present study, sdHDL-4 cholesterol, was positively associated with MI, which was in line with the results from Xu et al. [79]. Sacks et al. 2018 propose the hypothesis that mainly medium and large sized HDL are responsible for RCT as cholesterol enters and exits without changing size [81]. If their hypothesis is correct, the small and dense HDL particles are potentially not as atheroprotective as the larger HDL particles. To verify this, the exact mechanism for RCT remains to be fully elucidated. However, HDL is not just a carrier of cholesterol and RCT is not the only atheroprotective function of HDL. The results presented for cholesterol concentration within the HDL subfractions in men, supports previous statements that measurement of cholesterol in HDL differentiated by size may be more valuable than the traditional HDL-C [78, 80].

In total, the data presented in our study support the potential for HDL subfractions to reveal the functionality of HDL, suggested by previous studies [29, 32]. Especially for men, it appears relevant to further explore the HDL subfractions for improving MI risk prediction. The traditional measurement of HDL-C does not seem to fully capture the properties of HDL related to cardiovascular risk. Neither does it encounter the potential effect by HDL subfractions

differed by size and density. According to our results, HDL subfractions serves a great potential for possible risk markers in men, and their exact role should be further investigated.

4.1.2 Triglycerides in LDL: 2-year observation period

Apparently healthy cases experiencing a MI within 2 years after baseline is a small, but interesting group that we believed would display a more prominent risk profile. A subgroup analysis was therefore performed, revealing a significant positive association between LDL-TG and MI. The association did not discriminate between large and small LDL subfractions. A significantly higher concentration in cases is observed in both large and small LDL-subfractions, which is also seen in previous studies [93]. These results suggested that LDL-TG might be a potential biomarker of imminent MI. Interestingly, analysis of the whole study population also showed that the mean concentration of LDL-TG was higher in cases compared to controls, although not significant ($p = 0.11$).

The identified association between LDL-TG and future MI indicated that LDL-TG might be a better risk marker than total triglyceride levels. Current clinical measurements show total triglyceride concentration in lipoproteins, including triglyceride in LDL, VLDL, IDL and HDL. Serum triglycerides are positively associated with atherosclerotic CVD [28, 94], and recent research have also stated that triglycerides represent a causal risk factor for CVD [95, 96]. It is widely accepted that cholesterol concentration varies within the different groups of lipoproteins, reflecting different association with cardiovascular risk [20]. The groups of cases and controls in the present study did not differ on total triglyceride concentration, but interestingly, they differed in LDL-TG. Hence, LDL-TG quantification might provide better risk prediction compared to total triglyceride. These results highlight the relevance of measuring triglyceride concentration within different lipoprotein classes. Supporting these results, another study also identified diverse associations with CAD in different lipoprotein subfractions [93]. However, Holmes et al. identified a strong positive association between triglyceride concentrations in all four lipoprotein subclasses and the risk of MI [71]. On the other hand, Albers et al. did not detect any association between LDL-TG and CVD [97].

Previous research on LDL related to CVD risk have primarily focused on cholesterol levels. The results presented in this current study suggested that triglyceride levels might add additional value. LDL particles are key components for the development of atherosclerosis, and elevated LDL-C has for a long time been an established biomarker [76]. Other components of

LDL particles have received less attention, but a few studies have investigated the relation between LDL-TG and cardiovascular risk. März et al. compared LDL-TG levels in cases with stable angiographic CAD with control subjects. The results suggested that levels of LDL-TG (including IDL-TG) were associated with stable CAD independently of LDL-C, implying that LDL-TG could add additional value for prediction of CAD [93]. Additionally, in line with the current study, a more recent study published by the same research group verified the positive association between LDL-TG levels and cardiovascular mortality. By adding LDL-TG to a risk prediction model, the prediction of cardiovascular mortality was improved [98]. Other recent studies also support this, identifying a positive association between LDL-TG and incident CVD [99, 100]. In contrast to our study, fasting blood samples were used for quantification of LDL-TG in the previously mentioned studies [93, 97-100]. Traditionally, lipid profile measurements have been performed fasting in the clinic. This was believed to present standardized measurements that best estimated the total amount of atherogenic lipoproteins [101]. However, recent research has pinpointed that non-fasting measurements are more relevant as this state predominates most of the 24-h cycle. A non-fasting measurement will therefore better reflect the average serum triglyceride concentration, which is more relevant for estimation of CVD risk [76, 95, 101]. In addition, we performed a correlation analysis between total triglyceride levels and fasting time (Appendix VII). There was no correlation, supporting the reliability of using non-fasting serum samples.

