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Assessment of LDL and HDL Subfractions and Metrics of HDL Function in High-Risk Patients of Cardiovascular Disease: Evaluation of Four Different Interventions

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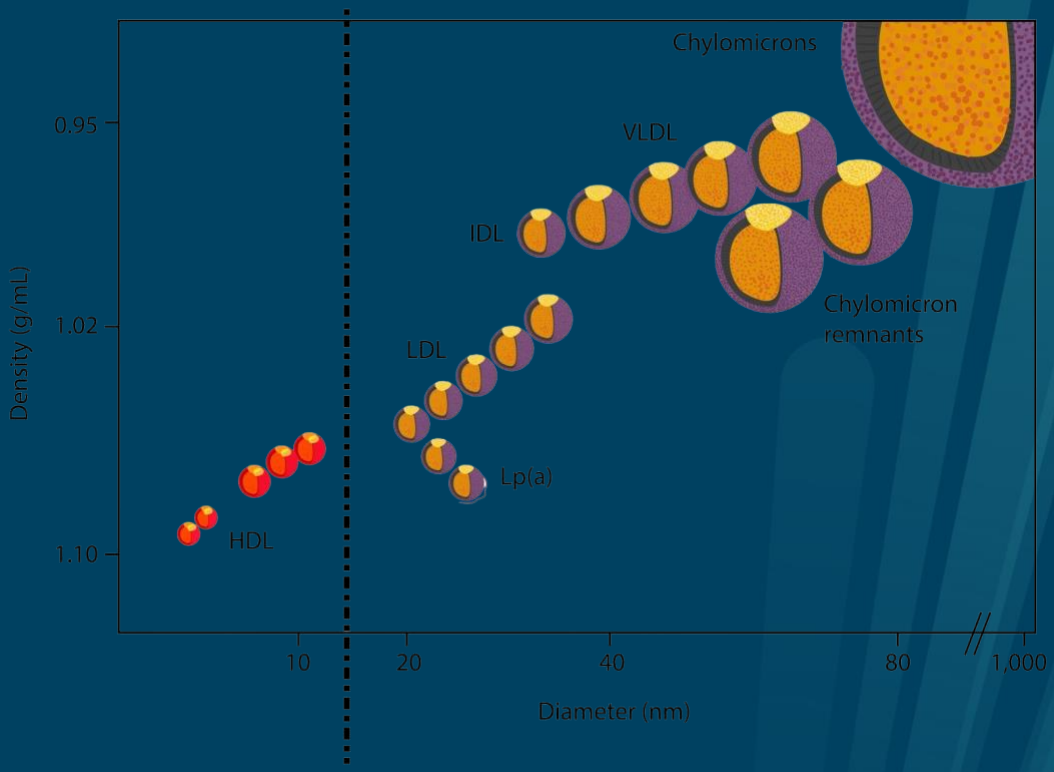
A dissertation for the degree of Philosophiae Doctor



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1 Acknowledgments

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2 Abbreviations

ABCA1	Adenosine triphosphate-binding cassette sub-family A member 1
ApoB	Apolipoprotein B
ApoA1	Apolipoprotein A1
ASCVD	Atherosclerotic cardiovascular disease
CEC	Cholesterol efflux capacity
CETP	Cholesteryl ester transport protein
CVD	Cardiovascular disease
FH	Familial hypercholesterolemia
GE	Gel electrophoresis
HDL	High-density lipoproteins
HDL-C	HDL cholesterol
HDL-P	HDL particles
IM	Ion mobility
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoproteins
LDL-C	LDL cholesterol
LDL-P	LDL particles
LDL-R	LDL-receptor
lbLDL	Large, boyant LDL
MI	Myocardial infarction
NMR	Nuclear magnetic resonance
N3-PUFA	N-3 polyunsaturated fatty acids
PCSK9	Proprotein convertase subtilisin/kexin type 9
PON1	Paraoxonase-1
RCT	Reverse cholesterol transportation
SAA1	Serum amyloid A1
SR-B1	Scavenger receptor class B type 1
sdLDL	Small, dense LDL
TC	Total cholesterol
TRL	Triglyceride-rich lipoproteins
UC	Ultracentrifugation
VLDL	Very-low-density lipoproteins

3 Abstract

Introduction

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of death and morbidity worldwide. Despite significant advances in risk prediction, prevention, and treatment, many patients do not receive adequate risk factor management, and even in patients with optimal risk factor management, there is a considerable residual risk of recurrent cardiovascular events. Advanced lipoprotein testing, such as measuring LDL and HDL subfractions or metrics of HDL function, has been suggested to improve ASCVD risk prediction and could help select high-risk patients for novel and expensive lipid-lowering therapy.

Methods and materials

We included patients from four different clinical intervention trials that the Cardiology research group at Nordland Hospital, Bodø had initiated or collaborated on; a trial of an intensive lifestyle intervention followed by bariatric surgery in 34 morbidly obese patients, a pilot study were lipoprotein apheresis was switched to a PCSK9 inhibitor in 3 patients with familial hypercholesterolemia, a randomized, placebo-controlled trial of a statin + ezetimibe in 30 elderly patients with atrial fibrillation and a randomized, placebo-controlled, cross-over trial of an n3-PUFA supplement in 34 patients with familial hypercholesterolemia. LDL and HDL subfractions were measured using a simple, in-house system (Lipoprint ®). Assays measuring serum amyloid A1 (SAA1), paraoxonase-1 (PON1) arylesterase activity, and cholesterol efflux capacity (CEC) was performed by our collaborators at Linköping University and the Swedish Toxicology Sciences Research Center in Södertälje, Sweden.

Results

In our study populations, including the morbidly obese patients, we discovered low baseline concentrations of sdLDL, and we found no effect on sdLDL from bariatric surgery, a combination of statins + ezetimibe, PCKS9 inhibitor or n3-PUFA. An intensive lifestyle intervention, bariatric surgery, and n3-PUFA increased the large HDL subfraction, and n3-PUFA reduced the small HDL subfraction. Despite significant changes in the composition of HDL subfractions, there were no changes in HDL efflux capacity from these interventions, The lifestyle intervention and bariatric surgery reduced PON1 and SAA1.

Conclusion

Our studies found no significant baseline levels of sdLDL in any study population and observed no notable impact on sdLDL from the interventions studied. Therefore, Lipoprint LDL does not appear to provide additional clinically relevant information compared to standard lipoprotein metrics in these high-risk groups. The clinical significance of changes in HDL composition and HDL subfractions in predicting ASCVD risk remains uncertain, as there is no consensus in the published literature. Furthermore, despite significant alterations in HDL composition, no changes in CEC were observed, suggesting the limited relevance of

Lipoprint HDL® as a surrogate for CEC.

4 Introduction

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of morbidity and mortality in the world today, with most deaths occurring in low- and medium-income countries. [1] At the beginning of the 20th century, the clinical manifestations of ASCVD were relatively rare causes of death in the U.S. [2] By the 1950s, it had become the most common cause of death, an unprecedented epidemic of cardiovascular disease (CVD) observed simultaneously in many western countries. Autopsies confirmed that most deaths were caused by coronary heart disease due to coronary atherosclerosis. [2]

Several societal trends that began during the first half of the 20th century have probably contributed to this increase in ASCVD; a massive increase in tobacco smoking, diet changes with increased intake of processed foods, saturated fats, sugars, and a decrease in exercise and physical activity. [2] The widespread utilization of the electrocardiogram (ECG) to diagnose myocardial infarction also increased the recognition of CVD as a cause of death.

As deaths from CVD peaked in the Western world around the 1960s, with some countries reporting over half of all deaths being attributed to CVD [3], cardiovascular research began to make significant progress. With the development of primary and secondary prevention strategies, improved diagnostics, and better treatments, the following decades initiated a steady decline in CVD mortality that has continued to this day in high-income countries. [4] Despite this, CVD remains the leading cause of death and morbidity worldwide.

ASCVD is a multifactorial disease with several modifiable factors being proven to be associated with an increased risk of developing the clinical manifestations of ASCVD. [5] Low-density lipoprotein (LDL) is the most extensively researched of these risk factors. An overwhelming body of evidence has established that LDL and other apolipoprotein B (apoB)-containing lipoproteins are causally implicated in the development of ASCVD. [6] Targeting and lowering LDL reduces ASCVD risk and is central in all ASCVD prevention guidelines in both primary and secondary prevention settings. [7]

ASCVD prevention guidelines are not unified in their recommendations on what lipoprotein measurement to be used in ASCVD risk assessment. [8, 9] Most guidelines recommend using an estimator of the 10-year risk of ASCVD events, such as the Framingham Risk Score, ACC/AHA Pooled Cohort Equations, or the Systemic Coronary Risk Evaluation (SCORE) estimator, for risk stratification. These calculators incorporate total cholesterol (TC) and high-

density lipoprotein-cholesterol (HDL-C) as lipoprotein metrics. LDL-cholesterol (LDL-C) is the most widely recommended target for therapy in dyslipidemia guidelines.

Despite apoB-containing lipoproteins playing a causal role in the initiation and progression of ASCVD, the value of the standard lipoprotein metrics such as TC, HDL-C, and LDL-C in CVD risk stratification is limited. Most people at risk have serum levels of these lipoprotein measurements within the "normal range" [10] (**Figure 1**) and they do not accurately reflect the cumulative exposure of apoB-containing lipoproteins to the arterial wall the individual has experienced over their lifespan. [11] Due to this, there has been an extensive effort in the cardiovascular research community to find new and improved lipid markers for CVD risk stratification.

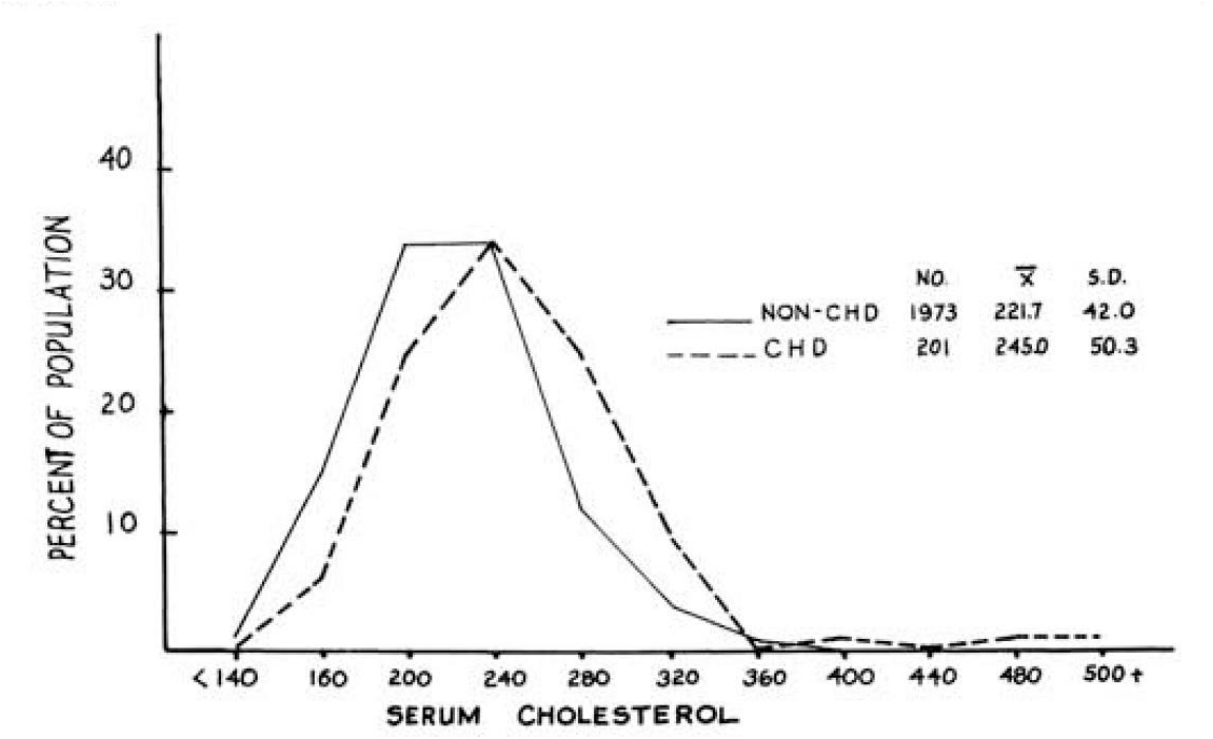

Figure 1 *Distribution of serum cholesterol in subjects free of coronary heart disease versus those developing coronary heart disease in 10 years: men, 30 to 59 years at entry. Adapted from "Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham study" by W. B. Kannel, 1977, Annals of Internal Medicine. Reprinted with permission. [12]*

4.1 Pathophysiology of atherosclerosis

In a landmark study from 1955, John Gofman used the newly developed analytical ultracentrifuge to identify two fractions of cholesterol-carrying lipoproteins in plasma: low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Looking at hospitalized patients with myocardial infarction, he found increased LDL and low levels of HDL in their plasma. [13] Gofmans findings sparked an intensive effort in lipoprotein research, particularly

in LDL, paving the way for the deep understanding of the pathogenesis of atherosclerosis we have today. His findings have been replicated numerous times, and the association between LDL and the risk of ASCVD is one of the most robust associations in medical science.

Subendothelial retention and accumulation of cholesterol-rich apoB-containing lipoprotein particles, predominantly LDL, in the arterial wall is the first key step in initiating atherosclerosis. The pathophysiology of atherosclerosis is complex, but the most prevalent theory today is that the retained apoB-containing particles in the arterial wall initiate a process where the lipids within, mainly cholesterol, become oxidized and otherwise modified. Modified apoB is internalized via endocytosis in macrophages, turning them into cholesterol-laden foam cells. The foam cells, oxidized LDL particles, and cholesterol crystals initiate an inflammatory immune response that enlarges the plaque, which eventually can rupture into the vessel's lumen. The ruptured plaque initiates platelet adhesion and aggregation on the exposed vascular surface and activates the clotting cascade leading to the formation of an occluding blood clot, inducing a cardiovascular event. [14, 15] (**Figure 2**)

While LDL is necessary to initiate atherosclerosis, cumulative exposure to several other risk factors influences the time it takes to develop a clinically relevant atherosclerotic plaque. Unless LDL levels are extraordinarily high or low, it is impossible to predict the risk of cardiovascular disease by lipid measurements alone. Hypertension, smoking, diabetes, obesity, psychosocial factors, and physical activity are among the most important and best understood of these risk factors. [5] Also, many factors related to inflammation and poorly understood genetic factors contribute to atherosclerosis. [16, 17]

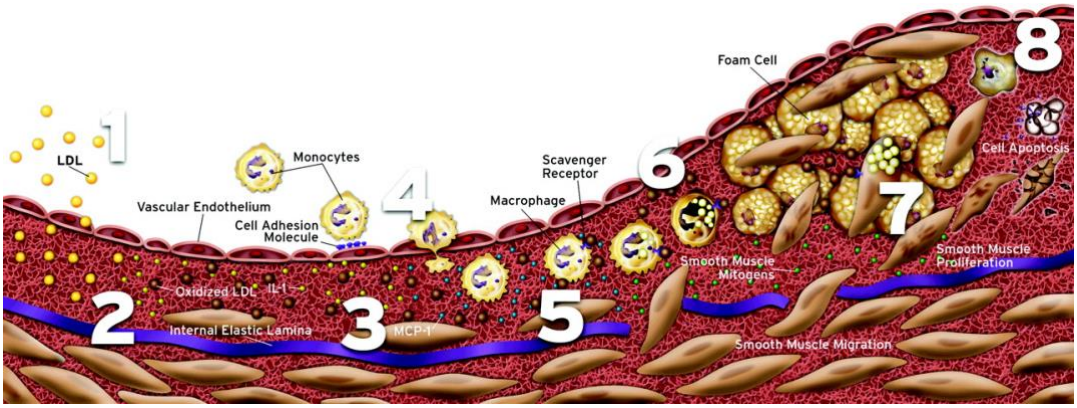

Figure 2 *Development of an atherosclerotic plaque. First, LDL moves into the subendothelium and is oxidized. (1 and 2). The release of growth factors and cytokines attracts additional monocytes (3 and 4). Foam cell accumulation and smooth muscle cell proliferation result in the growth of plaque (6, 7, and 8). Adapted from "Atherosclerotic Vascular Disease Conference Writing Group III: Pathophysiology" by David Faxon, 2004, Circulation. Reprinted with permission. [18]*

The risk of developing clinical manifestations of ASCVD increases with increasing concentrations of LDL particles (LDL-P) and duration of exposure. [6] Early evidence of this came from studies of patients with familial hypercholesterolemia (FH) who have mutations in genes coding for the LDL-receptor (LDL-R), apoB, or the proprotein convertase subtilisin/kexine type 9 (PCSK9)-protein, that results in elevated LDL-concentrations from

early childhood. To date, almost 4000 mutations in the LDL-R gene have been described. [19] Patients with monogenic FH are at a high to very risk of developing premature CVD, even as early as the first decade of life in the most severe forms of homozygous FH. [20]

On the flip side, genetic mutations that lead to lower LDL concentrations throughout life have been proven to be highly protective against developing CVD. PCSK9 is an enzyme that binds to hepatic LDL-R and disrupts LDL-R recycling back to the cell surface in hepatocytes after internalization. This results in a decline of hepatic LDL-R, reduced hepatic LDL clearance, prolonged LDL half-life, and increased plasma LDL concentration. Gain-of-function mutations in the PSCK9 gene result in a rare type of monogenic FH. In contrast, the more common loss-of-function mutations lead to moderate life-long reductions in LDL levels that significantly reduce the risk of developing CVD. [21] These observations strongly support that life-long reductions in LDL are much more effective in reducing CVD risk than reducing LDL with lipid-lowering therapy later in life when clinical or subclinical atherosclerosis has already developed. LDL lowered by 30% with statins reduces the 5-year risk of developing CVD by approximately 30%, while similar LDL reductions from PCSK9-mutations reduce the lifetime risk of ASCVD by as much as 90%. [22]

In the last decade, several Mendelian randomization studies [23-25] and studies calculating the cumulative exposure of LDL-C in large prospective cohorts [11] have added compelling evidence to support that both the concentration and the total duration of exposure of LDL to the arterial wall is critical in CVD pathogenesis.

It took two decades from Gofman discovered the HDL fraction and noted a negative correlation with CVD until these findings were replicated in the Framingham study [26] and the discouraging quest to unravel the mechanistic role of HDL in CVD pathogenesis began. Despite almost half a century of research and a vast amount of data correlating low HDL-Cholesterol (HDL-C) levels to increased risk of CVD, a causal relationship has yet to be established.

The complexity of the HDL particle is probably an important reason why it has been so challenging to elucidate the role of HDL in CVD pathogenesis. Several rigorous clinical trials of agents that significantly increase HDL-C have failed to improve cardiovascular outcomes [27] and Mendelian randomization studies of genetic variants linked to naturally elevated HDL-C found no association with reduced risk of CVD. [28-30] Consequently, the focus in published literature for the last decade has shifted from HDL-C quantity to the atheroprotective functions of HDL particles and the distribution of various HDL subfractions.

The mechanisms by which HDL might exert its atheroprotective function have been extensively researched. These include but are not limited to; reverse cholesterol transportation (RCT), anti-inflammatory and antioxidant effects, improved endothelial function, prevention of apoptosis, and antithrombotic effects. [31] Reverse cholesterol transport (RCT), the removal of cholesterol from peripheral cells to the liver for excretion, is considered the most important of HDLs atheroprotective actions. In the last decade, several methods for measuring

or quantifying aspects of HDL function have been developed, including assays measuring cholesterol efflux capacity (CEC), a key step in RCT.

4.2 LDL metabolism

Cholesterol is a vital component of cell membranes and serves as a precursor for steroid hormones, vitamin D and bile acids. Almost all cholesterol in plasma is transported to peripheral cells by apoB-containing lipoproteins, with LDL being the most abundant of these lipoproteins. (**Figure 6**) LDL particles are shaped as a sphere, have a core filled mostly with cholesteryl esters and are covered by a layer of phospholipids and one molecule of apolipoprotein B. (**Figure 3**) Two-thirds of the LDL in circulation originates from very low-density lipoprotein particles (VLDL) that have been secreted by the liver and shrunk in size due to the delivery of fatty acids and cholesterol to muscle and adipose tissue trough interaction with lipoprotein lipase (LPL), the rest of circulating LDL is secreted directly from the liver. [32]

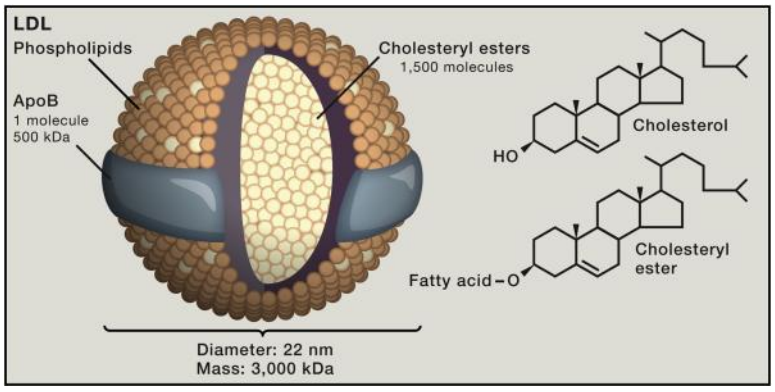

Figure 3 The LDL particle is spherical, contains cholesteryl esters in an oily core with a hydrophilic coat consisting of phospholipids and one molecule of apolipoprotein B. Adapted from "A Century of Cholesterol and Coronaries: From Plaques to Genes to Statins" by Joseph Goldstein and Michael Brown, 2016, Cell. Reprinted with permission. [14]

LDL uptake in cells is regulated through the LDL-R, a surface protein that binds to apoB. LDL-R internalizes LDL in the cell via endocytosis, the LDL particle is delivered to lysosomes that release the cholesterol for membrane or hormone synthesis. Because cholesterol is such a vital part of membrane stability all cells can synthesize its own cholesterol from acetyl CoA, with the rate-controlling step being catalyzed by HMG-CoA reductase. (**Figure 4**) LDL-R and HMG-CoA reductase synthesis are subjected to coordinated feedback suppression. When cellular cholesterol concentrations are low the cells increase the production of LDL-R and HMG-CoA reductase and when cholesterol levels are rising the production of both these proteins is suppressed. LDL particles in plasma are removed from circulation by LDL-R in the liver. With non- or dysfunctional LDL-R the uptake of LDL in liver cells is reduced, leading to higher LDL concentrations in plasma. Statins work by inhibiting HMG-CoA reductase, this reduces the *de novo* biosynthesis of cholesterol in the liver and thus reduces the production of VLDL and LDL particles. Through the feedback

system, the number of LDL-Rs on the cell surface of hepatocytes is increased, augmenting uptake of LDL and further lowering the plasma concentration of LDL.

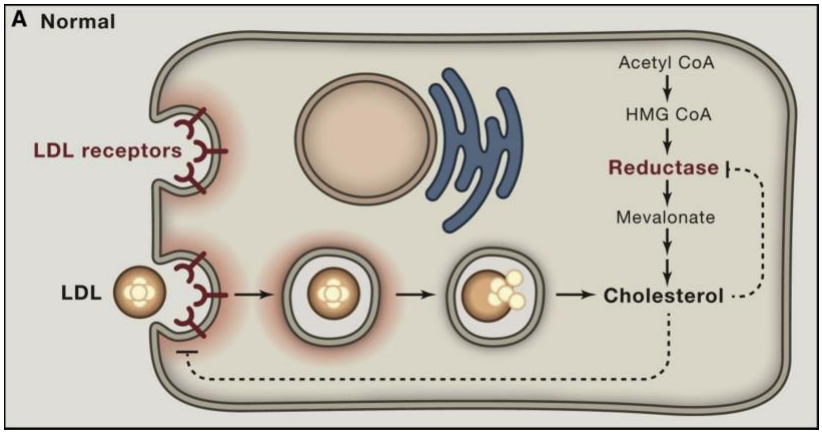

Figure 4 *Feedback regulation of de novo biosynthesis of cholesterol and LDL receptors in normal subjects. Adapted from "A Century of Cholesterol and Coronaries: From Plaques to Genes to Statins" by Joseph Goldstein and Michael Brown, 2016, Cell. Reprinted with permission. [14]*

4.3 Metrics of LDL and apolipoprotein B-containing lipoproteins

The apoB-containing particles with atherogenic potential are usually divided into chylomicron remnants, very large LDL (VLDL), intermediate LDL (IDL), LDL, and lipoprotein(a) (Lp(a)). (**Figure 5**) VLDL, IDL, chylomicrons and chylomicron remnants are also called triglyceride-rich lipoproteins (TRL). To initiate atherosclerosis the apoB-containing particle must be able to enter the arterial intima and to achieve this they must be smaller than 70 nm. [33]

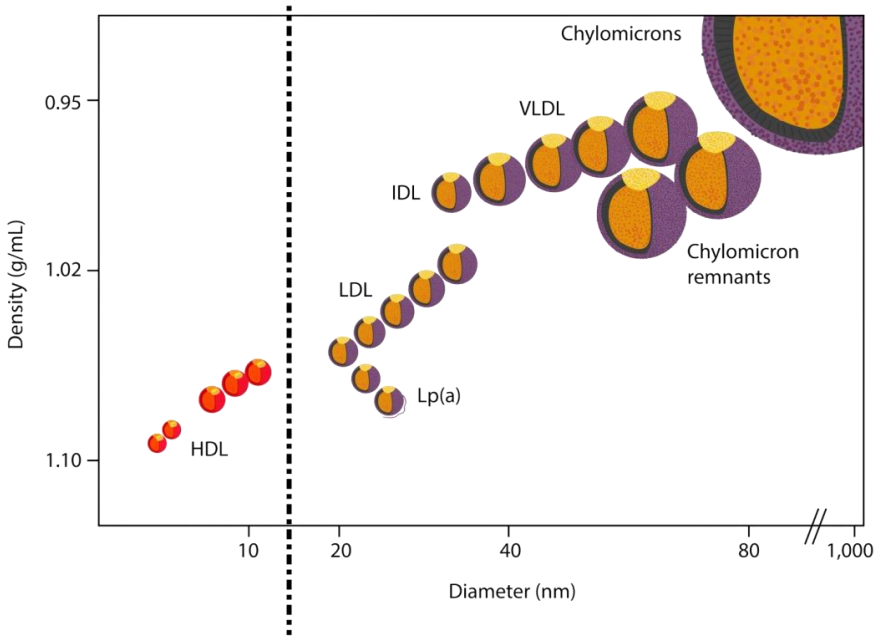

Figure 5 *Relative size and density of the major plasma lipoproteins and their subfractions [34]*

The LDL particles constitute approximately 90% of the circulating apoB-containing particles in a normolipidemic population (**Figure 6**). The most used metric of LDL in clinical practice is LDL-C, a measure of the cholesterol content within all LDL particles.

