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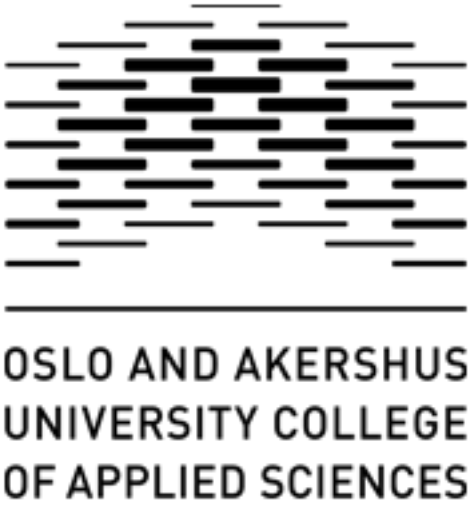
Plasma Fatty Acids and Expression of Genes Related to Lipid Metabolism



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Kine Næss Dagsland

Abstract

Background: The amount and type of fatty acids in the diet may influence the pathogenesis of cardiovascular disease. Fatty acids is known to modulate the expression of genes, which could have an impact on cardiovascular disease risk. However, the underlying mechanisms are not fully established.

Aim: The main aim of this study was to examine the impact of plasma fatty acids on expression of genes related to lipid metabolism in peripheral blood mononuclear cells in healthy subjects.

Method: Blood samples and expression level of 43 genes from 54 healthy subjects from a previous randomized controlled intervention trial were used. Subjects were divided into high and low groups based on the 70^{th-} and 30^{th} percentiles of total plasma omega-3 level, omega-6/omega-3 ratio and ratio of saturated fatty acid to polyunsaturated fatty acid.

Results: The results showed that nine of the 43 included genes were significantly different regulated when comparing subjects with high and low plasma omega-3 level and fatty acid ratios. The ratio of saturated fatty acid to polyunsaturated fatty acid was associated with the highest number of differently expressed genes (FABP5, CPT1A, INSIG2, SOAT1, CRAT, ACOT1 and PLIN5), followed by the plasma omega-6/omega-3 ratio (CETP and PPARG) and total plasma omega-3 level (CETP).

Conclusion: A high ratio of saturated fatty acid to polyunsaturated fatty acid modulated the expression of several genes associated with increased cardiovascular disease risk. A high level of plasma omega-3 and low plasma omega-6/omega-3 ratio modulated one gene associated with reduced cardiovascular disease risk, whereas a high omega-6/omega-3 ratio increased the expression of one gene associated with reduced cardiovascular disease risk. These results suggest that the level of plasma FAs may modulate and expression of genes related to lipid metabolism and thereby influence the CVD risk.

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Abbreviations

AA: arachidonic acid

Acetyl coenzyme A: acetyl-CoA

ALA: alpha-linolenic acid

BMI: body mass index

CD36: CD36 molecule

CE: cholesteryl ester

CETP: cholesteryl ester transfer protein

CHD: coronary heart disease

CRP: C-reactive protein

CVD: cardiovascular disease

DHA: docosahexaenoic acid

DNA: deoxyribonucleic acid

DPA: docosapentaenoic acid

EPA: eicosapentaenoic acid

ER: endoplasmic reticulum

FA: fatty acid

FA CoA: fatty acyl CoA

FAS: fatty acid synthase

FATP: fatty acid transport protein

HDL: high-density lipoprotein

HMG-CoA: hycroxymethylglutaryl CoA reductase

HSL: hormone sensitive lipase

LA: linoleic acid

LDL: low-density lipoprotein

LDLR: low-density lipoprotein receptor

LPL: lipoprotein lipase

LXR: liver X receptor

mRNA: messenger ribonucleic acid

MUFA: monounsaturated fatty acid

NEFA: non-esterified fatty acid

NNR: Nordic Nutrition Recommendations

Omega-3: n-3

Omega-6: n-6

PBMC: peripheral mononuclear blood cell

PL: phospholipids

PPAR: peroxisome-proliferator activated receptor

PPARA: peroxisome proliferator activated receptor alpha

PPARB: peroxisome proliferator-activated receptor beta

PPARG: peroxisome proliferator-activated receptor gamma

PPRE: PPAR response element

PUFA: polyunsaturated fatty acid

RCT: randomized controlled trial

RNA: ribonucleic acid

RXR: retinoid X receptor

SD: standard deviation

SFA: Saturated fatty acid

SREBP: sterol regulatory element-binding protein

TAG: triacylglycerol

TC: total cholesterol

TF: transcription factors

VLDL: very low-density lipoprotein

WHO: World health organization

1 Introduction

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is a group of disorders of the heart and blood vessels, and is the number one cause of death among humans, representing 31% of all deaths worldwide (World Health Organization [WHO], 2015a). CVD include stroke, coronary heart disease (CHD) and disease of other arteries (Stanner, 2008). The most important risk factors of stroke and heart disease are physical inactivity, an unhealthy diet, use of tobacco and harmful use of alcohol. These risk factors may lead to elevated blood lipids, raised blood pressure and raised blood glucose, eventually increasing the risk of CVD (WHO, 2015a).

Prevention of CVD is a major focus in nutrition research, and research has shown that development of CVD can be prevented by changes towards a healthier diet. Nutritional habits, especially regarding dietary fat are involved in the progress of CVD (Michas, Micha, & Zampelas, 2014; Perk et al., 2012). The amount and type of fatty acids (FAs) in the diet determines the relative amount of FA in the tissue of the body, and may influence the pathogenesis of CVD (Jakobsen et al., 2009; A. Simopoulos, 2006; Wall, Ross, Fitzgerald, & Stanton, 2010). A high intake of saturated fatty acids (SFA) has been shown to increase the CVD risk, whereas a high intake of polyunsaturated fatty acids (PUFA) has been shown to decrease the CVD risk (Deckelbaum, 2010; Michas et al., 2014).

1.2 Fatty acids

1.2.1 Structure of fatty acids

The majority of naturally existing lipids are a combination of different triacylglycerol molecules (TAG) (Nordic Counsil of Ministers [NCM], 2014). TAG consist of three individual FAs, each esterified to a glycerol molecule (Frayn, 2010). TAG is stored in fat cells, and is the major form of stored energy in the body (Schaefer, 2002). A FA consists of a hydrophobic hydrocarbon chain with a terminal carboxyl group. At physiologic pH, the terminal carboxyl group (-COOH) ionizes, becoming $–COO^-$. This anionic group has an affinity for water, giving the FAs its amphipathic nature (Champe, Harvey, & Ferrier, 2008).

The metabolic effects of FAs depend on the length of the carbon chain, the number and location of double bonds and the degree of saturation (Champe et al., 2008; NCM, 2014). The

most important FAs in metabolism are mainly unbranched, long-chain (12 carbons or more) with an even number of carbon atoms (Frayn, 2010). The double bonds are counted either from the carboxyl (delta) or the methyl (omega) end (NCM, 2014).

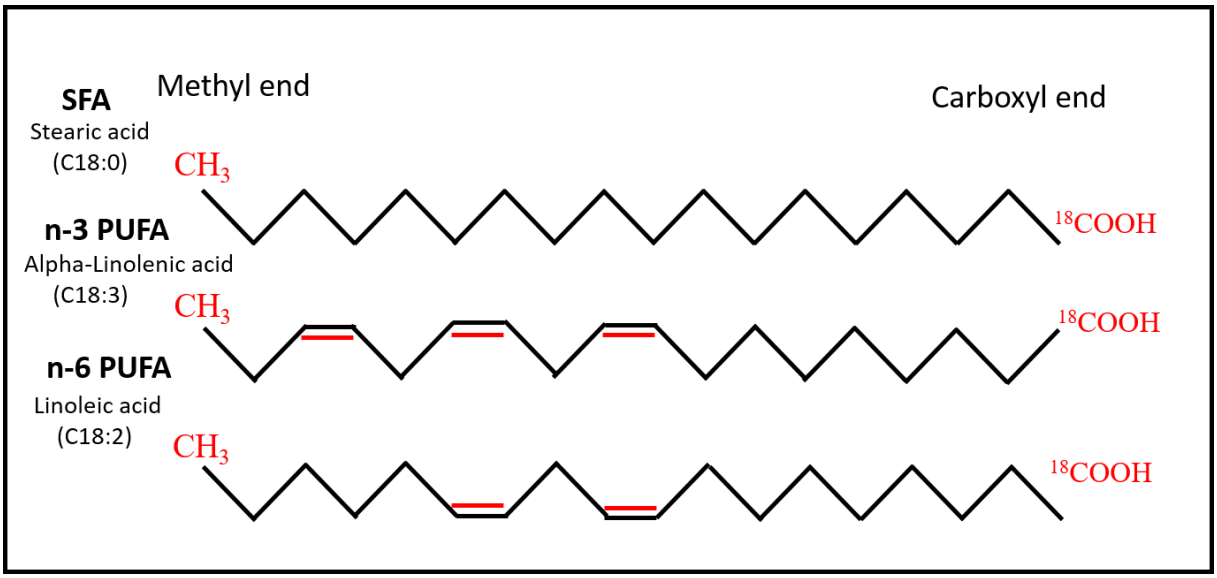


Figure 1. *Chemical structure of stearic acid, alpha-linolenic acid and linoleic acid. Adapted from Valenzuela & Valenzuela (2013).*

SFA, saturated fatty acid; n-3 PUFA, omega 3 polyunsaturated fatty acid; n-6 PUFA, omega 6 polyunsaturated fatty acid.

FAs containing no double bonds are referred to as SFAs, FAs containing one double bond are referred to as monounsaturated fatty acids (MUFA), whereas FAs containing several double bonds are referred to as polyunsaturated fatty acids (PUFA) (Frayn, 2010; Jump, 2002b). The PUFAs are divided into the omega-6 (n-6) and omega-3 (n-3) classes based on the positioning of the first double bond. n-6 have their first double bond beginning at the sixth bond position from the methyl end, whereas long chain n-3 have the first double bond beginning at the third position from the methyl end (Figure 1) (Champe et al., 2008; Frayn, 2010).

1.2.2 Synthesis of fatty acids

The n-6 and n-3 precursors, linoleic acid (C18:2 n-6; LA) and alpha-linolenic acid (C18:3 n-3; ALA) are essential FAs because humans lack the enzymes to introduce double bonds at the n-6 and n-3 positions (Seo, Blaner, & Deckelbaum, 2005). An adequate intake of the essential PUFAs is necessary for normal growth in humans, and needs to be included in the diet (Calder et al., 2010). The essential LA and ALA can be elongated and desaturated to other members of the same family, but cannot be converted from n-6 to n-3. LA is mainly metabolised to arachidonic acid (C20:4 n-6, AA), whereas ALA is mainly metabolised to the

n-3 FAs eicosapentaenoic acid (C20:5 n-3, EPA), docosapentaenoic acid (22:5 n-3, DPA) and docosahexaenoic acid (C22:6 n-3, DHA) (Figure 2) (Frayn, 2010; NCM, 2014; Seo et al., 2005). The n-6 and n-3 FAs compete for the same enzymes for further metabolism in the body, with n-3 having a higher affinity than n-6 (NCM, 2014; A. Simopoulos, 2006).

The major types of SFA in the diet and bloodstream are palmitic acid (C16:0), stearic acid (C18:0) and myristic acid (C14:0) (Schaefer, 2002). In addition to being supplied from the diet, SFAs can be synthesized within the human body. FA synthase (FAS) produces mainly palmitic acid from acetyl coenzyme A (acetyl-CoA) molecules. Several pathways can produce a range of SFAs and MUFAs, but not the essential n-6 and n-3 PUFAs (Dongol, Shah, Kim, Gonzalez, & Hunt, 2007; Frayn, 2010; NCM, 2014).

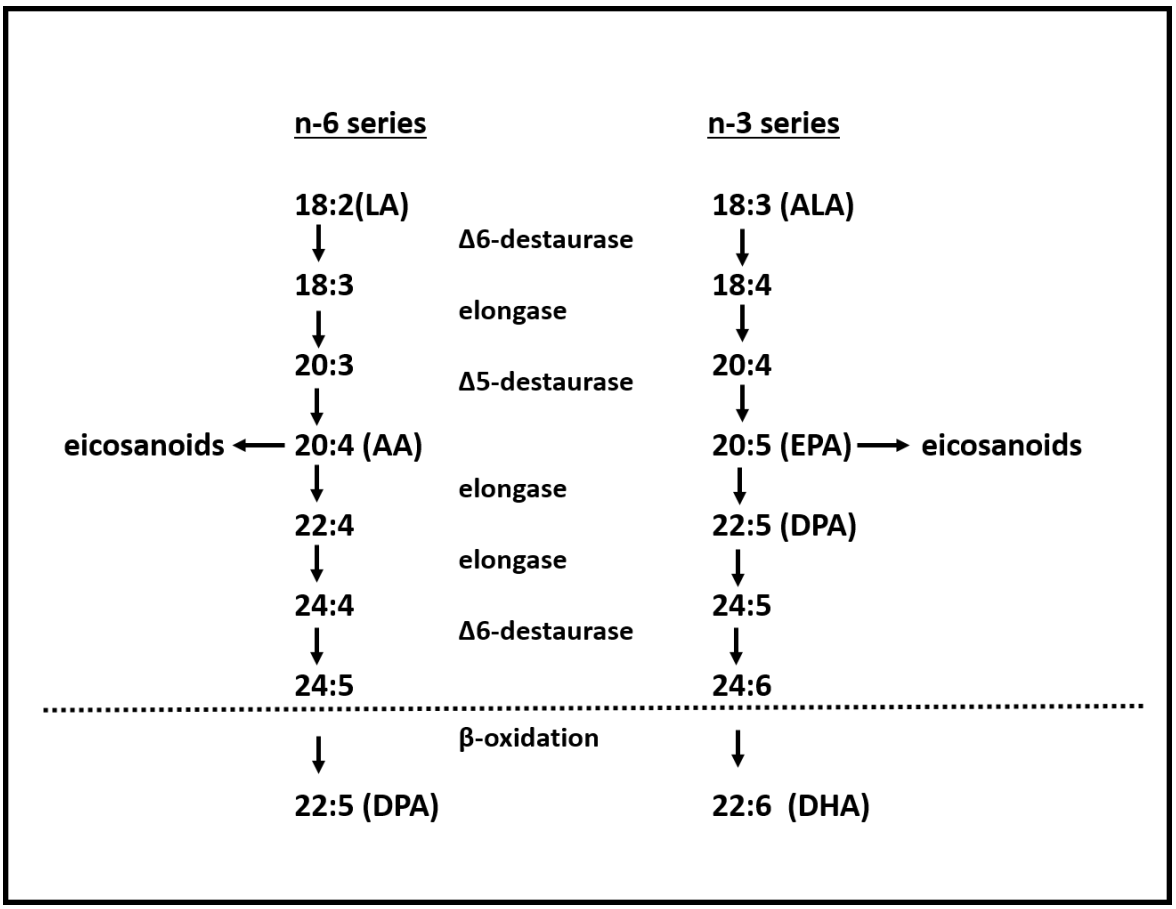


Figure 2. *Metabolism of polyunsaturated fatty acids. Adapted from Arteburn, Hall & Oken (2006).*

n-6, omega 6; n-3, omega 3; LA, linoleic acid; AA, arachidonic acid; DPA, docosapentaenoic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

1.2.3 Fatty acids as components in cell membranes

FAs are an important part of the cell membrane, both as structural components in membrane lipids, such as glycolipids and phospholipids (PL), and as integrated lipids in the cell membrane. FAs in the cell membrane are involved in several processes, such as changing the membrane fluidity, substrate availability for lipid synthesis and cell signalling to gene expression (Calder, 2002; Frayn, 2010; Jump, 2002a).