The elevated LDL-TG levels in MI cases in this study suggested that LDL-TG is a stronger marker for subclinical atherosclerosis than LDL-C and serum triglycerides. The exact molecular mechanism is still not known, but it has been hypothesized that elevated levels of LDL-TG reflect alterations of LDL metabolism leading to a higher atherogenic potential of LDL [100]. However, it is of importance to specify that this subgroup analysis is based on a small study population with a short follow-up time. There is a restricted, but unique, group of patients that are clinically healthy at baseline, but experience a MI within 2 years. It cannot be excluded that there have been life changing factors involving physiological stress that have contributed to the participants MI. The potential atherogenic contribution of LDL-TG is very interesting and should be further investigated in larger studies. Future research should investigate if there is a causal relationship between LDL-TG and atherosclerotic CVD.

4.2 Protein biomarkers

4.2.1 MMP-9

Current literature proposes MMP-9 as a promising biomarker for subclinical atherosclerosis [52, 53, 102]. However, this is not supported by our results. Garvin et al. performed a case-control study, investigating the association between plasma MMP-9 concentration and first-time CAD. The study identified a significant positive association [102]. Hence, similar results were expected for this present study. Differentiating from this present study, most other studies have measured MMP-9 in plasma. This is explained by the assumption that MMP-9 levels in serum is artificially high, while plasma samples reflect a more accurate concentration [103]. Contradictory, a study using serum samples have indicated a positive association between MMP-9 levels and MI risk [104]. That particular study, performed by Jefferis et al., investigated apparently healthy participants and patients with pre-existing CVD. They found a strong association between serum levels of MMP-9 and MI risk in patients with pre-existing CVD. An argument for using serum samples compared to plasma samples in apparently healthy subjects is that levels of MMP-9 are often too low to be detected in plasma, making serum levels a more appropriate choice [104]. This supports the use of serum samples in our study as apparently healthy participants were investigated. Another challenge that might have influenced the results is the hypothesis that MMP-9 will degrade over time when stored in -80 °C [105]. Our samples have been stored for 14-16 years; it can therefore not be excluded that this affected the MMP-9 concentration. Despite promising studies suggesting MMP-9 as a cardiovascular risk marker, our study was unable to support this conclusion.

4.2.2 ALDH4A1

A recently published article by Lorenzo et al. suggested ALDH4A1 as a potential disease biomarker, having higher concentration in mice and humans with atherosclerosis [55]. The results from this present study does not support that conclusion, as the statistical analysis detected no significant differences between cases and controls in ALDH4A1 concentration. The number of participants is almost the same for both studies (110 and 100, respectively). Differentiating from our study, Lorenzo et al. included cases with carotid atherosclerosis. These cases underwent carotid endarterectomy, having carotid artery stenosis higher than 70%. This analysis is a clinical evidence of atherosclerosis [55]. This present study investigated participants with subclinical atherosclerosis, as they are apparently healthy at baseline but later experience

a MI. Inclusion of clinical different cases might explain the divergent results. Further analysis should be performed to establish the potential use of ALDH4A1 as biomarker of subclinical atherosclerosis.

4.3 Reuse of serum samples

This study investigated if samples used for NMR lipidomics could be reused for ELISA. A total of 49 serum samples were used for both analyses, where two different ELISA kits were applied. Results from both MMP-9 and adiponectin showed that data from reused and fresh serum sample correlated. The reused samples were diluted 1:1 with a buffer prior to NMR analysis, and further diluted for ELISA according to the manufacturer's protocol. However, it does not seem like the buffer used for NMR analysis affected the ELISA-results. To our knowledge, this is the first study to investigate if serum samples used for NMR analysis can be reused for protein quantification by ELISA. Reusing serum samples is of special advantage when using serum from biobanks, where material is restricted. The possibility to reuse the same material for several analysis produces much valuable data from less material. For both proteins analysed, adiponectin and MMP-9, differences in protein levels were detected between fresh and reused samples within the upper range of the standard curve used in the ELISAs. The results were conflicting whether fresh or reused samples had highest protein concentration, which support the assumption that this was most likely caused by technical challenges in the analysis. In specific, it might seem like the standard curve was more unspecific at higher concentrations for both ELISA kits.

