Use of transcriptomics to explore the role of dietary fat on cardiovascular disease risk

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Oslo, May 2022
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Summary

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality world-wide. Preventive treatment that reduces CVD risk can substantially reduce the number of people developing CVD, as well as the economic burden on the society. Lifestyle changes, including dietary modification, is a key strategy in CVD prevention. In particular, improving the dietary fat quality by reducing saturated fatty acids (SFA) and increasing polyunsaturated fatty acids (PUFA) is associated with reduced CVD risk. This effect is largely mediated through lowering of serum low-density lipoprotein (LDL) cholesterol, which is a well-characterized CVD risk factor. Intake of n-3 PUFAs may also lower serum triglycerides (TG), and is proposed to have anti-inflammatory effects. However, despite many years of research, we yet do not fully understand the molecular mechanisms that link dietary fatty acids and such physiological effects. Also, studies show inter-individual variation in TG response to n-3 PUFA intake which should be further investigated. The overall aim of this PhD project was therefore to explore the molecular mechanisms underlying the effect of dietary fat quality on CVD risk by use of transcriptome profiling of peripheral blood mononuclear cells (PBMC) in human dietary intervention studies. More specifically, we investigated: 1) associations between plasma fatty acid levels and gene expression related to lipid metabolism in healthy subjects, 2) differences in gene expression profiles and pathways between TG responders and non-responders to n-3 PUFA supplementation, and 3) effects of replacing dietary SFAs with PUFAs on gene expression profiles and pathways in subjects with moderate hypercholesterolemia.

We used data and material from two randomized controlled dietary intervention studies. Study 1 was designed to investigate effects of fish oils (1.6 g/day n-3 PUFAs) with different quality on several selected CVD risk markers. In this study, healthy subjects were randomly assigned into one of three intervention groups receiving either high-quality fish oil (*n* = 17), oxidized fish oil (*n* = 18) or high-oleic sunflower oil (*n* = 19) for seven weeks. Study 2 was designed to investigate effects of replacing dietary SFAs with PUFAs on serum cholesterol levels and inflammatory markers. In this study, subjects with moderate hypercholesterolemia were randomly assigned into one of two intervention groups receiving either a control diet (C-diet, *n* = 49) or an experimental diet (Ex-diet, *n* = 43) with an improved fatty acid composition (SFAs were replaced by mostly n-6 PUFAs) for eight

weeks. The dietary difference during the intervention was 6.5 energy % (E%) lower intake of SFAs and 6.4 E% higher intake of PUFAs in the Ex-diet group compared to the C-diet group. Pre-registered secondary outcomes in both studies included transcriptome profiling of PBMCs by use of microarray technology.

In paper I and II (study 1) in the current thesis, we used an exploratory approach and regrouped the study subjects based on respectively plasma fatty acid levels at end of study (paper I) and TG response to n-3 PUFA supplementation during the intervention (paper II). In paper I, we found that particularly plasma SFA to PUFA ratio was associated with differences in gene expression, including gene transcripts related to cholesterol homeostasis, using a cross-sectional design. Further, in paper II, we found that TG responders and non-responders to n-3 PUFA supplementation had different baseline gene expression profiles and differentially altered gene expression profiles after seven weeks of intervention, which may partly explain the variation in TG response to n-3 PUFA intake. Finally, using a whole transcriptome approach in paper III (study 2), we found that gene expression associated with processes related to CVD risk were favorably altered in the Ex-diet group compared to the C-diet group. Moreover, pathways particularly related to immune response were differentially modulated between the groups. These novel findings may offer new mechanistic insight regarding the effect of dietary fat quality on CVD risk, and should be further investigated in future studies.

In conclusion, our findings support the hypothesis that dietary fat quality is associated with PBMC gene expression and pathways, which may contribute to extend our understanding of how dietary fat affects CVD risk. Hence, use of transcriptomics in human dietary intervention studies may be a useful approach to explore the role of dietary fat on CVD risk at a molecular level. Furthermore, this approach may also extend our understanding of why individuals respond differently to n-3 PUFA intake.

Sammendrag

Hjerte- og karsykdom (HKS) er den ledende årsaken til sykdom og dødelighet på verdensbasis. Forebyggende tiltak som reduserer risiko for HKS kan bidra til at færre mennesker utvikler sykdom, og at kostnader knyttet til behandling av denne sykdomsgruppen går ned. Livsstilsendring som inkluderer å legge om kosten til et mer hjertevennlig kosthold er sentralt i forebygging av HKS. Flere studier har vist at å redusere inntaket av mettede fettsyrer (SFA) og samtidig øke inntaket av flerumettede fettsyrer (PUFA) reduserer risikoen for HKS. Denne effekten skyldes i stor grad reduksjon av serum LDL-kolesterol, som er en vel etablert risikofaktor for HKS. Inntak av n-3 PUFAs kan også redusere serum triglyserider (TG), og er vist å kunne ha en anti-inflammatorisk effekt. Til tross for at det i mange år har vært forsket på sammenhengen mellom fett i kosten og fysiologiske effekter, er de underliggende molekylære mekanismene mindre kjent. Studier viser også at det er interindividuell variasjon i serum TG respons på n-3 PUFA inntak som bør bli videre undersøkt. Hovedformålet i dette PhD-prosjektet var derfor å undersøke de molekylære mekanismene bak effekten av fettkvalitet i kosten på HKS risiko ved bruk av heltranskriptom data fra mononukleære celler i perifert blod i humane kostintervensjonsstudier. Mer spesifikt ønsket vi å undersøke: 1) sammenhengen mellom plasma fettsyrenivåer og genuttrykk knyttet til lipidmetabolisme i friske individer, 2) forskjeller i genekspresjonsprofil og signalveier hos TG respondere sammenlignet med ikke-respondere på n-3 PUFA inntak, og 3) effekten av å bytte ut SFAs med PUFAs i kosten på endring i genekspresjonsprofil og signalveier hos individer med moderat hyperkolesterolemi.

I dette prosjektet har vi brukt data og materiale fra to randomiserte kontrollerte kostintervensjonsstudier. Studie 1 var designet for å undersøke effekter av fiskeolje (1.6 g/d n-3 PUFAs) med ulik kvalitet på ulike risikomarkører for HKS. I denne studien ble friske individer randomisert til en av tre intervensjonsgrupper som enten fikk høykvalitets fiskeolje (*n* = 17), oksidert fiskeolje (*n* = 18) eller solsikkeolje (*n* = 19) i 7 uker. Studie 2 var designet for å undersøke effekter av å bytte ut SFAs med PUFAs i kosten på serum kolesterolnivåer og ulike inflammasjonsmarkører. I denne studien ble individer med moderat hyperkolesterolemi randomisert til en av to intervensjonsgrupper som enten fikk en kontroll-diett (*n* = 49) eller en eksperimentell diett (*n* = 43) med en forbedret fettkvalitet

(SFAs ble byttet ut med hovedsakelig n-6 PUFAs) i 8 uker. Forskjellen mellom de to gruppene var 6.5 energiprosent (E%) lavere inntak av SFAs og 6.4 E% høyere inntak av PUFAs i den eksperimentelle gruppen sammenlignet med kontroll-gruppen. Pre-registrerte sekundære utfallsmål i begge studiene inkluderte heltranskriptom analyser av mononukleære celler i perifert blod ved bruk av microarray teknologi.

I artikkel I og II (studie 1) i denne avhandlingen brukte vi en eksploratorisk tilnærming ved å gruppere deltakerne i nye grupper basert på henholdsvis plasma fettsyrenivåer etter 7 uker (artikkel I) og TG respons på n-3 PUFA inntak etter gjennomført intervensjon (artikkel II). I artikkel I fant vi at spesielt plasma SFA/PUFA ratio var assosiert med forskjeller i genuttrykk, inkludert genuttrykk knyttet til kolesterol homeostase, ved bruk av tverrsnittsdata. Videre fant vi i artikkel II at TG respondere og ikke-respondere på n-3 PUFA inntak hadde forskjellig baseline genekspresjonsprofil og forskjellig endret genekspresjonsprofil og signalveier etter 7 uker, som kan bidra til å forklare noe av variasjonen i TG respons på n-3 PUFA inntak. Til slutt fant vi ved bruk av heltranskriptom data i artikkel III (studie 2) at genuttrykk assosiert med prosesser relatert til HKS risiko var gunstig endret og at signalveier relatert til spesielt immunrespons var forskjellig modulert i den eksperimentelle gruppen sammenlignet med kontroll-gruppen etter endt intervensjon. Disse funnene kan bidra med ny mekanistisk innsikt vedrørende effekten av fettkvalitet i kosten på HKS risiko, og bør bli videre undersøkt i andre studier.

For å oppsummere fant vi i denne avhandlingen at fettkvalitet i kosten er assosiert med genuttrykk og signalveier som kan bidra til å utvide vår forståelse for hvordan fett i kosten påvirker HKS risiko. Bruk av heltranskriptom data fra mononukleære celler i perifert blod i humane kostintervensjonsstudier kan derfor være en nyttig tilnærming for å undersøke molekylære mekanismer bak effekten av fett i kosten på HKS risiko. Videre kan en slik tilnærming også kunne gi oss bedre forståelse for hvorfor individer responderer ulikt på n-3 PUFA inntak.

Scientific papers

Paper I

Larsen SV, Holven KB, Ottestad I, Dagsland KN, Myhrstad MC and Ulven SM. *Plasma fatty acid levels and gene expression related to lipid metabolism in peripheral blood mononuclear cells: a cross-sectional study in healthy subjects.* Genes nutr. 2018 Apr 10;13:9

Paper II

Rundblad A, Larsen SV, Myhrstad MC, Ottestad I, Thoresen M, Holven KB and Ulven SM. *Differences in peripheral blood mononuclear cell gene expression and triglyceride composition in lipoprotein subclasses in plasma triglyceride responders and non-responders to omega-3 supplementation.* Genes nutr. 2019 Apr 25;14:10

Paper III

Larsen SV, Holven KB, Christensen JJ, Flatberg A, Rundblad A, Leder L, Blomhoff R, Telle-Hansen VH, Kolehmainen M, Carlberg C, Myhrstad MC, Thoresen M and Ulven SM. *Replacing saturated fat with polyunsaturated fat modulates peripheral blood mononuclear cell gene expression and pathways related to cardiovascular disease risk using a whole transcriptome approach*. Mol nutr food res. 2021 Dec;65(24)

Abbreviations

AA	arachidonic acid
ABCA1	ATP binding cassette A1
ABCA2	ATP binding cassette subfamily A member 2
ABCG1	ATP binding cassette G1
ALA	α-linolenic acid
apo	apolipoprotein
BMI	body mass index
C-diet	control diet
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
COX	cyclooxygenase
CVD	cardiovascular disease
DHA	docosahexaenoic acid
DNAJC10	DnaJ heat shock protein family (Hsp40) member C10
E%	energy percent
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ERLIN2	ER lipid raft associated 2
Ex-diet	experimental diet
FDR	false discovery rate
FGF18	fibroblast growth factor 18
GEO	Gene Expression Omnibus
GPR	G protein-coupled receptor
GUCY1B1	guanylate cyclase 1 soluble subunit beta 1
GWAS	genome-wide association study
HDL	high-density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HOSO	high-oleic sunflower oil
hsCRP	high-sensitivity C-reactive protein

IL	interleukin
INSIG2	insulin induced gene 2
IκB	inhibitor of κB
IκK	IκB kinase
LA	linoleic acid
LCAT	lecithin-cholesterol acyl transferase
LDL	low-density lipoprotein
LDLR	LDL receptor
LPA	lysophosphatidic acid
LPAR	LPA receptor
LPL	lipoprotein lipase
LXR	liver X receptor
MI	myocardial infarction
MUFA	monounsaturated fatty acid
NF-κB	nuclear factor kappa B
NMR	nuclear magnetic resonance
NNR	Nordic nutrition recommendations
PBMC	peripheral blood mononuclear cell
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
RCT	randomized controlled trial
RIN	RNA integrity number
RNA-seq	RNA sequencing
RT-qPCR	reverse transcription quantitative polymerase chain reaction
RXR	retinoid X receptor
SCAP	SREBP cleavage-activating protein
SEPTIN4	septin 4
SFA	saturated fatty acid
SRB1	scavenger receptor B1
SREBP	sterol regulatory element-binding protein
TG	triglyceride

TLR	toll-like receptor
TNF	tumor necrosis factor
TRL	TG-rich lipoprotein
VLDL	very low-density lipoprotein
VSMC	vascular smooth muscle cell
WHO	World Health Organization

Table of contents

1 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity worldwide [1]. Preventive treatment that reduces CVD risk can substantially reduce the number of people developing CVD, as well as the economic burden on the society. Lifestyle changes, including dietary modification, is a key strategy in prevention of CVD. In particular, improving the dietary fat quality is associated with reduced CVD risk [2-5], although the molecular mechanisms underlying this effect are not fully understood. One approach to further elucidate the effects of dietary fat on CVD risk at a molecular level is by use of transcriptomics. The following chapters focus on CVD, dietary fat, and use of transcriptomics in human dietary intervention studies.

1.1 Cardiovascular disease

According to the World Health Organization (WHO), CVD comprise diseases of the major arteries including coronary heart diseases (CHD) and cerebrovascular diseases such as stroke [6]. Common to several of these conditions is the stenosis of major arteries supplying blood to the heart muscle or the brain, as a result of atherosclerotic lesions, leading to obstruction, ischemia and ultimately occlusion with subsequent necrosis of the supplied tissues (infarction). Heart disease due to obstruction of the epicardial coronary blood vessels with impaired supply of oxygen, is commonly termed ischemic heart disease and includes angina pectoris and acute myocardial infarction (MI).

CVD is a major concern for both the affected individuals and the society, and accounts for more than 18 million global deaths annually [1]. Over the past decades, preventive strategies and improved treatment have led to a decline in CVD burden in many countries [7, 8]. In Norway, reports show that the incidence of acute MI continuous to decline, and now also involves individuals aged 25-44 years [9]. However, according to the Norwegian Institute of Health, more than 1/5 of the Norwegian population is living with established CVD or increased risk of CVD [10].

As first reported in the Framingham Heart and Seven Countries cohorts in the mid last century, CVD is highly responsive to lifestyle and therefore represents a preventable

disease [11, 12]. In these studies, three major behavioral CVD risk factors were identified; high serum total cholesterol, hypertension and smoking. Today these risk factors remain the most important modifiable risk factors of CVD, in addition to dyslipidemia, diabetes, abdominal obesity, psychosocial factors, consumption of fruits and vegetables, regular alcohol consumption, and physical inactivity [13]. In the INTERHEART study, a global case-control study including subjects from 52 different countries, the above-mentioned risk factors were associated with more than 90 % of the risk of an acute MI, consistent across geographic region and ethnic groups of the world, sex, and age [13]. This indicates that modification of currently known risk factors has the potential to prevent most premature cases of MI worldwide.

1.2 Atherosclerosis

The most common etiology of CVD is atherosclerosis. This is a life-long, progressive process within the arteries, initiated by accumulation of cholesterol rich apolipoprotein (apo) B-containing lipoproteins in the intimal space, followed by a non-resolving low-grade inflammation [14]. ApoB-lipoproteins, like low-density lipoproteins (LDL) and triglyceride (TG)-rich lipoprotein (TRL) remnants normally fluctuate in and out of the arterial wall, with small particles passing more easily through. In the intima, some apoB-lipoproteins will be retained by proteoglycans and modified in a manner that increases their retention and aggregation [15]. Oxidized lipoproteins activate endothelial cells that induce the expression of adhesion molecules and chemokines leading to immune cell migration into the intima. In a fatty streak, monocytes differentiate into macrophages that engulf aggregated apoB-lipoproteins and become foam cells that sustain and aggravate the inflammatory response of the atherosclerotic lesion [16]. In addition, smooth muscle cells migrate from the media to the intima, and together with extracellular proteins they form a fibrous cap that covers the lipid core of the lesion [17]. Over time, driven by the continuous influx of lipids, the sub-endothelial fatty streak develops into a complex lesion and further to an advanced plaque that protrudes into the lumen of the vessel and results in lumen occlusion and distal tissue hypoxia. Ultimately, unless the arterial wall can properly adapt, the plaque can rupture, causing blood clot formation, arterial clogging and infarction [14].

1.3 Diet and CVD risk

According to the Global Burden of Disease Project, an unhealthy diet is considered to be among the most important contributors to years of life lost due to premature cardiovascular deaths [18]. This has been supported by several studies investigating the effect of diet on CVD risk. In the Seven Countries study, Keys et al. found that populations in different countries had widely diverse incidence and mortality rates from CHD, as well as other CVDs and overall mortality, and that these differences were strongly associated with saturated fatty acid (SFA) intake and average serum cholesterol levels [19, 20]. The Oslo study, performed in the 1970s, was one of the first randomized controlled trials (RCT) that aimed at preventing CHD by lowering serum lipids through improving lifestyle. This study included healthy men who were randomized to a control diet group or an intervention diet group receiving advice to eat food with less SFAs and more polyunsaturated fatty acids (PUFA), low fat meat, fish, fiber rich bread, fruit, vegetables, and less sugar and alcohol, and stop smoking. After five years of intervention, serum cholesterol level was reduced by 13 % and the incidence of fatal and non-fatal MI and sudden death was reduced by 47 % in the intervention group compared to the control group [21]. Importantly, after 40 years follow up, there was a reduced risk of coronary mortality in the intervention group, indicating that the five-year intervention provided a lifelong benefit [22]. More recently, RCTs investigating the effect of a Mediterranean diet, a diet rich in whole grain, vegetables, fruits, nuts, olive oil and fish, have found a beneficial effect on both primary and secondary CVD prevention [23, 24]. Similarly, cohort studies have found that a micronutrient- and fiber-dense, unprocessed whole food based, healthy Nordic diet associates with lower all-cause and CVD mortality [25, 26].

1.4 Dietary fat and CVD risk

TG consisting of one molecule of glycerol esterified with three fatty acid molecules are the main contributors of fat in the human diet. Fatty acids are hydrocarbon chains with a methyl group at one end and a carboxyl group at the other end (Figure 1). The carbon atom at the carboxylic group is called the alpha-carbon and the carbon atom at the methyl group is

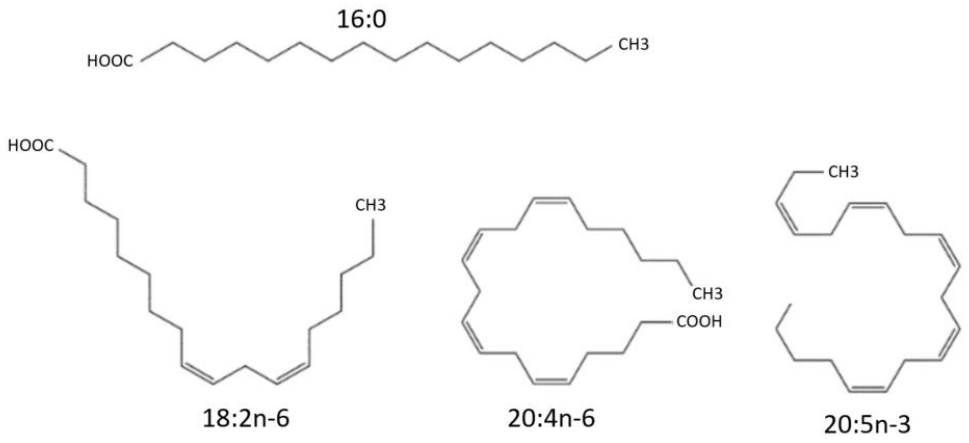

Figure 1. Structure of some common dietary fatty acids. Arachidonic acid (20:4n-6); eicosapentaenoic acid (20:5n-3); linoleic acid (18:2n-6); palmitic acid (16:0).

called the omega-carbon. The fatty acids in the diet are mainly long chain fatty acids, which have at least 16 carbon atoms in their hydrocarbon chain. Depending on the number and position of double bonds between the carbon atoms, fatty acids can be classified into SFAs which have no double bonds and are thus saturated with hydrogen atoms, monounsaturated fatty acids (MUFA) which contain one double bond, and PUFAs which contain two or more double bonds (Figure 1) [27]. There are two major classes of PUFAs, n-3 and n-6 PUFAs, in which the first double bond occupies the third or the sixth position from the omega-end of the fatty acid, respectively. The n-6 PUFA linoleic acid (LA, 18:2n-6) and the n-3 PUFA α-linolenic acid (ALA, 18:3n-3) are essential PUFAs since they are fundamental for the organism and cannot be synthesized in humans. Endogenously, LA and ALA can be elongated to respectively arachidonic acid (AA, 20:4n-6), and eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3).

A large body of evidence supports that reducing dietary SFAs and increasing dietary PUFAs is associated with reduced CVD risk [2-5]. Consequently, the Nordic nutrition recommendations (NNR) 2012 recommend an intake of 5-10 energy percent (E%) from PUFAs, including at least 1 E% from n-3 PUFAs, and less than 10 E% from SFAs [28]. Furthermore, it is recommended to replace dietary SFAs by particularly PUFAs. These recommendations are also supported by many other countries and health authorities [29]. Foods that are rich in PUFAs are fatty fish, nuts and seeds, and vegetables oils. SFAs are

mainly found in animal food sources, such as red meat and dairy products, but can also be found in vegetable palm oil and coconut oil. According to NNR 2012, the dietary intake of SFAs is above the recommendations and the ratio of unsaturated fatty acids to SFAs is below the recommendations in the Nordic countries [28].

The beneficial health effects of n-3 PUFAs first gained attention in the 1970s when Bang and Dyerberg found that Inuits living in Greenland had lower incidence of CHD compared to the Danish population. Since the Inuit diet consisted largely of fatty fish, seal and whale, Bang and Dyerberg suggested that the marine n-3 PUFAs found in these dietary sources were the mediators of this effect [30-33]. These observations were further replicated in other native Arctic populations and the Japanese population [34, 35], and subsequently, epidemiological and case-control studies in Western populations found that intake of fish, fatty fish or EPA and DHA was associated with reduced CVD risk [36]. Since these discoveries, many studies have investigated the health effects of n-3 PUFAs on CVD risk. In a meta-analysis summarizing prospective cohort studies published between 1947 and 2015, increased intake of EPA and DHA from foods or taken as supplements was associated with 18 % risk reduction for any CHD event [37]. Correspondingly, several large RCTs with fish or fish oil containing the marine n-3 PUFAs EPA and DHA have found reduced total mortality and major coronary event including fatal and nonfatal MI [38-41]. In a recently updated meta-analysis including data from 13 RCTs involving 127 477 participants, marine n-3 PUFA supplementation significantly lowered the risk of MI, CHD death, total CHD, CVD death and total CVD, in a dose-response relationship for several outcomes [42]. In contrast, recent evidence from other RCTs show little or no effect of n-3 PUFA supplementation on CVD risk and events [43-45].

Reducing dietary SFAs has been a cornerstone of recommendations for reducing CVD risk for decades. This recommendation is based on the totality of evidence that links dietary SFA intake to increased serum cholesterol levels and subsequently increased risk of CVD [4]. Prospective observational studies in many populations have found that lower intake of SFAs coupled with higher intake of PUFAs and MUFAs is associated with lower rates of CVD and other major causes of death and all-cause mortality [4]. In a pooled analysis of 11 cohort studies, Jacobsen et al. found that replacing 5 E% of SFAs with PUFAs was associated with 13 % lower risk of coronary events and 26 % lower risk of coronary deaths [2]. These findings are also confirmed in a meta-analysis of seven RCTs, with an estimated 19 %

reduction in CHD events and 10 % reduction in CHD risk for each 5 % increase in PUFA consumption [3]. Furthermore, RCTs that lowered the intake of dietary SFAs and replaced it with polyunsaturated vegetable oil reduced CVD by approximately 30 %, similar to the reduction achieved by statin treatment [4]. In contrast, replacing SFAs with mostly refined carbohydrates and sugars is not associated with lower rates of CVD and did not reduce CVD in clinical trials [4]. However, despite the overwhelming evidence linking saturated fat intake to CVD, controversies still exist [46-48].

The most common PUFA in the modern human diet is the n-6 PUFA LA, most likely due to the increased use of vegetable oils rich in LA. Since LA cannot be synthesized *in vivo* in humans, plasma LA may to some extent reflect dietary LA intake [49]. In a prospective cohort study, high circulating LA was inversely associated with total and CHD mortality [50]. Furthermore, in a pooled analysis of prospective studies, higher circulating and tissue levels of LA were associated with lower risk of major CVD events [51]. A meta-analysis of prospective cohort studies showed that dietary LA was inversely associated with CHD risk in a dose-response manner [52]. In contrast, some studies have found that intervention with n-3 and n-6 PUFAs together reduce CHD risk, whereas intervention with n-6 PUFAs alone tend to increase CHD risk [53]. However, the overall evidence indicates that higher n-6 PUFA intake lowers CHD risk [54, 55].

1.4.1 Dietary fat and serum lipids

Dyslipidemia, defined as elevated serum total cholesterol, LDL cholesterol or TG, or low levels of high-density lipoprotein (HDL) cholesterol, is a major risk factor of CVD. Dietary fatty acid composition regulates lipoprotein metabolism, which may affect serum lipids and thereby CVD risk [56]. One mechanism by which n-3 PUFAs reduce CVD risk is through reduction of serum TG [57, 58]. A meta-analysis published in 2017 that analyzed 171 RCTs examining the effect of EPA and DHA on CVD risk factors reported an overall significant TG reduction of 0.4 mmol/L [59]. This effect size is in line with a previous systematic review [60]. The TG-lowering effect of n-3 PUFAs is dose dependent, and individuals with elevated baseline TG have the greatest TG reduction [59, 60]. Other effects of n-3 PUFAs include

lowering of blood pressure, reduced heart rate and increased heart rate variability and reduced platelet aggregation [61].

Replacing dietary SFAs with PUFAs reduces serum LDL cholesterol, linking biological evidence with incidence of CVD in populations and in clinical trials [4]. This effect has been demonstrated in several RCTs [4, 62-66], and is mediated both by the reduction of SFAs and the increase of PUFAs. In particular, the SFAs lauric acid (12:0), myristic acid (14:0) and palmitic acid (16:0) have a total and LDL cholesterol raising effect, whereas stearic acid has a more neutral effect on LDL cholesterol level [67-69]. In a meta regression analysis including 84 studies and 2353 participants, replacing 1 E% of total daily calories from SFAs with PUFAs, chiefly in the form of LA, reduced serum LDL cholesterol by an average of 0.055 mmol/L [70]. It has been well demonstrated in RCTs that LA lowers serum total and LDL cholesterol, particularly when it replaces SFAs in the diet [71]. Also, in a more recent study it has been shown that replacing 9.5 E% from SFAs with n-6 PUFAs leads to significantly lower serum total cholesterol (-9.5 %) and LDL cholesterol (-13.6 %) and total to HDL cholesterol ratio (-8.5 %) after 4 months [72].

1.4.2 Dietary fat and inflammation

Subclinical chronic inflammation is known to play an important role in the progression of atherosclerosis and development of CVD [73]. n-3 PUFAs are associated with anti-inflammatory effects, and it has been shown in humans that intake of n-3 PUFAs may reduce serum levels of the inflammatory markers high-sensitivity C-reactive protein (hsCRP), interleukin (IL) 6 and tumor necrosis factor (TNF) [59, 74]. In humans on a Western diet, the n-6 PUFA AA makes a significant contribution to the fatty acids present in the membrane phospholipids of cells involved in inflammation. It has been reported that peripheral blood mononuclear cells (PBMC) have an average AA content of 16-20 % of total fatty acids [75-79]. AA is a precursor to a number of potent pro-inflammatory mediators (chapter 1.5). Thus, it is commonly believed that increasing the dietary intake of AA or its precursor LA will increase inflammation. However, studies in healthy humans have found that increased intake of AA or LA does not increase the concentration of many inflammatory markers, and epidemiological studies have even suggested that these n-6 PUFAs may be linked to reduced

inflammation [80]. In contrast, there is also evidence that a diet high in n-6 PUFAs inhibits the anti-inflammatory and inflammation-resolving effects of the n-3 PUFAs [80]. Hence, the interaction of n-3 and n-6 PUFAs and their lipid mediators in the context of inflammation is complex and still not fully understood. In vitro and animal studies suggest a pro-inflammatory role of SFAs, in particular lauric acid and palmitic acid [68]. In a human observational study, the relationship between SFA exposure and circulating inflammatory markers has been investigated reporting a positive association for hsCRP and IL-6 [81]. Moreover, the ratios of SFAs to n-6 PUFAs or SFAs to n-3 PUFAs were positively associated with hsCRP and IL-6 concentrations in overweight subjects [82].

1.5 Fatty acids and mechanisms of action

Fatty acids have a wide variety of biological effects. In addition to playing a central role in energy metabolism, fatty acids are involved in membrane formation, cell signaling, and regulation of gene expression [27, 83], the latter being the main focus of this thesis.
The effects depend on the fatty acids length of the carbon chain, the degree of unsaturation, the number, position and structure of the double bonds, and to some extent on the fatty acids position within the TG molecule. Fatty acids esterified in phospholipids play an essential role in the composition of cell membranes, and many of the effects are linked to changes in cell membrane lipid composition affecting cell signaling [83]. SFAs are in general assimilated into the sn-1 position of phospholipids and decrease fluidity, whereas unsaturated fatty acids are assimilated into the sn-2 position and seem to stabilize membranes in which they maintain or even increase fluidity [27, 83]. PUFAs also serve as substrates for enzymes involved in generation of eicosanoids from 20-carbon PUFAs (Figure 2). Eicosanoids include prostaglandins, thromboxanes, leukotrienes and other oxidative

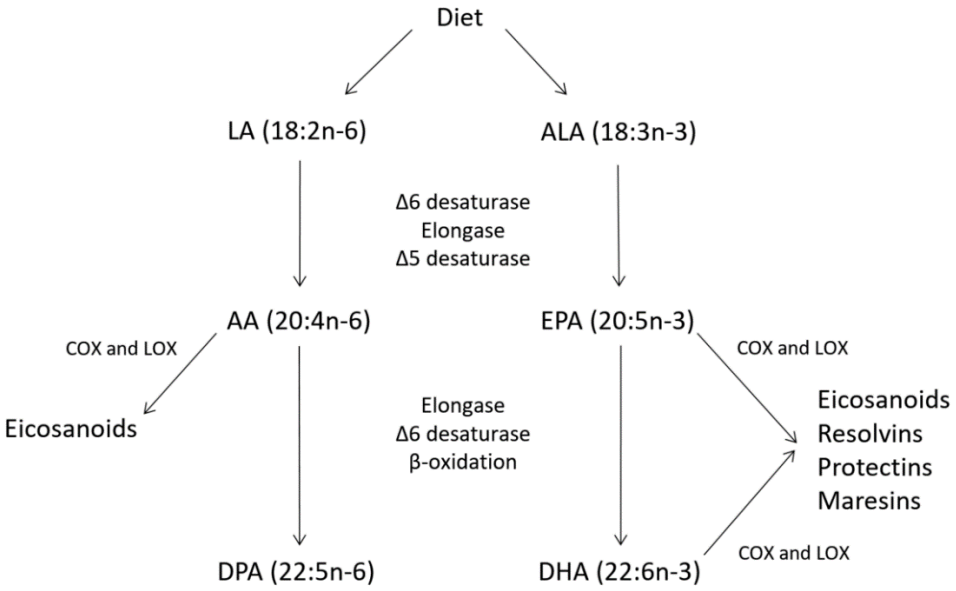

Figure 2. Overview of n-6 and n-3 PUFA metabolism and eicosanoid production. AA, arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LOX, lipoxygenase.

derivatives, and function as paracrine hormones with multiple opposing effects, e.g. they can alter the size and permeability of blood vessels, alter the activity of platelets and contribute to blood clotting, and modify the processes of inflammation. Because inflammatory cells typically contain a high proportion of the n-6 PUFA AA and low proportions of other 20-carbon PUFAs, AA is usually the major substrate for eicosanoid synthesis. Some of the eicosanoids produced from AA are pro-inflammatory, whereas others are anti-inflammatory. Increased proportion of n-3 PUFAs in inflammatory cell phospholipids is partly at the expense of AA, thus limiting synthesis of eicosanoids from AA. The n-3 PUFA EPA also act as substrate for cyclooxygenase (COX) and 5-lipoxygenase, giving rise to eicosanoids that are less potent than those formed from AA. In addition, EPA and DHA give rise to resolvins, protectins and maresins which mediate the resolution of an inflammatory response [84, 85].

In addition to being metabolized into lipid mediators, dietary fatty acids may act as signal molecules and mediate the response to varying fatty acid levels by regulating gene expression. This regulation occurs either directly by binding and activating nuclear

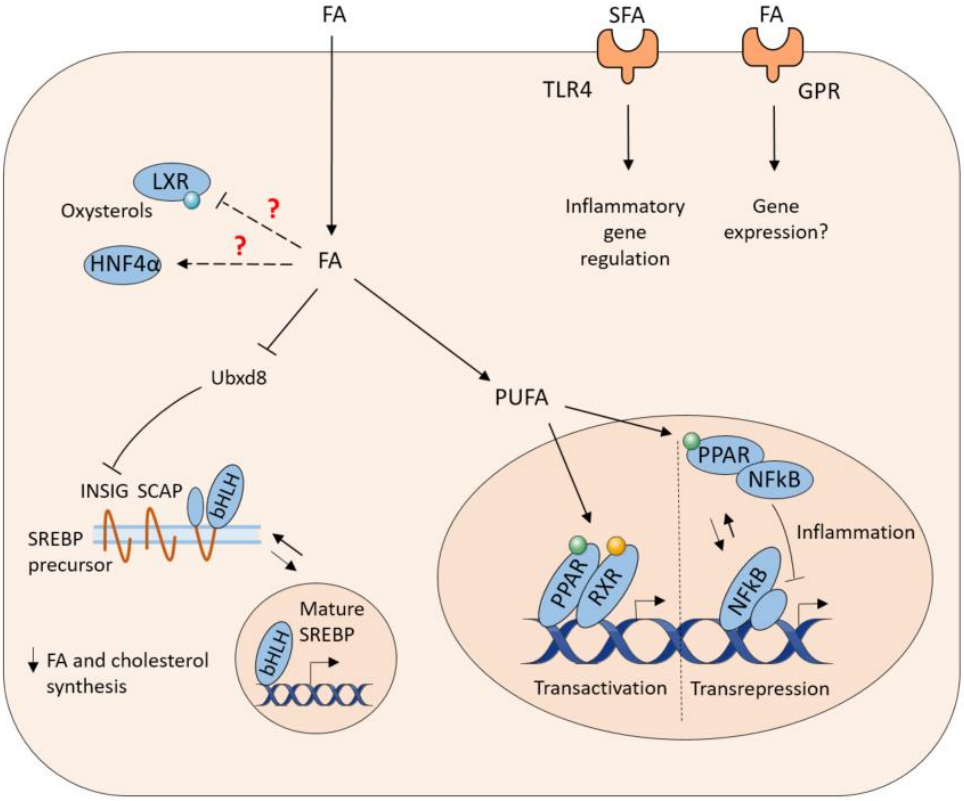

Figure 3. Mechanisms of gene regulation by fatty acids. In the liver, PUFAs may bind and activate UBXD8 and thereby inhibit proteolytic processing of SREBP leading to inhibition of fatty acid and cholesterol synthesis. Activation of PPAR by PUFAs may induce transactivation of genes involved in fatty acid catabolism or transrepression of genes involved in inflammation through inhibition of NF-κB. Fatty acid binding of LXR and HNF4α is not entirely clear. Furthermore, fatty acids bind to TLR4 and GPR receptors. Whereas TLR4 activation of SFAs promote expression of inflammatory genes, it is uncertain to what extent GPR activation of fatty acids directly influences genes expression. bHLH, basic-helix-loop-helix; FA, fatty acid; HNF4α, hepatocyte nuclear factor 4 alpha; INSIG; insulin induced gene; LXR, liver X receptor; NF-κB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; RXR, retinoid X receptor; SCAP, SREBP cleavage activating protein; SFA, saturated fatty acid; SREBP, sterol regulatory element binding protein; TLR4, toll-like receptor 4. The figure was created using free images from Servier Medical Art (Creative Common Attribution License, creativecommons.org/liscences/by/3.0/). Inspired by [86, 87].

receptors, or indirectly by altering the nuclear abundance of transcription factors (Figure 3) [83, 88, 89]. The latter can include phosphorylation, ubiquitination, or proteolytic cleavage of transcription factors. In particular, unsaturated fatty acids modulate gene transcription by regulating the activity of numerous transcription factors, including nuclear receptors such as the peroxisome proliferator-activated receptors (PPAR) and the liver X receptors (LXR), or by suppressing the nuclear abundance of the sterol regulatory element binding proteins (SREBP) [86]. In addition, activation of PPAR and LXR inhibit the nuclear factor kappa B (NF-κB) signaling pathway [90]. SFAs, on the other hand, have been shown to activate the membrane receptor toll-like receptor (TLR) 4 leading to activation of the NF-κB signaling pathway [91] (Figure 3).

PPARs include the subtypes PPARα, -γ and –δ, that can all bind and be activated by fatty acids, with a preference for long chain PUFAs [86]. The binding affinity of fatty acids is shown to increase with increasing unsaturation [92]. Additionally, PPARs can be activated by some fatty acid derivatives such as eicosanoids and acyl coenzyme A [93]. Each PPAR subtype is characterized by a unique tissue-dependent expression and function. While PPARδ is ubiquitously expressed, PPARα is expressed mainly in brown adipose tissue, heart and skeletal muscle and liver, and PPARγ is predominantly expressed in white adipose tissue and macrophages [86]. PPARs regulate the transcription of genes involved in lipid metabolism, cell differentiation, proliferation, glucose metabolism and inflammation [83, 86, 89]. Similarly to many other nuclear receptors, PPARs heterodimerize with retinoid X receptor (RXR) and bind DNA at PPAR response elements located in promoters of target genes [86]. Upon ligand binding they are activated, corepressors dissociate and coactivators are recruited which induces gene transcription.

