Effects of omega-3 polyunsaturated fatty acids on gene expression profiles of normo- and dyslipidemic men

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Abstract

Background and aims: The beneficial effects of omega-3 polyunsaturated fatty acids (n-3 PUFAs) and their oxidized metabolites, especially for the prevention of cardiovascular disease and dyslipidemia, are widely described in the literature, but the underlying molecular mechanisms in humans are largely unknown. However, it is believed that n-3 PUFAs exert many effects via direct or indirect regulation of gene expression. A double-blind and placebo-controlled intervention trial was undertaken to analyze gene expression changes of normo- and dyslipidemic male subjects after fish oil (FO) supplementation and to find possible indications for a molecular mechanism of action. Dyslipidemic male subjects were included in the study population to consider the higher risk profile of developing cardiovascular disease in these subjects, as well as to focus on the gene regulatory effects of n-3 PUFAs on lipid metabolism-related genes in regard to the triacylglycerol (TG) lowering effect of n-3 PUFAs.

Methods: Twenty normo- and twenty dyslipidemic men were supplemented for twelve weeks with six FO capsules per day (2.7 g n-3 PUFAs, 1.14 g docosahexaenoic acid and 1.56 g eicosapentaenoic acid) or six corn oil capsules (3 g linoleic acid). The gene expression levels were determined by whole genome microarray analysis at baseline, after four hours, one week, and twelve weeks of supplementation and pathway analyses of differential regulated genes were performed. In addition, selected transcripts were quantified by quantitative real-time polymerase chain reactions at baseline and after twelve weeks of supplementation.

Results and conclusions: This proof of concept study revealed significant differences in gene expression profiles between normo- and dyslipidemic male subjects after FO supplementation. Dyslipidemic male subjects presented substantially more regulated genes and pathways, of which several were related to the immune system, inflammation, lipid metabolism, and cardiovascular disease. Additionally, the study showed that n-3 PUFAs activate and repress several transcription factors resulting in the regulation of numerous target genes. In this case, lipid metabolism-related transcription factors, such as the hepatic nuclear factor (HNF) 6 and HNF1ß, which could affect HNF4α, an additional important regulator of the lipid metabolism, were repressed by n-3 PUFA supplementation. Otherwise, peroxisome proliferator-activated receptor (PPAR) α was activated by n-3 PUFA supplementation. The regulation of named transcription factors may induce expression changes of target genes resulting in TG lowering, which could be observed in normo- and dyslipidemic male subjects. Moreover, the activation of PPARα appears to play a significant role in various other cardioprotective effects of n-3 PUFAs. In this case, antioxidative genes were up-regulated and pro-inflammatory genes were down-regulated after n-3 PUFA supplementation, suggesting that n-3 PUFA may induce anti-inflammatory and antioxidative effects via PPARα activation.

Although this study revealed several indications of possible molecular mechanisms of action in what extent n-3 PUFAs mediate their cardioprotective effects, further studies analyzing a combination of gene expression, metabolic markers and clinical end-points are needed to clarify the mechanisms by which n-3 PUFAs trigger gene regulation and affect various regulatory networks.

Trial registration: ClinicalTrials.gov (ID: NCT01089231)

Keywords: omega-3 fatty acids, dyslipidemia, gene regulation

Zusammenfassung

Hintergrund und Ziele der Arbeit: Omega-3 Fettsäuren (n-3 FS) und ihren oxidierten Metaboliten werden zahlreiche gesundheitsfördernde Effekte in der Prävention von kardiovaskulären Erkrankungen und Dyslipidämien nachgesagt. Die zu Grunde liegenden molekularen Mechanismen, besonders innerhalb des menschlichen Organsimuses, sind jedoch unzureichend geklärt. Allerdings wird vermutet, dass n-3 FS eine Vielzahl ihrer Effekte direkt oder indirekt über die Regulation der Genexpression ausüben. Daher wurde eine doppel-blinde und placebo-kontrollierte Interventionsstudie mit dem Ziel durchgeführt, Genexpressionsveränderungen nach einer Fischölsupplementierung in gesunden Probanden sowie in Probanden mit erhöhten Blutfettwerten zu analysieren, um Ansatzpunkte möglicher molekularer Wirkmechanismen identifizieren zu können. Der Einschluss von Probanden mit erhöhten Blutfettwerten sollte zum einen das höhere Risikoprofil dieser Probanden für die Entstehung kardiovaskulärer Erkrankungen berücksichtigen und zum anderen die gezielte Untersuchung der Triacylglycerol (TG)-senkenden Eigenschaften von n-3 FS ermöglichen.

Methodik: Zwanzig gesunde männliche Probanden, sowie zwanzig männliche Probanden mit erhöhten Blutfettwerten wurden über zwölf Wochen mit sechs Fischölkapseln pro Tag (2.7 g n-3 FS, 1.14 g Docosahexaesäure und 1.56 g Eicosapentaensäure) oder sechs Maiskeimölkapseln (3 g Linolsäure) supplementiert. Mittels whole genome Microarrays wurden die Genexpression zum Startzeitpunkt (t_0) der Studie sowie nach vier Stunden (t_{4h}), einer Woche (t_1) und zwölf Wochen (t_{12}) Supplementierung gemessen. Die auf diese Weise identifizierten regulierten Gene wurden Pathway Analysen unterzogen. Zusätzlich wurden einige Transkripte mittels quantitativer Echtzeit-Polymerase-Kettenreaktion zu t_0 und t_{12} quantifiziert.

Ergebnisse und Schlussfolgerungen: Im Rahmen dieser Proof of concept Studie konnten signifikante Unterschiede im Genexpressionsprofil nach Fischölsupplementierung von gesunden Probanden und Probanden mit erhöhten Bluttfettwerten festgestellt werden. Die Probanden mit erhöhten Bluttfettwerten zeigten wesentlich mehr regulierte Gene und Pathways, von denen viele mit dem Immunsystem, Entzündungen, dem Fettstoffwechsel und kardiovaskulären Erkrankungen in Verbindung stehen. Des Weiteren zeigte die Studie, dass n-3 FS die Expression verschiedener Transkriptionsfaktoren sowie dessen Zielgene induzieren und unterdrücken können. Eine reduzierte Expression ist beispielsweise bei zwei Transkriptionsfaktoren des Fettstoffwechsels, dem hepatic nuclear factor (HNF) 6 und dem HNF1ß zu beobachten, die wiederum Einfluss auf HNF4α – ein ebenfalls wichtiger Schlüsselregulator des Fettstoffwechsels, nehmen können. Die Expression des Transkriptionsfaktors peroxisome proliferator-activated receptor (PPAR) α hingegen wurde durch die n-3 FS Supplementierung verstärkt. Die Regulation dieser genannten Transkriptionsfaktoren könnte für die Veränderung der Expressionsraten von Zielgenen verantwortlich sein, die wiederum eine TG Senkung induzieren, was sowohl in gesunden als auch in Probanden mit erhöhten Blutfettwerten beobachtet wurde. Des Weiteren scheint die Aktivierung von PPARα zur Umsetzung weiterer zahlreicher gesundheitsfördernder Effekte eine große Rolle zu spielen. So führt eine n-3 FS Supplementierung beispielsweise neben der PPARα Aktivierung gleichzeitig zu einer verstärkten Expression von antioxidativen Genen und einer Reduktion der Expression pro-inflammatorischer Gene. Dies lässt vermuten, dass anti-inflammatorische- sowie antioxidative Effekte von n-3 FS über eine PPARα Aktivierung vermittelt werden.