There is an increasing need for developing a standard analytical method for lipoprotein classification [66, 74, 75, 106]. The opportunity for reusage of NMR samples is an advantage pointing towards NMR analyses as the standard lipidomics method. Results presented in this study were based on a relatively small sample. Further investigation should be performed to verify if the samples can be reused for ELISA, including testing of several different proteins. However, these preliminary data are very promising, showing a significant positive correlation between fresh and reused samples for both MMP-9 and adiponectin.

4.4 Limitations

There were several limitations to this study. First, the study consisted of a small study population. This was partly a result of the selection criteria of cases, which limited the number of potential cases that could be included. The probability of errors increases substantially with a low number of participants, which affects the reliability of the results. To minimize the limitations of a small study population, two controls were included for each case. Because NMR lipidomic analysis test for multiple possible biomarkers simultaneously, multiplicity issues need to be addressed as a possible limitation. Multiple comparisons increase the probability of finding false positives, i.e., Type I errors, for one or more of the biomarkers tested. Adjustments for multiple comparisons, e.g., Bonferroni correction [107], is often performed. However, this present study did not for account for multiplicity. Supporting our decision, it has been suggested that the Bonferroni correction becomes very conservative for lipidomic analyses [107]. Application of Bonferroni correction will thus come at the expense of increasing the probability of Type II errors (false negatives) [108, 109]. Therefore, by applying a significance level of 0.05 for each test and discussing p-values of 0.05-0.1 as trends, we reduced the chance for overlooking potential biomarkers of interest.

Another limitation was the method used for excluding participants using lipid-lowering medication. The HUNT3 study had no questions regarding this, and the selection was therefore based on the participants' answers in HUNT4. Hence, it cannot be fully excluded that some of the cases used lipid-lowering medication at blood sampling, when HUNT3 was collected. A MI is categorized as type I or type II, whereas atherosclerosis mainly contributes to type I. The results presented does not discriminate between the two, which is another limitation. At last, the study reflects a homogenous Norwegian population, and the results may therefore not be generalizable to other populations.

5. Conclusion

This study investigated potential biomarkers of subclinical atherosclerosis, by analysing lipoprotein subfractions and proteins in serum from apparently healthy individuals that later experienced a MI. In conclusion, several lipoprotein subfractions were associated with future MI, potentially reflecting subclinical atherosclerosis. Analysis of the two proteins, MMP-9 and ALDH4A1, did not support their previously suggested potential as promising cardiovascular risk markers.

The potential biomarkers of subclinical atherosclerosis suggested through this study was concentration of ApoA1 in HDL-4 and triglyceride in LDL. Both lipoprotein subfractions had a positive association with MI. LDL-TG was apparent in the subgroup analysis with a 2-year observation period, suggesting its potential as an imminent risk marker. The results indicated that LDL-TG was a better biomarker of future MI compared to the traditionally used total serum triglyceride levels. Increased ApoA1 levels in the sdHDL subfraction in cases indicated a possible unexplored proatherogenic role of ApoA1. Furthermore, HDL subfractions were detected as potential gender-specific risk markers for men. Larger HDL subfractions (HDL-1 and HDL-2) were negatively associated with MI. Opposing trends were observed in sdHDL (HDL-4), being positively associated with MI. Larger HDL subfractions might therefore possess the cardioprotective properties of HDL, which is partly masked by sdHDL when measuring total HDL. The positive association of sdHDL with future MI point towards a possible proatherogenic role of sdHDL. Altogether, results from the NMR lipidomic analysis supported the increasing evidence that lipoprotein subfractions might provide additional information overseen by traditional lipid biomarkers. Protein quantification of MMP-9 and adiponectin indicated that serum samples used for NMR analysis could be reused to quantify circulating proteins. These initial results are promising for efficient utilization of serum samples collected from biobanks.

5.1 Future perspectives

Future studies containing a larger population is necessary to verify the results from this present study. This applies to all the analysis performed. Regarding lipoprotein subfractions, it is beneficial if these future studies also apply NMR lipidomic analysis. Additionally, the potential of the biomarkers suggested in this present study should be further investigated. By adding them

to the current risk prediction model for MI, it can be estimated whether they provide additional predictive value. With an increasing prevalence of CAD in the years to come, more refined analysis of lipoprotein subfractions seems promising to complement the assessment of traditional risk factors. For implementation of lipoprotein subfractions in the clinical risk evaluation, determination of a standard method for lipidomic analyses is required. The methods separates lipoproteins based on different properties [106], making the published literature challenging to directly compare[110]. As an increasing number of studies support the association between lipoprotein subfractions and CAD, the need for a standardized lipidomics method becomes apparent.