SREBP1 and SREBP2 are transcription factors that control the expression of genes involved in de novo lipogenesis and cholesterol synthesis and uptake, respectively [94]. To induce transcription, SREBP needs to be transported to the Golgi apparatus by SREBP cleavage-activating protein (SCAP) before it can be proteolytically cleaved and translocate to the nucleus. PUFAs suppress the nuclear abundance of SREBP1 by binding and inhibiting the activity of UBXD8, an endoplasmic reticulum (ER)-bound protein that promotes the degradation of insulin-induced gene-1 (Insig-1) which in general holds the SCAP-SREBP complex back in the ER and prevents its movement to the Golgi apparatus for cleavage and maturation [95]. In addition to the mechanism described above, it is suggested that DHA

stimulate the removal of SREBP from the nucleus and increase SREBP mRNA degradation [96, 97]. Moreover, some studies have raised the possibility that PUFAs may compete with oxysterols and act as antagonists for LXR, thereby inhibiting LXR-induced transcription of SREBP [98]. However, this mechanism is not entirely clear. There are two subtypes of LXRs; LXRα with a dominant expression in the liver, adipose tissue and macrophages, and LXRβ more widely expressed among all tissues. These receptors carry out essential functions in lipid metabolism; they promote the synthesis of bile acid and cholesterol, lipogenesis, reverse cholesterol transport, and fatty acids and glucose uptake [87]. Similar to PPAR, LXR bind to RXR as a heterodimer.

NF-κB is a major transcription factor regulating the expression of genes involved in inflammation. In its inactive form, NF-κB is bound to inhibiter of κB (IκB) which retains it in the cytoplasm. Upon an inflammatory signal, IκB kinase (IκK) phosphorylates IκB which leads to dissociation of IκB from NF-κB promoting nuclear translocation of the active transcription factor [99]. In the nucleus, NF-κB binds promoters and enhancers and activate transcription of cytokines, adhesion molecules and chemoattractants [89]. PUFAs reduce the nuclear translocation of NF-κB through several different mechanisms, including activation of PPARs as well as inhibition of TLR4 signaling by modulation of lipid rafts [89, 100]. In contrast, SFAs have been shown to activate TLR4 leading to activation of the NF-κB signaling pathway [91]. Furthermore, n-3 PUFAs have been shown to stimulate G protein-coupled receptor (GPR) 120 in macrophages and adipocytes, and thereby mediate an anti-inflammatory effect via inhibition of NF-κB signaling [100, 101].

1.6 Lipoproteins, their metabolism and relation to disease

Lipids, such as TG and cholesterol, are insoluble in aqueous solutions and are therefore transported in lipoproteins in the circulation [102]. Lipoproteins are complex emulsion particles that create an interphase between hydrophobic and hydrophilic environment in blood. The outer layer of the lipoprotein is hydrophilic and consist mainly of free cholesterol, phospholipids and apolipoproteins, whereas the core is hydrophobic and consist mainly of TG and cholesteryl esters. The lipoproteins associate with apolipoproteins, enabling them to bind specific receptors and enzymes. Based on their size, lipid

Table 1. Characteristics of the major lipoprotein classes

Fraction	Density range (g/ml)	Diameter (nm)	Major lipids	Major apo-lipoproteins	Composition (percentage by weight) Protein	TG	Chol	PL
Chylomicron	<0.950	80-1000	Dietary TG	B48, AI, AIV, C, E	1	90	5	4
VLDL	0.950-1.006	30-80	Endogenous TG	B100, C, E	10	65	13	13
LDL	1.019-1.063	20-25	Cholesterol and CE	B100	20	10	45	23
HDL	1.063-1.210	9-15	CE and PL	AI, AII, C, E	50	2	18	30

VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high density lipoprotein; TG, triglyceride; CE, cholesteryl ester; PL, phospholipid; B48, apolipoprotein B48; B100, apolipoprotein B100; AI, apolipoprotein AI; AII, apolipoprotein AII; AIV, apolipoprotein AIV; C, apolipoprotein CI, CII and CIII; E, apolipoprotein E. Table reproduced after [102].

composition, and apolipoproteins, lipoproteins are differentiated into four major classes; chylomicrons, very low-density lipoproteins (VLDL), LDL and HDL (Table 1) [102, 103]. However, this categorization is somewhat simplified as lipoproteins shrink and increase in size as they are depleted of an acquire lipids and proteins, and therefore exist as a continuum of different sizes. A simplified overview of lipoprotein metabolism is given in Figure 4.

Chylomicrons are assembled from dietary lipids and apoB48 in the intestine, predominantly in the postprandial state, before secretion into the lymph and further into the circulation. In the circulation, chylomicrons acquire apoE, apoCII and CIII, mainly delivered by HDL [104, 105]. ApoCII makes chylomicrons substrate for the action of lipoprotein lipase (LPL) which is situated at the endothelial cell surface of the capillaries. This activation initiates the hydrolysis of TG inside the chylomicrons and release of fatty acids that are used as an energy source by peripheral tissues, such as muscle and adipose tissue [106-108]. ApoCIII, on the other hand, inhibits LPL activity [106, 107]. As the chylomicrons are depleted in TG, they disassociate some unesterified cholesterol and phospholipids, and some apolipoproteins, which are taken up by other particles such as HDL, and the lipoprotein particle shrinks in size. The chylomicron remnant is taken up by the liver via a process mediated by apoE [102, 107].

VLDL particles are formed in the liver in a similar way as chylomicrons, brought together with apoB100. The VLDL particles are less TG-rich than the chylomicrons, and thus less voluminous, however, their function is still to deliver fatty acids to peripheral tissues [109]. Hence, chylomicrons and VLDL particles are commonly called TRLs. Similar to the chylomicrons, VLDL particles receive apoC and apoE from HDL in the circulation. As the VLDL particle is depleted in TG by the action of LPL, the particle shrinks in size and becomes a lipoprotein particle that is richer in cholesterol than in TG [106]. Eventually, the VLDL

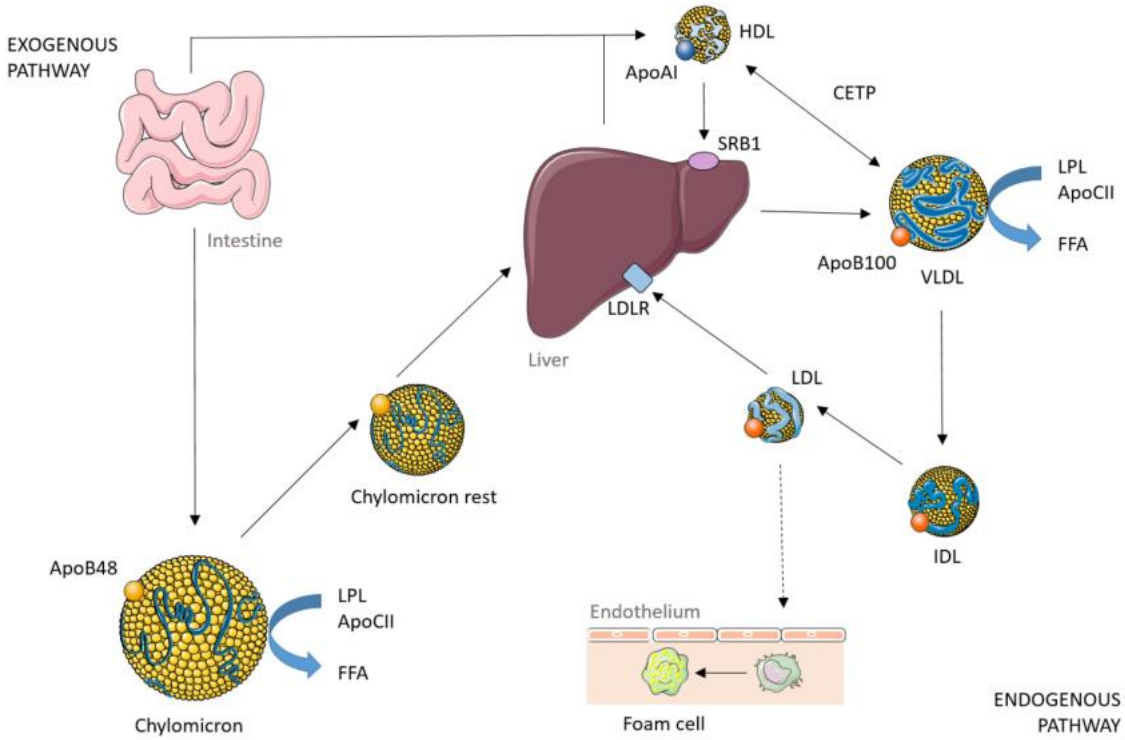

Figure 4. Simplified overview of lipoprotein metabolism. ApoAI, apolipoprotein AI; ApoB48, apolipoprotein B48; ApoB100, apolipoprotein B100; ApoCII, apolipoprotein CII; CETP, cholesteryl ester transfer protein; FFA, free fatty acids; HDL, high-density lipoprotein; IDL, intermediate density lipoprotein; LDL, low-density lipoprotein; LDLR, LDL receptor; LPL, lipoprotein lipase; VLDL, very low-density lipoprotein, SRB1, scavenger receptor B1. The figure was created using free images from Servier Medical Art (Creative Common Attribution License, creativecommons.org/liscences/by/3.0/).

remnant shed all apolipoproteins, except apoB100, and becomes an LDL particle that circulates until uptake by the liver via the LDL receptor (LDLR). VLDL remnants and LDL particles can also be taken up by macrophages, a central process in the development of atherosclerosis [110]. In contrast to LDL that needs to be modified before uptake by macrophages, VLDL remnants can be taken up directly [111]. Some VLDL remnants may also be taken up by the liver via the same receptors as the chylomicron remnants [106]. The higher uptake of VLDL remnants, the lower production of LDL particles, and thus fewer particles that can accumulate, oxidize and be taken up by macrophages in the intima. PPARα activation upregulates LPL transcription in liver and muscle cells, leading to increased lipolysis and clearance of TRL remnants [112]. Furthermore, PPARα agonists reduces synthesis of apoCIII, thereby favoring VLDL lipolysis and generation of large LDL particles that are more efficiently cleared via the LDLR. Serum cholesterol is highly dependent on both the secretion of apoB100-containing particles, and quantity and quality of the LDLR on the surface of hepatocytes [103, 113, 114]. PPARα activation reduces apoB-secretion and regulates the expression of several genes promoting mitochondrial fatty acid β-oxidation resulting in decreased substrate availability for TG formation, likely contributing to decreased VLDL particle production and serum TG levels [112]. This is also accompanied by suppression of SREBP1, leading to reduced transcription of genes involved in de novo lipogenesis [86]. Furthermore, PUFAs have been linked to increased whereas SFAs have been linked to decreased *LDLR* expression level in animal and *in vitro* studies [115], and in some human postprandial and long term dietary intervention studies [116-118].

Finally, HDL is formed by apoA1 secretion from the liver and intestine, followed by acquisition of free cholesterol and phospholipids from TRLs as well as free cholesterol from cells via ATP binding cassette A1 and G1 (ABCA1 and ABCG1). This free cholesterol is esterified to cholesteryl esters by lecithin-cholesterol acyl transferase (LCAT) to maintain a concentration gradient and enable continuous acquisition of free cholesterol. Via the process of reverse cholesterol transport, HDL transfer its cholesteryl ester content to the liver by binding to the scavenger receptor B1 (SRB1). Additionally, HDL transfer cholesteryl esters into apoB-containing lipoproteins in exchange for TG via the enzyme cholesteryl ester transfer protein (CETP) before hepatic uptake of the apoB-containing particle. The CETP-mediated pathway is estimated to be the most important route for reverse cholesterol transport [119]. The expression of *CETP, ABCA1 and ABCG1* is regulated by activation of LXR

[120]. PPARα upregulates the expression level of several genes involved in HDL metabolism, including apoA1 and apoAII. PPARα agonists also induce *ABCA1* and *SCARB1* (SRB1) expression in macrophages, thereby enhancing the first steps of macrophage reverse cholesterol transport [112].

1.7 Nutrigenomics in nutrition research

One approach to explore the effect of dietary fat quality on CVD risk at a molecular level is to use transcriptome profiling in dietary intervention studies [121]. Transcriptomics is one of multiple high-throughput omics approaches that have evolved as a result of methodological advances in molecular biology and genetics over the past few decades [122, 123]. Together these omics approaches, collectively termed nutrigenomics, aim to understand the effects of diet on health or disease development [124, 125]. From a nutrigenomics perspective, nutrients are dietary signals that are detected by cellular sensors, such as membrane proteins and nuclear receptors, leading to changes in omics measures such as the transcriptome, proteome and metabolome, i.e the complete set of all mRNA molecules, proteins and metabolites in a cell or in a biological sample. Subsequently, patterns of gene expression, protein expression and metabolite production in response to diets or dietary components can be viewed as dietary "signatures". Nutrigenomics aims to examine these dietary signatures in specific cells, tissues and organisms, and to understand how nutrition influences homeostasis and the risk of diet-related diseases on a genome-wide scale [124].

Transcriptomics is the study of the transcriptome, using high throughput methods such as microarray technology and RNA sequencing (RNA-seq). In nutrition research, transcriptomics is mainly used for three different purposes; 1) to provide information about the mechanisms underlying the effects of a certain nutrient or diet, 2) to identify genes, proteins or metabolites that are altered in the pre-disease state and might therefore act as molecular biomarkers, and 3) to identify and characterize molecular pathways regulated by nutrients [124]. Comparison of transcriptomes allows the identification of genes that are differentially expressed in distinct cell populations or in response to different treatments. Applied together with traditional biochemical methods, transcriptomics may provide

extensive information about nutritional status and metabolic responses to diet. Furthermore, since changes in the transcriptome profile occur prior to changes in protein levels, transcriptomics may be considered as a valuable and sensitive technique measuring early metabolic changes related to dietary challenges [126, 127].

1.8 Transcriptomics in human dietary intervention studies

In order to study the molecular mechanisms underlying the effects of diet and dietary components on target tissues, metabolically active tissues such as liver, adipose- and muscle tissues are of primary interest. However, since study populations mostly consist of healthy volunteers, the availability of samples from these tissues are limited. PBMCs are immune cells consisting mostly of monocytes and lymphocytes. These cells circulate around in the blood and are exposed to many of the same environmental factors as metabolically active tissues and the arterial wall [127]. Furthermore, PBMCs seem to reflect changes occurring in metabolically active organs, including fasting [128-130]. Hence, PBMCs may provide information on how diet influences metabolic regulation and may to a certain extent reflect systemic health. Given the notion that PBMCs are central in the process of inflammation, these cells may also constitute a link between diet, metabolic changes and systemic inflammation that underlie many lifestyle-related diseases, including atherosclerosis. Monocytes and lymphocytes are immune cells of the innate and adaptive immune systems, and are both actively involved in the development of atherosclerotic plaques. Furthermore, leukocytes respond to changes in plasma lipid levels and systemic inflammation by regulating a network of genes, including genes involved in immune response and lipid metabolism [131].

Several human dietary intervention studies have successfully used transcriptomics to demonstrate that diets or dietary components induce alterations in PBMC gene expression [126, 127, 132, 133]. These include studies investigating health effects of dietary patterns such as the Mediterranean diet [134] and a Healthy Nordic diet [135]. Furthermore, several postprandial studies [117, 118, 136-142] and long term dietary intervention studies [116, 143-149] have investigated effects of dietary fat on targeted gene expression and the whole transcriptome response in PBMCs. These studies have reported changes in gene expression

related to inflammation, oxidative stress and lipid metabolism, and demonstrate that the fatty acids ability to alter gene expression may be important in relation to their health effects. However, the molecular mechanisms underlying the effect of dietary fat on CVD risk at gene expression level are incompletely understood. Furthermore, studies show inter-individual variation in TG response to n-3 PUFA intake which may be partly explained by differences in gene expression and should be further investigated. Hence, transcriptomics studies exploring the role of dietary fat on CVD risk are warranted.

2 Study aims

The overall aim of this PhD project was to investigate the molecular mechanisms underlying the effect of dietary fat quality on CVD risk by use of transcriptomics in human dietary intervention studies.

The specific aims were to:

- Investigate differences in PBMC gene expression of specific predefined genes related to lipid metabolism depending on plasma fatty acids levels and ratio in healthy subjects (Paper I)

- Investigate differences in PBMC gene expression profiles and pathways in TG responders and non-responders to n-3 PUFA supplementation (Paper II)

- Investigate effects of replacing dietary SFAs with PUFAs on PBMC gene expression profiles and pathways in subjects with moderate hypercholesterolemia (Paper III)

3 Subjects and methods

The work in this thesis is based on data and material from two human dietary intervention studies; study 1 was conducted at Akershus University College (OsloMet) in 2009 [150], and study 2 was conducted at Oslo and Akershus University College (OsloMet) and the University of Oslo between 2012-2014 [66]. An overview of the two studies and the papers included in the thesis is presented in Table 2.

Table 2: Overview of study population, study design, interventions and aim for paper I, II and III

Study	Design	Population	Intervention	Duration	Aim	Paper
1	Randomized controlled double-blinded study	Healthy men and women 18-50 years	High-oleic sunflower oil group (*n* = 19) Oxidized fish oil group; 1.6 g/d EPA + DHA (*n* = 18) High-quality fish oil group; 1.6 g/d EPA + DHA (*n* = 17)	7 weeks	To investigate differences in expression of specific pre-defined genes related to lipid metabolism in PBMCs depending on plasma n-3 and n-6 PUFA levels and SFA to PUFA ratio	I
					To investigate baseline differences and differences in changes in PBMC gene expression and lipoprotein subclass TG levels between TG responders and non-responders to n-3 PUFA supplementation	II
2	Randomized controlled double-blinded study	Healthy men and women with moderate hypercholesterolemia 25-75 years	Experimental diet group (*n* = 43) Control diet group (*n* = 49)	8 weeks	To investigate effects of replacing dietary SFAs with PUFAs on gene expression profiles and pathways in PBMCs	III

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PBMC, peripheral blood mononuclear cell; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TG, triglyceride.

3.1 Study 1

In paper I and II, we used data from a seven week double-blinded randomized controlled three arm parallel study conducted at the Akershus University College (OsloMet) from September to December 2009 [150]. In this study, 69 subjects were randomized at baseline to receive high-quality fish oil, oxidized fish oil or high-oleic sunflower oil (HOSO) capsules. The EPA and DHA content of the high quality and oxidized fish oils was equal and corresponded to an intake of 1.6 g/d EPA + DHA. Intake of n-3 supplements, fish and food items enriched with n-3 PUFAs was not allowed 4 weeks prior to the baseline visit and throughout the intervention. In total, 17, 18, and 19 subjects completed the intervention in the high-quality fish oil-, oxidized fish oil- and HOSO group, respectively. A schematic overview of the study design is given in Figure 5. The primary outcome of the study was several selected CVD risk markers, and predefined secondary outcomes included metabolic and transcriptome profiling.

In paper I, we used cross-sectional data from end of study (*n* = 54). The study population was grouped into tertiles three times by arranging samples from the highest to the lowest value according to 1) plasma level of n-3 PUFAs, 2) plasma level of n-6 PUFAs, and 3) plasma SFA to PUFA ratio. Within each grouping, the subjects in the highest (*n* = 18) and the lowest (*n* = 18) tertile were compared.

In paper II, we used data from baseline and end of study. The two groups receiving fish oils were merged (*n* = 35) and then subjects were regrouped into new groups depending on serum TG response during the intervention, whereas the HOSO group was excluded (Figure 6). TG responders were defined as subjects having a larger TG reduction than the 20 % day-to-day variation [151] (*n* = 8) and non-responders as having a TG change between –20 % and +20 % (*n* = 16) after n-3 PUFA supplementation. Participants with a higher TG increase than the 20 % day-to-day variation were excluded (*n* = 11).

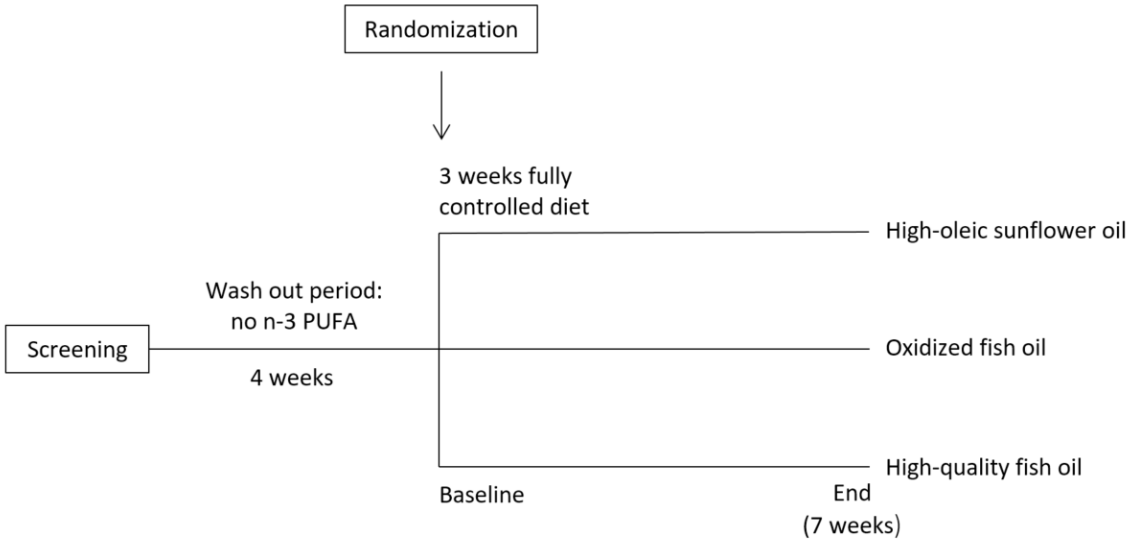

Figure 5. Study design of study 1. Subjects were randomized to receive high-oleic sunflower oil, oxidized fish oil or high-quality fish oil. The study duration was 7 weeks, with the first three weeks being a fully controlled diet period.

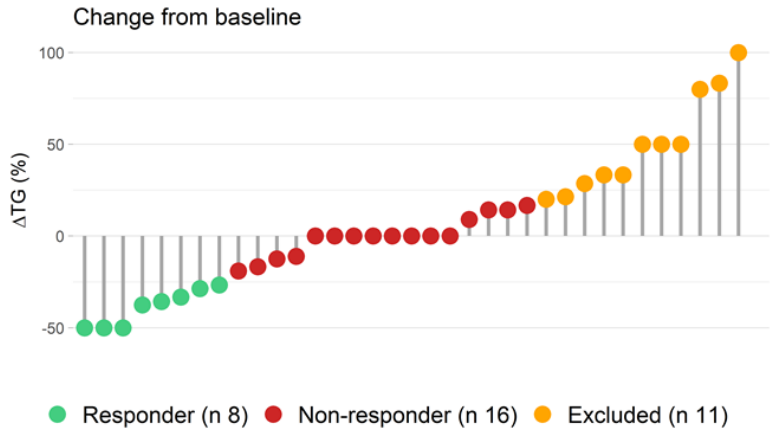

Figure 6. Individual changes in fasting TG. The relative change in fasting TG levels were used to categorize subjects as responders (ΔTG ≤ −20 %, green) and non-responders (−20 % < ΔTG < +20 %, red). Subjects with ΔTG ≥ +20 % (yellow) were excluded. TG, triglycerides.

3.2 Study 2

In paper III, we used data and material from an eight week double-blinded randomized controlled parallel study conducted at the Oslo and Akershus University College (OsloMet) and the University of Oslo from July 2012 to April 2014 [66]. At baseline, 115 subjects were stratified by sex and age, and randomly assigned into one of two intervention groups receiving either control food items or experimental food items (Figure 7). The experimental food items were the same type of food products as the control food items, but with an improved fatty acid composition. The experimental food items and the control food items were, respectively, butter-based spread or margarine-based spread, butter or liquid margarine, and olive oil or rapeseed and sunflower oil. In addition, products such as liver paté, cheese, bread, muesli cereals, cream, mayonnaise, and crème fraîche were given to the participants, in which some of the SFAs were replaced with particularly n-6 PUFAs from rapeseed and sunflower oils in the products in the experimental diet (Ex-diet) group. Based on the minimum intake of the food items, the n-6 PUFA content was 4.2 g/d in the control food items and 12.9 g/d in the experimental food items, and the SFA content was 19.2 g/d in the control food items and 5.7 g/d in the experimental food items. The dietary difference during the intervention was 6.5 E% lower intake of SFAs and 6.4 E% higher intake of PUFAs in the Ex-diet group compared to the control diet (C-diet) group. Before the baseline visit, all subjects underwent a 2 week run-in period where they had to include the control food items in their habitual diet. A total of 100 subjects completed the study. In paper III, we included data and material from baseline and end of study for 92 subjects.

The primary outcome of the study was serum cholesterol levels and inflammatory markers. Predefined secondary outcomes included transcriptome profiling.

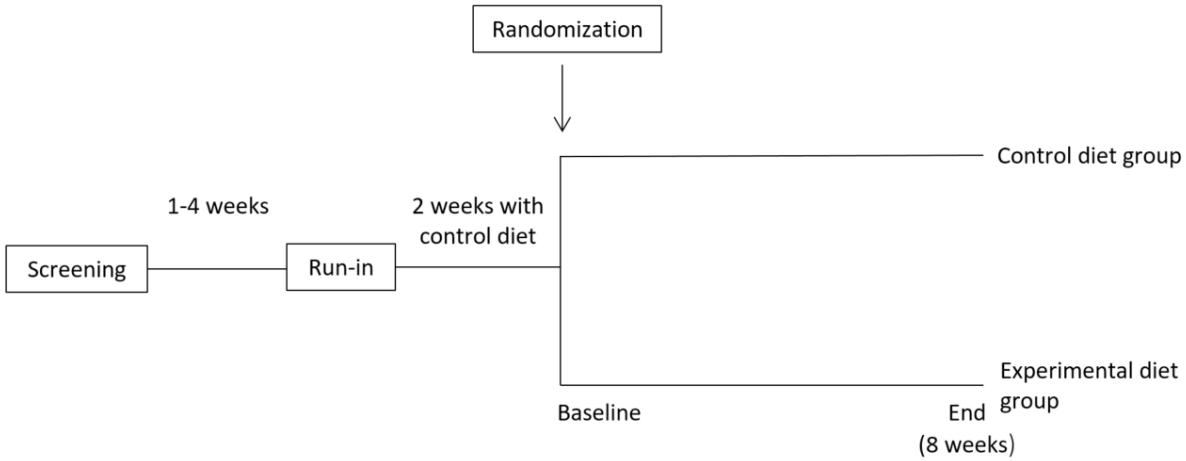

Figure 7. Study design of study 2. In the run-in period, all participants consumed food products of the control diet for two weeks before they were randomized at baseline to either the experimental diet group or the control diet group. The study duration was eight weeks.

3.3 Plasma fatty acid analysis

Plasma fatty acid concentrations were measured by Nofima (Ås, Norway), a collaborator partner, in study 1 (paper I and II), and a commercial laboratory (Vitas Analytical Service) in study 2 (paper III), using gas chromatography. A more detailed description of the methods used is included in the respective papers. The concentration of the individual fatty acids was measured as µg fatty acid/ml plasma and presented as percentage of total plasma fatty acids.

3.4 Lipoprotein subclass analysis

Analysis methods such as nuclear magnetic resonance (NMR) spectroscopy enable subclass identification of VLDL-, LDL- and HDL particles based on their diameter. This methodology can be used to identify and map the distribution of atherogenic lipoprotein particles in blood samples, and to make more precise risk analyses regarding the link between lipoprotein concentrations and CVD. In paper II, lipoprotein subclass particles and their lipid constituents were measured using a well-validated and widely applied commercial NMR platform (Nightingale Health, formerly Brainshake, Finland) [152]. This platform classifies lipoproteins into 14 different subclasses based on their average diameter; extremely large (XXL) VLDL with a possible contribution of chylomicrons (> 75 nm); extra large (XL-), large (L-), small (S-), and extra small (XS-) VLDL (64.0, 53.6, 44.5, 36.8, and 31.3 nm); intermediate-density lipoprotein (IDL, 28.6 nm); L-, M-, and S-LDL (25.5, 23.0 and 18.7 nm); and XL-, L-, M-, and S-HDL (14.3, 12.1, 10.9 and 8.7 nm). Furthermore, the platform enables the measurement of total lipid, TG, cholesterol, cholesteryl ester and phospholipid content of each subclass particle [152].

3.5 Gene expression profiling in PBMCs

In this thesis, we used PBMCs as a model system for studying gene expression differences and differences in changes between groups. PBMCs were isolated from blood at baseline and end of study, using the BD Vacutainer Cell Preparation tubes according to the manufacturer's instructions (Becton, Dickinson San Jose, CA, USA). This is a well-documented and standardized method to collect mononuclear cells with high purity (above 90 %), and according to the manufacturer approximately 80 % of the cells are lymphocytes and 12 % are monocytes. Total RNA was isolated from all PBMCs using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). RNA quantity measurements were performed using a Nanodrop ND-1000 Spectrometer (Thermo Fisher Scientific, Gothenburg, Sweden), and RNA integrity number (RIN) value was measured with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to check the RNA quality. Samples with RIN value above eight were labeled and served as templates for microarray hybridization.

Total RNA was analyzed by use of Illumina microarray technology (also known as BeadArray technology). This technology is based on a hybridization strategy, in which mRNA expressed in a cell is copied into cRNA using reverse transcriptase and hybridized to known sequences of DNA oligonucleotides in microarray platforms (Figure 8). After washing away unbound material, mRNA abundance is quantitated by scanning and image analysis. Bead level data is transformed to probe level intensity values which are extracted for bioinformatics analysis. The microarray analyses were carried out by an external partner at the Genomics Core Facility (Norwegian University of Science and Technology), and followed standard Illumina protocol (Illumina Inc., CA, USA) as described in the respective papers. Further, the intensity values were filtered to improve the statistical power (detection *p*-value < 0.01), and quantile normalized. In order to select one probe per gene (paper III), the probe with the largest variance (interquartile range (IQR)) was selected. Gene expression changes were obtained by calculating log2 ratios between the baseline and end of study intensity values.

The raw data are available from the Gene Expression Omnibus (GEO) database (accession number GSE111567 and GSE176043).

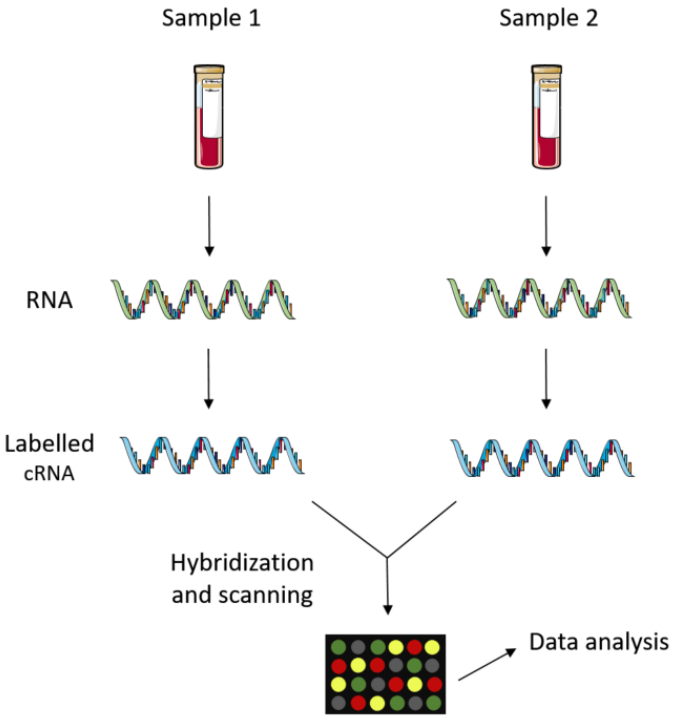

Figure 8. Basic principle of hybridization-based microarrays. The figure was created using free images from Servier Medical Art (Creative Common Attribution License, creativecommons.org/liscences/by/3.0/).

3.6 Selection of genes

In paper I, 285 genes encoding proteins related to cholesterol- and TG metabolism were selected for exploratory analysis. The genes were selected based on relevant gene sets related to cholesterol- and TG metabolism (26 gene sets) in the Molecular Signatures Database (MSigDB) v6.0 [153] limited to collection C5 (Gene ontology, GO), and literature summarizing loci associated with different lipid traits [154].

3.7 Statistical methods

Statistical analyses were performed using R. Details of the analyses are described in the respective papers. In paper I, we analyzed differences in gene expression by use of independent samples t-tests. The probe with the lowest *p*-value was reported for gene transcripts with multiple probe sets. In paper II, we analyzed baseline differences and differences in changes in gene expression by use of a linear regression model adjusted for age and sex. Due to the exploratory design in paper I and II, we did not adjust for multiple testing. In these papers, gene transcripts with a p-value < 0.05 (paper I) and ≤ 0.05 (paper II) were considered differentially expressed or differentially altered between the groups. In paper III, we analyzed differences in changes in gene expression by a linear regression model adjusted for age, sex, smoking, study center and log(baseline gene expression) level. To adjust for the large number of tests, the false discovery rate (FDR) was controlled by the Benjamini-Hochberg procedure. Gene transcripts with an FDR adjusted p-value < 0.25 were considered differentially altered between the groups.

Functional analysis at the pathway level was carried out in paper II and III by use of Metacore from Clarivate Analytics (GeneGo, division of Thomson Reuters, St. Joseph, MI, USA). Metacore is a knowledge database suitable for functional analyses of experimental data. Gene transcripts with a p-value ≤ 0.05 (paper II) and < 0.05 (paper III) were included in the pathway analyses.

3.8 Ethics

Study 1 and 2 were conducted according to the Declaration of Helsinki. All participants provided written informed consent and all procedures, including transcriptome profiling, were approved by the Regional Ethics Committee (approval no. 6.2008.2215) and the Norwegian Social Science Data Services in study 1, and the Regional Ethics Committee for Medical Research in South East Norway (approval no. 2011/1951 D) in study 2. Both trials were registered at www.clinicaltrials.gov (identification number study 1: NCT01034423 and study 2: NCT01679496).

4 Summary of results

Paper I: Plasma fatty acid levels and gene expression related to lipid metabolism in peripheral blood mononuclear cells: a cross-sectional study in healthy subjects

In paper I, we aimed to explore differences in PBMC gene expression of specific predefined genes related to lipid metabolism, depending on plasma levels of n-3 and n-6 PUFAs and SFA to PUFA ratio in healthy subjects.

Of 285 genes selected for this exploratory study, 161 genes were defined as expressed in the PBMCs and further included in the statistical analyses. Plasma SFA to PUFA ratio was associated with the largest number of differentially expressed genes (25 gene transcripts) followed by plasma n-6 (15 gene transcripts) and n-3 (8 gene transcripts) PUFA level. Differentially expressed genes between subjects with the highest compared to subjects with the lowest SFA to PUFA ratio included several genes related to cholesterol homeostasis, including insulin induced gene 2 (*INSIG2*) and ER lipid raft associated 2 (*ERLIN2*) which were higher expressed, and 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) and ATP binding cassette subfamily A member 2 (*ABCA2*) which were lower expressed, among subjects in the former group.

In conclusion, we found that PBMCs express genes involved in hepatic lipid metabolism and that the expression of several of the genes may be influenced by plasma fatty acid levels. These findings support the use of PBMCs as a model system for exploring the role of dietary fat on gene expression related to lipid metabolism. The differential expression of genes involved in cholesterol homeostasis between subjects with the highest and lowest plasma SFA to PUFA ratio may reflect an intracellular status of excess cholesterol among the former group. The current findings should, however, be further investigated in well-controlled human dietary intervention studies.

Paper II: Differences in peripheral blood mononuclear cell gene expression and triglyceride composition in lipoprotein subclasses in plasma triglyceride responders and non-responders to omega-3 supplementation

In paper II, we aimed to explore baseline differences and differences in changes in PBMC gene expression and lipoprotein subclass TG levels between TG responders and non-responders to n-3 PUFA supplementation.

In the current study, eight subjects were defined as responders with a median TG reduction of 37 %, and 16 subjects were defined as non-responders with a median TG change of 0 %. At baseline, responders had higher TG levels in two of four HDL subclasses compared to non-responders, and 909 gene transcripts were differentially expressed between the groups. During the intervention, the plasma TG reduction among responders was reflected in TG reductions in four of six different VLDL subclasses and three of four different HDL subclasses. Furthermore, the expression of 454 transcripts was differentially altered in responders compared to non-responders. Pathway analyses revealed that responders had altered pathways related to development and immune function. In addition, two of the top 10 enriched pathways in responders compared to non-responders were related to lysophosphatidic acid (LPA) signaling.

To conclude, TG responders and non-responders to n-3 PUFA supplementation have different baseline lipoprotein subclass and PBMC gene expression profiles. Furthermore, they differentially alter their lipoprotein subclass and PBMC gene expression profiles. The differentially altered PBMC gene expression may partly explain the variation in TG response to n-3 PUFA intake.

Paper III: Replacing saturated fat with polyunsaturated fat modulates peripheral blood mononuclear cell gene expression and pathways related to cardiovascular disease risk using a whole transcriptome approach

In paper III, we aimed to explore the effect of replacing dietary SFAs with PUFAs for eight weeks on PBMC gene expression profiles and pathways in subjects with moderate hypercholesterolemia.

