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

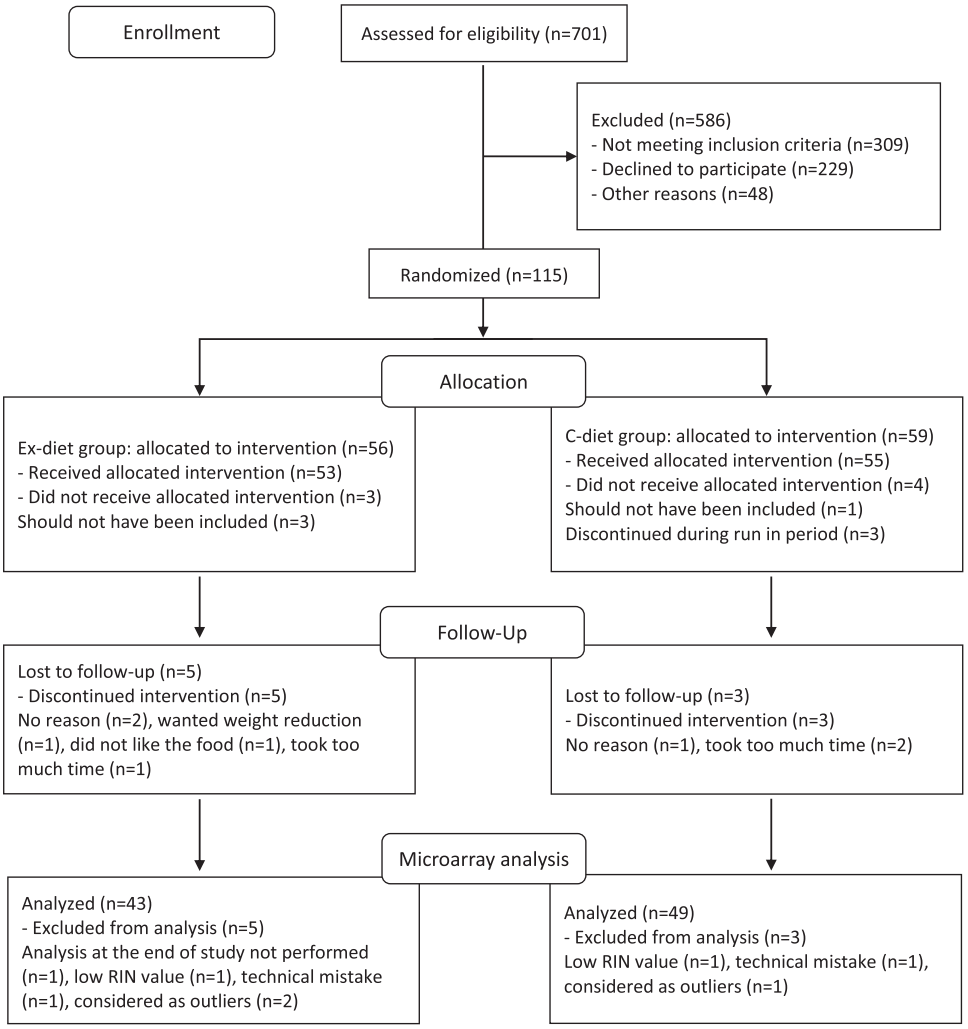

Figure 1. Flowchart showing the number of subjects included in the microarray analyses. C-diet, control diet; Ex-diet, experimental diet.

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.