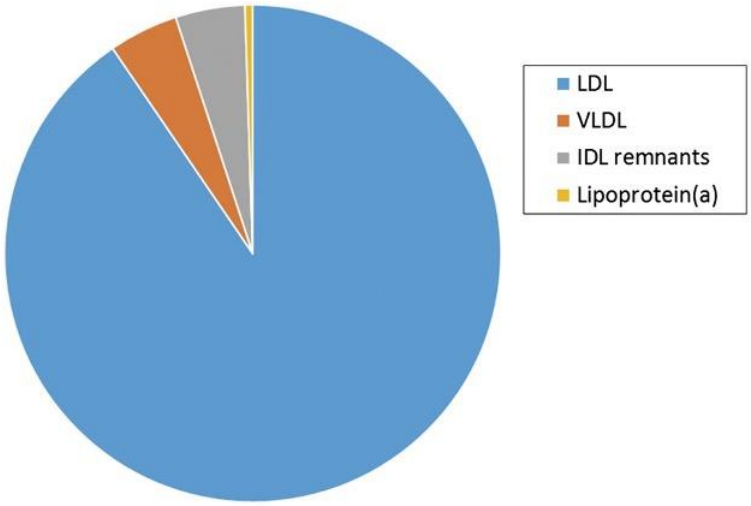

Figure 6 *Relative concentration of the various apoB-containing particles in circulating lipoproteins in normolipidaemic individuals. Adapted from "Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel" by Ference et.al, 2017, Eur Heart J. Reprinted with permission* **[6]**

The gold standard for measuring LDL-C is ultracentrifugation, a laborious and expensive method, so for almost 50 years, the Friedewald equation has been used as the primary method for estimating LDL-C in clinical practice. [35] The Friedewald equations subtracts HDL-C and a fixed ratio of triglycerides/2.2 from total cholesterol (TC) to estimate LDL-C (mmol/L). This leads to potential inaccuracies, specifically an underestimation of LDL-C when LDL levels are low, and triglycerides are elevated. [36] With treatment targets for LDL-C constantly being pushed to lower levels and hypertriglyceridemia becoming more common due to the increasing prevalence of obesity, diabetes mellitus, and insulin resistance, these issues with the Friedewald equation have led to the development of alternative LDL-assays and equations. Several direct enzymatic LDL-C assays are now in widespread clinical use and have been endorsed in the latest dyslipidemia guidelines as a more reliable measurement compared to the Friedewald equation, especially in patients with low LDL-C and elevated triglycerides. [37-39]

As an alternative to LDL-C is it possible to calculate non-HDL-C by subtracting HDL-C from total cholesterol (TC). This is an estimate of all the cholesterol carried by atherogenic apoB-containing particles, including TRLs, and has been suggested to be superior to LDL-C in ASCVD risk stratification. [40]

LDL-C and the LDL particle number (LDL-P) are usually highly correlated, but in certain circumstances – notably in high-risk patients with diabetes and metabolic syndrome – LDL-C

and LDL-P may become discordant due to the presence of small, cholesterol-depleted LDL-particles (sdLDL). [41] (**Figure 7**) *In-vitro* studies of sdLDL have suggested that these small LDL particles have increased atherogenic potential compared to larger LDL particles. The circulation time of sdLDL has been proposed to be increased due to impaired interaction with the LDL-R. [42] It has also been suggested that sdLDL has an increased susceptibility to undergo atherogenic modifications such as desialyation, glycation, and oxidation. [43, 44] Small, dense LDL particles have been shown to be more avidly taken up by macrophages, they are more easily transported into the arterial wall and have a greater binding potential to proteoglycans in the arterial intima. [44]

Due to this, it has been proposed that measurements of LDL particle number or LDL subfractions, including sdLDL, might improve ASCVD risk stratification and guide therapy in high-risk patients. [34]

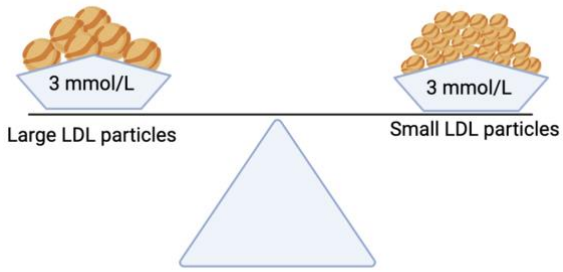

Figure 7 *Discordance between LDL-C and LDL-P due to a high number of smaller, cholesterol-depleted LDL particles. [45]*

LDL particles can be separated and measured based on different physicochemical properties depending on the protein purification technique. Gel electrophoresis (GE), nuclear magnetic resonance (NMR), ultracentrifugation (UC), or ion mobility (IM) are the most commonly used methods in published literature, but other methods also exist. [34]

These methods report different terms to describe LDL particles and their distribution, including, but not limited to: LDL subfractions, LDL subclasses, LDL particle and subfraction concentration, LDL particle number, LDL particle diameter, LDL peak diameter, and others. These terms describe overlapping attributes of the LDL subfractions. The potential for confusion is significant and, in this thesis, the generic term LDL subfractions will be used. The recognition of the different LDL subfractions has led to the description of two distinct patterns of phenotypes that are reported by most LDL subfractioning methods available today: Phenotype "A", with a predominance of large, buoyant LDL particles and phenotype "B", with a predominance of small, dense LDL particles, with the phenotype B being suggested as a more atherogenic phenotype. [46]

LDL-P is a measurement of the total number of LDL particles across all subfractions. As mentioned before, LDL-C and LDL-P may be discordant, and it has been suggested that LDL-P might be superior to LDL-C in ASCVD risk stratification because it more accurately reflects

the total number of atherogenic particles in plasma. LDL-P can be measured directly with both NMR and IM, while GE and UC do not take LDL-P into account. As each atherogenic LDL particle contains one single apoB molecule, quantifying apoB is a direct estimate of LDL-P. Methods for measuring apoB are standardized, inexpensive, and readily available at clinical laboratories. The analytical performances of the apoB measurement methods are superior to directly or indirectly measuring LDL-C or non-HDL-C. [47] In individuals with discordant LDL-P/apoB and LDL-C, only LDL-P/apoB is predictive of future ASCVD events, while LDL-C is not. [48, 49] Important dyslipidemia guidelines have begun to emphasize apoB in the latest editions and it is likely that this metric will become more important in ASCVD risk stratification in the future. [37, 39]

Targeting and lowering LDL-C significantly reduces the risk of future cardiovascular events across various patient populations, in primary or secondary prevention and regardless of the type of lipid lowering therapy used. [6] Despite unprecedented advances in ASCVD prevention there is still significant residual risk in patients with optimal risk factor management. In the latest trials of PCSK9 inhibitors (PSCK9i) the recurrent cardiovascular event rate remained high around 10% despite reductions in LDL-C of almost 50%. [50, 51] Several possible pathways beyond traditional risk factors have been suggested to play an important role in determining residual risk; inflammatory, genetic, pro-thrombotic, and metabolic risk factors. Among the metabolic risk factors, the sdLDL subfraction could be relevant.

4.4 HDL metabolism

The HDL particles consist of a lipid core with a mix of cholesterol, triglycerides, phospholipids, sphingolipids and free fatty acids. The protein surface is highly diverse and complex. The most abundant protein is apolipoprotein A-1 (apoA1) and unlike apoB-containing lipoproteins, the number of apoA1 proteins on each HDL particle varies. HDL is mainly produced and secreted from the liver and the small intestine as apoA1 in a lipid free state, apoA1 is then lipidated to form a particle with first a discoidal and later a spherical shape. ApoA1 makes up the framework of the HDL particle to carry cholesterol and phospholipid and acts as the acceptor of cholesterol from cells. Several transport proteins and enzymes are involved in the formation, maturing and catabolism of HDL particles. (**Figure 8**) Among the most important are:

ABCA1

Adenosine triphosphate-binding cassette sub-family A member 1 (ABCA1) has a pivotal role in the formation of pre-beta HDL by transporting cholesterol and phospholipids to lipid-free or lipid-poor apoA1. As ABCA1-mediated cholesterol efflux is the most important in HDL synthesis, several assays measuring ABCA1-mediated cholesterol efflux has been developed and these have been used in large, prospective studies showing that cholesterol efflux capacity is inversely associated with the risk of developing ASCVD. [52, 53] Patients with Tangiers disease, familial HDL deficiency, have a loss-of-function mutation in the ABCA1 protein and have absent or extremely low HDL levels in plasma, illustrating the rate-limiting importance of ABCA1-mediated cholesterol efflux in HDL biogenesis. Patients with Tangiers disease have moderately increased risk of premature ASCVD. [54]

LCAT

Lecithin-cholesterol acyltransferase (LCAT) is an important enzyme in lipoprotein metabolism with several functions. LCAT binds to HDL in plasma and converts cholesterol to cholesterol esters (CE), leading to the maturing of pre-beta HDL to larger, spherical HDL particles. LCAT deficiency is a potentially severe hereditary metabolic disease that causes very low HDL-C levels and two distinct phenotypes: Fisheye disease (FED) and familial LCAT deficiency (FLD). While both phenotypes have very low HDL-C levels, the risk of premature cardiovascular disease is predominantly seen among those with FED [55]

SR-B1

Scavenger receptor class B type 1 (SR-B1) regulates the bidirectional transport of free cholesterol in cells. In the liver and steroidogenic tissues SR-B1 facilitates the uptake of CE from HDL and endocytosis of HDL particles for production of steroid hormones (steroidogenic tissue) and delivery of cholesterol back to the liver.

CETP

Cholesteryl ester transport protein (CETP) is a protein produced in the liver and adipose tissue whose main function is replacing cholesterol esters in HDL with triglycerides from VLDL and LDL particles, to form triglyceride-rich HDL. Triglyceride-rich HDL is an important substrate of hepatic lipase (HL), an enzyme that promotes HDL clearance. CEPT inhibitors are a class of drugs that substantially increase HDL by inhibiting HDL clearance. Several of these drugs have been studied in clinical trials aiming at reducing cardiovascular risk, but despite significant increases in HDL-C the results have generally been neutral or even caused a marked increase in CVD mortality in one study. [56]

In addition to apoA1, almost 100 different proteins with different properties, such as anti-oxidative and anti-inflammatory functions, have been associated with HDL. [57] These proteins are not evenly distributed among HDL particles and the proteins may also be present in different isoforms with disparate biological properties. Several of the suggested atheroprotective functions of HDL are related to these surface proteins on the HDL particle.

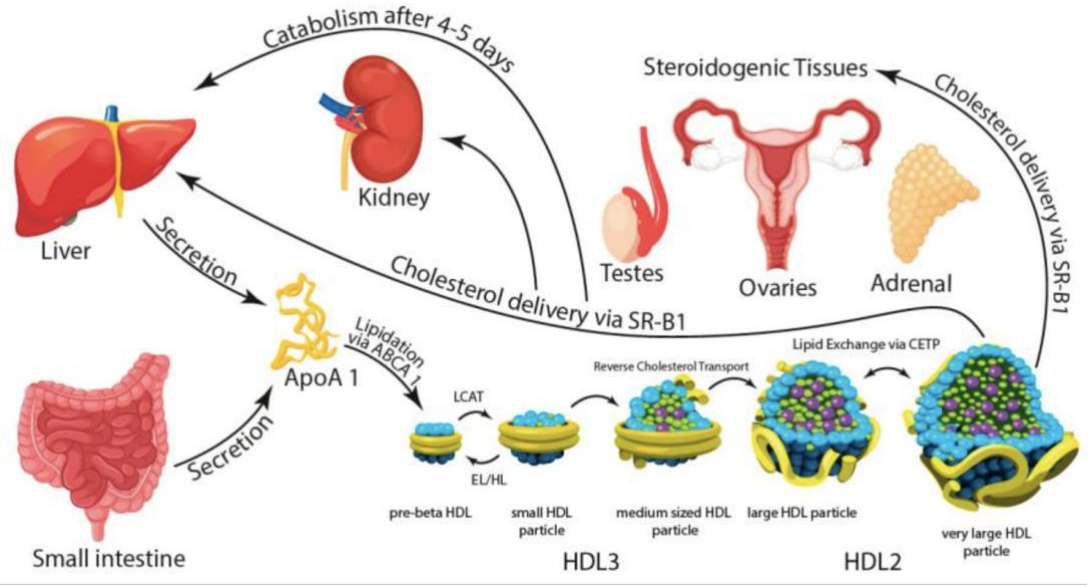

Figure 8 *Apolipoprotein A-1 (ApoA1) is synthesized in the liver and small intestine and secreted in a lipid-free state. Lipidation of apoA1 via adenosine triphosphate-binding cassette sub-family A member 1 (ABCA1)-mediated cholesterol efflux initiates the assembly of the High density lipoprotein (HDL)-particle, creating discoidal prebeta HDL. Lipidation of prebeta-HDL via lecithin-cholesterol acyltransferase (LCAT) matures it into a larger, spherically shaped particle. Further re-modelling and metabolism of HDL is a complex process that involves several enzymes. Reverse cholesterol transport results in the accumulation of cholesterol and an increase in size of the HDL particle. Cholesterol delivery via scavenger receptor class B type 1 (SR-B1), interaction with endothelial or hepatic lipases (EL/HL), or lipid exchange via cholesteryl ester transport protein (CEPT) to apoB-containing lipoproteins will remodel the HDL particle further. After a life cycle of 4-5 days HDL particles are catabolized in the liver or kidneys. Adapted from "High density lipoprotein subfractions – Much ado about nothing or clinically relevant" by Knut Tore Lappegård et al., 2021, Biomedicines. Reprinted with permission.*

4.5 Metrics of HDL quantity and function

The standard measurement of HDL in clinical practice is HDL-C, a measurement of the cholesterol content within all HDL particles. HDL subfractions has traditionally been divided in two using ultracentrifugation; HDL2 and HDL3, with HDL2 being the larger, more lipid-rich subfraction and HDL3 being the smaller, denser HDL particle. The same methods for separating LDL subfractions can be used for separating HDL particles, usually reporting small, intermediate and large HDL based on size.

Cholesterol efflux describes the uptake of cholesterol from cells, most importantly macrophages, to HDL particles and is the first key step of RCT. Several different assays measuring the cholesterol efflux capacity (CEC) from macrophages to HDL particles have been developed. [58] CEC has been shown to be inversely associated with the risk of developing hard cardiovascular outcomes in 3 large prospective studies [52, 53, 59] and CEC was independently associated with the presence of ASCVD in a cohort of patients with heterozygous FH. [60] As CEC assays are laborious and difficult to standardize, they are not suitable for routine use in a clinical setting. CEC can however be used as a reference to develop other biomarkers that can be measured in clinical laboratories. *In-vitro* studies of the cholesterol efflux capacity of different HDL subgroups have shown that smaller HDL particles seem to be more efficient in mediating cholesterol efflux compared to larger HDL

particles. [61] An algorithm based on the information from NMR-measured HDL subfractions that estimates CEC has been developed. Estimated-CEC showed good correlation with *in-vitro* measured CEC and estimated CEC was found to be associated with a lower risk of incident CVD-events in a population-based study of 7603 individuals. [62] This suggests that measuring subfractions of HDL might provide useful insight in evaluating HDL function.

Paranoxonase-1 (PON1) is an HDL-associated surface protein with antioxidant properties. Reduced HDL-PON1 activity has been associated with an increased risk of CVD, [63, 64] suggesting it could be a potential biomarker for CVD. [65] PON1-activity has been shown to be more strongly correlated with small and medium-sized HDL particles compared to large HDL particles, [66, 67] further suggesting that HDL subfractions could provide useful insight into HDL function.

Serum amyloid A1 (SAA1) is an acute-phase protein that increases during inflammation and harbors pro-inflammatory effects. [68] SAA1 in circulation is predominantly bound to HDL, and increased SAA1 is associated with CVD mortality, possibly by impairing the anti-inflammatory properties of HDL. [68, 69] The ratio of SAA1 and PON1 activity has been proposed as a possible marker for dysfunctional and pro-inflammatory HDL [68]

4.6 LDL and HDL subfractions and their association with increased risk of cardiovascular disease

Several prospective observational studies and randomized trials of lipid-lowering therapies have found significant associations between elevated sdLDL and increased risk of CVD. Ip et al. published a systematic review of 52 such studies in 2009, reporting that 37 of these trials found a statistically significant association between a metric of LDL subfractions and various cardiovascular outcomes. [70] However, only 26 of these studies adjusted for the standard lipoprotein metrics, and of those, 12 reported statistically significant associations between LDL subfractions and CVD outcomes.

Our research group published a review article on LDL subfractions in 2018 [34] and summarized the results from 6 large prospective, population-based studies on LDL subfractions or metrics of LDL particle number and cardiovascular outcomes published after 2009. [41, 71-75] (**Figure 9**) These studies are not directly comparable as they have used different methods for measuring or quantifying LDL subfractions or LDL particle number, but several LDL subfractions and metrics of LDL-P were independently associated with increased risk of CVD.
A systematic review and meta-analysis on the association between sdLDL-C or sdLDL particle concentration and coronary heart disease (CHD) was published in 2020 and included a total of 30,628 subjects and 5,693 incident CHD events from 21 studies. [76] Both higher sdLDL and sdLDL-C levels were significantly associated with a higher risk of CHD; the pooled estimate for the high vs. low categorization of sdLDL was 1.36 (95% CI: 1.21, 1.52) and 1.07 (95% CI: 1.01, 1.12) for comparing the top quartiles versus the bottom of sdLDL-C, with several studies suggesting a dose-response relationship.

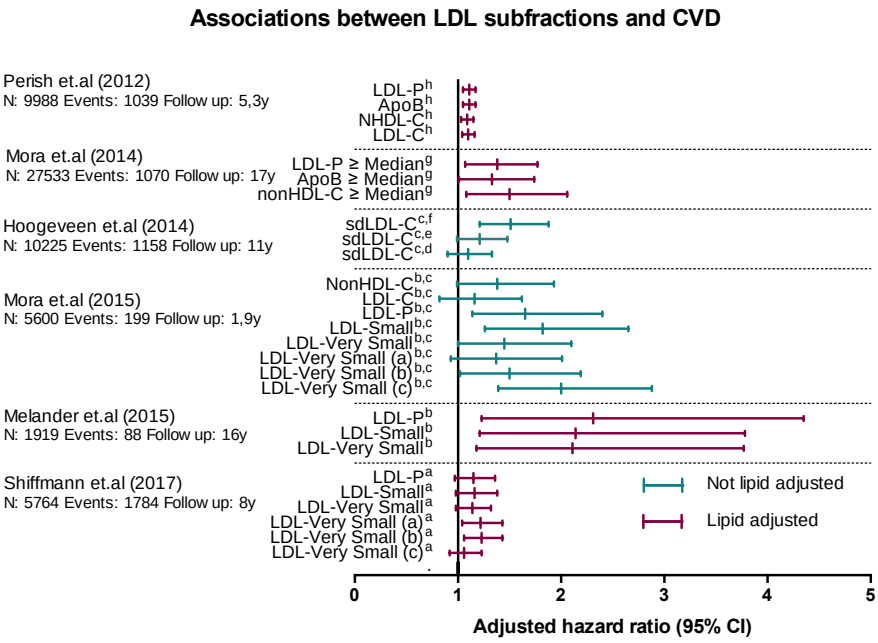

Figure 9 *Associations between low-density lipoprotein (LDL) subfractions and cardiovascular disease (CVD) reported in prospective population-based studies published after 2009 and adjusted for confounding factors, such as age, smoking, gender, hypertension, etc. a Top vs. bottom quartile analysis. b Top vs. bottom tertile analysis. c Not significant in a model adjusted for lipids. d 1st vs. 2nd quartile analysis. e 1st vs. 3rd quartile analysis. f 1st vs. 4th quartile analysis. g Associations with CVD in patients with discordant levels of LDL particle number (LDL-P), apolipoprotein B (apoB), non-high density lipoprotein cholesterol (non-HDL-C) (≥median), and LDL cholesterol (LDL-C) (<median). h Hazard ratio (95% CI) pr. 1 SD higher. Hazard ratios for the outcome "major occlusive event" in the placebo arm are depicted in the graph. Adapted from "CVD Risk Stratification in the PCSK9 Era: Is there a role for CVD Subfractions" by Kjellmo et al., 2018, Diseases. Reprinted with permission.*

Statistically significant associations between different HDL subfractions and risk of developing cardiovascular disease have been reported in several prospective studies, but the results are conflicting on which subfraction is the most important in CVD risk stratification. [57, 77-80] Obesity and diabetes are associated with an HDL-subfraction composition skewed toward higher levels of small HDL-particles (HDL3) and lower levels of the larger HDL particles (HDL2), [81-84] and this could be an important confounding factor in the epidemiological studies that suggest that the larger HDL particles (HDL2) are superior to HDL-C or small HDL (HDL3) in CVD risk prediction. Later trials and a recent meta-analysis have suggested that the small HDL subfraction (HDL3) might be superior to other HDL metrics in risk stratification. [77, 78]

4.7 Therapeutic modification of LDL and HDL subfractions, SAA1, CEC, and PON1 activity

Knowledge about how to target and modulate the LDL and HDL subfractions is lacking. Statins have been suggested to be less effective in lowering sdLDL compared to larger LDL particles [85]. Still, the studies are conflicting, and there seems to be a significant variation between the available statins. [86] Most studies evaluating statins' effects on LDL subfractions were conducted decades ago during the era of placebo-controlled statin trials, and ultracentrifugation was the preferred method to measure the sdLDL subfraction at that

time.
Fibrates have been suggested to be more effective in lowering sdLDL compared to other interventions, but the studies are conflicting [87, 88] and this is clinically less relevant today as fibrates are no longer recommended in ASCVD prevention. Compared to statins, a PCSK9 inhibitor (Repatha ®) was effective in lowering sdLDL in 54 patients with acute coronary syndrome (ACS). [89] A couple of studies evaluating the effects of bariatric surgery on LDL subfractions have been published; gastric banding did not affect sdLDL after 13 months in 20 obese patients [90] and Roux-en-Y gastric bypass surgery significantly reduced sdLDL-apoB measured by ultracentrifugation at 6 and 12 months. [91]

CETP inhibitors have been shown to significantly increase the large HDL particles and the smallest, pre-beta HDL particles, [92] without affecting hard cardiovascular outcomes. Statins are known to produce a slight increase in HDL-C (approximately 5-10%) due to an increase in the large HDL subfraction, [93] but there is no evidence to suggest that this effect accounts for any of the benefits of statins in ASCVD prevention. Other published interventional trials on modification of HDL subfractions have small sample sizes and measure secondary, non-clinical outcomes.

The CANTOS trial found that reducing vascular inflammation using canakinumab, a monoclonal antibody against IL-1β, decreased cardiovascular events without affecting lipids. [94] Increased levels of SAA are associated with an increased risk of ASCVD, and SAA exhibits several pro-inflammatory effects that could contribute to the development of atherosclerosis. [68] This suggests that SAA could be a therapeutical target to consider in ASCVD prevention, but no interventional trials have been conducted so far.
While evidence suggests that CEC and Paraoxonase 1-activity might be useful as a risk factor for CVD, there is currently no evidence available to suggest that targeting these metrics will improve outcomes.

5 Aims of the thesis

The first aim of this thesis was to investigate the baseline composition of LDL subfractions in high-risk populations measured by a simple, in-house system (Lipoprint ®) and the effects on LDL subfractions by interventions that can or have been suggested to reduce CVD risk.

The second aim of this study was to investigate the baseline composition of HDL subfractions and metrics of HDL function (cholesterol efflux capacity, HDL-PON1 activity, and SAA1) in high-risk patients and the effects by interventions that can or have been suggested to reduce CVD risk on these metrics.

6 Hypotheses

Paper 1:

We hypothesized that morbidly obese patients would exhibit significantly elevated levels of small dense LDL (sdLDL) when compared to control subjects. Furthermore, we anticipated that both lifestyle intervention and bariatric surgery would lead to a substantial reduction in sdLDL. Additionally, we expected the lifestyle intervention and bariatric surgery to result in increased levels of all HDL subfractions, decreased levels of serum amyloid A1 (SAA1), increased paraoxonase 1 (PON1) activity, and enhanced cholesterol efflux capacity (CEC).

Paper 2:

Our hypothesis suggested that lipoprotein apheresis would effectively decrease all LDL and HDL subfractions, as well as metrics of HDL function, when compared to baseline measurements. In contrast, we anticipated that the PCSK9 inhibitor, evolocumab, would reduce LDL subfractions but would not have an impact on HDL subfractions or metrics of HDL function, relative to baseline values.

Paper 3:

We hypothesized that a combination of a statin and ezetimibe would significantly reduce all LDL subfractions, without affecting HDL subfractions, when compared to a placebo.

Paper 4:

Our hypothesis proposed that n-3 polyunsaturated fatty acids (n3-PUFA) would modify the composition of HDL subfractions and improve markers of HDL function, such as SAA1, PON1 activity, and CEC. However, we did not expect n3-PUFA to influence LDL subfractions.

7 List of papers

Paper 1:

Kjellmo, C. A.; Karlsson, H.; Nestvold, T. K.; Ljunggren, S.; Cederbrant, K.; Marcusson-Stahl, M.; Mathisen, M.; Lappegard, K. T.; Hovland, A., Bariatric surgery improves lipoprotein profile in morbidly obese patients by reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity. *J Clin Lipidol* **2018,** *12* (1), 193-202.

Paper 2:

Lappegard, K. T.; Kjellmo, C. A.; Ljunggren, S.; Cederbrant, K.; Marcusson-Stahl, M.; Mathisen, M.; Karlsson, H.; Hovland, A., Lipoprotein apheresis affects lipoprotein particle subclasses more efficiently compared to the PCSK9 inhibitor evolocumab, a pilot study. *Transfus Apher Sci* **2018,** *57* (1), 91-96.

Paper 3:

Kjellmo, C. A.; Pop, G.; Lappegard, K. T.; Hovland, A., Intensive lipid-lowering therapy reduces large, but not small, dense low-density lipoprotein particles measured by gel electrophoresis, in elderly patients with atrial fibrillation. *Eur J Prev Cardiol* **2019,** *26* (18), 2017-2018.

Paper 4:

Hande, L. N.; Kjellmo, C.; Pettersen, K.; Ljunggren, S.; Karlsson, H.; Cederbrant, K.; Marcusson-Stahl, M.; Hovland, A.; Lappegard, K. T., Effect of N-3 Polyunsaturated Fatty Acids on Lipid Composition in Familial Hypercholesterolemia: A Randomized Crossover Trial. *Biomedicines* **2022,** *10* (8).

8 Materials and Methods

8.1 Study subjects and study design

The patients included in our studies were enrolled in 4 different clinical intervention trials that the Cardiology research group at Nordland Hospital, Bodø had initiated or collaborated on.

Paper 1 was a collaboration with Torunn K. Nestvold at the Department of Surgery, Nordland Hospital Bodø. Thirty-four morbidly obese patients were evaluated before and after lifestyle changes and then one year after bariatric surgery. They were compared with 17 lean subjects. LDL and HDL subfractions, standard lipoprotein metrics, serum amyloid A (SAA), serum paraoxonase-1 (PON1)-activity, and macrophage cholesterol efflux capacity (CEC) were assessed.

Paper 2 was a pilot study of two women and one man with genetically confirmed FH. They had angiographically verified coronary artery disease, were intolerant to statins due to myalgia, and did not take any type of lipid-lowering medication. The patients had been in lipoprotein apheresis on average for 11 years (11-13 years) at the Nordland Hospital, Bodø. LDL and HDL subfractions, serum amyloid A (SAA), serum paraoxonase-1 (PON1)-activity, and macrophage cholesterol efflux capacity (CEC) was assessed during apheresis and after apheresis was switched to a PSCK9 inhibitor (Evolocumab).

Paper 3 was a collaboration with the cardiology research group at the Radboud University Medical center in Nijmegen, Netherlands. Thirty elderly patients with atrial fibrillation (69-85 years) were randomized to double-blind treatment with atorvastatin 40 mg plus ezetimibe 10 mg (n = 14) or double placebo (n = 16). No patients with known coronary heart disease were included, as determined by clinical history, 12-lead electrocardiogram, and transthoracic echocardiography. All were anticoagulated with warfarin (target INR 2.5-3.5). LDL and HDL subfractions and LDL-C were assessed at inclusion and after six months of treatment.

Paper 4 was a double-blind, placebo-controlled cross-over study with 34 FH patients on statin treatment (mean age 46.6 years) conducted at Nordland Hospital, Bodø. In random order, all individuals were treated for three months with high-dose n-3 PUFA (2 g ×2) and three months placebo (olive oil, 2 g ×2), separated by a 3-month washout period. LDL and HDL subfractions, standard lipoprotein metrics, serum amyloid A (SAA), serum paraoxonase-1 (PON1)-activity, and macrophage cholesterol efflux capacity (CEC) were assessed.

8.2 Blood sampling procedures and laboratory measurements

8.2.1 Blood sampling and standard lipoprotein measurements

Paper 1, *Bariatric surgery improves lipoprotein profile in morbidly obese patients by*

reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity.:
Fasting blood samples were obtained by standard venipuncture on three occasions: At first admission, the day before surgery (after three months of lifestyle intervention), and one year after surgery. Routine blood analyses were performed on the sampling day at Nordland Hospital's laboratory. Serum, EDTA, and citrate plasma were frozen in aliquots at -80° C and analyzed in batch at the end of the study. TG, TC, LDL- and HDL-C serum levels were measured using an ADVIA®1800 system (Siemens Medical Solutions Diagnostics, Japan). ApoB and apoA-1 were measured using an ADVIA 1800 system from Siemens Healthcare Diagnostics (Deerfield, IL, USA)

Paper 2, *Lipoprotein apheresis affects lipoprotein particle subclasses more efficiently compared to the PCSK9 inhibitor evolocumab, a pilot study:*
Fasting blood samples were obtained by venipuncture immediately before and after apheresis, and before each injection with PCKS9-inhibitor at weeks 1, 3, 5 and 7. Serum LDL and HDL cholesterol, Lp(a), and triglycerides were analyzed on ADVIA 1800 from Siemens Healthcare Diagnostics (Deerfield, IL) immediately after collection. The historically highest, untreated lipid profile was retrieved from the digital laboratory system. Serum, EDTA plasma and citrate plasma were frozen in aliquots at -80° C and analyzed in batch at the end of the study.

Paper 3, *Intensive lipid lowering therapy reduces large, but not small, dense low-density lipoprotein particles measured by gel electrophoresis, in elderly patients with atrial fibrillation:*
Fasting blood samples were obtained by venipuncture (vacutainer tubes) at baseline and at the 6 months follow-up appointment. All blood samples were taken in the morning hours between 10:00 and 12:00 AM and after at least 1 hour of fasting. Blood samples were frozen in aliquots at -80° C and analyzed in batch. Serum levels of LDL-C were measured using an ADVIA®1800 system (Siemens Medical Solutions Diagnostics, Japan).

Paper 4, *Effect of N-3 Polyunsaturated Fatty Acids on Lipid Composition in Familial Hypercholesterolemia: A Randomized Crossover Trial*:
Fasting blood samples were obtained by venipuncture (vacutainer tubes) at baseline, after the first treatment period, after washout, and after the second treatment period. Serum tubes were centrifugated at 2000 g for 10 min. The citrate vacutainers had 3.2% sodium citrate and were centrifugated at 3000 g for 20 min at 4 °C. Serum levels of triglycerides and total, LDL, and HDL cholesterol were analyzed on an ADVIA 1800 system (Siemens Medical Solutions Diagnostics, Tokyo, Japan). The procedure was performed according to the manufacturer. Apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB) were measured by the ADVIA 1800 system (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

8.2.2 Lipoprint ® LDL and HDL subfractions

LDL and HDL subfractions were determined with the Lipoprint® system (Lipoprint LDL & Lipoprint HDL system, Quantimetrix Corporation, Redondo Beach, CA). Lipoprint® is an in-house diagnostic device for measuring LDL and HDL subfractions using polyacrylamide gel electrophoresis.