A total of 13 148 genes were defined as expressed in the PBMCs and included in the statistical analyses of this study. Of these, 1105 gene transcripts were differentially altered between the groups when comparing the relative change from baseline to 8 weeks of intervention. After adjusting for multiple testing, 14 gene transcripts were differentially altered between the groups. These included gene transcripts associated with vascular smooth muscle cell (VSMC) proliferation (fibroblast growth factor 18 (*FGF18*), septing 4 (*SEPTIN4*)), regulation of blood pressure (ATPase plasma membrane Ca2+ transporting 1 (*ATP2B1*), guanylate cyclase 1 soluble subunit beta 1 (*GUCY1B1*)), and LDLR folding (DnaJ heat shock protein family (Hsp40) member C10 (*DNAJC10*)). Change in *ATP2B1* expression was positively correlated to change in total cholesterol and LDL cholesterol (borderline) for the whole study population (*n* = 92). Furthermore, there was a positive correlation between change in *FGF18* expression and change in total cholesterol, and a negative correlation between change in *GUYC1B1* expression and change in LDL cholesterol. Pathway analyses revealed that pathways mainly related to immune response and inflammation were enriched in the Ex-diet group compared to the C-diet group. Furthermore, pathways related to platelet aggregation were among the top 10 most differentially regulated pathways.

In conclusion, we found that replacing dietary SFAs with PUFAs for 8 weeks modulates PBMC gene expression and pathways related to CVD risk in subjects with moderate hypercholesterolemia. The current findings may offer new mechanistic insight regarding the effect of dietary fat quality on CVD risk, and should be further investigated in future studies.

5 Discussion

5.1 Methodological considerations

5.1.1 Subjects and study design

The work in this thesis is based on two dietary RCTs; study 1 (paper I and II) was designed to investigate effects of fish oils with different quality on several CVD risk markers, and study 2 (paper III) was designed to investigate effects of replacing dietary SFAs with PUFAs on serum cholesterol levels and several inflammatory markers. Pre-registered secondary outcomes included transcriptome profiling of PBMCs. In paper I and II we used an exploratory approach and regrouped the study subjects based on respectively plasma fatty acid levels at end of study (paper I) and serum TG response to n-3 PUFA supplementation during the intervention (paper II). The main outcome in all three papers was gene expression differences or differences in changes between groups. In paper I, we examined the expression of pre-specified genes using a cross-sectional design, whereas in paper II and III we examined the whole transcriptome response using an interventional design.

The subjects included in the studies were respectively healthy (study 1, paper I and II) and moderately hypercholesterolemic (study 2, paper III) men and women between the age of 18-50 and 25-70 years. Inclusion criteria that were common across the studies included no chronic disease, fasting blood glucose < 6 mmol/L and hsCRP < 10 mg/L. Furthermore, in study 1, subjects had to have body mass index (BMI) < 30 kg/m^2, fasting TG ≤ 4.0 mmol/L and total cholesterol ≤ 7.5 mmol/L. In study 2, subjects were included based on age-specific serum cholesterol values set by the commercially laboratory used for analysis; 5–7.8 mmol/L for those between 50 and 70 years, 5.0–6.9 mmol/L for those between 30 and 49 years, and 5.0–6.1 mmol/L for those between 25 and 29 years, as we wanted to include subjects with cholesterol values at the upper range of normal serum cholesterol and these values vary among age-groups. In addition, inclusion criteria in study 2 was BMI between 20 and 35 kg/m^2, fasting TG ≤ 2.6 mmol/L and LDL cholesterol ≥ 3.5 mmol/L. The latter eligibility criteria differed in the two studies, as the primary outcome of study 2 was change in serum LDL cholesterol, whereas study 1 was designed to investigate health effects of fish oils with different quality.

A major challenge when studying gene expression in human dietary intervention studies is the large inter- and intra-individual variability in baseline expression and response to intervention. Thus, procedures to minimize this variability should be applied. In the studies included in this thesis, PBMCs were isolated from fasted blood samples since short term (postprandial) food intake influences gene transcription, and this effect on gene expression level may be larger than the effect of the intervention [155]. Furthermore, in study 2, all subjects were provided and consumed the control products for two weeks prior to intervention to ensure that the participants had lower variability in dietary intake and serum cholesterol prior to entering the study at baseline. To reduce the day-to-day variability in TG measurements [111], subjects were instructed to avoid alcohol consumption and vigorous physical activity the day prior to blood sampling. Physical activity may also alter gene expression [155-157], and should therefore be kept constant throughout the study as well as before sampling. Finally, the samples were collected at the same time point of the day at baseline and end of study taking into account that gene expression is altered by our circadian rhythm and therefore may vary throughout the day [158, 159].

In paper III, subjects were randomly assigned into one of two intervention groups, eliminating selection bias that arises when the groups differ because of systematic errors [160]. The aim of the randomization process is to ensure an equal distribution of known and unknown confounding factors between groups [161]. Thus, a cause-effect relationship between the intervention and the outcome can be inferred with a higher degree of confidence. In cases were the groups are small and cannot rely on randomization alone to produce equal groups, stratification should be applied to ensure an equal distribution of important confounding factors [160]. In study 1 and 2, subjects were stratified by sex and age as these variables influence most outcomes, including gene expression levels [162, 163]. However, in paper I and II, the groups were regrouped into new exploratory groups as described above. In paper III, block randomization ensured approximately equal sizes of the groups. The RCT design applied in paper III is defined as the gold standard of intervention studies, and only a few, if any, double-blinded RCTs have investigated the effect of exchanging a few regularly consumed and commercially available key food items with similar food items with improved fat quality on the whole transcriptome response for eight weeks. However, several studies investigating variation in the transcriptome profile of

PBMCs have shown that the inter-individual variation in these cells is larger than the intra-individual differences [164], and it is therefore suggested that within group analysis and cross-over design are more appropriate designs for such studies which are likely to elicit only small changes in gene expression. In a cross-over design, each subject is their own control. A limitation of this design is, however, the carry over effect; the potential persistence of an effect of a manipulation after it has been removed [165], necessitating a sufficiently long washout period. On the other hand, dietary gene expression studies reporting within group changes have been criticized for not having a control group at all. In the papers included in the present thesis, one must take into account that the studies were designed for primary outcomes other than transcriptome profiling, supporting the use of the RCT design.

In paper I and II we used an exploratory approach to examine gene expression differences and differences in changes between groups. As described above, the subjects in paper I were regrouped into tertiles based on plasma n-6 and n-3 PUFA levels and SFA to PUFA ratio at end of study, and the highest and lowest tertile within each grouping were compared with regard to gene expression differences. Since the subjects included in this paper were healthy individuals, and two of the three original groups consumed fish oil, the differences in plasma fatty acid levels defining the groups were subtle. Furthermore, due to the small number of subjects, the subjects were separated into groups based on tertiles. Thus, the gene expression results in paper I may have been different if larger variation in plasma fatty acid levels between groups. However, because of the cross-sectional design we cannot assume any causal relationship between plasma fatty acids levels and gene expression in paper I. The cross-sectional design is limited by the fact that the exposure and the outcome is measured at the same time point, in which the temporal sequence of their occurrence cannot be elucidated. Furthermore, the design is prone to confounding. However, a main function and strength of exploratory and descriptive studies is the ability to generate new hypotheses, which may be further investigated in studies with other designs.

In paper II, one must take into account that the study is limited by the low baseline TG levels; 1.5 mmol/L in responders and 0.8 mmol/L in non-responders. The TG reduction observed after n-3 PUFA supplementation depends on the baseline TG level [60], hence, the low baseline TG level in this study may have resulted in a lower percentage of responders

compared to other studies. Furthermore, the power of the study was challenged by the TG responder definition applied in paper II, in which only subjects with a clinically relevant TG reduction larger than the assumed 20 % day-to-day variation [151] were defined as responders. In contrast, other studies have defined all subjects with a TG reduction as responders [166, 167], enabling the inclusion of a larger number of participants in the responder group. A limitation in these studies is, however, that day-to-day variation in TG measurements is not accounted for. Furthermore, in responder studies in general it is difficult to distinguish whether the response is a result of the intervention or a result of random variation (regression to the mean). Future studies investigating TG responders to n-3 PUFA intake should therefore include participants with high enough baseline TG levels to ensure a clinically relevant TG response and a sufficient number of responders, and should be tested in well-designed studies for this purpose.

5.1.2 Microarray analysis

In all three papers of this thesis, PBMC gene expression was analyzed by use of microarray technology. This technology is a comprehensive, sensitive and well-validated technique, which enables the expression of thousands of genes to be determined simultaneously, within a sample or across a study [155]. Although RNA-seq technology is now more widely used, microarray-based gene expression profiling is still used in dietary intervention studies due to its ease of use, robust performance, reproducibility, and low per-sample cost. Furthermore, open gene expression databases, such as GEO [168], contain a vast amount of microarray experiments, which can be re-analyzed, integrated or combined with newly generated data in the context of modern integrated systems biology research. However, despite the promising opportunities of microarray technology, there are also challenges regarding this methodology which must be considered. These include pre-analytical issues, the technology itself, data analysis and interpretation of the results, as discussed in the next sections.

Variance associated with laboratory procedures is well known to influence microarray results [169]. Once a transcriptomic study has been performed, RNA quantity and quality need to be verified. The quality of the RNA is largely dependent upon sampling

and RNA isolation methods. We isolated RNA from PBMCs using the RNeasy Mini Kit (Qiagen), and measured RNA quantity and quality using the ND 1000 Spectrophotometer (Seven Werner AB) and Agilent bioanalyser (Agilent Technologies Inc.), as recommended by the Genomics Core Facility employed in this project. The procedures related to RNA isolation were performed at one study center and by one individual, minimizing variation related to sampling. Moreover, to minimize variation between arrays, subsequent labelling and hybridization of RNA was performed by one individual at the Genomics Core Facility [155].

Microarray technology has been the most used technology for transcriptome profiling in the subsequent years. Problems reported from early microarray studies included limited reproducibility and comparability between platforms, which generated skepticism against use of this technology. Indeed, microarray technology has shown some drawbacks regarding quantification [121]. In microarray analysis, the levels of hybridization are quantified using fluorescence that is converted into expression measurements. Since the fluorescent readout of hybridization intensities may change between different laser scanners, this resulted in low reproducibility between different laboratories. However, this issue was later overcome with the introduction of the MicroArray Quality Control consortium [170], which led to the development of quality control standards to establish a framework for the use of microarray technology in clinical and experimental settings [171]. Subsequently, quantitative reverse transcription polymerase chain reaction (RT-qPCR) has often been applied to validate the results from high-throughput platforms. RT-qPCR is a well-established method for analysis of gene expression that allows for the analysis of genes that vary greatly in abundance. Over the past decade, RNA-seq has emerged as an alternative method for gene expression profiling. The main difference between RNA-seq and microarrays is that the former allows full sequencing of the whole transcriptome while the latter only profiles predefined genes through hybridization [172]. This implies that RNA-seq may identify more differentially modulated transcripts, splice variants, and non-coding transcripts, and that these additional data may be informative for disease prediction, mechanistic investigations or biomarker discovery. An important advantage of this technology is the possibility of also detecting and quantifying low-expressed genes that could not be revealed by microarray analyses [121].

One of the major challenges to providing reproducible microarray data involves the lack of consensus regarding data analysis methods, challenging the effectiveness of the research process and making it less interactive. Data analysis is carried out in multiple consecutive steps, such as preprocessing and normalization, quality control, data filtering, imputation of missing values, identification of differentially expressed genes, and pathway-level analysis. Each step involves making choices regarding methods, cut-offs etc., which will influence the reported results; selecting a different method may result in a different list of genes. In this thesis, total RNA was analyzed by use of Illumina microarray technology, which is a common platform used for microarray based analysis of gene expression. Further, the Illumina intensity values were quality controlled, filtered to improve the statistical power (detection *p*-value < 0.01), and quantile normalized. The purpose of normalization is to eliminate or minimize technical variation [173]. We applied quantile normalization which was first developed for microarray array based gene expression and is now widely used across high-dimensional/-throughput omics platforms, including RNA-seq. Quantile normalization is a global adjustment normalization method that transforms the statistical distributions across samples to be the same and assumes that global differences in the distribution are induced by technical variation [174]. To determine gene expression differences between groups, we calculated log2 ratios between the baseline and end of study intensity values. The significance levels applied, and the gene lists included in the pathway analysis, were chosen based on the methodology of similar dietary gene expression studies in the field.

5.1.3 PBMCs as a model system

Although transcriptomics can be applied to any tissue, a major challenge in human dietary studies is the collection of appropriate analysis material. Transcriptomic profiling requires accurate sampling of material for the extraction of sufficient, high quality RNA. However, due to practical and ethical reasons, human dietary studies, which usually include healthy volunteers, are restricted to easily accessible samples of e.g blood. One way to obtain analysis material from relevant tissues or organs from healthy individuals is to biopsy relatively accessible tissues including muscle and subcutaneous adipose tissue.

Transcriptome profiling of such tissues is useful to explore important physiological functions, however, the method is limited by the invasive nature of the procedure and the low RNA yield.

PBMCs is a widely used surrogate tissue for studying gene expression and identify early risk markers in human dietary intervention studies [127]. Gene expression in PBMCs changes in response to dietary intervention, however, often it is not known whether these changes are comparable to other cells or tissues. PBMCs and hepatic cells seem to share similarities in terms of cholesterol homeostasis and may reflect hepatic regulation of cholesterol metabolism [175]. Thus, these immune cells may be a useful model system for hepatic lipid metabolism. Furthermore, it is well documented that dietary intervention studies affect the expression of various inflammatory genes and genes involved in lipid metabolism [143, 147, 148, 176]. Finally, in study 1 and 2, only one and two samples, respectively, had RIN value < 8, indicating that PBMCs provide RNA with high quality.

Since PBMCs is a heterogeneous immune cell population, it is important to control that the distribution of the different cell types is not altered by the intervention, as the expression level of a given gene might vary between e.g. lymphocytes and monocytes. In paper I, the percentage number of monocytes and lymphocytes did not significantly differ between the groups that were compared. Even though the percentage number was constant in the three comparisons, we also checked if cell subset-specific genes were differentially expressed between the groups. In paper III, there was no difference in change in white blood cell counts between the two intervention groups, implying that the changes in gene expression was not an effect of altered PBMC composition.

Human dietary intervention studies are known for their relatively small effect sizes [177]. This is probably because biological systems are quite robust and try to maintain homeostasis. Dietary interventions generally result in subtle changes in gene expression, and, as expected, we observed rather low fold changes in PBMC gene expression in the papers of this thesis. However, subtle changes may lead to profound biological effects, especially in the long term. Moreover, subtle changes in several gene transcripts involved in the same biological pathway are of high interest, as this could lead to alterations of pathways involved in central processes related to health and disease.

5.1.4 Statistical considerations

Sample size calculation in study 1 and 2 has been described previously [66, 150], and was not relevant for the papers in this thesis since the studies were designed for primary outcomes other than the outcomes reported in the respective papers. Furthermore, in paper I and II (study 1), we applied an exploratory design. In paper III, we adjusted for multiple comparisons in the gene expression analyses to decrease the probability of type I errors. The risk of type I errors, or false positive findings, arise when multiple outcomes are reported without any adjustment of the *p*-values [178]. Due to the explorative approach in paper I and II, we did not adjust for multiple comparisons in these papers. This was because we wanted to decrease the probability of type II errors, the reporting of false negative results, which could arise because of small sample sizes [178].

5.2 Discussion of results

In this thesis, we explored effects and associations of dietary fat and plasma fatty acid levels on gene expression of specific predefined genes, the whole transcriptome response and pathways in PBMCs (paper I and III). Further, we explored differences in the whole transcriptome response and pathways in PBMCs of TG responders and non-responders to n-3 PUFA supplementation (paper II). In the following, the main findings of the thesis is discussed in light of previous literature and studies in the field, as well as future perspectives.

5.2.1 Dietary fat, gene expression and CVD risk

The CVD reducing effect of improving the dietary fat quality is largely mediated through modulation of serum lipids [4]. Serum LDL cholesterol is a well-established biomarker, or surrogate end point, reflecting CVD risk [179-181], and it has been estimated that a reduction in serum LDL cholesterol of 1 mmol/L reduces CVD risk by 22 % over a median of 5 years [182]. Our research group has previously reported a reduction in serum total and LDL cholesterol of respectively 9 % and 11 % when replacing SFAs with mostly n-6 PUFAs in the diet for eight weeks (study 2) [66]. These results were consistent in paper III, where we included a slightly lower number of the study sample included in the previous analyses. In line with our results, reductions in serum LDL cholesterol has been observed in studies investigating the effect of a healthy Nordic diet, which includes an improved fatty acid composition, on CVD risk factors [183-185]. Furthermore, the results are in accordance with the results observed in the Dietary Intervention and VAScular function study, where a successful implementation of a food-exchange model achieved the dietary target intake for exchanging SFAs with MUFAs or n-6 PUFAs, leading to reduced serum total and LDL cholesterol in a free-living population [72, 186].

Serum LDL cholesterol is highly dependent on hepatic LDLR abundance and activity [103, 113, 114]. This is well demonstrated in humans with familial hypercholesterolemia; an autosomal dominant disease caused by a mutation in one of three genes; *LDLR, APOB* or proprotein convertase subtilisin/kexin type 9 (*PCSK9*), leading to impaired clearance of LDL cholesterol from the circulation [187]. Animal studies and *in vitro* studies have shown that

dietary fatty acids regulate LDL receptor activity, protein, and mRNA abundance [115]. Data from studies with hamsters and pigs showed that dietary SFAs decrease while dietary PUFAs increase membrane-associated LDLR in the liver [188, 189]. These distinct effects of dietary fatty acids were also paralleled by changes in *LDLR* mRNA levels. In human hepatic cells, LA has been shown to up-regulate LDLR gene and protein expression [190], and it has been suggested that this effect is probably the main mechanism by which increased intake of LA lowers serum cholesterol levels [68]. Furthermore, reducing dietary SFAs has been associated with increased LDL receptor abundance of magnitude similar to the decrease in serum LDL cholesterol in humans [191]. However, few studies have investigated the effect of dietary fat quality on *LDLR* expression in humans. Our research group has recently reported an increase in PBMC *LDLR* expression when replacing dietary SFAs by mostly n-6 PUFAs in study 2, analyzed by RT-qPCR [116]. Although not significant, we also observed a small increase in PBMC *LDLR* expression analyzed by use of microarray analysis in this study (paper III). In line with these findings, two postprandial studies have found a reduction in the PBMC expression level of *LDLR* after intake of a high SFA challenge compared to a high PUFA or MUFA challenge [117, 118]. Furthermore, in paper III, we observed a differential expression of *DNAJC10*, a gene that has been associated with LDLR folding [192]. The significance of this finding in relation to dietary fat intake in humans should however be further investigated in other studies.

Interestingly, several gene transcripts associated with other processes related to atherosclerosis and CVD risk, including regulation of blood pressure (*ATP2B1, GUCY1B1*) and vascular smooth muscle cell (VSMC) proliferation (*FGF18, SEPTIN4*) [193-197], were differentially altered between the groups in paper III. *ATP2B1* genomic regions have also been associated with hyperlipidemia [198], and in a candidate gene association study this gene was associated with coronary artery calcification in chronic kidney disease and MI in the general population [199]. Coronary artery calcification is a quantitative estimate of coronary atherosclerosis and a useful predictor of CHD [200, 201]. In contrast, *GUCY1B1* has been associated with cardio-protective effects against MI [202]. Consequently, the down-regulation of *ATP2B1* and the up-regulation of *GUCY1B1* reported in paper III support the CVD reducing effect of replacing dietary SFAs with PUFAs. VSMC proliferation plays key role in the development of atherosclerosis. A down-regulation of *FGF18* and an up-regulation of *SEPTIN4*, as reported in paper III, has been associated with reduced VSMC proliferation.

Furthermore, the expression of *ATP2B1* and *FGF18* was paralleled by an increase in serum total cholesterol, whereas the expression of *GUCY1B1* was paralleled by a decrease in serum LDL cholesterol, further implicating an association between these genes and atherosclerosis.

Although atherosclerosis was formerly considered as a lipid storage disease, it is now well established that inflammation has a fundamental role in mediating all stages of this disease [16, 73, 203]. SFAs and n-3 PUFAs are associated with opposing effects on inflammation, in which n-3 PUFAs may reduce whereas SFAs may increase inflammation. However, controversy exist regarding the effects of n-6 PUFAs on inflammation, as these fatty acids (AA) are linked to pro-inflammatory eicosanoid production and may also inhibit the synthesis of anti-inflammatory eicosanoids from EPA and DHA [80]. Furthermore, studies investigating the relationship between n-6 PUFA intake and hsCRP level are inconsistent [204]. In a meta-analysis of RCTs, the consumption of a healthy dietary pattern such as the Mediterranean diet and a healthy Nordic diet, which include an increased proportion of n-6 PUFAs, decreased circulating hsCRP levels and attenuated the inflammatory state [205]. Moreover, in several RCTs, intake of n-6 PUFAs was not related to inflammation, oxidative stress or endothelial activation [72, 206, 207]. Despite an increased intake of n-6 PUFAs in the Ex-diet group compared to the C-diet group in study 2 (paper III), we did not observe any differences in serum levels of hs-CRP, IL-6, soluble tumor necrosis factor receptor 1 (sTNFR1), and interferon (IFN)-γ. However, the majority of the differentially enriched pathways observed between the groups was related to immune response and inflammation. This was expected as we analyzed the whole transcriptome response of PBMCs consisting mostly of monocytes and lymphocytes. Nevertheless, modulation of gene expression and pathways related to inflammation has also been observed in other studies investigating the effect of a healthy diet or fat intake on gene expression in PBMCs, as well as in other tissues, implying that the observed effects on pathway level may also be caused by changes in dietary fat quality [133, 135, 143, 208]. Furthermore, since changes in gene expression occur prior to changes at protein level, the observed changes may be an early effect of alterations in dietary fat quality. As described in the introduction, long chain PUFAs may reduce inflammation by binding to PPARα and subsequently inhibit NF-κB activity and transcription of inflammatory-related genes. Furthermore, the anti-inflammatory effects of n-3 PUFAs may be mediated through the

GPR120 [209, 210]. SFAs, on the other hand, have been proposed as non-microbial TLR agonists that promote inflammatory activation [91]. Studies have shown that the SFA lauric acid stimulates pro-inflammatory gene expression by binding to TLR2 and TLR4, thereby mediating NF-κB and COX-2 activation and expression. In contrast, consumption of fish oil rich in n-3 PUFAs inhibits the TLR4-induced signaling pathways and target gene expression [211, 212].

The most regulated pathways between the groups in paper III were related to immune response, apoptosis and survival, and platelet aggregation, which are processes related to atherosclerosis. In line with our results, modulation of PBMC gene expression related to apoptosis has previously been observed in other dietary intervention studies in which the fat quality was improved or fish oil supplementation was given [135, 148]. Furthermore, we also observed modulation of pathways related to apoptosis in paper II where we compared TG responders and non-responders to n-3 PUFA supplementation. Interestingly, several pathways related to platelet aggregation were differentially modulated between the groups, and the up-regulation of *GUCY1B1* contributed to the modulation of one of these pathways. A previous study has reported that knockout of *GUCY1B1* causes lack of nitric oxide effect on platelet aggregation [202], leading to increased platelet aggregation. Thus, an increased expression of this gene, as observed in paper III, may have a beneficial effect on platelet aggregation supporting a possible CVD reducing effect of *GUCY1B1*.

5.2.2 Plasma fatty acids, gene expression and CVD risk

Since LA and ALA are essential fatty acids, and the conversion of these fatty acids into long chain PUFAs is limited, plasma PUFA levels may be objective biomarkers of dietary intake [213, 214]. Hence, in paper I, we used a cross-sectional and exploratory approach examining the association of plasma n-6 and n-3 PUFA levels and SFA to PUFA ratio to PBMC gene expression related to lipid metabolism. The main finding in this paper was that SFA to PUFA ratio was associated with the largest number of differentially expressed genes, followed by n-6 and n-3 PUFA level. Interestingly, several genes involved in regulation of cholesterol homeostasis were differentially expressed depending on SFA to PUFA ratio, supporting the

hypothesis that PBMCs reflect hepatic cholesterol metabolism at a molecular level and that plasma fatty acid levels may affect the expression of these genes.

Cholesterol uptake and synthesis is tightly regulated by cholesterol sensors in cellular membranes [113, 114]. When cholesterol content falls below approximately 5 % of total lipids in ER membranes, the SREBP pathway is activated, resulting in transcription of *LDLR* and cholesterol synthesis genes. In paper I, we observed a lower expression of *HMGCR*, which is the rate controlling enzyme in cholesterol biosynthesis and also the target of statins drugs, among subjects with the highest compared to subjects with the lowest SFA to PUFA ratio. Furthermore, genes involved in the proteolytic regulation of SREBP (*INSIG2 and ERLIN2*) were higher expressed among subjects with the highest SFA to PUFA ratio. Insig-2 binds to SCAP in the ER and prevents the movement of the SCAP-SREBP complex to the Golgi apparatus for further processing and eventually transcription of *HMGCR* and other SREBP target genes. In addition, Insig proteins play an important role in oxysterol-regulated cleavage of SREBP [215]. *ERLIN2* encodes a cholesterol-sensing protein which has been suggested to stabilize the Insig-SCAP-SREBP complex in the ER [216] . Thus, a higher expression of *INSIG2* and *ERLIN2* is in line with the lower expression of *HMGCR* observed in paper I. In accordance to our results, Esser et al. found that a high SFA challenge decreased the postprandial expression of genes involved in cholesterol biosynthesis and uptake, including *LDLR* and *HMGCR* [118]. Although we did not observe a significant difference in the expression of *LDLR* in paper I, we observed a lower expression of *ABCA2*, a gene that has been associated with increased *LDLR* expression and decreased cellular cholesterol levels [217, 218], in the highest compared to the lowest SFA to PUFA ratio group. Together, these findings may reflect an intracellular status of excess cholesterol among subjects with the highest SFA to PUFA ratio.

Although study 1 was designed to investigate health effects of fish oil supplementation, and we therefore at least could expect inter-individual differences in plasma n-3 PUFA levels at end of intervention, we observed rather few differentially altered gene transcripts associated with plasma n-3 PUFA level in paper I. Further, we observed no significant differences in serum lipids between subjects with the highest and lowest plasma n-3 PUFA level. In support to these findings, the baseline level and the change in plasma n-3 PUFA level did not differ between TG responders and non-responders to n-3 PUFA supplementation in paper II, indicating that n-3 PUFA levels in plasma are not necessarily

important for the TG-lowering effect. Indeed, n-3 PUFAs mediate many of their biological effects after being incorporated into plasma membrane phospholipids [219]. Thus, the level of n-3 PUFAs in red blood cells, also called the omega-3 index, may be a better measure of an individual's n-3 PUFA status. On the other hand, the n-3 PUFA dose provided in study 1 was quite low (1.6 g/d EPA + DHA), and the study population consisted of healthy individuals with TG levels within the recommended range, which may have had an impact on the gene expression results.

In study 2, the subjects received either a C-diet or an Ex-diet with an improved fatty acid composition, substituting SFAs with mainly n-6 PUFAs. After eight weeks of intervention, we observed that the plasma LA level was significantly increased in the Ex-diet group compared to the C-diet group. Thus, we performed additional gene expression analyses depending on change in plasma LA level for the whole study population. In paper III, we report a large number of differentially altered gene transcripts depending on change in plasma LA level. Interestingly, there was a large covariation between the gene expression results from this analysis and the between group analysis, including changes in *FGF18, SEPTIN4* and *GUCY1B1*, implying that changes in plasma LA level may be the major driver behind the gene expression differences observed between the groups. However, as this analysis may also be confounded by the intervention, one should interpret the results with caution.

5.2.3 Inter-individual differences in response to dietary fat

Dietary guidelines recommend to increase the consumption of fish or fish oil containing the long chain n-3 PUFAs EPA and DHA. This recommendation is based on numerous studies demonstrating that n-3 PUFAs have cardio-protective effects [42]. One of the main CVD-reducing effects of n-3 PUFAs is the TG-lowering effect, which has been demonstrated in many studies with n-3 PUFA supplementation [59, 60]. However, there are large inter-individual variation in the TG-lowering effect of n-3 PUFAs, and studies have shown that about 30–40 % of participants do not obtain reduced TG levels following n-3 PUFA supplementation [166, 220, 221]. In study 1 in this thesis, an even higher number (about 65%) of participants receiving fish oil did not reduce their TG levels. This may be explained

by the relatively low n-3 PUFA dose supplemented as well as the low baseline TG level in this study including healthy subjects, since the TG reduction is dependent on these parameters [59, 60]. Correspondingly, the participants who had a TG reducing effect in paper II (study 1) had higher baseline TG level than non-responders. In line with these results, Rudkowska et al. found that non-responders had lower fasting baseline TG level than responders [167]. Furthermore, they found that non-responders had lower baseline glucose and insulin, and higher HDL cholesterol, than responders, suggesting that responders may have a less healthy phenotype.

It has previously been shown in a genome-wide association study (GWAS) that genotype explains about 20 % of the variation in TG response to n-3 PUFA supplementation in the Fatty Acid Sensor (FAS) study [222]. However, in the same study, a more refined and improved genetic risk score (GRS) has recently showed that GRS may explain almost 50 % of the variation in TG response [223]. Among others, polymorphisms in the genes apolipoprotein E (*APOE*), acetyl-CoA carboxylase α (*ACACA*), ATP citrate lyase (*ACLY*), cluster of differentiation 36 (*CD36*), retinoid X receptor α (*RXRA*), and acyl-CoA oxidase 1 (*ACOX1*), have been found to affect the TG response to n-3 PUFA intake [224-227]. These genes are involved in processes important for the TG-lowering effect of n-3 PUFAs, including *de novo* lipogenesis and β-oxidation. Furthermore, factors such as age, gender, baseline n-3 PUFA level, diet, and other lifestyle factors, are probably important [228].

Gene-environment interactions leading to gene expression differences may explain some of the variation in TG response to n-3 PUFA intake. Thus, in paper II we explored baseline differences and differences in changes in PBMC gene expression profiles between TG responders and non-responders to n-3 PUFA supplementation. TG responders were defined as subjects having a larger TG reduction than the assumed 20 % day-to-day variation and non-responders as having a TG change between –20 % and +20 % after n-3 PUFA supplementation. By use of pathway analysis, we found that particularly pathways related to development and apoptosis, immune function, and LPA signaling were differentially altered in responders compared to non-responders. In line with our results, immune-related pathways were altered in PBMCs of elderly subjects allocated to fish oil supplementation [143]. Furthermore, it has previously been reported that pathways related to apoptosis and immune function were altered among subjects receiving fish oil compared to the control group in study 1 [148]. As discussed above, this may be expected as PBMCs

are cells of the immune system. Furthermore, n-3 PUFAs may modulate immune responses through altering NF-κB and PPAR-induced gene expression, and by acting as precursors for the production of anti-inflammatory and pro-resolving lipid mediators [83, 84]. Interestingly, we also observed differences in pathways related to LPA signaling between non-responders and responders in paper II. LPA is a lipid containing a fatty acid that may vary in length and degree of unsaturation, and different forms of LPA may differentially affect LPA signaling through binding and activation of LPA receptors (LPAR) [229]. LPARs are expressed in lymphocytes where they affect cytokine secretion, chemotaxis, and proliferation, and LPA signaling may be involved in the development of atherosclerosis [229, 230]. In paper II, responders and non-responders had different levels of various plasma fatty acids that may reflect different plasma levels of various chemical forms of LPA [229]. Similarly, Rudkowska et al. found that responders had a greater increase in unsaturated fatty acids in glycerol-phosphatidylcholines, which can be used as LPA precursors, compared to non-responders [167, 229].

One of the key mechanistic links between dietary fat and CVD risk is the LDL cholesterol raising effect of SFAs. Thus, it is recommended to limit intake of dietary SFAs to < 10 E% [29]. As described above, replacing dietary SFAs by particularly PUFAs reduces serum LDL cholesterol [4]. However, also the serum LDL cholesterol response to dietary fat intake displays inter-individual variation [46]. In study 2, 12 individuals (25.5 %) receiving the Ex-diet with an improved fatty acid composition did not reduce or even increase their serum LDL cholesterol levels. This finding lend further support to the results observed in the National Cholesterol Education Programme's (NCEP) Step 2 diet, a diet low in total fat and SFAs, which reduced serum LDL cholesterol levels within the range of +3 to -55 % in men and +39 to -39 % in women [231]. Furthermore, inter-individual variation in serum LDL cholesterol has also been observed in response to increased intake of SFAs in two dietary RCTs [72, 232]. After controlling for confounding, extrinsic factors and dietary compliance, these studies suggest that the variation is highly attributed to intrinsic factors such as polymorphisms and gene expression. In particular apoE genotype is associated with variation in serum LDL cholesterol. Two polymorphisms (rs429358 and rs7412) in this gene produce different isoforms of apoE with variable capacity to function as ligands for the binding of TRLs and their remnants, and HDL, to cell surface receptors, including LDL receptors. These polymorphisms are reported to account for up to 8-10 % of the variance in

serum LDL cholesterol [233], primarily by influencing the regulatory pool of intracellular free cholesterol and LDLR activity. ApoE genotype has also been linked to variation in serum LDL cholesterol response to changes in dietary SFAs [234, 235]. In particular, carriers of the apoE4 isoform tend to have elevated serum LDL cholesterol (5–10 %) and are consistently more responsive to changes in SFAs. Few dietary intervention studies have however investigated effects of dietary fat quality on serum LDL cholesterol, as well as other serum lipids, by including and allocating subjects into groups based on genotype. Furthermore, studies investigating gene expression differences between LDL responders and non-responders to interventions with dietary fat may reveal new molecular mechanisms explaining the variation in LDL response to dietary fat intake.

6 Conclusion

The present thesis concludes with the following:

- Plasma SFA to PUFA ratio is associated with PBMC gene expression related to cholesterol homeostasis in healthy subjects.

- TG responders and non-responders to n-3 PUFA supplementation have different baseline PBMC gene expression profiles. Furthermore, they have differentially altered PBMC gene expression profiles and pathways after seven weeks, which may partly explain the variation in TG response to n-3 PUFA intake.

- Replacing dietary SFAs with PUFAs for eight weeks alters PBMC gene expression and pathways related to CVD risk in subjects with moderate hypercholesterolemia.

In summary, our findings support the hypothesis that dietary fat quality is associated with PBMC gene expression and pathways, which may contribute to extend our understanding of how dietary fat affects CVD risk. Hence, use of transcriptomics in human dietary intervention studies may be a useful approach to explore the role of dietary fat on CVD risk at a molecular level. Furthermore, this approach may also extend our understanding of why individuals respond differently to n-3 PUFA intake.

7 Future perspectives

Several aspects discussed in this thesis should be further examined in future studies. First, despite many years of research, controversies still exist regarding the effect of dietary fat on CVD risk. Thus, molecular studies investigating the underlying mechanisms are needed, including studies combining different omics approaches to explore how changes at different levels interact to produce a phenotype. Trials that focus on effects in one single person, the n-of-1 trials, may also be useful when integrating data from different omics technologies to understand how interventions affect entire molecular pathways [236].

Furthermore, studies show inter-individual variation in response to dietary fat intake, which harbor significant potential to confound the relationship between dietary fat and CVD risk. Thus, well-designed and sufficiently powered studies are needed to further investigate this variation. This includes variation in TG response, as well as LDL response which has been investigated to a lesser extent. Moreover, identification and application of simple biomarkers of this phenomenon would make it possible to tailor dietary guidelines to responsive individuals, who stand to gain a greater benefit to their cardiovascular health, leading us towards personalized nutrition.

Finally, in a GWAS it was found that 20 % of the variability in serum TG response to n-3 PUFA supplementation might be explained by genetic polymorphisms [222]. In particular, apoE polymorphisms have been associated with variation in TG response to n-3 PUFA intake. Furthermore, apoE genotype has also been associated with variation in serum LDL cholesterol response to SFA intake [46]. In our studies, we do not have permission to investigate genetic polymorphisms. However, it will be important to apply for these permissions in future studies. Consequently, we may investigate effects of dietary fat on CVD risk, and the determinants of the inter-individual variation, by including participants based on genotype.

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Papers I-III

I

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Plasma fatty acid levels and gene expression related to lipid metabolism in peripheral blood mononuclear cells: a cross-sectional study in healthy subjects

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Abstract

Background: Solid evidence indicates that intake of marine n-3 fatty acids lowers serum triglycerides and that replacing saturated fatty acids (SFA) with polyunsaturated fatty acids (PUFA) reduces plasma total cholesterol and LDL cholesterol. The molecular mechanisms underlying these health beneficial effects are however not completely elucidated. The aim of this study was therefore to investigate the expression of genes related to lipid metabolism in peripheral blood mononuclear cells (PBMC) depending on the plasma levels of n-6 and n-3 fatty acids and the SFA to PUFA ratio.

Methods: Fifty-four healthy subjects were grouped into tertiles ($n = 18$) based on plasma levels of n-6 and n-3 fatty acids and the SFA to PUFA ratio. The PBMC gene expression levels among subjects in the highest versus the lowest tertiles were compared. In total, 285 genes related to cholesterol and triglyceride metabolism were selected for this explorative study.

Results: Among the 285 selected genes, 161 were defined as expressed in the PBMCs. The plasma SFA to PUFA ratio was associated with the highest number of significantly different expressed genes (25 gene transcripts), followed by plasma n-6 fatty acid level (15 gene transcripts) and plasma n-3 fatty acid level (8 gene transcripts). In particular, genes involved in cholesterol homeostasis were significantly different expressed among subjects with high compared to low plasma SFA to PUFA ratio.