Obwohl diese Studie zahlreiche Hinweise auf mögliche Wirkmechanismen zur Ausübung kardioprotektiver Effekte liefert, sind weitere Studien, die die Messung der Genexpression, metabolischer Marker und klinischer Endpunkte vereinen, nötig, um die Mechanismen zu klären, wie n-3 FS Genexpresionsveränderungen induzieren und verschiedene regulative Netzwerke beeinflussen.

Trial Registrierung: ClinicalTrials.gov (ID: NCT01089231)

Schlagwörter: omega-3 Fettsäuren, Dyslipidämie, Genregulation

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Schmidt S, Stahl F, Mutz KO, Scheper T, Hahn A, Schuchardt JP (2012). Transcriptome-based identification of antioxidative gene expression after fish oil supplementation in normo- and dyslipidemic men. *Nutrition & Metabolism* 1:45

Schmidt S, Willers J, Stahl F, Mutz KO, Scheper T, Hahn A, Schuchardt JP (2012). Regulation of lipid metabolism-related gene expression in whole blood cells of normo- and dyslipidemic men after fish oil supplementation. *Lipids in Heath and Disease*, submitted

Conference contributions

Posters

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Schmidt S, Schuchardt JP, Stahl F, Mutz KO, Scheper T, Hahn A (2012): Identification of genes related to antioxidative mechanisms after fish oil supplementation in normolipidemic and dyslipidemic men, 49. Wissenschaftlicher Kongress der DGE, Bonn, 14.-16.03.2012

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Abbrevations

℃	Degree Celsius
%	Percent
AA	Arachidonic acid (20:5n-6)
ABCG5	ATP-binding cassette sub-family G member 5
ACACB	Acetyl-CoA carboxylase beta
ACC	Acetyl-CoA carboxylase
ADMA	Asymmetric dimethylarginine
ALA	α-linolenic acid
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Apo	Apolipoprotein
ARVC	Arrhythmogenic right ventricular cardiomyopathy
BK(Ca)channel	Large-conductance calcium and voltage-activated potassium channel
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
CAM	Cell adhesion molecule
CAT	Catalase
cDNA	Complementary desoxyribonucleic acid
CHD	Coronary heart disease
ChREBP	Carbohydrate response element binding protein
CO	Corn oil
CO-D	Dyslipidemic corn oil group
CO-N	Normolipidemic corn oil group
COX	Cyclooxygenases
CRP	C reactive protein
CVD	Cardiovascular disease
CYP	Cytochrome P450 enzyme
DCM	Dilated cardiomyopathy
DGAT	Diacylglycerol O-acyltransferase
DHA	Docosahexaenoic acid (22:6 n-3)
dl	Deciliter
ECI2	Enoyl-CoA delta isomerase 2
ECM	Extracellular matrix
EPA	Eicosapentaenoic acid (20:5 n-3)
et al.	Et alii (lat.: and others)

FA	Fatty acid
FAS	FA synthase
FM	Foam cell
FO	Fish oil
FO-D	Dyslipidemic fish oil group
FO-N	Normolipidemic fish oil group
FXR	Farnesoid X receptor
g	Gram
gamma-GCL	Gamma-glutamylcysteine synthetase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCP	Good clinical practice
GEO	Gene Expression Omnibus
GISSI	Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico
GPX	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione-S-transferase
H_2O_2	Hydrogenperoxide
HADH	Hydroxyacyl-CoA dehydrogenase
HADHA	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit
HDL-C	High-density lipoprotein cholesterol
HepG2	Hepatocellular carcinoma, human
HMOX	Heme oxygenase
HNF	Hepatic nuclear factor
HTG	Hypertriglyceridemia
ICAM	Intracellular cell adhesion molecule
ID	Identifer
IDL	Intermediate-density lipoproteins
i.e.	In example
IκB	Inhibitor of κB
IL	Interleukin
iNOS	Inducible nitrit oxid synthase
ISSFAL	International Society for the Study of Fatty Acids and Lipids
KEGG	Kyoto Encyclopaedia of Genes and Genomes
kg	Kilogram
LA	Linoleic acid (18:2 n-6)

LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LOX	Lipoxygenase
LP	Lipoprotein
LPL	Lipoprotein lipase
LT	Leukotriene
LXR	Liver X receptor
m²	square meter
mg	Milligram
µg	Microgram
MAPK	Mitogen-activated-protein kinase
MIAME	Minimum information about a microarray experiment
MMP	Matrix metalloproteinase
MOGAT	2-acylglycerol O-acyltransferase
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acids
n-3 PUFA	Omega-3 polyunsaturated fatty acid
n-6 PUFA	Omega-6 polyunsaturated fatty acid
NFκB	Nuclear factor kappa b
NO	Nitric oxide
NOS	Nitrit oxide synthase
oxEPA	Oxidized EPA
oxLDL	Oxidized low-density lipoprotein
p	Error probability
PBMC	Peripheral blood mononuclear cells
PG	Prostaglandin
PGI	Prostacyclin
PL	Phospholipids
PLA2G2E	Phospholipase 2 group 2 E
PLB1	Phospholipase B1
PLTP	Phospholipid transfer protein
PPAR	Peroxisome proliferator-activated receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPS2	Ribosomal protein S2

RXR	Retinoid X receptor
SCD1	Stearyl-CoA desaturase-1
SFA	Saturated fatty acid
SMC	Smooth muscle cell
SOAT1	Sterol O-acyltransferase 1
SOD	Superoxide dismutase
SREBP	Sterol regulatory element-binding protein
t	Time point
t_0	Baseline
t_{4h}	Four hours after the first ingestion of the study capsules
t_1	After one week of supplementation
t_{12}	After twelve weeks of supplementation
TC	Total cholesterol
TG	Triacylglycerol
TNF	Tumor necrosis factor
TSA	Tyramide signal amplification
TX	Thromboxane
VCAM	Vascular cell adhesion molecule
VLDL	Very low-desity lipoprotein