Gel electrophoresis is a technique to separate proteins based on size and charge. The gel used in electrophoresis is usually made of agarose or polyacrylamide which forms a solid, homogenous, and porous matrix. A buffer is added to the gel to provide ions for the passage of the electric current. By applying an electric field to the gel, the proteins added at the top of the gel will move through due to an electromotive force. Separation occurs because they will move at different rates depending on their size and charge, a phenomenon called sieving. **(Figure 10**) Smaller proteins will travel further down the gel compared to larger ones, positively charged proteins will migrate toward the cathode and negatively charged proteins will migrate toward the anode. After the electrophoresis is done, molecules of the same size and charge will form a band in the gel. (**Figure 11**)

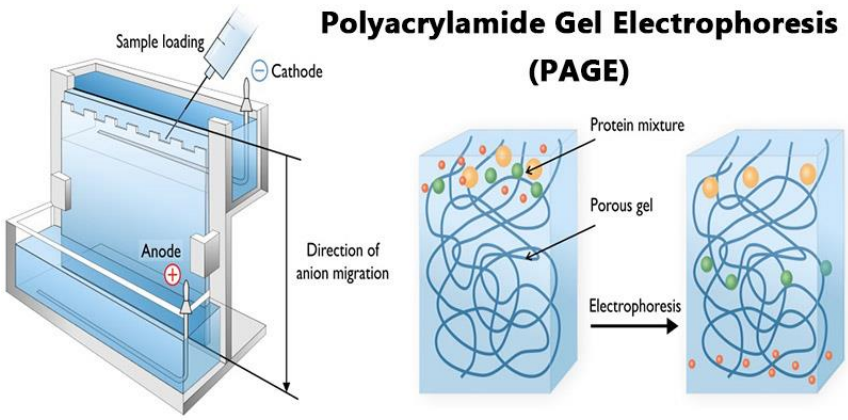

Figure 10 PAGE

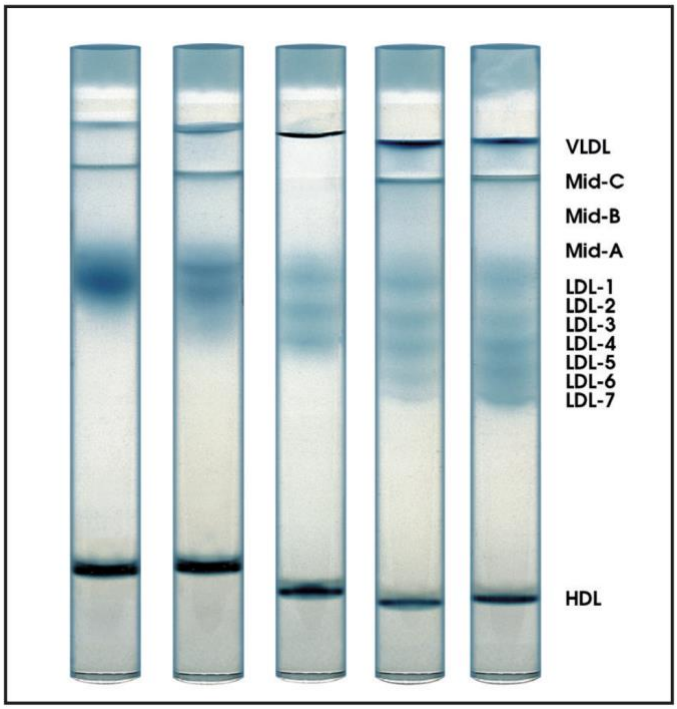

Figure 11 *LDL bands after electrophoresis from 5 individuals using the Lipoprint LDL system ®*

The Quantimetrix Lipoprint LDL and HDL system use a loading gel containing Sudan Black B dye to stain the lipoproteins. This dye binds proportionally to the relative amount of cholesterol in each lipoprotein. [95] After electrophoresis, the gels are analyzed using densitometry, a quantitative measurement of optical density. The densitometric scan (**Figure *12*** and **Figure *13***) is then analyzed using computer software (Lipoware ®) to calculate the area under the curve for each subfraction. [96] Total-Cholesterol (Lipoprint LDL) and HDL-C (Lipoprint HDL) in the sample are measured independently and added to the software. Using the relative area for each lipoprotein fraction, the software then calculates the concentration of cholesterol of each fraction.

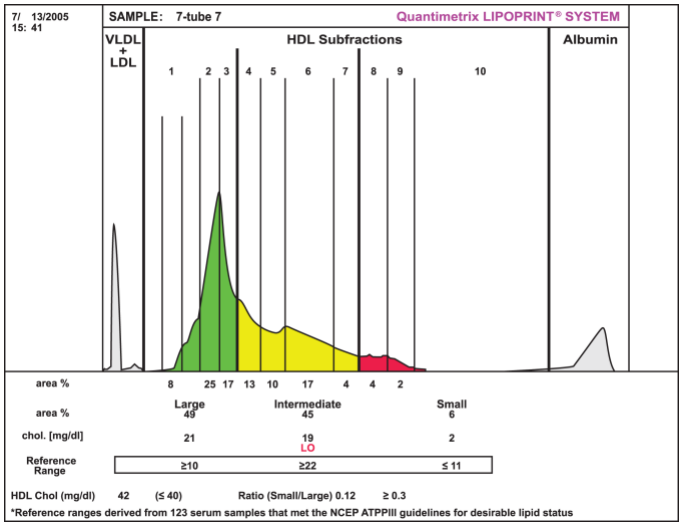

Figure 12 *Densitometric scan of the HDL subfractions using the Lipoprint HDL system®*

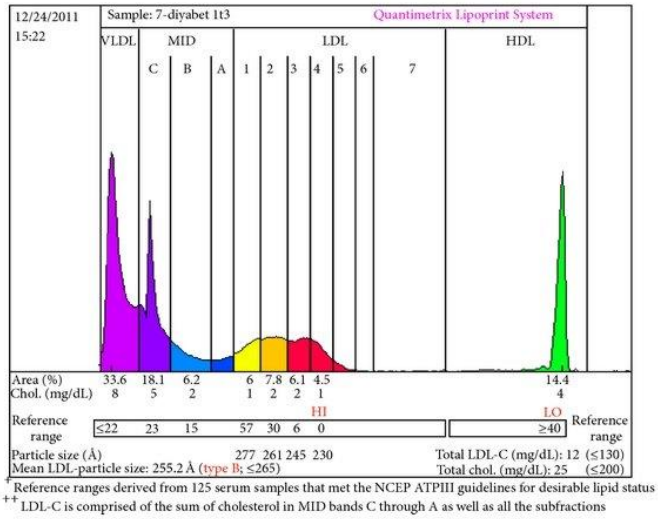

Figure 13 *Densitometric scan of the LDL subfractions using the Lipoprint LDL system®*

Reference ranges for the LDL and HDL subfractions were provided by the manufacturer and are based on 125 serum samples from volunteers who met the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) Guidelines for a desirable lipid status. [97]

We ran two samples of quality control with each batch of patient samples: LipoSure, the quality control from the manufacturer, and one sample of a serum mixture from the Blood Bank of Nordland Hospital. Running one batch of 10 patient samples and two quality controls requires one dedicated technician and takes approximately 3 hours. The cost of each test is approximately 20$ or 200 NOK.

LDL subfractions are divided into LDL-1 and LDL-2 (large, buoyant LDL, lbLDL) and LDL-3 to LDL-7 (small, dense LDL, sdLDL). Based on these results, a lipoprotein profile is provided; Type A (predominance of lbLDL), intermediate, and type B (predominance of sdLDL). HDL subfractions are divided into HDL 1-3 (large HDL), HDL 4-7 (intermediate HDL), and HDL 8-10 (small HDL).

8.2.3 PON1 arylesterase activity

PON1 arylesterase activity was measured in citrate plasma. Briefly, plasma was diluted 1:80 with a salt buffer (20 mM Tris–HCl and 1.0 mM $CaCl_2$). A triplicate of 20 µl diluted plasma was added to the wells in a UV-transparent 96-well plate (Sigma-Aldrich). 200 µl of phenyl acetate solution, containing 3.26 mM phenyl acetate in salt buffer, was added to each well, and the absorbance of produced phenol was measured at 270 nm with 250 nm as background in a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). The initial period when the reaction was linear was used for the calculation of activity, expressed as U/ml, using an extinction coefficient of phenol of 1310 M−1 cm1. This analysis was conducted by our collaborators at Linköping University and the Swedish Toxicology Sciences Research Center in Södertälje, Sweden.

8.2.4 SAA1 ELISA

To investigate the acute phase response by SAA, plasma SAA1 levels were measured by an ELISA (DY3019-05, R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. In short, citrate plasma was added to the plate and incubated for 2 hours at room temperature. Following wash, a detection antibody was added and incubated for 2 hours. The plate was washed, and streptavidin-horseradish peroxidase was added, followed by incubation for 20 min. The plate was then washed a final time before a substrate solution was added before 20 min incubation. At the end of the incubation, stop solution was added, and absorbance was measured at 450 nm with correction at 570 nm using a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). This analysis was conducted by our collaborators at Linköping University and the Swedish Toxicology Sciences Research Center in Södertälje, Sweden.

8.2.5 Cholesterol efflux capacity

Cholesterol efflux was measured with a commercial kit from Sigma-Aldrich (MAK192) according to the description. Briefly, a human monocyte cell line, THP-1, was differentiated into macrophages with 10 ng/ml phorbol myristate acetate (PMA) for 24 hours at 37° and 5% CO2 in a 96-well plate. The PMA-containing medium was replaced with complete cultivation medium (RPMI1640 including 10% fetal bovine serum, 2 mM Glutamine) and incubated for another 30 hours. The serum-containing medium was removed and then washed with serum-free medium. A reaction mix, containing equilibration buffer and fluorescence-labeled LDL, was added to the cells and incubated for 16 hours. The reaction mix was removed, and wells were washed with serum free medium. Patient serum samples were precipitated with a reaction mix from the kit, and the clear supernatant was added to the plate and incubated for 5

hours. After the incubation, supernatants were transferred to a new plate, and the fluorescence was measured (482 ex/515 em). The cell layer was solubilized with a cell lysis buffer, incubated for 30 minutes on a shaker. The cell lysate was then transferred to the plate with supernatants, and the fluorescence of the mixture was measured. Percent efflux was calculated as follows: 100 x fluorescence intensity of the medium / x fluorescence intensity of the medium and cell lysate. This analysis was conducted by our collaborators at Linköping University and the Swedish Toxicology Sciences Research Center in Södertälje, Sweden.

8.3 Statistics

Paper 1

A repeated measures one-way analysis of variance (RM One-way ANOVA) was used to calculate the longitudinal effects of the lifestyle changes followed by surgery on the different lipids, LDL/HDL subfractions, and parameters of HDL function and composition when appropriate. An unpaired t-test was used to calculate the differences between patients and controls. If the data was not normally distributed, non-parametric tests were applied; the Friedman test for the longitudinal effects in the patients and the Mann-Whitney test for testing differences between patients and controls. All tests were two-tailed, and results with a $p<0.05$ were considered statistically significant. Analyses were performed using PRISM 6 (Graph Pad Software Inc, La Jolla, CA).

Paper 2

Numerical data are presented with mean and standard deviation (SD), age and duration of treatment are presented as mean and range. To calculate the longitudinal effects of the evolocumab treatment (week one to week seven), a repeated measures one-way analysis of variance (RM one-way ANOVA) was used. A paired t-test was used to compare levels before and after lipoprotein apheresis treatment (week 0) and before apheresis vs. after the last evolocumab injection. All tests were two-tailed, and results with a $p < 0.05$ were considered statistically significant. Analyses were performed using PRISM 6 (Graph Pad Software Inc, La Jolla, CA, USA).

Paper 3

The baseline values and values after 6 months in the treatment group and placebo group were compared by an unpaired t-test, after normality was assessed by the Shapiro–Wilk normality test. The differences between baseline values and after six months in the treatment and placebo group were compared by a paired t-test. A 2-tailed p-level < 0.05 was considered statistically significant. Analyses were performed using PRISM 6 (Graph Pad Software Inc, La Jolla, CA, USA).

Paper 4

Statistical work was performed using Prism version 8.4.3 (GraphPad Software Inc., La Jolla, CA, USA). Before the trial registration, a sample size calculation based on the primary outcome was performed. The period effect was tested by a two-sample t-test or Mann–Whitney test comparing the differences between the treatments in the two sequence order groups. Treatment–period interaction was evaluated by a t-test or a Mann–Whitney test comparing the average response in each sequence order group. The baseline values in the treatment sequence groups are presented as mean and standard deviation if normally distributed or as median and first and third quartile if not normally distributed. The normality in differences between treatment periods was assessed by the Shapiro–Wilk normality test. The values after n-3 PUFAs treatment and after placebo was compared by a paired t-test or Wilcoxon matched-pairs signed-rank

test when appropriate. Confidence intervals (95%) were computed when the differences were symmetrically distributed. Correction for multiple comparisons was not performed. A 2-tailed p-level < 0.05 was considered statistically significant.

9 Summary of Results

9.1 Paper 1

Bariatric surgery improves lipoprotein profile in morbidly obese patients by reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity

In this paper, we assessed changes in lipoprotein profile and HDL function in 34 morbidly obese patients who first underwent a lifestyle intervention and then bariatric surgery. The average BMI at inclusion was 44,4 kg/m^2. They were eligible for surgery when they reached a weight loss from the lifestyle intervention of 10% of body weight. Two surgical methods for bariatric surgery were used: 27 patients with a BMI < 50 kg/m^2 underwent laparoscopic Roux-en-Y gastric bypass, and 7 patients with a BMI > 50 kg/m^2 underwent biliopancreatic diversion with duodenal switch. The control group consisted of 17 patients with a BMI <28 kg/m^2 and no established cardiovascular disease, scheduled to undergo elective laparoscopic cholecystectomy or laparoscopic fundoplication.

Anthropometric characteristics of the controls and the patients at baseline, after the lifestyle intervention, and 12 months after bariatric surgery are presented in Table 1. The average total weight loss (TWL) at the 1-year follow-up after surgery was 44,4kg. Seventeen patients had diabetes mellitus type II at inclusion, and at the 1-year follow-up after surgery, 5 patients still had HbA1c > 6,5% and/or had to remain on anti-diabetic medication. Eight patients used statins at the inclusion of the study, and 4 patients remained on statins at the 1-year follow-up.

Table 1 The effect of lifestyle changes and bariatric surgery on anthropometric characteristics compared with controls

Variable	Inclusion	Lifestyle intervention	12 mo after surgery	Controls
n, No.	34	34	34	17
Age, median (range)	43 (30–58)			45 (27–70)
Female	20 (59%)			12 (71%)
Weight (kg), mean ± SD	130.4 ± 24.7	117.4 ± 21.9	86.0 ± 17.3	73.0 ± 7.5
BMI (kg/m^2), mean ± SD	44.8 ± 6.9	40.1 ± 5.7	29.3 ± 4.2	25.2 ± 2.0
TWL (kg), mean ± SD		13 ± 5.7	44.4 ± 18.0	
Diabetes, No. (%)	17 (50)		5 (15)	1 (6)
Hypertension, No. (%)	14 (41)		N.D	3 (18)
Statins, No. (%)	8 (24)		4 (12)	2 (11)
Smoking, No. (%)	3 (9)		3 (9)	1 (6)
HbA1c% ± SD	6.5 ± 1.1	N.D	5.7 ± 0.5	5.4 ± 0.2

BMI, body mass index; HbA1c, glycated hemoglobin; N.D, not determined; TWL, total weight loss.

Table 1 *Adapted from* "*Kjellmo, C. A.; Karlsson, H.; Nestvold, T. K.; Ljunggren, S.; Cederbrant, K.; Marcusson-Stahl, M.; Mathisen, M.; Lappegard, K. T.; Hovland, A., Bariatric surgery improves lipoprotein profile in morbidly obese patients by reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity." J Clin Lipidol* ***2018,*** *12 (1), 193-202.*

Compared with controls, the morbidly obese patients had lower HDL-C, higher triglycerides, and similar levels of LDL-C and apoB at inclusion. The lifestyle intervention resulted in significant reductions in LDL-C, apoB, triglycerides, lbLDL, sdLDL, HDL-C, and the small HDL subfraction, while large HDL subfraction was increased. At the 1-year follow-up, bariatric surgery resulted in sustained reductions in LDL-C, TG, apoB, and the lbLDL

subfraction compared to at inclusion. HDL-C was increased due to increases in the large HDL subfraction, while the small HDL subfraction was unchanged compared to inclusion. (**Figure 14 and Figure 15**) The morbidly obese patients had significantly lower baseline PON1 arylesterase activity compared to controls. The lifestyle intervention reduced significantly reduced PON1 activity, and bariatric surgery had no further effect. The patients had significantly higher baseline levels of SAA compared to controls. After the lifestyle intervention, there was a significant reduction in SAA in the patients, and bariatric surgery had no further effect on SAA. The patients had a higher SAA/PON1 ratio at inclusion compared to controls. The lifestyle intervention significantly reduced SAA/PON1 ratio, and a further reduction in SAA/PON1 ratio was observed after bariatric surgery. We found no differences in cholesterol efflux capacity between patients and controls at inclusion, and no effect from the lifestyle intervention or bariatric surgery. (**Figure 16**)

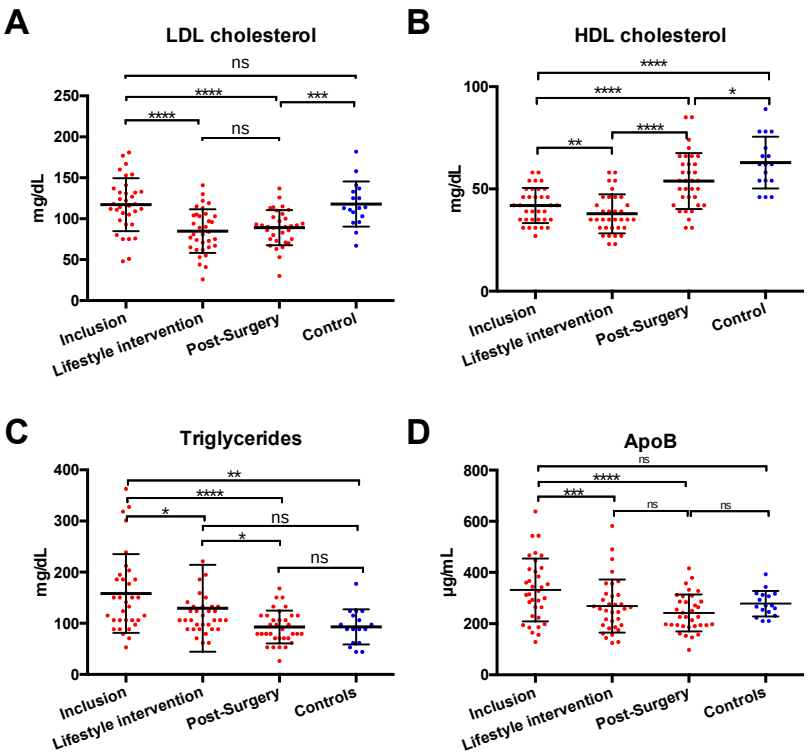

Figure 14 Serum levels of (A) LDL cholesterol, (B) HDL cholesterol, (C) triglycerides (TGs), and (D) apoB at inclusion, after a lifestyle intervention (ca. three months) and one year after bariatric surgery in 34 morbidly obese patients, and at inclusion for controls. Values are mean with standard deviation. *P, .05, **P , .01, ***P , .001, ****P , .0001, ns, not statistically significant. *Adapted from "Kjellmo, C. A.; Karlsson, H.; Nestvold, T. K.; Ljunggren, S.; Cederbrant, K.; Marcusson-Stahl, M.; Mathisen, M.; Lappegard, K. T.; Hovland, A., Bariatric surgery improves lipoprotein profile in morbidly obese patients by reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity." J Clin Lipidol* ***2018,*** *12 (1), 193-202*

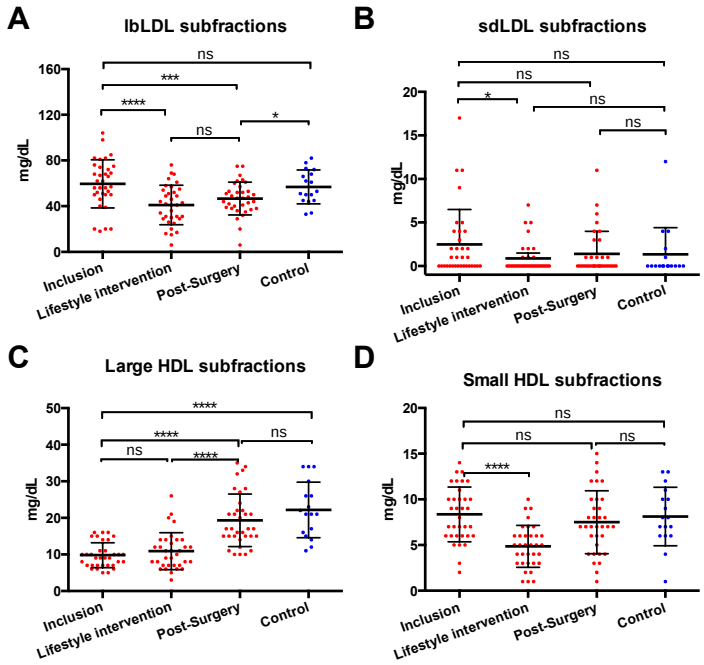

Figure 15 *Serum levels of the (A) large, buoyant LDL (lbLDL) subfractions, (B) the small, dense LDL (sdLDL) subfractions, (C) large HDL subfractions, (D) small HDL subfractions at inclusion, after a lifestyle intervention (w3 months) and one year after bariatric surgery in 34 morbidly obese patients, and at inclusion for controls. Values are mean with standard deviation. *P , .05, **P , .01, ***P , .001, ****P , .0001, ns, not statistically significant. Adapted from "Kjellmo, C. A.; Karlsson, H.; Nestvold, T. K.; Ljunggren, S.; Cederbrant, K.; Marcusson-Stahl, M.; Mathisen, M.; Lappegard, K. T.; Hovland, A., Bariatric surgery improves lipoprotein profile in morbidly obese patients by reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity." J Clin Lipidol* ***2018,*** *12 (1), 193-202*

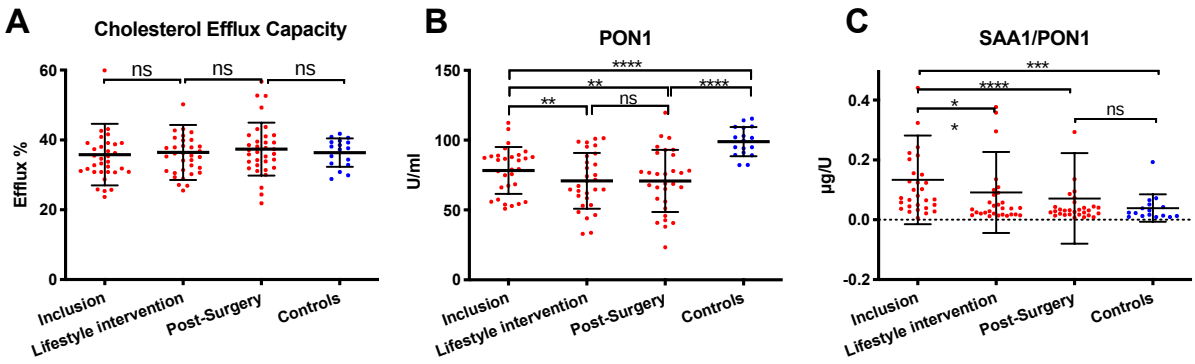

Figure 16 *A) Cholesterol efflux capacity (CEC), (B) Serum paraoxonase-1 (PON1) activity, and (C) serum amyloid A1/PON1 (SAA1/PON1) ratio determined at inclusion, after a lifestyle intervention and one year after bariatric surgery in 34 morbidly obese patients, and at inclusion for controls. Values are mean with standard deviation. *P, .05, **P, .01, ***P, .001, ****P, .0001, ns, not statistically significant. Adapted from "Kjellmo, C. A.; Karlsson, H.; Nestvold, T. K.; Ljunggren, S.; Cederbrant, K.; Marcusson-Stahl, M.; Mathisen, M.; Lappegard, K. T.; Hovland, A., Bariatric surgery improves lipoprotein profile in morbidly obese patients by reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity." J Clin Lipidol* ***2018,*** *12 (1), 193-202*

9.2 Paper 2

Lipoprotein apheresis affects lipoprotein particle subclasses more efficiently compared to the PCSK9 inhibitor evolocumab, a pilot study.

In this pilot study, we assessed changes in lipoprotein profile and HDL function in 3 patients with FH and established cardiovascular disease during lipoprotein apheresis after treatment was switched to a PSCK9-inhibitor (evolocumab). The patients had been on weekly lipoprotein apheresis for an average of 11 years due to statin intolerance at Nordland Hospital, Bodø. Evolocumab was administered according to the manufacturers' instructions in weeks one, three, five, and seven with the recommended dose of 140 mg subcutaneously (autoinjector). The patients were examined immediately before and after their last apheresis treatment (week 0), after one week (before starting evolocumab), and then biweekly before the administration of the next dose of evolocumab. Lipoprotein apheresis reduced all subfractions of LDL and HDL, with statistically significant reductions observed in the large LDL and intermediate LDL subfractions. During evolocumab treatment, there was no significant reductions in LDL subfractions compared to one week after apheresis, although a possible slight reduction in the large and intermediate LDL subfraction was noted over time. (**Figure 18 and Figure 17**) PON1 arylesterase activity was significantly reduced after apheresis, but not affected by evolocumab. We did not observe any changes in SAA1 or CEC from apheresis or during the evolocumab treatment. (**Figure 19**)

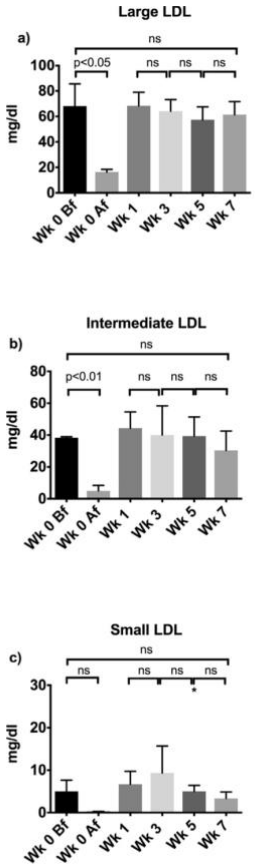

Figure 17 LDL-subfraction concentrations during lipoprotein apheresis and after starting evolocumab. Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1–7: Week 1–7 (samples taken before evolocumab administration).

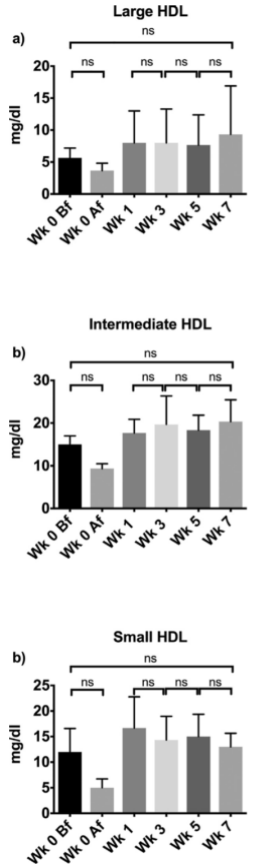

Figure 18 HDL-subfraction concentrations during lipoprotein apheresis and after starting evolocumab. Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1–7: Week 1–7 (samples taken before evolocumab administration)

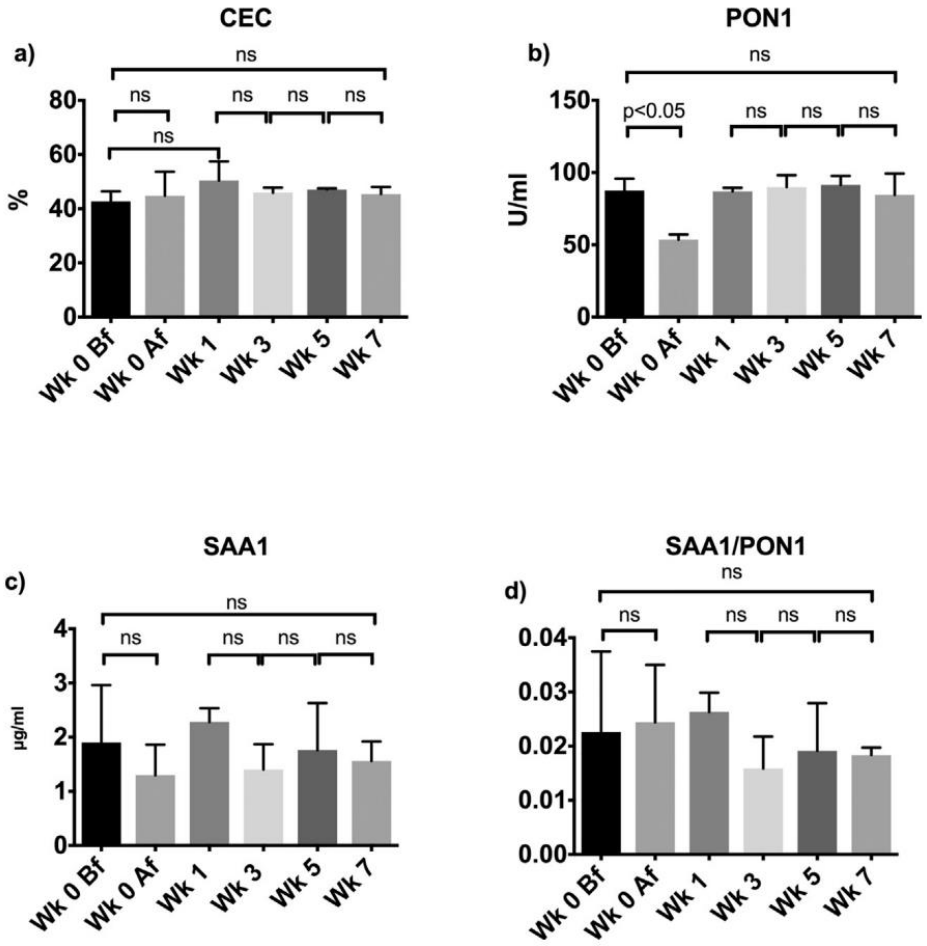

Figure 19 Cholesterol efflux capacity, PON1 activity, SAA1 concentration, and PON1/SAA1 ratio during lipoprotein apheresis and after starting evolocumab. CEC: Cholesterol efflux capacity. PON1: paraoxonase-1. SAA1: serum amyloid A1. Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1–7: Week 1–7 (samples taken before evolocumab administration). *Adapted from Lappegard, K. T.; Kjellmo, C. A.; Ljunggren, S.; Cederbrant, K.; Marcusson-Stahl, M.; Mathisen, M.; Karlsson, H.; Hovland, A., Lipoprotein apheresis affects lipoprotein particle subclasses more efficiently compared to the PCSK9 inhibitor evolocumab, a pilot study. Transfus Apher Sci* ***2018,*** *57 (1), 91-96.*

9.3 Paper 3

Intensive lipid-lowering therapy reduces large, but not small, dense low-density lipoprotein particles measured by gel electrophoresis, in elderly patients with atrial fibrillation

In this study, we assessed changes in LDL-C and LDL subfractions in 30 elderly patients with atrial fibrillation who were enrolled in a double-blind, placebo-controlled trial of a combination of atorvastatin 40mg x 1 and ezetimibe 10mg x 1 vs. placebo. The patients had no known atherosclerotic disease at enrollment. Atorvastatin and ezetimibe significantly reduced LDL-C and the large LDL subfractions but had no effect on the small LDL subfraction. (**Figure 20**) Atorvastatin and ezetimibe had no effect on the HDL subfractions (data not shown).