Conclusion: Genes involved in lipid metabolism were differentially expressed in PBMCs depending on the plasma fatty acid levels. This finding may increase our understanding of how fatty acids influence lipid metabolism at a molecular level in humans.

Keywords: Cardiovascular risk factors, Nutrition, Fat quality, Plasma fatty acids, Lipid metabolism, Gene expression, Peripheral blood mononuclear cells

Background

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide [1]. Dyslipidemia, including elevated levels of plasma total cholesterol (total-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG), is a major risk factor for CVD. Dietary fatty acids play a significant role in modulating plasma lipids, thereby influencing the CVD risk [2]. Solid evidence indicates that intake of marine n-3 fatty acids, and replacing saturated fatty acids (SFA) with polyunsaturated fatty acids (PUFA), prevents CVD and CVD mortality [3–7]. One of the CVD reducing effects of marine n-3 fatty acids is the TG lowering effect, while replacing SFAs with PUFAs has been shown in several randomized controlled trials to reduce plasma total- and LDL-C [8–10]. Animal studies and in vitro experiments have demonstrated different molecular mechanisms of how marine n-3 fatty acids reduce hepatic very low-

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density lipoprotein (VLDL) production and increase the VLDL clearance [11, 12]. The molecular mechanisms behind the total- and LDL-C lowering effects of replacing SFAs with PUFAs are however less clear. Therefore, studies investigating the molecular mechanisms underlying the health effects of SFAs and PUFAs in humans are warranted.

In humans, linoleic acid (LA; 18:2n-6) and alpha-linolenic acid (ALA; 18:3n-3) are not biosynthesized de novo. Since the conversion of these fatty acids into long chain PUFAs is limited, plasma PUFA levels have been shown to be objective biomarkers of dietary intake [13, 14]. Hence, using plasma fatty acids is an alternative approach to examine the association between dietary fat quality and CVD risk.

The ability of fatty acids to regulate gene transcription may account for their effects on lipid metabolism. Fatty acids regulate gene transcription directly by binding as ligands to specific transcription factors, thereby controlling the activity of the transcription factor, or indirectly by regulating different signaling pathways controlling the nuclear abundance of transcription factors [15–17]. In particular, there is considerable evidence that PUFAs modulate the transcription of genes involved in lipid metabolism by regulating the activity of the nuclear receptors peroxisome proliferator activated receptors (PPAR) and liver x receptors (LXR) or by suppressing the nuclear abundance of the sterol regulatory binding proteins (SREBP) [17, 18]. Our understanding of how SFAs modulate the expression of genes encoding proteins related to lipid metabolism is however more limited [19].

In order to get a comprehensive understanding of how dietary fat quality affect lipid metabolism, to prevent dyslipidemia in humans, we need a suitable model system. Changes in gene expression occur prior to changes in protein levels, and gene expression analysis is therefore a valuable and sensitive technique measuring early changes related to diet [20, 21]. Peripheral blood mononuclear cells (PBMC) include lymphocytes and monocytes which circulate around in the body and are exposed to both environmental factors and metabolic tissues. Studies have shown that PBMCs may be used as a surrogate model for liver metabolism since these cells reflect hepatic regulation of cholesterol metabolism as well as metabolic and immune responses [22–26].

Some postprandial studies have examined the effect of fat intake on the mRNA level of genes involved in lipid metabolism in PBMCs [26, 27]. To our knowledge, no studies have particularly focused on the impact of plasma fatty acid levels on PBMC gene expression related to lipid metabolism. The aim of the present study was therefore to investigate the relation of plasma levels of n-6 and n-3 fatty acids, and SFA to PUFA ratio, to PBMC gene expression specifically related to lipid metabolism using cross-sectional data from a human intervention study [28].

Methods

Study design and participants

Fifty-four healthy, non-smoking men and women aged 18–50 years were included in this cross-sectional sub-study of a randomized controlled trial designed to investigate the health effects of fish oil with different quality focusing on lipids, oxidative stress, and inflammation [28, 29]. In addition, we have analyzed the plasma lipidomic profile, the PBMC gene expression profile, and the effects on lipoprotein subclasses from this dietary intervention study [30–33]. A detailed description of the protocol, participant recruitment and enrolment, inclusion and exclusion criteria, and compliance is described elsewhere [28]. In the present study, data from the end of intervention was utilized.

The study population was grouped into tertiles three times according to the plasma fatty acid levels and the SFA to PUFA ratio by arranging samples from the highest to the lowest value. First, the subjects were grouped according to the plasma level of total n-3 fatty acids which included ALA, eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3), and docosahexaenoic acid (DHA; 22:6n-3). Second, the subjects were grouped according to the plasma level of total n-6 fatty acids which included LA and arachidonic acid (AA; 20:4n-6). Finally, the subjects were grouped according to the plasma SFA to PUFA ratio, which included the SFAs myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0), and the PUFA included the sum of plasma total levels of n-3 and n-6 fatty acids. The subjects in the highest ($n = 18$) and the lowest ($n = 18$) tertile were compared.

The intervention study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Regional Committee of Medical Ethics (approval no. 6.2008.2215) and the Norwegian Social Science Data Services (approval no. 21924). Written informed consent was obtained from all participants. The study was registered at www.clinicaltrials.gov (ID no. NCT01034423).

Clinical and biochemical measurements

Procedures regarding clinical and biochemical measurements have been described earlier [28]. In brief, fasting venous blood samples were collected after an overnight fast (≥ 12 h). Serum was obtained from silica gel tubes (Becton Dickinson Vacutainer Systems, UK) and kept at room temperature for 30 min before centrifugation (1500*g*, 12 min). Plasma was obtained from EDTA tubes (Becton Dickinson Vacutainer Systems, UK), immediately placed on ice and centrifuged within 10 min (1500*g*, 4 °C, 10 min). EDTA tubes with whole blood

were kept at room temperature for a maximum of 48 h before counting the total number of lymphocytes and monocytes. Fasting serum concentrations of total-C, LDL-C, HDL-C, and TGs, as well as lymphocyte and monocyte counts, were measured by standard methods in a routine clinical laboratory (Fürst Medical Laboratory, Oslo, Norway).

Fatty acid analysis

Plasma lipids were extracted by use of the Bligh and Dyer method [34], as described by Ottestad et al. [28]. Fatty acids in Bligh and Dyer extract were derivatized and analyzed as methyl esters on a GC (HP 6890) equipped with a BPX-70 column (SGE Analytical Science Private Limited, Melbourne, Australia). The plasma level of the individual fatty acids is expressed as mass percentage (%wt) of total fatty acids in plasma.

PBMC and RNA isolation

PBMCs were isolated by using the BD Vacutainer Cell Preparation tubes according to the manufacturer's instructions (Becton, Dickinson San Jose, CA, USA), as described previously [31]. This is a well-documented and standardized method to collect mononuclear cells with high purity (above 90%), and according to the manufacturer, approximately 80% of the cells are lymphocytes and 12% are monocytes. Pellets were stored at − 80 °C for further RNA analysis. Total RNA was isolated from the PBMC samples using RNeasy Mini Kit (Qiagen). RNA quantity and quality were measured using the ND 1000 Spectrophotometer (Seven Werner AB) and Agilent bioanalyser (Agilent Technologies Inc.), respectively.

Microarray analysis and selection of genes

Gene expression was analyzed by hybridization to an Illumina HumanHT-12 v4 Expression BeadChip and scanned on an Illumina HiSCan microarray scanner (Illumina Inc., CA 92122). Illumina GenomeStudio was used to transform bead-level data to probe-level intensities and statistics, which were exported raw for bioinformatics analysis. Quantile normalization of the Illumina intensities was performed, and probes without a detectable expression (detection $P > 0.01$) in at least 10% of the samples were excluded from further analyses. From the 48,000 probes presented on the Illumina array, 21,236 probes were defined as expressed in the current study. A more detailed description of the protocol is given elsewhere [31]. The raw data are available from the Gene Expression Ominbus (GEO) (accession number GSE111567).

A total of 285 genes encoding proteins related to cholesterol and TG metabolism were selected for this explorative study. The genes were selected based on relevant gene sets related to cholesterol and TG metabolism (26 gene sets) in the Molecular Signature Database v6.0 [35] limited to collection C5 (Gene ontology, GO), as well as literature summarizing loci associated with different lipid traits [36]. Out of the 285 genes, 161 genes were defined as expressed on the HumanHT-12 v4 microarray and included in the statistical analyses (Additional file 1). The list of the differentially expressed genes was based on the lowest P values for genes containing multiple probe set. The expression levels of the differentially expressed genes which were expressed by more than one probe are shown in Additional files 2, 3, and 4.

Statistical analysis

Differences in Log2 gene expression between subjects in the highest and lowest tertiles were compared by independent samples t test. No adjustment for multiple testing was performed because of the explorative design of the study. Significantly different expressed genes were further correlated with clinical and biochemical parameters by Pearson's correlation. Differences in subject characteristics and plasma fatty acid levels between subjects in the highest and lowest tertiles were compared by independent samples t test or Mann-Whitney U test when normally and not normally distributed, respectively. All statistical analyses were performed using R open source software version 3.4.1 [37]. P values < 0.05 were considered significant.

Results

Subject characteristics and plasma fatty acid levels

The subjects included in the study were young and middle-aged adults with a median age of 25 (22-30) years and BMI (22.6 ± 2.6 kg/m^2) and serum lipids within the normal range as shown in Table 1. There was a skewed distribution of men and women (15 men and 39 women) among the subjects. The plasma levels of fatty acids in the study population are shown in Table 1.

The characteristics and plasma fatty acid levels of subjects in the highest ($n = 18$) and lowest ($n = 18$) tertiles based on plasma levels of n-6 and n-3 fatty acids, and SFA to PUFA ratio, are presented in Table 2. The serum level of TG was significantly lower among subjects in the highest compared to subjects in the lowest plasma n-6 fatty acid tertile ($P < 0.01$). In contrast, the serum level of TG was significantly higher among subjects in the highest compared to subjects in the lowest plasma SFA to PUFA ratio tertile ($P < 0.01$). There were no significant differences in any markers between subjects in the highest and the lowest plasma n-3 fatty acid tertile.

As expected, the plasma levels of EPA, DPA, and DHA were significantly higher among subjects in the highest compared to subjects in the lowest plasma n-3 fatty acid tertile ($P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively) (Table 2). The plasma levels of myristic and palmitic acid were significantly higher among subjects in the highest

Table 1 Characteristics and plasma fatty acid profile of the study population

	n 54
Male/female (*n*)	15/39
Age (years)	25 (22-30)
BMI (kg/m^2)	22.7 ± 2.6
Total-C (mmol/l)	4.8 ± 0.9
LDL-C (mmol/l)	2.7 ± 0.8
HDL-C (mmol/l)	1.5 ± 0.4
TG (mmol/l)	0.9 (0.7–1.1)
Plasma level of fatty acids (wt%)	
Total SFA	30.7 (29.5–31.9)
Myristic acid (14:0)	0.9 (0.7–1.1)
Palmitic acid (16:0)	21.5 (20.6–23.3)
Stearic acid (18:0)	7.9 ± 1.0
Total n-6	34.7 ± 3.9
LA (18:2n-6)	28.8 ± 3.6
AA (20:4n-6)	5.9 ± 1.1
Total n-3	6.3 ± 2.4
ALA (18:3n-3)	0.5 (0.5–0.6)
EPA (20:5n-3)	1.94 (0.7–2.7)
DPA (22:5n-3)	0.6 ± 0.2
DHA (22:6n-3)	3.2 ± (2.1–4.1)
Total PUFA	40.1 (38.9–48.1)
SFA to PUFA ratio	0.8 ± (0.7–0.8)

Values are presented as frequency, mean ± SD or median and 25th–75th percentiles

BMI body mass index, *Total-C* total cholesterol, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *TG* triglyceride, *SFA* saturated fatty acid, *LA* linoleic acid, *AA* arachidonic acid, *ALA* alpha-linolenic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid, *DHA* docosahexaenoic acid, *PUFA* polyunsaturated fatty acid

compared to subjects in the lowest plasma SFA to PUFA ratio tertile ($P < 0.01$ and $P < 0.01$, respectively) and significantly lower among subjects in the highest compared to subjects in the lowest plasma n-6 fatty acid tertile ($P < 0.01$ and $P < 0.01$, respectively). In addition, the plasma levels of LA and AA were significantly higher among subjects in the highest compared to subjects in the lowest plasma n-6 fatty acid tertile ($P < 0.01$ and $P = 0.02$, respectively). The percentage of monocytes and lymphocytes did not significantly differ among subjects in the highest compared to the lowest plasma n-3 fatty acid tertile, n-6 fatty acid tertile, and the SFA to PUFA ratio tertile, respectively (Table 2).

PBMC gene expression

Out of the 161 mRNA transcripts included in the study, 41 were significantly differently expressed depending on plasma fatty acid levels and the SFA to PUFA ratio (Fig. 1). The plasma SFA to PUFA ratio was associated with the highest number of significantly different expressed genes (25 gene transcripts, $P < 0.05$), followed by plasma n-6 fatty acid level (15 gene transcripts, $P < 0.05$), and n-3 fatty acid level (8 gene transcripts, $P < 0.05$), as shown in Fig. 1. Seven gene transcripts were associated with both plasma n-6 fatty acid level and plasma SFA to PUFA ratio, and one gene transcript was associated with both plasma n-3 fatty acid level and plasma SFA to PUFA ratio (Fig. 2). No gene transcripts were shared across plasma n-6 and n-3 fatty acid levels and plasma SFA to PUFA ratio. Differentially expressed genes associated with the plasma levels of n-6 and n-3 fatty acids and the SFA to PUFA ratio are presented in Tables 3, 4, and 5, respectively.

Differentially expressed genes between subjects in the highest and lowest plasma SFA to PUFA ratio tertile included genes encoding proteins involved in cholesterol homeostasis (Table 5). The mRNA levels of *insulin-induced gene 2* (INSIG2), *ER lipid raft associated 2* (ERLIN2), *Caveolin 1* (CAV1), and *COPII subunit SEC24* (SEC24) were significantly higher expressed among subjects in the highest compared to subjects in the lowest plasma SFA to PUFA ratio tertile. The mRNA levels of *scavenger receptor class B member 1* (SCARB1,) *ATP binding-cassette subfamily A member 2* (ABCA2), and *3-hydroxy-3-methylglutaryl-CoA reductase* (HMGCR) were significantly lower expressed among subjects in the highest compared to subjects in the lowest plasma SFA to PUFA ratio tertile. Interestingly, several of the differentially expressed genes associated with plasma SFA to PUFA ratio, including CAV1 and HMGCR, were opposite differentially expressed when comparing subjects in the highest versus the lowest plasma n-6 fatty acid tertile.

In order to examine whether the differentially expressed genes were associated with BMI or serum lipids, correlation analyses were performed (Table 6). BMI was significantly positively correlated to the expression levels of two genes (LACTB and SNX13) and significantly negatively correlated to the expression levels of five genes (ERLIN2, GSK3B, KLHL8, SCARB1, and SELS). The serum level of TG was significantly negatively correlated to the expression levels of five genes (DAGLB, GSK3B, KAT5, KLHL8, and SCARB1). The serum levels of total-C and LDL-C were significantly negatively correlated to the expression levels of two genes (FAM117B and KAT5) and one gene (LRP5), respectively.

Even though the percentage number of lymphocytes and monocytes was constant in the three comparisons, we also checked if cell subset-specific genes were differently expressed between the groups (data not shown). We did not observe any significant alterations in the gene expression levels of the B cell-specific gene *CD20*, the T helper cell-specific gene *CD4*, and the monocyte-specific gene *CD14* between the subjects in any of the groups. These findings suggest that there is no indication that there is a

Table 2 Characteristics and plasma fatty acid profile of subjects in the highest and lowest tertiles

	n-6 level			n-3 level			SFA/PUFA ratio		
	Highest tertile (n 18)	Lowest tertile (n 18)	*P*	Highest tertile (n 18)	Lowest tertile (n 18)	*P*	Highest tertile (n 18)	Lowest tertile (n 18)	*P*
Male/female	5/13	5/13		3/15	5/13		5/13	6/12	
Age (years)	28 (23–33)	26 (21–29)	0.31	27 (21–31)	27 (21–28)	0.80	27 (22–29)	29 (23–33)	0.38
BMI (kg/m^2)	22.5 ± 2.9	22.6 ± 2.6	0.94	22.2 ± 2	22.7 ± 3.3	0.57	23.3 ± 2.7	22.6 ± 2.6	0.41
Total-C (mmol/l)	4.9 ± 0.9	4.7 ± 1.1	0.65	4.9 ± 1	4.9 ± 1	0.96	4.7 ± 0.8	5.0 ± 0.9	0.25
LDL-C (mmol/l)	2.7 ± 0.8	2.6 ± 0.9	0.79	2.6 ± 0.9	2.8 ± 0.6	0.71	2.5 ± 0.6	2.8 ± 0.9	0.16
HDL-C (mmol/l)	1.6 ± 0.4	1.4 ± 0.3	0.15	1.6 ± 0.3	1.4 ± 0.4	0.28	1.5 ± 0.4	1.6 ± 0.3	0.74
TG (mmol/l)	0.8 (0.6–0.9)	1.4 (0.9–1.7)	*0.01*	0.9 (0.6–1.1)	1.2 (0.7–1.2)	0.30	1.3 (0.8–1.6)	0.8 (0.6–0.9)	*0.01*
Lymphocytes (%)	38.4 ± 6.8	43.0 ± 8.5	0.08	42.2 ± 10.0	37.6 ± 4	0.09	42.1 ± 8.7	37.4 ± 8.4	0.11
Monocytes (%)	8.5 ± 1.8	8.1 ± 1.9	0.49	8.1 ± 1.7	8.6 ± 2.3	0.42	8.8 ± 2.2	8.48 ± 2.0	0.71
Plasma level of fatty acids (wt%)									
Total SFA	29.6 (28.8–30.5)	32.7 (31.1–33.2)	*< 0.01*	30.7 (29.3–32.3)	31.3 (29.8–31.8)	0.78	33.0 (31.5–33.2)	29.1 (28.7–29.5)	*< 0.01*
Myristic acid (14:0)	0.8 (0.6–1.0)	1.2 (0.8–1.2)	*< 0.01*	0.9 (0.6–1.1)	1.0 (0.7–1.1)	0.57	1.2 (0.9–1.2)	0.7 (0.6–0.9)	*< 0.01*
Palmitic acid (16:0)	20.8 (20.2–21.6)	23.5 (21.4–24.2)	*< 0.01*	21.9 (20.4–23.7)	22.2 (20.9–22.6)	0.39	23.8 (21.8–24.5)	20.7 (20.2–20.7)	*< 0.01*
Stearic acid (18:0)	8.1 ± 0.7	8.1 ± 1.1	0.86	8.2 ± 1.1	8.0 ± 1.0	0.65	8.0 ± 1.1	7.7 ± 0.8	0.37
Total n-6	38.6 ± 1.3	30.3 ± 2.9	*< 0.01*	31.0 ± 4.0	31.5 ± 4.5	0.74	30.8 ± 3.4	38.0 ± 2.0	*< 0.01*
LA (18:2n-6)	32.3 ± 1.6	24.9 ± 2.5	*< 0.01*	27.8 ± 3.8	29.2 ± 4.1	0.29	24.8 ± 2.5	31.8 ± 2.1	*< 0.01*
AA (20:4n-6)	6.2 ± 1.1	5.4 ± 0.9	*0.02*	5.8 ± 0.7	6.2 ± 1.2	0.32	5.9 ± 1.3	6.2 ± 1. 1	0.58
Total n-3	5.8 ± 2.5	7.0 ± 2.3	0.12	9.0 ± 0.9	3.6 ± 0.49	*< 0.01*	6.1 ± 2.5	6.8 ± 2.4	0.44
ALA (18:3n- 3)	0.6 (0.5–0.6)	0.5 (0.4–0.6)	0.46	0.5 (0.5–0.6)	0.5 (0.5–0.6)	0.79	0.5 (0.5–0.6)	0.5 (0.5–0.5)	0.95
EPA (20:5n-3)	1.7 (0.6–2.3)	2.3 (1.8–2.9)	0.14	3.2 (2.8–3.7)	0.6 (0.5–0.6)	*< 0.01*	1.8 (0.7–2.6)	2.2 (1.3–3.2)	0.46
DPA (22:5n-3)	0.6 ± 0.1	0.7 ± 0.2	0.26	0.8 ± 0.1	0.5 ± 0.1	*< 0.01*	0.6 ± 0.2	0.6 ± 0.1	0.80
DHA (22:6n-3)	3.0 (1.9–4.1)	3.6 (3.1–4.1)	0.14	4.3 (4.1–4.7)	1.9 (1.8–2.1)	*< 0.01*	3.2 (2.0–4.1)	3.4 (2.8–4.3)	0.48
Total PUFA	44.4 (42.1–47.3)	37.3 (35.8–39.7)	*< 0.01*	42.6 (39.5–46.7)	40.4 (37.7–41.5)	*0.05*	36.9 (35.8–39.1)	44.7 (43.1–47.3)	*< 0.01*
SFA to PUFA ratio	0.7 (0.6–0.7)	0.8 (0.8–0.9)	*< 0.01*	0.7 (0.6–0.8)	0.8 (0.7–0.8)	0.13	0.9 (0.8–0.9)	0.7 (0.6–0.7)	*< 0.01*

Lymphocytes and monocytes are given as percentage of total white cell count. Differences between tertiles were analyzed using the independent samples *t* test when normally distributed or the Mann-Whitney *U* test when not normally distributed. *P* values < 0.05 were considered significant and are presented in italic. Values are presented as mean ± SD or medians and 25th–75th percentile

BMI body mass index, *Total-C* total cholesterol, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *TG* triglycerides, *SFA* saturated fatty acid, *LA* linoleic acid, *AA* arachidonic acid, *ALA* alpha-linolenic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid, *DHA* docosahexaenoic acid, *PUFA* polyunsaturated fatty acid

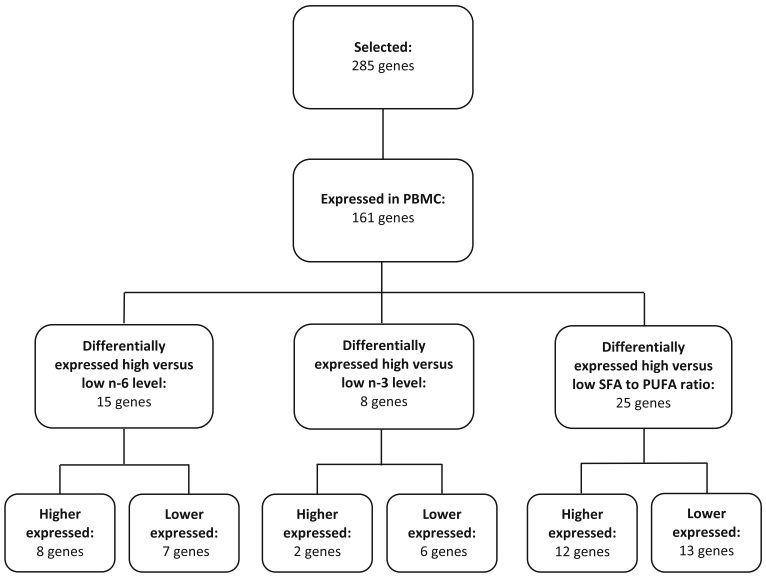

Fig. 1 Flowchart of gene selection and number of differentially expressed genes between subjects in the highest and lowest tertile of plasma n-6 level, plasma n-3 level and plasma SFA to PUFA ratio. Differences in Log2 gene expression between subjects in the highest and lowest tertiles were compared by independent samples *t* test. *P* values < 0.05 were considered significant

difference in B lymphocyte/monocyte ratio in the comparisons. However, we observed a significant lower mRNA level of the cytotoxic T lymphocyte-specific gene *CD8A* in the high versus low plasma n-6 level group, and significant higher mRNA levels of *CD8A* and *CD8B* in the high versus low plasma SFA to PUFA ratio group. This may indicate that plasma fatty acids may influence the T lymphocyte/monocyte ratio.

Discussion

In this explorative study, we investigated the potential relation of plasma n-6 and n-3 fatty acid levels, and plasma SFA to PUFA ratio, to PBMC gene expression related to lipid metabolism in healthy subjects. The plasma SFA to PUFA ratio was associated with the highest number of differentially expressed genes, followed by plasma n-6 and n-3 fatty acid level. In particular, genes involved in cholesterol metabolism were differentially expressed.

PBMCs have previously been shown to reflect hepatic lipid metabolism during fasting [23]. In addition, genes related to lipid metabolism have been shown to be differentially expressed in PBMCs after acute meal studies with different fat qualities [26, 27]. In the present study, we used a targeted approach to investigate whether 285 genes encoding proteins related to cholesterol and TG metabolism were differentially expressed depending on plasma fatty acid levels. Among the 285 genes, a total of 161 genes were expressed in the PBMCs. Interestingly, it seems like the plasma SFA to PUFA ratio is a stronger determinant than the plasma levels of n-6 and n-3 fatty acids alone regarding the potential of influencing gene expression levels in PBMCs. PBMCs may therefore function as a good model system to get a better understanding of how genes involved in lipid metabolism are regulated by the plasma fatty acid levels and in particular the SFA to PUFA ratio.

Genes involved in the regulation of cholesterol homeostasis were differentially expressed among subjects with high compared to low plasma SFA to PUFA ratio. ABCA2 encodes a member of the ATP-binding cassette (ABC) transporters, a subfamily of transporters that have been functionally linked to intracellular lipid transport [38]. The mRNA level of ABCA2 was significantly lower among subjects with high compared to low plasma SFA to PUFA ratio. It has been demonstrated that ABCA2 positively regulates low-density lipoprotein receptor (LDLR) mRNA expression and negatively regulates cholesterol esterification in hamster ovary cells [39]. In addition, it has been shown that overexpression of ABCA2 in neuroblastoma cells results in decreased cellular cholesterol levels [40]. Thus, our findings suggest that a lower ABCA2 expression level may lead to lower uptake and lower synthesis of cholesterol.

Because most cells in the periphery of the body do not express pathways for catabolizing cholesterol, efflux of cholesterol is critical for maintaining cholesterol homeostasis. SCARB1 and CAV1 are genes encoding proteins involved in cholesterol efflux. The mRNA level of CAV1 was significantly higher among subjects with high compared to low plasma SFA to PUFA ratio. It has been shown that CAV1 regulates the ATP-binding cassette subfamily G member 1 (ABCG1)-mediated efflux of

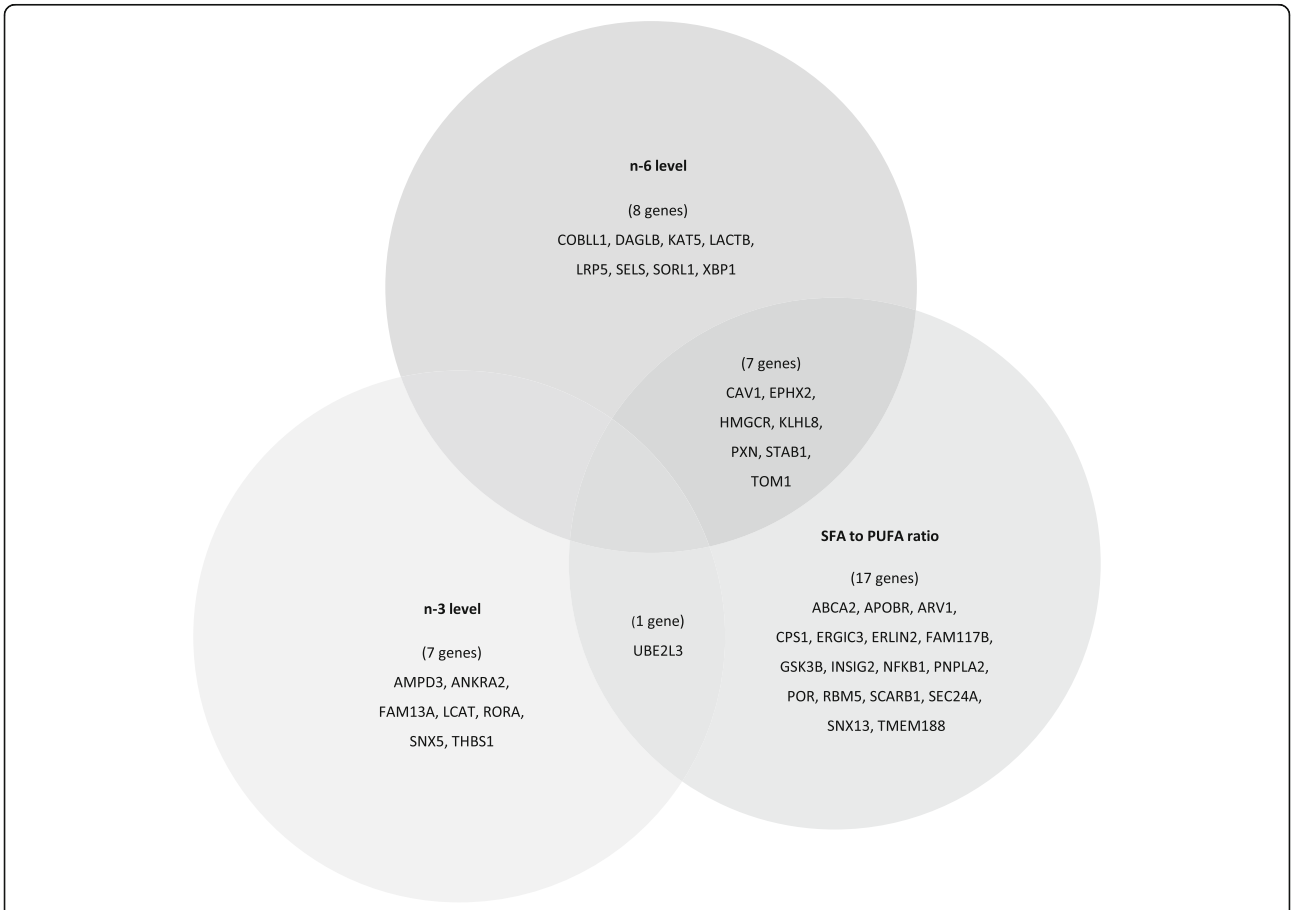

Fig. 2 Differently expressed genes associated with plasma fatty acid levels and SFA to PUA ratio. Number of differentially expressed genes associated with n-3 level (8 gene transcripts), n-6 level (15 gene transcripts), and SFA to PUFA ratio (25 gene transcripts). Seven differentially expressed genes were associated with both n-6 level and SFA to PUFA ratio, and one differently expressed gene was associated with both n-3 level and SFA to PUFA ratio. Differences in Log2 gene expression between subjects in the highest and lowest tertiles were compared by independent samples *t* test. *P* values < 0.05 were considered significant

cholesterol, probably by regulating ABCG1 trafficking to the cell surface [41], and a potential increase in CAV1 will subsequently lead to increased cholesterol efflux through ABCG1. Interestingly, there was also a significantly lower mRNA level of CAV1 among subjects with high compared to low plasma n-6 fatty acid level. As n-6 fatty acids are the majority of total plasma PUFAs, this finding may suggest that SFAs and PUFAs may exert different effects on cholesterol efflux pathways. We did not observe a significant difference in the mRNA level of ABCG1 among subjects with high compared to low plasma SFA to PUFA ratio. However, there was a significantly lower mRNA level of SCARB1, which encodes another plasma membrane receptor mediating cholesterol transfer to and from HDL. In contrast to ABCG1, which mediate cholesterol efflux via active transport, SCARB1 mediates cholesterol transport to and from HDL via passive facilitated diffusion [42]. In cholesterol-loaded mouse macrophages incubated with diluted human serum, it has been shown that cholesterol efflux is mainly mediated by active transport [43]. We therefore speculate that there may be a higher efflux of cholesterol via active transport than by passive transport in response to a high intracellular cholesterol load, which may explain the lower expression level of SCARB1 in subjects with high compared to low plasma SFA to PUFA ratio.

In addition, there was a significantly lower mRNA level of HMGCR among subjects with high compared to low SFA to PUFA ratio, and the mRNA level of HMGCR was significantly higher among subjects with high compared to low plasma n-6 PUFA level. HMGCR encodes the rate limiting step in the cholesterol biosynthesis pathway, and a lower expression of this gene will subsequently lead to reduced synthesis of cholesterol. The finding in the present study is in line with a recent postprandial study investigating the effects of SFA in lean and obese subjects [27]. It is interesting that although the abovementioned study investigated the acute effects of a high fat meal, we were able to observe a similar change in the mRNA level of HMGCR among subjects

Table 3 Differentially expressed genes between subjects in the highest and lowest plasma n-6 tertile

Gene	Highest tertile (n 18)	Lowest tertile (n 18)	Mean difference	*P*
STAB1*	8.66 ± 0.30	8.30 ± 0.47	0.36	0.011
SORL1	*10.25 ± 0.27*	*10.06 ± 0.27*	*0.19*	*0.039*
*KLHL8**	*9.13 ± 0.25*	*8.94 ± 0.19*	*0.18*	*0.020*
DAGLB	9.21 ± 0.30	9.03 ± 0.19	0.18	0.040
*TOM1**	*9.78 ± 0.24*	*9.62 ± 0.20*	*0.16*	*0.037*
PXN*	8.14 ± 0.19	8.00 ± 0.18	0.14	0.030
HMGCR*	8.85 ± 0.14	8.76 ± 0.11	0.09	0.036
KAT5	*7.45 ± 0.09*	*7.39 ± 0.09*	*0.06*	*0.047*
LRP5	6.79 ± 0.09	6.87 ± 0.09	− 0.08	0.013
LACTB	*7.15 ± 0.12*	*7.24 ± 0.12*	*− 0.09*	*0.027*
SELS	*9.66 ± 0.12*	*9.83 ± 0.27*	*− 0.18*	*0.020*
XBP1	*10.63 ± 0.23*	*10.85 ± 0.37*	*− 0.22*	*0.042*
EPHX2*	8.18 ± 0.32	8.40 ± 0.30	− 0.22	0.043
CAV1*	6.88 ± 0.11	7.13 ± 0.32	− 0.25	0.005
COBLL1	7.98 ± 0.23	8.28 ± 0.49	− 0.30	0.028

Expression of genes is given as mRNA level. Values are presented as mean ± SD and are log2 transformed. Differences between tertiles were analyzed using the independent samples *t* test. *P* values < 0.05 were considered significant
*Genes differentially expressed between subjects in the highest and lowest n-6 tertile and between subjects in the highest and lowest SFA to PUFA ratio tertile. The genes presented in italics are expressed by more than one probe (shown in Additional file 2: Table S2). The probe with lowest *p*-value is shown in Table 3

with higher plasma SFA to PUFA ratio compared to subjects with lower plasma SFA to PUFA ratio. The transcription of HMGCR is regulated by sterol regulatory element-binding proteins (SREBPs) [44]. SREBP2 stimulates the transcription of genes involved in cholesterol biosynthesis and uptake [45]. We did not observe a

Table 4 Differentially expressed genes between subjects in the highest and lowest plasma n-3 tertile

Gene	Highest tertile (n 18)	Lowest tertile (n 18)	Mean Difference	*P*
SNX5	*8.53 ± 0.13*	*8.43 ± 0.14*	*0.10*	*0.029*
AMPD3	6.94 ± 0.06	6.88 ± 0.06	0.06	0.012
LCAT	6.93 ± 0.07	6.99 ± 0.07	− 0.06	0.013
RORA	*7.11 ± 0.13*	*7.21 ± 0.10*	*− 0.10*	*0.018*
FAM13A	*7.33 ± 0.14*	*7.43 ± 0.15*	*− 0.10*	*0.049*
*UBE2L3**	*7.16 ± 0.16*	*7.28 ± 0.17*	*− 0.12*	*0.035*
ANKRA2	9.10 ± 0.20	9.25 ± 0.17	− 0.15	0.017
THBS1	7.29 ± 0.32	7.62 ± 0.43	− 0.32	0.016

Expression of genes is given as mRNA level. Values are presented as mean ± SD and are log2 transformed. Differences between tertiles were analyzed using the independent samples *t* test. *P* values < 0.05 were considered significant
*Genes differentially expressed between subjects in the highest and lowest n-3 tertile and between subjects in the highest and lowest SFA to PUFA ratio tertile. The genes presented in italics are expressed by more than one probe (shown in Additional file 3: Table S3). The probe with lowest *p*-value is shown in Table 4

Table 5 Differentially expressed genes between subjects in the highest and lowest plasma SFA to PUFA ratio tertile

Gene	Highest tertile (n 18)	Lowest tertile (n 18)	Mean difference	*P*
EPHX2*	8.41 ± 0.33	8.18 ± 0.31	0.23	0.036
FAM117B	*8.58 ± 0.33*	*8.36 ± 0.09*	*0.22*	*0.012*
CAV1*	7.09 ± 0.27	6.87 ± 0.23	0.22	0.014
SEC24A	7.72 ± 0.26	7.56 ± 0.17	0.16	0.038
*UBE2L3***	*7.24 ± 0.18*	*7.09 ± 0.13*	*0.15*	*0.010*
ARV1	8.07 ± 0.16	7.95 ± 0.11	0.12	0.011
NFKB1	11.09 ± 0.10	11.00 ± 0.15	0.09	0.037
SNX13	7.49 ± 0.15	7.40 ± 0.10	0.09	0.045
INSIG2	8.14 ± 0.09	8.07 ± 0.07	0.06	0.025
TMEM188	7.01 ± 0.07	6.95 ± 0.09	0.06	0.034
ERLIN2	*6.99 ± 0.06*	*6.93 ± 0.06*	*0.05*	*0.034*
CPS1	6.84 ± 0.07	6.79 ± 0.07	0.05	0.033
*TOM1**	*7.04 ± 0.08*	*7.13 ± 0.10*	*− 0.09*	*0.006*
POR	7.75 ± 0.14	7.85 ± 0.11	− 0.10	0.037
GSK3B	8.22 ± 0.15	8.32 ± 0.13	− 0.10	0.021
HMGCR*	8.76 ± 0.19	8.88 ± 0.13	− 0.12	0.015
RBM5	10.81 ± 0.12	10.93 ± 0.15	− 0.12	0.033
ERGIC3	*10.54 ± 0.18*	*10.67 ± 0.13*	*− 0.12*	*0.031*
PXN*	8.00 ± 0.14	8.13 ± 0.15	− 0.13	0.014
SCARB1	7.49 ± 0.23	7.62 ± 0.12	− 0.14	0.025
APOBR	8.11 ± 0.18	8.25 ± 0.17	− 0.14	0.038
*KLHL8**	*8.96 ± 0.21*	*9.13 ± 0.25*	*− 0.17*	*0.042*
ABCA2	7.41 ± 0.25	7.61 ± 0.30	− 0.20	0.042
PNPLA2	8.28 ± 0.28	8.48 ± 0.30	− 0.20	0.050
STAB1*	8.31 ± 0.50	8.67 ± 0.38	− 0.35	0.023

Expression of genes is given as mRNA level. Values are presented as mean ± SD and are log2 transformed. Differences between tertiles were analyzed using the independent samples *t* test. *P* values < 0.05 were considered significant
*Genes differentially expressed between subjects in the highest and lowest n-6 tertile and between subjects in the highest and lowest SFA to PUFA ratio tertile
**Genes differentially expressed between subjects in the highest and lowest n-3 tertile and between subjects in the highest and lowest SFA to PUFA ratio tertile. The genes presented in italics are expressed by more than one probe (shown in Additional file 4: Table S4). The probe with lowest *p*-value is shown in Table 5

significant difference in the mRNA level of SREBP2 among subjects with high compared to low SFA to PUFA ratio, nor did we observe a significant difference in the expression level of the *LDL receptor* (LDLR) which is also regulated by SREBP2. However, several genes involved in the proteolytic regulation of SREBP2, including INSIG2 and ERLIN2, were significantly higher expressed among subjects with high compared to low SFA to PUFA ratio. INSIG2 binds to cholesterol-loaded sterol binding proteins (SCAPs) in the endoplasmic reticulum (ER) and prevents the movement of the SCAP-SREBP complex to the Golgi apparatus for further

Table 6 Correlations between subject characteristics and differentially expressed genes associated with plasma n-6 level and/or SFA to PUFA ratio (n 54)

	BMI	TG	Total-C	LDL-C
DAGLB		− 0.31 (*P* 0.020)		
ERLIN2	− 0.31 (*P* 0.021)			
FAM117B			− 0.29 (*P* 0.034)	
GSK3B	− 0.28 (*P* 0.042)	− 0.31 (*P* 0.024)		
KAT5		− 0.33 (*P* 0.016)	− 0.32 (*P* 0.017)	
KLHL8	− 0.29 (*P* 0.034)	− 0.30 (*P* 0.026)		
LACTB	0.31 (*P* 0.023)			
LRP5				− 0.30 (*P* 0.027)
SCARB1	− 0.26 (*P* 0.059)	− 0.27 (*P* 0.045)		
SELS	− 0.32 (*P* 0.033)			
SNX13	0.42 (*P* < 0.01)			

Correlations were analyzed using Pearson's *r*. *P* values < 0.05 were considered significant
BMI body mass index, *TG* triglycerides, *Total-C* total cholesterol, *LDL-C* low-density lipoprotein cholesterol

processing and eventually transcription of HMGCR and other SREBP target genes. In addition, INSIG proteins play an important role in oxysterol-regulated cleavage of SREBPs [46]. ERLIN2 encodes a cholesterol-sensing protein which has been suggested to stabilize the SREBP-SCAP-INSIG complex in the ER [47]. This gene was also significantly negatively correlated with BMI, thereby implicating that BMI impact on intracellular cholesterol levels. Thus, a higher mRNA level of INSIG2 and ERLIN2 is in line with the lower mRNA level of HMGCR observed in the present study. Additionally, we observed a higher mRNA level of SEC24 among subjects with high compared to low plasma SFA to PUFA ratio. SEC24 is a component of the COP11-coated vesicles, which transport the SREBP-SCAP complex to the Golgi. When ER cholesterol rises above a threshold of total lipids, the cholesterol binds to SCAP, which triggers a conformational change in the protein that occludes the binding of the COPII proteins [48]. We speculate that a higher expression level of SEC24 may be a response to a higher intracellular cholesterol level among subjects with high compared to low plasma SFA to PUFA ratio.