1. General introduction

1.1 Cardiovascular disease and atherosclerosis

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide (Roger et al. 2012), and frequently appears as a result of complications of atherosclerosis. Atherosclerosis is a chronic inflammatory disease of the artery system and is characterized by progressive degeneration of the large- and medium-sized arteries and alterations of the vascular wall. Blood lipids, especially free and esterified cholesterol, chalk, collagen and proteoglycans accumulate within the vascular wall, leading to the formation of arterial plaques, resulting in thickening, stiffness and decreased elasticity. These alterations are often followed by several cardiovascular events, such as sudden cardiac death, cardiac infarction and stroke, which are generally the result of plaque rupture and thrombosis. After plaque rupture, lipids and tissue factors are exposed to blood components, initializing the coagulation cascade, platelet adherence and thrombosis (Glass and Witztum 2001). The pathogenesis of atherosclerosis is very complex and several epidemiological and clinical studies evaluate factors which increase the risk of developing this disease. These known risk factors include influenceable factors, such as arterial hypertension, obesity, metabolic syndrome, diabetes, insulin resistance, dyslipidemia, and lifestyle factors (smoking, stress, high-fat diet), but also non-influenceable factors, such as male gender, age, family history, genetic disorders, and infectious agents.

1.1.1 Pathogenesis of atherosclerosis

Two main hypotheses were formulated in the past concerning the initial causes of the development of atherosclerosis: The “response-to-injury hypothesis” and the “lipoprotein-induced atherosclerosis hypothesis” (Ross and Glomset 1973; Ross 1993). The response-to-injury hypothesis generally implies that an endothelial injury induces the initiation of atherosclerosis, and that this injury could be produced by mechanical (trauma, hypertension), chemical (toxins, viral attacks, antigen-antibody reactions) or physical events (damage on a molecular level). A more specified approach, the “lipoprotein-induced atherosclerosis hypothesis”, assumes that endothelial injury is caused by oxidized low-density lipoprotein (oxLDL) (Brown and Goldstein 1983). In agreement with both hypotheses, the pathogenesis of atherosclerosis begins with an alteration of the endothelium, called endothelial activation, which could be induced by the factors named above. In addition, within these factors oxLDL is probably unique in initiating atherosclerosis without any other factors. Endothelial activation leads to an inflammatory response, resulting in recruitment and increased adhesion of leucocytes and monocytes which, in turn, lead to a release of cytokines,

chemokines, cell adhesion molecules (CAMs), and growth factors by endothelial cells, leucocytes and smooth muscle cells. These inflammatory proteins are involved in facilitating the migration of monocytes, which become macrophages after migration. Macrophages accumulate lipids in response to inflammatory proteins and transmute to foam cells, which compose, together with leucocytes, the so-called fatty streak (Mangge et al. 2004). In addition, the formation of foam cells increases the inflammatory response, resulting in additional migration and proliferation of smooth muscle cells, which secrete extracellular matrix (ECM) proteins that initiate tissue reorganization, resulting in plaque formation. Atherosclerosis-promoting risk factors and the various steps during the progression of atherosclerosis are displayed in **Figure 1.1**. The role of dyslipidemia, chronic inflammation and oxidative stress in the pathogenesis of atherosclerosis will be described in detail within the next section.