SFAs and PUFAs have opposing effects on membrane fluidity, membrane cholesterol content, and lipid raft organization (Soni et al., 2008; Wassall & Stillwell, 2009). SFA intake decreases the fluidity in cell membranes, resulting in less low-density lipoprotein receptor (LDLR) activity and increased levels of low-density lipoprotein cholesterol (LDL-C) in the blood stream (Ooi et al., 2012). Cell membranes with a high content of PUFAs are more fluid than those with high amount of SFAs. This may make them better able to regulate metabolic processes (Frayn, 2010).

1.2.4 Eicosanoid production

n-6 and n-3 FAs are precursors for eicosanoids, which are important immune modulators (Calder, 2002; NCM, 2014). Prostaglandins, and the related compounds thromboxanes and leukotrienes, are eicosanoids (Champe et al., 2008; A. P. Simopoulos, 2001). AA and EPA are incorporated in the cell membrane, where they compete for the same (phospholipase) enzymes, which are able to release them for conversion to different forms of eicosanoids. EPA has a higher affinity for the enzymes (Calder, 2002; NCM, 2014; A. Simopoulos, 2006; A. P. Simopoulos, 1991). The eicosanoids produced from AA are considered pro-inflammatory, whereas the eicosanoids from EPA are considered less pro-inflammatory compared to eicosanoids from AA. Since EPA have higher affinity for the enzymes, they are believed to antagonize the pro-inflammatory effects of n-6, and it has been hypothesized that the n-6/n-3 ratio in the diet is important to prevent inflammation and CVD (Calder, 2012; Harris, 2006; Ristić & Ristić, 2002; Schmitz & Ecker, 2008).

1.2.5 Dietary sources and recommendations

There are more sources of n-6 than n-3 in the western diet. n-6 consumption is mainly from LA, obtained from vegetable oils, whereas n-3 is found in plants sources (flaxseed oils and canola oils), or by direct intake of EPA and DHA, primarily from marine sources (Champe et

al., 2008; Russo, 2009; Seo et al., 2005). The dietary SFAs is found mainly in animal fat (meat and dairy products) and some plant oils (palm oils and coconut oils) (Champe et al., 2008; Michas et al., 2014).

As part of a healthy diet, WHO recommends to restrict the total fat intake to under 30 % of the daily energy intake, and SFA intake to under 10 % (WHO, 2015b; WHO/FAO expert consultation, 2003). Nordic Nutrition Recommendations (NNR) recommend a total fat intake between 25-40 % of the daily energy intake, and to restrict the SFA intake to under 10 % of the daily energy intake. PUFAs should contribute 5-10 % of the daily energy intake, with at least 1% from n-3. However, recommendations regarding the intake of the marine n-3, EPA and DHA, or the n-6/n-3 ratio are not quantified (NCM, 2014). The Norwegian Directorate of Health's nutrition recommendations are based on the NNR, and the same total daily intake of total fat, SFA and PUFAs are recommended. In Norway, the average PUFA intake meets the recommendations, but the average consumption of SFA is approximately 15% of the daily intake, and exceeds the recommended intake (The Norwegian Directorate of Health, 2015b).

1.3 Cardiovascular disease and fatty acids

Early clinical trials, including the Oslo Diet-Heart Study and the Finnish Mental Hospital Study showed reduced CVD related events when replacing SFA with PUFAs in the diet (Siri-Tarino, Sun, Hu, & Krauss, 2010). A summary of evidence from 15 randomized controlled trials (RCT) showed reduced CVD risk by 17% after reducing SFA intake (Hooper et al., 2015). A meta-analysis by Mozaffarian et al. estimated a 10% reduction in CVD risk for every 5% of SFAs replaced with PUFAs (Mozaffarian, Micha, & Wallace, 2010). Furthermore, a cohort study published in 2015 showed that replacing SFA with PUFAs lowered CHD risk, suggesting that recommendations should include replacing SFA with unsaturated fat to reduce the risk of CHD (de Souza et al., 2015). However, a summary of evidence from 28 cohorts and 19 RCTs showed no associations between SFA intake and CHD (Skeaff & Miller, 2009). Another study showed that greater SFA intake was associated with less progression of coronary disease in postmenopausal women (Mozaffarian, Rimm, & Herrington, 2004). A systematic review and meta-analysis concluded that current evidence does not clearly support cardiovascular guidelines encouraging high PUFA intake and low SFA intake (Chowdhury et al., 2014).

1.3.1 Fatty acids and biomarkers for cardiovascular disease risk

Elevated levels of LDL-C and TAG are biomarkers associated with increased risk of atherosclerosis, which is the underlying cause of CVD in most cases, whereas increased levels of high-density lipoprotein cholesterol (HDL-C) are associated with decreased risk of atherosclerosis and CVD (Epstein & Ross, 1999; Frayn, 2010; Nabel & Braunwald, 2012). High levels of LDL-C is a strong, but treatable, risk factor for CVD (Go et al., 2014). On the contrary, epidemiological studies have demonstrated a strong inverse relationship between high levels of HDL-C and CVD risk, which is independent of LDL-C (P. Barter et al., 2007; Castelli, 1988; Kosmas, Christodoulidis, Cheng, Lerakis, & Vittorio, 2014).

As early as in 1953, Ancel Keys launched an epidemiological longitudinal study called the seven countries study. He investigated the relationship between diet and CHD in middle-aged men from seven different countries. This study was the first to find that serum cholesterol increases in proportion to intake of total fat, and more interestingly intake of SFA. In addition, the fatal coronary heart events increased in proportion to serum cholesterol (Keys, 1980). Plasma FAs affect levels biomarkers for CVD risk, and PUFAs have been shown to lower plasma TAG levels, whereas SFA have been shown to increase the plasma TAG levels (Frayn, 2010; Lottenberg, da Silva Afonso, Lavrador, Machado, & Nakandakare, 2012). Furthermore, SFA intake is associated with increased plasma cholesterol and LDL-C, resulting in increased CVD risk. n-6 intake is associated with decreased plasma cholesterol and LDL-C when substituted for SFA. However, the plasma HDL-C is also lowered. n-3 reduces serum TAG, which reduces CVD risk, but is shown to have little effect on LDL-C and HDL-C (Champe et al., 2008).

Furthermore, SFA can suppress the activity of LDL receptors causing reduced LDL clearance, and also increased LDL production rate (Berneis & Krauss, 2002; Dreon et al., 1998). On the contrary, n-6 FAs may increase the activity of the LDL receptor, reducing LDL production rate (Cortese et al., 1983; Shepherd et al., 1980; Turner, Le, & Brown, 1981). Replacement of SFA with n-6 FAs is shown to decrease total LDL-C and HDL-C, as well as decrease the total cholesterol (TC)/HDL ratio (Mensink & Katan, 1992; Mensink, Zock, Kester, & Katan, 2003).

1.4 Lipid metabolism

Abnormalities or deficiencies in the lipid metabolism can lead to atherosclerosis and CVD (Chang, Li, Chang, & Urano, 2009; Millen, Wolongevicz, de Jesus, Nonas, & Lichtenstein, 2014; Nguyen et al., 2008; Parekh & Anania, 2007).

The digestion of dietary lipids (TAG, PL and cholesteryl ester (CE)) starts in the stomach and continues in the small intestine, where they are degraded by enzymes before being absorbed and re-esterified in the cells in the intestinal wall. Then the lipids are assembled into chylomicrons and carried to the circulation (Champe et al., 2008; Frayn, 2010). The lipids are delivered to cells as very low density lipoproteins (VLDL) or chylomicrons. Lipoprotein lipase (LPL) hydrolyses TAG, and the non-esterified FAs (NEFAs) are released and taken up by metabolic tissues. The NEFAs enter the cells via diffusion and transporters of FA (fatty acid transfer protein (FATP) and CD36 molecule (CD36) (Jump, 2002b; Stahl, Gimeno, Tartaglia, & Lodish, 2001).

After entering the cell, NEFAs are immediately converted to fatty acyl CoA thioesters (FA CoAs), which are necessary in nearly all metabolic processes. Intracellular FAs are bound by a fatty acid binding protein (FABP) which transports the FA through the cytosol (Figure 3) (Frayn, 2010). FAs are synthesized in the cytosol of the liver. Citrate is the activator, whereas long chain FA CoA is the inhibitor. The rest of the FA synthesis is regulated by FAS. FAs can be elongated and desaturated in endoplasmic reticulum (ER), incorporated into the PL of cell membranes, converted to eicosanoids or degraded by β-oxidation. The carnitine shuttle, a translocase and carnitine-palmitoyltransferase 1 and 2 (CPT1 and CPT2) is essential for transporting long chain FAs from cytosol to the mitochondrial matrix, where β-oxidation occurs (Champe et al., 2008; Frayn, 2010). When FAs are required for energy, hormone-sensitive lipase (HSL) are activated by glucagon which starts lipolysis, which is the breakdown of TAG. NEFA is released to the circulation and transported to the needed tissue bound to albumin (Figure 3) (Champe et al., 2008).

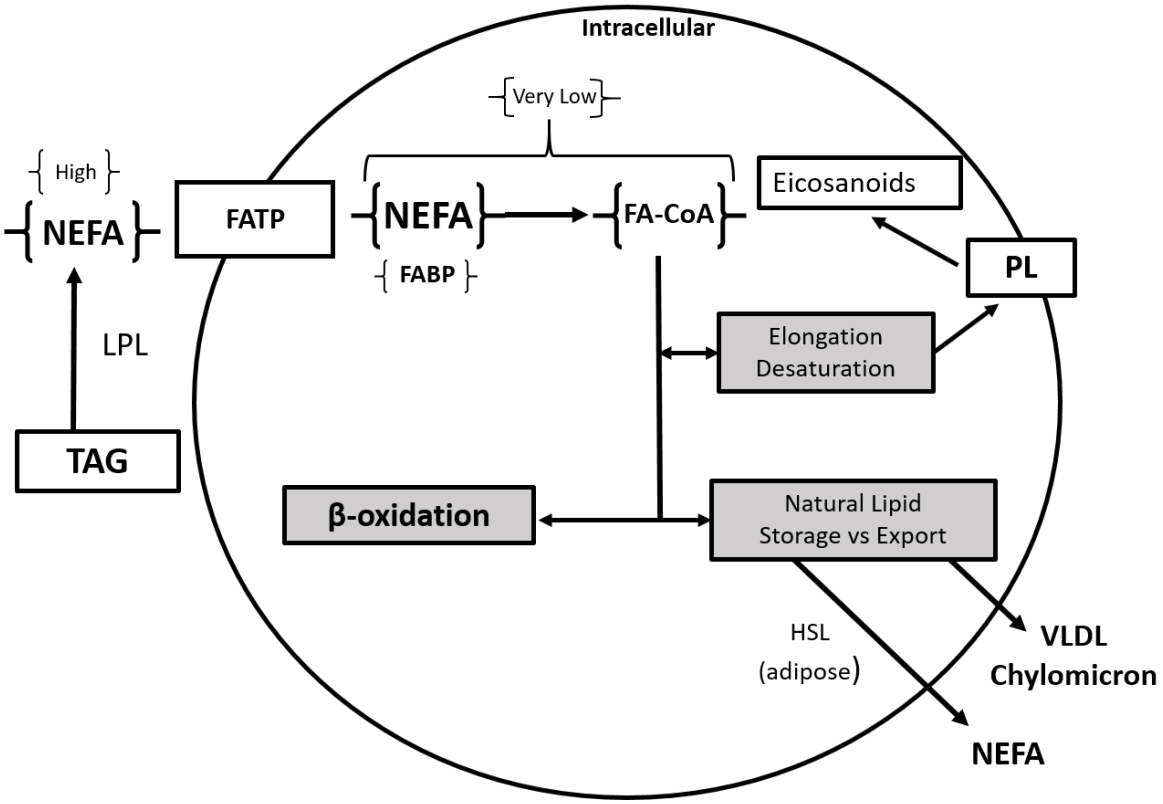


Figure 3. *Cellular lipid metabolism. Adapted from Jump (2004).*

NEFA, non-esterified fatty acid; TAG, triacylglycerol; LPL, lipoprotein lipase; FATP, fatty acid transport protein; FABP, fatty acid binding protein; FA-CoA, fatty acid coenzyme A; PL, phospholipids; HSL, hormone sensitive lipase; LDL, very-low density lipoprotein.

Cholesterol is formed in several types of cells in the human body, and is used for the production of steroid hormones, bile salts and structures for cell membranes (NCM, 2014). Cholesterol is a hydrophobic compound, consisting of a single hydroxyl group located at carbon number 3. A FA may attach to the carbon, producing CE, which is even more hydrophobic. Cholesterol can be synthesized in more or less all human tissue, and the synthesis takes place in the cytoplasm (Champe et al., 2008). Elimination of cholesterol from the body can occur by conversion to bile salts or secretion intro bile salts (Champe et al., 2008).

After VLDL have delivered lipids to tissues, they can either be endocytosed in the liver by the LDLR or they can become LDL particles (Frayn, 2010). The main function of LDL is to deliver cholesterol to peripheral tissue, and LDL leave the circulation by being endocytosed by the LDLR in various tissues (Champe et al., 2008; Frayn, 2010). By contrast, HDL removes cholesterol by delivering excess cholesterol from peripheral tissue to the liver, which is the only organ that can eliminate it. This process is known as reversed cholesterol transport,

and happens with help of cholesteryl ester transfer protein (CETP) (Figure 4) (Champe et al., 2008; Frayn, 2010).

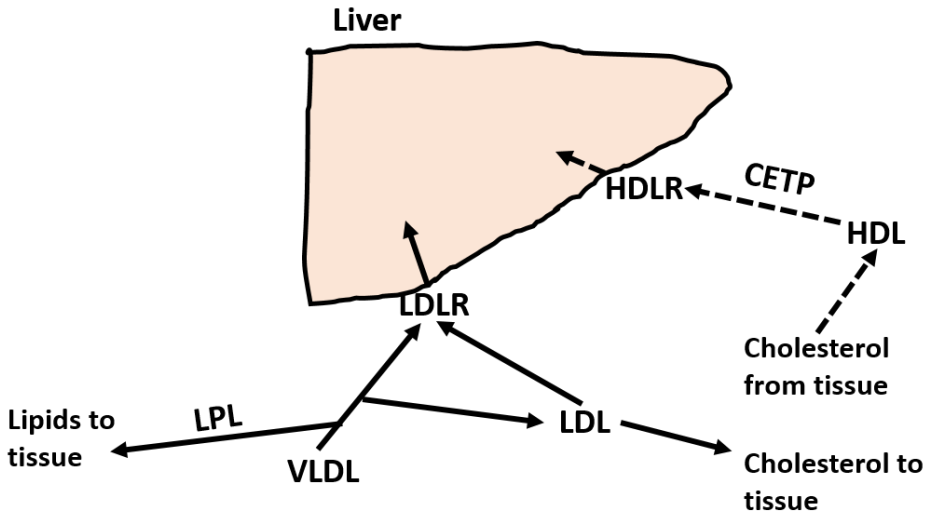


Figure 4. *Cholesterol and lipoprotein metabolism.*

LPL, lipoprotein lipase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; HDL, high-density lipoprotein; CETP, cholesteryl ester transfer protein; HDLR, high-density lipoprotein receptor.

1.5 Regulation of Lipid Metabolism by Fatty Acids

Whereas the metabolic flux in lipid metabolism can be adjusted by enzymes and proteins over a short period of time, e.g. after a meal when nutrients enter the bloodstream, long-term changes in metabolic flux may need to be regulated over longer periods, allowing the body to adapt to changes in dietary patterns (Frayn, 2010). This involves changes in the amount of protein, mainly by changes in the rate of transcription of genes into messenger RNA (mRNA). The overall process leading from a gene to its product is called gene expression (Frayn, 2010).