Although we did not observe differences in serum cholesterol levels among subjects with high compared to low plasma SFA to PUFA ratio, the differentially expressed genes observed in the present study may reflect an intracellular status of excess cholesterol. Changes in the expression of genes occur prior to changes at protein level, and these findings may therefore reflect early changes related to diet. The question remains how the abovementioned genes are potentially regulated by changes in the plasma SFA to PUFA ratio. It is well known that dietary intake of PUFA, in particular of LA, AA, EPA, and DHA, correlate with their respective percentages in plasma total fatty acids [14]. However, the total plasma fatty acid profile may not reflect the total dietary intake of fat as the fatty acid composition in different lipid fractions differs depending on the fat intake [49]. However, we have shown recently in a dietary intervention study where SFA were replaced with PUFA that the total plasma fatty acid profile reflected dietary fat intake changes [8]. It is clear that fatty acids have the ability to regulate the expression of genes involved in lipid metabolism. PUFAs have been shown to decrease nuclear SREBP-1 protein levels in part by inhibition of the interaction of oxysterols with LXR; however, this mechanism does not seem to affect SREBP2 [50]. The findings in the present study may therefore be explained by the plasma levels of SFAs. Recent findings suggest that SFAs may decrease SREBP activity directly, but the exact mechanisms whereby SFAs may exert their effects on SREBP and its downstream targets remain to be established [19].

The present study has several strengths. Gene expression profiling in PBMCs has been shown to be more sensitive to dietary changes than the traditional biochemical parameters in the circulation, and we specifically choose genes involved in biological processes related to TG and cholesterol metabolism. In the present study, we have compared the mRNA levels with plasma levels of n-3, n-6, and the SFA to PUFA ratio. Since intake of the n-3 and the n-6 fatty acids are reflected in the plasma total fatty acid composition, our data indicate that intake of these fatty acids may cause differences in gene expression. The major limitation of the present study is the limited number of subjects. Due to the small number of subjects, the subjects were separated into groups based on tertiles, which may have had an impact on the number of significantly differentially expressed genes observed between the highest and lowest tertiles. However, since we used end of study samples from a fish oil intervention, we could at least expect a larger variation in plasma n-3 fatty acid level among the subjects. Another limitation is that that there may be a different T lymphocyte/monocyte ratio in the n-6 tertile groups and the SFA to PUFA tertile groups. Whether the mRNA levels of CD8A and CD8B are linked to the plasma level of the fatty acids or by the change in the number of subset cells cannot be determined by this

cross-sectional study. Another limitation is the lack of PBMC material to perform protein measurements to validate our mRNA results. Since this was an explorative study, we did not adjust for multiple testing. Although no causal relationship can be made due to the cross-sectional design of the study, the current study shows that the plasma fatty acid levels can influence the PBMC expression of genes involved in lipid metabolism.

Conclusion

In conclusion, the main findings in the present study were that PBMCs express genes involved in hepatic lipid metabolism and that the expression of several of the genes was influenced by plasma fatty acid levels. This finding supports the use of PBMCs as a model system for investigating the role dietary n-3 and n-6 fatty acids on gene expression related to lipid metabolism. The plasma SFA to PUFA ratio seems to be more important than the plasma n-6 and n-3 fatty acid level alone with regard to influencing mRNA levels. In particular, genes involved in cholesterol homeostasis were significantly differently expressed among subjects with high compared to low plasma SFA to PUFA ratio. This may reflect an intracellular status of excess cholesterol among subjects with high plasma SFA to PUFA ratio. The current findings should be further studied in experimental studies and tested in well-controlled human dietary intervention studies.

Additional files

Additional file 1: Selection of genes related to triglyceride- and cholesterol metabolism (285 genes). Genes expressed in peripheral blood mononuclear cells are in bold (161 genes). (DOCX 31 kb)

Additional file 2: Differentially expressed genes associated with plasma n-6 level and expressed by more than one probe. (DOCX 19 kb)

Additional file 3: Differentially expressed genes associated with plasma n-3 level and expressed by more than one probe. (DOCX 16 kb)

Additional file 4: Differentially expressed genes associated with plasma SFA to PUFA ratio and expressed by more than one probe. (DOCX 17 kb)

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Authors' contributions

SVL designed the research, selected the genes, performed the statistical analyses, analyzed the data, and drafted the manuscript. KBH designed the research, analyzed the data, and drafted the manuscript. IO designed the research, coordinated the intervention study, and drafted the manuscript. KND designed the research and selected the genes. MCWM designed the research, performed the microarray analysis, analyzed the data, and drafted the manuscript. SMU designed the research, was responsible for the intervention study, analyzed the data, and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The intervention study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Regional Committee of Medical Ethics (approval no. 6.2008.2215) and the Norwegian Social Science Data Services (approval no. 21924). Written informed consent was obtained from all participants. The study was registered at www.clinicaltrials.gov.gov (ID no. NCT01034423).

Competing interests

Dr. Holven has received research grants and/or personal fees from Tine SA, Mills SA, Olympic Seafood, Kaneka, Amgen, Sanofi, and Pronova, none of which are related to the content of this manuscript. Dr. Ulven has received research grants from Tine DA, Mills DA, and Olympic Seafood, none of which are related to the content of this manuscript. The other authors declare that they have no competing interests.

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CORRECTION

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Correction to: Plasma fatty acid levels and gene expression related to lipid metabolism in peripheral blood mononuclear cells: a cross-sectional study in healthy subjects

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Correction

Unfortunately, after publication of this article [1], it was noticed that some corrections to Table 2 were not carried out. The corrected Table 2 can be seen below and the original article has also been updated to reflect this.

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1. Larsen SV, Holven KB, Ottestad I, Dagsland KN, Myhrstad MCW, Ulven SM. Plasma fatty acid levels and gene expression related to lipid metabolism in peripheral blood mononuclear cells: a cross-sectional study in healthy subjects. Genes Nutr. 2018;13:9. https://doi.org/10.1186/s12263-018-0600-z.

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Table 2 Characteristics and plasma fatty acid profile of subjects in the highest and lowest tertiles

	n-6 level			n-3 level			SFA/PUFA ratio		
	Highest tertile (n 18)	Lowest tertile (n 18)	*P*	Highest tertile (n 18)	Lowest tertile (n 18)	*P*	Highest tertile (n 18)	Lowest tertile (n 18)	*P*
Male/female	5/13	5/13		3/15	5/13		5/13	6/12	
Age (years)	25 (23–33)	25 (21–29)	0.31	25 (21–31)	24 (21–28)	0.80	25 (22–29)	28 (23.0–32.8)	0.38
BMI (kg/m2)	22.5 ± 2.9	22.6 ± 2.6	0.94	22.2 ± 2	22.7 ± 3.3	0.57	23.3 ± 2.7	22.6 ± 2.6	0.41
Total-C (mmol/l)	4.9 ± 0.9	4.7 ± 1.1	0.65	4.9 ± 1	4.9 ± 1	0.96	4.7 ± 0.8	5.0 ± 0.9	0.25
LDL-C (mmol/l)	2.7 ± 0.8	2.6 ± 0.9	0.79	2.6 ± 0.9	2.8 ± 0.6	0.71	2.5 ± 0.6	2.8 ± 0.9	0.16
HDL-C (mmol/l)	1.6 ± 0.4	1.4 ± 0.3	0.15	1.6 ± 0.3	1.4 ± 0.4	0.28	1.5 ± 0.4	1.6 ± 0.3	0.74
TG (mmol/l)	0.8 (0.6–0.9)	1.2 (0.9–1.7)	*0.01*	0.8 (0.6–1.1)	1.0 (0.7–1.2)	0.30	1.1 (0.8–1.6)	0.8 (0.6–0.9)	*0.01*
Lymphocytes (%)	38.4 ± 6.8	43.0 ± 8.5	0.08	42.2 ± 10.0	37.6 ± 4	0.09	42.1 ± 8.7	37.4 ± 8.4	0.11
Monocytes (%)	8.5 ± 1.8	8.1 ± 1.9	0.49	8.1 ± 1.7	8.6 ± 2.3	0.42	8.8 ± 2.2	8.48 ± 2.0	0.71
Plasma level of fatty (wt%)									
Total SFA	29.4 (28.8–30.5)	32.1 (31.1–33.2)	*< 0.01*	30.7 (29.3–32.3)	30.8 (29.8–31.8)	0.78	32.6 (31.5–33.2)	29.1 (28.7–29.5)	*< 0.01*
Myristic acid (14:0)	0.6 (0.6–1.0)	1.0 (0.8–1.2)	*< 0.01*	0.8 (0.6–1.1)	0.9 (0.7–1.1)	0.57	1.1 (0.9–1.2)	0.6 (0.6–0.9)	*< 0.01*
Palmitic acid (16:0)	20.5 (20.2–21.6)	23.4 (21.4–24.2)	*< 0.01*	20.9 (20.4–23.7)	21.5 (20.9–22.6)	0.39	23.9 (21.8–24.5)	20.4 (20.2–20.7)	*< 0.01*
Stearic acid (18:0)	8.1 ± 0.7	8.1 ± 1.1	0.86	8.2 ± 1.1	8.0 ± 1.0	0.65	8.0 ± 1.1	7.7 ± 0.8	0.37
Total n-6	38.6 ± 1.3	30.3 ± 2.9	*< 0.01*	31.0 ± 4.0	31.5 ± 4.5	0.74	30.8 ± 3.4	38.0 ± 2.0	*< 0.01*
LA (18:2n-6)	32.3 ± 1.6	24.9 ± 2.5	*< 0.01*	27.8 ± 3.8	29.2 ± 4.1	0.29	24.8 ± 2.5	31.8 ± 2.1	*< 0.01*
AA (20:4n-6)	6.2 ± 1.1	5.4 ± 0.9	*0.02*	5.8 ± 0.7	6.2 ± 1.2	0.32	5.9 ± 1.3	6.2 ± 1. 1	0.58
Total n-3	5.8 ± 2.5	7.0 ± 2.3	0.12	9.0 ± 0.9	3.6 ± 0.49	*< 0.01*	6.1 ± 2.5	6.8 ± 2.4	0.44
ALA (18:3n- 3)	0.5 (0.5–0.6)	0.5 (0.4–0.6)	0.46	0.5 (0.5–0.6)	0.5 (0.5–0.6)	0.79	0.5 (0.5–0.6)	0.5 (0.5–0.5)	0.95
EPA (20:5n-3)	1.7 (0.6–2.3)	2.4 (1.8–2.9)	0.14	3.2 (2.8–3.7)	0.6 (0.5–0.6)	*< 0.01*	2.0 (0.7–2.6)	2.2 (1.3–3.2)	0.46
DPA (22:5n-3)	0.6 ± 0.1	0.7 ± 0.2	0.26	0.8 ± 0.1	0.5 ± 0.1	*< 0.01*	0.6 ± 0.2	0.6 ± 0.1	0.80
DHA (22:6n-3)	3.0 (1.9–4.1)	4.0 (3.1–4.1)	0.14	4.3 (4.1–4.7)	1.9 (1.8–2.1)	*< 0.01*	3.5 (2.0–4.1)	3.3 (2.8–4.3)	0.48
Total PUFA	43.8 (42.1–47.3)	38.6 (35.8–39.7)	*< 0.01*	42.2 (39.5–46.7)	40.4 (37.7–41.5)	*0.05*	37.8 (35.8–39.1)	44.6 (43.1–47.3)	*< 0.01*
SFA to PUFA ratio	0.7 (0.6–0.7)	0.8 (0.8–0.9)	*< 0.01*	0.7 (0.6–0.8)	0.8 (0.7–0.8)	0.13	0.9 (0.8–0.9)	0.7 (0.6–0.7)	*< 0.01*

Lymphocytes and monocytes are given as percentage of total white cell count. Differences between tertiles were analyzed using the independent samples *t* test when normally distributed or the Mann-Whitney *U* test when not normally distributed. *P* values < 0.05 were considered significant. Values are presented as mean ± SD or medians and 25th–75th percentile

BMI body mass index, *Total-C* total cholesterol, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *TG* triglycerides, *SFA* saturated fatty acid, *LA* linoleic acid, *AA* arachidonic acid, *ALA* alpha-linolenic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid, *DHA* docosahexaenoic acid, *PUFA* polyunsaturated fatty acid

II

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Differences in peripheral blood mononuclear cell gene expression and triglyceride composition in lipoprotein subclasses in plasma triglyceride responders and non-responders to omega-3 supplementation

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Abstract

Background: Intake of the marine omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduces fasting triglyceride (TG) levels and may thereby lower cardiovascular disease risk. However, there are large inter-individual differences in the TG-lowering effect of omega-3 supplementation. Genotype differences partly explain this variation, but gene-environment interactions leading to gene expression differences may also be important. In this study, we aimed to investigate baseline differences and differences in the change in peripheral blood mononuclear cell (PBMC) gene expression and lipoprotein subclass TG levels between TG responders and non-responders to omega-3 fatty acid supplementation.

Methods: In a previous randomized controlled trial, healthy normotriglyceridemic subjects ($n = 35$, 71% women) received 1.6 g EPA + DHA/day for 7 weeks. In this exploratory sub-study, we defined TG responders as subjects having a TG reduction beyond the 20% day-to-day variation and non-responders as having a TG change between − 20% and + 20% after omega-3 supplementation. PBMC gene expression was measured using microarray, and lipoprotein subclasses were measured using nuclear magnetic resonance spectroscopy.

Results: Eight subjects were defined as responders with a median TG reduction of 37%, and 16 subjects were defined as non-responders with a median TG change of 0%. At baseline, responders had higher TG levels in two of four high-density lipoprotein (HDL) subclasses and 909 gene transcripts ($p \leq 0.05$) were differentially expressed compared to non-responders. During the intervention, the plasma TG reduction among responders was reflected in TG reductions in four of six different very low-density lipoprotein subclasses and three of four different HDL subclasses. Compared to non-responders, the expression of 454 transcripts was differentially altered in responders ($p \leq 0.05$). Pathway analyses revealed that responders had altered signaling pathways related to development and immune function. In addition, two of the top 10 enriched pathways in responders compared to non-responders were related to lysophosphatidic acid signaling.

(Continued on next page)

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Conclusion: TG responders and non-responders to omega-3 supplementation have different lipoprotein subclass and PBMC gene expression profiles at baseline and different lipoprotein subclass and PBMC gene expression responses to omega-3 supplementation. These gene expression differences may partially explain the variability in TG response observed after omega-3 supplementation.

Keywords: EPA, DHA, Omega-3, Transcriptomics, Microarray, Triglycerides, Lipoprotein subclasses

Background

Intake of the marine omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may reduce the risk of cardiovascular disease (CVD), especially in high-risk populations [1]. This risk reduction is linked to the anti-inflammatory, anti-arrhythmic, blood pressure-lowering, and lipid-modifying effects of omega-3 fatty acids [2–5]. The reduction in triglyceride (TG) levels is one of the most important effects of omega-3 fatty acids, and this effect is dependent on pre-supplementation TG levels and the omega-3 fatty acid dose [2, 3]. However, there are large inter-individual differences in the TG-lowering effect of omega-3 fatty acids, with several studies showing that about 30–40% of participants do not obtain reduced TG levels following omega-3 supplementation (non-responders) [6–8]. Those who have a TG-lowering effect of omega-3 supplementation (responders) seem to have a less healthy biochemical profile, such as higher TG and glucose levels and lower HDL-C. In addition, responders have more favorable changes in total cholesterol and HDL-C in response to omega-3 supplementation than non-responders [9]. However, the distribution of TG in lipoprotein subclasses in responders and non-responders is less characterized.

Furthermore, polymorphisms in the genes apolipoprotein E (*APOE*), acetyl-CoA carboxylase α (*ACACA*), ATP citrate lyase (*ACLY*), cluster of differentiation 36 (*CD36*), retinoid X receptor α (*RXRA*), and acyl-CoA oxidase 1 (*ACOX1*), among others, have been found to affect the TG-lowering effect of omega-3 fatty acids [10–13]. It has previously been shown in a genome-wide association study (GWAS) that genotype explains only 20% of the variation in TG response to omega-3 fatty acids in the Fatty Acid Sensor (FAS) study [14]. However, in the FAS study, a more refined and improved genetic risk score (GRS) has recently showed that GRS can explain almost 50% of the TG response variance [15].

Omega-3 fatty acids mediate their effects largely at the cellular level, such as by their ability to alter gene expression. This can happen directly, by binding and activating nuclear receptors such as peroxisome proliferator-activated receptors (PPARs), or indirectly by inhibiting the nuclear translocation of transcription factors (TFs) including nuclear factor kappa B (NF-κB) and sterol regulatory element binding protein (SREBP) [16–18]. These TFs affect the transcription of genes that, among others, are involved in lipid metabolism and inflammation [19].

Peripheral blood mononuclear cells (PBMC) are cells of both the innate and adaptive immune system and are mainly composed of lymphocytes and monocytes. Because they are circulating cells, they are exposed to nutrients, metabolites, and peripheral tissues and may therefore reflect whole-body health [20, 21]. It has been shown that PBMC gene expression reflects liver and adipose tissue expression of genes involved in lipid metabolism and inflammation [22–25], and omega-3 fatty acids have been shown to alter PBMC gene expression [26–28]. Hence, PBMCs may be a good a model system for exploring the underlying mechanisms of the TG-lowering effect of omega-3 fatty acids.

In this exploratory study, we aimed to analyze the baseline differences and the difference in change in PBMC transcriptome and the TG content in lipoprotein subclasses between TG responders and non-responders to omega-3 supplementation. Since the effect of omega-3 fatty acids on lipid metabolism is partly mediated through effects on gene expression, we hypothesized that intake of omega-3 fatty acids would differentially affect PBMC gene expression in TG responders and non-responders.

Results

Characteristics of responders and non-responders, biochemical parameters, plasma fatty acids, and estimated omega-3 index

After 7 weeks of omega-3 supplementation, 8 participants who received omega-3 supplementation were defined as responders with a median fasting TG reduction of 37% and 16 participants were defined as non-responders with a median change in fasting TG of 0% (Fig. 1a). The median compliance estimated by capsule count was 100% in responders and 99.5% in non-responders, and the difference was not significant. At baseline, the age of the participants, the distribution of men and women, the intervention group allocation, and BMI did not differ between responders and non-responders (Table 1). Nonetheless, responders had higher baseline levels of fasting TG ($p = 0.01$) and plasma oleic acid (OA; $p = 0.007$) and lower baseline levels of plasma linoleic acid (LA; $p = 0.009$) compared to non-responders (Table 1). The baseline levels of plasma EPA, docosapentaenoic acid (DPA), and DHA did

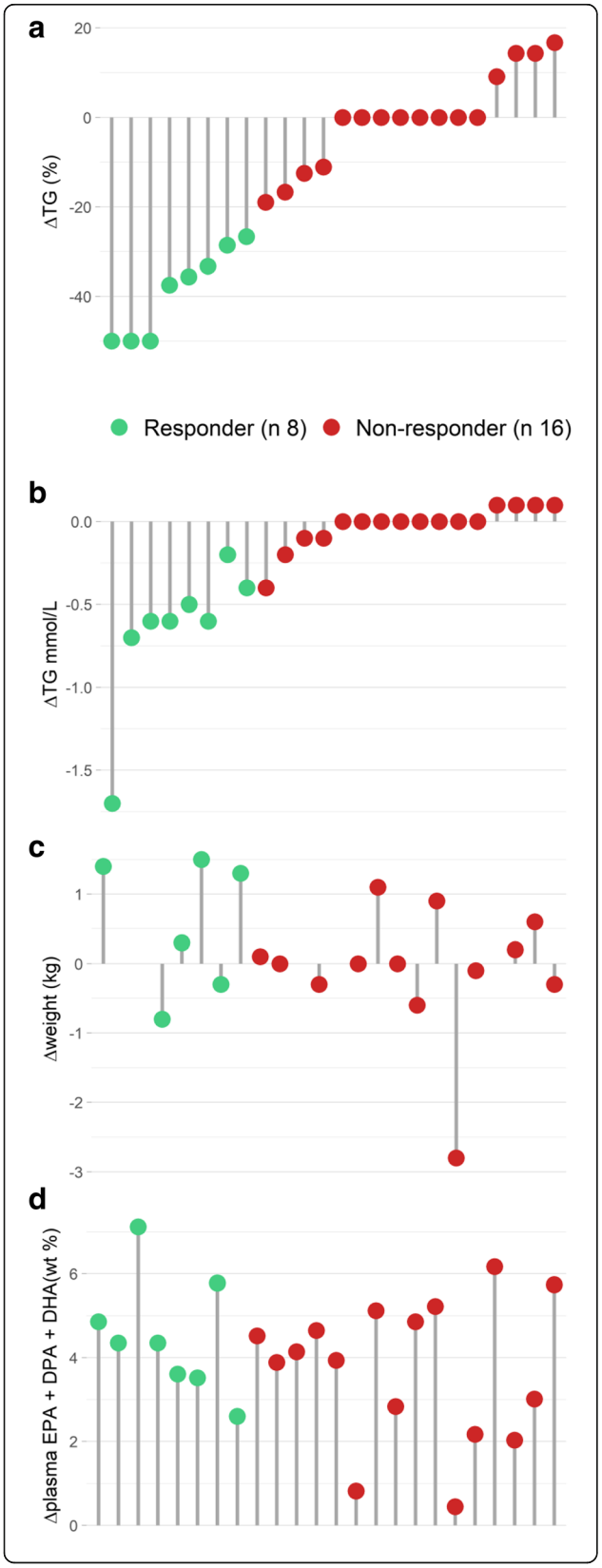

Fig. 1 Individual changes in fasting TG, weight, and plasma levels of omega-3 fatty acids. The relative change in fasting TG levels (**a**) were used to categorize participants as responders (ΔTG ≤ − 20%, green) and non-responders (− 20% < ΔTG < + 20%, red). The participants are presented in the same order for the absolute change (mmol/L) in fasting TG levels (**b**), weight change (**c**), and the change in plasma levels of marine omega-3 fatty acids (**d**)

not differ between responders and non-responders. However, the baseline estimated omega-3 index was 4.6% in responders and 5.6% in non-responders and the baseline difference was significant (Table 1).

In whole blood, there were 19% lymphocytes at baseline in both responders and non-responders, while at the 7-week visit there was 22% and 20% lymphocytes in responders and non-responders, respectively. There were 4% monocytes in both responders and non-responders at the baseline and 7-week visits. The change of lymphocytes and monocytes during the study did not differ between responders and non-responders.

During the intervention, there was no significant weight change within or between responders and non-responders (Fig. 1c). Evidently, the change in TG levels in responders and non-responders was significantly different ($p = 0.0001$) (Table 1), and as shown in Fig. 1b, the TG change ranged from − 1.7 to − 0.2 mmol/L in responders and from − 0.4 to 0.1 mmol/L in non-responders. The change in plasma EPA, DPA, and DHA did not differ between responders and non-responders (Table 1), as also illustrated by the individual changes in total plasma omega-3 levels in Fig. 1d. Nonetheless, there were significant differences between responders and non-responders in the change of several plasma fatty acids. The plasma level of OA was more reduced in responders compared to non-responders ($p = 0.004$), LA increased in responders while it decreased in non-responders ($p = 0.04$), the reduction in α-linolenic acid (ALA) was greater in responders than non-responders ($p = 0.03$), and the reduction in arachidonic acid (AA) was greater in non-responders than responders ($p = 0.01$) (Table 1).

Lipoprotein subclasses

To investigate the baseline differences and the difference in change in TG levels in more detail, we analyzed TG levels in 14 different lipoprotein subclasses. At baseline, responders had higher levels of TG in the two smallest HDL subclasses, M-HDL and S-HDL (Fig. 2). However, there were no significant differences between responders and non-responders in the baseline levels of TG in any of the very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), or LDL subclasses (not shown). Furthermore, we found that responders had a greater reduction of TG in extra large (XL-), large (L-), medium (M-), and small

Table 1 Baseline characteristics, biochemical parameters, plasma fatty acids, and omega-3 index of responders and non-responders to omega-3 supplementation

	Responders ($n = 8$)							Non-responders ($n = 16$)								
	Baseline			Change 7 weeks				Baseline			Change 7 weeks				P‡	P‡
	Median/*n*	25th	75th	Median	25th	75th	P†	Median/*n*	25th	75th	Median	25th	75th	P†	Baseline	Δ 7 weeks
Gender (male/female)	3/5							5/11							1§	
Intervention group (FO/oxFO)	2/6							10/6							0.19§	
Age (years)	23.5	21.5	25.8					26.5	22.5	33					0.17	
BMI (kg/m^2)	22.4	21.2	22.9					22.0	21.2	23.6					0.98	
Biochemical parameters																
Total-C (mM)	4.6	4.1	5.0	0.0	− 0.3	0.4	1	4.8	4.4	5.2	0.1	− 0.2	0.3	0.8	0.5	0.9
LDL-C (mM)	2.4	2.0	3.1	0.2	− 0.3	0.5	0.5	2.8	2.3	3.1	0.1	− 0.2	0.2	0.8	0.7	0.6
HDL-C (mM)	1.3	1.2	1.5	0.0	− 0.1	0.1	0.7	1.3	1.3	1.6	0.0	0.0	0.1	0.2	0.4	0.8
TG (mM)	1.5	1.4	1.7	− 0.6	− 0.6	− 0.5	0.01*	0.8	0.7	1.1	0.0	0.0	0.0	0.6	0.01*	0.0001*
Glucose (mM)	4.7	4.7	4.8	0.0	− 0.1	0.4	0.5	4.6	4.5	5.0	4.8	4.6	5.1	0.2	0.9	1
18:1 *n-9* (OA) (wt%)	24.74	21.40	26.09	− 3.86	− 5.51	− 2.25	0.01*	19.18	18.28	20.35	− 0.81	− 1.92	− 0.03	0.01*	0.007*	0.004*
18:2 *n-6* (LA) (wt%)	25.34	23.31	26.78	0.94	− 0.58	3.22	0.5	32.12	29.77	33.41	− 1.59	− 2.78	0.59	0.04*	0.009*	0.04*
18:3 *n-3* (ALA) (wt%)	0.71	0.52	0.76	− 0.23	− 0.34	− 0.04	0.01*	0.57	0.52	0.63	− 0.05	− 0.14	0.01	0.1	0.3	0.03*
20:4 *n-6* (AA) (wt%)	6.30	5.68	6.45	0.01	− 0.25	0.16	1	6.20	5.62	7.05	− 0.54	− 1.29	− 0.38	0.0004*	0.9	0.01*
20:5 *n-3* (EPA) (wt%)	0.50	0.44	0.70	1.90	1.79	2.27	0.01*	0.69	0.53	0.82	2.08	1.41	2.41	0.0005*	0.3	0.9
22:5 *n-3* (DPA) (wt%)	0.42	0.37	0.61	0.15	0.11	0.24	0.01*	0.53	0.47	0.58	0.15	0.13	0.25	0.0009*	0.6	0.9
22:6 *n-3* (DHA) (wt%)	1.70	1.46	2.01	2.35	1.57	2.67	0.01*	2.15	1.74	2.35	1.67	1.19	2.34	< 0.0001*	0.4	0.1
omega-3 index (wt%)	4.6	4.5	4.9	2.9	2.25	3.25	0.02*	5.6	4.9	6.0	2.35	1.38	3.03	0.0004*	0.03*	0.2

25th 25th percentile, *75th* 75th percentile, *FO* fish oil, *oxFO* oxidized fish oil, *BMI* body mass index, *C* cholesterol, *TG* triglycerides,, *n-* omega-, *OA* oleic acid, *LA* linoleic acid, *ALA* alpha-linolenic acid, *AA* arachidonic acid, *EPA* eicosapentaenoic acid, *DPA* docosapentaenoic acid, *DHA* docasahexaenoic acid, *wt%* weight%.

† Change from baseline to 7 weeks tested with paired Wilcoxon signed rank test

‡ Difference between responder and non-responder at baseline and change after 7 weeks tested with Mann-Whitney *U* test

§ Difference between responders and non-responders is tested with a Fisher's exact test

*$p < 0.05$

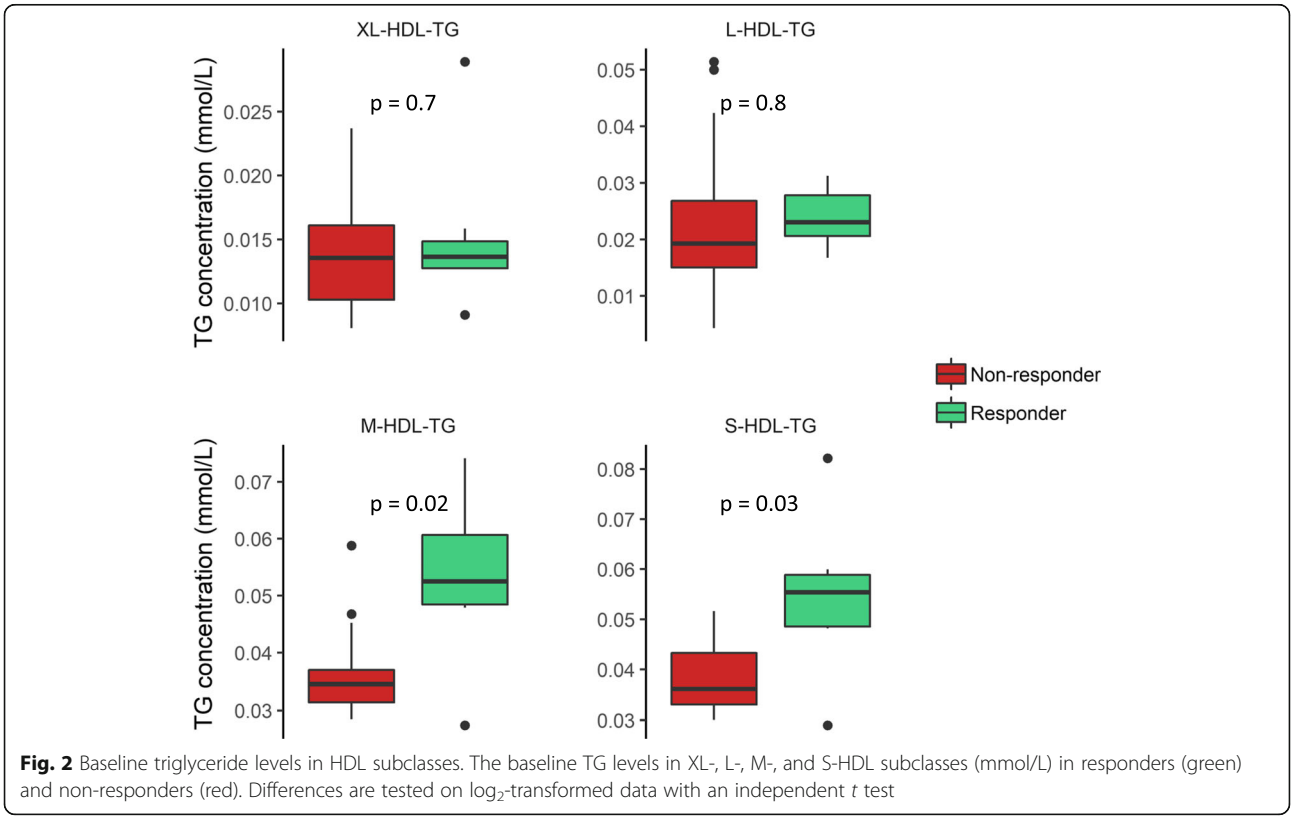

Fig. 2 Baseline triglyceride levels in HDL subclasses. The baseline TG levels in XL-, L-, M-, and S-HDL subclasses (mmol/L) in responders (green) and non-responders (red). Differences are tested on $\log_2$-transformed data with an independent *t* test

(S-)VLDL subclasses (Fig. 3) as well as in the XL-, M-, and S-HDL subclasses (Fig. 4), but there were no changes in the TG levels in IDL or the LDL subclasses (not shown).

PBMC gene expression

We analyzed the change in whole genome PBMC transcriptome at baseline and after 7 weeks of omega-3 supplementation. As previously described, all arrays fulfilled the quality criteria and were included in the analyses [27]. At baseline, 909 transcripts were differentially expressed in responders compared to non-responders ($p \leq 0.05$). Of these transcripts, 458 transcripts had a lower expression while 451 transcripts had a higher expression in responders compared to non-responders (Additional file 1). Only one of these transcripts, the long non-coding RNA *LINC00473*, was expressed at a significantly higher level in responders after adjusting for multiple comparisons (FDR < 25%). The transcripts with the largest difference in expression at baseline ($\log_2$ ratio < − 0.4 and $\log_2$ ratio > 0.4) encoded ribosomal and cytoskeleton proteins, among others (Table 2).