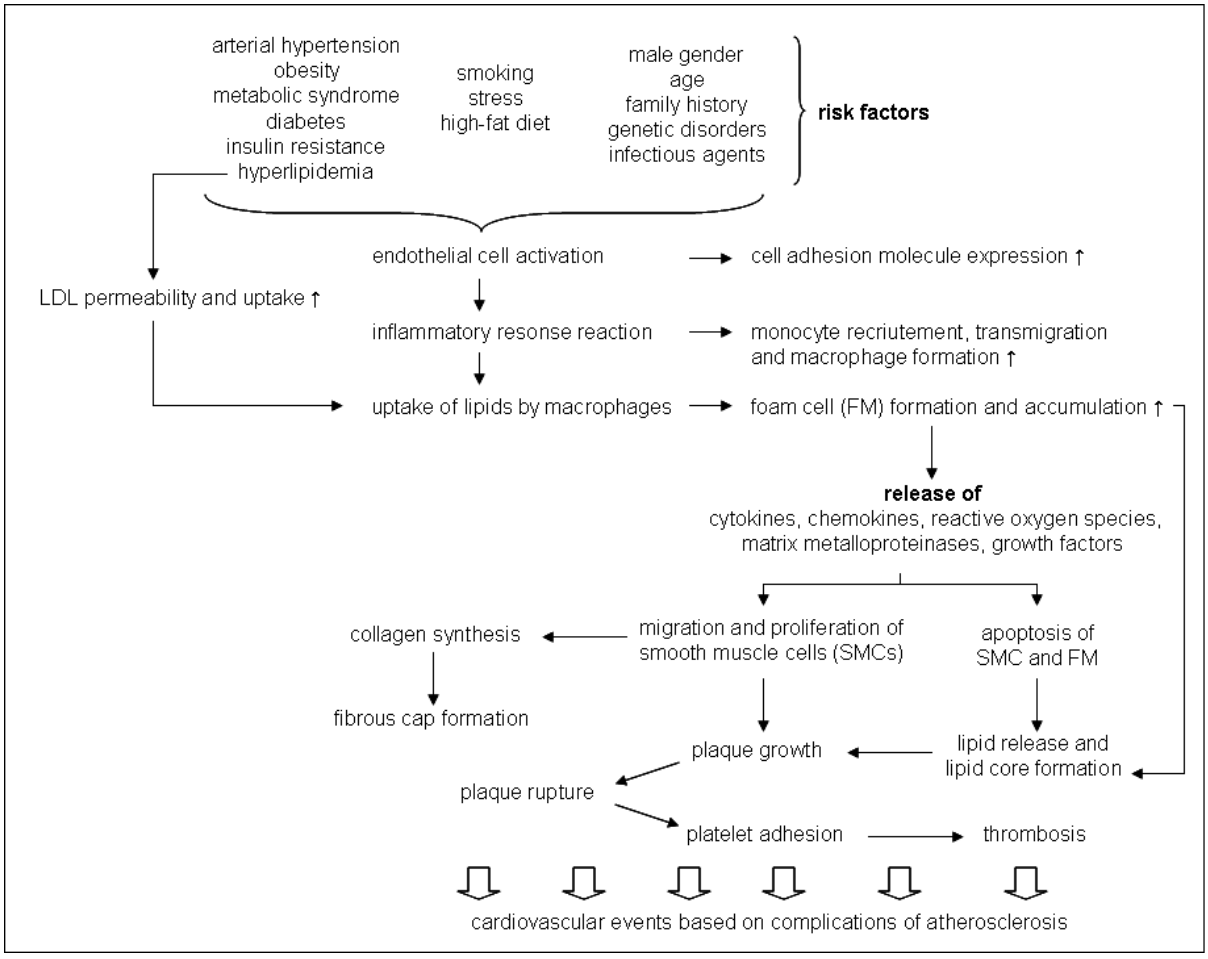

Figure 1.1: Simplified schematic representation of the pathogenesis of atherosclerosis. The figure was designed in accordance with Da Luz and Favarato 1999, and two figures found at http://journals.prous.com/journals/dot/19993508/html/dt350641/images/Marimon1.gif and http://ocw.tufts.edu/data/51/673632/673700_xlarge.jpg.

1.1.2 Role of dyslipidemia

An unbalanced lipid profile, i.e. hypercholesterolemia, hypertriglyceridemia (HTG) or combined hyperlipidemia, has a considerable atherogenic potential and increases the cardiovascular risk (Yusuf et al. 2004). Hypercholesterolemia, in particular, which is characterized by an elevated level of cholesterol-transporting lipoproteins, plays a key role in the development and progression of atherosclerosis (Tóth 2009). In this context, an elevated low-density lipoprotein cholesterol (LDL-C) level is associated with a high risk of CVD (Klag et al. 1993), as well as with the extent of atherosclerosis in the aorta and coronary circulation (McGill et al. 1997). It is believed that high amounts of cholesterol-transporting lipoproteins could undergo oxidative modifications (see section 1.1.3), leading to the injury of vascular endothelial cells, which subsequently promotes the development of atherosclerosis. In addition, the risk of coronary disease is markedly increased if both levels of LDL-C and lipoprotein(a) (Lp(a)) are elevated (Armstrong et al. 1986). The positive correlation between the Lp(a) level and coronary heart disease (CHD) (Craig et al. 1998; Luc et al. 2002) may be caused by the reduced activity of plasminogen (Eaton et al. 1987) and altered LDL-C mediated delivery of cholesterol to the vessel wall (Armstrong et al. 1986).

Similarly, HTG, which is characterized by an elevated triacylglycerol (TG) level, is also known as an independent risk factor of CHD (Assmann et al. 1998; Austin et al. 1998; Davingnon and Chon 1996; Hokanson and Austin 1996; Sarwar et al. 2010). TG in the blood circulation is carried inside chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoproteins (IDL), LDL-C, and high-density lipoprotein cholesterol (HDL-C), of which chylomicrons and VLDL are the primary TG-rich lipoproteins (Dunbar and Rader 2005). Subsequently, associations between TG-rich lipoproteins and coronary events could be observed (Sacks et al. 2000).

In addition to the hyperlipidemias described, low levels of HDL-C are associated with increased risk of CVD. Furthermore, the progression of atherosclerosis is inversely correlated with the level of HDL-C (McGill et al. 1997). This correlation and atheroprotective function of HDL-C could be partly caused by facilitating reverse cholesterol transport and transporting antioxidant enzymes, which can break down oxidized lipids and neutralize their pro-inflammatory effects (Libby et al. 2002).

Because of the important role of the lipid profile for cardiovascular health, general guidelines for optimal total cholesterol (TC), LDL-C, HDL-C, and TG levels, as well as guidelines for the LDL-C level based on a person's global risk of CHD, have been published. In general, the following levels for named lipids are recommended: LDL-C < 160 mg/dl (4.14 mmol/L), HDL-C > 40 mg/dl (1.03 mmol/L), TC < 250 mg/dl (6.47 mmol/L), and TG < 150 mg/dl (3.88

mmol/L). In addition, high-risk individuals with CHD or CHD-risk equivalents should not feature a LDL-C level above 100 mg/dL (5.55 mmol/L), while an LDL-C goal of < 130 mg/dL (3.36 mmol/L) was recommended for those at moderate-risk who had ≥ 2 risk factors but no CHD or CHD-risk equivalents (Grundy et al. 2004).

1.1.3 Role of chronic inflammation

Inflammation is part of the body's defense system in response to injury or infection with the goal of eliminating pathogens or toxins and to repair damaged tissue. The typical major symptoms, redness, swelling, heat, and pain, are the results of an increased transmigration of leucocytes (granulocytes, monocytes, macrophages, and lymphocytes) and the production of inflammatory mediators, such as cytokines, CAMs, chemokines, acute phase proteins, eicosanoids, nitric oxide (NO), matrix metalloproteinases (MMPs), and reactive oxygen species (ROS), which lead to local tissue damage, systemic effects on the central nervous system (fever, loss of appetite), skeletal muscle (proteolysis), adipose tissue (lipolysis) and liver (synthesis of acute phase proteins), as well as to pathogen destruction (Galli and Calder 2009). Afterwards, the inflammatory process undergoes resolution and recovery, which is characterized by the repair of lesions and down-regulation of inflammatory mediators.

Pathological chronic inflammation is characterized by an increased number of leucocytes in circulation and by an increased expression of inflammatory mediators, especially cytokines and CAMs, which are known and partly above-mentioned contributors to the development of atherosclerosis. Prospective epidemiological studies evaluated a positive correlation between vascular risk and the levels of cytokines, such as interleukin (IL) 6 and tumor necrosis factor (TNF) α; CAMs, such as intracellular (I) CAM-1, vascular (V) CAM-1, P-selectin, and E-selectin; and downstream acute-phase reactants, such as C reactive protein (CRP), fibrinogen and serum amyloid A (Calder 2003; Libby et al. 2002). In addition, chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis and inflammatory bowel disease (Crohn's disease, ulcerative colitis), have been shown to be associated with a risk of CVD and atherosclerosis (Avina-Zubieta et al. 2012; Siegel et al. 2012; Yarur et al. 2011). Taken as a whole, chronic inflammation is able to promote the initiation and progression of atherosclerosis, which is visualized in **Figure 1.2**.