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Appendix

Appendix I. Densities of lipoproteins

Table I**:** Densities (in kg/L) of lipoprotein main fractions.

VLDL	IDL	LDL	HDL
0.950-1.006	1.006-1.019	1.019-1.063	1.063-1.210

VLDL: Very low-density lipoprotein, IDL: Intermediate-density lipoprotein, LDL: Low-density lipoproteins, HDL: High-density lipoprotein.

Table 2: Densities (in kg/L) of LDL subfractions.

LDL-1	LDL-2	LDL-3	LDL-4	LDL-5	LDL-6
1.019-1.031	1.031-1.034	1.034-1.037	1.037-1.040	1.040-1.044	1.044-1.063

LDL: Low-density lipoproteins

Table 3: Densities (in kg/L) of HDL subfractions.

HDL-1	HDL-2	HDL-3	HDL-4
1.063-1.100	1.100-1.112	1.112-1.125	1.125-1.210

HDL: High-density lipoprotein

Appendix II. Lipoprotein subfractions

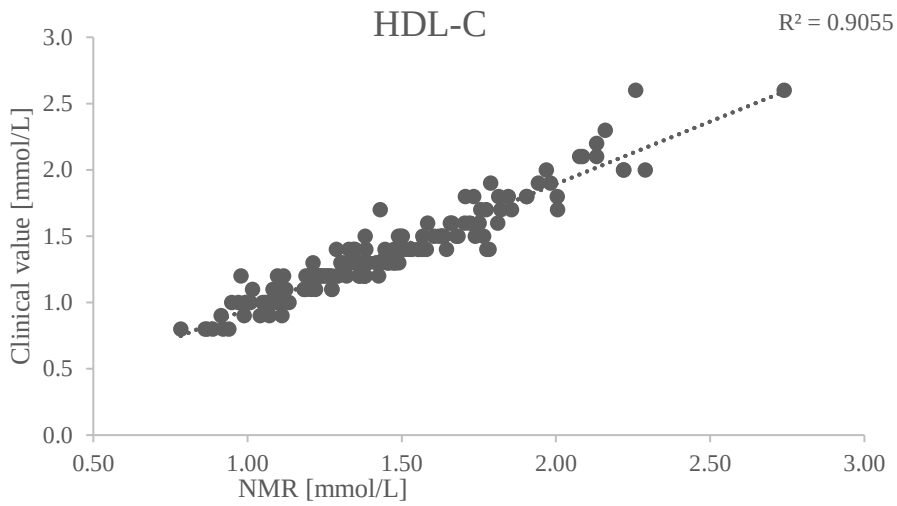
Table II: Overview of lipoprotein subfractions deduced from NMR lipidomics.

Lipoprotein subfraction	Lipid concentration	Lipoprotein
TPTG	Triglyceride	Total plasma
TPCH	Cholesterol	Total plasma
LDCH	Cholesterol	LDL
HDCH	Cholesterol	HDL
TPA1	Apolipoprotein-A1	Total plasma
TPA2	Apolipoprotein-A2	Total plasma
TPAB	Apolipoprotein-B100	Total plasma
LDHD	LDL-C/HDL-C	Total plasma
ABA1	Apo-B100/Apo-A1	Total plasma
TBPN	Total particle number	Total plasma
VLPN	Particle number	VLDL
IDPN	Particle number	IDL
LDPN	Particle number	LDL
L1PN	Particle number	LDL-1
L2PN	Particle number	LDL-2
L3PN	Particle number	LDL-3
L4PN	Particle number	LDL-4
L5PN	Particle number	LDL-5
L6PN	Particle number	LDL-6
VLTG	Triglyceride	VLDL
IDTG	Triglyceride	IDL
LDTG	Triglyceride	LDL
HDTG	Triglyceride	HDL
VLCH	Cholesterol	VLDL
IDCH	Cholesterol	IDL
VLFC	Free cholesterol	VLDL
IDFC	Free cholesterol	IDL
LDFC	Free cholesterol	LDL
HDFC	Free cholesterol	HDL
VLPL	Phospholipids	VLDL
IDPL	Phospholipids	IDL
LDPL	Phospholipids	LDL
HDPL	Phospholipids	HDL
HDA1	Apo-A1	HDL
HDA2	Apo-A2	HDL
VLAB	ApoB	VLDL
IDAB	ApoB	IDL
LDAB	ApoB	LDL
V1TG	Triglycerides	VLDL-1
V2TG	Triglycerides	VLDL-2
V3TG	Triglycerides	VLDL-3
V4TG	Triglycerides	VLDL-4
V5TG	Triglycerides	VLDL-5
V1CH	Cholesterol	VLDL-1
V2CH	Cholesterol	VLDL-2
V3CH	Cholesterol	VLDL-3
V4CH	Cholesterol	VLDL-4
V5CH	Cholesterol	VLDL-5
V1FC	Free cholesterol	VLDL-1
V2FC	Free cholesterol	VLDL-2
V3FC	Free cholesterol	VLDL-3
V4FC	Free cholesterol	VLDL-4
V5FC	Free cholesterol	VLDL-5
V1PL	Phospholipids	VLDL-1