After 7 weeks of omega-3 supplementation, 454 transcripts were differentially altered ($p \leq 0.05$) in responders compared to non-responders. Of these, 246 transcripts were reduced and 208 were increased (Additional file 2). However, after correction for multiple testing, there were no differences between responders and non-responders in change in mRNA expression from baseline to 7 weeks (FDR < 25%). Among the significantly altered transcripts, those that had the greatest reduction ($\log_2$ ratio < − 0.25) and the highest increase ($\log_2$ ratio > 0.25) in responders compared to non-responders encode proteins involved in regulation of gene expression, apoptosis, and immune function (Table 3).

Pathway and transcription factor analyses

To examine processes differentially altered in responders compared to non-responders after 7 weeks of omega-3 supplementation, we performed functional analyses for the 454 altered transcripts. Of the 73 enriched pathways (FDR < 25%, Additional file 3), many were related to signaling pathways involved in development and immune function. Two of the top 10 enriched pathways (Table 4) were related to lysophosphatidic acid (LPA) signaling. In addition, some pathways related to regulation of lipid metabolism, adipogenesis, blood coagulation, and thromboxane A2 signaling were enriched in responders compared to non-responders (Additional file 3).

To further examine the mechanisms behind the differences in transcription levels between responders and non-responders, transcription factor (TF) analyses were performed. We found that genes with binding sites for 78 TFs were overrepresented in the list of genes that were significantly differentially altered in responders

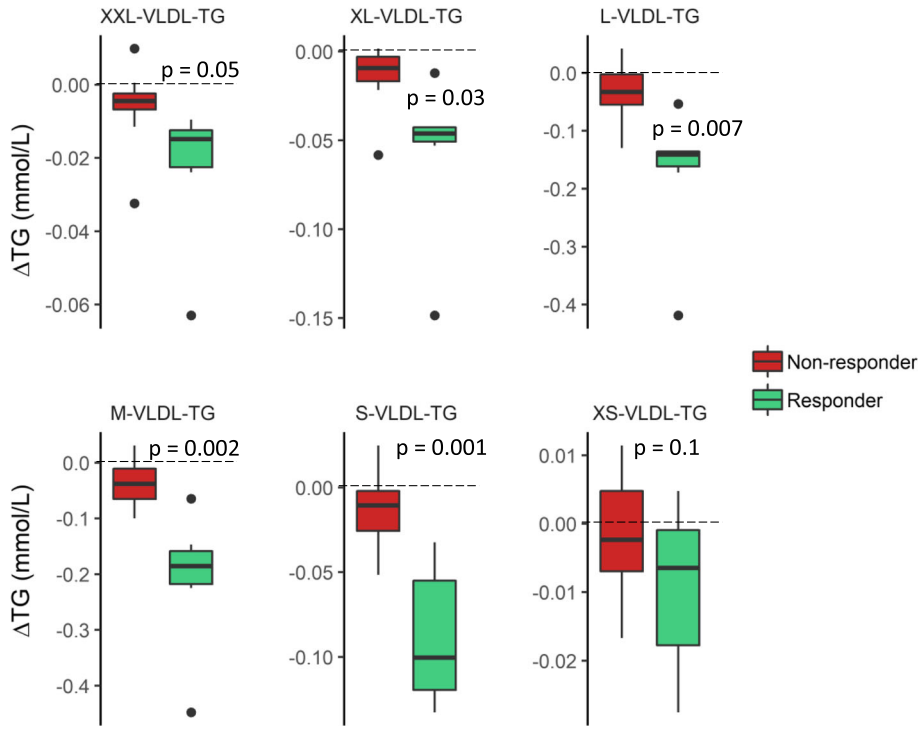

Fig. 3 Change in triglyceride levels in VLDL subclasses. The change in TG levels in XXL-, XL-, L-, M-, S-, and XS-VLDL subclasses from baseline to end of study (Δmmol/L) in responders (green) and non-responders (red). Differences are tested on the $\log_2$ ratio with an independent *t* test

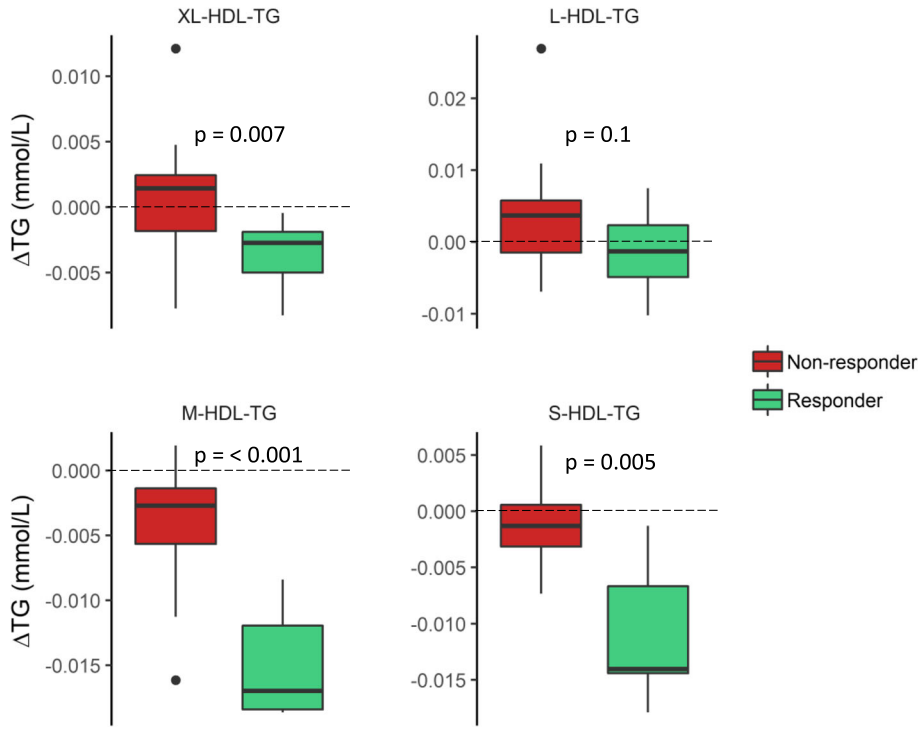

Fig. 4 Change in triglyceride levels in HDL subclasses. The change in TG levels in XL-, L-, M-, and S-HDL subclasses from baseline to the end of study (Δmmol/L) in responders (green) and non-responders (red). Differences are tested on the $\log_2$ ratio with an independent *t* test

Table 2 Genes with the highest and lowest baseline expression (log_2 ratio > |0.4|) in responders compared to non-responders to omega-3 supplementation

Gene	Probe ID	Log ratio*	95% CI	*P* value	Biological function/process
HBB	ILMN_2100437	− 0.74	(− 1.47 to − 0.01)	0.05	Hemoglobin
KRT72	ILMN_1695812	− 0.66	(− 1.28 to − 0.05)	0.04	Cytoskeleton
ACRBP	ILMN_1784203	− 0.57	(− 1.08 to − 0.06)	0.03	Acrosin condensation
UTS2	ILMN_1732198	− 0.54	(− 1.08 to − 0.01)	0.05	Vasoconstriction
RPL23AP7	ILMN_2222750	− 0.52	(− 0.95 to − 0.08)	0.02	Unknown
C21orf7	ILMN_1699071	− 0.48	(− 0.9 to − 0.07)	0.02	Unknown
RPL23AP7	ILMN_1750273	− 0.43	(− 0.8 to − 0.06)	0.02	Unknown
OST4	ILMN_1739335	− 0.43	(− 0.77 to − 0.09)	0.01	Glycosylation of polypeptides
RPS12	ILMN_1679920	− 0.43	(− 0.85 to − 0.01)	0.05	Ribosomal protein
TNNC2	ILMN_1693428	− 0.42	(− 0.83 to − 0.01)	0.05	Cytoskeleton
MIR130A	ILMN_3308808	0.42	(0.05 to 0.80)	0.03	Regulation of proliferation
GNLY	ILMN_1708779	0.43	(0.01 to 0.85)	0.04	Inflammation
RPS26	ILMN_3299955	0.67	(0.01 to 1.33)	0.05	Ribosomal protein
RPS26	ILMN_1657950	0.68	(0.03 to 1.33)	0.04	Ribosomal protein
RPS26	ILMN_3291511	0.80	(0.05 to 1.55)	0.04	Ribosomal protein
RPS26	ILMN_1750636	1.03	(0.02 to 2.04)	0.05	Ribosomal protein

*mRNA levels in responders relative to non-responders adjusted for age and gender

Table 3 Genes with the most altered expression (log_2 ratio > |0.25|) in responders compared to non-responders to omega-3 supplementation

Gene	Probe ID	Difference in log ratio*	95% CI	*P* value	Biological function/process
LINC01000	ILMN_3234967	− 0.47	(− 0.76 to − 0.19)	0.003	Unknown
TGFBR3	ILMN_1784287	− 0.40	(− 0.71 to − 0.09)	0.01	Growth factor and cytokine signaling
CCDC88C	ILMN_3248352	− 0.37	(− 0.74 to 0.0)	0.05	Wnt signaling
SDF4	ILMN_2378257	− 0.33	(− 0.66 to − 0.01)	0.05	Ca^{2+} binding
PKN1	ILMN_2367710	− 0.31	(− 0.6 to − 0.02)	0.04	Signal transduction in apoptosis
SNORD33	ILMN_1682354	− 0.30	(− 0.55 to − 0.05)	0.02	Small nucleolar RNA
NBPF10	ILMN_2155719	− 0.29	(− 0.54 to − 0.04)	0.02	Unknown
HNRNPL	ILMN_2389582	− 0.28	(− 0.48 to − 0.09)	0.007	mRNA processing
CYFIP2	ILMN_2354478	− 0.28	(− 0.53 to − 0.03)	0.03	T cell adhesion
PRRC2A	ILMN_2408179	− 0.28	(− 0.55 to 0.0)	0.05	Associated with IDDM and RA development
RFFL	ILMN_1753819	− 0.28	(− 0.4 to − 0.15)	0.0002	Apoptosis
FYN	ILMN_2380801	− 0.26	(− 0.49 to − 0.03)	0.03	Proliferation
GPKOW	ILMN_1684197	− 0.26	(− 0.49 to − 0.02)	0.04	mRNA processing
ZNF683	ILMN_1678238	− 0.25	(− 0.5 to − 0.01)	0.04	T cell differentiation
SNN	ILMN_1788251	0.27	(0.04 to 0.51)	0.03	Regulation of growth and apoptosis
RPL7	ILMN_1815292	0.30	(0.01 to 0.58)	0.04	Ribosomal protein
FTH1	ILMN_1696911	0.32	(0.05 to 0.59)	0.02	Iron storage
RPS3A	ILMN_1679025	0.34	(0.03 to 0.66)	0.04	Ribosomal protein

*Change in mRNA levels from baseline to the 7-week visit in responders relative to non-responders adjusted for age and gender
IDDM insulin-dependent diabetes mellitus, *RA* rheumatoid arthritis

Table 4 Top 10 enriched pathways in responders compared to non-responders after 7 weeks of omega-3 supplementation

Pathway maps	Ratio	*P* value	FDR	Gene transcripts
Immune response: lysophosphatidic acid signaling via NF-κB	5/53	0.0003	0.14	*ROCK2, IL6, CARD10, ROCK, TRIP6*
Inhibition of TGF-beta signaling in lung cancer	4/31	0.0003	0.14	*SERPINE1 (PAI1), TAK1 (MAP3K7), TGFBR3, SMAD6*
Immune response: IL-11 signaling pathway via MEK/ERK and PI3K/AKT cascades	5/67	0.0008	0.18	*YES1, HRAS, IL6, FYN, NCOA1*
Activation of Cortisol production in major depressive disorder	4/40	0.0009	0.18	*ASAH1, IL6, ABCB1, CYP11A1*
Development: ACM2 and ACM4 activation of ERK	4/43	0.001	0.18	*HRAS, CALD1, FYN, PLCB1*
Development: Angiotensin II/ AGTR1 signaling via RhoA and JNK	5/77	0.001	0.18	*ROCK2, RECK, SERPINE1 (PAI1), PLCB1, ROCK*
Transcription: Androgen Receptor nuclear signaling	4/46	0.001	0.18	*HRAS, NCOA1, IL6, DVL3*
Immune response: M-CSF-receptor signaling pathway	5/81	0.002	0.19	*YES, CLB, HRAS, MSR1, FYN*
Chemotaxis: lysophosphatidic acid signaling via GPCRs	6/129	0.003	0.21	*HRAS, ROCK, PREX1, PKN1, TRIP6, PLCB*
Development: angiotensin II/ AGTR1 signaling via p38, ERK and PI3K	5/94	0.003	0.21	*RECK, SERPINE1 (PAI1), HRAS, IL6, FYN*

The ratio indicates the number of altered transcript out of the total number of transcripts in the pathway

compared to non-responders including the TF hepatic nuclear factor 4 alpha (HNF4-α; Additional file 4).

Discussion

In the present exploratory study, we found that intake of omega-3 fatty acids differentially altered PBMC gene expression in TG responders and non-responders. Specifically, enriched pathways in responders compared to non-responders were related to development and apoptosis, immune function, and LPA signaling. These results lend further support to the findings of Rudkowska et al. who investigated transcriptomic and metabolomics profiles of TG responders and non-responders to omega-3 supplementation. They reported that 1020 transcripts were altered within the non-responder group, and 252 transcripts were altered within the responder group with only 10 transcripts overlapping between the groups [9]. In the current study, we also report that responders had higher baseline TG in M- and S-HDL subclasses and a greater reduction in TG levels in four of six VLDL and three of four HDL subclasses than non-responders.

Among the enriched pathways in responders compared to non-responders, several were related to development signaling and immune response. In line with this, omega-3 supplementation in elderly subjects altered immune-related pathways in PBMCs [26]. This may be expected, as PBMCs are cells of the immune system, and omega-3 fatty acids alter immune responses through altering NF-κB- and PPAR-induced gene expression and by acting as precursors for the production of anti-inflammatory and pro-resolving lipid mediators [4, 29]. In addition, pathways related to immune response and apoptosis have previously been found to be altered in the group that received fish oil compared to the control oil in this study [27]. This supports that responders had altered pathways related to apoptosis and immune function compared to non-responders in this sub-study.

Pathways related to LPA signaling were enriched in responders compared to non-responders to omega-3 supplementation. LPA is a lipid containing a fatty acid that may vary in length and degree of unsaturation. LPA can be produced from membrane phospholipids; it binds and activates LPA receptors (LPAR) and different forms of LPA may differentially affect LPA signaling [30]. In addition to their role in development of the central nervous system, LPARs are expressed in lymphocytes where they affect cytokine secretion, chemotaxis, and proliferation, and LPA signaling may be involved in the development of atherosclerosis and adipocyte differentiation [30–32]. We found that responders and non-responders had different levels of various plasma fatty acids that may reflect different plasma levels of various chemical forms of LPA [30]. Similarly, Rudkowska et al. found that compared to non-responders, responders had a greater increase in unsaturated fatty acids in glycerophosphatidylcholines, which can be used as LPA precursors [9, 30].

Transcripts with binding sites for HNF4-α were over-represented in the list of gene transcripts differentially altered in responders compared to non-responders. Long-chain PUFAs have been shown to suppress HNF4-α activity [33]. HNF4-α is a TF that induces the expression of genes involved in lipid metabolism, and a decreased HNF4-α activity has been suggested to explain decreases in serum TG levels [34]; hence, an altered activity of HNF4-α in responders may be involved in the TG-lowering effect of omega-3 fatty acids.

The baseline level and the change in plasma omega-3 fatty acid levels did not differ between responders and non-responders, indicating that omega-3 fatty acid levels in plasma are not important for the TG-lowering effect. Indeed, omega-3 fatty acids mediate many of their biological effects after being incorporated into plasma membrane phospholipids [35]. Thus, the level of omega-3 fatty acids in red blood cells (RBC), also called the omega-3 index, that indicate the level of biologically

available omega-3 fatty acids may be a better measure. We report that responders had a lower estimated omega-3 index than non-responders at baseline. This may imply that responders had a lower habitual fish intake before the start of the intervention than non-responders. In line with this, only participants with a low habitual fish intake had a reduced risk of major cardiovascular events in the VITAL trial [36]. Finally, only responders had an increase in RBC DHA in the study by Rudkowska et al., indicating that the level of omega-3 fatty acids in cell membranes may be important for the TG-lowering response [9]. Coupled with the higher baseline TG levels in responders, the lower omega-3 index at baseline may explain why responders lowered their TG levels after omega-3 supplementation.

At baseline, responders had higher plasma OA levels and lower plasma LA levels than non-responders, which may reflect different dietary patterns at baseline. This may have affected the ability of omega-3 fatty acids to lower TG levels. To further understand the variation of the TG-lowering effect after omega-3 supplementation, the role of other nutrients, such as other fatty acids, in modulating this effect should be elucidated in future studies.

As expected, the TG reduction in responders was reflected by reduced TG levels in almost all VLDL subclasses. However, there was also an unexpected lowering of HDL-TG in the XL-, M-, and S-HDL subclasses. Changes in HDL-TG levels in the current study may indicate that cholesteryl ester transfer protein (CETP) could be involved, as CETP facilitates the transfer of cholesteryl esters from HDL to VLDL and LDL in the exchange for TG. Although we did not find a difference between responders and non-responders in the change in *CETP* expression, CETP activity has been shown to increase after omega-3 intake [37]. In future TG responder studies, measurements of CETP activity may provide valuable insight into possible mechanisms behind the TG-lowering effect of omega-3 fatty acids.

The classification of subjects into responders and non-responders was based on previous studies that have suggested and employed a classification of responders as those with a TG reduction, while subjects who increase or do not alter their TG levels have been classified as non-responders [7, 9]. To be more confident that the group we defined as responders actually reduced their TG levels as a response to increased omega-3 intake, we chose to only include responders with a clinically relevant TG reduction. Day-to-day variation in TG levels has been reported to be 20%; hence, a TG reduction greater than 20% in responders in this study is likely to be an effect beyond day-to-day variations [38]. Another approach would be to use the actual day-to-day TG variation in the control group in our previous study to define responders and non-responders. However, the day-to-day variation in the control group was as high as 40%, which would result in only three participants who would be defined as responders, and the statistical power of this sub-study would be low. Nonetheless, the use of actual TG variation may be useful in future studies. Moreover, the baseline TG level in this study was low, 1.5 mmol/L in responders and 0.8 mmol/L in non-responders. The TG reduction observed after omega-3 supplementation depends on the baseline TG level [3]; hence, the low baseline TG level in this study may have resulted in a lower percentage of responders compared to other studies [6, 8, 39]. Future studies investigating TG responders to omega-3 intake should include participants with high enough baseline TG levels to ensure a clinically relevant TG response.

This study is limited by the low baseline TG levels and the low number of participants. As we included fewer subjects in this sub-study compared to the main study, this study may be underpowered. In addition, no gene expression changes were significant after adjusting for multiple testing. Hence, we did not expect any detectable differences between responders and non-responders in analyses of single genes using qPCR. Therefore, the microarray results in this study are not validated by qPCR. Furthermore, an FDR limit of 25% for the enriched pathways implies that 1 in 4 of the significant results is a false positive. However, a high FDR limit was chosen to avoid losing interesting results. Hence, the results in this study needs to be interpreted with caution and validated in a study designed and powered to investigate differences between TG responders and non-responders to omega-3 fatty acid supplementation. Despite these limitations, this exploratory study found differences between responders and non-responders, and it shows that this study design may be useful for investigating the underling mechanisms of the TG-lowering effect of omega-3 fatty acids.

Conclusions

TG responders and non-responders to omega-3 supplementation have different baseline lipoprotein subclass and PBMC gene expression profiles. Furthermore, they differentially alter their lipoprotein subclass and PBMC gene expression profiles. The differentially altered PBMC gene expression may partially explain the variability in TG response to omega-3 intake.

Methods

Study design and subjects

In this exploratory study, we used data from a previous double-blind randomized controlled parallel-group trial that aimed to investigate health effects of fish oils with different qualities [40]. The study was performed at Akershus University College in 2009. Healthy and

non-smoking men and women aged 18–50 years with BMI < 30 kg/m^2, TG ≤ 4 mmol/L, total cholesterol ≤ 7.5 mmol/L, C-reactive protein ≤ 10 mg/L, glucose ≤ 6 mmol/L, and blood pressure < 160/100 mmHg were included. Participants were stratified by gender and randomized in a 1:1:1 ratio to receive 1.6 g EPA + DHA per day from fish oil or oxidized fish oil or 0 g EPA + DHA per day from high-oleic sunflower oil. In total, 54 participants completed the 7-week intervention. Four weeks prior to the baseline visit and throughout the study, participants were instructed to avoid consumption of fish, omega-3 supplements, or foods enriched in omega-3 fatty acids. The first 3 weeks of the intervention was a fully controlled isoenergetic diet period, and for the remaining 4 weeks, subjects returned to their habitual diet, still avoiding consumption of omega-3 fatty acids. We have previously described subject characteristics, protocol, blinding, compliance and side effects, study products, and the fully controlled diet period in detail [40].

In the current sub-study, we combined data from the two groups that received fish oil (n = 35). From baseline to the 7-week visit, participants increased their intake of omega-3 fatty acids while their remaining diet was unchanged. Hence, we used data from these two visits to investigate differences in TG responders and non-responders to omega-3 fatty acids. In other studies, TG responders have been defined as all subjects with a TG reduction and non-responders as subjects who have no change or an increase in TG levels following omega-3 supplementation [7, 9]. Here, we define responders as subjects having a larger reduction in fasting TG than the 20% day-to-day variation [38] (n = 8) and non-responders as having a TG change between − 20 and 20% (n = 16). Because we wanted to compare participants with a TG reduction with those with no change in TG levels following omega-3 supplementation, participants with a higher TG increase than the 20% day-to-day variation were excluded from analyses in this study (n = 11).

This study was performed according to the guidelines laid down by the Declaration of Helsinki, and all procedures involving human subjects were approved by the Regional Committee for Medical Ethics (approval no. 6.2008.2215) and the Norwegian Social Science Data Services (approval no. 21924). All subjects provided written informed consent, and the study was registered at www.clinicaltrials.gov (ID no. NCT01034423).

Blood sampling and routine laboratory analyses

Blood samples were drawn at the baseline and 7-week visits after an overnight fast (≥ 12 h). Participants were instructed to avoid alcohol consumption and strenuous physical activity the day before blood sampling. Whole blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes that were kept at room temperature for maximum 48 h. Serum was obtained from silica gel tubes that were kept at room temperature for at least 30 min before centrifugation (1500***g***, 12 min), and plasma was obtained from EDTA tubes that were immediately placed on ice and centrifuged within 10 min (1500*g*, 4 °C, 10 min). Routine laboratory analyses, such as serum TG, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), as well as white blood cell count in whole blood were performed at a clinical routine laboratory (Fürst Medical Laboratory, Oslo, Norway). Plasma fatty acids were extracted by the Bligh and Dyer method [41] as previously described [40]. The level of plasma fatty acids is expressed as the percentage of total plasma fatty acids.

Dried blood spot omega-3 index

Whole blood from EDTA tubes were transferred to a filter paper that was dried, sealed in plastic bags, and stored at − 80 °C until analysis. The level of omega-3 fatty acids in the dried blood spots was analyzed by Vitas analytical service, Oslo, Norway. The omega-3 index was estimated from the level of whole blood EPA and DHA using an equation derived by a Norwegian population by Vitas AS (omega-3 index = whole blood EPA + DHA (%) * 0.95 + 0.35).

Analysis of lipoprotein subclasses

The EDTA plasma concentrations of 14 different lipoprotein subclass particles and their lipid constituents, including TG levels, were measured with a commercially available NMR platform (Nightingale Health Ltd). The different lipoprotein subclasses were defined based on their average diameter: extremely large very low-density lipoprotein (VLDL) with a possible contribution of chylomicrons (> 75 nm); extra large (XL-), large (L-), small (S-), and extra small (XS-) VLDL (64.0, 53.6, 44.5, 36.8, and 31.3 nm); intermediate-density lipoprotein (IDL, 28.6 nm); L-, M-, and S-LDL (25.5, 23.0 and 18.7 nm); and XL-, L-, M-, and S-HDL (14.3, 12.1, 10.9 and 8.7 nm). Details of the NMR metabolomics platform have previously been described [42].

Isolation of PBMC and RNA and microarray hybridization

PBMC were isolated using BD Vacutainer Cell Preparation tubes (Becton, Dickinson San Jose, CA, USA), a well-documented method for PBMC isolation with more than 90% purity. According to the manufacturer, about 80% of the isolated PBCMs are lymphocytes and 12% are monocytes. The PBMCs were isolated according to the manufacturer's instructions and cell pellets were stored at − 80 °C. Total RNA was isolated using the Qiagen's RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA quality and quantity were measured with the NanoDrop ND-1000

Spectrophotometer (Thermo Fisher Scientific, Gothenburg, Sweden) and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The average RNA integrity number (RIN) was 9.6. Gene expression was analyzed by hybridization to an Illumina HumanHT-12 v4 Expression BeadChip that was scanned on an Illumina HiSCan microarray scanner (Illumina Inc., San Diego, CA, USA). Illumina GenomeStudio was used to transform bead-level data to probe-level intensities, which were exported raw for bioinformatics analysis as previously reported [27].

Analyses of microarray data

The Illumina intensities were quantile normalized, and probes without a detectable expression in at least 10% of the samples were excluded (detection $P > 0.01$). Of the 48,000 probes, 21,236 probes on the Illumina array were defined as expressed. A more detailed protocol of the microarray analyses has previously been reported [27]. We calculated the change in gene expression as the $\log_2$ ratio between intensities at baseline and after 7 weeks. The differences in gene expression changes between responders and non-responders were tested with multiple regression analyses adjusted for age and gender. Differentially expressed genes ($p \leq 0.05$) were subjected to pathway analyses and transcription factor (TF) analyses using MetaCore (GeneGo, division of Thomson Reuters, St Joseph, MI, USA), and enriched pathways with a false discovery rate (FDR) < 25% were considered significant.

Other statistical analyses

Sample size calculation for the main study was based on the expected change in plasma omega-3 levels and has previously been described [40]. Lipoprotein subclass TG levels were $\log_2$-transformed before baseline differences and the difference in change from baseline ($\log_2$ ratio) between responders and non-responders were tested with a t test. Differences in categorical data were tested with Fisher's exact test. All other data are presented as median and 25th–75th percentiles. The differences from baseline to the 7-week visit within a group were tested with a paired Wilcoxon signed rank test and the differences between responders and non-responders at baseline and the differences in change from baseline to the 7-week visit were tested with a Mann-Whitney U test. All statistical analyses were performed in R [43].

Additional files

Additional file 1: List of genes with a significantly ($p \leq 0.05$) different baseline expression in responders compared to non-responders adjusted for age and gender. (XLSX 671 kb)

Additional file 2: List of significantly ($p \leq 0.05$) differentially altered gene transcripts in responders compared to non-responders adjusted for age and gender. (XLSX 622 kb)

Additional file 3: Enriched pathways (FDR < 25%) among the significantly altered transcripts in responders compared to non-responders. (XLSX 14 kb)

Additional file 4: Results from the transcription factor analyses. (XLSX 16 kb)

Abbreviations

AA: Arachidonic acid; ACACA: Acetyl-CoA carboxylase α; ACLY: ATP citrate lyase; ACOX1: Acyl-CoA oxidase 1; ALA: α-Linolenic acid; APOE: Apolipoprotein E; BMI: Body mass index; C: Cholesterol; CD36: Cluster of differentiation 36; CETP: Cholesteryl ester transfer protein; CVD: Cardiovascular disease; DGAT2: Diacylglycerol O-acyltransferase 2; DHA: Docosahexaenoic acid; EDTA: Ethylenediaminetetraacetic acid; EPA: Eicosapentaenoic acid; FDR: False discovery rate; FO: Fish oil; GWAS: Genome-wide association study; HDL: High-density lipoprotein; HNF4A: Hepatic nuclear factor 4α; IDL: Intermediate-density lipoprotein; L-: Large; LA: Linoleic acid; LDL: Low-density lipoprotein; LPA: Lysophosphatididic acid; LPAR: Lysophosphatididic acid receptor; M-: Medium; NF-κB: Nuclear factor kappa B; NMR: Nuclear magnetic resonance; OA: Oleic acid; oxFO: Oxidized fish oil; PBMC: Peripheral blood mononuclear cells; PPAR: Peroxisome proliferator-activated receptors; RBC: Red blood cell; RXRA: Retinoid X receptor α; S-: Small; SREBP: Sterol regulating element binding protein; TF: Transcription factor; TG: Triglycerides; TGF-beta: Transforming growth factor β; VLDL: Very low-density lipoprotein; XL-: Extra large; XS-: Extra small; XXL-: Extremely large

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Authors' contributions

AR designed the research, performed the statistical analyses, interpreted the data, and drafted the manuscript. SVL designed the research, performed the statistical analyses, interpreted the data, and drafted the manuscript. MCM designed the research, performed the microarray analysis, interpreted the data, and drafted the manuscript. IO designed the research, coordinated the intervention study, and drafted the manuscript. MT contributed to the analysis and interpretation of the data and drafted the manuscript. KBH designed the research, interpreted the data, and drafted the manuscript. SMU designed the research, was responsible for the intervention study, interpreted the data, and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Given in the methods section.

Consent for publication

Not applicable.

Competing interests

Dr. Holven has received research grants and/or personal fees from Tine SA, Mills SA, Olympic Seafood, Kaneka, Amgen, Sanofi, and Pronova, none of which are related to the content of this manuscript. Dr. Ulven has received research grants from Tine DA, Mills DA, and Olympic Seafood, none of which are related to the content of this manuscript. The other authors declare that they have no competing interests.

Publisher's Note

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III

RESEARCH ARTICLE

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Replacing Saturated Fat with Polyunsaturated Fat Modulates Peripheral Blood Mononuclear Cell Gene Expression and Pathways Related to Cardiovascular Disease Risk Using a Whole Transcriptome Approach

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Scope: The aim of this study is to explore the molecular mechanisms underlying the effect of replacing dietary saturated fat (SFA) with polyunsaturated fat (PUFA) on cardiovascular disease (CVD) risk using a whole transcriptome approach.

Methods and Results: Healthy subjects with moderate hypercholesterolemia ($n = 115$) are randomly assigned to a control diet (C-diet) group or an experimental diet (Ex-diet) group receiving comparable food items with different fatty acid composition for 8 weeks. RNA isolated from peripheral blood mononuclear cells (PBMCs) at baseline and after 8 weeks of intervention is analyzed by microarray technology ($n = 95$). By use of a linear regression model ($n = 92$), 14 gene transcripts are differentially altered in the Ex-diet group compared to the C-diet group. These include transcripts related to vascular smooth muscle cell proliferation, low-density lipoprotein receptor folding, and regulation of blood pressure. Furthermore, pathways mainly related to immune response and inflammation, signal transduction, development, and cytoskeleton remodeling, gene expression and protein function, are differentially enriched between the groups.

Conclusion: Replacing dietary SFA with PUFA for 8 weeks modulates PBMC gene expression and pathways related to CVD risk in healthy subjects with moderate hypercholesterolemia.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death and disability worldwide.[1] Lifestyle changes, including dietary modification, is a key strategy in primary prevention of CVD.[2] Replacing dietary saturated fat (SFA) with polyunsaturated fat (PUFA) is associated with reduced CVD risk,[3–5] and randomized controlled trials have shown that this effect is largely attributed to lowering of serum low-density lipoprotein (LDL) cholesterol,[6–10] which is a well-characterized CVD risk factor. The molecular mechanisms underlying the health effect of replacing SFA with PUFA on CVD risk are, however, incompletely understood. Furthermore, the CVD reducing effect of the n-6 PUFAs is debated, as these fatty acids are suggested to promote inflammation.[11,12]

Atherosclerosis is the underlying process leading to most CVDs. Dyslipidemia and chronic low-grade inflammation are

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major players involved in the progression of atherosclerosis and development of CVD.[13] Dietary fatty acids may influence CVD risk through transcriptional regulation of genes encoding proteins involved in lipid metabolism and inflammation. There is considerable evidence that PUFAs modulate the transcription of genes encoding proteins involved in lipid metabolism by regulating the activity of the nuclear receptors peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs), or by suppressing the nuclear abundance of the sterol regulatory binding proteins (SREBPs).[14] In addition, activation of PPARs and LXRs reduces inflammation by inhibiting the nuclear factor kappa B (NF-kB) signaling pathway.[15] By contrast, SFAs have been shown to activate the membrane receptor toll-like receptor 4 (TLR4) leading to activation of the NF-kB signaling pathway.[16]

Peripheral blood mononuclear cells (PBMCs) are immune cells consisting mostly of monocytes and lymphocytes. These cells are exposed in vivo to many of the same environmental factors as metabolically active tissues and the arterial wall, and play a central role in the inflammatory process of atherosclerosis.[17] Hence, PBMCs may provide information about how fatty acids affect CVD risk. Previously, our group and others have investigated the effect of a healthy Nordic diet and a traditional Mediterranean diet, which include an improved fatty acid composition, on the PBMC whole transcriptome response.[18,19] Others have also investigated the postprandial effect of high fat challenges with different fatty acids on the PBMC whole transcriptome response.[20] In the LIPGENE study, the postprandial effect of fat challenges after a 12 week intervention of diets with different fat quality on targeted PBMC gene expression focusing on inflammation and oxidative stress was studied.[21,22] However, to our knowledge, no studies have investigated the effect of replacing dietary SFA with PUFA on the whole transcriptome response in PBMCs for a longer study duration.

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Recently, we performed an 8 week double-blind, randomized, controlled dietary intervention study exchanging commercially available and regularly consumed food items with similar food items with improved fat quality (replacing SFAs with mostly n-6 PUFAs) among subjects with moderate hypercholesterolemia. The primary finding of this study was that serum total- and LDL cholesterol was reduced by 9% and 11% in the experimental diet (Ex-diet) group compared to the control diet (C-diet) group.[6] Furthermore, the lipoprotein concentration of the atherogenic particles was reduced and many metabolites associated with CVD risk were favorably altered in the Ex-diet group compared to the C-diet group.[23] Using a targeted approach, the PBMC gene expression of the LDL receptor *(LDLR)* and *LXRA*, some LXR target genes, and several gene transcripts involved in inflammation, was increased.[23]

The aim of this sub-study was to further explore the molecular mechanisms underlying the effect of replacing dietary SFA with PUFA on CVD risk, using a whole transcriptome approach examining differences in changes in PBMC gene expression profiles and pathways between the Ex-diet group and the C-diet group during the intervention.

2. Results

2.1. Subject Characteristics and Clinical and Biochemical Measurements

A total of 92 subjects (C-diet group; $n = 49$, Ex-diet group; $n = 43$) were included in the statistical analyses of this sub-study, as three subjects were excluded because they were considered as outliers (**Figure 1**). Subject characteristics and changes in clinical and biochemical measurements are presented in **Table 1**, and were in agreement with the results obtained for the whole study population ($n = 99$).[6] There were no significant differences between the C-diet group and the Ex-diet group at baseline (data not shown). As reported previously,[6] serum total- and LDL cholesterol concentrations were significantly reduced (p-value ≤ 0.001 for both), and the plasma level of linoleic acid (LA) was significantly increased (p-value ≤ 0.001), in the Ex-diet group compared to the C-diet group. In addition, there was a significant reduction in serum HDL cholesterol concentration (p-value $= 0.003$) in the Ex-diet group compared to the C-diet group. There were no significant differences in changes in weight and body mass index (BMI), serum concentrations of triglycerides (TG), lipoprotein (a) (Lp(a)), glucose and high-sensitive C-reactive protein (hs-CRP), and number of lymphocytes and monocytes, between the groups (Table 1), and no significant differences in changes in serum levels of interleukin 6 (IL-6), soluble tumor necrosis factor receptor 1 (sTNFR1), and interferon (IFN)-γ (data not shown).