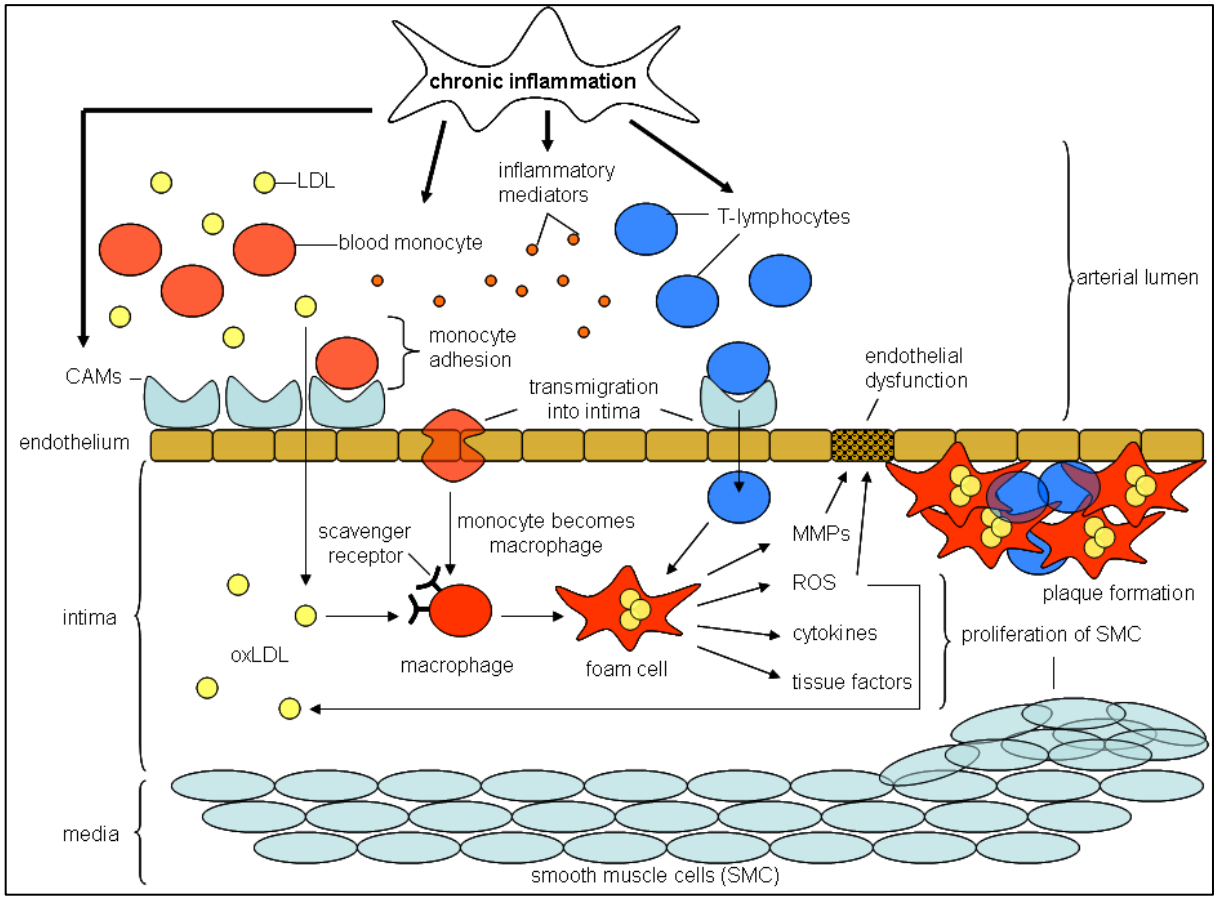

Figure 1.2: Role of chronic inflammation for the progression of atherosclerosis. This figure was designed in accordance with Libby et al. 2010.

1.1.4 Role of oxidative stress

Oxidative stress is defined as a state in which the level of ROS exists in excess, which could result from overproduction, a decreased antioxidative defense system, or a combination of both. An imbalance of the redox status in the vasculature could lead to pathophysiological consequences mainly caused by ROS-mediated oxidative modification of lipids and proteins (Leopold and Loscalzo 2009; Yung et al. 2006). In this context, several studies have shown an association between oxidative stress and the pathogenesis of atherosclerosis, endothelial dysfunction and CVD (Heinecke 2003; Jung et al. 2004; Knight 1995; Landmesser et al. 2000; Madamanchi et al. 2005; Sydow et al 2003; Tyagi et al. 2005; Wang et al. 1998). ROS-mediated oxidative modification of lipids and especially an increased level of oxLDL (Navab et al. 2004) plays a key role in the initiation and progression of arteriosclerotic vascular diseases (El-Melegy et al. 2008; Huang et al. 2008; Navab et al. 2002) due to several reasons. Firstly, elevated absorption of oxLDL into vascular cells enhances the formation of foam cells and atherogenic plaque (Lankin et al. 2007), and promotes inflammatory processes (Ehara et al. 2002) by the induction of gene expression of CAMs, chemokines and pro-inflammatory cytokines (Libby et al. 2002). In general, inflammatory processes are

promoted by the apoprotein moieties of all lipoprotein particles, which can undergo modification in the artery wall, resulting in inciting T-cell responses (Stemme et al. 1995). However, an increased oxLDL level elevates the concentration of asymmetric dimethylarginine (ADMA) (Böger and Bode-Böger 2000), an inhibitor of the nitrit oxide synthase (NOS), resulting in reduced vasodilation (Zhang et al. 2008) and increased risk of thrombosis (Anayeva et al. 1997; Mehta and Li 1998). Besides the ROS-mediated increase of oxLDL, ROS also increase the concentration of homocystein and decrease the concentration of fetuin A. An elevated homocystein level inhibits dimethylarginine dimethylaminohydrolase, an enzyme which catabolizes ADMA, resulting in an increased ADMA level and, in turn, decreased vasodilation by decreased NO production (Jia et al. 2006; Jin et al. 2001; Stühlinger et al. 2001; Tyagi et al. 2005). Last but not least, a decreased level of fetuin A (Moe and Chen 2005), an inhibitor of vascular calcification, results in increased stiffness of arteries and is correlated with increased mortality of CVD patients (Ford et al. 2010; Jenkins et al. 2011).