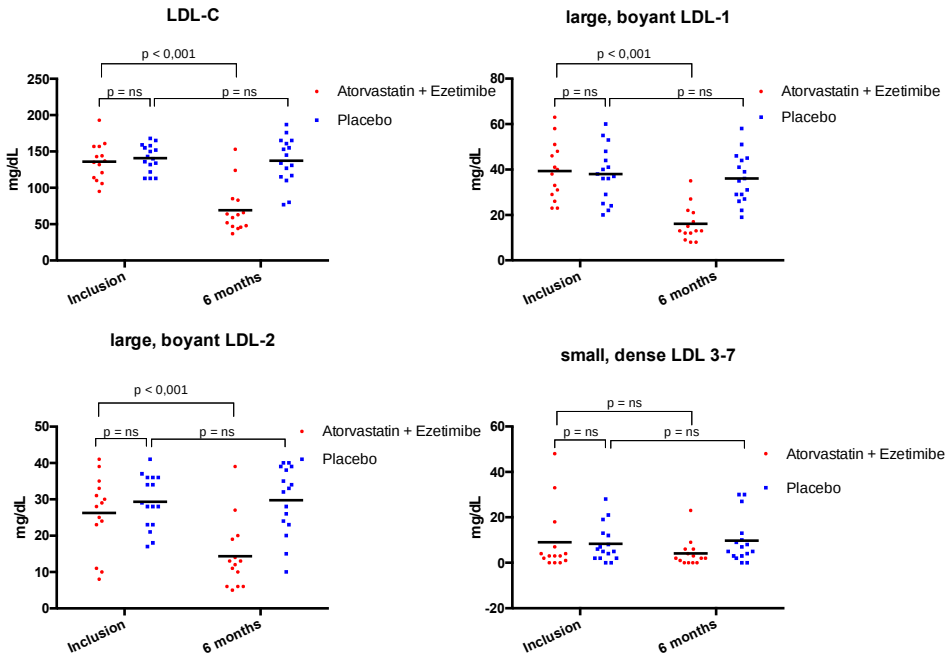

Figure 20 The effects of atorvastatin and ezetimibe versus placebo on LDL-C and various LDL-subfractions in 30 elderly patients with atrial fibrillation. *Adapted from Kjellmo, C. A.; Pop, G.; Lappegard, K. T.; Hovland, A., Intensive lipid-lowering therapy reduces large, but not small, dense low-density lipoprotein particles measured by gel electrophoresis, in elderly patients with atrial fibrillation. Eur J Prev Cardiol* ***2019,*** *26 (18), 2017-2018.*

9.4 Paper 4

Effect of N-3 Polyunsaturated Fatty Acids on Lipid Composition in Familial Hypercholesterolemia: A Randomized Crossover Trial

In this study, we assessed the effect of a supplement of omega-3 polyunsaturated fatty acids (n-3 PUFA) on LDL and HDL subfractions and metrics of HDL function in patients with monogenic FH. The study had a randomized, placebo-controlled, double-blinded, cross-over design, and we included 34 patients with genetically confirmed, monogenic FH from the lipid clinic at Nordland Hospital, Bodø. The study lasted over nine months with two treatment periods of 3 months that were separated by a 3-month wash-out period to minimize a potential carry-over effect. N-3 PUFA and placebo capsules were administrated in the same manner; 4 capsules a day for each treatment period. Each n-3 PUFA capsule contained 460 mg eicosatetraenoic acid (EPA) and 380 mg docosahexaenoic acid (DHA), while the placebo capsule contained olive oil. The primary outcome of this study was change in reactive hyperemia index assessed by peripheral arterial tonometry and these results had been previously reported. [98] The inclusion criteria were age between 18 and 75 years, genetically verified FH, clinically stable disease, and statin treatment for at least 12 months. Exclusion criteria were noncompliance, pregnancy or fertility treatment, breastfeeding, cancer, and/or severe illness.

Thirty-eight patients were included in the study. Three patients left the study, and one patient was excluded from the statistical analysis due to pregnancy. Thirty-four patients (17 men and 17 women) with a mean age of 46,6 years completed the study.

n3-PUFA significantly reduced total cholesterol (median difference of -0,24 mmol/L), LDL-C (median difference of -0,2 mmol/L), and triglycerides (-0,14 mmol/L) compared to placebo, while there was no change in HDL-C. (**Figure 21**) n3-PUFA did not affect the large or small LDL subfractions, but significantly increased the large HDL subfraction and decreased the small HDL subfraction. (**Figure 22**) n-3 PUFA did not affect apoB, apoA1, cholesterol efflux capacity, SAA1 or PON1 activity. (**Table 2**)

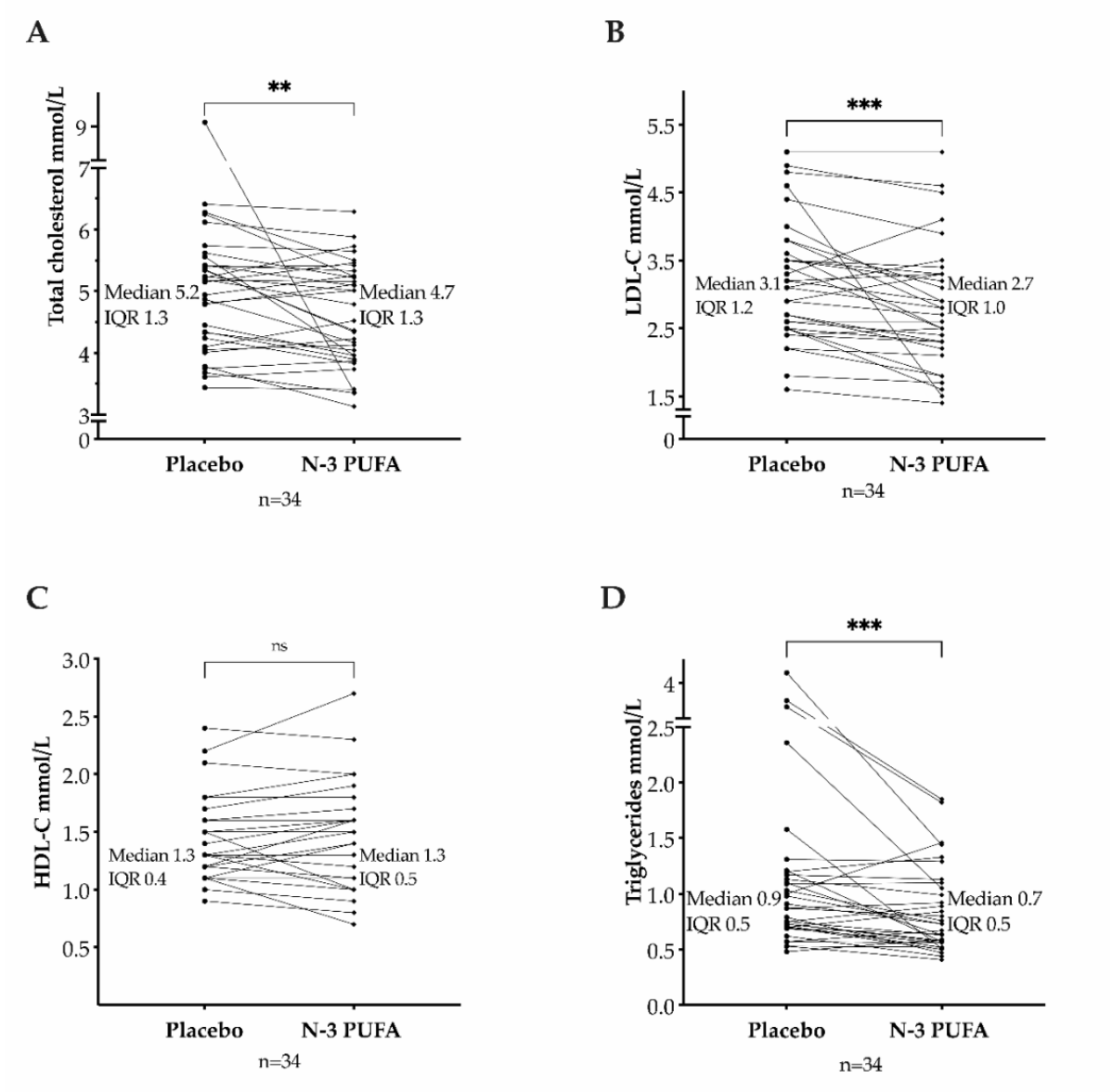

Figure 21 Total cholesterol (A), low-density lipoprotein cholesterol (LDL-C) (B), high-density lipoprotein cholesterol (HDL-C) (C), and triglycerides (D) after omega-3 polyunsaturated fatty acids (n-3 PUFAs) and placebo. IQR: interquartile range. ns = $p > 0.05$. ** = $p \leq 0.01$. *** = $p \leq 0.001$. *Adapted from Hande, L. N.; Kjellmo, C.; Pettersen, K.; Ljunggren, S.; Karlsson, H.; Cederbrant, K.; Marcusson-Stahl, M.; Hovland, A.; Lappegard, K. T., Effect of N-3 Polyunsaturated Fatty Acids on Lipid Composition in Familial Hypercholesterolemia: A Randomized Crossover Trial. Biomedicines* ***2022,*** *10 (8).*

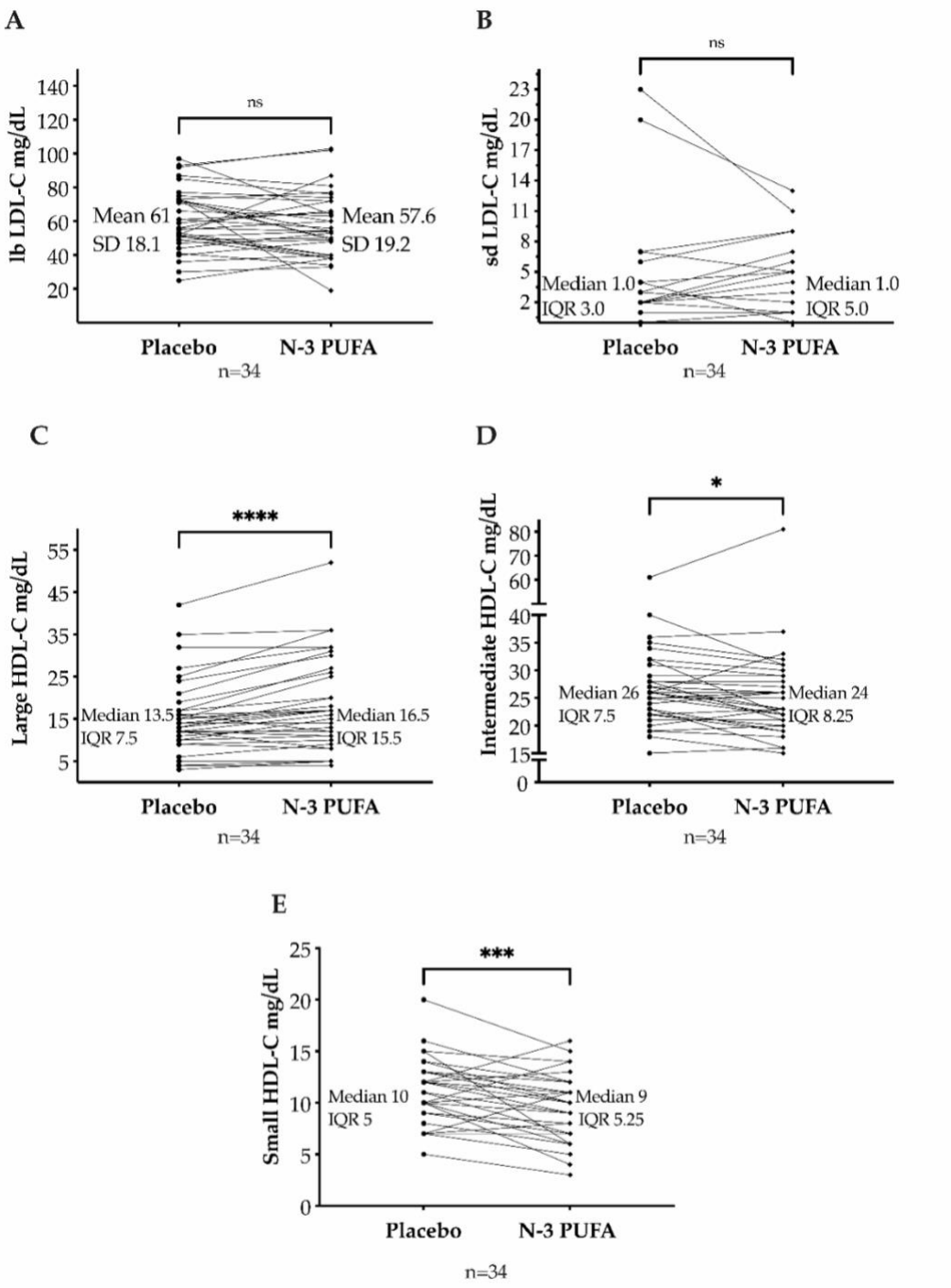

Figure 22 Large, buoyant low-density lipoprotein cholesterol (lbLDL-C) (A) and small, dense low-density lipoprotein cholesterol (sdLDL-C) (B) after omega-3 polyunsaturated fatty acids (n-3 PUFAs) and placebo. Large high-density lipoprotein cholesterol (large HDL-C) (C), intermediate HDL-C (D), and small HDL-C (E) after n-3 PUFAs and placebo. SD: standard deviation. IQR: interquartile range. ns = p > 0.05; * = p ≤ 0.05 *** = p ≤ 0.001; **** = p ≤ 0.0001. Adapted from *Hande, L. N.; Kjellmo, C.; Pettersen, K.; Ljunggren, S.; Karlsson, H.; Cederbrant, K.; Marcusson-Stahl, M.; Hovland, A.; Lappegard, K. T., Effect of N-3 Polyunsaturated Fatty Acids on Lipid Composition in Familial Hypercholesterolemia: A Randomized Crossover Trial. Biomedicines* ***2022,*** *10 (8).*

Table 2. Apolipoproteins and high-density lipoprotein function presented by treatment.

	n-3 PUFA n=34	Placebo n=34	Treatment difference (n-3 PUFA - Placebo) with 95% confidence interval	p
ApoA1 (µg/L)	1.51 (1.3, 1.7)	1.55 (1.3, 1.9)	-0.07 [-0.12 to 0.05]	0.29
ApoB (µg/L)	0.91 (0.8, 1.2)	1.03 (0.8, 1.2)	-0.06 [-0.13 to 0.01]	0.09
ApoB/ApoA1	0.57 (0.5, 0.7)	0.58 (0.5, 0.8)	-0.009 [-0.04 to 0.04]	0.91
SAA1 (µg/mL)	1.36 (1.0, 2.6)	1.68 (1.0, 2.7)	-0.11 [-0.59 to 0.11]	0.15
PON1 (U/mL)	107 ± 25.5	113 ± 31.0	-5.9 [-12.9 to 1.1]	0.10
SAA1/PON1	0.01 (0.01, 0.02)	0.02 (0.01, 0.2)	-0.002 [-0.006 to 0.003]	0.38
CEC (%)	38.5 ± 3.2	38.9 ± 3.5	-0.41 [-1.8 to 1.0]	0.57

n-3 PUFA; omega-3 polyunsaturated fatty acids. Values are presented as median (interquartile range) or mean ± standard deviation. ApoA1; apolipoprotein A1. ApoB; apolipoprotein B. SAA1; serum amyloid A1. PON1; serum paraoxonase-1. CEC; cholesterol efflux capacity. *Adapted from Hande, L. N.; Kjellmo, C.; Pettersen, K.; Ljunggren, S.; Karlsson, H.; Cederbrant, K.; Marcusson-Stahl, M.; Hovland, A.; Lappegard, K. T., Effect of N-3 Polyunsaturated Fatty Acids on Lipid Composition in Familial Hypercholesterolemia: A Randomized Crossover Trial. Biomedicines* ***2022,*** *10 (8).*

10 Discussion

In paper 1, we studied changes in the standard lipoproteins, LDL, and HDL subfractions and metrics of HDL function in 34 morbidly obese patients after an intensive lifestyle intervention and then one year after bariatric surgery. Increased risk of cardiovascular morbidity and mortality is well described in morbidly obese patients, [99] and bariatric surgery has been proven to reduce cause-specific mortality from coronary cardiovascular disease in morbidly obese patients. [100]

The typical dyslipidemia observed in obese patients, which includes elevated triglycerides, low HDL-C, and normal or slightly elevated LDL-C, was also observed in our patients at

baseline compared to lean controls. (**Figure 14**) Surprisingly, the morbidly obese patients had low levels of sdLDL at baseline, comparable to the controls (**Figure *15***). Out of the 34 morbidly obese patients included in this study, only two (6%) exhibited a lipoprotein phenotype pattern B with a predominance of sdLDL at the time of inclusion (data not shown). In contrast, other studies have reported a significantly higher prevalence of sdLDL in obese patients. [101, 102] For example, Kulanuwat et al. found that 58% of morbidly obese patients exhibited a lipoprotein phenotype B using ultracentrifugation (UC) to measure sdLDL. [103] These contrasting results may have been influenced by the absence of known cardiovascular disease (CVD) or the fact that only half of the patients had diabetes, and 41% were male, two factors associated with elevated levels of sdLDL. Nevertheless, as will be discussed later, we suspect that the use of Lipoprint® to quantify sdLDL may have had the greatest impact on these unexpected findings.

The lifestyle intervention significantly reduced LDL-C, apoB, TG, and HDL-C. A small, but statistically significant reduction in sdLDL was also observed. (**Figure 15**) The lifestyle intervention reduced the small HDL particles and did not affect the larger ones. (**Figure 15**) However, the clinical relevance of these findings is questionable as this was an intensive lifestyle intervention over a limited period with a clear, prespecified goal as they had to lose 10% of their body weight to be eligible for surgery.

One year after bariatric surgery, the lipid status of the patients had improved. ApoB and LDL-C were significantly reduced compared to baseline. The observed reduction in LDL-C was, on average 24%, comparable to treatment with a statin. [104] (**Figure 14**) The observed decrease in LDL-C was primarily due to a reduction in the larger LDL particles and we found no effect from bariatric surgery on the sdLDL particles compared to baseline values. (**Figure 15**). Only a few studies evaluating the effects of bariatric surgery on LDL subfractions have been published; Coimbra et al. reported no effect on sdLDL at 13 months after gastric banding [90], while Li. et al. and Yadav et al. reported significant reductions in sdLDL-C and sdLDL-apoB, respectively, at six months after bariatric surgery. [91, 105]

HDL-C levels increased after surgery, but the patients still had HDL-C slightly lower than the controls after one year. Despite being an important marker of CVD risk, there is currently no evidence to suggest that increasing HDL-C by drugs or other interventions will improve hard cardiovascular outcomes. [106] The increase in HDL-C was due to increases in the large HDL-subfraction, while the small HDL-subfraction was unaffected by bariatric surgery compared to baseline measurements. This is in line with other studies showing that weight loss by bariatric surgery increases the larger HDL particles, while the smaller HDL particles are unaffected or reduced. [90, 107, 108] The published literature on HDL subfractions is conflicting regarding which subfraction is most relevant in CVD risk stratification. [57] Obesity and diabetes are associated with an HDL-subfraction composition skewed toward higher levels of small HDL particles (HDL3) and lower levels of the larger HDL particles (HDL2) [81-84], a pattern also observed in our patients at baseline. Early epidemiological studies suggested that the larger HDL particles (HDL2) were superior to HDL-C or small

HDL (HDL3) in CVD risk prediction, [57] while later trials and a recent meta-analysis have suggested that the small HDL-subfraction (HDL3) might be superior to other HDL-metrics in risk stratification. [77, 78]

In this study, we found no difference in CEC between patients and controls at baseline, and we did not observe any change in CEC from the lifestyle intervention or bariatric surgery. (**Figure 16**) Reverse cholesterol transportation (RCT) is considered the most important aspect of HDL function, and cholesterol efflux capacity (CEC) is a vital and quantifiable aspect of RCT. [109] CEC is inversely correlated with the risk of developing CVD in large, prospective studies, [59, 110]. Still, there is currently insufficient evidence to suggest that targeting and improving CEC impacts CVD outcomes. [111] *In-vitro* studies have found that the smaller HDL particles are the most efficient in promoting cholesterol efflux from macrophages via the important ABCA1 pathway. [112] Large HDL particles correlate with increased CEC, while increased levels of the smaller HDL subfraction negatively correlate with CEC. [110, 112-114] Taken together, this has been suggested to indicate that increased levels of small HDLs are a marker of dysfunctional HDL with reduced CEC and delayed maturing from small to larger HDL particles. [113] The fact that we did not see any changes in CEC despite significant changes in the composition of the HDL subfractions throughout this study runs contrary to the belief that the size of the HDL particle is important for cholesterol efflux capacity, and it does not support that targeting HDL composition will affect CEC. [61] Published literature on the effect of bariatric surgery on CEC is scarce and conflicting. A study comparing RYGB with sleeve gastrectomy (SG) found no impact on CEC at 12 months after surgery, [108] while others have reported improvements in CEC after bariatric surgery. [115, 116]

PON1 is an HDL-associated enzyme that reduces oxidative stress, and prospective studies have shown that reduced PON1-activity is an independent risk factor for CVD. [117, 118] Reduced PON1-activity has been observed in patients with obesity and diabetes [119] and we found significantly lower PON1-activity in our patients compared to controls at baseline. Unexpectantly, we observed a reduction in PON1-activity from the lifestyle intervention, an effect that remained at 1 year after bariatric surgery. (**Figure 17**) Previous studies have reported increases in PON1-activity after bariatric surgery. [120, 121] Differences in the patient population, method of surgery, changes in HDL composition, or the time between the intervention and the measurements could possibly explain these divergent results.

Serum amyloid A1 (SAA1) is an acute-phase protein that increases during inflammation and SAA1 in circulation is predominantly bound to HDL. Increased SAA1 is associated with CVD mortality, possibly by impairing the anti-inflammatory properties of HDL. [68, 69] Chronic inflammation and elevated SAA1 is well described in patients with obesity and diabetes, and evidence from *in-vitro* studies suggests that mature adipocytes can produce SAA1. [122-124] SAA1 was significantly higher in our patients compared to controls at baseline, and there was a significant reduction in SAA1 by both the lifestyle intervention and by bariatric surgery (data not shown). Due to this, the SAA1/PON1-ratio, a proposed marker

of HDL function, was improved from both lifestyle intervention and bariatric surgery. **(Figure 17)** It is well documented that bariatric surgery reduces inflammation in obese patients, [125] but published literature on the effects of SAA1 levels are conflicting. Bratti et al. found no effect on SAA1 in 43 morbidly obese patients at 6 months after bariatric surgery, [126] while others have reported significant reductions in SAA1 following bariatric surgery. [127, 128]

This study has several important limitations. First, the sample size was small with 34 patients included and this could mean that the included patients were not sufficiently representative of the morbidly obese population. The patients had low levels of sdLDL at baseline and this could explain why we did not see any changes in sdLDL from the intervention. Secondly, the Lipoprint® system for measuring LDL and HDL subfractions and the Sigma-Aldrich MAK192-kit for measuring CEC are only two methods within a heterogenous set of technologies to measure these metrics. Using NMR or IM to measure lipoprotein subfractions also accounts for the lipoprotein particle numbers and could have provided additional information. The Sigma-Aldrich MAK192-kit measures total CEC from whole serum, while the prospective studies that have found an inverse correlation between CEC and CVD risk have used assays that primarily measures ABCA1-mediated efflux from apoB-depleted serum. [59, 110]

Paper 2 was a pilot study of three patients with genetically confirmed, monogenic, heterozygous familial hypercholesterolemia with established CVD. Due to statin intolerance, they had been on lipoprotein apheresis for several years at our hospital. Lipoprotein apheresis (LA) is an extra-corporeal therapy that physically removes lipoproteins from the blood. Apheresis is usually repeated weekly or bi-weekly and is highly effective at reducing apoB-containing lipoproteins. LA is an expensive and time-consuming treatment, and only recommended for selected patients at very high risk for CVD, where maximal lifestyle and drug treatments have failed to achieve acceptable plasma levels of apoB-containing lipoproteins. No randomized trials of lipoprotein apheresis have been conducted, but the effectiveness in lowering LDL-C and the available observational studies supports that LA improves CVD outcomes in high-risk patients. [129]

PCSK9 inhibitors (PCSK9i) are novel lipid-lowering agents that have been shown to reduce CVD-related morbidity and mortality in both primary and secondary prevention. [130] PSCK9 inhibitors are monoclonal antibodies that block the effect of the PCSK9 protein. By binding to the LDL-receptor, PCSK9 promotes degradation of the LDLR. By blocking PCSK9, PCSK9i increases the number of LDL-Rs on the surface of hepatocytes, augmenting LDL uptake and reducing plasma levels of LDL and other apoB-containing lipoproteins.

In this study we measured LDL and HDL subfractions and measurements of HDL function immediately before and after apheresis and then during the first 7 weeks of treatment with a PCSK9i (evolocumab). The effects on LDL-C and HDL-C in this study had already been published. [131] The patients had a historically highest LDL-C of 10,3 ± 0.8 mmol/L pre-

treatment. LDL-C was reduced to 1,2 ± 0.2 mmol/L immediately post-apheresis and increased to 6,1 ± 0.7 mmol/L one week after apheresis (during weekly apheresis). Treatment with evolocumab stabilized LDL-C around 5,0 ± 0.7 mmol/L. Apheresis transiently reduced HDL-C from 1,0 ± 0.2 mmol/L to 0,5 ± 0.1 mmol/L, while there was no effect on HDL-C from the PCSK9 inhibitor.

As anticipated, lipoprotein apheresis substantially reduced LDL subfractions across all sizes and we also observed a reduction in the different HDL subfractions, although not statistically significant due to the small number of patients included. (**Figure 17** and **Figure 18**) These results are in line with two other studies evaluating the effects of apheresis on LDL subfractions. [132, 133] Compared to pre-apheresis levels, evolocumab treatment did not affect the composition of LDL subfractions. (**Figure 17**) As no historical data on LDL or HDL subfractions was available, we could not evaluate the effect of evolocumab on LDL subfractions compared to when the patients had their historically highest levels of LDL-C. The lipid-lowering effect of PCSK9 inhibitors in heterozygous or polygenic FH is well documented and has been shown to be similar to patients without FH, with expected reductions in LDL-C of 50-60%. [134] Patients with homozygous FH generally have a reduced effect from PCSK9i, depending on the residual function of the LDL-R. [134] Few studies have looked at the effects of PCSK9i on LDL subfractions. Li et al. published a randomized trial of 99 patients with acute coronary syndrome and reported significant reductions in sdLDL, measured with NMR, from PCSK9i compared to treatment with a statin. [89] Di Minno et.al found that PCSK9i significantly reduced sdLDL (-52%), measured by Lipoprint®, in 20 patients with heterozygote, monogenic FH. [135] Comparing these results to our study is difficult as they also experienced a significant reduction in LDL-C (-56%) from the PCSK9i.