2.2. Whole Genome Gene Expression Profiling in PBMCs

Microarray hybridization was performed on RNA isolated from PBMCs collected at baseline and after 8 weeks of intervention. Among 48 000 probe sets presented on the Illumina HumanHT-12 v4 microarray, 13 148 unique gene transcripts were defined as

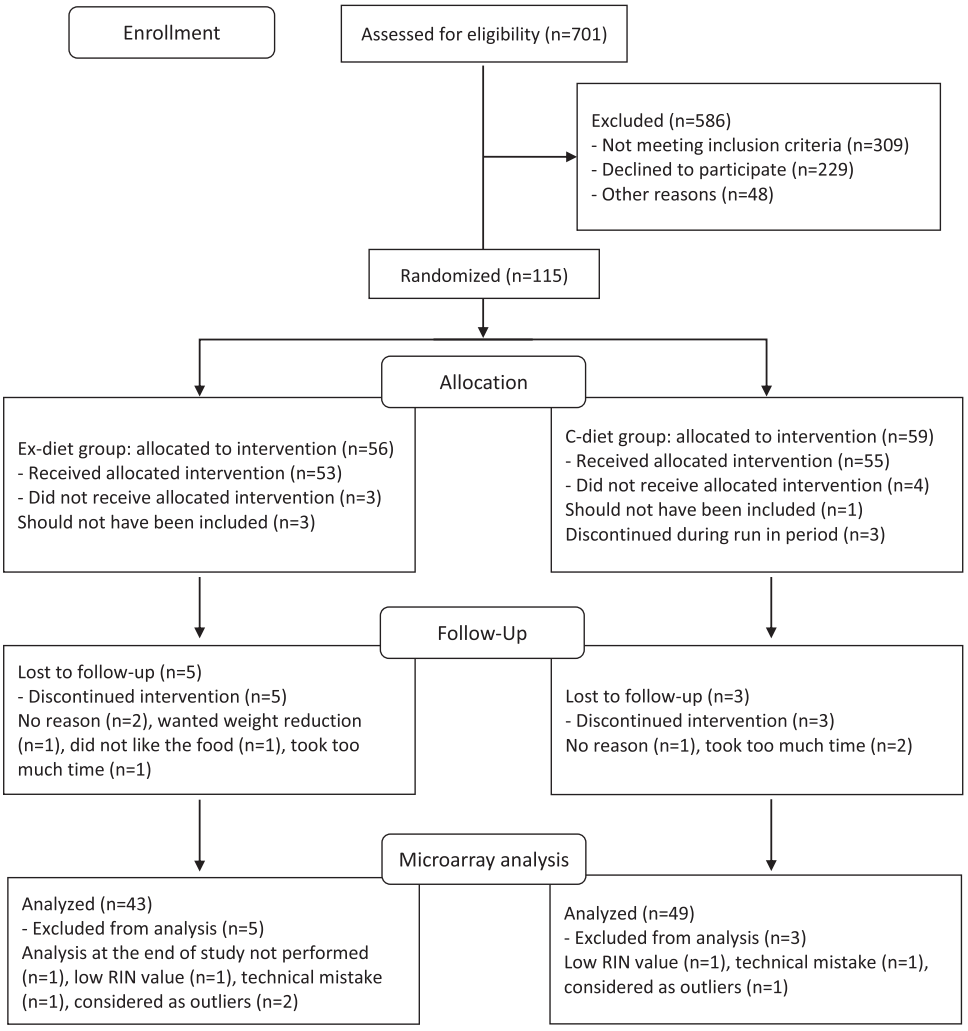

Figure 1. Flowchart showing the number of subjects included in the microarray analyses. C-diet, control diet; Ex-diet, experimental diet.

expressed and included in the statistical analyses of the present study. Of these, 1105 (8.4%) gene transcripts were differentially altered between the groups when comparing the relative change from baseline to 8 weeks of intervention (p-value < 0.05) (**Figure 2**A, Table S1, Supporting Information), and subjected to further pathway and transcription factor analyses in Metacore. After adjusting for multiple testing, 14 gene transcripts were significantly differentially altered in the Ex-diet group compared to the C-diet group (false discovery rate (FDR) q-value < 0.25), in which eight transcripts were up-regulated and six transcripts were down-regulated (Figure 2B, **Table 2**).

Since we observed a reduction in serum total- and LDL cholesterol concentration in the Ex-diet group compared to the C-diet group during the intervention, we examined whether changes in the expression level of the 14 differentially altered gene transcripts between the groups were correlated to change in serum concentration of total- and LDL cholesterol among all subjects (n = 92). The correlation analyses revealed a significant positive correlation between the change in *ATP2B1* expression and the change in total cholesterol concentration (r = 0.27, p-value = 0.009), and a borderline positive correlation between the change in *ATP2B1* expression and the change in LDL cholesterol concentration (r = 0.20, p-value = 0.05). In addition, there was a significant positive correlation between the change in *FGF18* expression and the change in total cholesterol concentration (r = 0.22, p-value = 0.032), and a significant negative correlation between the change in *GUYC1B1* expression and the change in LDL cholesterol concentration (r = −0.21, p-value = 0.046).

To further explore the effect of dietary fat quality on PBMC gene expression, we analyzed the effect of change in plasma LA level on changes in gene expression during the intervention (n = 92). These analyses showed that 169 gene transcripts were significantly altered depending on change in plasma LA level during the intervention (FDR q-value < 0.25), in which 58 transcripts were up-regulated and 111 transcripts were down-regulated (Table S2, Supporting Information). As shown in **Figure 3**, there was a strong covariation between the group effect and the LA effect on gene expression changes. Eight of 14 differentially altered gene transcripts between the groups (FDR q-value < 0.25) were also significantly altered depending on change in plasma LA level (Figure 3).

Table 1. Baseline characteristics and changes in clinical and biochemical measurements from baseline to 8 weeks of intervention.

	C-diet group ($n = 49$)		Ex-diet group ($n = 43$)		p-value
	Baseline	Change	Baseline	Change	
Clinical measurements					
Age [years]	55.7 ± 9.7	–	53.9 ± 9.8	–	
Gender: female, *n* (%)	30 (61.2)	–	25 (58.1)	–	
Smokers, *n* (%)	8 (16.3)	–	3 (7)	–	
Weight [kg]	74.3 ± 13.3	0.4 ± 1.1	76.1 ± 12.1	0 ± 1.1	0.094
BMI [kg m^{-2}]	24.7 ± 3	0.1 ± 0.3	25.5 ± 3	0 ± 0.4	0.092
Plasma fatty acids					
18:2-n6, %	27.1 ± 3	−0.6 ± 2.0	27.4 ± 2.9	3.0 ± 2.2	<0.001
Biochemical measurements					
Total cholesterol [mmol L^{-1}]	6.6 ± 0.8	0.1 ± 0.5	6.5 ± 0.8	−0.5 ± 0.5	<0.001
LDL cholesterol [mmol L^{-1}]	4.1 ± 0.7	0.1 ± 0.5	4.1 ± 0.6	−0.4 ± 0.4	<0.001
HDL cholesterol [mmol L^{-1}]	1.7 ± 0.4	0 ± 0.2	1.7 ± 0.5	−0.1 ± 0.1	0.003
TG [mmol L^{-1}]	1.2 (0.5)	−0.1 (0.9)	1.3 (0.7)	0.2 (1.2)	0.063
Lipoprotein (a) [mg dL^{-1}]	144 (338)	0.5 (21.5)	115.5 (465.8)	1.5 (23.5)	0.844
Glucose [mmol L^{-1}]	5.2 (0.4)	−0.1 (0.4)	5.3 (0.5)	0 (0.4)	0.320
hs-CRP [mg L^{-1}]	1.1 (1.4)	0.1 (0.5)	1.2 (1.3)	−0.1 (1)	0.147
Lymphocytes, %	36.2 (10.7)	−0.5 (5)	34.7 (8.9)	1 (5.5)	0.248
Monocytes, %	8.6 (2.4)	0.8 (2.5)	8.3 (2.2)	−0.2 (2.1)	0.060

Data are presented as mean ± SD or median (IQR). Fatty acids are given as percentage of plasma total fatty acids. Lymphocytes and monocytes are given as percentage of total white blood cell counts. p-value: between group changes; independent t-test or Mann–Whitney U test. C-diet, control diet; Ex-diet, experimental diet; hs-CRP, high-sensitivity C-reactive protein; TG, triglyceride.

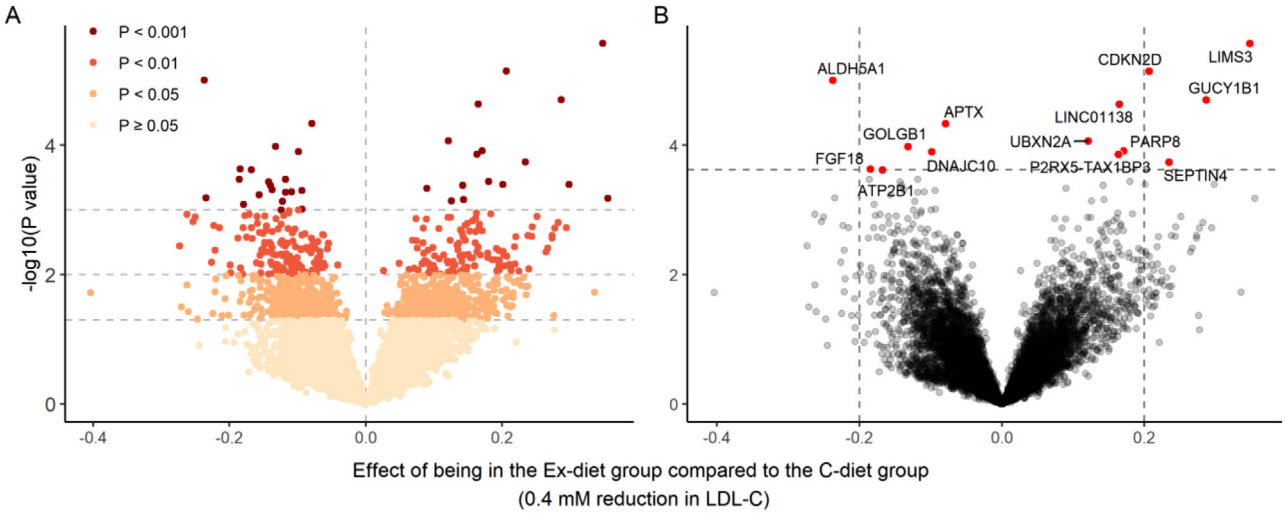

Figure 2. Difference in log2 fold change between groups (x axis) versus -log10(p-value) (y axis) for 13 148 gene transcripts analyzed with a linear regression model adjusted for sex, age, study center, smoking, and log(baseline gene expression) level. A) The vertical line represents no difference between the Ex-diet group and the C-diet group, and the three horizontal lines represent cutoffs for p-value < 0.05, <0.01, and <0.001. B) The horizontal line represents a cutoff for FDR q-value < 0.25. Gene transcripts significantly differentially altered within this cutoff are highlighted in red. C-diet, control diet; Ex-diet, experimental diet; LDL-C, low-density lipoprotein cholesterol.

2.3. Pathway and Transcription Factor Analyses

To examine biological processes differentially altered across the two groups during the intervention, we analyzed functional relationships among 1105 differentially altered gene transcripts between the groups (p-value < 0.05) using the software tool Metacore. In total, 101 pathways were significantly enriched in the Ex-diet group compared to the C-diet group (FDR q-value < 0.01, Table S3, Supporting Information). The majority of the enriched pathways were related to immune response and

Table 2. Differentially altered gene transcripts in the Ex-diet group compared to the C-diet group from baseline to 8 weeks of intervention.

Illumina Id	Gene symbol	C-diet group ($n = 49$)		Ex-diet group ($n = 43$)		Log2 FC	FDR	Biological process/function
		Baseline	Change	Baseline	Change			
2372403	*ALDH5A1*	6.68 ± 0.32	0.13 ± 0.22	6.72 ± 0.32	−0.08 ± 0.31	−0.24	0.04	Acetate metabolic process
1797030	*FGF18*	5.07 ± 0.28	0.12 ± 0.31	5.09 ± 0.23	−0.08 ± 0.29	−0.18	0.22	Growth factor activity
2401933	*ATP2B1*	7.78 ± 0.23	0.12 ± 0.18	7.89 ± 0.24	−0.07 ± 0.25	−0.17	0.22	Calcium transporting ATPase activity
1747935	*GOLGB1*	8.22 ± 0.33	0.09 ± 0.16	8.22 ± 0.38	−0.05 ± 0.15	−0.13	0.16	DNA binding transcription factor activity
2151541	*DNAJC10*	9.43 ± 0.13	0.05 ± 0.13	9.43 ± 0.13	−0.05 ± 0.13	−0.10	0.16	Protein folding in ER
2317348	*APTX*	8.49 ± 0.11	0.04 ± 0.12	8.52 ± 0.1	−0.05 ± 0.09	−0.08	0.10	DNA ligation
1776038	*UBXN2A*	8.09 ± 0.17	−0.03 ± 0.17	8.09 ± 0.19	0.08 ± 0.14	0.12	0.16	Regulation of gene expression
1803392	*P2RX5-TAX1BP3*	8.51 ± 0.26	−0.04 ± 0.27	8.56 ± 0.28	0.1 ± 0.19	0.16	0.16	Naturally occurring read-through transcription
1810953	*LINC01138*	7.34 ± 0.25	−0.07 ± 0.19	7.29 ± 0.28	0.08 ± 0.2	0.16	0.06	Translation
1806651	*PARP8*	8.44 ± 0.32	−0.07 ± 0.26	8.4 ± 0.34	0.08 ± 0.23	0.17	0.16	Protein ADP ribosylation
1748883	*CDKN2D*	10.03 ± 0.29	−0.14 ± 0.22	10.07 ± 0.26	0.05 ± 0.21	0.21	0.04	DNA synthesis involved in DNA repair
1776157	*SEPTIN4*	6.88 ± 0.42	−0.11 ± 0.29	6.98 ± 0.5	0.09 ± 0.32	0.23	0.20	Apoptotic process
1782567	*GUCY1B1*	4.99 ± 0.32	−0.13 ± 0.34	5.13 ± 0.42	0.1 ± 0.33	0.29	0.06	Blood circulation
1799569	LIMS3	6.01 ± 0.31	−0.24 ± 0.33	6.04 ± 0.38	0.07 ± 0.38	0.35	0.04	Metal ion binding

Data are presented as log transformed mean values and standard deviations, and log2 fold change (FC). Statistical analyses have been performed using multiple regression analysis adjusted for sex, age, study center, smoking, and log(baseline gene expression) level. Gene transcripts with FDR q-value < 0.25 were defined as differentially altered. C-diet, control diet; Ex-diet, experimental diet; FC, fold change.

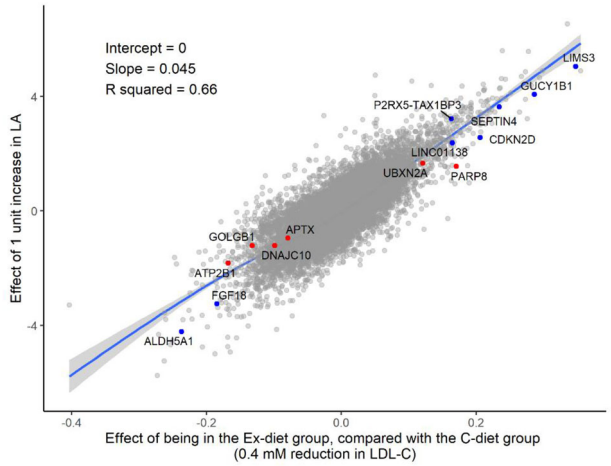

Figure 3. Covariation between group effect and LA effect on gene expression changes. Gene transcripts highlighted in blue or red are significantly differentially altered (FDR q-value < 0.25) in the Ex-diet group compared to the C-diet group. Gene transcripts highlighted in blue are significantly altered (FDR q-value < 0.25) depending on change in plasma LA level. C-diet, control diet; Ex-diet, experimental diet; LA, linoleic acid; LDL-C, low density lipoprotein cholesterol.

inflammation, signal transduction, development, and cytoskeleton remodeling, gene expression and protein function (**Figure 4**). The top 10 differentially enriched pathways were particularly related to immune response, apoptosis and survival, and blood coagulation (**Table 3**). Furthermore, we analyzed the up-regulated (517 transcripts, p-value < 0.05) and down-regulated (588 transcripts, p-value < 0.05) gene transcripts for functional relationships separately. Among the up-regulated gene transcripts, 131 pathways were significantly enriched (FDR q-value < 0.01) (Table S4, Supporting Information). The majority of these pathways were related to the biological processes described above. By contrast, no pathways were significantly enriched (FDR q-value < 0.01) among the down-regulated gene transcripts.

To further explore the changes in gene expression, transcription factor analyses were performed in Metacore to examine transcription factor binding sites within the regulatory regions of the 1105 differentially altered gene transcripts between the groups (p-value < 0.05). The up-regulated and down-regulated gene transcripts were analyzed separately. Gene transcripts with binding sites for 145 and 111 transcription factors were overrepresented among the up-regulated and down-regulated transcripts, respectively (Tables S5 and S6, Supporting Information). Gene transcripts with binding sites for many of the same transcription factors were overrepresented among both the up-regulated and down-regulated transcripts. However, there was also an increased occurrence of gene transcripts with binding sites for several unique transcription factors. Of particular interest was the increased occurrence of gene transcripts with binding sites for NRF2, PPARB, PPARG, LXRB, SREBP2, IRF2, c-Rel, NFKB2, and NFKB1 among the up-regulated transcripts, and the increased occurrence of gene transcripts with binding sites for

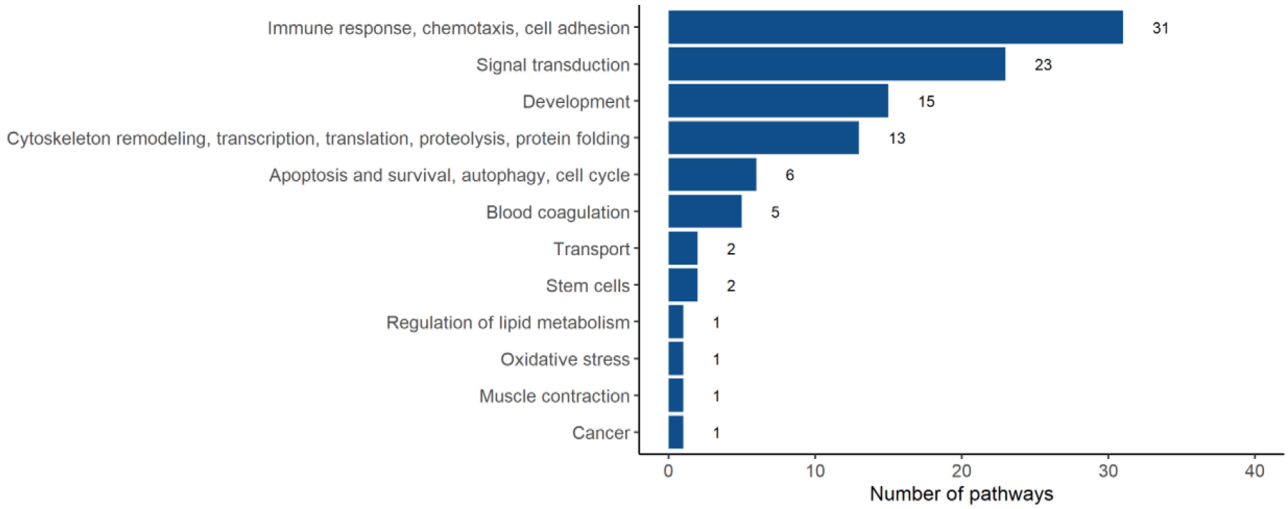

Figure 4. Number of differentially enriched pathways between the groups within different biological processes analyzed in Metacore. A total of 1105 gene transcripts (p-value < 0.05) were included in the analyses. Pathways with FDR q-value < 0.01 were defined as significantly differentially enriched.

ChREBP, RELB, NRF1, HNF3, and HNF6 among the down-regulated transcripts.

3. Discussion

In this sub-study of a randomized, controlled, dietary intervention study, we investigated the effect of replacing SFA with PUFA for 8 weeks on the PBMC whole transcriptome response in healthy subjects with moderate hypercholesterolemia. In total, 14 gene transcripts were differentially altered in the Ex-diet group compared to the C-diet group during the intervention, including transcripts involved in several processes related to CVD risk. Furthermore, functional analyses revealed that pathways mainly related to immune response and inflammation, signal transduction, development, and cytoskeleton remodeling, gene expression and protein function, were differentially enriched between the groups.

In the current study, the expression of *FGFG18* was down-regulated in the Ex-diet group compared to the C-diet group. This gene encodes a member of the fibroblast growth factor (FGF) family, which possesses broad mitogenic and cell survival activities and is involved in a variety of biological processes.[24] Furthermore, pathways related to apoptosis and survival, and cell cycle, were differentially modulated between the groups. In line with these findings, modulation of PBMC gene expression related to apoptosis and cell cycle has previously been observed in other dietary intervention studies in which the fat quality was improved or fish oil supplementation was given.[18,25] A down-regulation of *FGF18* has been linked to reduction of vascular smooth muscle cell (VSMC) proliferation.[26] Furthermore, we observed an up-regulation of *SEPTIN4*, encoding a member of the septin family of nucleotide binding proteins, which is also associated with reduced VSMC proliferation.[27] Since VSMC proliferation plays a key role in the development of atherosclerosis, the observed changes in *FGF18* and *SEPTIN4* expression are interesting as the changes in the expression of these genes may lead to reduced VSMC proliferation and subsequently prevent atherosclerosis. In addition, we detected a positive correlation between change in *FGF18* expression and change in serum total cholesterol concentration, further implicating an association between *FGF18* and atherosclerosis. In the present study, the fatty acid composition of the Ex-diet consisted mostly of LA, and statistical analyses showed that the expression of *FGF18* was also down-regulated depending on change in plasma LA level, which may indicate that this fatty acid is involved in the gene regulation of *FGF18*. However, further analysis is needed to clarify the causal role of LA.

We observed a down-regulation in the expression of *ATP2B1* in the Ex-diet group compared to the C-diet group. *ATP2B1* encodes the plasma membrane calcium ATPase isoform 1 (PMCA1), which plays a critical role in intracellular calcium homeostasis.[28] Genome wide association studies and *ATP2B1* knockout mouse studies have shown that *ATP2B1* may play an important role in the regulation of blood pressure and hypertension through alterations of calcium handling and vasoconstriction in VSMCs.[29,30] *ATP2B1* genomic regions have also been associated with hyperlipidemia and diabetes,[31] and in a recent study, *ATP2B1* gene silencing increased insulin sensitivity in endothelial cells.[32] Furthermore, in a candidate gene association study, *ATP2B1* was associated with coronary artery calcification in chronic kidney disease and myocardial infarction in the general population.[33] Since coronary artery calcification is a quantitative estimate of coronary atherosclerosis[34] and a useful predictor of coronary heart disease,[35] our findings are interesting as we know that replacing SFAs with PUFAs reduces the CVD risk. We have previously reported a significant reduction in serum total and LDL cholesterol concentration in the Ex-diet group compared to the C-diet group, subsequently affecting the risk of atherosclerosis and development of CVD. In the current study, the change in *ATP2B1* expression was positively correlated to the change in serum total cholesterol concentration, and there was a borderline positive correlation between the change in *ATP2B1* expression and the change in serum LDL cholesterol concentration.

Interestingly, we observed a down-regulation in the expression of *DNAJC10* in the Ex-diet group compared to the C-diet group. This gene encodes an endoplasmic reticulum (ER) co-chaperone,

Table 3. Top 10 enriched biological pathway maps among gene transcripts differentially altered (p-value < 0.05) in the Ex-diet group compared to the C-diet group from baseline to 8 weeks of intervention.

Pathway maps	p-value	FDR q-value	Ratio	Network object (gene symbol)
Immune response, B cell antigen receptor (BCR) pathway	4.331E-08	5.075E-05	18/110	GSK3 alpha/beta (GSK3B), IKK-gamma (IKBKG), Actin cytoskeletal (ACTB), ORAI1 (ORAI1), GSK3 beta (GSK3B), p70 S6 kinase1 (RPS6KB1), NF-kB (NFKB1), Calmodulin (CALM3), GRB2, CD79A (CD79A), NF-kB1 (p50) (NFKB1), SOS1, CalDAG-GEFIII (RASGRP3), FKHR (FOXO1), Lyn, PLC-gamma 2 (PLCG2), PLC-gamma (PLCG2), Rac1
Cytoskeleton remodeling, reverse signaling by ephrin-B	7.899E-08	5.075E-05	10/132	G-protein beta/gamma (GNB5, GNG11, GNG8), Actin cytoskeletal (ACTB), GSK3 beta (GSK3B), Tubulin alpha (TUBA1A, TUBA1C, TUBA3D), SOS (SOS1), F-Actin (ACTB), Ephrin-B receptors (EPHB4), G-protein alpha-i family (GNAZ), ILK, Tubulin (in microtubules) (TUBA1A, TUBA1C, TUBA3D, TUBB4B)
Apoptosis and survival, BAD phosphorylation	1.340E-07	5.740E-05	11/42	G-protein beta/gamma (GNB5, GNG11, GNG8), PKA-reg (cAMP-dependent) (PRKAR1A), p70 S6 kinase1 (RPS6KB1), SOS (SOS1), PP2C (PPM1B), G-protein alpha-s (GNAS), GRB2, PP1-cat alpha (PPP1CA), Bcl-2 (BCL2), Beclin 1 (BECN1), 14-3-3 (YWHAG)
Blood coagulation, GPCRs in platelet aggregation	8.827E-07	2.836E-04	13/71	Talin (TLN1), G-protein beta/gamma (GNB5, GNG11, GNG8), Actin cytoskeletal (ACTB), PKA-reg (cAMP-dependent) (PRKAR1A), P2Y12 (P2RY12), G-protein alpha-z (GNAZ), G-protein alpha-s (GNAS), PI3K reg class IB (p101) (PIK3R5), ITGB3, G-protein alpha-i family (GNAZ), Adenylate cyclase (ADCY7), MRLC (MYL9), G-protein alpha-13 (GNA13)
Blood coagulation, GPVI-dependent platelet activation	2.053E-06	3.814E-04	11/54	Talin (TLN1), von Willebrand factor (VWF), VAV-3 (VAV3), GRAP2, ITGB3, GP-IB beta (GP1BB), Lyn (LYN), PLC-gamma 2 (PLCG2), GP-IX (GP9), Rac1 (RAC1), Glycoprotein VI (GP6)
Blood coagulation, GPIb-IX-V-dependent platelet activation	2.307E-06	3.814E-04	13/77	Talin (TLN1), Alpha-actinin (ACTN1), von Willebrand factor (VWF), Calmodulin (CALM3), ITGB3, GP-IB beta (GP1BB), cPLA2 (PLA2G4C), Lyn (LYN), Actin (ACTB), PLC-gamma 2 (PLCG2), GP-IX (GP9), Guanylate cyclase (alpha-1/beta-1) (GUCY1A1, GUCY1B1), Glycoprotein VI (GP6)
Apoptosis and survival, NGF/TrkA PI3K-mediated signaling	2.307E-06	3.814E-04	13/77	Actin cytoskeletal (ACTB), GSK3 beta (GSK3B), p70 S6 kinase1 (RPS6KB1), SOS (SOS1), VAV-3 (VAV3), Calmodulin (CALM3), GRB2, FKHR (FOXO1), ILK, MRLC (MYL9), Bcl-2 (BCL2), Rac1 (RAC1), Tubulin (in microtubules) (TUBA1A, TUBA1C, TUBA3D, TUBB4B)
Immune response, IL-3 signaling via ERK and PI3K	2.374E-06	3.814E-04	15/102	GSK3 alpha/beta (GSK3B), Talin (TLN1), p70 S6 kinases (RPS6KB1), GSK3 beta (GSK3B), SOS (SOS1), Calmodulin (CALM3), GRB2, A-Raf-1 (ARA), p27KIP1 (CDKN1B), cPLA2 (PLA2G4C), Lyn (LYN), PLC-gamma 2 (PLCG2), Bcl-2 (BCL2), LPCAT2, Rac1 (RAC1)
High shear stress-induced platelet activation	3.219E-06	4.597E-04	10/46	Talin (TLN1), G-protein beta/gamma (GNB5, GNG11, GNG8), Actin cytoskeletal (ACTB), Alpha-actinin (ACTN1), P2Y12 (P2RY12), von Willebrand factor (VWF), ITGB3, GP-IB beta (GP1BB), GP-IX (GP9), G-protein alpha-i family (GNAZ)
Apoptosis and survival, HTR1A signaling	7.159E-06	8.697E-04	10/50	G-protein beta/gamma (GNB5, GNG11, GNG8), PKA-reg (cAMP-dependent) (PRKAR1A), SOS (SOS1), NF-kB (NFKB1), Calmodulin (CALM3), GRB2, PI3K reg class IB (p101) (PIK3R5), G-protein alpha-i family (GNAZ), Adenylate cyclase (ADCY7), Bcl-2 (BCL2)

Pathway maps with FDR q-value < 0.01 were defined as significantly differentially enriched. The ratio indicates the number of network objects associated with the differentially altered gene transcripts between the groups (p-value < 0.05) compared to the total number of network objects given in the pathway.

which is part of the ER-associated degradation complex involved in recognizing and degrading misfolded proteins and removal of incorrect disulfide bonds in misfolded glycoproteins.[36] In a recent study, it was demonstrated that this protein is also required for efficient folding of the LDL receptor (LDLR).[37] Using a targeted approach, we have previously reported that the expression of *LDLR* was up-regulated in the Ex-diet group compared to the C-diet group.[23] A down-regulation of *DNAJC10* in the present study may indicate that this gene is involved in cholesterol sensing, which should be further investigated in future studies.

In the present study, the expression of *GUCY1B1* was up-regulated in the Ex-diet group compared to the C-diet group. *GUCY1B1* encodes the beta subunit of the soluble guanylate cyclase (sGC), which catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP).[38] A previous study has shown that *GUCY1B1* exerts cardio-protective effects against myocardial infarction.[39] Furthermore, knockout of *GUCY1B1* in murine models caused hypertension and lack of nitric oxide effect on platelet aggregation.[40] In the present study, pathways related to blood coagulation were among the most differentially enriched pathways between the groups, and *GUCY1B1* was among the genes leading to the modulation in one of these pathways. This finding is interesting since the n-6 PUFA arachidonic acid (AA) that derives from LA is a precursor for eicosanoids, including thromboxanes that are involved in platelet aggregation and processes related to blood coagulation.[41] Furthermore, one pathway related to stimulation of AA was differentially enriched between the groups, and we have previously reported a significant increase in the plasma level of AA in the Ex-diet group compared to the C-diet group after the intervention.[6] Interestingly, the expression of *GUCY1B1* was also associated with plasma LA level, and negatively correlated to serum LDL cholesterol concentration. The latter may link the expression of this gene to prevention of atherosclerosis.

The expression of *ALDH5A1*, encoding a mitochondrial NAD(+)-dependent succinic semialdehyde dehydrogenase located in the mitochondria, was down-regulated in the Ex-diet group compared to the C-diet group. *ALDH5A1* is involved in the acetate metabolic process, and acetate has previously been reported to be increased during the intervention,[23] and reduced in children with familial hypercholesterolemia compared to healthy children.[42] Furthermore, genes with binding sites for NRF1 were overrepresented among the down-regulated gene transcripts. NRF1 is a transcription factor that, together with the transcriptional co-activator PGC1-a, stimulate the expression of a broad set of genes involved in mitochondrial biogenesis and functions.[43] These findings are in accordance with the results obtained in a study investigating the effect of a healthy Nordic diet on the whole transcriptome in PBMCs.[18] Moreover, the intake of a diet rich in monounsaturated fat or a Mediterranean diet decreased the expression of genes linked to mitochondrial function in PBMCs compared with a diet rich in SFA.[44]

We observed an enrichment of 101 pathways in the Ex-diet group compared to the C-diet group, analyzed by use of Metacore. As expected, the most prevalent pathways were related to immune response and inflammation, as we have analyzed the whole transcriptome response of PBMCs, which mostly consist of lymphocytes and monocytes. Others who have investigated the effect of healthy diets or fat intake on PBMC gene expression have also observed changes in gene expression related to immune response and inflammation.[17–19,21,45–48]

PBMCs may serve as a surrogate tissue for metabolically active tissues.[49] Fasting induces the expression of genes involved in processes related to lipid metabolism, such as fatty acid beta-oxidation, and PPARα is an important transcription factor mediating this effect in the liver. In PBMCs, this pattern has also been shown during fasting, and therefore it is conceivable that PBMC gene expression profiles may also reflect nutrition-related metabolic changes.[49] Additionally, it has been shown that PPARα activation is involved in the regulation of these genes in PMBCs.[49,50] PPARα is ubiquitously expressed, including in endothelial cells and VSMCs in the vasculature.[51,52] We may therefore speculate that the changes in PBMC gene expression observed in our study is reflecting gene expression changes in the liver and the vasculature.

The present study has several strengths, including the large number of subjects and the double-blinded, randomized, controlled study design. Furthermore, in the present study, changes in gene expression in the Ex-diet group were compared to changes in a C-diet group, and the reported effects may therefore be directly linked to the intervention and not the ritual of the intervention or daily fluctuations in the transcriptome. Other dietary intervention studies that have examined the transcriptome profile have often reported solely within-group changes. A limitation of the study is that the power calculations were performed with regard to finding an effect of the intervention on serum LDL cholesterol concentration, and not the whole transcriptome response, which was a secondary outcome. Furthermore, we cannot exclude the possibility that the plasma LA effect on gene expression changes is confounded by the intervention, and the results obtained from this analysis should therefore be interpreted with caution. The gene expression changes observed in the study are small, but in line with the findings in other dietary transcriptome studies.[17,18,25] It is also recognized that this effect over time will have an impact on disease development.[53] In addition, we observed a significant enrichment of a large number of pathways in the Ex-diet group compared to the C-diet group, supporting important changes in biological pathways and processes despite small changes in gene expression.

In conclusion, we have shown that replacing dietary SFA with PUFA for 8 weeks modulates PBMC gene expression and pathways related to CVD risk in healthy subjects with moderate hypercholesterolemia. Of particular interest were changes in the expression of genes related to VSMC proliferation, LDLR folding, and regulation of blood pressure, and the enrichment of pathways related to blood coagulation. To our knowledge, this is the first study investigating the effect of replacing SFA with PUFA for 8 weeks on the whole transcriptome response of PBMCs. The current findings may offer new mechanistic insight regarding the effect of fat quality on CVD prevention, and should be further investigated in future studies.

4. Experimental Section

Study Design and Subjects: In this study, we utilize data and total RNA from an 8 week, randomized, controlled, dietary intervention study, designed to investigate health effects of exchanging a few commercially

available and regularly consumed food items with comparable food items with improved fat quality.[6] The study was conducted at the Oslo and Akershus University College of Applied Sciences (OsloMet) and the University of Oslo, Norway, from July 2012 to April 2014. A detailed description of the protocol and the participant recruitment and enrolment has been published previously.[6] In short, 701 subjects were assessed for eligibility, 115 were randomly assigned, and 100 completed the study. Before the baseline visit, all subjects underwent a 2 week run-in period where they had to include the control food items in their habitual diet. At baseline, the subjects were stratified by sex and age, and randomly assigned into one of two intervention groups in a 1:1 ratio. The C-diet group continued with the control food items and the Ex-diet group received experimental food items. The experimental food items were the same type of food products as the control food items, but with a different fatty acid composition (SFAs were replaced with mostly n-6 PUFAs). Clinical and blood laboratory assessments were performed at baseline and after 8 weeks follow-up.

The subjects included in the study were healthy, moderately hypercholesterolemic, non-statin treated men and women between the age of 25 and 75 years. Inclusion and exclusion criteria have been described in detail previously.[6] In brief, inclusion criteria were hs-CRP < 10 mg L^{-1}, LDL cholesterol ≥ 3.5 mmol L^{-1}, fasting TG ≤ 2.6 mmol L^{-1}, and a stable body weight during the last 3 months (±5%). Furthermore, the subjects had to have age-specific serum total cholesterol values of 5–7.8 mmol L^{-1} (for those between 50 and 70 years), 5.0–6.9 mmol L^{-1} (for those between 30 and 49 years), and 5.0–6.1 mmol L^{-1} (for those between 25 and 29 years), as the authors wanted to include subjects with cholesterol values at the upper range of normal serum cholesterol.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki. Written informed consent was obtained from all participants, and the study was approved by the Regional Ethics Committee for Medical Research in South East Norway (2011/1951). The study was registered at ClinicalTrials.gov (identification number NCT 01679496).

Diet: The food items in the C-diet group and the Ex-diet group were, respectively, butter-based spread or margarine-based spread, butter or liquid margarine, and olive oil or rapeseed and sunflower oil. In addition, products such as liver paté, cheese, bread, muesli cereals, cream, mayonnaise, and crème fraîche were given to the participants, in which some of the SFAs were replaced with particularly n-6 PUFAs from rapeseed and sunflower oils in the products in the Ex-diet group. Based on the minimum intake of the food items, the n-6 PUFA content was 4.2 g d^{-1} in the control food items and 12.9 g d^{-1} in the experimental food items, and the SFA content was 19.2 g d^{-1} in the control food items and 5.7 g d^{-1} in the experimental food items. The dietary difference during the intervention was 6.5 energy % (E%) lower intake of SFAs and 6.4 E% higher intake of PUFAs in the Ex-diet group compared to the C-diet group.

Blood Sampling and Standard Laboratory Analyses: Blood samples were drawn after an overnight fast (≥12 h) at the baseline and 8 week visits. The participants were instructed to avoid alcohol consumption and vigorous physical activity the day prior to blood sampling. Serum was obtained from silica gel tubes (BD Vacutainer Systems, Plymouth, UK) and kept at room temperature for at least 30 min until centrifugation (1500 × *g*, 15 min). Plasma was obtained from EDTA tubes (BD Vacutainer Systems, Plymouth, UK), immediately placed on ice, and centrifuged within 10 min (2000 × *g*, 4 °C, 15 min). EDTA tubes with whole blood were kept at room temperature for a maximum of 48 h before counting the total number of lymphocytes and monocytes (Fürst Medical laboratory, Oslo, Norway). As previously described, fasting serum concentrations of hs-CRP, total cholesterol, LDL cholesterol, HDL cholesterol, Lp(a), TG, and glucose were measured by standard methods at a routine laboratory (Fürst Medical Laboratory, Oslo, Norway). Serum IL-6 and sTNFR1 were measured by Quantikine high sensitivity and Quantikine ELISA kit from R&D Systems (Minneapolis, MN, USA), and IFN-γ was measured by high sensitivity ELISA from eBioscience (San Diego, CA, USA), according to the manufacturer's instructions.[6]

Total Plasma Fatty Acid Analysis: The plasma fatty acid composition was measured by a commercial laboratory (Vitas Analytical Service), as described previously.[6] Analyses were performed using a 7890 N GC with a split/splitless injector, a 7683B automatic liquid sampler, and a flame ionization detector (Agilent Technologies). Separations were performed with a SP-2380 (30 m × 0.20 mm i.d. × 0.25 μm film thickness) column from Supelco. The concentration of the individual fatty acids was measured as μg fatty acid per mL plasma (Vitas Analytical Service) and presented as percentage of total fatty acids.