1.2 Omega-3 polyunsaturated fatty acids

1.2.1 Structure and elongase/desaturase pathway

Fatty acids (FAs) are composed of a carbon chain and vary in the chain length and number of double bonds. Depending on the number of double bonds, saturated fatty acids (SFAs) with no double bond, monounsaturated fatty acids (MUFAs) with one double bond and polyunsaturated fatty acids (PUFAs) with at least two double bonds are distinguished. PUFAs are subdivided into short-, medium- and long-chain PUFAs depending on the number of carbon atoms and thus, the chain length. The name “omega” indicates the position of the first double bond from the methyl terminal. Therefore, omega-3 polyunsaturated fatty acids (n-3 PUFAs) show the first double bond at the third carbon atom from the methyl terminal. The position of the first double bond is an important factor which influences the properties of FAs. The simplest n-3 PUFA is α-linolenic acid (ALA; 18:3), which can be converted to longer and most bioactive n-3 PUFAs, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Flachs et al. 2009). The most bioactive n-6 PUFA, which shows the first double bond at the sixth carbon atom from the methyl terminal, is arachidonic acid (AA; 20:4). The chemical structures of these named FAs are shown in **Figure 1.3**. In general, FAs present the most energetic substrates of the diet and can be stored in the form of TGs or incorporated into phospholipids (PLs) of cellular membranes. The transport of FAs into the cell is achieved by FA transporters, and intracellular FAs are rapidly converted to fatty acyl-CoA thioesters by acyl-CoA synthetases, which serve as substrates for the synthesis of neutral (triglycerides, cholesterol esters) and polar lipids (PLs, sphingolipids and

plasmalogens), as well as for elongation, desaturation, ß-oxidation, and protein acylation reactions (Jump 2002). The conversion of the essential FA ALA to EPA and DHA mentioned above is characterized by several elongations and desaturations catalyzed by rate-limiting Δ6 and Δ5 desaturases. The different steps of conversion of n-3 PUFAs and n-6 PUFAs are displayed in **Figure 1.4**.

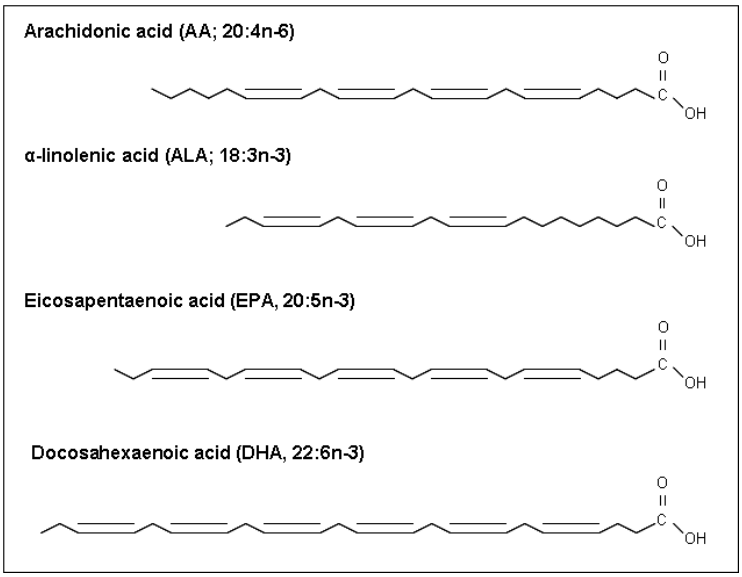

Figure 1.3: Chemical structure of α-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, and arachidonic acid

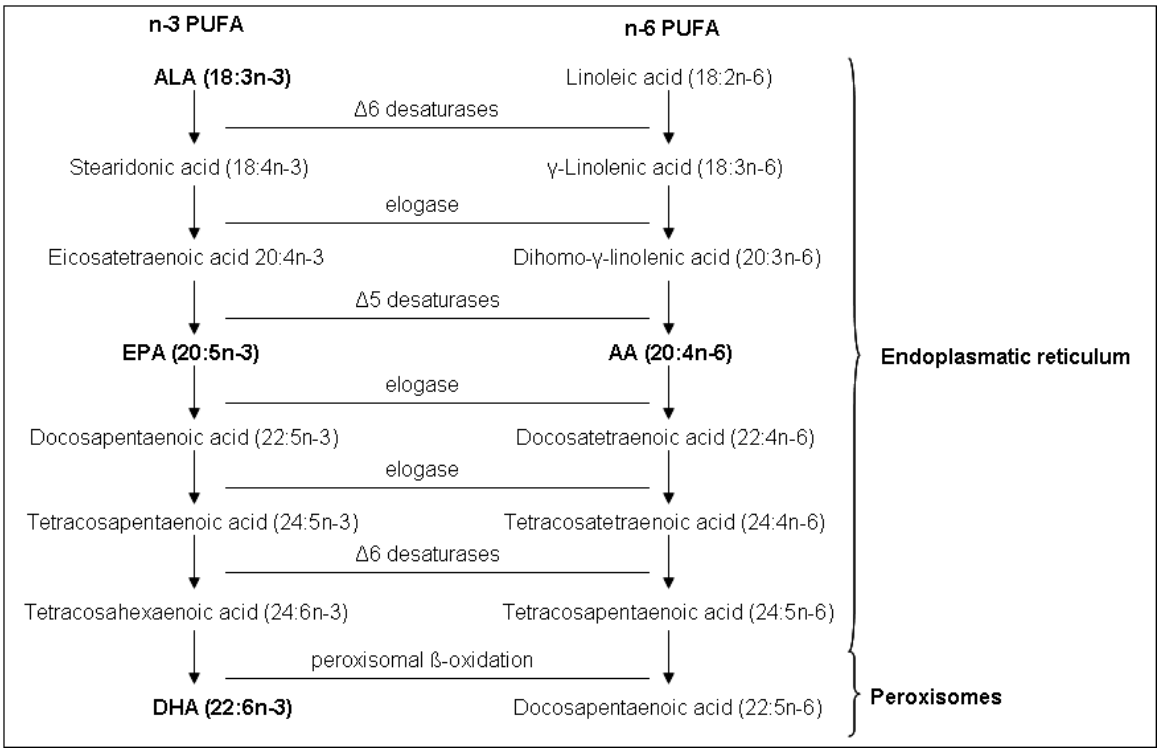

Figure 1.4: Conversion of n-3 PUFAs and n-6 PUFAs *in vivo*. The figure was designed in accordance with Jump 2002.

Although the conversion from ALA to EPA and DHA is possible, exogenous sources of EPA and DHA are needed, because the conversion rate is low. Studies using isotope-labeled ALA showed conversion rates ranging between 0.2 and 8% for EPA and between 0 and 4% for DHA in males (Burdge 2004). Additionally, genetic, epigenetic and dietary co-factors, including magnesium, zinc and vitamin B6, could additionally modulate the conversion (Das 2005; Devlin et al. 2007). Therefore, an adequate accommodation of body tissues is almost achieved by dietary intake of exogenous sources of EPA and DHA.