V2PL	Phospholipids	VLDL-2
V3PL	Phospholipids	VLDL-3
V4PL	Phospholipids	VLDL-4
V5PL	Phospholipids	VLDL-5
L1TG	Triglycerides	LDL-1
L2TG	Triglycerides	LDL-2
L3TG	Triglycerides	LDL-3
L4TG	Triglycerides	LDL-4
L5TG	Triglycerides	LDL-5
L6TG	Triglycerides	LDL-6
L1CH	Cholesterol	LDL-1
L2CH	Cholesterol	LDL-2
L3CH	Cholesterol	LDL-3
L4CH	Cholesterol	LDL-4
L5CH	Cholesterol	LDL-5
L6CH	Cholesterol	LDL-6
L1FC	Free cholesterol	LDL-1
L2FC	Free cholesterol	LDL-2
L3FC	Free cholesterol	LDL-3
L4FC	Free cholesterol	LDL-4
L5FC	Free cholesterol	LDL-5
L6FC	Free cholesterol	LDL-6
L1PL	Phospholipids	LDL-1
L2PL	Phospholipids	LDL-2
L3PL	Phospholipids	LDL-3
L4PL	Phospholipids	LDL-4
L5PL	Phospholipids	LDL-5
L6PL	Phospholipids	LDL-6
L1AB	ApoB	LDL-1
L2AB	ApoB	LDL-2
L3AB	ApoB	LDL-3
L4AB	ApoB	LDL-4
L5AB	ApoB	LDL-5
L6AB	ApoB	LDL-5
H1TG	Triglycerides	HDL-1
H2TG	Triglycerides	HDL-2
H3TG	Triglycerides	HDL-3
H4TG	Triglycerides	HDL-4
H1CH	Cholesterol	HDL-1
H2CH	Cholesterol	HDL-2
H3CH	Cholesterol	HDL-3
H4CH	Cholesterol	HDL-4
H1FC	Free cholesterol	HDL-1
H2FC	Free cholesterol	HDL-2
H3FC	Free cholesterol	HDL-3
H4FC	Free cholesterol	HDL-4
H1PL	Phospholipids	HDL-1
H2PL	Phospholipids	HDL-2
H3PL	Phospholipids	HDL-3
H4PL	Phospholipids	HDL-4
H1A1	Apolipoprotein-A1	HDL-1
H2A1	Apolipoprotein-A1	HDL-2
H3A1	Apolipoprotein-A1	HDL-3
H4A1	Apolipoprotein-A1	HDL-4
H1A2	Apolipoprotein-A2	HDL-1
H2A2	Apolipoprotein-A2	HDL-2
H3A2	Apolipoprotein-A2	HDL-3
H4A2	Apolipoprotein-A2	HDL-4

Appendix III. Validation of NMR analysis

A.



B.