1.5.1 Gene expression

Gene expression is the production of a functional gene product, either ribonucleic acid (RNA) or protein, processed by the steps of replication, transcription and translation. The genetic information is copied and transferred to daughter cells via DNA replication. The process where a DNA strand serves as a pattern for the synthesis of ribonucleic acid (RNA), is the transcription (Champe et al., 2008). The transcription is the first stage in the expression of genetic information. Further, the code contained in the nucleotide sequence of mRNA is translated to a protein, completing the gene expression. Regulation of gene expressions

happens primarily at the level of transcription, and is mediated by the binding of proteins to regulatory elements on the DNA (Champe et al., 2008).

Transcription involves polymerases in addition to a great amount of proteins called transcription factors (TFs). The TFs are necessary both for the assembly of a transcription complex at the promoter as well as for determination of which genes are to be transcribed (Champe et al., 2008). The enzyme RNA polymerase II synthesizes the nuclear precursors of mRNA that are later translated to protein production. General TFs are the minimal requirements for a promoter to be recognized, and require RNA polymerase II and initiation of transcription (Champe et al., 2008). Specific TFs, also called transcriptional activators, are needed to modulate the frequency of initiation, to mediate the response to signals, and regulate which genes are expressed. Specific TFs also bind to other proteins, referred to as coactivators, recruiting them to the transcription complex (Champe et al., 2008).

1.5.2 Fatty acids and transcription factors

FAs may regulate gene transcription through several mechanisms. Some involve direct binding of FAs to nuclear receptors, whereas other act indirect and rely on changes in activity or nuclear abundance of the TFs (Figure 5) (Jump, 2004, 2008).

One of the most important groups of nutrient sensors is the nuclear receptor superfamily of TFs, comprising of 48 different members in humans (Zhang & Mangelsdorf, 2002). The nuclear receptors are ligand-inducible TFs that regulate target genes related to development, metabolism and reproduction (Hermanson, Glass, & Rosenfeld, 2002). Several nuclear receptors have been identified as targets for FA binding, including peroxisome proliferator activated receptors (PPARs), liver X receptor (LXR) and retinoid X receptor (RXR) (Jump, 2004).

PPARs are a group of nuclear receptors consisting of the peroxisome proliferator activated receptor alpha (PPARA), Peroxisome proliferator-activated receptor beta (PPARB) and PPAR gamma (PPARG). The PPARs form heterodimers with RXR and bind to PPAR response elements (PPRE) in the promotor region of target genes. The PPARs act as sensors for FAs and modulate expression of genes related to lipid metabolism (Mandard, Müller, & Kersten, 2004; Rakhshandehroo, Knoch, Müller, & Kersten, 2010; Yuriko & Kelley, 2010). FAs are regulators of PPARs, and activation leads to introduction of genes involved in FA synthesis and oxidation (Desvergne, Michalik, & Wahli, 2006).

LXRs are closely related to PPARs and are shown to bind to, and have their activity affected by, FAs (Mak et al., 2002; Ou et al., 2001; Pawar, Xu, Jerks, Mangelsdorf, & Jump, 2002; Yoshikawa et al., 2002). LXRs are important regulators of cholesterol homeostasis and play an important role in the interface between inflammation and metabolism. They also regulate the expression of genes involved in reverse cholesterol transport (Im & Osborne, 2011; Mak et al., 2002; Joyce J Repa et al., 2002; Tangirala et al., 2002). Ligands regulating LXR activity therefore have the potential to regulate blood lipid levels and development of atherosclerosis (Joseph, Castrillo, Laffitte, Mangelsdorf, & Tontonoz, 2003; Joseph et al., 2002; J. J. Repa et al., 2000).

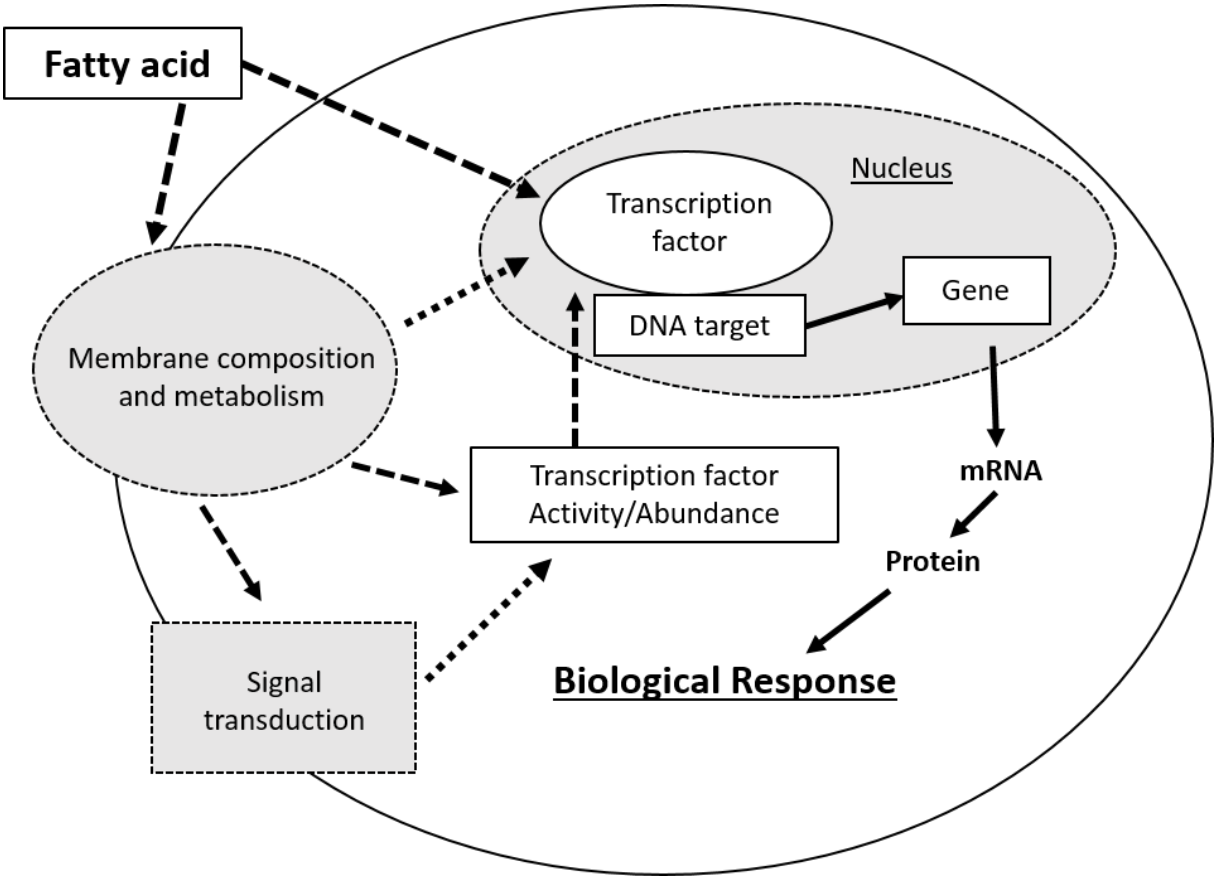


Figure 5. *Fatty acids effects on regulation of gene expression. Adapted from Jump (2004). mRNA, messenger ribonucleic acid; DNA: deoxyribonucleic acid.*

FAs of varying compositions affect the abundance of many transcription factors in the nucleus. These effects are not due to direct binding of the FA to the transcription factor, as described above. Instead, they involve mechanisms that affect the nuclear content of the transcription factor (Figure 5) (Jump, 2004).

SREBPs are TFs involved in the synthesis of FAs, complex lipids and cholesterol (Brown & Goldstein, 1997; Horton et al., 2002). Two genes encodes SREBPs. SREBP-1 has appeared as a regulator of TAG and FA synthesis, whereas SREBP-2 regulates cholesterol synthesis. PUFAs are believed to suppress the nuclear abundance of SREBP-1, as opposed to SFAs. PUFAs suppress its target genes involved in lipogenesis and FA desaturation and elongation, whereas cholesterol prevents SREBP-2 from inducing genes involved in cholesterol synthesis and transport, e.g. LDLR (Horton et al., 2002; Jump, Tripathy, & Depner, 2013; Xu et al., 1999).

FA regulation of PPARs of nuclear receptors is so far the best-characterized model of nuclear receptor regulation, whereas mechanisms for FA control of other molecular targets, such as SREBPs, remain poorly understood at the molecular level (Jump et al., 2013).

1.5.3 Peripheral blood mononuclear blood cells as a modelling system for studying gene expression

To understand the underlying molecular mechanisms of FAs, peripheral blood mononuclear cells (PBMCs) may provide a model system for studying expression of genes involved in lipid metabolism. PBMCs consist of monocytes and lymphocytes which is important cells in the inflammatory process, and are directly related to atherosclerosis (Pasterkamp & Daemen, 2008; Visvikis-Siest et al., 2007).

Several studies have shown that FAs regulate genes in PBMCs. A RCT study showed that supplementation of fish oil may modulate expression of genes involved in normal cellular function in PBMCs in healthy subjects (Myhrstad et al., 2014). A RCT study examined the postprandial effects of intake of PUFA and SFA on expression of gene profiles in PBMCs. PUFAs were found to decrease expression of genes related to LXR, whereas SFAs increased the expression of these genes, suggesting that differences in FAs regulate gene expression differently in PBMCs (Bouwens, Bromhaar, Jansen, Müller, & Afman, 2010). A RCT study on gene expression in PBMCs showed that a high EPA and DHA intake changed the expression of 1040 genes, whereas expression of genes involved in atherogenic and inflammatory pathways was found to be lower expressed (Bouwens et al., 2009). Furthermore, a follow-up study showed that plasma FA ratios consisting of different n-6 and n-3 FAs had differential impact on expression of genes related to inflammation and lipid metabolism in PBMCs (Olsen et al., 2013).

Collectively, it is well known that dietary FAs have an impact on lipid metabolism and CVD development. However, the exact underlying mechanisms on how different plasma FAs regulate expression of genes related to lipid metabolism in healthy subjects are not fully established.

2 Aim of this study

The present study is a continuation of a previous double-blinded, randomized, controlled parallel-group study on the health effect of fish oil intake. Fish oil was given to the subjects as a dietary supplement for seven weeks (Ottestad et al., 2012). A microarray analysis of expression of genes in PBMCs was later conducted with the same subjects (Myhrstad et al., 2014).

The main aim of the present study is to examine the impact of plasma fatty acids on expression of genes related to lipid metabolism in PBMCs in healthy subjects using the gene expression data from the previous microarray analysis.

The objectives can be described as follows:

i. To investigate the expression of genes related to lipid metabolism in PBMC in subjects with high plasma n-3 levels compared to subjects with low plasma n-3 levels.
ii. To investigate the expression of genes related to lipid metabolism in PBMC in subjects with high n-6/n-3 ratio compared to subjects with low plasma n-6/n-3 ratio.
iii. To investigate the expression of genes related to lipid metabolism in PBMC in subjects with SFA/PUFA ratio compared to subjects with low SFA/PUFA ratio.

3 Subjects and methods

This study is a cross-sectional, exploratory study. Data from a previous double-blind, randomized, controlled parallel-group study performed at Akershus University College from September to December 2009 was used. 83 subjects were screened for eligibility, whereof 58 received allocated interventions, and four were excluded at the three-week follow-up. A total of 54 subjects completed the seven-week intervention. The subjects were randomly assigned and stratified by gender into three groups at baseline. One group received 8 g/d fish oil (1.6 g/d EPA/DHA), another received 8 g/d oxidized fish oil (1.6 g/d EPA/DHA) and the last group received 8 g/d of high-oleic sunflower oil. The original study was a collaboration between UiO, HiOA and Matforsk (Ottestad et al., 2012).

3.1 Subjects, inclusion and exclusion criteria

Healthy men and women between 18 and 50 years were recruited among students and employees at Akershus University College. Inclusion criteria was non-smoking and stable weight for the last three months (±5%). Exclusion criteria were fasting serum level of TC (<7.5mm), TAG (>4 mm), Glucose (>6.0 mm), C-reactive protein (CRP) (>10 mg/l), body mass index (BMI) (>30kg/m2), hypertension (>106/100 mmHg) lactation, pregnancy and chronic illness. In the four weeks leading up to baseline, subjects were not allowed to consume dietary supplements, fish, marine n-3 enriched food or fish products (Ottestad et al., 2012).

3.2 Data collection in the previous study

3.2.1 Venous blood sampling

Subjects were told to refrain from alcohol consumption and high intensity physical activity the day before blood sampling. Venous blood samples were collected after a 12 hour long overnight fast. Serum samples were kept in room temperature for 30 minutes before centrifugation. Plasma was obtained from EDTA tubes and kept in room temperature for maximum 48 hours before counting the total number of monocytes and lymphocytes. Fasting serum concentrations of high-sensitivity CRP, TC, HDL-C, LDL-C and TAG were measured by standard methods in a routine laboratory (Fürst Medical Laboratory, Oslo, Norway). The Bligh and Dyer method was used to extract plasma lipids (Bligh & Dyer, 1959).

Concentrations of the individual plasma FA was expressed in percentage (%) of total FA (Ottestad et al., 2012).

3.2.2 Isolation and preparation of mRNA from peripheral mononuclear blood cells

In the original study, PBMCs were isolated after blood sampling. BD Vacutainer Cell Preparation tubes were used to isolate total mRNA from all PBMC samples, according to the manufacturer´s instructions (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). RNeasy Mini Kit was used to isolate total mRNA from all PBMC samples (qiagen, Hilden, Germany). Experimental procedures are described in detail in another study (Myhrstad et al., 2014).

The RNA were labelled, and extracts were hybridized to an Illumnia HumanHT-12 v4 Expression BeadChip and scanned on an Illumina HiScan microarray scanner. Bead-level data was transformed to probe-level values and statistics using Illumina GenomeStudio, which was transported raw (non-normalized and unfiltered) for analysis. A manual quality control step was performed after hybridization and scanning. All samples displayed good characteristics and were included in further analysis. In the end, from 48 000 probes presented on the Human HT-12 v4 microarray, a total of 21 236 genes expressed in PBMC were filtrated and normalized for further studies (Myhrstad et al., 2014).

3.3 Grouping of subjects based on plasma fatty acids

Only subjects that completed the intervention were included in this cross-sectional study (n=54). Data on age, BMI, serum lipids, plasma FAs, and gene expression at the end of intervention were used (Myhrstad et al., 2014; Ottestad et al., 2012).

Subjects were grouped according to plasma levels of FAs. To calculate the total plasma n-3 level, the plasma values of EPA, DPA, DHA and ALA after end of intervention were summed for each subject. The n-6/n-3 ratio was calculated by adding together the plasma values of the n-6 FAs (LA and ALA) and dividing the sum by the total level of plasma n-3 (EPA, DPA, DHA and ALA). The SFA/PUFA ratio was calculated by adding together the plasma values of the SFAs (Myristic acid, palmitic acid and stearic acid) and dividing by the sum of the total level of plasma PUFAs (EPA, DPA, DHA, ALA, LA and AA) (Table 1).