1.2.2 Dietary intake recommendations and supply situation

Dietary sources of ALA are mainly nuts, soy and rapeseed oils, whereas fatty fish especially is the primary food source of EPA and DHA. Nowadays, guidelines for the daily intake of n-3 PUFAs and, in particular, EPA and DHA are recommended by different health organizations (i.e. Food and Agriculture Organization/World Health Organisation, American Dietetic Association, American Heart Association, International Society for the Study of Fatty Acids and Lipids (ISSFAL), German Association for Nutrition). Recommendations for EPA and DHA were established firstly to prevent an n-3 FA deficiency, while more recent recommendations are typically based on the prevention of CHD mortality. Therefore, ISSFAL approved the intake of 500 mg n-3 PUFAs per day for the primary prevention of subjects without CHD (ISSFAL 2004). Individuals with diagnosed CHD should consume 1 g EPA and DHA per day (Kris-Etherton et al. 2002), and subjects with HTG should obtain 2 – 4 g EPA and DHA per day for the secondary prevention of CHD (Kris-Etherton et al. 2003). Dietary guidelines for n-3 PUFA consumption are assessed by CHD disease status, but several other influencing factors, such as interindividual variability (gender, ethnicity, genetic disorders, epigenetic modifications, does not like fish), availability of fish products, food contaminants (heavy metals, polychlorinated biphenyls, dioxins), and particularly the dietary composition, are mostly not under consideration. The last named factor addresses the high intake of n-6 PUFAs in the typical western diet. As a result of this diet, the n-6 to n-3 ratio increased from 1:1 in ancestral nutrition to 20:1 (Sanders 2000; Simopoulus 2002). Thus, an aspired n-6 to n-3 ratio should be 4:1.

A large percentage of the population cannot consume sufficient amounts of EPA and DHA only by eating fish (Meyer et al. 2003, Roger et al. 2012). High-concentrated or enriched products such as fish oil (FO) capsules are alternative sources to cover the daily demand. Beside this traditional supplement, other enriched dairy products, such as algal oil or powder, krill oil, plant oils (Tur et al. 2012), and also newly engineered oils yielded from transgenic plants, are a possibility to supplement n-3 PUFAs (Napier et al. 2004; Ruiz-López et al. 2009).

1.2.3 Role in cardiovascular health

Many epidemiologic studies have shown that n-3 PUFA intake influences the risk of developing CVD. First indications for the cardioprotective effects of n-3 PUFAs were found within the Greenland Eskimo population, who consumed diets very rich in n-3 PUFAs and showed a low rate of CHD events (Bang et al. 1971; Bang et al. 1980; Dyerberg et al. 1978). Furthermore, several observational studies revealed a decreased risk for CHD by a higher intake of fish or fish FA, while a few reported no association between high fish intake and decreased risk of CHD (for a study overview, see Wang et al. 2006). Numerous randomized intervention studies with CHD patients were conducted to analyze the effect of n-3 PUFAs for secondary prevention, resulting in reduced mortality (Burr et al. 1989; GISSI 1999; Schacky von et al. 1999). A meta-analysis suggested reduced rates of all-cause mortality, cardiac and sudden death, and possibly stroke after increased consumption of n-3 PUFAs from fish or fish-oil supplements, whereas the benefits of FO are stronger in secondary than in primary prevention settings (Wang et al. 2006).

The physiological effects of n-3 PUFAs, which are linked to the prevention of CHD, are well-documented. Besides anti-hypertensive (Cicero et al. 2009, 2010) and anti-thrombotic effects (Adkins and Kelley 2010), arrhythmias and atrial fibrillation are reduced (Leaf et al. 2003; Mozaffarian et al. 2005), vascular function and the lipid profile are improved (Nestel et al. 2002), and inflammation is diminished. In the following sections, the effects of n-3 PUFAs on the lipid metabolism, as well as on inflammation and oxidative stress, are described in more detail.

1.2.3.1 Effects on lipid metabolism

The main clinical effect of an n-3 PUFA supplementation on the lipid profile is to reduce both elevated fasting and non-fasting TG levels (Seo et al. 2005). The TG-lowering effect of n-3 PUFAs appears to be in a dose-dependent manner (Harris 1996; Musa-Veloso et al. 2010). Concerning this reduction, initial TG value is crucial for the extent of the reduction and the decrease is more pronounced at higher baseline TG levels (Musa-Veloso et al. 2010). Therefore, subjects with HTG benefit particular well from n-3 PUFA supplementation (Balk et al. 2006; Skulas-Ray et al. 2008; Tremoli et al. 1994). It is believed that TG reduction by n-3 PUFAs is achieved by reduced VLDL-TG synthesis and secretion from the liver caused by several interrelated processes. An increased ß-oxidation of FAs as well as a decreased de novo lipogenesis (process of converting carbohydrates into fat) reduces FA availability for TG synthesis in the liver. Furthermore, the activity of hepatic enzymes for TG synthesis is reduced and hepatic synthesis of PLs rather than TGs is increased. In addition, with the decreased VLDL-TG synthesis and secretion from the liver, the TG clearance of circulating

VLDL and chylomicron particles is enhanced (Mozzaffarian and Wu 2011; Shearer et al. 2012).

Whereas total cholesterol levels are mostly not affected by n-3 PUFA supplementation (Harris 1989), cholesterol lipoproteins showed changes in many studies. Although existing results are inconsistent, two meta-analyses revealed that n-3 PUFAs could lead to a slight increase of HDL-C levels by 1 to 10% (Harris 1996; Balk et al. 2006). It is suspected that this increase is induced by a shift of the HDL-C particle composition from the TG-rich HDL3 subtype to the cholesterol-rich HDL2 subtype (Mori et al. 1999, 2000). Studies investigating the effects of n-3 PUFAs on LDL-C levels also contributed heterogeneous results. However, most studies have shown that an n-3 PUFA supplementation results in increased LDL-C levels (Balk et al. 2006; Kelley et al. 2007). In this case, it is assumed that the LDL-C rise is caused by an enhanced conversion of VLDL to LDL-C (Lu et al. 1999) or an increased LDL particle size after modification of the LDL particle composition (Calabresi et al. 2000; Minihane et al. 2000).

1.2.3.2 Effects on inflammation

As mentioned in section 1.1.2, inflammation is a part of the body's defense system and is characterized by the production of several inflammatory mediators. It is believed that PUFAs affect inflammation mainly via so-called oxylipins, which are oxidized metabolites of PUFAs produced by specific oxygenases, such as cyclooxygenases (COX) and lipoxygenases (LOX), as well as by cytochrome P450 enzymes (CYPs) (**Figure 1.5**). The family of oxylipins, which can be generated by n-3 PUFAs, comprise specific eicosanoids, lipoxins, resolvins, protectins, and maresin 1 (Figure 1.5).