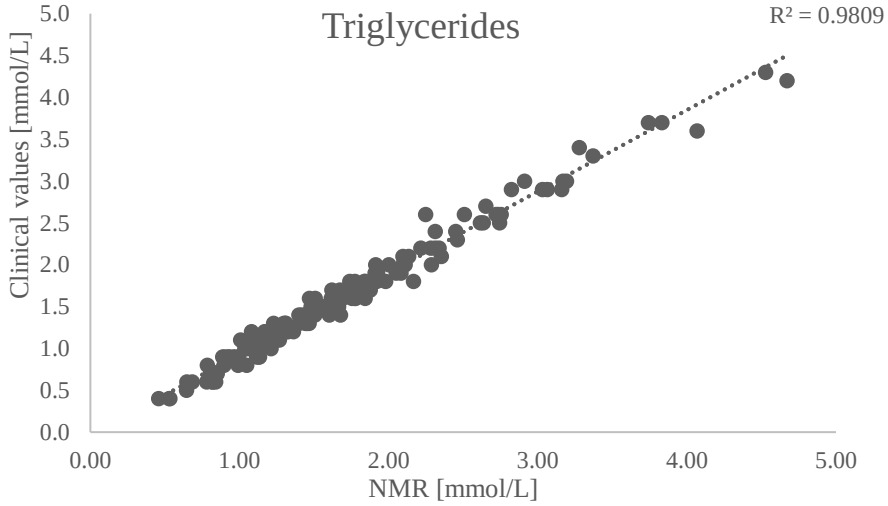


Figure I: *Correlation plots comparing routine clinical chemistry analysis measurements to the concentration quantified by NMR lipidomics.* R-squared is indicated in the plot. A. Correlation plots for serum HDL-C concentration. B. Correlation plot for serum triglyceride concentration. NMR: Nuclear magnetic resonance, HDL-C: High-density lipoprotein - cholesterol.

Appendix IV. PCA-plot

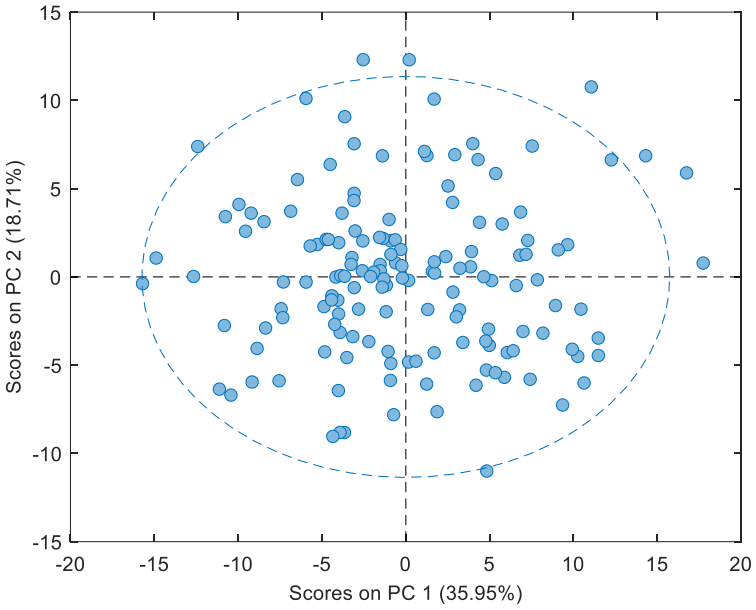


Figure II: *A PCA-plot of lipoprotein subfractions results from the study population (n=150).* Each of the participants in the study population is illustrated with a blue dot. The participants are relatively clustered together, indicating that there are not obvious differences within the population. None of the participants deviated considerable from the others, so not outliers were detected. PCA: Principal component analysis.