Apheresis or evolocumab did not affect CEC or SAA1 in our patients, but we did observe a significant reduction in PON1 activity after apheresis, possibly due to the reduction in HDL-C. This effect on PON1 was transient and probably not clinically relevant, as there was no difference between the pre-apheresis levels and PON1 activity during treatment with a PCSK9 inhibitor. Similar effects on the HDL subfractions and PON1 activity were found in a study on 11 patients on lipoprotein apheresis. [136] Previous, larger studies have reported marked, but transient reductions in CEC after lipoprotein apheresis in FH patients. [137, 138] The small sample size in our pilot study or differences in the assays used to measure CEC could explain these divergent results.

In paper 3 we evaluated the effect of an intensive lipid-lowering treatment regimen with atorvastatin 40mg + ezetimibe 10mg x 1 on LDL and HDL subfractions in a randomized, placebo-controlled trial of 30 elderly patients with atrial fibrillation without known cardiovascular disease. Atorvastatin + ezetimibe significantly reduced LDL-C compared to placebo, and the observed reduction in LDL-C was due to a significant reduction in the large LDL subfraction. (**Figure 20**) All patients had low levels of sdLDL at inclusion and we did not see any effect on sdLDL from the atorvastatin + ezetimibe regime. (**Figure 20**)

Statins reduce LDL-C by inhibiting de-novo synthesis of cholesterol in cells by inhibiting HMG-CoA reductase and have been the most important class of drug in preventive cardiovascular medicine for the last decades. A meta-analysis of 26 statin trials with over 170,00 individuals has shown that statin treatment is associated with a 22% relative risk reduction of major cardiovascular events pr. mmol/L reduction in LDL-C. [139] This effect is independent of baseline LDL-C and equal between patients with and without known cardiovascular disease at baseline. These studies also confirmed that LDL-C is a satisfactory surrogate for LDL-P as a target for therapy, as apoB and LDL-C showed similar improvements in ASCVD risk pr. mmol/L reduction. Ezetimibe inhibits intestinal absorption of cholesterol by blocking the NPC1L1 transporter in the jejunum and reducing uptake of cholesterol into the enterocytes. IMPROVE-IT, a large, randomized trial demonstrated significant reductions in hard cardiovascular outcomes when ezetimibe was used as an add-on therapy to statins in high-risk patients. [140]

The effect of statins on LDL subfractions has been evaluated in several studies and the results are conflicting. Statins can potentially reduce all LDL subfractions, but wide variations between the different statins are reported [86] and observational studies have suggested sdLDL remains elevated in statin users. [85] Using Lipoprint LDL ®, Pinto et.al reported significant reductions in sdLDL from a treatment combination of simvastatin and ezetimibe, but no effect on sdLDL from monotherapy with rosuvastatin in 101 patients with ST-segment elevation myocardial infarction (STEMI). [141]

Due to the unequivocal benefit of statins in both primary and secondary ASCVD prevention it is ethically problematic to conduct new placebo-controlled trials of statins in patients at risk of CVD. This study included 30 elderly patients without established ASCVD, who did not have an indication for statin treatment at the time when the study was conducted. This is an important limitation to our study, as the composition of LDL subfractions in these patients could differ from very high-risk patients who would be more relevant for advanced lipoprotein testing. The patients had low levels of sdLDL at baseline and this could explain why we did not see any effect on sdLDL from the statin + ezetimibe treatment.

In paper 4 we evaluated the effect of a n3-PUFA supplement on lipoprotein measurements and metrics of HDL function in 34 FH patients in a randomized, placebo-controlled, cross-over trial. The role of n3-PUFA in the prevention of ASCVD in the general population has been studied for decades and the potential benefit of these supplements in an era of highly effective lipid-lowering therapies is fiercely debated. [142-144] The triglyceride-lowering effect of n3-PUFA is undisputed, but it has yet to be proven that reducing triglycerides improves cardiovascular outcomes. [37] PROMINENT, a large RCT study of the triglyceride-lowering agent pemafibrate, was recently published and found no reduction in cardiovascular events from pemafibrate in patients with DM2 and mild to moderate hypertriglyceridemia. [145]

Three large RCT trials of n3-PUFA in ASCVD prevention have recently been published; REDUCE-IT found a significant reduction of cardiovascular events from icosapent ethyl supplement, [146] while STRENGTH and RESPECT-EPA found no differences in major adverse cardiovascular events, [147, 148] ensuring that this matter will remain disputed until further trials are conducted. Only a few trials of n3-PUFA supplements in FH populations have been conducted and these have reported divergent results on secondary outcomes such as lipoprotein composition and endothelial function. [98, 149-151] As expected, n3-PUFAs are currently not routinely recommended to FH patients in European guidelines. [37]

Our study found that n3-PUFA resulted in a modest, but statistically significant, reduction in TC, LDL-C and triglycerides. (**Figure 21**) The effect of n3-PUFA on LDL-C and TC has been investigated in several studies. A Cochrane review concluded that there is high-certainty evidence that n3-PUFAs have little to no impact on these lipoprotein metrics. [144] The LDL-C-lowering effect from n-3 PUFA found in our trial differs from the results in previous trials investigating n-3 PUFA supplementation in heterozygous FH individuals, [149-151] where no effect on LDL-C has been reported. The short duration of these studies, sample sizes, use of DHA only, and lack of placebo comparison are possible explanations for the disparate results.

The FH patients included in this study had low levels of sdLDL at inclusion and we found no effect on the composition of the LDL subfractions from n3-PUFA in this study. (**Figure 22**) Two randomized diet trials of n3-PUFAs in healthy volunteers [152, 153] and a randomized trial of n3-PUFA supplement in 44 hyperlipidemic, statin-users [154] have reported significant reductions in sdLDL, but as no trials on the effect of n3-PUFA on LDL subfractions in FH patients has been conducted, these divergent results are difficult to interpret due to the difference in the study populations and the fact that our patients had low levels of sdLDL at baseline.
We found no effect from n3-PUFA on HDL-C, but the composition of the HDL subfractions changed significantly with an increase in the large HDL subfraction and a decrease in the small HDL subfraction. (**Figure 22**) This aligns with published literature; a meta-analysis of 43 trials with 15106 participants found no effect on HDL-C from n3-PUFA supplements. [155] A review of 20 trials on the effect of n3-PUFA on HDL subfractions concluded that the majority of these studies have reported increases in the large HDL subfraction and reductions in the small HDL subfraction from n-3 PUFA, [156] including a trial of 147 individuals at high ASCVD risk where the HDL subfractions were measured using the Lipoprint HDL ® system. [157] As in **paper 1**, we observed no change in cholesterol efflux capacity despite significant changes in HDL subfraction composition, (**Table 2**) suggesting that the HDL particle size might not be a critical determinant of CEC and that targeting HDL subfraction composition might not be a viable pathway to improve CEC. n-3 PUFA did not affect apoB, apoA1, SAA1 or PON1 activity in our study. (**Table 2**) Few studies on the effect of n3-PUFA on SAA1 have been published. A small, randomized trial of 11 healthy volunteers with moderate obesity reported no effect on SAA1 from n3-PUFA-supplements [158]

The effect of n3-PUFA on PON1 activity has been investigated in a few studies and the results are divergent. Two randomized trials, one study of n3-PUFA in 83 patients with rheumatoid arthritis (RA) and one study of an EPA supplement in 36 patients with DM2 reported significant increases in PON1 activity from n3-PUFA supplement, [159, 160]. In contrast, a randomized trial of n3-PUFA in 147 individuals at high risk of ASCVD and a trial of high-dose EPA in 28 Japanese patients with dyslipidemia reported no effect on PON1 activity from n3-PUFA supplements. [157, 161] As these studies have included vastly different study populations compared to ours, it is difficult to draw any conclusions based on these results.

The strength of this trial is the robust cross-over design, the well-known study population, the duration of the intervention (three-month treatment and minimum three-month wash-out) and the small number of dropouts during the trial. There are however some important limitations. The sample size was small and as these FH patients were both in a primary and secondary prevention setting, we could not differentiate results between patients in the high-risk category and those in the very-high risk category.

10.1 Lipoprint® and the comparability between different methods of lipoprotein subfractioning

The studies in this thesis focus on the composition of LDL and HDL subfractions, metrics of HDL function and the effects from various ASCVD prevention interventions on these metrics. When comparing our results from the Lipoprint ® system to published literature it is important to note that there are important differences between the available methods for measuring LDL and HDL subfractions.

LDL and HDL particles can be separated based on various physicochemical properties depending on the protein separation technique used. In the published literature on LDL and HDL subfractions, the most used protein separation techniques are gel electrophoresis (GE), ultracentrifugation (UC), nuclear magnetic resonance (NMR), and ion mobility (IM). GE separates particles based on size and charge, UC separates particles based on density, NMR measures methyl group signals from the lipoprotein particles and calculates the particle number and size, while IM separates the lipoprotein particles using gas-phase electrophoresis and then quantifies the size-separated particles.

GE and UC are in general laborious and expensive methods, less suitable for regular use in clinical practice. Vertical Auto Profile II ® from Atherotec was a patented, commercially available lipoprotein subfraction test based on ultracentrifugation that was available up until 2018 at one laboratory in the U.S. Three patented, commercially available laboratory tests based on NMR (Lipoprofile ®, Vantera ®, LipoFit®) and one test based on IM (Cardio IQ®) are approved by the federal Food and Drug Administration (FDA) in the U.S for the subfractioning of LDL and HDL. The latest and largest prospective studies on lipoprotein subfractions and their association with ASCVD have used NMR and IM. [41, 71, 74] It is relevant to note that several of the authors behind these studies have vested interests in these

tests. [71-74, 162] In addition to these patented, laboratory-based tests using NMR and IM, two in-house systems for the determination of lipoprotein subfractions are available: A simple tube-gel electrophoresis system (Lipoprint LDL and HDL ®) [96] and a homogenous assay adaptable to auto analyzers for the measurement of sdLDL-C. [163]

As these methods separate the lipoprotein particles based on different characteristics and use different thresholds for size, density, and particle number when reporting the different LDL subfractions, it is impossible to compare measurements of individual subfractions between methods directly. [164] The only reasonable comparison that can be made between these methods is their ability to classify patients into LDL phenotype; pattern A (predominance of larger LDL-particles, "less atherogenic"), pattern B (predominance of smaller LDL particles, "more atherogenic") or intermediate (A/B).

Ensign et.al published a study comparing four methods for subfractionating LDL, including Lipoprint ®, in 2006. [165] Blood samples from 40 healthy individuals were analyzed using NMR (Lipoprofile®), gradient gel electrophoresis (GGE), tube-gel electrophoresis (Lipoprint ®), and ultracentrifugation (Vertical Auto Profile II®). Only three individuals of these 40 were classified as having the same phenotype by all four methods. While Lipoprint® only classified two individuals (8%) as type "B", with high levels of sdLDL, VAP and NMR classified 21 individuals (54%) as phenotype B.

Our group did a comparison between Lipoprint ® and VAPII ® (ultracentrifugation) on 34 blood samples from 6 healthy individuals used in a study of 3 different apheresis filters and found that Lipoprint ® classified 33 of these samples as type "A" and one as intermediate (A/B), while VAP assigned 22 samples as "A", nine as "A/B" and 3 as "B". (Data not shown)

Chung et al. published a review in 2009 on the comparability between the different methods used for measuring lipoprotein subfractions available at the time (IM was introduced later). They reported that Lipoprint® had good agreement with other tests based on gel electrophoresis - 93% concordance for classification of the sdLDL-subfraction – but a wide range of agreement (7 – 94% concordance) is reported between the other methods. [164] This review addressed that the inherent differences between these methods, combined with a lack of standardization or gold standard, make it impossible to determine if they are measuring the same lipoprotein subfraction. Chung et al argued that developing a gold standard or reference method was necessary to establish the clinical value of LDL subfractions. [164]
No reference method or gold standard has been established since 2009. IM has been introduced as another modality, adding to the complexity of LDL and HDL subfractioning. Williams et al. published a comparison of 4 methods for separating LDL subfractions (GE, UC, IM, and NMR) in 2014 that looked at 136 patients who received baseline and follow-up coronary angiographies. All four methods confirmed the independent association between sdLDL and the risk of greater progression of coronary atherosclerosis, but the correlation between methods varied significantly. [166]

As no reference method or gold standard exists, the Lipoprint® system was chosen for our studies as it is the only in-house system to analyze both LDL and HDL subfractions. As an in-house system at an affordable price, it is also the most relevant method for regular use outside of the U.S. However, the comparability studies indicate that Lipoprint ® classifies fewer samples as phenotype "B", thus measuring lower concentrations of sdLDL compared to other methods. We found low baseline concentrations of sdLDL in all 4 study populations and that Lipoprint ® reported lower sdLDL-concentrations compared to VAPII ®. This suggests a significant bias in our results. Ideally, we should have run further comparisons between Lipoprint ® and NMR and IM, including on HDL subfractions, but we could not do so due to the high costs of these tests and a lack of available serum from our studies. This is an important limitation of our findings. We did not find any effect on sdLDL from bariatric surgery, a combination of statins + ezetimibe or n3-PUFA, and thus no indication that sdLDL could be relevant as a target for these therapies.

Our studies have shown that it is possible to target and change the composition of HDL subfractions with an intensive lifestyle intervention, bariatric surgery and n3-PUFA. Despite significant changes in the HDL subfractions, we did not observe any change in CEC from these interventions, suggesting that the composition of HDL is not a critical determinator for CEC. Further studies are required to answer whether HDL subfractions could be a relevant surrogate for HDL function, or if modulating the HDL subfractions could improve ASCVD outcomes.

11 Conclusions

The use of advanced lipoprotein testing has been suggested to enhance the prediction of ASCVD risk and identify high-risk patients who could benefit from novel and expensive lipid-lowering therapies. However, such tests are generally not available in regular clinical practice outside of the US due to their limited accessibility and high costs. In four interventional studies, we utilized Lipoprint®, a simple, inexpensive in-house system, to measure LDL and HDL subfractions, as well as HDL function assays including CEC, SAA1, and PON1 activity, in high-risk patients undergoing interventions that are commonly used or proposed to lower the risk of ASCVD.

We found low baseline concentrations of sdLDL across all our patient populations. Although this may be due to small sample sizes, these results are likely influenced by the fact that Lipoprint LDL® measures lower levels of sdLDL compared to other LDL subfractioning methods. We did not find significant reductions of sdLDL from these interventions, except for a slight reduction with an intensive lifestyle intervention in morbidly obese patients. Overall, our research suggests that Lipoprint LDL® may not offer any significant insights beyond the conventional lipoprotein metrics already available in clinical labs.

In recent years, research has focused on HDL function rather than HDL-C quantity, as trials of drugs that increase HDL-C have failed to improve cardiovascular outcomes in large, randomized trials. HDL subfractions have been proposed to improve ASCVD risk prediction, but it is unclear which subfraction is most relevant. Our studies found significant changes in HDL subfraction composition from a lifestyle intervention followed by bariatric surgery in morbidly obese patients and by n-3 PUFA in patients with familial hypercholesterolemia. Several metrics of HDL function, including cholesterol efflux capacity (CEC), have been shown to be correlated with an increased risk of CVD. However, CEC assays are currently unsuitable for routine clinical use, and HDL subfractions have been suggested as a possible surrogate measurement for CEC. Our findings suggest that Lipoprint HDL® subfractions may not be relevant as a surrogate for CEC, as we observed no changes in HDL efflux capacity from interventions that significantly affected the composition of HDL subfractions. Additionally, we observed reductions in PON1 and SAA1, two markers of HDL function associated with increased ASCVD risk, from a lifestyle intervention and bariatric surgery in morbidly obese patients. However, further research is needed to determine the clinical significance of these observations.

12 Papers

Paper 1

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Bariatric surgery improves lipoprotein profile in morbidly obese patients by reducing LDL cholesterol, apoB, and SAA/PON1 ratio, increasing HDL cholesterol, but has no effect on cholesterol efflux capacity

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KEYWORDS:
Bariatric surgery;
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Lipoprotein particle subclasses;
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Serum amyloid A (SAA);
Cholesterol efflux capacity

BACKGROUND: Bariatric surgery has been shown to reduce cardiovascular events and cause-specific mortality for coronary artery disease in obese patients. Lipoprotein biomarkers relating to low-density lipoprotein (LDL), high-density lipoprotein (HDL), their subfractions, and macrophage cholesterol efflux have all been hypothesized to be of value in cardiovascular risk assessment.

OBJECTIVES: The objective of this study was to examine the effect of a lifestyle intervention followed by bariatric surgery on the lipid profile of morbidly obese patients.

METHODS: Thirty-four morbidly obese patients were evaluated before and after lifestyle changes and then 1 year after bariatric surgery. They were compared with 17 lean subjects. Several lipoprotein metrics, serum amyloid A (SAA), serum paraoxonase-1 (PON1), and macrophage cholesterol efflux capacity (CEC) were assessed.

RESULTS: Average weight loss after the lifestyle intervention was 10.5% and 1 year after bariatric surgery was 33.9%. The lifestyle intervention significantly decreased triglycerides (TGs; −28.7 mg/dL, $P < .05$), LDL cholesterol (LDL-C; −32.3 mg/dL, $P < .0001$), and apolipoprotein B (apoB; −62.9 μg/mL, $P < .001$). Bariatric surgery further reduced TGs (−36.7 mg/dL, $P < .05$), increased HDL cholesterol (+12 mg/dL, $P < .0001$), and reductions in LDL-C and apoB were sustained. Bariatric surgery reduced large, buoyant LDL ($P < .0001$), but had no effect on the small, dense LDL.

Conflict of interest: The authors declare that they have no conflicts of interest.

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The large HDL subfractions increased ($P < .0001$), but there was no effect on the smaller HDL subfractions. The ratio for SAA/PON1 was reduced after the lifestyle intervention ($P < .01$) and further reduced after bariatric surgery ($P < .0001$). Neither the lifestyle intervention nor bariatric surgery had any effect on CEC.

CONCLUSIONS: Lifestyle intervention followed by bariatric surgery in 34 morbidly obese patients showed favorable effects on TGs, LDL-C, and apoB. HDL cholesterol and apoA1 was increased, apoB/apoA1 ratio as well as SAA/PON1 ratio reduced, but bariatric surgery did not influence CEC.

Introduction

Obesity, defined as a body mass index (BMI) > 30 kg/m^2, is a growing health problem in large parts of the world. Worldwide obesity has doubled since 1980, and the prevalence of obesity in adults has passed 20% in most Western countries.[1] Similar increases in childhood obesity predict that we have not yet seen the peak of this epidemic.[2] Obesity is associated with known cardiovascular risk factors such as hypertension, dyslipidemia, type II diabetes, and chronic inflammation.[3] An increased risk of morbidity and mortality from cardiovascular disease (CVD) is well documented in obese patients.[4]

Bariatric surgery is considered a safe and effective treatment for obesity,[3] usually recommended to patients with severe obesity, defined as a BMI >40 kg/m^2 or BMI >35 kg/m^2 in the presence of substantial comorbidities such as hypertension, diabetes, and obstructive sleep apnea. Bariatric surgery results in significant and sustained weight loss and reduces all-cause mortality and cause-specific mortality from coronary artery disease.[5]

The typical dyslipidemia observed in obese patients consists of elevated fasting and postprandial triglycerides (TGs), reduced high-density lipoprotein cholesterol (HDL-C) and normal or just slightly elevated low-density lipoprotein cholesterol (LDL-C).[3] These routine lipoprotein biomarkers fail to identify a significant proportion of patients at risk of cardiovascular events,[6] and there has been intensive research into whether different advanced lipoprotein testing methods can improve cardiovascular risk prediction. LDL and HDL particles are heterogeneous with respect to size, density, composition, and function. Several LDL and HDL metrics have been investigated, with the most common being total and subfraction particle numbers, sizes, and lipid content. Small, dense LDL (sdLDL) has been associated with increased cardiovascular risk, obesity, and diabetes. sdLDL biomarkers include LDL diameter or phenotype, particle number (sdLDL-P), or cholesterol content (sdLDL-C).[7–9] Despite over 3 decades of research, the clinical utility of LDL sub-fractionation continues to be debated.[6]

Cholesterol efflux capacity (CEC), the ability of HDL to accept cholesterol from macrophages, a step in reverse cholesterol transport, has been inversely associated with cardiovascular events.[10] The relevance of HDL subfractions and subclasses as markers for HDL-related risk is uncertain,[11] but recent studies have shown that small, dense HDL particles seem to be more efficient in mediating ATP-binding casette transporter 1 (ABCA1)-mediated cholesterol efflux from macrophages.[12] Other aspects of HDL functionality include the ability to mitigate oxidative and inflammatory arterial wall responses. Paraoxonase-1 (PON1) is an HDL-associated enzyme capable of preventing LDL oxidation, and reductions in PON1 activity are believed to lead to dysfunctional HDL.[13] Serum amyloid A (SAA) is an acute-phase protein that increases during inflammation and impairs the anti-inflammatory properties of HDL, possibly by replacing protective proteins in HDL.[14] An increased ratio of SAA/PON1 activity has been proposed as a possible marker for dysfunctional and pro-inflammatory HDL.[15]

The effect of bariatric surgery on lipoprotein composition and function is largely unknown. Furthermore, the mechanisms through which bariatric surgery affects morbidity and mortality are not fully understood. We hypothesized that lifestyle changes followed by bariatric surgery would induce a more favorable lipoprotein profile in morbidly obese patients and, therefore, examined the effects of bariatric surgery on TGs, LDL-C, and HDL-C, apolipoprotein (apo) B, apoA1, apoB/apoA1-ratio, HDL and LDL subfractions, and HDL functions measured by SAA, PON1 activity, and macrophage CEC.

Materials and methods

Study participants and experimental design

In this prospective study, we included 34 patients admitted to the Regional Centre for Treatment of Morbid Obesity, Nordland Hospital, Norway. The patient population has been described previously.[16] Briefly, inclusion criteria in the morbidly obese group were as follows: aged >18 years, BMI > 40 kg/m^2 or BMI > 35 kg/m^2 with significant comorbidity such as hypertension, type II diabetes mellitus, or sleep apnea. The patients underwent

lifestyle changes for a mean period of 3 months before bariatric surgery. The control group consisted of 17 subjects with a BMI <28 kg/m^2 and no established CVD, scheduled to undergo elective laparoscopic cholecystectomy or laparoscopic fundoplication.

Ethics

The study was approved by the Regional Ethics Committee of Northern Norway and by the Norwegian Data Protection Authority and complied with the Helsinki II declaration. Written informed consent was obtained from all participants.

Blood sampling

Fasting blood samples were obtained by standard venipuncture on 3 occasions: At first admission, the day before surgery (after 3 months of lifestyle intervention), and 1 year after surgery. Routine blood analyses were performed on the day of sampling at the laboratory of Nordland Hospital. Serum, ethylenediaminetetraacetic acid plasma, and citrate plasma were frozen in aliquots at -80°C and analyzed in batch at the end of the study.

Serum levels of TGs, LDL-C, HDL-C, apoB, and apoA1 were measured using an ADVIA1800 system (Siemens Medical Solutions Diagnostics, Japan).

LDL and HDL subfractions

LDL and HDL subfractions were determined electrophoretically by the use of high resolution 3% polyacrylamide gel tubes and the Lipoprint system (Lipoprint LDL system & Lipoprint HDL system; Quantimetrix Corporation, Redondo Beach, CA) according to the manufacturer's instructions. LDL subfractions were divided into LDL-1 and LDL-2 (large, buoyant LDL [lbLDL]) and LDL-3 to LDL-7 (small, dense LDL [sdLDL]). Based on these results, a lipoprotein profile is provided; Type A (predominance of lbLDL), intermediate and type B (predominance of sdLDL). Furthermore, the mean LDL particle size (in Å) was determined. HDL subfractions were divided into HDL 1-3 (large HDL), HDL 4-7 (intermediate HDL) and HDL 8-10 (small HDL).

PON1 arylesterase activity

PON1 arylesterase activity was measured in citrate plasma. Briefly, plasma was diluted 1:80 with a salt buffer (20 mM Tris–HCl and 1.0 mM $CaCl_2$). A triplicate of 20 μL diluted plasma was added to the wells in an ultraviolet-transparent 96-well plate (Sigma-Aldrich). Two hundred microliter of phenyl acetate solution, containing 3.26 mM phenyl acetate in salt buffer, was added to each well and the absorbance of produced phenol was measured at 270 nm with 250 nm as background in a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA). The initial period when the reaction was linear was used for calculation of activity, expressed as U/mL, using an extinction coefficient of phenol of 1310 M^{-1} cm.

SAA enzyme-linked immunosorbent assay

To investigate the acute phase response by SAA, plasma SAA1 levels were measured by an enzyme-linked immunosorbent assay (DY3019-05; R&D systems, Minneapolis, MN) according to the manufacturers' instructions. In short, citrate plasma was added to the plate and incubated for 2 hours at room temperature. Following wash, a detection antibody was added and incubated for 2 hours. The plate was washed and streptavidin-horseradish peroxidase was added followed by incubation for 20 minutes. The plate was then washed a final time before a substrate solution was added before 20 minutes incubation. At the end of the incubation, stop solution was added and absorbance was measured at 450 nm with correction at 570 nm using a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA).

Cholesterol efflux capacity

Cholesterol efflux was measured with a commercial kit from Sigma-Aldrich (MAK192) according to the manufacturers' description. Briefly, a human monocyte cell line, THP-1, was differentiated into macrophages with 10 ng/mL phorbol myristate acetate for 24 hours at 37°C and 5% CO_2 in a 96-well plate. The phorbol myristate acetate containing medium was replaced with complete cultivation medium (RPMI1640 including 10% fetal bovine serum, 2 mM Glutamine) and incubated for another 30 hours. The serum containing medium was removed and then washed with serum-free medium. A reaction mix, containing equilibration buffer and fluorescence-labeled LDL, was added to the cells and incubated for 16 hours. The reaction mix was removed, and wells were washed with serum-free medium. Patient serum samples were treated with reaction mix from the kit, and the apoB depleted, clear supernatant was added to the plate and incubated for 5 hours. After the incubation, supernatants were transferred to a new plate, and the fluorescence was measured (482 ex/515 em). The cell layer was solubilized with a cell lysis buffer and incubated for 30 minutes on a shaker. The cell lysate was then transferred to the plate with supernatants, and the fluorescence of the mixture was measured. Percent efflux was calculated as follows: 100 $\times$ fluorescence intensity of the medium/fluorescence intensity of the medium and cell lysate.

Lifestyle changes

Patients had to undergo lifestyle changes preoperatively, and they were not accepted for surgery before they had achieved a 10% weight loss. It took patients on average 12 weeks to achieve this weight loss. The details of these lifestyle changes have been described previously.[17]

Surgery

All operations were performed by 2 experienced bariatric surgeons at the Department of Surgery, Nordland Hospital, Bodø. Two surgical methods were used:

1. Laparoscopic Roux-en-Y gastric bypass for patients with a BMI <50 kg/m^2, n = 27 (79%).
2. Biliopancreatic diversion with duodenal switch for patients with a BMI >50 kg/m^2, n = 7 (21%).

Details of these methods of surgery have been described previously.[17]

Among the controls, 15 patients had laparoscopic cholecystectomy, whereas 2 patients had laparoscopic fundoplication performed.

Statistics

Numerical data are presented with mean and standard deviation. A repeated measures 1-way analysis of variance was used to calculate the longitudinal effects of the lifestyle changes followed by surgery on the different lipids, LDL/HDL subfractions, and parameters of HDL function and composition when appropriate. An unpaired *t*-test was used to calculate the differences between patients and controls. If the data were not normally distributed, non-parametric tests were applied; Friedman test for the longitudinal effects in the patients and Mann–Whitney test for testing differences between patients and controls. All tests were 2-tailed, and results with a $P < .05$ were considered statistically significant. Analyses were performed using PRISM 6 (Graph Pad Software Inc, La Jolla, CA).

Results

Anthropometric data

Anthropometric characteristics of the controls and the patients at baseline, after the lifestyle intervention and 12 months after bariatric surgery, are presented in Table 1.

The patients were eligible for surgery when they reached a weight reduction of 10% from the lifestyle intervention. One year after the surgery, the patients had a mean total weight loss of 44.4 kg, correspondingly a mean reduction in BMI of 15.5 kg/m^2.

Seventeen patients had a diagnosis of diabetes mellitus type II at the inclusion of the study. Surgery was associated with better glycemic control as at 1-year follow-up only 5 patients still had HbA1c > 6.5% and/or had to remain on diabetes medication. Eight patients used statins at the time of inclusion; 4 patients used statins at 1 year after surgery (Table 1).

Lipoprotein profile

The patients had significantly higher baseline levels of TGs (158.3 vs 93.2 mg/dL, $P < .01$; Fig. 1), significantly lower baseline levels of HDL-C (41.9 vs 62.9 mg/dL, $P < .0001$; Fig. 1), and no difference in baseline levels of LDL-C compared with the controls (Fig. 1).

After lifestyle intervention, the patients had significant reductions in TGs (−28.7 mg/dL, $P < .05$), LDL-C (−32.3 mg/dL, $P < .0001$), and a small, but statistically significant reduction in HDL-C (−4 mg/dL, $P < .01$; Fig. 1). At the 1 year-follow-up after surgery, the patients had maintained the significantly lower level of LDL-C (89.1 vs 117.9 mg/dL, $P < .001$) compared with the controls, but there was no additional effect compared with the lifestyle intervention (Fig. 1). HDL-C had increased statistically significant (+12 mg/dL, $P < .0001$), but the levels of HDL-C were still lower than in the controls (53.9 vs 62.9 mg/dL, $P < .05$). There was no difference in TGs between the controls and the patients at this point (Fig. 1).