Table 1. *Included plasma fatty acids in the groups*

Group	Included plasma FAs
Total n-3 level	EPA + DPA + DHA + ALA
n-6/n-3 ratio	[LA + AA] / [EPA + DPA + DHA + ALA]
SFA/PUFA ratio	[Myristic acid + palmitic acid + stearicacid] / [EPA + DPA + DHA + ALA LA + AA]

LA, linoleic acid; ALA, Alpha-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

The subjects were thereafter divided into high and low groups based on the 70^{th-} and 30^{th} percentiles of total plasma n-3 level, n-6/n-3 ratio and SFA/PUFA ratio. The groups are hereafter referred to as the "high" and "low" groups, for the total plasma n-3 level, n-6/n-3 ratio and SFA/PUFA ratio. The highest 70^{th-} and lowest 30^{th} percentile level and ratio groups included 16 subjects in each group, whereas the middle 40^{th} percentile consisted of 18 subjects (Figure 6). The ranking by 70^{th-} and 30^{th} percentiles was conducted in Microsoft Excel (Excel 2011, version 14.4.5. Microsoft Corporation), and data was imported to *statistical package for the social sciences (*SPSS) for mac (SPSS, version 22.0 IBM Corp, Armonk, NY, USA) for statistical analysis.

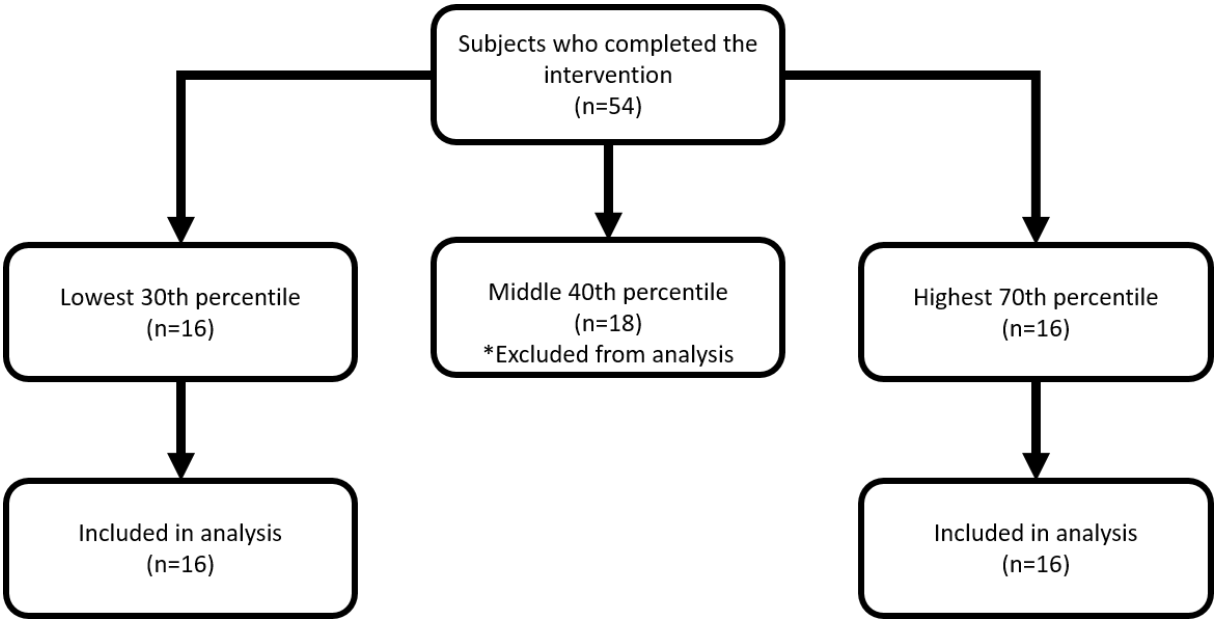


Figure 6. *Flowchart showing subjects completing the original study and excluded and included subjects in the statistical analysis in the present study.*

**Excluded from further analyses.*

3.4 Selection of genes related to lipid metabolism

A total of 21 236 genes were originally defined as expressed in PBMCs (Myhrstad et al., 2014). In accordance with the main objectives of the present study, a total of 43 genes related to various aspects of lipid metabolism were selected, and are shown in Table 2. Official gene symbols were used to denote the genes in the present study. The selected genes cover most of the pathways of lipid metabolism (cholesterol metabolism, FA oxidation, FA uptake, lipolysis, lipogenesis, elongation, desaturation, TAG metabolism, lipoprotein metabolism, glucose metabolism, hydrolysis of FA-CoAs, FA activation, lipid storage droplets, adipocyte differentiation and energy metabolism). In some cases, the same gene was measured with several probes, resulting in multiple measurements of the same gene. The genes measured in several probes were annotated with an ending letter, for example FABP5A, FABP5B and FABP5C.

Table 2. *Overview of included genes (n=43)*

Official gene symbol	Gene name (alias)	Function
CD36	CD36 molecule	Cholesterol metabolism
SCARB1	Scavenger receptor class B, member 1	Cholesterol metabolism
LDLR	LDL receptor	Cholesterol metabolism
ABCG1	ATP-binding cassette, sub-family G, member 1	Cholesterol efflux
SOAT1	Sterol O-acyltransferase 1	Cholesterol metabolism
PPARA	Peroxisome proliferator-activated receptor alpha	FA oxidation
CPT1A	Carnitine palmitoyltransferase 1A	FA oxidation
CPT1B	Carnitine palmitoyltransferase 1B	FA oxidation
CRAT	Carnitine O-acetyltransferase	FA oxidation
ACOX1	Acyl-CoA oxidase 1, palmitoyl	FA oxidation
FASN	Fatty acid synthase	FA synthesis
SCD1	Stearoyl-CoA desaturase-1	FA synthesis
SREBF1	Sterol regulatory element binding transcription factor 1	Lipogenesis
ELOVL5	ELOVL fatty acid elongase 5	VLCPUFA elongation
ELOVL6	ELOVL fatty acid elongase 6	LCPUFA elongation
FADS1	Fatty acid desaturase 1	LCPUFA desaturation
FADS2	Fatty acid desaturase 2	LCPUFA desaturation
LIPE	Hormone sensitive lipase	Lipolysis
DGAT1	Diacylglycerol O-acyltransferase 1	TAG metabolism
LPL	Lipoprotein lipase	Lipoprotein metabolism
APOBR	Apolipoprotein B receptor	Lipoprotein metabolism
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	Glucose metabolism
ACOT1	Acyl-CoA thioesterase 1	Hydrolysis of fatty acyl-CoAs
ACSL1	Acyl-CoA synthetase long-chain family member 1	Fatty acyl synthetase/FA activation
PLIN4	Perilipin 4	Lipid storage droplets

PLIN2	Perilipin 2	Lipid storage droplets
CETP	Cholestserol ester transfer protein	Cholesterol metabolism
PLIN5	Perilipin 5	FA uptake, lipolysis, FA oxidation
PPARG	Peroxisome proliferator-activated receptor gamma	Adipocyte differenation,
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Energy metabolism
PPARGC1B	peroxisome proliferator-activated receptor gamma, coactivator 1 b	Energy metabolism/fat oxidation
UCP2	Uncopling protein 2	Energy metabolism
NR1H3	Nuclear receptor subfamily 1, group H, member 3 (LXRA)	Lipid metabolism/cholesterol
NR1H2	Nuclear receptor subfamily 1, group H, member 2 (LXRB)	Lipid metabolism/cholesterol
RXRA	Retinoic X receptor alpha	Lipid metabolism
FABP5	Fatty acid binding protein 5	FA uptake
PCSK9	proprotein convertase subtilisin/kexin type 9	Cholesterol metabolism
SRGN	Serglycin	Lipid homeostasis
SORT1	Sortilin1	Trafficking of proteins
MYLIP	Myosin regulatory light chain interacting protein	Myosin regulatory light chain
INSIG1	Insulin Induced Gene 1	Regulation of cholesterol biosynthesis
INSIG2	Insulin Induced Gene 2	Regulation of cholesterol biosynthesis
SCAP	SREBF Chaperone	Regulation of cholesterol biosynthesis

FA: fatty acid, VLCPUFA: very long chain polyunsaturated fatty acid, LCPUFA: long chain polyunsaturated fatty acid, TAG: triacylglycerol.

3.5 Statistical methods

3.5.1 Variables

The plasma FA levels are continuous variables, but in the present study they are grouped and tested as categorical variables in the form of high and low groups based on plasma n-3 level, n-6/n-3 ratio and SFA/PUFA ratio. The included genes, measured as mRNA levels, are continuous variables. The normally distributed data is presented as mean ± standard deviations (SD), whereas the non-normally distributed data is presented as median and minimum and maximum values.

3.5.2 Statistical analysis

Processing of data and statistical analysis was performed using SPSS version 22 statistical package for Mac (IBM Corp, Armonk, NY, USA, 2013) and Microsoft Excel (Excel 2011, version 14.4.5, Microsoft Corporation). Parametric tests were performed on the normally distributed variables, whereas non-parametric tests were performed on the non-normal distributed variables. The parametric test, *independent samples t-test* is suitable for normally distributed variables when testing for differences in the mean value of two groups of the same variable. The *Mann-Whitney U test* is the non-parametric alternative (Pallant, 2013). For comparisons of two groups, *independent samples t-test* was performed when data was normally distributed, whereas *Mann-Whitney U test* was performed on the non-normally distributed variables.

Comparison of characteristics between subjects with low and high plasma n-3 level and fatty acid ratios was performed by comparing subjects with high and low plasma n-3 level, n-6/n-3 ratio and SFA/PUFA ratio using *independent samples t-test* or *Mann-Whitney U test*. The gene expression levels were log 2-transformed and *independent samples t-test* was performed to evaluate differences in gene expression between subjects with high and low plasma n-3 level, n-6/n-3 and SFA/PUFA ratio. *Spearman's Rho* analysis was performed to investigate the linear relationship between variables. The statistical significance level was defined as p-values <0.05 in all tests (2-sided).

3.6 Research ethics

In the original study written informed consent was obtained from all participants. The study was approved by the Regional Committee of Medical Ethics (approval no. 6.2008.2215) (Appendix 1) and by the Norwegian social Science Data Services (approval no. 21924). The original study was conducted in accordance with the declaration of Helsinki.

4 Results

4.1 Subject characteristics

4.1.1 Descriptive data

A total of 54 normal-weight healthy subjects were included in the present study. A majority of the participants were women (72.2% women and 27.8% men). Subjects were between 18 and 50 years (26.8±7.2 years) with a stable weight (±5%) for the last three months. Serum lipid levels were within the normal range in accordance to the reference values for serum lipids at Fürst Medical Laboratory (Fürst Medical Laboratory, Oslo, Norway) (Table 3).

Table 3. *Descriptive characteristics of the 54 participants*

	All (n=54)	Reference values
Male/Female (n)	15/39	
Age (years)	26.8±7.2	
BMI (kg/m^2)	22.6±2.6	
TC (mmol/L)	4.8±0.9	2.9-6.1/3.3-6.9*
TAG (mmol/L)	1.0±0.6	<2.60
LDL-C (mmol/L)	2.7±0.8	1.2-4.3/1.4-4.8*
HDL-C (mmol/L)	1.5±0.4	0.8-2.1 /1-2.8**

BMI, body mass index; TC, total cholesterol; TAG, triacylglycerol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol.
Values are presented as number of male and females and mean ±SD.
* Reference values for serum lipids at Fürst Medical Laboratory, for age group 0-29 years/30-49 years.
** Reference values for serum lipids at Fürst Medical Laboratory, for male/female.

4.1.2 Fatty acid profiles

The amount of individual FA is given as the percentage of total plasma FA (Table 4). The dominating individual FA was LA with a mean level of 28.8% of the total plasma FA, across subjects. The mean total plasma n-3 level, including ALA, EPA, DPA and DHA was 6.3% of the total plasma FAs. In addition, the mean of the FA ratios were also dominated by n-6 FAs, including LA and AA. The mean of the total n-6/n-3 ratio was 6.6, whereas the mean of the

SFA/PUFA ratio (myristic acid, palmitic acid and stearic acid/ALA, EPA, DPA, DHA, LA and ALA) was 0.8%.

Table 4. *Plasma fatty acid profile of the subjects (n=54)*

Fatty acid	Serum proportions (% of total fatty acids)
Myristic acid (14:0)	0.9±0.4
Palmitic acid (16:0)	22.1±2.1
Stearic acid (18:0)	7.9±1
Oleic acid (18:1)	19.5±2.6
LA (18:2n-6)	28.8±3.6
ALA (18:3n-3)	0.5±0.1
AA (20:4n-6)	5.9±1.1
EPA (20:5n-3)	1.9±1.2
DPA (22:5n-3)	0.6±0.2
DHA (22:6n-3)	3.2±1.1
Total n-3	6.3±2.4
n-6/n-3 ratio	6.6±2.9
SFA/PUFA ratio	0.8±0.2

LA, linoleic acid; ALA, Alpha-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Total n-3: ALA, EPA, DPA, DHA; total n-6: LA, ALA; total SFA: myristic acid, palmitic acid, stearic acid; total PUFA: ALA, EPA, DPA, DHA, LA and AA.

Values are presented as mean ±SD.

4.1.3 Inclusion of subjects with high and low plasma n-3 level and fatty acid ratios

Subjects were divided into groups based on high or low plasma n-3 level, high and low plasma n-6/n-3 ratio and high and low plasma SFA/PUFA ratio by arranging samples from highest to lowest values of these categories. The subjects were divided into three groups by using percentiles. Subjects in the highest 70^{th} and lowest 30^{th} percentile of level and ratio groups used for testing, and consisted of 16 subjects in each group. Mean values, cut-off

points and minimum and maximum values for the included subjects in high and low total plasma n-3 level, n-6/n-3 and SFA/PUFA ratio are listed in Table 5.

Table 5. *Mean, min.-max and cut-off values in subjects with low and high plasma n-3 level and fatty acid ratios*

	Total n-3 level		n-6/n-3 ratio		SFA/PUFA ratio	
	Lowest level (n=16)	Highest level (n=16)	Lowest ratio (n=16)	Highest ratio (n=16)	Lowest ratio (n=16)	Highest ratio (n=16)
Mean ±SD	3.5±0.4	9.1±1.0	3.6±0.4	10.5±1.4	0.6±0.0	0.9±0.2
Min. – max. values	2.4-4.3	8.9±11.0	2.9-4.1	8.4-13.3	0.6-0.7	0.8-1.7
Cut-off points	4.3	8.9	4.1	8.4	0.7	0.8

SD: standard deviation

Total n-3: ALA, EPA, DPA, DHA; total n-6: LA, ALA; total SFA: myristic acid, palmitic acid, stearic acid; total PUFA: ALA, EPA, DPA, DHA, LA and AA.

4.2 Comparison of characteristics between subjects with low and high plasma n-3 level and fatty acid ratios

Subjects with high plasma n-3 level had a significantly lower level of oleic acid compared to subjects with low plasma n-3 level ($P<0.01$). The levels of EPA, DPA and DHA were significantly higher in subjects with high plasma n-3 level and n-6/n-3 ratio compared to subjects with low plasma n-3 level and n-6/n-3 ratio ($P<0.01$ for both). The oleic acid and LA levels were higher in subjects with high n-6/n-3 ratio compared to subjects with low n-6/n-3 ratio ($P<0.01$ in both). The level of TAG, myristic acid, palmitic acid and oleic acid were higher in subjects with high SFA/PUFA ratio compared to subjects with low SFA/PUFA ratio (respectively, $P<0.01$, $P<0.01$, $P<0.01$, and $P<0.01$), whereas LA levels were lower in subjects with high SFA/PUFA ratio compared to subjects with low SFA/PUFA ratio ($P<0.01$). No other differences in characteristics and lipid profiles between subjects in with high and low plasma n-3 level and FA ratios were found (Table 6).