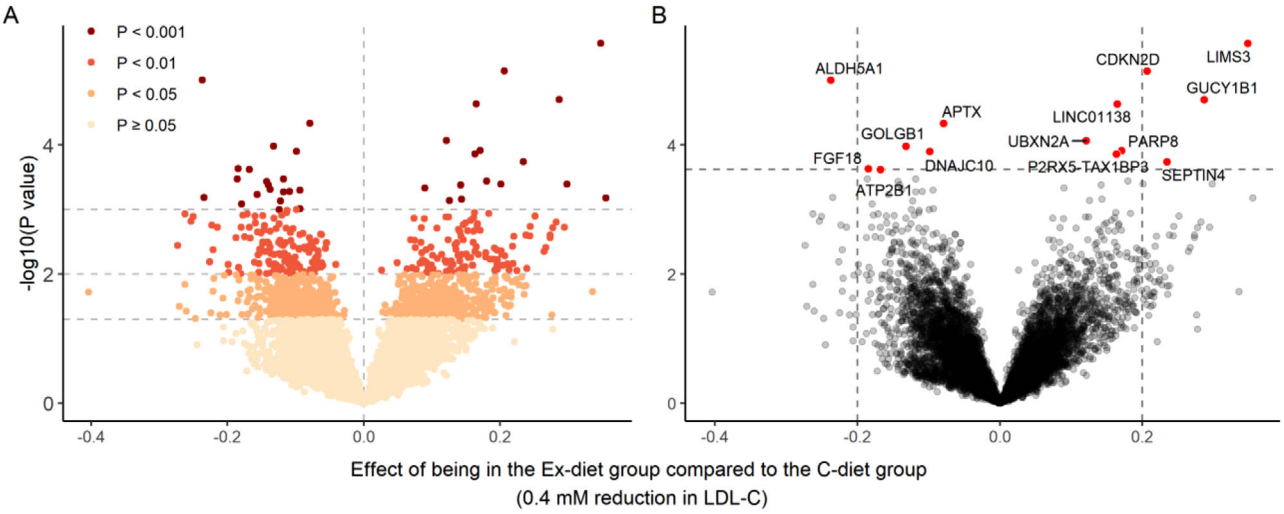

Figure 2. Difference in log2 fold change between groups (x axis) versus -log10(*p*-value) (y axis) for 13 148 gene transcripts analyzed with a linear regression model adjusted for sex, age, study center, smoking, and log(baseline gene expression) level. A) The vertical line represents no difference between 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.

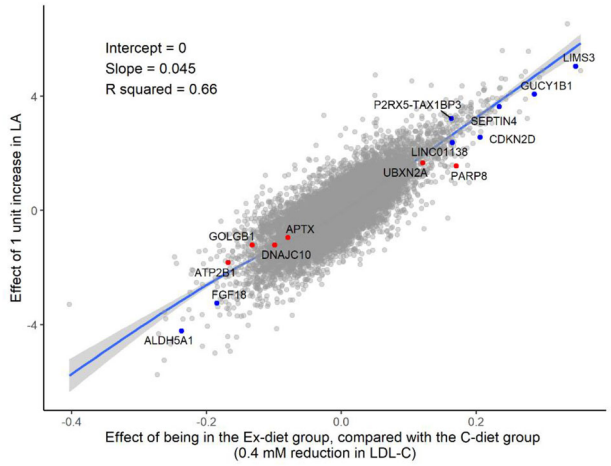

Figure 3. Covariation between group effect and LA effect on gene expression changes. Gene transcripts highlighted in blue or red are significantly differentially altered (FDR q-value < 0.25) in the Ex-diet group compared to the C-diet group. Gene transcripts highlighted in blue are significantly altered (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

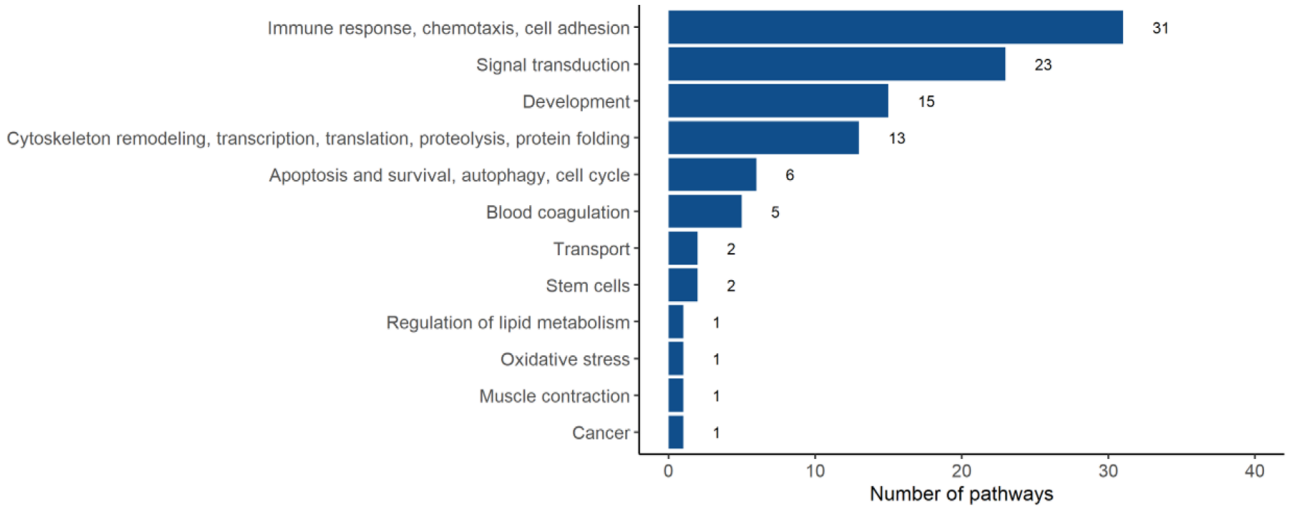

Figure 4. Number of differentially enriched pathways between the groups within different biological processes analyzed in Metacore. A total of 1105 gene transcripts (p-value < 0.05) were included in the analyses. Pathways with FDR q-value < 0.01 were defined as significantly differentially enriched.

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 ($\pm$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 ($\geq$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 $\times$ g, 15 min). Plasma was obtained from EDTA tubes (BD Vacutainer Systems, Plymouth, UK), immediately placed on ice, and centrifuged within 10 min (2000 $\times$ *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 $\times$ 0.20 mm i.d. $\times$ 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

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