Appendix V. PLSDA-plot

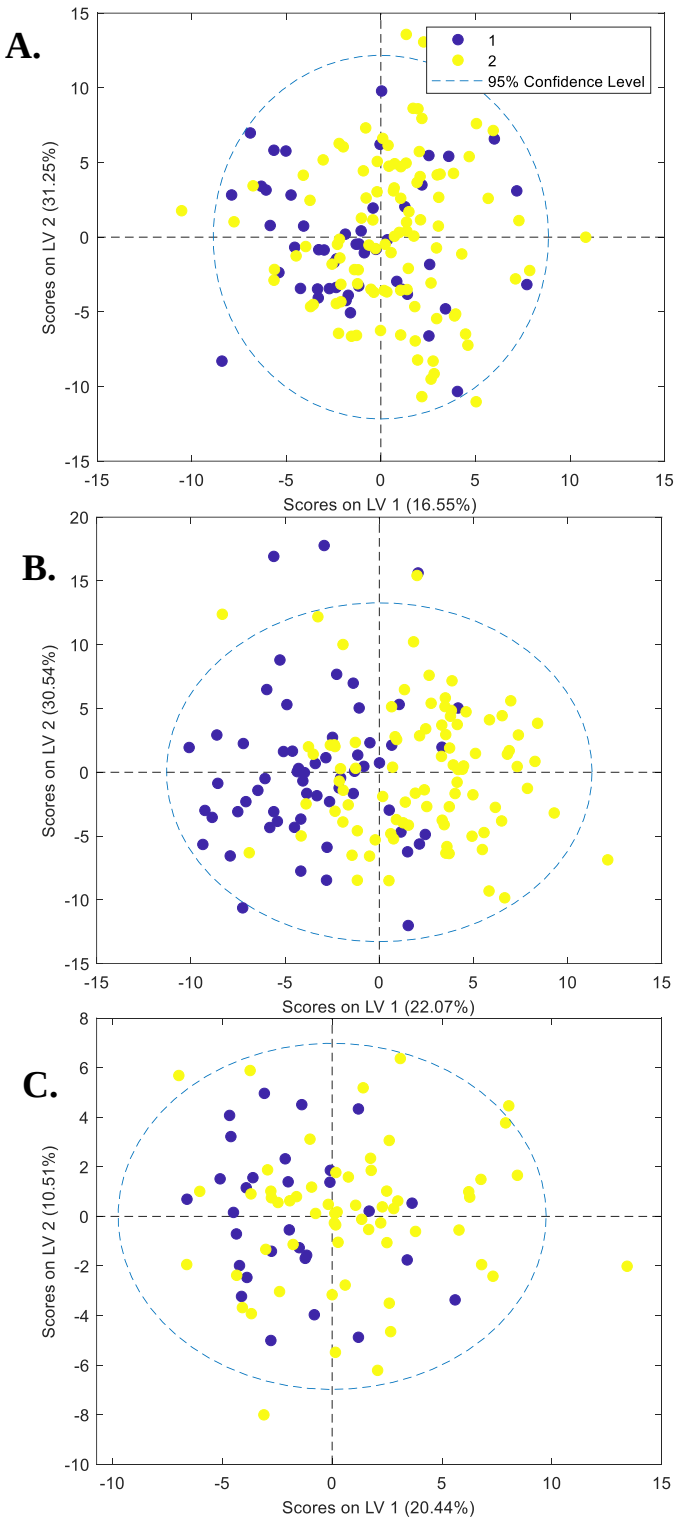


Figure III*: Different PLSDA-plots deduced from the NMR lipidomics dataset*. **A**. Differences in lipoprotein profile of cases (1) and controls (2). A p-value of 0.09 indicate that there is a slight difference in lipoprotein subfractions between cases and controls. **B**. Differences in lipoprotein profile for men (yellow) and women (blue). There is a significant ($p < 0.001$) difference in lipoprotein profile between men and women. **C.** Subgroup analysis of men, PLSDA-plot of differences in lipoprotein profile for cases (blue) and controls (yellow). PLSDA: Partial least squares discriminant analysis.