Table 6. *Comparison of characteristics between subjects with low and high plasma n-3 level and fatty acid ratios*

	Total n-3 level			n-6/n-3 ratio			SFA/PUFA ratio		
	Lowest level (n=16)	Highest level (n=16)	P	Lowest ratio (n=16)	Highest ratio (n=16)	P	Lowest ratio (n=16)	Highest ratio (n=16)	P
Age (years)	24.5 (21-49)	25.0 (20-47)	0.64	24.5 (20-47)	24.5 (20-49)	0.46	27.5 (20-35)	25.5 (20-47)	0.60
BMI (kg/m2)	23.1±3.2	22±2.0	0.24	21.9±1.9	23.3±3.2	0.13	22.3±2.4	23.2±3.1	0.41
TAG (mmol/L)	0.98 (0.6-4.4)	0.77 (0.5-4.4)	0.22	0.9 (0.5-1.9)	0.9 (0.6-1.8)	0.84	0.7 (0.5-1.1)	1.0 (0.5-4.4)	**<0.01****
LDL-C (mmol/L)	2.9±0.6	2.7±0.9	0.69	2.7±1.0	2.8±0.6	0.65	2.6±0.8	2.4±0.6	0.30
HDL-C (mmol/L)	1.5 (1.0-2.3)	1.6 (1.0-2.4)	0.68	1.5 (1.0-2.3)	1.4 (0.9-2.4)	0.75	1.6 (1.0-2.1)	1.5 (1.0-2.4)	0.59
TC (mmol/L)	5.1±0.9	4.9±1.0	0.66	4.8±1.1	4.9±0.9	0.77	4.8±0.7	4.6±0.8	0.92
Myristic acid (14:0)	1.0 (0.6-3.1)	1.0 (0.5-2.0)	0.71	0.9 (0.5-2.0)	0.9 (0.6-1.3)	0.79	0.6 (0.5-1.1)	1.0 (0.7-3.1)	**<0.01****
Palmitic acid (16:0)	21.5 (19.8-31.8)	20.9 (20.2-26.7)	0.37	21.5 (20.2-26.7)	21.5 (19.8-24.8)	0.73	20.4 (19.4-22.0)	24.1 (21.0-31.8)	**<0.01****
Stearic acid (18:0)	8.0±1.1	8.2±1.1	0.66	8.2±1.1	8.1±1.0	0.80	7.8±0.6	8.1±1.1	0.45
Oleic acid (18:1)	20.9±2.3	18±2.2	**<0.01****	18.6±2.3	20.7±2.2	**<0.01****	17.4±1.8	21±2.6	**<0.01****
LA (18:2n-6)	29.3±4.2	27.5±3.6	0.19	26.5±3.2	30.1±2.9	**<0.01****	31.8±2.0	24.6±2.6	**<0.01****
ALA (18:3n-3)	0.5 (0.4-0.9)	0.5 (0.4-0.8)	0.97	0.5 (0.4-0.9)	0.5 (0.4-0.8)	0.82	0.5 (0.4-0.9)	0.5± (0.4-0.8)	0.82
AA (20:4n-6)	6.2±1.2	5.8±0.8	0.27	5.7±0.8	6.3±1.1	0.11	6.4±0.9	5.9±1.4	0.26
EPA (20:5n-3)	0.6 (0.3-0.8)	3.3 (2.4-4.7)	**<0.01****	3.2 (2.3-4.7)	0.6 (0.3-0.8)	**<0.01****	2.4 (0.5-4.7)	1.7 (0.5-4.7)	0.24
DPA (22:5n-3)	0.5±0.1	0.8±0.1	**<0.01****	0.8±0.1	0.5±0.1	**<0.01****	0.7±0.1	0.6±0.2	0.54
DHA (22:6n-3)	1.9 (1.5-2.7)	4.3 (4.1-5.3)	**<0.01****	4.3 (3.6-5.3)	1.9 (1.5-2.7)	**<0.01****	0.24	3.2 (1.5-5.3)	4.0 (1.5-5.3)

BMI, body mass index; TAG, triacylglycerol; LDL-C, LDL cholesterol; HDL, HDL cholesterol; TC, total cholesterol; LA, linoleic acid; ALA, alpha-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Total n-3: ALA, EPA, DPA, DHA; total n-6: LA, ALA; total SFA: myristic acid, palmitic acid, stearic acid; total PUFA: ALA, EPA, DPA, DHA, LA and AA.

Values are presented as mean ±SD or median and min- and max.

* Differences between the groups were calculated using independent samples t-test or Mann-Whitney U Test. P-values <0.05 were considered significant.

** P-values <0.01.

4.3 Gene expression analysis

43 genes were selected for analysis based on their involvement in various aspects of lipid metabolism. The results showed that nine genes were significantly different expressed between subjects with high and low plasma n-3 level, n-6/n-3- and SFA/PUFA ratio (Table 7). The SFA/PUFA ratio was associated with the highest number of differentially expressed genes (seven genes), followed by the total n-6/n-3 ratio (two genes), and the total n-3 level (one gene). The level of six gene transcripts were increased in the high group, compared to the low level or ratio group. Three genes showed decreased gene transcript levels, compared to the low level or ratio group.

A summary of the results from the gene expression analysis is presented in Table 7. The mean mRNA values for the significantly different expressed genes in subjects with high and low n-3 level, n-6/n-3- or SFA/PUFA ratio is presented in Table 8.

Table 7. *Number of differentially expressed genes*

Group	Differentially expressed	Higher gene transcript level	Lower gene transcript level
Total n-3	1		1
n-6/n-3 ratio	2	2	
SFA/PUFA ratio	6	4	2

Total n-3: ALA, EPA, DPA, DHA; total n-6: LA, ALA; total SFA: myristic acid, palmitic acid, stearic acid; total PUFA: ALA, EPA, DPA, DHA, LA and AA.

Table 8. *Differentially expressed genes between subjects with high and low plasma n-3 level and fatty acid ratios. Data is given as mean ± SD of the normalized mRNA level.*

Official Gene symbol	Gene name	FA level or ratio	mRNA low level or ratio	mRNA high level or ratio	P
CETP	Cholesteryl ester transfer protein	Total n-3	128.9±1.1	123.0±1.1	0.02*
		n-6/n-3	123.6±1.1	129.0±1.1	0.04*
PPARG	Peroxisome proliferator-activated receptor gamma	n-6/n-3	108.0±1.0	112.2±1.1	0.04*
FABP5b	Fatty acid binding protein 5	SFA/PUFA	124.2±1.1	133.2±1.1	<0.01**
FABP5c		SFA/PUFA	281.9±1.2	332.0±1.2	<0.01**
CPT1A	Carnitine palmitoyltransferase 1A	SFA/PUFA	137.8±1.1	147.7±1.1	<0.01**
INSIG2	Insulin induced gene 2	SFA/PUFA	270.3±1.1	283.3±1.1	0.02*
SOAT1	Sterol O-acyltransferase 1	SFA/PUFA	152.1±1.1	165.9±1.1	0.02*
CRAT	Carnitine O-acetyltransferase	SFA/PUFA	145.3±1.1	136.8±1.1	0.01*
ACOT1	Acyl-CoA thioesterase 1	SFA/PUFA	201.6±1.1	219.1±1.1	0.02*
PLIN5	Perilipin 5	SFA/PUFA	1195.8±1.3	935.2±1.4	0.03*

CETP, Cholesteryl ester transfer protein; PPARG, peroxisome proliferator-activated receptor gamma; FABP5, fatty acid binding protein 5; CPT1A, carnitine palmitoyltransferase 1A; INSIG2, insulin induced gene 2; SOAT1, sterol O-acyltransferase 1; CRAT, carnitine O-acetyltransferase; ACOT1, acyl-CoA thioesterase 1, PLIN5: perilipin 5.

Total n-3: ALA, EPA, DPA, DHA; total n-6: LA, ALA; total SFA: myristic acid, palmitic acid, stearic acid; total PUFA: ALA, EPA, DPA, DHA, LA and AA.

* Differences between the groups were calculated using independent samples t-test. P-values <0.05 were considered significant.

** P-values <0.01.

Subjects with high plasma n-3 level had lower mRNA levels of CETP compared to subjects with low plasma n-3 level (P=0.02) (Table 8). The mRNA levels of CETP in the plasma n-3 level groups is shown in Figure 7.

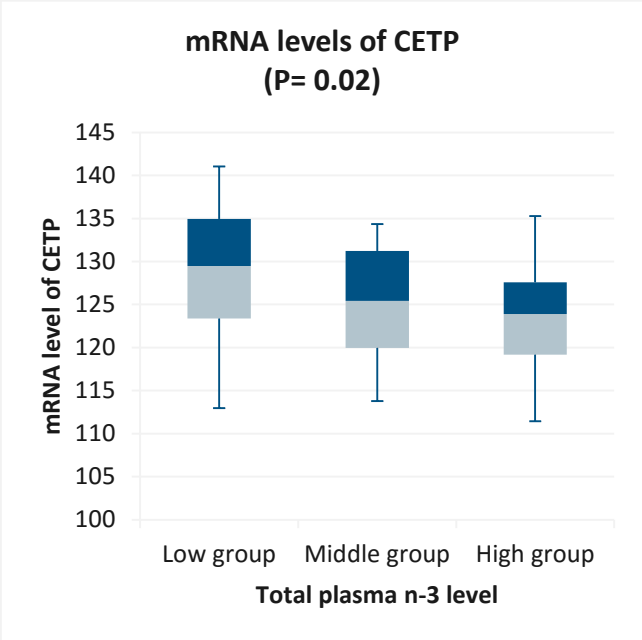


Figure 7. *Boxplot of the cholesteryl ester transfer protein (CETP) mRNA levels in the total plasma n-3 groups. The figure shows the mRNA level of CETP of subjects in the low, middle and high plasma n-3 groups. Median, 25- and 75 percentiles are set as lower, middle and upper line in the boxes. Minimum and maximum values is indicated by "whiskers". mRNA is given as normalized levels.*

Subjects with high n-6/n-3 ratio had higher mRNA levels of CETP compared to subjects in the low n-6/n-3 ratio (P=0.04) (Table 8). The mRNA level of PPARG were higher in subjects with high n-6/n-3 ratio compared to subjects with low n-6/n-3 ratio (P=0.04) (Table 8). The mRNA levels of CETP and PPARG in the plasma n-6/n-3 ratio groups is shown in Figure 8A and B.

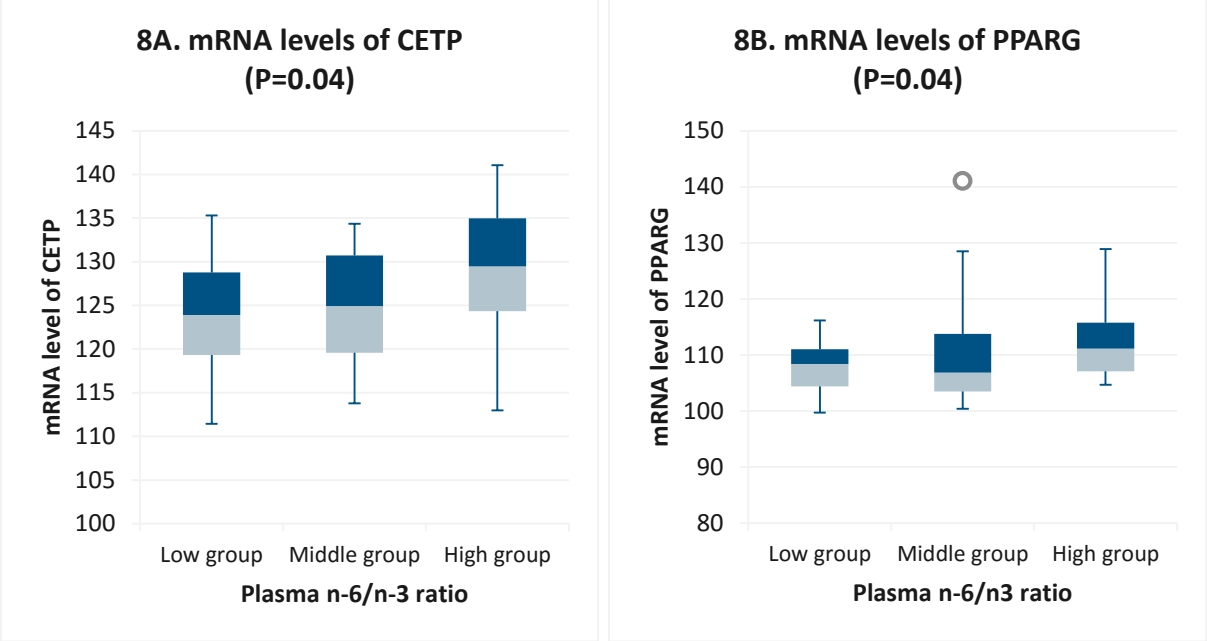
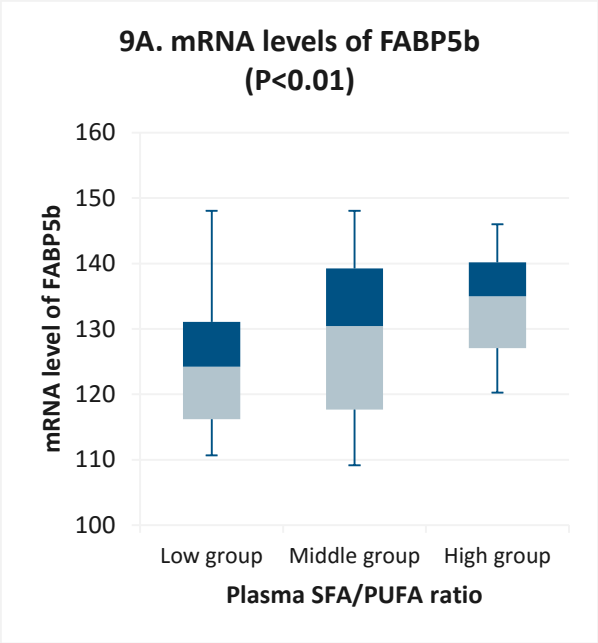
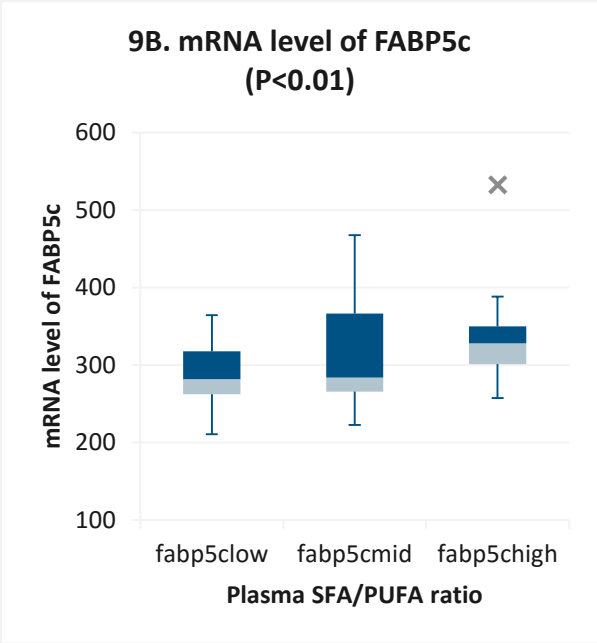
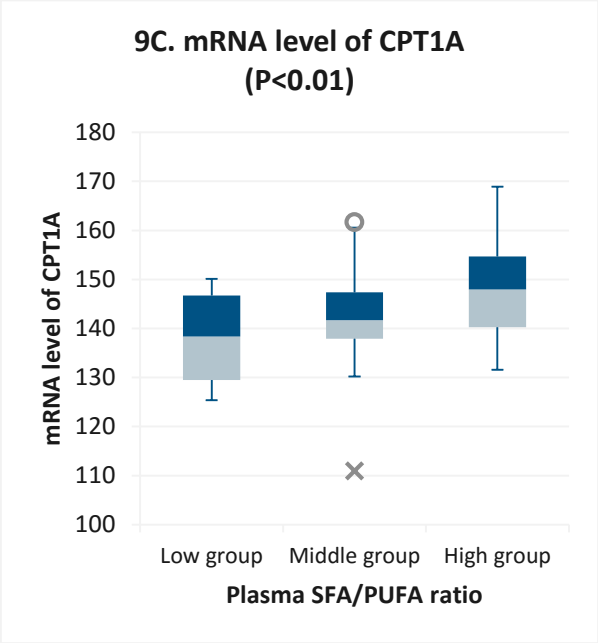