PBMC and RNA Isolation: PBMCs were isolated from blood by using the BD Vacutainer Cell Preparation tubes according to the manufacturer's instructions (Becton, Dickinson San Jose, CA, USA). This is a well-documented and standardized method to collect mononuclear cells with high purity (above 90%), and according to the manufacturer, approximately 80% of the cells are lymphocytes and 12% are monocytes. Pellets were stored at −80 °C until further RNA isolation. Total RNA was isolated from all PBMCs using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). RNA quantity measurements were performed using a Nanodrop ND-1000 Spectrometer (Thermo Fisher Scientific, Gothenburg, Sweden) and RNA integrity number (RIN) value was measured with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to check the RNA quality. As two samples were lost due to technical error and two samples were excluded because they had RIN values below eight, RNA samples from 95 subjects were labeled (cRNA) and served as templates for microarray hybridization.

Microarray Hybridization and Processing: The whole transcriptome was analyzed by use of microarray technology. The microarray gene expression analysis followed standard Illumina protocol (Illumina Inc., CA, USA). In brief, cRNA was prepared with Ambion's Illumina TotalPrep RNA Amplification Kit (Thermo Fisher Scientific, MA, USA), using 300 ng total RNA as input material. For each sample, the biotin-labeled cRNA concentrations were measured (NanoDrop, Thermo Fisher Scientific, MA, USA) and 750 ng hybridized overnight to HumanHT-12 Expression BeadChips (Illumina Inc., CA, USA). After washing, the BeadChips were scanned with the Illumina HiScann instrument (Illumina Inc.), according to the manufacturer's instructions. IlluminaGenome Studio was used to transform bead level data to probe level intensity values, which were extracted using Illumina's BeadStudio software (gene expression module v3.0.19.0) (Illumina Inc.) for bioinformatics analysis.

The intensity values were filtered to improve the statistical power (detection p-value < 0.01), and quantile normalized. In order to select one probe per gene, the probe with the largest variance (interquartile range (IQR)) was selected. Gene expression changes were obtained by calculating log2 ratios between the baseline and 8 week intensity values, and the two intervention groups were compared with regard to this ratio.

Statistical and Functional Analyses: The intervention study was designed based on the primary outcome of the study, which was change in serum LDL cholesterol concentration.[6] Power calculations estimated that 180 subjects (including a 20% drop-out rate) were required for obtaining 80% power with a type I error of 5% to detect a difference between the two groups of 8% in LDL cholesterol at the end of the study. Post-hoc analyses showed that the number of subjects recruited gave sufficient power with the observed 10% change in LDL cholesterol between the groups.[23] Pre-specified secondary outcomes included transcriptomics.

Differences in changes in clinical and biochemical measurements and white blood cell counts between the groups were tested by use of independent samples t-test when normally distributed or Mann–Whitney U test when not normally distributed. Continuous data were presented as mean and standard deviation (SD) when normally distributed or median and IQR when not normally distributed, and categorical data were presented as frequencies. Changes in gene expression levels were correlated to changes in serum total- and LDL cholesterol concentrations by use of Pearson's correlation.

Differentially altered gene transcripts between the groups were identified by a linear regression model, adjusted for age, sex, study center, smoking, and log(baseline gene expression) level, with log ratio as the dependent variable. To adjust for the large number of tests, the FDR was controlled by the Benjamini-Hochberg procedure. This method corresponds to a tail area-based FDR (or q-value), and can be interpreted in the usual frequentist way, just like the regular p-value. Gene transcripts with an FDR q-value < 0.25 were defined as differentially altered by the two diets. Gene transcripts with a nominal p-value < 0.05 were subjected to

further pathway- and transcription factor analyses by use of Metacore from Clarivate Analytics (GeneGo, division of Thomson Reuters, St. Joseph, MI, USA). Metacore is a knowledge database suitable for functional analyses of experimental data. Pathways with an FDR q-value < 0.01 were defined as differentially enriched in the Ex-diet group compared to the C-diet group.

LA was the most prevalent PUFA in the food items given to the Ex-diet group.[6] Since plasma LA is a biomarker of dietary LA intake[54] and was known to directly regulate gene expression by acting as ligand for PPAR, the effect of change in plasma LA level on gene expression changes in PBMCs was analyzed, using the same statistics as described above. Gene transcripts with an FDR q-value < 0.25 were defined as significantly altered depending on change in plasma LA level.

All statistical analyses were performed with R version 3.5.0 using RStudio.[55]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

S.M.U. and K.B.H. received research grants from Mills AS to partially fund the conduction of the dietary intervention study. None own any stocks or serves as advisory board in the company. L.L. and V.T.-H. are former employees at Mills AS and do not own stocks in the company. S.M.U. has received research grants from TINE BA and Olympic Seafood, none of which are related to the content of this manuscript. K.B.H. has received research grants and/or personal fees from TINE BA, Olympic Seafood, Kaneka, Amgen, Sanofi, and Pronova, none of which are related to the content of this manuscript. The other authors declare no conflict of interest.

Author Contributions

S.V.L., K.B.H., V.T.-H., and S.M.U. designed the research. K.B.H., J.J.C., L.L., V.T.-H., and S.M.U. conducted the intervention study. L.L. and A.F. performed laboratory analysis. S.V.L., J.J.C., and M.T. performed statistical analyses. All authors interpreted the data. S.V.L., K.B.H., J.J.C., A.R., and S.M.U. drafted the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The raw data are available from the Gene Expression Omnibus (GEO) (accession number GSE176043).

Keywords

cardiovascular disease risk, dietary fat quality, gene expression, inflammation, LDL cholesterol

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Supplementary material – Paper I-III

Table S1. Selection of genes related to triglyceride- and cholesterol metabolism (285 genes). Genes expressed in peripheral blood mononuclear cells are in bold (161 genes).

Official gene symbol	Official gene name
AACS	acetoacetyl-CoA synthetase
ABCA1	ATP-binding cassette sub-family A member 1
ABCA12	ATP-binding cassette sub-family A member 12
ABCA2	ATP-binding cassette sub-family A member 2
ABCA5	ATP-binding cassette sub-family A member 5
ABCA7	ATP-binding cassette sub-family A member 7
ABCA8	ATP binding cassette subfamily A member 8
ABCB11	ATP binding cassette subfamily B member 11
ABCB4	ATP-binding cassette sub-family B member 4
ABCG1	ATP-binding cassette sub-family G member 1
ABCG4	ATP-binding cassette sub-family G member 4
ABCG5	ATP-binding cassette sub-family G member 5
ABCG8	ATP-binding cassette sub-family G member 8
ABHD5	abhydrolase domain containing 5
ABO	ABO, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase
ACAD11	acyl-CoA dehydrogenase family member 11
ACADL	acyl-CoA dehydrogenase long chain
ACADVL	acyl-CoA dehydrogenase very long chain
ACOX1	acyl-CoA oxidase 1
ACSM1	acyl-CoA synthetase medium chain family member 1
ACSM2A	acyl-CoA synthetase medium chain family member 2A
ACSM3	acyl-CoA synthetase medium chain family member 3
ADH5	alcohol dehydrogenase 5 (class III), chi polypeptide
ADIPOQ	adiponectin, C1Q and collagen domain containing
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
AGTR1	angiotensin II receptor type 1
AKR1C1	aldo-keto reductase family 1 member C1
AKR1C4	aldo-keto reductase family 1 member C4
AMPD3	adenosine monophosphate deaminase 3
ANGPTL1	angiopoietin like 1
ANGPTL3	angiopoietin like 3
ANGPTL4	angiopoietin like 4
ANGPTL8	angiopoietin like 8
ANKRA2	ankyrin repeat family A member 2
APOA1	apolipoprotein A-1
APOA2	apolipoprotein A-2
APOA4	apolipoprotein A-4
APOA5	apolipoprotein A-5
APOB	apolipoprotein B
APOBR	apolipoprotein B receptor
APOC1	apolipoprotein C1
APOC2	apolipoprotein C2I
APOC3	apolipoprotein C3
APOC4	apolipoprotein C4
APOE	apolipoprotein E
APOF	apolipoprotein F
APOH	apolipoprotein H
APOL1	apolipoprotin L 1

APOM	apolipoprotein M
APOO	apolipoprotein O
ARL15	ADP ribosylation factor like GTPase 15
ARV1	sterol homeostasis protein ARV1
ASAP3	ArfGAP with SH3 domain, ankyrin repeat and PH domain 3
ATG7	autophagy related 7
BRAP	BRCA1 associated protein
BRCA2	BRCA2, DNA repair associated
C10orf112	chromosome 10 open reading frame 112
C19orf80	chromosome 19 open reading frame 80
C1QTNF3	C1q and tumor necrosis factor related protein 3
C3	complement component 3
C6orf106	chromosome 6 open reading frame 106
CAPN3	calpain 3
CAV1	caveolin 1
CAV3	caveolin 3
CD24	CD24 molecule
CD36	CD36 molecule
CDH13	cadherin 13
CETD2	SET domain containing 2
CETP	cholesteryl ester transfer protein
CIDEA	cell death-inducing DFFA-like effector a
CILP2	cartilage intermediate layer protein 2
CITED2	Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2
CLU	clusterin
CMIP	c-Maf inducing protein
CMTM5	CKLF like MARVEL transmembrane domain containing 5
COBLL1	cordon-bleu WH2 repeat protein like 1
COLEC12	collectin sub-family member 12
CPS1	carbamoyl-phosphate synthase 1
CRP	C-reactive protein
CSNK1G3	casein kinase 1 gamma 3
CTDNEP1	CTD nuclear envelope phosphatase 1
CTF1	cardiotrophin 1
CYP26A1	cytochrome P450 family 26 subfamily A member 1
CYP7A1	cytochrome P450 family 7 subfamily A member 1
DAGLB	diacylglycerol lipase beta
DGAT2	diacylglycerol O-acyltransferase 2
DHCR7	7-dehydrocholesterol reductase
DLG4	discs large MAGUK scaffold protein 4
DNAH11	dynein axonemal heavy chain 11
EGF	epidermal growth factor
EHBP1	EH domain binding protein 1
EHD1	EH-domain containing 1
EPHX2	epoxide hydrolase 2, cytoplasmic
ERGIC3	ERGIC and golgi 3
ERLIN1	ER lipid raft associated 1
ERLIN2	ER lipid raft associated 2
EVI5	ecotropic viral integration site 5
FABP3	fatty acid binding protein 3
FABP4	fatty acid binding protein 4
FADS1	fatty acid desaturase 1

FADS2	fatty acid desaturase 2
FADS3	fatty acid desaturase 3
FAM117B	family with sequence similarity 117 member B
FAM13A	family with sequence similarity 13 member A
FBXW7	F-box and WD repeat domain containing 7
FGF1	fibroblast growth factor 1
FGFR4	fibroblast growth factor receptor 4
FITM1	fat storage-inducing transmembrane protein 1
FITM2	fat storage-inducing transmembrane protein 2
FN1	fibronectin 1
FRK	fyn related Src family tyrosine kinase
FRMD5	FERM domain containing 5
FTO	FTO, alpha-ketoglutarate dependent dioxygenase
G6PC	glucose-6-phosphatase catalytic subunit
GALNT2	polypeptide N-acetylgalactosaminyltransferase 2
GCKR	glucokinase regulator
GIP	gastric inhibitory polypeptide
GPAM	glycerol-3-phosphate acyltransferase
GPIHBP1	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1
GPLD1	glycosylphosphatidylinositol specific phospholipase D1
GPR 146	G protein-coupled receptor 146
GSK3B	glycogen synthase kinase 3 beta
HAS1	hyaluronan synthase 1
HBS1L	HBS1 like translational GTPase
HDLBP	high density lipoprotein binding protein
Hfe	hemochromatosis
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)
HNF1A	hepatocyte nuclear factor 1 alpha
HNF4A	hepatocyte nuclear factor 4 alpha
HPR	haptoglobin-related protein
IKZF1	IKAROS family zinc finger 1
INHBA	inhibin beta A subunit
INSIG2	insulin induced gene 2
INSR	insulin receptor
IRF2BP2	interferon regulatory factor 2 binding protein 2
IRS1	insulin receptor substrate 1
JMJD1C	jumonji domain containing 1C
KAT5	lysine acetyltransferase 5
KCNK17	potassium two pore domain channel subfamily K member 17
KLHL8	kelch like family member 14
LACTB	lactamase beta
LAMTOR1	late endosomal/lysosomal adaptor, MAPK and MTOR activator 1
LCAT	lecithin-cholesterol acyltransferase
LDLR	low density lipoprotein receptor
LDLRAP1	low density lipoprotein receptor adaptor protein 1
LEP	leptin
LILRA3	leukocyte immunoglobulin like receptor A3
LIPC	Lipase C, hepatic type
LIPG	Lipase G, endothelial type
LMF1	lipase maturation factor 1
LPA	lipoprotein(a)

LPL	lipoprotein lipase
LRP1	low density lipoprotein receptor related protein 1
LRP4	low density lipoprotein receptor related protein 4
LRP5	low density lipoprotein receptor related protein 5
LRP6	low density lipoprotein receptor related protein 6
LRP8	low density lipoprotein receptor related protein 8
LRPAP1	LDL receptor related protein associated protein 1
LSR	lipolysis stimulated lipoprotein receptor
MAFB	MAF bZIP transcription factor B
MALL	mal, T-cell differentiation protein like
MAMSTR	MEF2 activating motif and SAP domain containing transcriptional regulator
MAP3K1	mitogen-activated protein kinase kinase kinase 1
MC4R	melanocortin 4 receptor
MED13	mediator complex subunit 13
MET	MET proto-oncogene, receptor tyrosine kinase
MIA2	melanoma inhibitory activity 2
MIR148A	microRNA 148a
MLC1	megalencephalic leukoencephalopathy with subcortical cysts 1
MLXIPL	MLX interacting protein-like
MOSC1	mitochondrial amidoxime reducing component 1
MPO	myeloperoxidase
MPP3	membrane palmitoylated protein 3
MSL2L1	MSL complex subunit 2
MSR1	macrophage scavenger receptor 1
MTMR3	myotubularin related protein 3
MVK	mevalonate kinase
MYLIP	myosin regulatory light chain interacting protein
NAT2	N-acetyltransferase 2
NFKB1	nuclear factor kappa B subunit 1
NFKBIA	NFKB inhibitor alpha
NPC1	NPC intracellular cholesterol transporter 1
NPC1L1	NPC1 like intracellular cholesterol transporter 1
NPC2	NPC intracellular cholesterol transporter 2
NR1D1	nuclear receptor subfamily 1 group D member 1
NR1H2	nuclear receptor subfamily 1 group H member 2
NR1H3	nuclear receptor subfamily 1 group H member 3
NR1H4	nuclear receptor subfamily 1 group H member 4
NR5A2	nuclear receptor subfamily 5 group A member 2
NUS1	NUS1 dehydrodolichyl diphosphate synthase subunit
NYNRIN	NYN domain and retroviral integrase containing
OR4C46	olfactory receptor family 4 subfamily C member 46
OSBPL11	oxysterol binding protein like 11
OSBPL7	oxysterol binding protein like 7
OSBPL8	oxysterol binding protein like 8
PABPC4	poly(A) binding protein cytoplasmic 4
PCSK9	proprotein convertase subtilisin/kexin type 9
PCYOX1	prenylcysteine oxidase 1
PDE3A	phosphodiesterase 3A
PEPD	peptidase D
PGS1	phosphatidylglycerophosphate synthase 1
PHLDB1	pleckstrin homology like domain family B member 1
PINX1	PIN2/TERF1 interacting telomerase inhibitor 1

PLA2G10	phospholipase A2 group X
PLA2G2A	phospholipase A2 group IIA
PLA2G6	phospholipase A2 group VI
PLA2G7	phospholipase A2 group VII
PLEC1	plectin
PLIN5	perilipin 5
PLSCR3	phospholipid scramblase 3
PLTP	phospholipid transfer protein
PNLIP	pancreatic lipase
PNPLA2	patatin like phospholipase domain containing 2
PON1	paraoxonase 1
POR	cytochrome p450 oxidoreductase
PPARA	peroxisome proliferator activated receptor alpha
PPARG	peroxisome proliferator activated receptor gamma
PPP1R3B	protein phosphatase 1 regulatory subunit 3B
PRKAA1	protein kinase AMP-activated catalytic subunit alpha 1
PTCH1	patched 1
PTCHD2	patched domain containing 2
PXN	paxillin
RAB3GAP1	RAB3 GTPase activating protein catalytic subunit 1
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
RBM5	RNA binding motif protein 5
RGN	regucalcin
RORA	RAR-related orphan receptor A
RSPO3	R-spondin 3 [*Homo sapiens*
SAA1	serum amyloid A1
SAA2	serum amyloid A2
SAA4	serum amyloid A4, constitutive
SBNO1	strawberry notch homolog 1
SCAP	SREBF chaperone
SCARB1	scavenger receptor class B member 1
SCARF1	scavenger receptor class F member 1
SCP2	sterol carrier protein 2
SEC14L2	SEC14 like lipid binding 2
SEC24A	SEC24 homolog A, COPII coat complex component
SELS	selenoprotein S
SERPINA12	serpin family A member 12
SESN2	sestrin 2
SHH	sonic hedgehog
SIK1	salt-inducible kinase 1
SIRT1	sirtuin 1
SLC39A8	solute carrier family 39 member 8
SMIM20	small integral membrane protein 20
SMO	smoothened, frizzled class receptor
SNX13	sorting nexin 13
SNX5	sorting nexin 5
SOAT1	sterol O-acyltransferase 1
SOAT2	sterol O-acyltransferase 2
SOD1	superoxide dismutase 1
SORL1	sortilin related receptor 1
SORT1	sortilin 1
SOX17	SRY-box 17
SPTLC3	serine palmitoyltransferase long chain base subunit 3

SPTY2D1	SPT2 chromatin protein domain containing 1
SREBF1	sterol regulatory element binding transcription factor 1
SREBF2	sterol regulatory element binding transcription factor 2
ST3GAL4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4
STAB1	stabilin 1
STAB2	stabilin 2
STARD3	StAR related lipid transfer domain containing 3
THBS1	thrombospondin 1
THRSP	thyroid hormone responsive
TIMD4	T-cell immunoglobulin and mucin domain containing 4
TMEM176A	transmembrane protein 176A
TMEM188	transmembrane protein 188
TMEM97	transmembrane protein 97
TOM1	target of myb1 membrane trafficking protein
TOP1	DNA topoisomerase I
TRIB1	tribbles pseudokinase 1
TRPS1	transcriptional repressor GATA binding 1
TSPO	translocator protein
TTC39B	tetratricopeptide repeat domain 39B
TYW1B	tRNA-yW synthesizing protein 1 homolog B
UBASH3B	ubiquitin associated and SH3 domain containing B
UBE2L3	ubiquitin conjugating enzyme E2 L3
UGT1A1	UDP glucuronosyltransferase family 1 member A1
VEGFA	vascular endothelial growth factor A
VLDLR	very low density lipoprotein receptor
XBP1	X-box binding protein 1
ZNF648	zinc finger protein 648
ZNF664	zinc finger protein 664

Table S2. Differentially expressed genes associated with plasma n-6 level and expressed by more than one probe

Gene	Highest tertile (n 18)		Lowest tertile (n 18)		Mean difference	P
SORL1_1	**10.25**	**0.27**	**10.06**	**0.27**	**0.19**	**0.039**
SORL1_2	10.37	0.18	10.33	0.23	0.04	0.585
KLHL8_1	8.12	0.27	8.18	0.14	-0.07	0.373
KLHL8_2	**9.13**	**0.25**	**8.94**	**0.19**	**0.18**	**0.020**
TOM1_1	**9.78**	**0.24**	**9.62**	**0.20**	**0.16**	**0.037**
TOM1_2	7.12	0.09	7.05	0.10	0.07	0.039
KAT5_1	7.27	0.09	7.21	0.10	0.06	0.055
KAT5_2	**7.45**	**0.09**	**7.39**	**0.09**	**0.06**	**0.047**
LACTB_1	7.57	0.27	7.61	0.20	-0.05	0.552
LACTB_2	8.76	0.35	8.85	0.37	-0.09	0.441
LACTB_3	**7.15**	**0.12**	**7.24**	**0.12**	**-0.09**	**0.027**
SELS_1	7.01	0.05	7.03	0.07	-0.02	0.265
SELS_2	**9.66**	**0.12**	**9.83**	**0.27**	**-0.18**	**0.020**
SELS_3	8.01	0.17	8.12	0.29	-0.12	0.150
XBP1_1	**10.63**	**0.23**	**10.85**	**0.37**	**-0.22**	**0.042**
XBP1_2	10.81	0.19	10.95	0.36	-0.14	0.164

Expression of genes is given as mRNA level. Values are presented as mean ± SD and are log2 transformed. Differences between tertiles were analysed using the Independent Samples t-test. P-values <0.05 were considered significant. The probes in bold are shown in Table 3.

Table S3. Differentially expressed genes associated with plasma n-3 level and expressed by more than one probe

Gene	Highest tertile (n 18)		Lowest tertile (n 18)		Mean difference	P
SNX5_1	7.29	0.20	7.17	0.14	0.12	0.054
SNX5_2	**8.53**	**0.13**	**8.43**	**0.14**	**0.10**	**0.029**
RORA_1	7.28	0.15	7.34	0.12	-0.06	0.195
RORA_2	**7.11**	**0.13**	**7.21**	**0.10**	**-0.10**	**0.018**
FAM13A_1	6.98	0.08	6.98	0.08	0.00	0.986
FAM13A_2	**7.33**	**0.14**	**7.43**	**0.15**	**-0.10**	**0.049**
FAM13A_3	7.40	0.15	7.44	0.18	-0.04	0.523
UBE2L3_1	8.14	0.20	8.11	0.18	0.03	0.687
UBE2L3_2	**7.16**	**0.16**	**7.28**	**0.17**	**-0.12**	**0.035**

Expression of genes is given as mRNA level. Values are presented as mean ± SD and are log2 transformed. Differences between tertiles were analysed using the Independent Samples t-test. P-values <0.05 were considered significant. The probes in bold are shown in Table 4.

Table S4. Differentially expressed genes associated with plasma SFA to PUFA ratio and expressed by more than one probe

Gene	Highest tertile (n 18)		Lowest tertile (n 18)		Mean difference	P
FAM117B_1	10.34	0.22	10.23	0.25	0.11	0.176
FAM117B_2	**8.58**	**0.27**	**8.36**	**0.23**	**0.22**	**0.012**
UBE2L3_1	8.12	0.22	8.17	0.19	-0.06	0.398
UBE2L3_2	**7.24**	**0.18**	**7.09**	**0.13**	**0.15**	**0.010**
ERLIN2_1	**6.99**	**0.07**	**6.93**	**0.07**	**0.05**	**0.034**
ERLIN2_2	7.02	0.08	6.99	0.09	0.03	0.269
ERLIN2_3	7.67	0.12	7.70	0.10	-0.03	0.400
TOM1_1	9.60	0.20	9.73	0.28	-0.13	0.118
TOM1_2	**7.04**	**0.08**	**7.13**	**0.10**	**-0.09**	**0.006**
ERGIC3_1	8.69	0.18	8.69	0.19	0.00	1.000
ERGIC3_2	**10.54**	**0.19**	**10.67**	**0.13**	**-0.12**	**0.031**
KLHL8_1	8.20	0.15	8.14	0.27	0.06	0.411
KLHL8_2	**8.96**	**0.21**	**9.13**	**0.25**	**-0.17**	**0.042**

Expression of genes is given as mRNA level. Values are presented as mean ± SD and are log2 transformed. Differences between tertiles were analysed using the Independent Samples t-test. P-values <0.05 were considered significant. The probes in bold are shown in Table 5.

The following supplementary material is available from the publisher:

Additional file 1. List of genes with a significantly ($p \leq 0.05$) different baseline expression in responders compared to non-responders adjusted for age and gender

Additional file 2. List of significantly ($p \leq 0.05$) differentially altered gene transcripts in responders compared to non-responders adjusted for age and gender

Additional file 3. Enriched pathways (FDR < 25%) among the significantly altered transcripts in responders compared to non-responders

Additional file 4. Results from the transcription factor analyses

Table S2. Differentially altered gene transcripts depending on change in plasma linoleic acid level during the intervention (*n* = 92, FDR < 0.25)

Illumina ID	Gene symbol	Estimate	2.50%	97.50%	FDR
ILMN_1673769	KCNG1	-5.75	-8.96	-2.54	0.20
ILMN_2060413	CD24	-4.67	-7.45	-1.90	0.23
ILMN_2337928	CXCR5	-4.53	-7.21	-1.86	0.22
ILMN_2371055	EFNA1	-4.44	-6.72	-2.16	0.20
ILMN_1695530	MS4A3	-4.42	-6.95	-1.88	0.20
ILMN_2372403	ALDH5A1	-4.22	-6.05	-2.40	0.19
ILMN_1791329	FCRL2	-4.05	-6.62	-1.49	0.24
ILMN_1692535	DPP4	-4.02	-6.11	-1.94	0.20
ILMN_1766718	LYSMD3	-3.80	-6.21	-1.40	0.24
ILMN_1655796	MARCH3	-3.69	-5.72	-1.66	0.20
ILMN_2107933	ZNF285	-3.68	-5.37	-1.99	0.19
ILMN_2058468	BACH2	-3.66	-5.65	-1.66	0.20
ILMN_3250446	ZNF626	-3.32	-5.14	-1.49	0.20
ILMN_1740490	ZFP82	-3.30	-4.98	-1.62	0.20
ILMN_1767322	EDAR	-3.28	-5.16	-1.39	0.20
ILMN_1797030	FGF18	-3.25	-4.98	-1.51	0.20
ILMN_2359287	ITGA6	-3.22	-5.03	-1.40	0.20
ILMN_2153466	FAM50B	-3.20	-5.02	-1.37	0.20
ILMN_1715131	CCR7	-3.17	-4.97	-1.37	0.20
ILMN_2389844	SP3	-3.12	-5.01	-1.23	0.23
ILMN_1660585	C15orf40	-3.11	-4.81	-1.41	0.20
ILMN_1865317	BX103864	-3.10	-4.89	-1.31	0.20
ILMN_1711087	EPPK1	-3.10	-5.01	-1.20	0.24
ILMN_1714756	YIPF5	-3.04	-4.95	-1.12	0.24
ILMN_2214278	ANKRD32	-3.02	-4.72	-1.31	0.20
ILMN_2150894	ALDH1B1	-2.97	-4.44	-1.49	0.20
ILMN_1790230	ZNF181	-2.94	-4.68	-1.19	0.23
ILMN_2196550	C13orf18	-2.90	-4.65	-1.14	0.23
ILMN_1799280	BDH1	-2.87	-4.75	-0.99	0.24
ILMN_2041222	FLJ40504	-2.82	-4.41	-1.23	0.20
ILMN_1713141	LOC389641	-2.81	-4.46	-1.16	0.21
ILMN_1712298	ANKRD46	-2.78	-4.55	-1.01	0.24
ILMN_2147993	ZNF23	-2.78	-4.44	-1.12	0.23
ILMN_1690999	MED23	-2.74	-4.10	-1.38	0.20
ILMN_1651950	TPST1	-2.68	-4.36	-1.01	0.24
ILMN_1688853	ZBTB9	-2.68	-4.38	-0.98	0.24
ILMN_1734346	IZUMO4	-2.63	-4.35	-0.91	0.24
ILMN_1694983	DDX20	-2.61	-4.23	-0.99	0.24
ILMN_1815154	MYH10	-2.61	-4.23	-0.98	0.24
ILMN_1682227	ZNF404	-2.60	-4.23	-0.97	0.24
ILMN_2118229	NAPEPLD	-2.59	-4.05	-1.14	0.20
ILMN_3263099	BC043177	-2.58	-4.24	-0.92	0.24

ILMN_2381064	TPD52	-2.55	-4.21	-0.89	0.24
ILMN_2246956	BCL2	-2.52	-3.96	-1.07	0.20
ILMN_1660852	ZC3H6	-2.50	-4.09	-0.91	0.24
ILMN_1654541	ATP6V1G2	-2.48	-4.10	-0.87	0.24
ILMN_3240586	PLD6	-2.45	-3.97	-0.94	0.24
ILMN_2131861	SOCS2	-2.44	-3.86	-1.01	0.21
ILMN_1696699	FAM184A	-2.43	-3.95	-0.91	0.24
ILMN_1749253	TUBD1	-2.42	-3.99	-0.85	0.24
ILMN_1691341	IL7R	-2.42	-3.76	-1.07	0.20
ILMN_1758811	IMPA1	-2.39	-3.62	-1.16	0.20
ILMN_1853631	BX115738	-2.37	-3.88	-0.87	0.24
ILMN_1748283	PIM2	-2.37	-3.91	-0.83	0.24
ILMN_2199022	SAP30BP	-2.30	-3.75	-0.86	0.24
ILMN_1760982	ZNF187	-2.30	-3.52	-1.08	0.20
ILMN_3270853	LOC100130298	-2.29	-3.60	-0.99	0.20
ILMN_1746784	SLAIN1	-2.29	-3.60	-0.97	0.20
ILMN_1674064	ZNF225	-2.22	-3.46	-0.98	0.20
ILMN_2098616	C5orf39	-2.21	-3.50	-0.92	0.21
ILMN_2169839	CNBP	-2.19	-3.61	-0.77	0.24
ILMN_1734596	TC2N	-2.18	-3.57	-0.78	0.24
ILMN_1675577	TRMT61A	-2.17	-3.58	-0.77	0.24
ILMN_1716272	KBTBD8	-2.16	-3.53	-0.80	0.24
ILMN_1759436	NOSIP	-2.16	-3.29	-1.04	0.20
ILMN_2347888	LARP4	-2.15	-3.54	-0.76	0.24
ILMN_3241127	LOC100128703	-2.13	-3.48	-0.78	0.24
ILMN_2139351	ZNF232	-2.12	-3.38	-0.85	0.23
ILMN_1665205	ZNF260	-2.11	-3.37	-0.84	0.23
ILMN_1815023	PIM1	-2.05	-3.29	-0.81	0.23
ILMN_1775423	C10orf88	-2.03	-3.33	-0.74	0.24
ILMN_2181992	MTRF1	-2.02	-3.23	-0.81	0.23
ILMN_1771689	EXD2	-2.01	-3.02	-1.00	0.20
ILMN_2187727	NOC3L	-1.98	-3.07	-0.89	0.20
ILMN_1671004	POLG2	-1.95	-3.12	-0.78	0.23
ILMN_1784428	MGC57346	-1.93	-3.17	-0.69	0.24
ILMN_1757408	ZNF256	-1.92	-3.16	-0.68	0.24
ILMN_1762003	SEC62	-1.92	-3.09	-0.76	0.23
ILMN_1653039	LOC441869	-1.90	-3.11	-0.68	0.24
ILMN_3240370	C11orf83	-1.89	-3.03	-0.75	0.23
ILMN_1913232	BM718830	-1.88	-3.11	-0.65	0.24
ILMN_2307450	ZNF302	-1.87	-2.89	-0.84	0.20
ILMN_1718069	MIS12	-1.81	-3.00	-0.63	0.24
ILMN_1707337	MSTO1	-1.81	-2.97	-0.65	0.24
ILMN_2323385	TRIM4	-1.80	-2.83	-0.77	0.20
ILMN_1778836	SRSF7	-1.80	-2.96	-0.64	0.24
ILMN_1692834	SWT1	-1.77	-2.76	-0.79	0.20
ILMN_1799890	ZFYVE20	-1.75	-2.71	-0.79	0.20

ILMN_1806713	ZNF18	-1.74	-2.82	-0.67	0.24
ILMN_1668498	CWF19L2	-1.64	-2.50	-0.78	0.20
ILMN_1722953	USP47	-1.63	-2.66	-0.60	0.24
ILMN_1844464	IL23A	-1.61	-2.66	-0.57	0.24
ILMN_3249406	URB2	-1.61	-2.54	-0.68	0.20
ILMN_1745152	UQCC	-1.54	-2.45	-0.62	0.23
ILMN_2349600	BRD8	-1.53	-2.51	-0.55	0.24
ILMN_1727184	WDR36	-1.48	-2.29	-0.67	0.20
ILMN_1689774	MRFAP1L1	-1.45	-2.33	-0.57	0.23
ILMN_1771411	ALG6	-1.45	-2.38	-0.52	0.24
ILMN_1737398	PTPLAD1	-1.44	-2.36	-0.52	0.24
ILMN_2066667	RRP8	-1.43	-2.37	-0.50	0.24
ILMN_2126239	SMG5	-1.40	-2.29	-0.50	0.24
ILMN_2225735	CRBN	-1.32	-2.15	-0.49	0.24
ILMN_1670420	METAP2	-1.31	-2.12	-0.50	0.24
ILMN_2361695	BAG5	-1.30	-2.07	-0.54	0.22
ILMN_1755909	C20orf11	-1.29	-1.91	-0.66	0.20
ILMN_1804834	C6orf130	-1.26	-2.04	-0.49	0.24
ILMN_1737588	C21orf33	-1.25	-1.87	-0.63	0.20
ILMN_2222984	NT5C1B	-1.18	-1.94	-0.43	0.24
ILMN_1671257	DKC1	-1.12	-1.84	-0.39	0.24
ILMN_1790136	C20orf20	-1.04	-1.68	-0.39	0.24
ILMN_2310589	DIABLO	-0.88	-1.37	-0.39	0.20
ILMN_1811373	FAM20B	1.17	0.42	1.92	0.24
ILMN_2360705	ACSL3	1.32	0.48	2.16	0.24
ILMN_1802203	ARF1	1.38	0.51	2.25	0.24
ILMN_1811551	DERA	1.44	0.52	2.37	0.24
ILMN_1724700	RIOK3	1.50	0.53	2.48	0.24
ILMN_2369580	C16orf35	1.55	0.62	2.48	0.23
ILMN_1685763	WDR45L	1.56	0.56	2.55	0.24
ILMN_1745962	FBXO7	1.77	0.74	2.81	0.21
ILMN_1781155	LYN	1.89	0.81	2.97	0.20
ILMN_1775692	EIF4G3	1.90	0.67	3.12	0.24
ILMN_1781819	PAPSS1	1.92	0.67	3.17	0.24
ILMN_2234016	FTH1	2.13	0.85	3.41	0.23
ILMN_1804530	ARPC1B	2.23	0.97	3.49	0.20
ILMN_1688322	ADIPOR1	2.32	1.13	3.52	0.20
ILMN_1714397	CRYL1	2.33	1.04	3.62	0.20
ILMN_1810953	FLJ39739	2.38	1.00	3.76	0.21
ILMN_1719286	CTSA	2.43	0.86	4.01	0.24
ILMN_1661537	LEPROT	2.46	0.88	4.04	0.24
ILMN_1695829	TTYH2	2.49	0.95	4.02	0.24
ILMN_1748883	CDKN2D	2.56	0.90	4.22	0.24
ILMN_2378100	FBXL5	2.57	0.96	4.17	0.24
ILMN_1658053	DYNLRB1	2.60	0.94	4.26	0.24
ILMN_1788268	NAPG	2.60	1.21	4.00	0.20

ILMN_1883473	BM678284	2.61	0.97	4.25	0.24
ILMN_1779752	CR627362	2.62	1.17	4.06	0.20
ILMN_1679520	AGPAT1	2.72	1.06	4.38	0.24
ILMN_1814998	POTEKP	2.72	1.15	4.29	0.20
ILMN_1898662	BX096254	2.73	0.95	4.50	0.24
ILMN_1815878	LAMTOR1	2.73	0.97	4.48	0.24
ILMN_1674941	ANO6	2.74	1.14	4.33	0.21
ILMN_3297455	LOC729082	2.74	1.09	4.39	0.23
ILMN_1831098	BX092776	2.78	1.07	4.49	0.24
ILMN_1810712	ARHGEF12	2.78	1.08	4.49	0.24
ILMN_1851244	AW205453	3.00	1.39	4.62	0.20
ILMN_1748797	GRB2	3.04	1.34	4.75	0.20
ILMN_1702447	IGF2BP2	3.05	1.20	4.90	0.23
ILMN_1742167	TUBA1C	3.18	1.24	5.12	0.24
ILMN_1803392	P2RX5-TAX1BP3	3.23	1.77	4.68	0.19
ILMN_1810785	RNF11	3.23	1.24	5.22	0.24
ILMN_1860753	BI831346	3.30	1.62	4.97	0.20
ILMN_1765208	GLUL	3.35	1.42	5.28	0.20
ILMN_1746137	ST7	3.39	1.67	5.11	0.20
ILMN_2048607	ANKRD9	3.58	1.26	5.90	0.24
ILMN_1679177	MARS2	3.60	1.67	5.53	0.20
ILMN_1671486	HOMER2	3.63	1.32	5.95	0.24
ILMN_1776157	SEPT4	3.63	1.43	5.84	0.23
ILMN_2086077	JUNB	3.74	1.51	5.96	0.23
ILMN_1782567	GUCY1B3	4.07	1.70	6.44	0.21
ILMN_1768577	D28514	4.08	1.42	6.75	0.24
ILMN_1701933	SNCA	4.21	1.51	6.92	0.24
ILMN_1713561	C20orf103	4.43	1.54	7.33	0.24
ILMN_1781966	OSBP2	4.54	1.70	7.38	0.24
ILMN_1717793	C19orf33	4.60	1.74	7.46	0.24
ILMN_1696183	HBQ1	4.62	1.88	7.36	0.23
ILMN_1691507	IL8RBP	4.86	1.71	8.02	0.24
ILMN_2227011	ACSBG1	4.90	1.95	7.85	0.23
ILMN_1799569	LIMS3L	5.05	2.43	7.68	0.20
ILMN_1675062	MYL9	5.60	2.50	8.69	0.20

The following supplementary material is available from the publisher:

Table S1, S3, S4, S5 and **S6**