ApoB, apoA1, and apoB/apoA1 ratio

There were no significant differences in baseline serum levels of apoB between patients and controls (331.8 vs 278 μg/mL, $P > .05$; Fig. 1), but the patients had lower baseline levels of apoA1 (860 vs 1061 μg/mL, $P < .05$, data not

Table 1 The effect of lifestyle changes and bariatric surgery on anthropometric characteristics compared with controls

Variable	Inclusion	Lifestyle intervention	12 mo after surgery	Controls
n, No.	34	34	34	17
Age, median (range)	43 (30–58)			45 (27–70)
Female	20 (59%)			12 (71%)
Weight (kg), mean ± SD	130.4 ± 24.7	117.4 ± 21.9	86.0 ± 17.3	73.0 ± 7.5
BMI (kg/m^2), mean ± SD	44.8 ± 6.9	40.1 ± 5.7	29.3 ± 4.2	25.2 ± 2.0
TWL (kg), mean ± SD		13 ± 5.7	44.4 ± 18.0	
Diabetes, No. (%)	17 (50)		5 (15)	1 (6)
Hypertension, No. (%)	14 (41)		N.D	3 (18)
Statins, No. (%)	8 (24)		4 (12)	2 (11)
Smoking, No. (%)	3 (9)		3 (9)	1 (6)
HbA1c% ± SD	6.5 ± 1.1	N.D	5.7 ± 0.5	5.4 ± 0.2

BMI, body mass index; HbA1c, glycated hemoglobin; N.D, not determined; TWL, total weight loss.

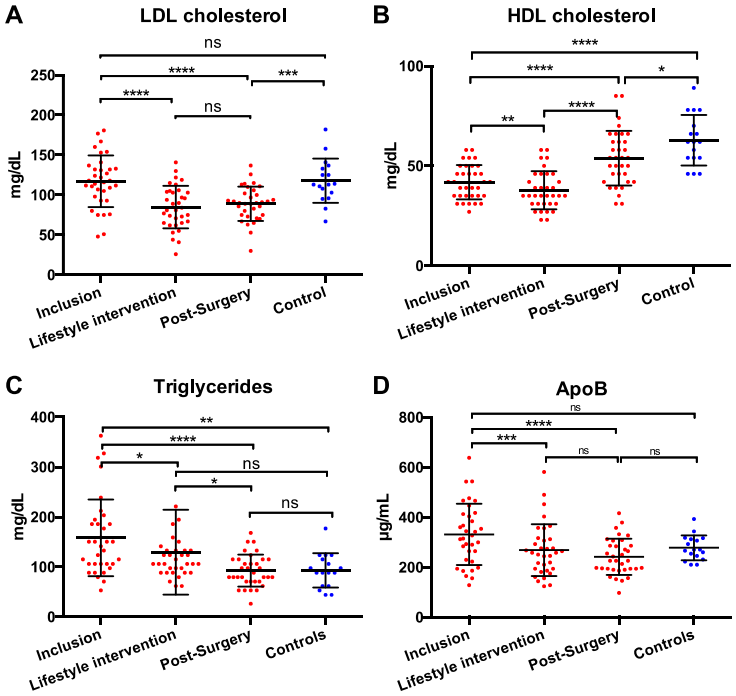

Figure 1 Serum levels of (A) LDL cholesterol, (B) HDL cholesterol, (C) triglycerides (TGs), and (D) apoB at inclusion, after a lifestyle intervention (~3 months) and 1 year after bariatric surgery in 34 morbidly obese patients, and at inclusion for controls. Values are mean with standard deviation. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$, ns, not statistically significant.

shown). The lifestyle intervention reduced apoB (−62.9 µg/mL, $P < .001$; Fig. 1) and apoA1 (−90.6 µg/mL, $P < .05$). At the 1-year follow-up, the patients had maintained significantly lower levels of apoB (−89.9 µg/mL, $P < .0001$; Fig. 1), but there was no additional effect compared with the lifestyle intervention. ApoA1 had at this point increased significantly (+137 µg/mL, $P < .001$), and there were now no significant differences between controls and patients. The patients had higher apoB/apoA1 ratio compared with controls at inclusion ($P < .001$, data not shown), and after the surgery, there was no difference between the groups. The lifestyle intervention had no effect on the apoB/apoA1 ratio, at the 1-year follow-up after surgery, the patients had a significant decrease in apoB/apoA1 ratio ($P < .0001$, data not shown).

LDL subfractions and LDL particle size

There were no significant differences in the baseline values of the lbLDL subfractions or the baseline values of the sdLDL subfractions between the patients and the controls (Fig. 2). After lifestyle intervention, the patients had a significant reduction in the lbLDL (−18.6 mg/dL, $P < .0001$) and a small, but statistically significant reduction in the sdLDL (−1 mg/dL, $P < .05$; Fig. 2). Compared with the controls, the patients had significantly lower lbLDL (41.0 vs 56.9 mg/dL, $P < .01$), but there were no significant differences in sdLDL (Fig. 2).

At the 1-year-follow-up after surgery, the patients had significantly lower levels of lbLDL (−12.9 mg/dL, $P < .001$) compared with baseline values, whereas the levels of sdLDL returned to baseline values (Fig. 2). Compared with the controls, the patients had lower levels of lbLDL (46.7 vs 56.9 mg/dL, $P < .05$), but there were no differences in sdLDL (Fig. 2).

There were no significant differences in baseline values of mean LDL particle size (Å) or distribution of lipid pattern (A, B, or intermediate) between controls and patients (data not shown). After the lifestyle changes, the patients had a small, but statistically significant increase in mean LDL particle size ($P < .01$, data not shown). At the 1-year follow-up after surgery, the mean particle size returned to baseline values.

HDL subfractions

The patients had a significantly lower concentration of the large ($P < .0001$, Fig. 2) and intermediate HDL ($P < .0001$, data not shown) subfractions at baseline

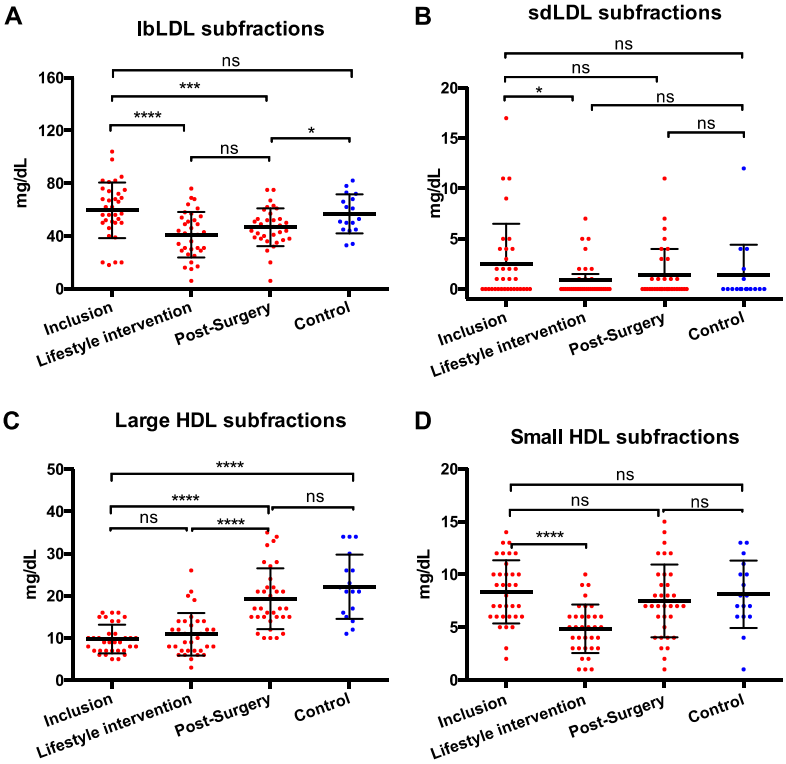

Figure 2 Serum levels of the (A) large, boyant LDL (lbLDL) subfractions, (B) the small, dense LDL (sdLDL) subfractions, (C) large HDL subfractions, (D) small HDL subfractions at inclusion, after a lifestyle intervention (~3 months) and 1 year after bariatric surgery in 34 morbidly obese patients, and at inclusion for controls. Values are mean with standard deviation. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$, ns, not statistically significant.

compared with the controls, but there were no significant differences in the small HDL subfractions (Fig. 2). After the lifestyle intervention, there was a significant reduction in the intermediate ($P < .05$, data not shown) and small HDL subfractions ($P < .0001$, Fig. 2); however, there was no change in the large HDL subfractions.

At the 1-year-follow-up after surgery, the patients had a significant increase in the large HDL subfractions (+9.5 mg/dL, $P < .0001$), and there was now no difference compared with the controls (Fig. 2). There was also a significant increase in the intermediate HDL subfractions ($P < .001$, data not shown), but the concentration was still significantly lower than compared with the controls. The small HDL subfractions returned to baseline values, with no significant difference compared with the controls (Fig. 2).

PON1 arylesterase activity, SAA1 abundance, and SAA/PON1 ratio

The patients had significantly lower baseline PON1 arylesterase activity compared with controls ($P < .0001$, Fig. 3). After the lifestyle changes, there was a significant reduction in PON1 activity ($P < .01$) in the patients, and bariatric surgery had no further effect on PON1 activity (Fig. 3). The patients had higher levels of SAA at inclusion compared with controls (10.4 vs 3.9 μg/mL, $P < .01$, data not shown). After the lifestyle intervention, there was a significant reduction in SAA in the patients (−4.6 μg/mL, $P < .01$, data not shown), and bariatric surgery had no further effect. The patients had higher SAA/PON1 ratio at inclusion compared with controls ($P < .001$, Fig. 3), and there was a statistically significant decrease in SAA/PON1 ratio after the lifestyle intervention ($P < .01$, Fig. 3) and further improvement at 1 year after the surgery compared with inclusion ($P < .0001$, Fig. 3).

Cholesterol efflux capacity

We found no significant differences in baseline values of macrophage CEC between patients and controls. Neither lifestyle changes nor bariatric surgery had any significant effect of CEC in the patients (Fig. 3).

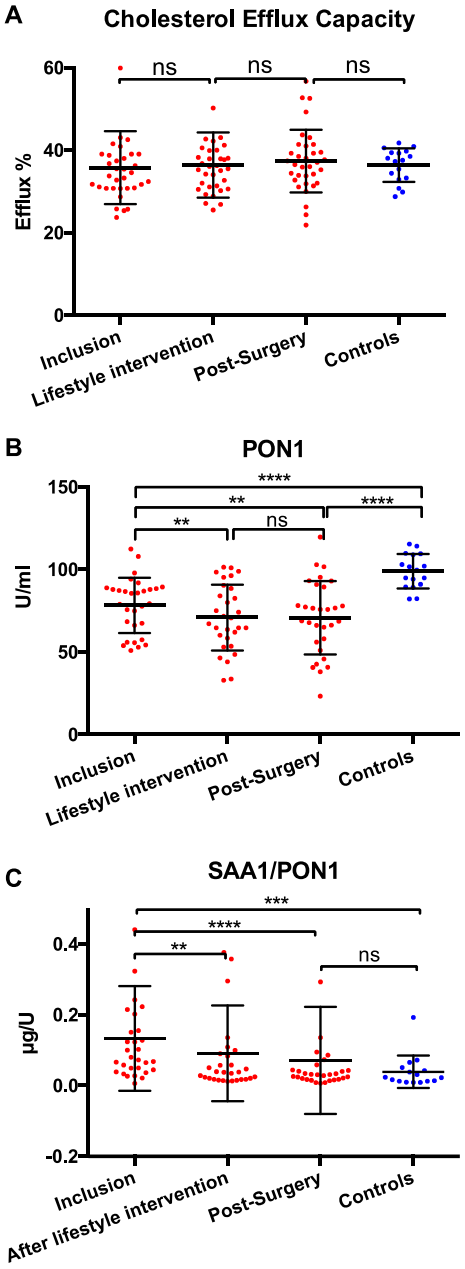

Figure 3 A) Cholesterol efflux capacity (CEC), (B) Serum paraoxonase-1 (PON1) activity, and (C) serum amyloid A1/PON1 (SAA/PON1) ratio determined at inclusion, after a lifestyle intervention (~3 months) and 1 year after bariatric surgery in 34 morbidly obese patients, and at inclusion for controls. Values are mean with standard deviation. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$, ns, not statistically significant.

Discussion

In the present study where 34 morbidly obese patients underwent bariatric surgery, we demonstrate anticipated reductions in BMI and total weight loss, in line with systematic reviews and meta-analyses of bariatric surgery.[18] The patients achieved reductions in TGs (41%), LDL-C (24%), and apoB (27%) at 12 months after surgery, results that are comparable with previously published literature.[19–23] The fact that bariatric surgery had no additional effect in reducing levels of LDL-C and apoB compared with the preoperative lifestyle intervention is probably because of the fact that the lifestyle intervention had already lowered them as low or even beyond what can be expected from bariatric surgery alone. A small study of pre-bariatric lifestyle changes showed a similar trend[24] and comparable improvements in LDL-C and TGs from a similar lifestyle intervention has been published earlier.[25] It is also important to note that 4 of the 8 patients using statins at inclusion of the study had discontinued this medication at the 12-month follow-up.

The patients in this study had low levels of HDL-C and apoA1 at admission, a hallmark of the obese dyslipidemia.[26] The reduction in HDL-C observed after the lifestyle intervention is expected, as reduced caloric intake during weight loss programs is associated with temporary declines in HDL-C, an effect that is reversed once a stable weight is achieved.[26] At 12 months after surgery, the patients had a significant increase in HDL-C (29%) and apoA1 (16%) compared with baseline, results that are in line with previously published literature.[19,20,22]

LDL and HDL subfractions

The patients in this study had an LDL profile at inclusion of the study dominated by pattern A (82%) and only 6% having a pattern B (data not shown), thus the measured levels of sdLDL were low. It is important to note that none of the included obese patients had known or symptomatic CVD. Another possible explanation for the low concentrations of sdLDL observed might be the fact that only half of the patients had diabetes and 41% were men, 2 important factors associated with elevated levels of sdLDL.[27,28] The lifestyle changes induced a small, but statistically significant reduction of sdLDL. We did not find any significant changes in pattern, concentrations of sdLDL or mean LDL-particle size after bariatric surgery compared with inclusion.

As drug trials with niacin and cholesteryl ester transfer protein inhibitors, both drugs that significantly increased HDL-C, failed to reduce the risk of CVD[29] and a Mendelian randomization study challenged the concept that raising HDL-C will lower CVD risk,[30] serious doubts have been raised about using the cholesterol content of HDLs (HDL-C) as relevant to any aspect of HDL function related to protection of CHD. Instead, hypotheses suggesting that the anti-atherogenic mechanisms of HDL are

related to HDL function and/or composition have gained momentum.[31–33]

As for HDL composition, epidemiologic and clinical studies are discordant regarding the prognostic value of measuring HDL subclasses and subfractions.[34] The reasons are suggested to be different assay methods used, subfraction heterogeneity or ethnic variation.[35] The pathophysiological aspect of HDL composition is still an evolving research field and a recent study indicates that the smaller HDL particles are more efficient mediators of ABCA1-mediated cholesterol efflux.[12] We did not observe any changes in macrophage CEC despite statistically highly significant changes in HDL composition and function, including the small HDL (Fig. 3).

The low levels of HDL-C observed in patients at admission compared with controls was because of significantly lower levels of the larger HDL-particles, while there was no significant difference in the concentration of smaller HDL-particles. Other studies of obese patients have observed a similar pattern.[36,37] At 12 months after surgery, the patients had a significant increase in HDL-C due to an increase in large HDL particles.

SAA, PON1 activity, and macrophage CEC

Reverse cholesterol transport, the transport of cholesterol from peripheral tissue to the liver for excretion, is 1 of many mechanisms by which HDL exerts its protective effect on the development of CVD.[34,38] Efflux of cholesterol from macrophages in the arterial wall is suggested to be an important aspect of HDL function,[39] and CEC is indeed inversely correlated with hard vascular endpoints.[10] We did not observe any significant differences in macrophage CEC between patients and controls and no significant effects of the lifestyle intervention or bariatric surgery despite significant changes in HDL-C concentration and HDL subfractions. However, it is important to keep in mind that using whole serum in an assay for CEC may not be a precise measurement of HDL-mediated efflux since cholesterol is transferred from macrophages to serum via several mechanisms that contain other possible acceptors than HDL. In addition, different assays, as well as apoB depletion methods, that may be more suitable have been described.[40,41]

PON1 is an HDL-associated protein that may protect against CVD by reducing oxidative stress.[42,43] Reduced PON1 enzyme activity has been observed in a number of diseases that increase the risk of CVD, including diabetes and metabolic syndrome,[43] and prospective studies have also established reduced PON1 activity as an independent risk factor for CVD.[44,45] The morbidly obese patients in this study had significantly lower PON1 activity levels compared with controls at inclusion, and surprisingly we observed a significant reduction in PON1 activity after the lifestyle intervention and bariatric surgery. Previous studies have found an increase in PON1 activity after bariatric surgery.[46,47] Differences in patient populations and methods of surgery, as well as other methodological factors such as time interval between intervention and analysis, might explain these discordant findings but further investigation is warranted. There is a possibility that the reduction in PON1 activity after the lifestyle intervention may be related to the decrease in HDL and regarding the bariatric surgery intervention; it is well known that toxic compounds, that are substrates for PON1, are released at weight loss and thereby may influence the PON1 measurements.[43,48] However, the acute phase protein SAA was significantly higher in patients at baseline compared with controls and SAA was also significantly reduced after the lifestyle intervention and further reduced after bariatric surgery.

Despite the observed reduction in PON1 activity, the recently proposed marker of HDL function, SAA/PON1 ratio,[15] did show a significant difference between controls and patients at baseline, followed by a significant decrease after the lifestyle intervention and bariatric surgery.

Study limitations

This study has several limitations. The sample size was small. The controls were patients hospitalized to undergo elective surgery (fundoplication or cholecystectomy), and they may not be representative of the healthy population. We also miss follow-up-data on the controls as they were only evaluated once. Measuring LDL and HDL subfractions with the Lipoprint system and macrophage CEC with the MAK192-kit from Sigma–Aldrich are only 2 methods of a heterogeneous set of technologies. Other methodologies such as nuclear magnetic resonance spectroscopy for measuring LDL and HDL subfractions[49] or a cholesterol efflux assay measuring ABCA1-mediated cholesterol efflux[40] may provide additional information, and there may be differences regarding the correlation between these methods. We also did not correct CEC for changes in TGs or apoA1, a possible confounder.

Conclusion

Lifestyle intervention, followed by bariatric surgery improved the lipoprotein profile of 34 morbidly obese patients by significantly reducing serum levels of TGs, LDL cholesterol, and apoB. The patients had an LDL profile at inclusion dominated by pattern A, with low concentrations of sdLDL-particles and the observed reductions in LDL cholesterol during the observation period were due to reduced concentrations of the large, buoyant LDL particles. After bariatric surgery, we observed a significant increase in HDL-C and apoA1, apoB/apoA1-ratio was reduced. Lifestyle invention followed by bariatric surgery reduced PON1 activity, but the SAA/PON1 ratio was decreased. Neither the lifestyle intervention nor bariatric surgery had any effect on macrophage CEC despite significant changes in serum levels of HDL-C and in HDL composition.

Acknowledgments

Authors' contributions: C.A.K., A.H., and K.T.L. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. C.A.K., A.H., K.T.L., and T.N. contributed to study concept and design. T.N., M.M., H.K., S.L., K.C., and M.M.-S contributed to acquisition or analysis. C.A.K., A.H., and K.T.L. drafted the manuscript. All authors made critical revision of the article for important intellectual content. C.A.K., A.H., and K.T.L. contributed to statistical analysis.

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Paper 2

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Lipoprotein apheresis affects lipoprotein particle subclasses more efficiently compared to the PCSK9 inhibitor evolocumab, a pilot study

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ABSTRACT

Lipoprotein apheresis and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors are last therapeutic resorts in patients with familial hypercholesterolemia (FH). We explored changes in lipoprotein subclasses and high-density lipoprotein (HDL) function when changing treatment from lipoprotein apheresis to PCSK9 inhibition.

We measured the levels of low-density lipoprotein (LDL) and HDL particle subclasses, serum amyloid A1 (SAA1), paraoxonase-1 (PON1) activity and cholesterol efflux capacity (CEC) in three heterozygous FH patients. Concentrations of all LDL particle subclasses were reduced during apheresis (large 68.0 ± 17.5 to 16.3 ± 2.1 mg/dL, (p = 0.03), intermediate 38.3 ± 0.6 to 5.0 ± 3.5 mg/dL (p = 0.004) and small 5.0 ± 2.6 to 0.2 ± 0.1 mg/dL (p = 0.08)). There were non-significant reductions in the LDL subclasses during evolocumab treatment. There were non-significant reductions in subclasses of HDL particles during apheresis, and no changes during evolocumab treatment. CEC was unchanged throughout the study, while the SAA1/PON1 ratio was unchanged during apheresis but decreased during evolocumab treatment.

In conclusion, there were significant reductions in large and intermediate size LDL particles during apheresis, and a non-significant reduction in small LDL particles. There were only non-significant reductions in the LDL subclasses during evolocumab treatment.

1. Introduction

Lipoprotein apheresis has been considered the last treatment option when conventional cholesterol lowering treatment is not tolerated or when treatment targets are not met, particularly in patients with familial hypercholesterolemia (FH) [1,2]. The proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors are effective in reducing LDL cholesterol in heterozygous FH patients [3], and they also reduce coronary atheroma size [4]. The first clinical endpoint study with evolocumab was published in March 2017 and showed significant reductions in hard clinical endpoints [5]. Our group has recently demonstrated that when switching from lipoprotein apheresis to PCSK9 inhibition the LDL reduction is partly maintained, while avoiding reduction in high-density lipoprotein (HDL) cholesterol [6], furthermore PCSK9 inhibition elicits less inflammatory response than lipoprotein apheresis [7].

In addition to focus on LDL and HDL cholesterol there has been a growing interest in subclasses of lipoprotein particles, and traditionally small dense LDL particles have been considered particularly atherogenic [8–10]. The clinical effect of subclassing HDL particle size is perhaps more uncertain and the results have been more conflicting [11]. However, HDL composition and function have in recent times been assigned greater importance in lipid metabolism [12–14]. Paraoxonase-1 (PON1) is a HDL associated protein with anti-atherosclerotic properties by preventing oxidation of LDL and cell membranes [15]. Another crucial component of HDL is the acute phase protein serum amyloid A (SAA). Increased expression of SAA, as a result of infection or inflammation, alters the HDL composition and reduces the anti-inflammatory effects of HDL [16]. Regarding HDL functionality, research in reverse cholesterol transport including efflux capacity, has increased in recent

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years, and indeed efflux capacity is inversely associated with cardiovascular endpoints [17]. Contrary to previous research, small HDL particles seem to be associated with more effective cholesterol efflux [18], underlining the difficulties in interpreting cholesterol efflux data.

In the present study, we explored subclasses of LDL and HDL particles, cholesterol efflux capacity, PON1 activity and SAA1, when switching from lipoprotein apheresis to PCSK9 inhibition with evolocumab.

2. Material and methods

The study design and results regarding lipid and inflammatory parameters have recently been published [6,7]. In brief, it was an observational study with three FH patients established in long-term lipoprotein apheresis. They were all heterozygous for the C210G missense mutation in the LDL receptor gene [19]. Treatment was converted to a PCSK9 inhibitor (evolocumab), and the patients were examined immediately before and after their last apheresis treatment (week 0), after one week (immediately before the first evolocumab injection (week 1)), then biweekly before administration of evolocumab (weeks 3, 5 and 7)

2.1. Patients and ethics

There were two women (52 and 53 years) and one man (49 years) with genetically confirmed FH, and they all had angiographically verified coronary artery disease. They were intolerant to statins due to myalgia, and they did not take any type of lipid lowering medication. The patients had been in lipoprotein apheresis on average for 11 years (11–13 years). All patients signed informed consent and the regional ethics committee approved the study. These were currently the only heterozygous FH patients in long term lipoprotein apheresis in Northern Norway, but further patients may be recruited if found eligible, in order to confirm our findings.

2.2. Lipoprotein apheresis

The patients were established in weekly filtration lipoprotein apheresis, and the last lipoprotein apheresis in week 0 was performed with the semi-selective LDL filtration column Cascadeflo-EC-50W (Asahi Kasei Medical Europe) after previous plasma separation with Plasmaflo OP-50 (Asahi Kasei Medical Europe), using the Infomed HF440 apheresis machine (Infomed SA, Geneva, Switzerland). For all three patients, a plasma volume of 4000 mL was treated, average treatment time was 2 h and 15 min. Anticoagulation of the priming solution (2 liters) was obtained by unfractionated heparin; 7500 units per liter. An individualized infusion of heparin was administered in each case in order to avoid clotting.

2.3. Evolocumab treatment

Evolocumab was administered according to the manufacturers' instructions in week one, three, five and seven with the recommended dose of 140 mg subcutaneously (autoinjector). The injections were performed by the patients in the hospital, supervised by experienced nurses, after demonstrations with dummy autoinjectors.

2.4. Blood samples and analyses

Fasting blood samples were obtained by standard venipuncture (or from the AV&HIPHEN;fistulas during apheresis). Blood samples for plasma preparation were placed on ice before centrifugation for 20 min, 3000 ×g at 4 °C. Serum, EDTA plasma and citrate plasma were frozen in aliquots at −80 °C and analyzed in batch at the end of the study.

LDL and HDL subfractions were determined electrophoretically by the use of lipid stained serum (Sudan Black), high-resolution 3% polyacrylamide geltubes and the Lipoprint® system (Quantimetrix Corporation, Redondo Beach, CA, USA, [20]). The different subfractions were identified by their migration distance – on basis of size – in the gel and the concentration of each subfraction was calculated using the Lipoprint analysis software. The Lipoprint LDL system can identify seven subfractions of LDL, (1 = larger, buoyant particles, 2 = intermediate and 3–7 = small, dense particles). The diameter of the LDL particles at the cut-off point separating subfractions 1–2 from 3 to 7 was 251 Å. The Lipoprint HDL system separates ten various HDL subfractions (1–3 = large HDL particles, 4–7 = intermediate HDL particles, 8–10 = small HDL particles). Hence the different subfractions of LDL and HDL were divided into three subclasses; large, intermediate and small.

Cholesterol efflux was measured with a commercial kit from Sigma-Aldrich (MAK192) according to the description. Briefly, a human monocyte cell line, THP-1, was differentiated into macrophages with 10 ng/ml phorbol myristate acetate (PMA) for 24 h at 37° and 5% CO2 in a 96-well plate. The PMA containing medium was replaced with complete cultivation medium (RPMI1640 including 10% foetal bovine serum, 2 mM Glutamine) and incubated for another 30 h. The serum containing medium was removed and then washed with serum free medium. A reaction mix, containing equilibration buffer and fluorescence labelled LDL, was added to the cells and incubated for 16 h. The reaction mix was removed and wells washed with serum free medium. Patient serum samples were precipitated with a reaction mix from the kit and the clear supernatant was added to the plate and incubated for 5 h. After the incubation, supernatants were transferred to a new plate and the fluorescence measured (482 ex/515 em). The cell layer was solubilized with a cell lysis buffer, incubated for 30 min on a shaker. The cell lysate was then transferred to the plate with supernatants and the fluorescence of the mixture was measured. Percent efflux was calculated as follows: 100× fluorescence intensity of the medium/x fluorescence intensity of the medium and cell lysate.

PON1 arylesterase activity was measured in citrate plasma. Briefly, plasma was diluted 1:80 with a salt buffer (20 mM Tris–HCl and 1.0 mM $CaCl_2$). A triplicate of 20 μl diluted plasma were added to the wells in an UV&HIPHEN;transparent 96-well plate (Sigma-Aldrich). 200 μl of phenyl acetate solution, containing 3.26 mM phenyl acetate in salt buffer, was added to each well and the absorbance of produced phenol was measured at 270 nm with 250 nm as background in a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). The initial period when the reaction was linear was used for calculation of activity, expressed as U/ml, using an extinction coefficient of phenol of 1310 M−1 cm1.

To investigate the acute phase response by SAA, plasma SAA1 levels were measured by an ELISA (DY3019-05, R&D systems, Minneapolis, MN, USA) according to the manufacturers' instructions. In short, citrate plasma was added to the plate and incubated for 2 h at room temperature. Following wash, a detection antibody was added and incubated for 2 h. The plate was washed and streptavidin-horseradish peroxidase was added followed by incubation for 20 min. The plate was then washed a final time before a substrate solution was added before 20 min incubation. At the end of the incubation, stop solution was added and absorbance was measured at 450 nm with correction at 570 nm using a Spectramax 190 plate reader (Molecular devices, Sunnyvale, CA, USA).

The measures of SAA and PON1 were integrated as SAA1/PON1 ratio, which has been proposed as a possible biomarker for dysfunctional HDL [21].

2.5. Statistics

Numerical data are presented with mean and standard deviation (SD). Age and duration of treatment are presented as mean and range. A repeated measures one-way analysis of variance (RM one-way ANOVA) was used to calculate the longitudinal effect of the evolocumab treatment (week one to week seven). Levels before and after lipoprotein apheresis treatment (week 0) and before apheresis vs. after the last evolocumab injection were compared by paired t-test *t*-tests. All tests were two-tailed and results with a $p < 0.05$ were considered statistically significant. Analyses were performed using PRISM 6 (Graph Pad Software Inc, La Jolla, CA, USA).