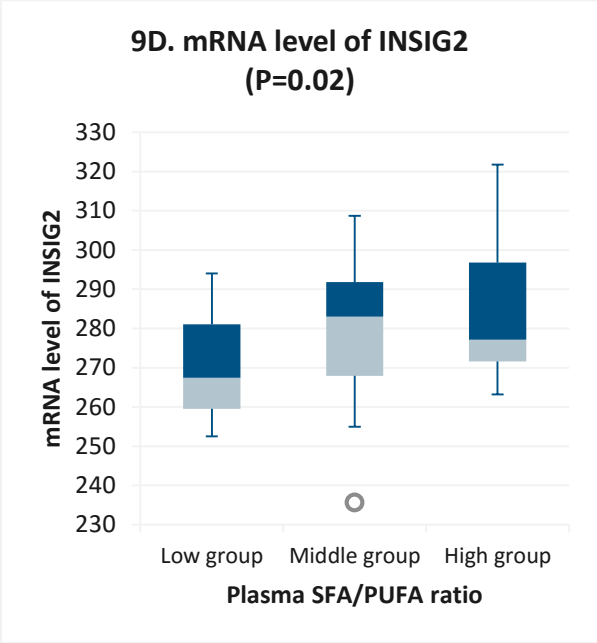
***Figure 8A and 8B**. Boxplot of the cholesteryl ester transfer protein (CETP) and peroxisome proliferator-activated receptor gamma (PPARG) mRNA levels in the n-6/n-3 ratio groups. The figure shows the mRNA levels of subjects in in the low, middle and high n-6/n-3 ratio. Median, 25- and 75 percentiles are set as lower, middle and upper line in the boxes. Minimum and maximum values is indicated by "whiskers". mRNA is given as normalized levels. Outliers, defined in SPSS as cases more than 1.5 box-lengths from the edge of the box, are marked by a circle. Extreme values, defined in SPSS as cases bore than three box-lengths from the edge of the box, are marked by a cross.*

Seven genes were significantly different expressed between subjects with high SFA/PUFA ratio compared with subjects with low SFA/PUFA ratio. FABP5b (P<0.01), FABP5c (P<0.01), CPT1A (P<0.01), INSIG2 (P=0.02), SOAT1 (P=0.02) and ACOT1 (P=0.02) showed higher mRNA levels in subjects with high SFA/PUFA ratio compared to subjects with low SFA/PUFA ratio. CRAT (P<0.01) and PLIN5 (P=0.03) showed lower mRNA levels in subjects with high SFA/PUFA ratio compared to subjects with low SFA/PUFA ratio. The mRNA levels of the significantly different expressed genes in the SFA/PUFA ratio groups is shown in Figure 9A-H.


9A. mRNA levels of FABP5b
(P<0.01)
mRNA level of FABP5b
160
150
140
130
120
110
100
Low group
Middle group
High group
Plasma SFA/PUFA ratio


9B. mRNA level of FABP5c
(P<0.01)
mRNA level of FABP5c
600
500
400
300
200
100
fabp5clow
fabp5cmid
fabp5chigh
Plasma SFA/PUFA ratio


9C. mRNA level of CPT1A
(P<0.01)
mRNA level of CPT1A
180
170
160
150
140
130
120
110
100
Low group
Middle group
High group
Plasma SFA/PUFA ratio


9D. mRNA level of INSIG2
(P=0.02)
mRNA level of INSIG2
330
320
310
300
290
280
270
260
250
240
230
Low group
Middle group
High group
Plasma SFA/PUFA ratio

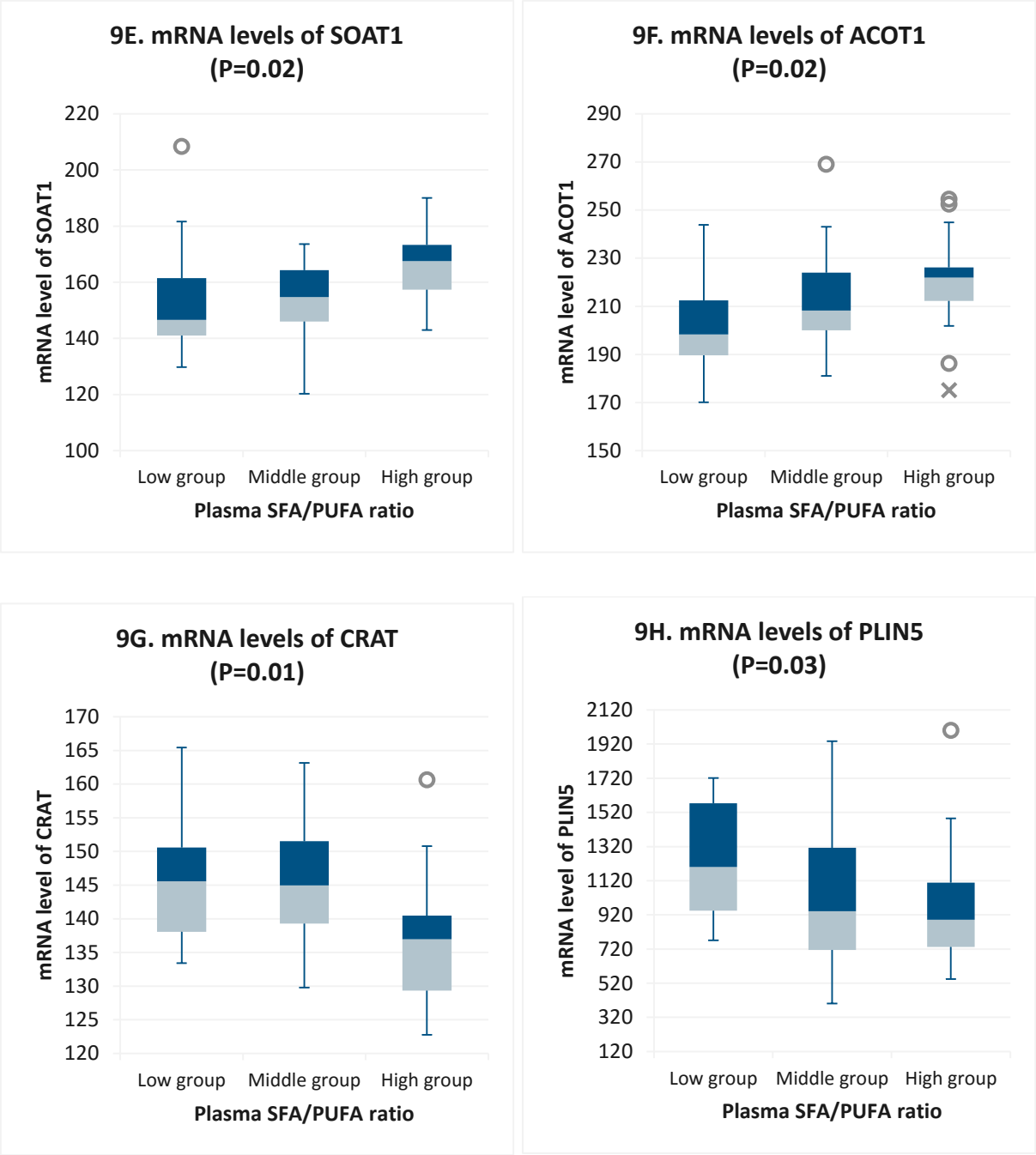


***Figure 9A-H**. Boxplot of the fatty acid binding protein 5 (FABP), carnitine palmitoyltransferase 1A (CPT1A), insulin induced gene 2 (INSIG2), sterol O-acyltransferase 1 (SOAT1), carnitine O-acetyltransferase (CRAT), acyl-CoA thioesterase 1 (ACOT1) and perilipin 5 (PLIN5) mRNA levels in the SFA/PUFA ratio groups. The figure shows the mRNA levels of subjects in the low, middle and high n-6/ n-3 ratio groups. Median, 25- and 75 percentiles are set as lower, middle and upper line in the boxes. Minimum and maximum values is indicated by "whiskers". mRNA is given as normalized levels. Outliers, defined in SPSS as cases more than 1.5 box-lengths from the edge of the box, are marked by a circle. Extreme values, defined in SPSS as cases bore than three box-lengths from the edge of the box, are marked by a cross.*

4.4 Correlation analysis

The correlation between mRNA level of CETP and plasma n-3 level was found to be negative (P=0.05). Positive correlation was found between mRNA levels of CETP and the n-6/n-3 ratio (P=0.04), and negative correlation were seen between the mRNA level of FABP5 and the n-6/n-3 ratio (P=0.02). FABP5, SOAT1 and ACOT showed a positive correlation (respectively, P=0.04, P<0.01 and P=0.02), whereas CRAT and PLIN showed a negative correlation with the SFA/PUFA ratio (respectively, P=0.05 and P=0.02) (Table 9).

Table 9. *Correlations between significantly regulated gene transcripts and plasma n-3 level, n-6/n-3- or SFA/PUFA ratio.*

	Plasma n-3 level	n-6/n-3 ratio	SFA/PUFA ratio
CETP	-.273*	.278*	
FABP5		-.308*	.278*
SOAT1			.351**
CRAT			-.273*
ACOT1			.328*
PLIN5			-.328*

CETP, Cholesteryl ester transfer protein; FABP, fatty acid binding protein 5; SOAT1, sterol O-acyltransferase 1; CRAT, carnitine O-acetyltransferase; ACOT1, acyl-CoA thioesterase 1; PLIN5, perilipin 5.

Total n-3: ALA, EPA, DPA, DHA; total n-6: LA, ALA; total SFA: myristic acid, palmitic acid, stearic acid; total PUFA: ALA, EPA, DPA, DHA, LA and AA.

Values are presented as Spearman Rho.

* Correlations were considered significant with P-values <0.05 (2-tailed).

** P-values <0.01 (2-tailed).

The correlation between TAG levels and mRNA level of ACOT1 was found to be positive (P=0.01) (Table 10). No other significant correlations between significantly regulated gene transcripts and BMI or serum lipids were found.

Table 10. *Correlations between significantly regulated gene transcripts and BMI or serum lipids*

	ACOT1
TAG	.344*

ACOT1: acyl-CoA thioesterase 1; TAG, triacylglycerol.

Values are presented as Spearman Rho.

* Correlations were considered significant with P-values <0.05 (2-tailed)

5 Discussion

In the present study, the impact of plasma FAs on the expression of genes related to lipid metabolism in PBMCs in healthy subjects was investigated. The results showed that of the 43 genes included in the study, nine were differently expressed in subjects with high plasma n-3 level, n-6/n-3 ratio and SFA/PUFA ratio compared to low level or ratio. Furthermore, six of the regulated gene transcripts were also significantly correlated with either plasma n-3 level, n-6/n-3 ratio or SFA/PUFA ratio.

5.1 Impact of fatty acid levels and ratios on expression of gene transcripts

Nine of the 43 included genes were significantly different expressed between subjects in high and low groups. The SFA/PUFA ratio was associated with the highest number of differently expressed genes (seven), followed by the n-6/n-3 ratio (two) and total plasma n-3 level (one). To our knowledge there are few studies on the relation between plasma FA levels or ratios and the expression of genes related to lipid metabolism. The genes were selected on the basis of their representation of multiple pathways in lipid metabolism as shown in Figure 10.

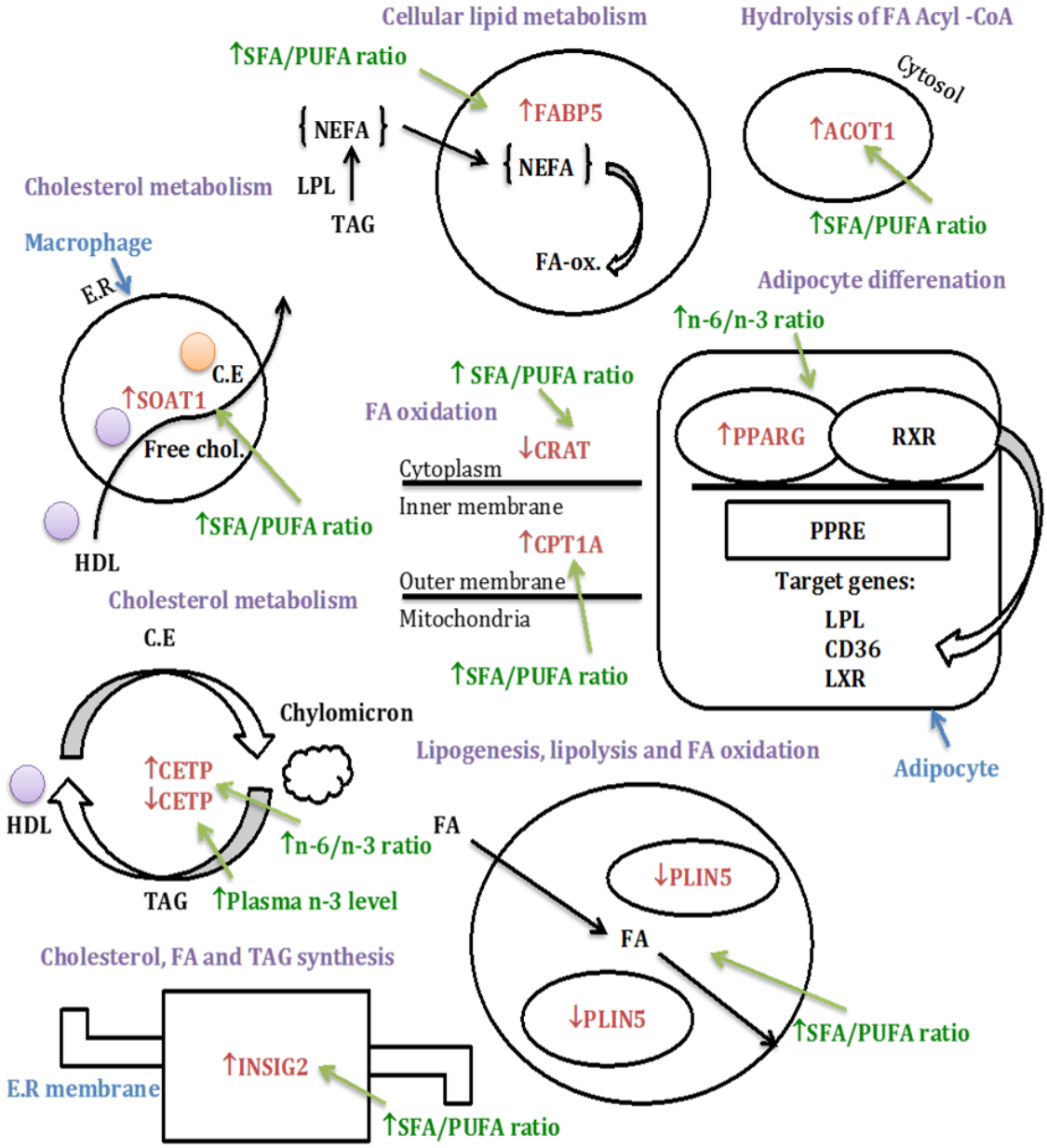


Figure 10. *Overview of significantly regulated genes in various aspects of lipid metabolism. The regulated genes are in red, whereas their role in lipid metabolism is in purple. The plasma FA group regulating the gene transcript is written in green.*

CETP, Cholesteryl ester transfer protein; PPARG, peroxisome proliferator-activated receptor gamma; FABP5, Fatty acid binding protein 5; CPT1A, carnitine palmitoyltransferase 1A; INSIG2, insulin induced gene 2; SOAT1, sterol O-acyltransferase 1; CRAT, carnitine O-acetyltransferase; ACOT1, acyl-CoA thioesterase 1; PLIN5: perilipin 5, NEFA: non-esterified fatty acids; TAG: triacylglycerol; LPL, lipoprotein lipase; HDL, high-density lipoprotein, CE, cholesteryl ester; RXR: retionid receptor alpha; LXR, liver X receptor; PPRE, PPAR response element; FA.ox, fatty acid oxidation; E.R, endoplasmic reticulum.