Appendix VI. Reuse of serum samples

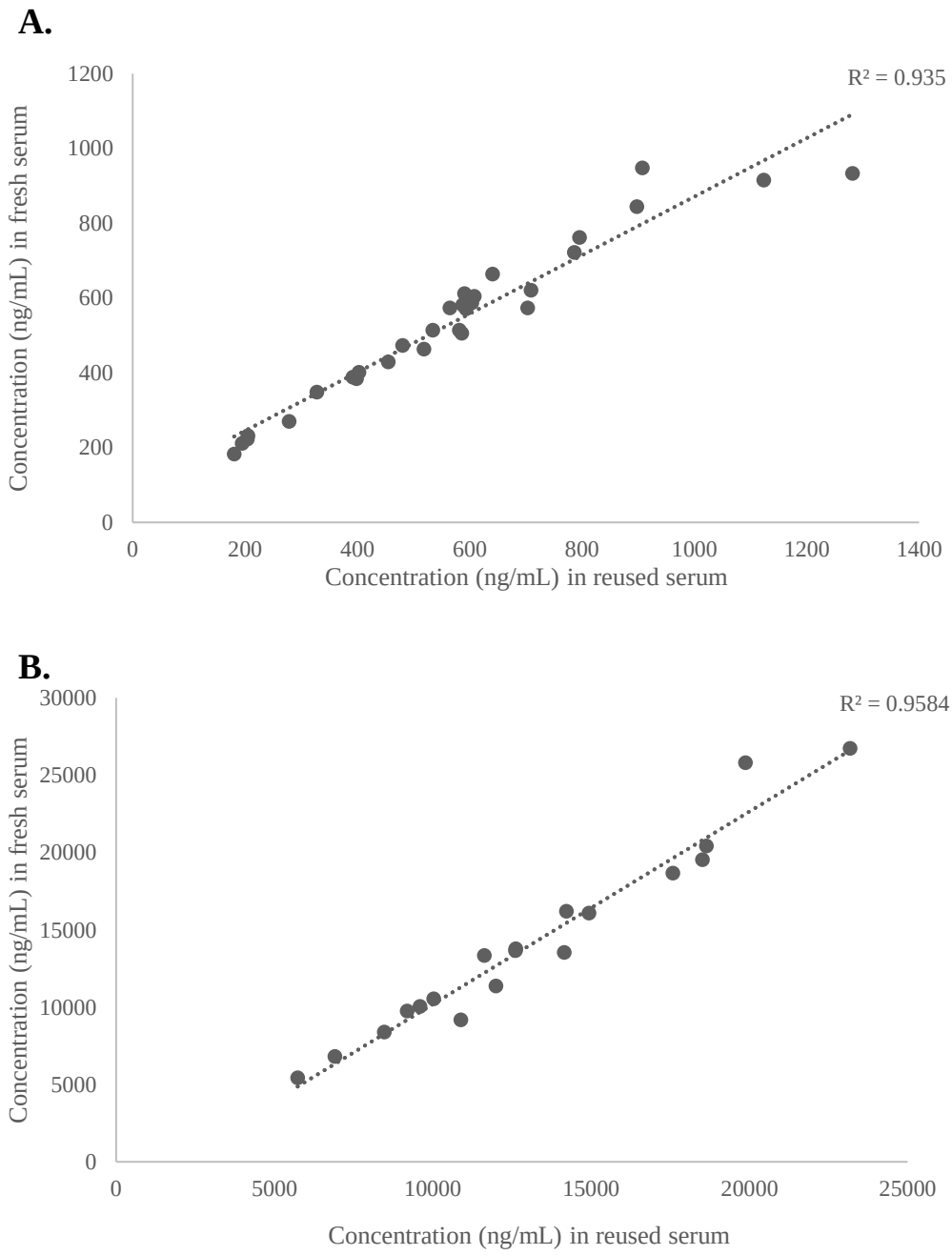


Figure IV: *Correlation plots of protein quantification by ELISA, measured in ng/mL, in fresh and reused serum samples.* NMR lipidomics are performed on the reused samples. R-squared is indicated in the plot. **A.** Correlation plot of MMP-9 concentration. **B.** Correlation plot of adiponectin concentration. NMR: Nuclear magnetic resonance. ELISA: Enzyme-linked immunosorbent assay, MMP-9: Matrix metalloproteinase-9

Appendix VII. Non-fasting triglycerides

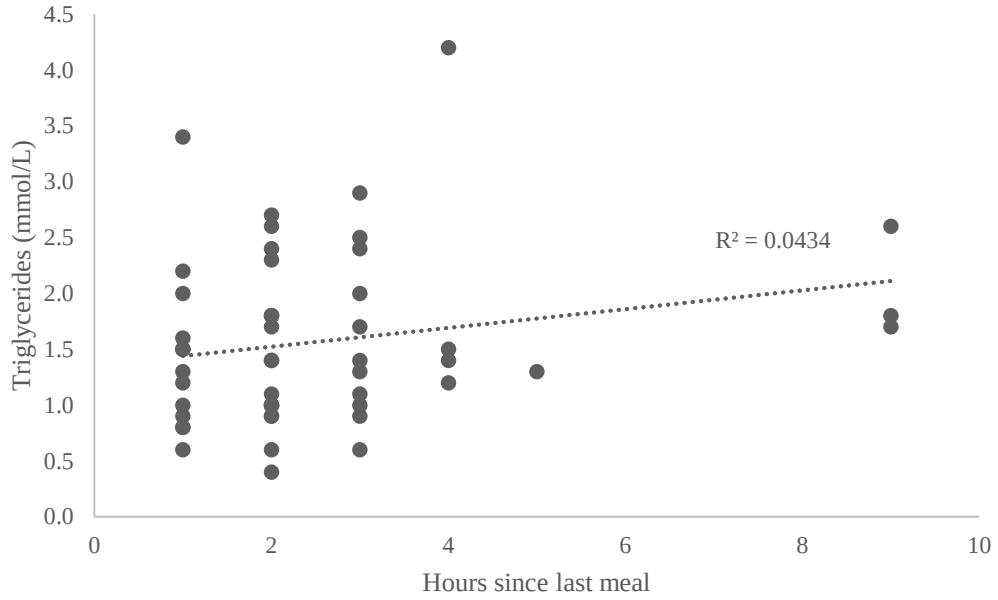


Figure V*: Correlation plot of total serum triglycerides and hours since last meal. R-squared is indicated in the plot.*

NTNU
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