3. Results

3.1. LDL particle subclasses (Fig. 1)

All subclasses of LDL particles were reduced during lipoprotein apheresis (Fig. 1). The concentration of large LDL particles was reduced from 68.0 ± 17.5 mg/dL to 16.3 ± 2.1 mg/dL during apheresis (p = 0.03) (Fig. 1a), intermediate LDL particles were reduced from 38.3 ± 0.6 mg/dL to 5.0 ± 3.5 mg/dL (p = 0.004) (Fig. 1b) and small LDL particles were reduced from 5.0 ± 2.6 mg/dL to 0.2 ± 0.1 mg/dL (p = 0.08) (Fig. 1c). During evolocumab treatment there were no significant changes in the large, intermediate or the small LDL particles, although there seemed to be a slight reduction over time for both large and intermediate particles. Average LDL particle diameter was unchanged during the course of study (data not shown).

3.2. HDL particle subclasses (Fig. 2)

All subclasses of HDL particles were reduced during lipoprotein apheresis (Fig. 2), however non-significantly. Large particles were reduced from 5.7 ± 1.5 mg/dL to 3.7 ± 1.2 mg/dL, (Fig. 2a), intermediate particles were reduced from 15.0 ± 2.0 mg/dL to 9.3 ± 1.2 mg/dL, (Fig. 2b), and small HDL particles were reduced from 12.0 ± 4.6 mg/dL to 5.0 ± 1.7 mg/dL, (Fig. 2c). All types of HDL particles increased during the week after lipoprotein apheresis, and were unchanged during treatment with evolocumab.

3.3. CEC, PON1 activity and SAA1 (Fig. 3)

There was no significant change in cholesterol efflux capacity during apheresis (42.6 ± 3.8% to 44.8 ± 8.8%, and no significant changes at week 1 (50.3 ± 7.1%), (Fig. 3a). Likewise, during evolocumab treatment there were no significant changes in efflux capacity.

There was a significant decrease in PON1 activity during apheresis from 87.5 ± 8.3 U/ml to 53.5 ± 3.6 U/ml (p = 0.03) (Fig. 3b). One week after apheresis PON1 activity had increased to levels comparable to pre-apheresis, and was unchanged during evolocumab treatment.

There was a non- significant reduction in SAA1 during apheresis (1.9 ± 1.1 μg/ml to 1.3 ± 0.6 μg/ml (p = 0.18), and SAA1 was unchanged during evolocumab treatment (Fig. 3c).

The SAA1/PON1 ratio was unchanged during apheresis but appeared to decrease during evolocumab treatment (Fig. 3d).

4. Discussion

We observed reductions in all subclasses of LDL particles after semi-selective lipoprotein apheresis. Although the reduction in small LDL particles did not reach statistical significance it seems numerically convincing, as there were virtually immeasurable amounts of small LDL particles present after apheresis.

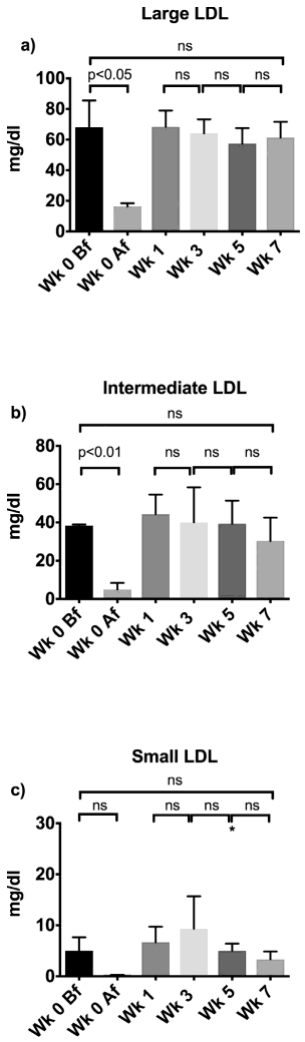

Fig. 1. Concentrations of subclasses of LDL particles during lipoprotein apheresis and after starting evolocumab.
LDL: Low-density lipoprotein. Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1–7: Week 1–7 (samples taken before evolocumab administration).

Subclasses of HDL particles were statistically unaffected by apheresis and evolocumab treatment, even though we previously have demonstrated significant reduction in total HDL cholesterol during apheresis [6]. This could be a result of the low number of participants. There was no significant change in cholesterol efflux capacity one week after apheresis compared to baseline before apheresis, and efflux was also unaffected by evolocumab.

The novel PCSK9 inhibitors have been shown in several clinical trials to lower LDL and coronary atherosclerosis significantly [4,22,23]. Clinical endpoints were reduced in a recent evolocumab study, however total mortality was not reduced [5]. Further long-

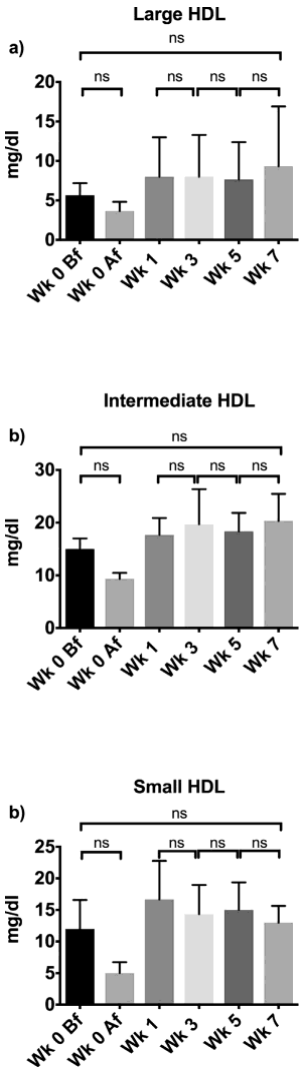

Fig. 2. Concentrations of subclasses of HDL particles during lipoprotein apheresis and after starting evolocumab.
HDL: High-density lipoprotein. Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1–7: Week 1–7 (samples taken before evolocumab administration).

term studies are expected to be reported in 2018. Lipoprotein apheresis, which – although time-consuming and expensive – also has a proven clinical effect [24]. Furthermore, whether the intermittent, very low values of LDL seen after apheresis with a rebound before next treatment is preferential to a more constant, moderate reduction seen with PCKS9 inhibition is also a matter of debate. A recent double-blind study has demonstrated that the rate of apheresis treatment can be reduced with PCSK9 inhibition [25]. However, it seems premature to recommend that all patients established in apheresis should be switched to PCSK9 inhibition.

4.1. Lipoprotein particle subclasses

Over the last decades, small LDL particles have been considered especially atherogenic [26], even if the clinical use of fractioning or subclassing of lipoprotein particles has been debated [27,28].

Otto et al. have previously demonstrated reduction in large, intermediate and small subfractions of LDL (measured with ultracentrifugation) during one session of lipoprotein apheresis [29], this finding was later confirmed by Geiss et al. [30]. The PCSK9 inhibitor alirocumab lowered large, intermediate and small LDL particles (measured by mass spectroscopy) when compared to placebo [31,32]. We demonstrate reductions in large, intermediate and small LDL particles during apheresis, and even if the latter was not statistically significant, virtually all of the small LDL particles were removed. This finding could be of particular importance for FH patients with high risk of atherosclerotic complications [33]. When changing the treatment from lipoprotein apheresis to evolocumab the findings are less clear; there was a trend for reductions in both large and intermediate LDL particles, while the findings regarding small LDL particles were less consistent and not in line with the findings cited above. This could be due to a number of factors including small sample size and short observation period.

Large HDL particles have previously been associated with better clinical outcome [34], however this notion has been challenged, and more recent studies indicate that smaller HDL particles may be more beneficial [35]. Small, dense HDL particles have also been identified as more efficient mediators of cholesterol efflux [18].

Orsoni et al. have demonstrated that lipoprotein apheresis reduces all sizes of HDL particles (mass spectroscopy), however relatively more of the larger subtypes of HDL [36]. Koren et al. noted a non-significant increase of all three particle sizes after 12 weeks of alirocumab compared to baseline [31]. We found non-significant reductions in all three subclasses of HDL cholesterol particles during apheresis, and no significant differences when changing the cholesterol lowering treatment from apheresis to PCSK9 inhibition.

4.2. CEC, PON1 activity and SAA1

Cholesterol efflux capacity is inversely related to atherosclerotic disease in FH patients [37]. Nenseter et al. did not find any change in cholesterol acceptor capacity after lipoprotein apheresis in patients with homozygous FH [38]. Adorni et al. demonstrated a reduction in cholesterol efflux capacity after lipoprotein apheresis in patients with hypercholesterolemia [39]. At present, there are few data on PCSK9 inhibition and cholesterol efflux capacity, however an increase in cholesterol efflux during PCSK9 inhibition has been hypothesized [40,41].

A number of previous studies have been performed in non-human cell-lines [39,42]. However, in order to avoid any possible species-related differences [43], we routinely use a human monocyte cell line. We demonstrate unchanged cholesterol efflux capacity moving from lipoprotein apheresis to PCSK9 inhibition, even if HDL cholesterol was reduced during lipoprotein apheresis and restored during PCSK9 inhibition. Our contradictory CEC results compared to Adorni et al. [39], may be explained by the fact that human monocytes and no acetyl-coenzyme A acetyltransferase inhibitor were used in the present study, but this has to be further investigated in a larger study.

PON1 activity is closely connected to HDL function, and could have an atheroprotective effect [44]. PON1 contributes to the antioxidative functions of HDL in the vascular wall but is also an important detoxifying agent [15], therefore techniques measuring enzyme activity may differ depending on the aim. Our finding of reduction of PON1 during apheresis could hence be of importance, also when taking into account the simultaneous reduction in HDL [6]. Furthermore, the return of PON1 to pre-apheresis levels

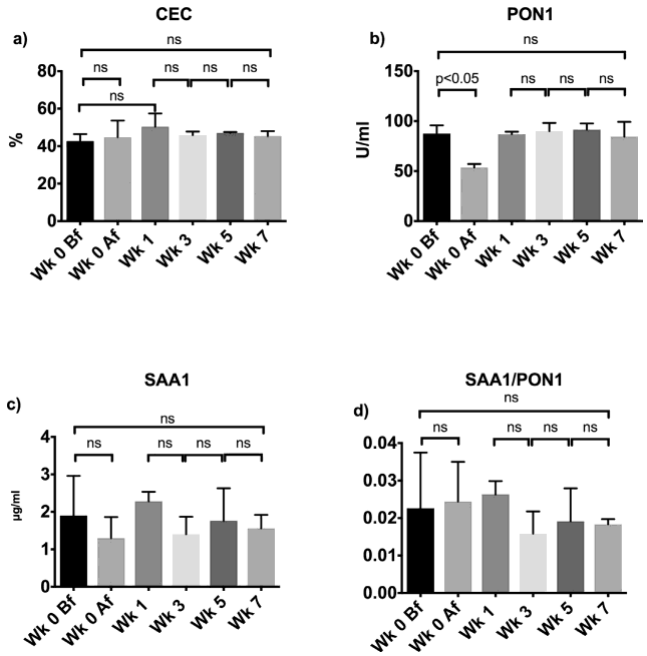

Fig. 3. Cholesterol efflux capacity, PON1 activity, SAA1 concentration and PON1/SAA1 ratio during lipoprotein apheresis and after starting evolocumab.
CEC: Cholesterol efflux capacity. PON1: paraoxonase-1. SAA1: serum amyloid A1. Wk 0 bf: Week 0 before apheresis. Wk 0 af: Week 0 after apheresis. Wk 1–7: Week 1–7 (samples taken before evolocumab administration).

at the start of PCSK9 inhibition at week 1 and during treatment of evolocumab is interesting and should be further explored in larger studies.

SAA is an acute phase protein, present in HDL in several isoforms [45] that has been associated with atherosclerotic disease [46], and from previous studies it is known that lipoprotein apheresis reduces serum amyloid A and serum amyloid P [47]. However, in the present study we only find a non-significant reduction in SAA1 during apheresis. Evolocumab treatment had no effect on SAA1 levels.

However, paired measurements of SAA and PON1 have been suggested as a possible marker for dysfunctional HDL since it combines the antioxidant capacity of PON1 with the marker of inflammatory responses SAA [21], and should therefore be investigated in a larger population before any conclusions could be drawn since we have previously shown that apheresis elicits an innate immune response [48,49], and we have recently reported that evolocumab seems inert in this respect [7]. In the present study, the ratio was unchanged after apheresis while treatment with evolocumab caused a non-significant reduction. This finding possibly indicates a favorable reduction of SAA1/PON1 with evolocumab treatment but further studies in larger populations are needed.

A very recent paper establishes the need for more data on combination of apheresis and new LDL lowering therapy, and international collaboration is established [50].

4.3. Limitations

There are several limitations to this study. It has a low number of participants (n = 3) and is merely observational. This limits generalizability. The apheresis method used is a semi-specific method, and may not reflect more specific lipoprotein apheresis systems. Furthermore, the effects seen when converting from lipoprotein apheresis to PCSK9 inhibition may not necessarily reflect the effects of PCSK9 inhibition in apheresis-naïve patients. Finally, some of the non-significant trends seen after seven weeks of treatment may reflect the relatively short observation time.

In conclusion, there were significant reductions in large and intermediate size LDL particles during lipoprotein apheresis, and a non-significant reduction in small LDL particles. There were non-significant reductions in all three subclasses of HDL particles during lipoprotein apheresis, while treatment with evolocumab did not significantly affect LDL or HDL particle subclasses. Cholesterol efflux capacity was not affected by lipoprotein apheresis or evolocumab treatment while the SAA1/PON1 ratio appeared to decrease slightly during evolocumab treatment.

Conflict of interest

The authors report no conflict of interest.

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Paper 3

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Research letter

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Intensive lipid lowering therapy reduces large, but not small, dense low-density lipoprotein particles measured by gel electrophoresis, in elderly patients with atrial fibrillation

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Low-density lipoprotein (LDL) cholesterol (LDL-C) is considered causative in atherosclerotic diseases, and LDL lowering therapy (LLT) has clearly demonstrated an effet on clinical endpoints. Despite numerous effetive LLTs being readily available, attainment to recommended LDL-C targets are low and a large number of high-risk patients would benefit from more intensive treatment.[1] LDL-C has significnt limitations as a risk marker, and measuring small, dense LDL (sdLDL) particles has been proposed as a novel tool to improve cardiovascular risk stratifiction. Epidemiological studies have found an association between elevated levels of sdLDL and increased risk of cardiovascular disease[2] and in-vitro studies suggest that sdLDL particles have increased atherogenic potential,[3] but no studies have yet proven that adjusting treatment according to sdLDL measurements improves clinical outcomes.[2,3] There are several methods for measuring and quantifying sdLDL; nuclear magnetic resonance, gel electrophoresis, ultracentrifugation and ion mobility are among the most commonly used. These methods are not directly comparable as they separate particles basedron diffeent physicochemical properties and the variability between methods is high.[4] As there is no method generally accepted as a reference or a gold standard, each method must be validated separately. With regard to lowering sdLDL, the clinical trials published have been contradictory,[5] possibly due to use of diffeent methods in quantifying sdLDL and the poor comparability between them.

The present study sought to evaluate the effets of a commonly used regime to lower LDL-C, atorvastatin 40 mg plus ezetimibe 10 mg, in a randomized, double blinded, placebo-controlled trial of 30 elderly patients with atrial fibillation. Patient characteristics and study design have already been published.[6] LDL subfractions were measured by a gel electrophoresis system (Lipoprint LDL) that separates LDL into LDL1 and LDL2 (larger, more buoyant particles) and LDL3 into seven (smaller, denser particles) based on size and electrical charge. Blood samples were collected at baseline and after six months of follow-up. At inclusion there were no significnt diffeences between the two groups. Atorvastatin/ezetimibe significnt ly reduced LDL-C ($p < 0.001$) and the larger, more buoyant particles LDL1 and LDL2 ($p < 0.001$), but had no effet on the sdLDL particles (Figure 1).

Should measuring sdLDL be important in ordereto defin risk and imply reductions in clinical outcomes, the results of this trial suggest that Lipoprint LDL, a commonly used gel electrophoresis system, would not be suitable to evaluate the treatment effet of a statin and ezetimibe. Further and larger studies are warranted, with regard to both identifying which method to use and whether measuring sdLDL yields clinically relevant information in the evaluation of patients at risk of atherosclerotic cardiovascular disease.

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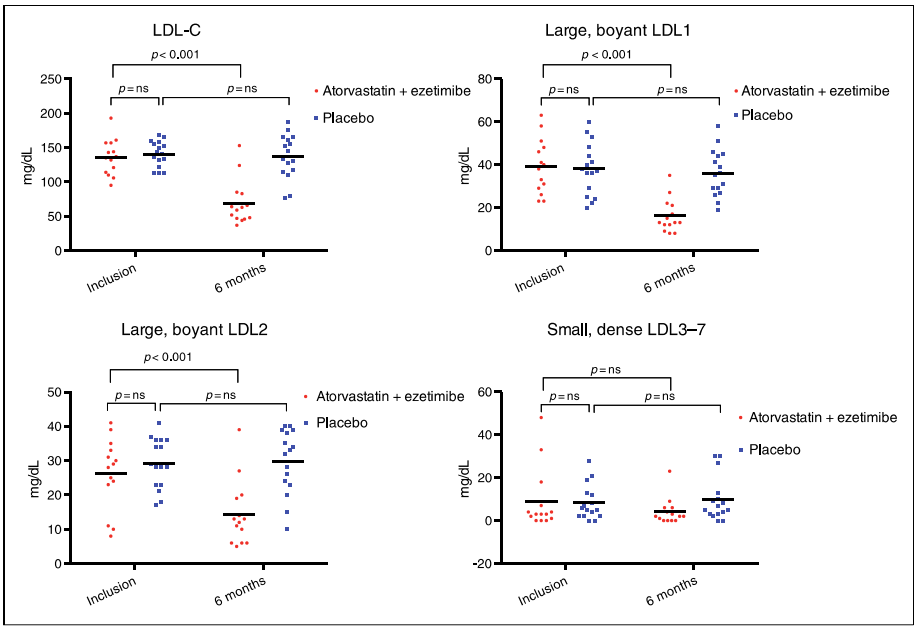

Figure 1. The effects of atorvastatin and ezetimibe versus placebo on LDL-C and various LDL-subfractions measured by a gel electrophoresis system (Lipoprint LDL®) in an elderly population with atrial fibrillation.
LDL: low-density lipoprotein; LDL-C: LDL cholesterol.

Declaration of conflicting interests

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Paper 4

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Article

Effect of N-3 Polyunsaturated Fatty Acids on Lipid Composition in Familial Hypercholesterolemia: A Randomized Crossover Trial

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Abstract: Individuals with familial hypercholesterolemia (FH) have an increased risk of cardiovascular disease. Treatment is mainly low-density lipoprotein cholesterol (LDL-C) reduction. How omega-3 polyunsaturated fatty acids (n-3 PUFAs) supplements affect lipoproteins in FH subjects is unknown. We hypothesized that a high-dose n-3 PUFA supplement would reduce atherogenic lipoproteins and influence the high-density lipoprotein cholesterol (HDL-C) function. We performed a randomized, double-blinded crossover study with 34 genetically verified FH individuals (18–75 years, clinically stable, statin treatment > 12 months). Treatment was 4 g n-3 PUFAs (1840 mg eicosapentaenoic acid and 1520 mg docosahexaenoic acid daily) or four capsules of olive oil for three months in a crossover design with a washout period of three months. The defined outcomes were changes in triglycerides, lipoproteins, lipoprotein subfractions, apolipoproteins, and HDL-C function. After treatment with n-3 PUFAs, total cholesterol, LDL-C, and triglycerides were reduced compared to placebo ($p \leq 0.01$ for all). Total HDL-C levels were unchanged, but the subfraction of large HDL-C was higher ($p \leq 0.0001$) after n-3 PUFAs than after placebo, and intermediate HDL-C and small HDL-C were reduced after n-3 PUFAs compared to placebo ($p = 0.02$ and $p \leq 0.001$, respectively). No changes were found in apolipoproteins and HDL-C function. N-3 PUFAs supplements reduced atherogenic lipoproteins in FH subjects, leaving HDL-C function unaffected.

Keywords: familial hypercholesterolemia; omega-3 fatty acids; lipids; cardiovascular disease; randomized trial

1. Introduction

Familial hypercholesterolemia (FH) is the most common monogenic disorder in the world [1]. Low-density lipoprotein cholesterol (LDL-C) reduction is the main treatment goal to prevent harmful long-term effects of LDL-C overload. However, only 50% of FH individuals reach their LDL-C treatment goal. Thus, many FH individuals live with a high residual risk of cardiovascular disease (CVD) [2]. All FH individuals are either in the high or very high cardiovascular risk category, and additional therapies to lower LDL-C in individuals with FH are needed [3].

The role of omega-3 polyunsaturated fatty acids (n-3 PUFAs) in cardiovascular health has been studied for decades. Early clinical trials indicated a reduction in CVD after n-3 PUFAs supplementation [4,5]. After the introduction of statins, the CVD-reducing effect of n-3 PUFAs has been debated [6,7]. The search for the most potent dosage and preparation of n-3 PUFAs is ongoing. The Reduction of Cardiovascular Events with Icosapent Ethyl—Intervention Trial (REDUCE-IT) showed an effect of icosapent ethyl supplement on cardiovascular outcomes [8]. In contrast, the Long-Term Outcomes Study to Assess Statin Residual Risk with Epanova in High Cardiovascular Risk Patients with Hypertriglyceridemia (STRENGTH) used a carboxylic acid preparation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and found no difference in major adverse cardiovascular events [9]. The effect of n-3 PUFAs on LDL-C is debated, ranging from potentially harmful to neutral in larger populations [10]. The triglyceride-lowering characteristics of n-3 PUFAs are less controversial, but the role of triglyceride-lowering in cardiovascular risk reduction has not been established [3,8,9,11]. The effect of n-3 PUFAs on lipoproteins is unclear for the FH population, as the trials available are limited and report divergent results [12–14].

It has been proposed that advanced lipoprotein testing can improve cardiovascular risk prediction, identify residual risk in high-risk patients, and guide lipid-lowering therapy. Available advanced lipoprotein testing ranges from subfractionating LDL-C and high-density lipoprotein cholesterol (HDL-C) particles and quantifying the particle number of LDL-C/HDL-C to a wide array of tests of HDL-C functionality.

In this study, we investigated the effects of n-3 PUFAs on LDL-C and HDL-C subfractions, paraoxonase-1 (PON1) arylesterase activity, serum amyloid A1 (SAA1) levels, and cholesterol efflux capacity (CEC) in a group of patients with genetically verified FH. We hypothesized that a high-dose n-3 PUFAs supplement in this population would reduce LDL-C and triglycerides, reduce small, dense LDL-C particles (sdLDL-C), and improve HDL-C level and function compared to placebo.

2. Materials and Methods

2.1. Trial Design and Interventions

The trial design and eligibility criteria have been previously reported [15]. In brief, the trial was randomized, double-blinded, and placebo-controlled and had a crossover design of nine months duration. Two treatment periods (three months each) were separated by a three-month washout period to minimize the potential carry-over effect. The inclusion criteria were age between 18 and 75 years, genetically verified FH, clinically stable disease, and statin treatment for at least 12 months. Exclusion criteria were noncompliance, pregnancy or fertility treatment, breastfeeding, cancer, and/or severe illness. Randomization was done at inclusion, with an allocation ratio of 1:1. We had a well-known study population with stable disease and expected a low drop-out rate; thus, a crossover design was chosen. The participants were patients at the lipid clinic at Nordland hospital (Bodø, Norway). An invitation letter was sent to the participants from the lipid outpatient clinic. Two research nurses collected blood samples and performed clinical tests. Three alternating physicians performed the physical examination.

The n-3 PUFAs and placebo were administered in the same manner in the two treatment periods; four capsules a day. The n-3 PUFAs capsule contained 460 mg EPA and 380 mg DHA (a daily dose of 1840 mg EPA and 1520 mg DHA). The placebo capsule contained olive oil. Both the n-3 PUFAs and the placebo were provided by BASF (Lysaker, Norway). The study medication was administered to the participants when they started each treatment period, and unused medicines were returned accordingly.

The primary outcome of this trial, as previously reported, was change in reactive hyperemia index assessed by peripheral arterial tonometry [15]. The outcome presented in this paper was a change in triglycerides, lipoproteins, and lipoprotein composition and function (predefined secondary outcomes).

The random allocation sequence of participants and the labeling of the study medication were provided by Apotekproduksjon AS (Oslo, Norway). To conceal the allocation

ager and a physician enrolled the participants in the trial. The study participants
providers were blinded to the series of interventions. Apotekproduksjon AS kep
domization key upon completion of the trial.

2.2. Blood Samples

Fasting blood samples were obtained by venipuncture (vacutainer tubes) at
after the first treatment period, after washout, and after the second treatment p
run tubes were centrifugated at 2000× *g* for 10 min. The citrate vacutainers had
dium citrate and were centrifugated at 3000× *g* for 20 min at 4 °C.

2.2. Blood Samples
Fasting blood samples were obtained by venipuncture (vacutainer tubes) at baseline,
after
sequence, the study medication was delivered in numbered containers. The project man-
ager and a physician enrolled the participants in the trial. The study participants and
care providers were blinded to the series of interventions. Apotekproduksjon AS kept the
randomization key upon completion of the trial.

2.3. Lipoprotein Measurements

Serum levels of triglycerides and total, LDL, and HDL cholesterol were ana
an ADVIA 1800 system (Siemens Medical Solutions Diagnostics, Tokyo, Japan).
cedure was performed according to the manufacturer. Apolipoprotein A1 (Ap
apolipoprotein B (ApoB) were measured by the ADVIA1800 system (Siemens H
Diagnostics, Deerfield, IL, USA).

l total, LDL, and HDL cholesterol were analyzed
on an ADVIA 1800 system (Siemens Medical Solutions Diagnostics, Tokyo, Japan). The
procedure was performed according to the manufacturer. Apolipoprotein A1 (ApoA1) and
apolipoprotein B (ApoB) were measured by the ADVIA1800 system (Siemens Healthcare
Diagnostics, Deerfield, IL, USA).

2.4. Lipoprotein Subfractions

LDL-C and HDL-C subfractions were estimated by serum electrophoresis
Lipoprint system (Lipoprint LDL system and Lipoprint HDL system, Quantime
poration, Redondo Beach, CA, USA). The analyses were performed according to
ufacturer's instructions. The Lipoprint system provides lipoprotein subfraction
into LDL-1 to LDL-7 and HDL-1 to HDL-10. LDL-1 and LDL-2 were classified
buoyant LDL-C (lbLDL-C) and LDL-3 to LDL-7 as small, dense LDL-C (sdLDL-
1 to HDL-3 were categorized as large HDL-C, HDL-4 to HDL-7 as intermediate
and HDL8–10 as small HDL-C, as in Figure 1.

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ration, Redondo Beach, CA, USA). The analyses were performed according to the manufac-
turer's instructions. The Lipoprint system provides lipoprotein subfractions divided into
LDL-1 to LDL-7 and HDL-1 to HDL-10. LDL-1 and LDL-2 were classified as large, buoyant
LDL-C (lbLDL-C) and LDL-3 to LDL-7 as small, dense LDL-C (sdLDL-C). HDL-1 to HDL-3
were categorized as large HDL-C, HDL-4 to HDL-7 as intermediate HDL-C,
and HDL 8–10 as small HDL-C, as in Figure 1.

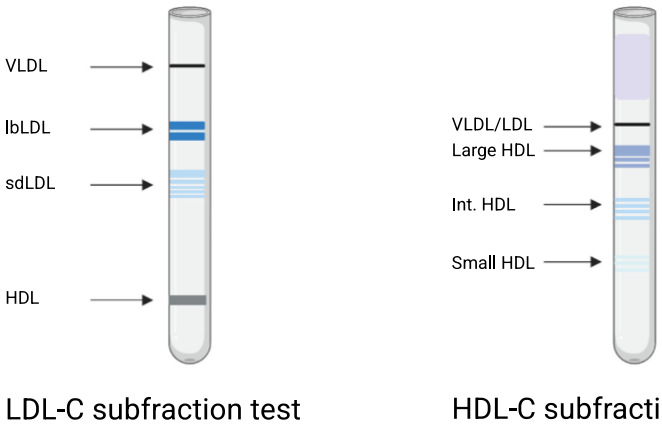

Figure 1. Graphical image of the Lipoprint LDL-C and HDL-C subfraction electrophoresis. VLDL: very low density lipoprotein. lbLDL: large, buoyant low-density lipoprotein cholesterol (LDL-C). sdLDL: small, dense LDL-C. HDL: high-density lipoprotein cholesterol. Int. HDL: intermediate HDL-C. Small, dense HDL-C: HDL: high-density lipoprotein cholesterol. Int. HDL: int
HDL-C. Created with BioRender.com (accessed on 5 July 2022).

Figure 1. Graphical image of the Lipoprint LDL-C and HDL-C subfraction electrophore
very low-density lipoprotein. lbLDL: large, buoyant low-density lipoprotein cholestero
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HDL-C. Small, dense HDL-C: HDL: high-density lipoprotein cholesterol. Int. HDL: int
HDL-C. Created with BioRender.com (accessed on 5 July 2022).