Total n-3: ALA, EPA, DPA, DHA; n-6: LA, ALA; SFA: myristic acid, palmitic acid, stearic acid; PUFA: ALA, EPA, DPA, DHA, LA and AA

5.1.1 Significantly regulated gene transcripts in subjects with high and low plasma n-3 level and n-6/n-3 ratio

The gene expression analysis showed that CETP was the only significantly different expressed gene transcript when the subjects with high or low plasma n-3 level were compared. Subjects with high plasma n-3 level had a lower mRNA level of CETP compared to subjects with low plasma n-3 level. An inverse tendency was observed in the n-6/n-3 ratio, where subjects with high plasma n-6/n-3 ratio had higher CETP mRNA level compared with subjects with low plasma n-6/n-3 ratio. In addition, the CETP gene was found to be significantly negatively correlated with the plasma n-3 level and significantly positively correlated with the plasma n-6/n-3 ratio.

The circulating transfer protein CETP plays an important role in the cholesterol metabolism by shuttling CE from HDL particles to lipoproteins, mainly in exchange for TAG (Barter, 2000; Frayn, 2010; Marotti et al., 1993; Plump et al., 1999). One consequence of these CETP-mediated transfers of CE from HDL is the reduction in the cholesterol content in HDL and size of HDL particles (Rye, Clay, & Barter, 1999). Evidence suggests that partial or complete CETP deficiency has a powerful effect on raising HDL-C as well as lowering LDL-C, resulting in a reduced risk of CVD (Clark et al., 2004; Van der Steeg et al., 2004). However, no significant correlation between CETP and plasma TC, HDL-C or LDL-C was found in the present study.

In a previous study on the effect of AA, EPA and DHA on the expression of CETP in HepG2 cells, a decrease in CETP mRNA was found to be correlated with increases in the degree of unsaturation of the acyl carbon chain in FAs (Hirano, Igarashi, Kondo, Itakura, & Matsumoto, 2001). This is partially in accordance with the results of the present study, with n-3 having a high degree of unsaturation. Furthermore, several supplementary studies have investigated the effect of FAs on CETP. A recently published RCT study on healthy males showed that intake of flaxseed oil, which is rich in the n-3 FA ALA, lowered CETP concentrations (Kawakami et al., 2015). Satoh et al. showed that men with diabetes had decreased CETP activity after supplementation of EPA, whereas PUFA, especially n-3 FA, was negatively correlated with CETP activity in women with diabetes in another study (Satoh et al., 2007; Smaoui et al., 2006). This is consistent with the results from the present study, where CETP expression level was significantly negatively correlated with plasma n-3 level. The level of plasma n-3 FAs seems to have an impact on CETP activity both in healthy subjects and in subjects with

diabetes. A high plasma n-3 FA level may decrease the expression of CETP, whereas a high plasma n-6/n-3 ratio may increase the expression of CETP. The role of n-6 FAs remains unclear.

Over the last decade, great effort has been made to develop CETP inhibitors due to its inverse association with CVD risk (Chapman, Le Goff, Guerin, & Kontush, 2010; Charles & Kane, 2012; Clark, 2006; Clark, Ruggeri, Cunningham, & Bamberger, 2006; Miyares & Davis, 2012). A RCT study a CETP inhibitor was given to 15 067 subjects with increased CVD risk. The results showed a 72.1% increase in HDL-C and 24.9% decrease in LDL-C. However, the study was terminated due to a 25% increase in CVD events and 58% increase in deaths (P. J. Barter et al., 2007). Another RCT study showed a 138% increase in HLD-C and a 39.8% reduction in LDL-C, with no significant differences in adverse events found (Cannon et al., 2010). The first trials with the CETP inhibitors failed to reduce CVD events. However, new CETP inhibitors with more favourable effects on lipids are studied in ongoing clinical trials, and may give an answer to whether the CETP inhibitors may be effective for reduced CVD risk (Kosmas, DeJesus, Rosario, & Vittorio, 2016).

Results from the present study indicate that a high plasma n-3 level decrease the expression of CETP, which is associated with reduced CVD risk, whereas a high n-6/n-3 ratio may have the opposite effect by increasing the expression of CETP, which may increase the CVD risk (Figure 11). These findings indicate that plasma FAs is potential targets for regulating genes related to CVD risk, such as CETP.

The mRNA levels of PPARG was significantly different expressed between subjects with high and low plasma n-6/n-3 ratio. PPARG agonists modulate genes involved in the whole lipid metabolism, including genes involved in FA uptake (FABP4), lipid uptake (CD36) and cholesterol efflux (LXRα) (Chawla et al., 2001; Tontonoz & Spiegelman, 2008). In the present study, the PPARG mRNA level was higher in subjects with high plasma n-6/n-3 ratio compared with subjects with low plasma n-6/n-3 ratio. A previous study showed that intake of EPA increased the mRNA level of PPARG in adipose tissue, whereas LA, DHA and total n-6 had no effect (Chambrier et al., 2002). Huang et al. recently published a study suggesting that diets enriched with fish oil up-regulated PPARG in mice (Huang, Hou, Yeh, & Yeh, 2015). These differences may be ascribed to different organs as we have utilized PBMC in the current study. Collectively, plasma level of PUFA may modulate the expression of PPARG. However, the exact impact of plasma n-6 level, n-3 level or the n-6/n-3 ratio on the expression

of PPARG is currently not clear. Furthermore, the PPARG has been shown to have antiatherogenic properties in macrophages and adipocytes, having protective effects against atherosclerosis and CVD development (Duval, Chinetti, Trottein, Fruchart, & Staels, 2002; Ricote, Valledor, & Glass, 2004).

Results from the present study indicate that a high plasma n-6/n-3 ratio increase the expression of PPARG, which is associated with antiatherogenic properties and possible reduced CVD risk (Figure 11). This is in accordance with previous studies which found that a high plasma n-6 level or n-6/n-3 ratio does not increase the CVD risk, as previously believed (Griffin, 2008; Lucas, 2015).

5.1.2 Significantly regulated gene transcripts in subjects with high and low SFA/PUFA ratio

The plasma SFA/PUFA ratio was associated with the highest number of significantly different expressed genes. Seven genes were differently regulated between subjects with high and low plasma SFA/PUFA ratio. The FABPs are expressed in adipocytes and are suggested to be central regulators of lipid metabolism through their control of FA metabolism, transport and storage (Storch & Thumser, 2010). In the present study, subjects with high plasma SFA/PUFA ratio had higher FABP5 mRNA level compared to subjects with low plasma SFA/PUFA ratio. These results were supported by the correlation analysis, where significant positive correlation between FABP5 and the plasma SFA/PUFA ratio was found. Literature on how FABP5 is regulated by plasma FAs is scarce. However, in a previous study the preferable ligand for FABP5 was stearic acid, whereas unsaturation seems to reduce affinity (Hohoff, Börchers, Rüstow, Spener, & van Tilbeurgh, 1999).

FABP5 has been shown to be upregulated in several states with disturbed lipid profiles as well as being involved in the pathologies of atherosclerosis (Hertzel & Bernlohr, 2000; Hong et al., 2011; Makowski & Hotamisligil, 2004; Yeung et al., 2008). In addition, a clinical research showed that FABP5 is a new circulating biomarker associated with increased CVD risk, and may contribute to atherosclerosis in humans (Hertzel & Bernlohr, 2000; Hong et al., 2011; Makowski & Hotamisligil, 2004; Yeung et al., 2008). Results from the present study indicate that a high plasma SFA/PUFA ratio may increase the expression of FABP5, which is associated with increased atherosclerosis and CVD risk.

ACOT1 is thought to play an important role in lipid metabolism, and is primarily responsible for the metabolism of long chain acyl-CoAs (Brocker, Carpenter, Nebert, & Vasiliou, 2010; Dongol et al., 2007; Hunt, Solaas, Kase, & Alexson, 2002). In the present study, the mRNA level of ACOT1 was higher in subjects with high plasma SFA/PUFA ratio compared to subjects with low plasma SFA/PUFA ratio. This is in accordance with previous animal studies, where diets rich in SFA showed to increase both protein and mRNA levels of ACOT1 (Cole et al., 2011; Durgan et al., 2006; Fujita et al., 2011). The mRNA levels of ACOT1 were also found to be increased after supplementation with oleic acid (Durgan et al., 2006). In the present study, the oleic acid level was significantly higher in subjects with high plasma SFA/PUFA ratio compared to subjects with low plasma SFA/PUFA ratio. Whether the effect on ACOT1 mRNA level could be ascribed the SFA/PUFA ratio or the oleic acid level or both is therefore not known.

Increased ACOT1 expression may result in accumulation of acyl-CoAs, causing production of free radicals and mitochondrial stress, possibly increasing the risk of CVD development (Mitchell et al., 2008; Schnabel & Blankenberg, 2007). This is supported by the correlation analysis in the present study, where significantly positive correlation between ACOT1 and TAG level was found. This may be because elevated plasma TAG levels is associated with increased CVD risk (Lottenberg et al., 2012). The results from the present study indicate that a high plasma SFA/PUFA ratio may increase expression of ACOT1, which is associated with elevated TAG levels and possible increased CVD risk.

The PLIN5 gene suppresses lipolysis, in addition to regulating intracellular FA fluxes and most likely reprogramming cells to a more ”oxidative phenotype” capable of dealing with high lipid loads (Kimmel & Sztalryd, 2014; Mason & Watt, 2015). In the present study, the mRNA level of PLIN5 was lower in subjects with high plasma SFA/PUFA ratio compared to subjects with low plasma SFA/PUFA ratio. In addition, significant negative correlation between PLIN5 and the plasma SFA/PUFA ratio was found. These results may indicate that a high SFA/PUFA ratio lowers the mRNA and protein level of PLIN5 and thereby preventing the PLIN5 in regulating FA fluxes to deal with high lipid loads. A recently published study identified a PPRE in the PLIN5 gene, which is essential for FA stimulated expression. This may indicate that PLIN5 is as a direct PPAR target gene (Bindesbøll, Berg, Arntsen, Nebb, & Dalen, 2013). Findings from the previous- and the present study makes the PLIN5 gene an interesting target for future research, since it seems to be regulated by plasma FAs.

The effect of plasma FAs on PLIN5 and CVD risk remains unclear. However, a recently published study observed defects in hepatic lipid storage and increased lipolysis when PLIN5 was deficient in mice. The increased lipolysis resulted in elevated intracellular NEFAs, and proposed that PLIN5 is required to maintain lipid metabolism in the liver (Wang et al., 2015). Results from the present study indicate that a high plasma SFA/PUFA ratio may decrease the expression of PLIN5, which is associated with elevated NEFAs and possible increased CVD risk.

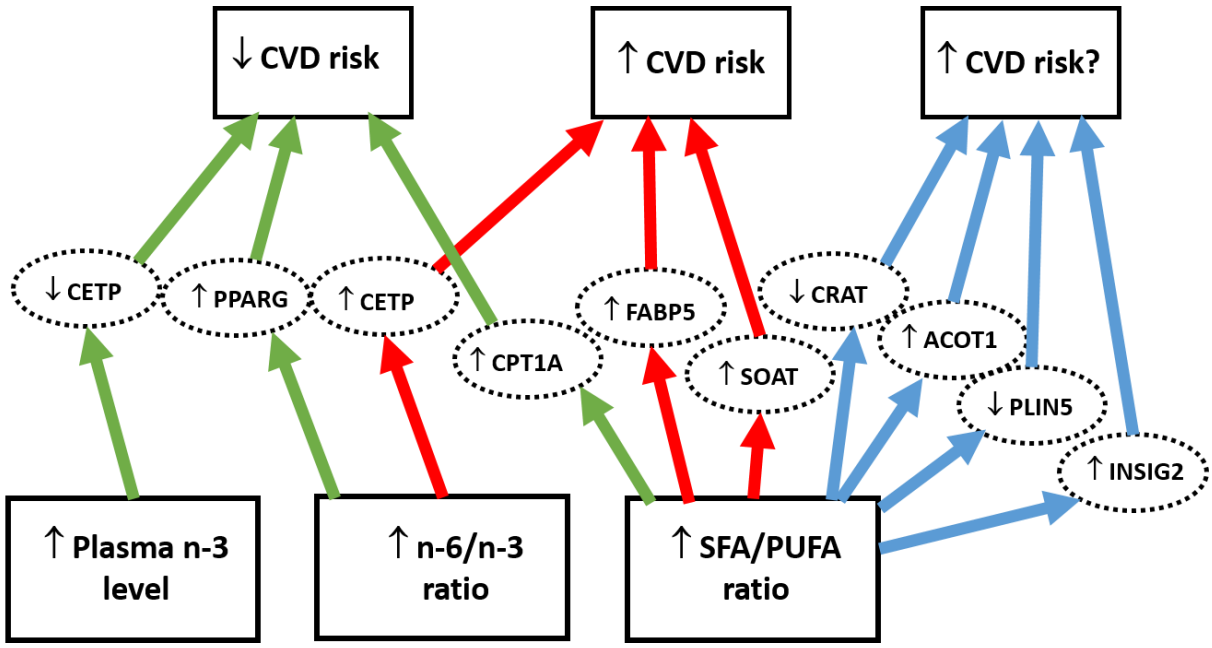


Figure 11. *Possible relations between significantly regulated genes and cardiovascular disease. The green arrows means there is a possible reduced CVD risk. The red arrows means there is a possible increased CVD risk. Blue arrows means there is a possible increased CVD risk, but the link between plasma FAs, gene and CVD risk remains unclear.*

CVD, cardiovascular disease; CETP, cholesteryl ester transfer protein; PPARG, peroxisome proliferator-activated receptor gamma; CPT1A, carnitine palmitoyltransferase 1A; FABP5, Fatty acid binding protein 5; SOAT1, sterol O-acyltransferase 1; CRAT, carnitine O-acetyltransferase; ACOT1, acyl-CoA thioesterase 1; PLIN5: perilipin 5; INSIG2, insulin induced gene 2.

Total n-3: ALA, EPA, DPA, DHA; n-6: LA, ALA; SFA: myristic acid, palmitic acid, stearic acid; PUFA: ALA, EPA, DPA, DHA, LA and AA.

CPT1A is found in the liver, and is essential for β-oxidation in the mitochondria (McGarry et al., 1991). In the present study, mRNA level of CPT1A was significantly higher in subjects with high plasma SFA/PUFA ratio compared to subjects with low plasma SFA/PUFA ratio. Several previous studies have shown that ALA, DHA and fish oil increase mRNA and protein levels of CPT1A in HepG2 cells, adipocytes and PBMCs (Bouchard-Mercier, Rudkowska, Lemieux, Couture, & Vohl, 2014; Fukumitsu et al., 2013; Popeijus et al., 2014; Zhou, Wu,

Chen, Wang, & Wang, 2015). This is contrary to results from the present study, and may be due to differences is study population and design. Additionally, none of the previous studies investigated the effect of the SFA/PUFA ratio on the gene expression level.