2.5. HDL-C Function

PON1 arylesterase activity was assessed in citrate plasma. Plasma was diluted at 1:80 using a salt buffer (20 mM Tris-HCl and 1.0 mM $CaCl_2$ with pH 8.0). Twenty microliters of diluted plasma and 200 µL of phenylacetate solution (3.26 mM phenylacetate in salt buffer) were added to each well in a UV-transparent 96-well plate. The absorbance of produced phenol was measured at 270 nm in a FLUOstar plate reader (BMG Labtech, Ortenburg, Germany). The activity (U/mL) was calculated from the initial linear reaction, and an extinction coefficient of phenol of 1310 M-1 cm1 was used.

The plasma SAA1 levels were measured by an enzyme-linked immunosorbent assay (DY3019-05, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a FLUOstar plate reader (BMG Labtech, Ortenburg, Germany). The cholesterol efflux capacity (CEC) was quantified with a MAK192 assay kit from Sigma-Aldrich (Saint-Louis, MO, USA), as previously described [16].

2.6. Statistical Analysis

Statistical work was performed using Prism version 8.4.3 (GraphPad Software Inc., La Jolla, CA, USA). Before the trial registration, a sample size calculation based on the primary outcome was performed. The period effect was tested by a two-sample *t*-test or Mann–Whitney test comparing the differences between the treatments in the two sequence order groups. Treatment–period interaction was evaluated by a *t*-test or a Mann–Whitney test comparing the average response in each sequence order group. The baseline values in the treatment sequence groups are presented as mean and standard deviation if normally distributed or as median and first and third quartile if not normally distributed. The normality in differences between treatment periods was assessed by the Shapiro–Wilk normality test. The values after n-3 PUFAs treatment and after placebo were compared by a paired *t*-test or Wilcoxon matched-pairs signed-rank test when appropriate. Confidence intervals (95%) were computed when the differences were symmetrically distributed. Correction for multiple comparisons was not performed. A 2-tailed p-level < 0.05 was considered significant.

3. Results

Of 65 subjects assessed for eligibility, 38 individuals were randomized to the sequence. The trial was conducted from September 2012 to July 2016. Three subjects left the trial, and one person was excluded from statistical analysis due to pregnancy, as shown in the participant flow diagram (Figure 2). Thirty-four participants (17 females and 17 males) with a mean age of 46.6 years completed the trial. The trial inclusion ended when the prespecified sample size (16 in each group) was reached. Sixteen started with n-3 PUFAs, and 18 started with placebo. Population characteristics and lipid changes from baseline have been previously published [15]. No important harms were detected.

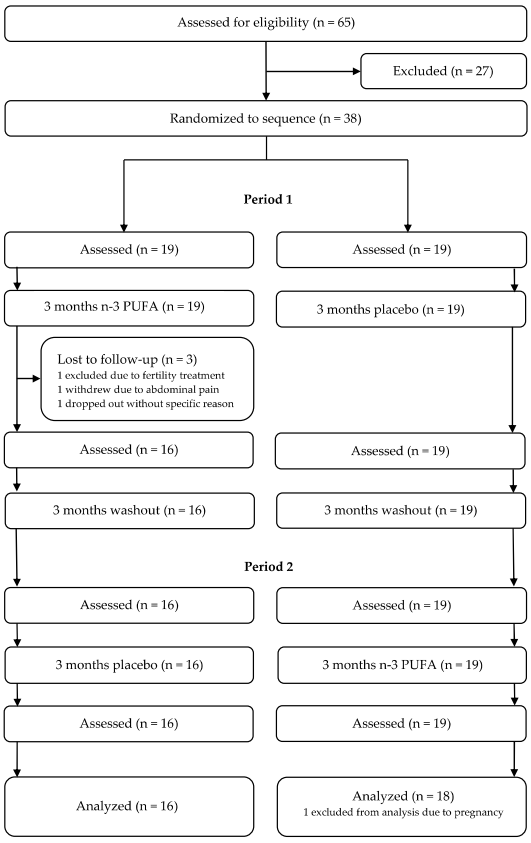

Figure 2. Participant flow diagram. n-3 PUFAs—omega-3 polyunsaturated fatty acids.

3.1. Total Cholesterol, Triglycerides, and Lipoproteins

The values in Table 1 were obtained from the electrophoresis strip analysis by densitometric scanning using the Lipoware software. As shown in Table 1, the lipoproteins levels in the two sequence groups were comparable at baseline for treatment one and post-washout before the second treatment. The baseline distribution of study participants by intervals of LDL-C and triglycerides is presented in Table 2. Lipoprotein levels were compared after n-3 PUFAs treatment and after placebo. In median, the total cholesterol level was lower after n-3 PUFAs (median: 4.7 mmol/L; interquartile range (IQR): 4.0–5.3) than after placebo (median: 5.2 mmol/L; IQR: 4.2–5.3). This reduction was statistically significant (p = 0.006, median of differences: −0.24 mmol/L, Figure 3A). The LDL-C levels decreased after n-3 PUFAs (median: 2.7 mmol/L; IQR: 2.3–3.3) compared to placebo (median: 3.1 mmol/L; IQR: 2.5–3.7) (p < 0.001, with a median of differences of −0.2 mmol/L, 95% CI [−0.4, −0.1], Figure 3B). No difference in HDL-C levels was found between n-3 PUFAs treatment (median: 1.3 mmol/L; IQR: 1.1–1.6) and placebo (median: 1.3 mmol/L; IQR: 1.1–1.5) (p = 0.71, median of differences 0, with a 95% CI [−0.1, 0.1], Figure 3C). Triglycerides were lower after treatment with n-3 PUFAs (median: 0.7 mmol/L; IQR: 0.6–1.1) than after placebo (median: 0.9 mmol/L; IQR: 0.7–1.2) (p < 0.001, the median of differences −0.14 mmol/L, Figure 3D).

Table 1. Lipoproteins at baseline presented by sequence, treatment period, and total.

	Baseline					
	Treatment Sequence			Treatment Sequence		
Variable	**N-3 PUFAs First, Then Placebo *n* = 16**	**Placebo First, Then N-3 PUFAs *n* = 18**	**Total *n* = 34**	**N-3 PUFAs First, Then Placebo *n* = 16**	**Placebo First, Then N-3 PUFAs *n* = 18**	**Total *n* = 34**
	Before Treatment 1 (Month 0)			**Before Treatment 2 (Month 6)**		
TC mmol/L	4.8 ± 1.0	4.8 (4.4, 5.4)	4.7 (4.4, 5.4)	5.0 ± 1.2	5.0 ± 1.1	5.0 ± 1.2
LDL-C mmol/L	3.0 (2.7, 3.3)	3.1 (2.9, 3.5)	3.0 (2.8, 3.3)	3.2 ± 0.9	3.1 (2.6, 3.6)	3.2 (2.5, 3.7)
HDL-C mmol/L	1.3 (1.1, 1.5)	1.2 (1.0, 1.5)	1.2 (1.1, 1.5)	1.3 (1.1, 1.6)	1.2 (1.0, 1.6)	1.2 (1.1, 1.6)
TG mmol/L	0.7 (0.6, 1.3)	1.0 (0.8, 1.6)	0.8 (0.7, 1.4)	0.8 (0.6, 1.4)	0.9 (0.8, 1.3)	0.9 (0.7, 1.4)
LbLDL-C mmol/L	1.45 (1.2, 1.7)	1.45 (1.2, 1.6)	1.45 (1.2, 1.7)	1.55 ± 0.6	1.58 (1.0, 1.8)	1.58 (1.1, 1.9)
LbLDL-C mg/dL	56 (47, 67)	56 (46, 63)	56 (47, 65)	60 ± 22.3	61 (39, 68)	61 (41, 74)
SdLDL-C mmol/L	0.04 (0, 0.1)	0.05 (0.002, 0.1)	0.05 (0, 0.1)	0.03 (0, 0.1)	0 (0, 0.1)	0.01 (0, 0.1)
SdLDL-C mg/dL	1.5 (0, 3.0)	2.0 (0.8, 2.3)	2.0 (0, 3.0)	1.0 (0, 3.8)	0 (0, 4)	0.5 (0, 4.0)
Large HDL-C mmol/L	0.38 (0.2, 0.5)	0.34 (0.2, 0.5)	0.34 (0.2, 0.5)	0.44 ± 0.2	0.28 (0.2, 0.5)	0.3 (0.2, 0.6)
Large HDL-C mg/dL	14.5 (9.3, 20.5)	13 (6.5, 18.8)	13 (8.0, 19.5)	17.1 ± 8.8	11 (6.5, 17.8)	12 (9, 22)
Int. HDL-C mmol/L	0.68 ± 0.1	0.65 ± 0.2	0.66 ± 0.2	0.63 (0.6, 0.8)	0.65 ± 0.2	0.62 (0.5, 0.8)
Int. HDL-C mg/dL	26.3 ± 5.1	25.1 ± 7.9	25.7 ± 6.6	24.5 (22, 30)	25.1 ± 7.2	24.0 (21, 30)
Small HDL-C mmol/L	0.25 ± 0.1	0.3 ± 0.1	0.28 ± 0.1	0.25 ± 0.1	0.28 ± 0.1	0.27 ± 0.1
Small HDL-C mg/dL	9.7 ± 2.9	11.6 ± 3.2	10.7 ± 3.2	9.8 ± 4.2	10.9 ± 2.5	10.4 ± 3.4
ApoA1 (µg/L)	1.52 (1.3, 1.9)	1.49 (1.3, 1.8)	1.50 (1.3, 1.8)	1.48 (1.3, 1.8)	1.52 (1.2, 1.8	1.5 (1.3, 1.8)
ApoB (µg/L)	0.94 (0.8, 1.1)	1.0 (0.9, 1.1)	0.99 (0.8, 1.1)	0.95 (0.7, 1.2)	1.01 (0.9, 1.2)	1.0 (0.8, 1.2)
ApoB/ApoA1	0.61 (0.5, 0.7)	0.65 (0.5, 0.9)	0.62 (0.5, 0.8)	0.62 (0.5, 0.8)	0.64 (0.5, 0.9)	0.6 (0.5, 0.9)

Values presented are mean ± standard deviation or median (first quartile, third quartile) by normal or non-normal distribution. N-3 PUFAs: omega-3 polyunsaturated fatty acids. TC: total cholesterol. LDL-C: low-density lipoprotein cholesterol. HDL-C: high-density lipoprotein cholesterol. TG: triglycerides. LbLDL-C: large, buoyant low-density lipoprotein cholesterol. SdLDL-C: small, dense low-density lipoprotein cholesterol. Int. HDL-C: intermediate high-density lipoprotein cholesterol. From mg/dL to mmol/L, conversion factor 0.02586 was used. ApoA1: apolipoprotein A1. ApoB: apolipoprotein B.

Table 2. Baseline characteristics and intervals of LDL-C and triglycerides.

	Total	**Group Starting with N-3 PUFAs**	**Group Starting with Placebo**
Number of patients, *n* (*n* female)	34 (17)	16 (7)	18 (10)
LDL-C < 1.8 mmol/L	1 (0)	0	1 (0)
LDL-C 1.8 to <2.6	5 (2)	3(1)	2(1)
LDL-C 2.6 to <3.0	7 (5)	5 (3)	2 (2)
LDL-C 3.0 to <4.9	19 (9)	7 (3)	12 (6)
LDL-C ≥ 4.9	2 (1)	1 (0)	1(1)
Triglycerides < 1.7 mmol/L	29 (14)	15 (6)	14 (8)
Triglycerides ≥ 1.7 mmol/L	5 (3)	1(1)	4 (2)
ApoB/ApoA1 > 0.9	4 (2)	1 (0)	3 (2)
Established ASCVD	9 (4)	4 (1)	5 (3)

N-3 PUFAs: omega-3 polyunsaturated fatty acids. LDL-C: low-density lipoprotein cholesterol. Established ASCVD: established atherosclerotic cardiovascular disease defined as previous acute coronary syndrome (myocardial infarction or unstable angina), stable angina, coronary revascularization, other arterial revascularization procedures, stroke and transitory ischemic attack, and peripheral arterial disease.

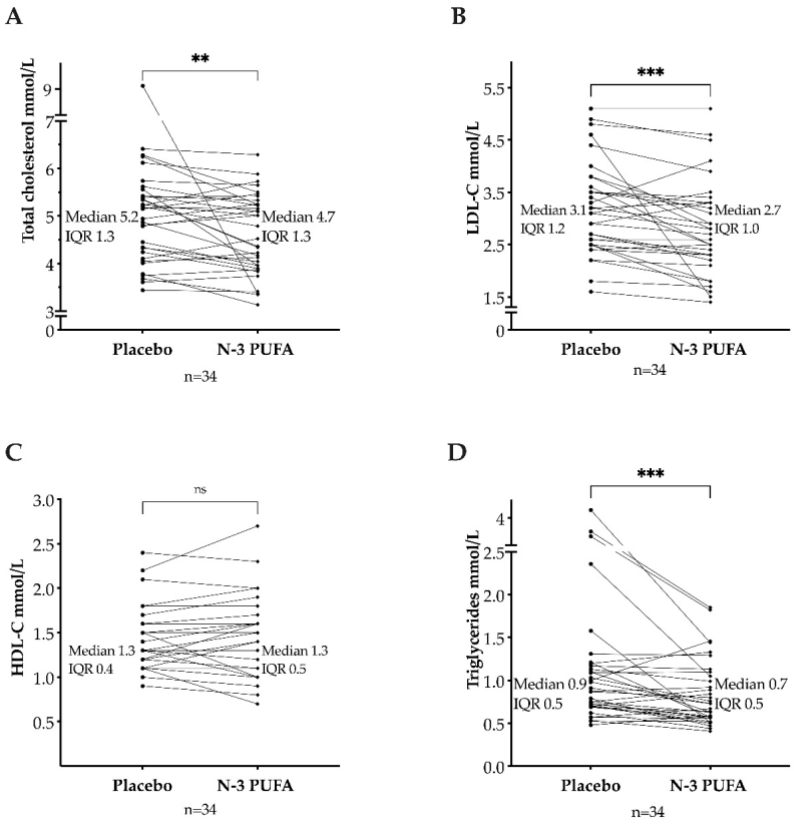

Figure 3. Total cholesterol (**A**), low-density lipoprotein cholesterol (LDL-C) (**B**), high-density lipoprotein cholesterol (HDL-C) (**C**), and triglycerides (**D**) after omega-3 polyunsaturated fatty acids (n-3 PUFAs) and placebo. IQR: interquartile range. ns > 0.05; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3.2. LDL and HDL Cholesterol Subfractions

The large, buoyant LDL-C subfraction was lower after n-3 PUFAs (mean: 57.5 mg/dL; SD: 19.2 mg/dL) than after placebo (mean: 61 mg/dL; SD: 18.1 mg/dL), but not statistically significant ($p = 0.22$, mean of differences = −3.4 mg/dL, 95% CI [−8.8; 2.1]). The small, dense LDL-C subfraction was unaffected by n-3 PUFAs (median: 1 mg/dL; IQR: 0–3) compared to placebo (median: 1 mg/dL; IQR: 0–3) ($p = 0.33$, with median of differences of 0. Figure 4A,B).

In median, the large HDL-C subfraction was higher after n-3 PUFAs (median; 16.5 mg/dL; IQR: 10.8–26.3) than after placebo (median; 13.5 mg/dL; IQR: 10–17.5). This increase was statistically significant ($p < 0.001$, median of differences = 2.0 mg/dL with a 95% CI [1.0, 4.0]). The intermediate HDL-C subfraction was lower after n-3 PUFAs (median; 24 mg/dL; IQR: 20.8–29) than after placebo (median; 26 mg/dL; IQR: 22–29.5) ($p = 0.02$, median of differences = −1 mg/dL). Moreover, the small HDL-C subfraction decreased after n-3 PUFAs treatment (median; 9 mg/dL; IQR: 6–11.3) compared to placebo (median; 10 mg/dL; IQR: 8–13) ($p < 0.001$, median of differences = −2 mg/dL, Figure 4C–E).

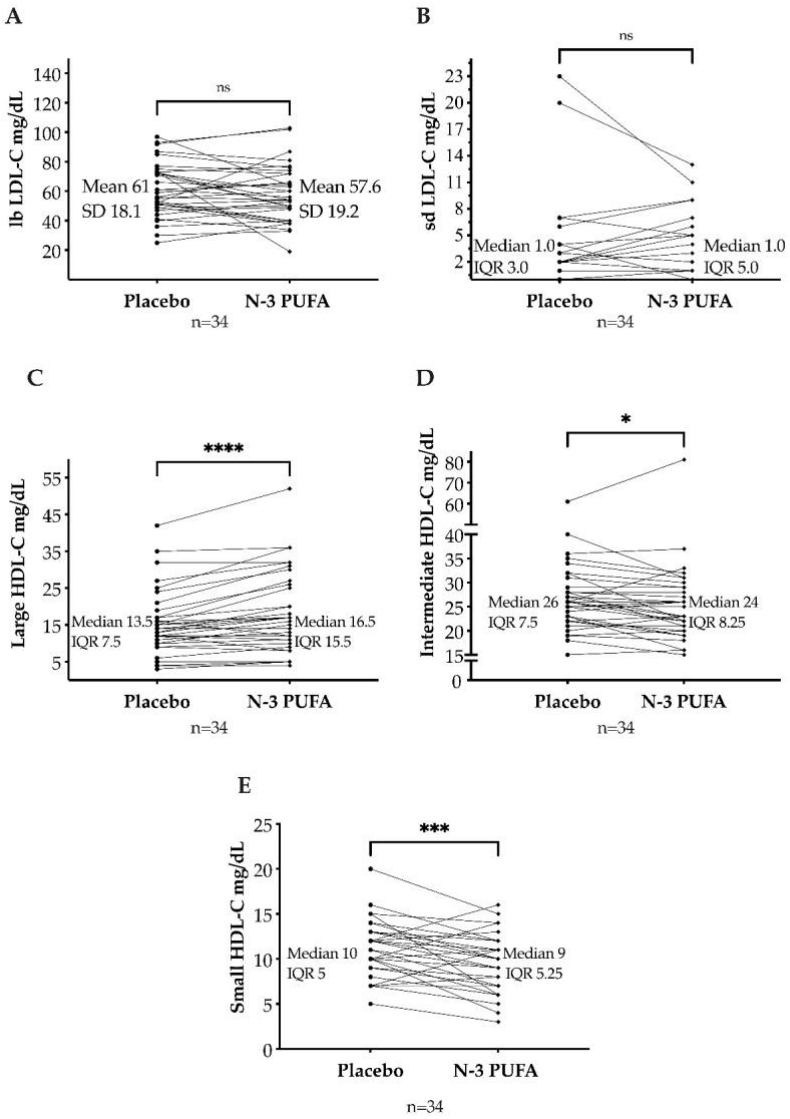

Figure 4. Large buoyant low-density lipoprotein cholesterol (lbLDL-C) (**A**) and small, dense low-density lipoprotein cholesterol (sdLDL-C) (**B**) after omega-3 polyunsaturated fatty acids (n-3 PUFAs) and placebo. Large high-density lipoprotein cholesterol (large HDL-C) (**C**), intermediate HDL-C (**D**), and small HDL-C (**E**) after n-3 PUFAs and placebo. SD: standard deviation. IQR: interquartile range. ns = $p > 0.05$; * = $p \leq 0.05$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

3.3. Apolipoproteins and HDL-C Function

The parameters for apolipoproteins and HDL function at baseline before treatment and post-washout are presented in Table 3. The N-3 PUFAs supplement did not affect the levels of ApoA1 and ApoB when compared to the placebo, Table 4. No difference in

SAA1, PON1, and CEC was found after the n-3 PUFAs supplement compared to placebo; Table 4.

Table 3. High-density lipoprotein function at baseline presented by sequence, treatment period, and total.

Variable	Baseline					
	Treatment Sequence			Treatment Sequence		
	N-3 PUFAs First, Then Placebo n **= 16**	**Placebo First, Then N-3 PUFAs** n **= 18**	**Total** n **= 34**	**N-3 PUFAs First, Then Placebo** n **= 16**	**Placebo First, Then N-3 PUFAs** n **= 18**	**Total** n **= 34**
	Before Treatment 1 (Month 0)			**Before Treatment 2 (Month 6)**		
SAA1 (µg/mL)	0.99 (0.5, 2.4)	2.2 (0.9, 4.1)	1.4 (0.7, 3.1)	1.1 (0.9, 1.8)	2.4 (1.4, 3.7)	1.7 (0.9, 2.5)
PON1 (U/mL)	111 ± 33.2	109.9 ± 28.8	110.4 ± 30.5	115.7 ± 29.2	108.7 ± 23.3	112.0 ± 26.1
SAA1/PON1	0.01 (0.005, 0.02)	0.02 (0.01, 0.04)	0.01 (0.01, 0.03)	0.01 (0.01, 0.02)	0.02 (0.01, 0.04)	0.02 (0.01, 0.03)
CEC (%)	37.5 ± 4.2	38.6 ± 5.0	39.1 ± 4.6	38.1 ± 4.0	39.5 ± 3.9	38.9 ± 4.0

Values shown are median (interquartile range) or mean ± standard deviation. N-3 PUFAs: omega-3 polyunsaturated fatty acids. ApoA1: apolipoprotein A1. ApoB: apolipoprotein B. SAA1: serum amyloid A1. PON1: serum paraoxonase-1. CEC: cholesterol efflux capacity.

Table 4. Apolipoproteins and high-density lipoprotein function presented by treatment.

	N-3 PUFAs n **= 34**	**Placebo** n **= 34**	**Treatment Difference (N-3 PUFAs−Placebo) with 95% Confidence Interval**	p
ApoA1 (µg/L)	1.51 (1.3, 1.7)	1.55 (1.3, 1.9)	−0.07 [−0.12 to 0.05]	0.29
ApoB (µg/L)	0.91 (0.8, 1.2)	1.03 (0.8, 1.2)	−0.06 [−0.13 to 0.01]	0.09
ApoB/ApoA1	0.57 (0.5, 0.7)	0.58 (0.5, 0.8)	−0.009 [−0.04 to 0.04]	0.91
SAA1 (µg/mL)	1.36 (1.0, 2.6)	1.68 (1.0, 2.7)	−0.11 [−0.59 to 0.11]	0.15
PON1 (U/mL)	107 ± 25.5	113 ± 31.0	−5.9 [−12.9 to 1.1]	0.10
SAA1/PON1	0.01 (0.01, 0.02)	0.02 (0.01, 0.2)	−0.002 [−0.006 to 0.003]	0.38
CEC (%)	38.5 ± 3.2	38.9 ± 3.5	−0.41 [−1.8 to 1.0]	0.57

N-3 PUFAs; omega-3 polyunsaturated fatty acids. Values are presented as median (interquartile range) or mean ± standard deviation. ApoA1: apolipoprotein A1. ApoB: apolipoprotein B. SAA1: serum amyloid A1. PON1: serum paraoxonase-1. CEC: cholesterol efflux capacity.

4. Discussion

In this study, we found that n-3 PUFAs supplements in FH individuals reduced total cholesterol, LDL-C, and triglycerides. After the n-3 PUFAs supplement, the proportions of HDL-C subfractions changed. However, as assessed by SAA1, PON1, and CEC, the HDL-C function was unchanged during the trial.

Treatment with n-3 PUFAs reduced the LDL-C and TG levels in our FH population, followed by decreased total cholesterol. The LDL-C-lowering effect from n-3 PUFAs found in our trial differs from the results in previous trials investigating n-3 PUFAs supplementation in heterozygous FH individuals [12–14]. Two FH trials with four and eight weeks of combined EPA and DHA supplementation (5.1 g and 4 g) found no effect on LDL-C levels [12,14]. In contrast, six weeks of 1.2 g DHA supplement increased the LDL-C levels in children with FH and familial combined hyperlipidemia [13]. Short intervention periods, low sample sizes, use of DHA only, and lack of placebo comparison are possible explanations for the disparate results. In clinical trials, increased LDL-C levels after n-3 PUFAs supplements have been a concern. There are indications that the increase in LDL-C is related to DHA supplementation and not treatment with EPA [17]. This increase in LDL-C can reflect an increase in particle size rather than an increase in LDL-C concentration [18]. However, we found a reduction in LDL-C but no effect on particle size as large, buoyant LDL-C and small, dense LDL-C proportions were unchanged.

N-3 PUFAs supplements are known for their triglyceride-reducing capacity. Increased triglycerides are not a hallmark of FH, and only 15% of our study population had triglycerides ≥1.7 mmol/L (150 mg/dL) at baseline. Hypertriglyceridemia is associated with increased atherosclerotic cardiovascular disease (ASCVD) risk [19], and triglycerides can be considered a marker for triglyceride-rich lipoproteins. Although epidemiological and genetic evidence supports a causal role for triglyceride-rich lipoproteins in the ASCVD pathway [11], evidence from clinical studies with triglyceride-reducing drugs is lacking. The triglyceride levels attained after treatment with an n-3 PUFAs supplement in the REDUCE-IT and the STRENGTH were comparable. However, only the REDUCE-IT showed a reduction in primary composite endpoint (CVD death, nonfatal myocardial infarction, nonfatal stroke, coronary revascularization, or unstable angina) after n-3 PUFAs treatment [8,9]. Therefore, it is suggested that the favorable effect found in the REDUCE-IT was not from a triglyceride-lowering pathway alone. N-3 PUFAs supplements are not recommended routinely in FH in the current European guidelines [3].

Small, dense LDL-C particles (sdLDL-C) are the advanced lipoprotein metric that has received the most attention over the last decades. Several large population-based studies found an association between elevated sdLDL-C and increased risk of CVD [20]. A higher atherogenic potential from small, dense LDL-C particles could be explained by an increased circulation time due to impaired interaction with the LDL-receptor, an increased susceptibility to undergo atherogenic modification (i.e., oxidization), and a greater propensity for transport into the arterial wall. Despite decades of research, the clinical significance of measuring sdLDL-C or other LDL-C subfractions is unknown, including among FH patients. Randomized diet trials of n-3 PUFAs in healthy volunteers have shown a reduction in sdLDL-C and an increase in LDL size [21,22]. In the present study, the concentration of sdLDL-C in the included patients was low at baseline, and we found no effect of n-3 PUFAs on either the small or large LDL-C subfractions.

Epidemiological and clinical trials are discordant regarding the prognostic value of measuring HDL subfractions. In different studies, both the smaller and the larger HDL-C subfractions have been proposed to be superior to HDL-C in CVD risk prediction. However, these studies have not been conducted in patients with FH. In the present study, we observed a significant change in the composition of HDL-C particles with a decrease in the smaller HDL-C particles and an increase in the larger HDL-C particles. The reverse transport of cholesterol (RCT) from peripheral tissue to the liver is probably the most critical mechanism for the protective effect of HDL-C on the development of CVD [23,24]. An early step in RCT is the efflux of cholesterol from macrophages to HDL particles [25]. Cholesterol efflux capacity is inversely correlated with hard vascular endpoints [26]. Versmissen et al. found that FH patients without CVD had higher CEC compared with non-FH siblings [27]. Ogura et al. found that CEC was independently and inversely associated with ASCVD in patients with heterogenous FH. The authors suggested that CEC could be a therapeutic target for preventing CVD in FH patients [28]. The smallest HDL particles have been proposed to be the most efficient mediators of cholesterol efflux [29,30]. Our data did not support this, as we observed significant changes in the large and small HDL subfractions without any changes in the cholesterol efflux capacity from n-3 PUFAs.

PON1 is an HDL-associated protein that has been proposed to have a protective effect on the development of CVD by reducing oxidative stress [31,32]. Prospective studies have shown that reduced PON1 activity is an independent risk factor for CVD [33,34]. PON1 activity is decreased in patients with FH compared to healthy controls [35]. We did not observe any differences in PON1 activity from n-3 PUFAs compared to placebo.

SAA1 is an acute-phase protein that has been suggested to impair the anti-inflammatory properties of HDL-C, possibly by replacing its protective proteins [36]. SAA1 levels are elevated in patients with FH [37]. We did not observe any differences in serum SAA1 concentrations after n-3 PUFAs compared to placebo.

The strengths of this trial are the crossover design, the well-known study population (single-center), and the treatment lengths (three-months treatment and at least a three-

months washout). Several limitations need consideration. First, these are secondary endpoints and should be interpreted with care. Second, our study population was a combination of individuals in a primary and secondary prevention setting, placing our FH subjects in the high or very high cardiovascular risk category. Due to the sample size, we could not differentiate between the effect of n-3 PUFAs in the high-risk and the very high risk category. Third, the median LDL-C level at baseline was higher than recommended in the current European Guidelines for the management of dyslipidemia [3]. Thus, it is possible that the LDL-C-reducing effect of the n-3 PUFAs supplement would be nuanced in an FH population with lower LDL-C. However, we believe the LDL-C levels in our study population are representative of a clinical setting.

5. Conclusions

In this study of FH individuals, we found that n-3 PUFAs supplements reduced LDL-C and triglycerides. N-3 PUFAs did not change the LDL-C subfractions, but the large HDL-C subfractions increased, and the small HDL subfractions decreased. Despite changes in the HDL-C composition, we did not detect any alteration in the HDL function after the n-3 PUFAs supplement. N-3 PUFAs treatment has the potential to reduce LDL levels and change the HDL composition in FH subjects. The clinical benefit from these lipoprotein modifications remains to be elucidated, and further research is needed.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Ethics Committee of Northern Norway P REK 2011/899 and by the Norwegian Medicines Agency (EUDRACTNR 2012-000505-68; ClinicalTrials.gov NCT01813006).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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