Several studies have shown that increased CPT1A activity is associated with an elevated level of HDL-C, reduced adiposity and reduction in TAG level, resulting in reduced CVD risk (Bouchard-Mercier et al., 2014; Lemas et al., 2012; Rajakumar et al., 2009). In the present study, a high SFA/PUFA ratio increased the expression of CPT1A, which is associated with possible reduced CVD risk.

Another differently regulated gene related to β-oxidation was CRAT. In addition to buffer mitochondrial acetyl-CoA pool, CRAT regenerates free CoA (Seiler et al., 2014). In the present study, the mRNA level of CRAT was significantly lower in subjects with high plasma SFA/PUFA ratio compared to subjects with low plasma SFA/PUFA ratio. These results were supported by the correlation analysis, where significant negative correlation between CRAT and plasma SFA/PUFA ratio was found. There is a limited number of studies published on the effect of FAs and regulation of CRAT. One study showed that chronic overexposure of a 1:1 mixture of oleic and palmitic acid restricted the availability of CRAT (Seiler et al., 2014). This is consistent with results in the present study, which show a lower mRNA level in subjects with high plasma SFA/PUFA ratio compared to subjects with low plasma SFA/PUFA ratio. Palmitic acid is included in the ratio, and this may indicate that SFA not only antagonizes CRAT activity, but also reduces its mRNA level. The biological relevance of this finding needs to be investigated.

A previous study suggested that chronic inhibition of CRAT in the context of overnutrition may contribute to glucose intolerance, which has shown to increase CVD risk (Ford, Zhao, & Li, 2010; Seiler et al., 2014). Although the previous study did not establish a direct link between reduced CRAT activity and CVD risk, findings in the present study indicate that high plasma SFA/PUFA ratio may increase the expression of CRAT, which is associated with glucose intolerance and a possible increased CVD risk.

In the present study, the mRNA level of SOAT1 was significantly higher in subjects with high plasma SFA/PUFA ratio compared to subjects with low plasma SFA/PUFA ratio. In addition, positive correlation between SOAT1 and the plasma SFA/PUFA ratio was found. SOAT1 is the enzyme that catalyses the conversion of cholesterol to CE, and is allosterically regulated

by cholesterol. Accumulation of CE in macrophages causes these cells to appear foamy, and is an indication of early stages in atherosclerosis (Chang et al., 2009). Previous studies have shown that n-3 intake decreases SOAT1 expression in dyslipidemic men and breast cancer cells, suggesting a cholesterol-lowering effect (Antalis, Arnold, Lee, Buhman, & Siddiqui, 2009; Schmidt et al., 2012). This is consistent with results from the present study, where high SFA/PUFA ratio was found to increase the expression of SOAT1. This may indicate that high plasma SFA/PUFA ratio increases cholesterol levels.

Inhibition of SOAT1 is shown to directly or indirectly diminish foamy macrophage formation, thus reducing incidents of atherosclerotic CVD (Chang et al., 2009). Several studies support the concept that upregulation of SOAT1 expression is closely associated with the beginning and progression of atherosclerosis in animals and perhaps in humans as well (Daugherty, Webb, Rateri, & King, 2005; Furukawa et al., 2004; Kalbak, 1972; Li et al., 2004; Yang et al., 2001). The results from the present study indicate that a high plasma SFA/PUFA ratio may increase the expression of SOAT1 which is associated with development of atherosclerosis and possible increased CVD risk.

Another significantly different expressed gene related to cholesterol metabolism was INSIG2, which plays a key role in cholesterol homeostasis (Engelking et al., 2005). The INSIG2 binds to HMG-CoA reductase, thereby inhibiting SREBP activity, which prevents transcription of genes involved in cholesterol uptake and synthesis (Radhakrishnan, Ikeda, Kwon, Brown, & Goldstein, 2007; Sever et al., 2003; Yabe, Brown, & Goldstein, 2002). In the present study, the mRNA level of INSIG2 was higher in subjects with high plasma SFA/PUFA ratio compared to subjects with low plasma SFA/PUFA ratio. There is a limited amount of literature on how plasma FAs regulate expression of INSIG2. However, a recent transcriptomic study published in 2016 showed that intake of fish oil corrected the increased expression of INSIG2 caused by feeding rats a high fat and cholesterol diet (Yuan et al., 2016). This is in accordance with the present study, as the total PUFA group contains several of the same FAs as fish oil. No studies on the direct link between plasma FAs effect on INSIG2 and CVD risk were found. However, INSIG2 has been associated with obesity and peripheral arterial disease. Peripheral arterial disease and CVD are both results of the atherosclerotic process (Engelking et al., 2005). Based on results from previous studies and the present study, a high plasma SFA/PUFA ratio may increase the expression of INSIG2, which is associated with obesity and peripheral disease, and potentially increased CVD risk.

Results in the present study indicate that a high plasma SFA/PUFA ratio may regulate expression of genes associated with reduced CVD risk (CPT1A), increased CVD risk (FABP5, SOAT1) and genes potentially associated with increased CVD risk, but where the link has not yet been fully established (CRAT, ACOT1, PLIN5, INSIG2). (Bouchard-Mercier et al., 2014; Daugherty et al., 2005; Hertzel & Bernlohr, 2000) (Figure 11).

5.1.3 Differences in plasma FA level and ratio across subjects and reference groups

In the present study, the mean plasma n-6/n-3 ratio for the whole study population was 6.6. This is in accordance with a similar study investigating plasma FA ratios and blood gene expression in Norwegian women, where the mean plasma n-6/n-3 ratio was 6.1 (Olsen et al., 2013). According to NNR 2004 the n-6/n-3 ratio in the Norwegian population was estimated 5.5, and considered healthy (NCM, 2004). A typical western diet is estimated to be between 15/1 and 20/1, and is associated with increased CVD risk (Hibbeln, Nieminen, Blasbalg, Riggs, & Lands, 2006; A. Simopoulos, 2006; Wall et al., 2010). The plasma n-6/n-3 ratio in the present study may be due to the use of healthy subjects with a normal diet.

Subjects with a high plasma n-6/n-3 ratio had a mean ratio of 10.5, whereas subjects with low plasma n-6/n-3 had a mean ratio of 3.6. Subjects with a high plasma SFA/PUFA ratio had a mean ratio of 0.9, whereas subjects with high SFA/PUFA ratio had a mean ratio of 0.6. Although the differences between subjects in high and low plasma SFA/PUFA ratio were small compared to the n-6/n-3 ratio, most of the significant findings in the present study were related to the plasma SFA/PUFA ratio. This indicates that small changes in the ratio of SFA to PUFA may have significant biological impact.

Furthermore, TAG level was significant higher in subjects with high SFA/PUFA ratio compared to subjects with low SFA/PUFA ratio. This is however not surprising, as high SFA intake is shown to increase plasma TAG levels (Frayn, 2010; Lottenberg et al., 2012). The oleic acid level was higher in subjects with high compared to low plasma n-6/n-3 and SFA/PUFA ratio and lower in subjects with high compared to low plasma n-3 levels. These differences indicate that other plasma FAs, such as oleic acid may have affected the results, and that the results do not necessarily relate only to plasma n-3 level, n-6/n-3 and SFA/PUFA ratios.

5.2 Discussion of methods

5.2.1 Study design and subjects

The data used in the present study was from an RCT originally designed to investigate health effects after intake of fish oil (Ottestad et al., 2012). Data after end of intervention was used in this cross-sectional study. As this is an explorative, cross-sectional study, no conclusions on causality can necessarily be drawn (Laake, Olsen, & Benestad, 2008). However, the result can be useful at identifying associations which can then be more rigorously studied using RCT and cohort studies (Mann, 2003).

A total of 54 healthy subjects were included in this follow-up study. Subjects in the present study were arranged into groups based on levels of plasma n-3, n-6/n-3 and SFA/PUFA ratio. The level of plasma n-3, n-6 FAs or SFA may vary between subjects within the group. Due to this, the study shows the relationship between the overall plasma levels or ratios and not the specific FAs. Furthermore, when using ratios it is a possibility that extremely high or low intake of plasma FAs are disguised (Harris, 2006). An example is that you can have a low intake of fish oil, but still have a decent n-6/n-3 ratio (Olsen et al., 2013).

By using healthy subjects, it is desirable to generalize to the healthy population, which is a major proportion of the overall population. The relatively large age span also increases the generalizability. However, in order to generalize to the population the sample size is of importance. In the present study, the external validity is relatively low due to strict inclusion criteria's and few participants. On the other side, because of the exploratory design, the aim is not to generalize the conclusion, but rather enlighten the topic for future research.

Fat intake in the Norwegian diet is measured as % of daily energy intake, whereas plasma FA was measured as % of total plasma fat in the current study (NCM, 2014). It is therefore not possible to directly compare plasma FA levels in the present study with the Norwegian population. However, the subjects in the present study are healthy with normal serum lipids, and their health could thus be comparable to the Norwegian population, given that the Norwegian population are healthy also. The data of FAs used to calculate the FA level and ratio groups was measured from plasma. This is a more accurate measurement method than reported energy intake, which is often used in studies investigating the exposure to different types of FAs. The human body can synthesize FAs, except from the essential FAs, which makes plasma measurements more accurate compared to reported dietary intake (Frayn,

2010). In addition, reported FA intake do not reflect interindividual differences in metabolism, genetic variations and absorption leading to differences in circulating FA concentrations (Sun, Ma, Campos, Hankinson, & Hu, 2007). Bias in form of misreporting intake is also avoided (Cantwell, 2000).

PBMCs were used as a modelling system to study expression of genes in relation to plasma FAs in the present study. The PBMCs are exposed to many of the same environmental factors as the arterial wall. They are easy available, which makes them suitable for studying gene expression (Pasterkamp & Daemen, 2008). mRNA expression of genes related to lipid metabolism is shown to be regulated by dietary factors in several studies using PBMCs as a modelling system (Boucher et al., 2000; Bouwens et al., 2010; Bouwens et al., 2009; Radler et al., 2011). A challenge when studying gene expressions is the individual variations in response to nutritional intervention. This may be due to sex, age and genotype differences (Bouwens, Afman, & Müller, 2008). These individual variations make it difficult to detect changes in gene expression, and may have prevented some findings in this study. That there were few significant results is not surprising as it is also evident that changes in gene expression induced by nutrients are relatively small compared to e.g. drugs. However, the small changes may lead to development of disease over time (Afman & Müller, 2012; Sanderson et al., 2008).

5.2.2 Statistical analysis

In the present study, both parametric and nonparametric tests were used in the present study. Parametric tests are more powerful than nonparametric tests, and increase the power of the statistical analysis (Pallant, 2013). For comparisons of two groups in the test for differences of the subjects, *independent samples t-test* were performed when data were normally distributed, whereas *Mann-Whitney U test* was performed on the non-normally distributed variables. The genes were log2-transformed, considered normally distributed, and ready for use in parametric tests. *Independent samples t-test* were used in the gene expression analysis, and since this is a parametric test, the power of the analysis is higher compared to nonparametric tests (Pallant, 2013). *Spearman's Rho* was used in the correlation analyses, and since this is a nonparametric test, it has a lower statistical power compared to a parametric test. Other factors like sample size also influence the power of the tests, and the few participants weaken the power.

It was considered to do multiple regression analysis, but this was not conducted due to the small sampling size and type of study. Multiple regression can be used to tell how well a set of variables is able to predict an outcome, in addition to control for additional variables when examining the predictive ability of the individual subscale (Pallant, 2013). It is therefore not possible to rule out that confounding variables has affected the results in the current study. Confounding is when outsider variables correlates with both the independent and dependent variable in the statistical test (Laake et al., 2008).

The variables are not independent, which makes it difficult to correct for multiple testing. Multiple testing is any instance where several tests are conducted at once. When decision about an individual hypothesis are based on an unadjusted p-value, there is a chance that some of the null hypothesis will be rejected (Romano & Wolf, 2007; Veazie, 2006). When testing 43 genes and nine are significantly different regulated, two of them will be false positive. It would be possible to adjust the statistical confidence measures by using e.g. the Bonferroni adjustment, which considers the number of tests performed (Noble, 2009). However, the Bonferroni-adjustment is conservative and may diminish power by setting strict significance levels (Veazie, 2006). Since this is an explorative study, it was preferred get some false positive rather than to reject them.

6 Conclusion

The results in the present study indicates that plasma FA level and ratios may modulate gene expressions and thereby influence the CVD risk. A lower expression level of CETP was found in subjects with high plasma n-3 level and low n-6/n-3 ratio. Inhibition of this gene has been suggested in research to reduce CVD incidents. Subjects with a high plasma n-6/n-3 ratio had increased expression of PPARG, which is associated with reduced CVD risk. A high SFA/PUFA ratio modulated the expression of several genes associated with increased CVD risk, such as ACOT1, PLIN5, SOAT1 and FABP. Larger studies are however warranted to further establish the relationship and causality between plasma FAs and expression of genes related to lipid metabolism and CVD risk.

7 Literature

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Appendix 1. Approval from the Regional Committee of Medical Ethics.

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DET MEDISINSKE FAKULTET

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Dato: 05.12.08
Deres ref.:
Vår ref.: **S-08764c 2008/20195** (oppgis ved henvendelse)

Helseeffekter av marine omega-3 fettsyrer fra fiskeolje med ulik kvalitet - Forskningsbiobank nr 2592

Komiteen behandlet søknaden 20.11.2008. Prosjektet er vurdert etter lov om behandling av etikk og redelighet i forskning av 30. juni 2006, jfr. Kunnskapsdepartementets forskrift av 8. juni 2007 og retningslinjer av 27. juni 2007 for de regionale komiteer for medisinsk og helsefaglig forskningsetikk.

Søknaden gjelder en randomisert kontrollert hoststudie med parallell design. Hensikten med studien er å belyse hvilken betydning kvaliteten på fiskeolje rik på EPA og DHA har for helse, bl.a. ved å måle markører for inflammasjon, antioksidantstatus og oksidativ stress, genekspresjonsprofil og modulering av lipider. Markørene kan være til hjelp for å identifisere de molekylære mekanismer bak de helsegunstige effektene av omega-3. I studien vil det bli undersøkt hvorvidt fiskeolje, som er oksidert til et nivå med hva man finner i kosttilskuddsmarkeder på det norske markedet, påvirker markører for helse på samme måte som den samme fiskeoljen av god kvalitet.

Komiteen har ingen innvendinger mot at prosjektet gjennomføres, men ber om at følgende bemerkninger til informasjonsskjema til deltakere etterkommes: Det opplyses i søknad om biobank at analyseresultatene vil bli oppbevart avidentifisert til 2022. Denne informasjonen fremkommer ikke i informasjonsskrivet under delkapittel *Hva skjer med prøvene og informasjonen om deg*. Det bes om at informasjonen inkluderes i dette delkapitelet.

Det opplyses heller ikke hvorvidt informasjonen som samles inn har kommersielt utnyttelsespotensial i informasjonsskrivet. Det bes derfor om at det redegjøres for dette i informasjonsskrivet under samme delkapittel.

Vedtak
Komiteen godkjenner prosjektet under forutsetning av at de merknadene til informasjonsskrivet som er anført blir innarbeidet før prosjektet settes i gang. Revidert informasjonsskriv bes oversendt til orientering.

Søknad om opprettelse av forskningsbiobank, informasjonsskriv samt komiteens vedtak vil bli oversendt til Helsedirektoratet for endelig behandling.

Komiteens avgjørelse var enstemmig.