The effects of n-3 PUFAs on the eicosanoid synthesis and the accompanied impact on inflammation have been investigated in many studies so far. In this context, it was shown that the FA membrane composition is an important factor for the eicosanoid synthesis, because eicosanoids are produced by PUFAs released from membrane PLs. While, inflammatory cells contain a higher content of n-6 PUFAs, especially AA than n-3 PUFAs (EPA) (Calder 2003), an increased n-3 PUFA intake results in a dose-dependent decrease of AA and increase of n-3 PUFAs in inflammatory cells. Eicosanoids derived from n-3 PUFAs are related to more favorable effects (anti-thrombotic, vasodilative and anti-inflammatory) than eicosanoids derived from n-6 PUFAs (pro-thrombotic, vasocontrictive and inflammatory) (Calder 2003; Molendi-Coste et al. 2011; Mozzaffarian and Wu 2011). Supplementation of humans with n-3 PUFAs results in a decreased production of prostaglandine (PG) E_2, thromboxane (TX) B_2, leukotriene (LT) B_4, 5-hydroxyeicosatetraenoic acid, and LTE_4, as well as in an increased production of LTB_5, LTE_5 and 5-hydroxyeicosapentaenoic acid. More

favorable effects of the n-3 PUFA-derived eicosanoids occur, because these eicosanoids are believed to be less potent than those formed from AA (Calder 2008). Furthermore, some eicosanoids, such as prostacyclin $(PGI)_3$, may serve as an endogenous inhibitor of the angiotensin converting enzyme and 3-hydroxy-3-methylglutaryl-coenzyme A reductase, as well as a NO enhancer to produce anti-hypertensive, anti-inflammatory and anti-atherosclerotic effects by acting on vascular cells, leukocytes and platelets (Lagarde et al. 2010). Accordingly, an increased amount of n-3 PUFAs in membranes is related to anti-inflammatory effects.

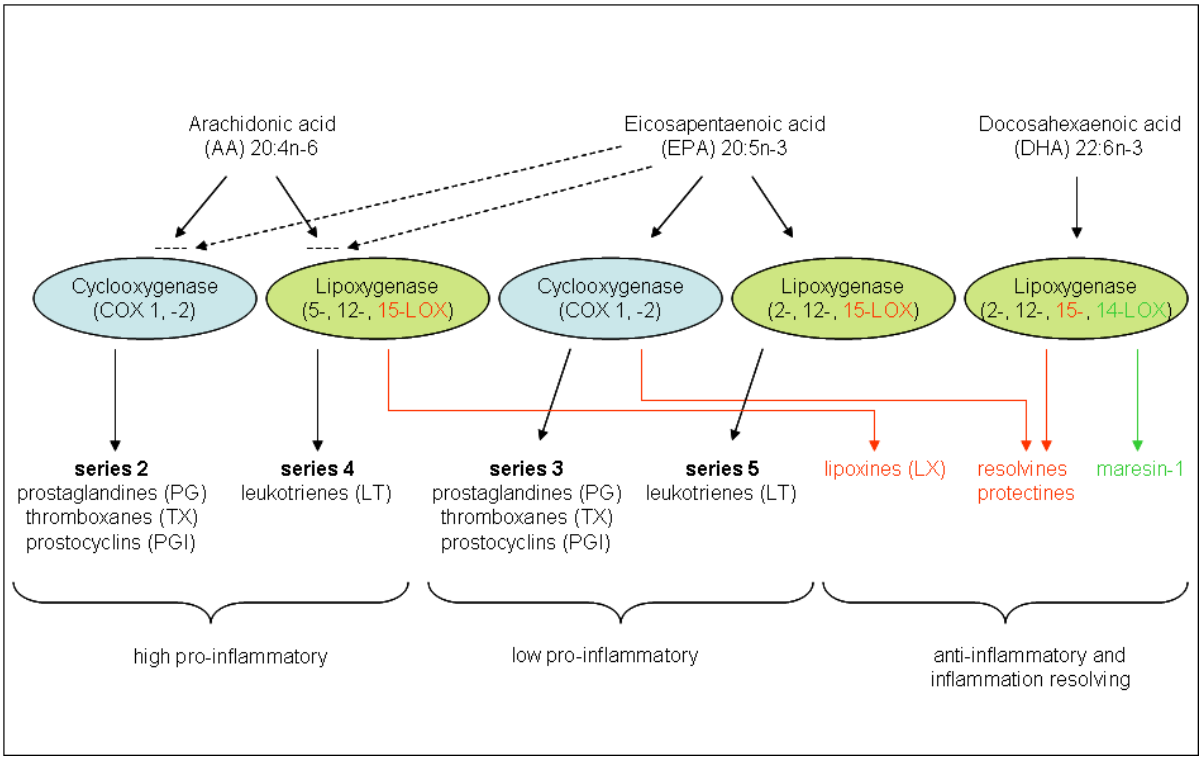

Figure 1.5: Synthesis and actions of lipid mediators produced from arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid. The Figure was designed in accordance with Calder 2009 and Molendi-Coste et al. 2011.

Besides a changed eicosanoid production, other oxylipins have been identified as specialized mediators which stimulate host defense, attenuate inflammation, prevent platelet aggregation, lower blood pressure, have anti-arrhythmic action, reduce LDL-C, activate telomerase, and have cyto-protective properties (Molendi-Coste et al. 2011). These include epoxides, diols, triols, alcohols, CYP epoxygenase products, ketones, lipoxines, resolvines, and protectines (Shearer et al. 2010; Stables and Gilroy 2011). Because of the complexity of these metabolites produced, little is known about the individual effects of each metabolite, and studies focusing on the effects of these different metabolites are still under investigation. Nevertheless, some metabolites, such as resolvines and protectines, could be discovered which are responsible for reducing the duration of inflammation (Serhan 2010). In this case, it was shown that resolvin E1, resolvin D1 and protectin D1 inhibit transendothelial migration of

neutrophils and thus prevent neutrophilic infiltration at sites of inflammation. In addition, both resolvin D1 and protectin D1 inhibit IL-1ß production, which is an important mediator of inflammatory signaling processes (Serhan et al. 2008).

1.2.3.3 Effects on oxidative stress

N-3 PUFAs are very susceptible to oxidation, which was observed to increase lipid peroxidation of n-3 PUFAs in membranes *in vivo* (Palozza et al. 1996). However, some *in vitro* and also *in vivo* studies have shown that n-3 PUFAs appear to increase antioxidative capacity and thus reduce oxidative stress (Bouzidi et al. 2010; Richard et al. 2008), while the effectiveness of n-3 PUFAs in reducing the production of ROS is probably dependent on given doses and existing ROS-producing stimuli (Calder 2006). Investigations in patients with chronic renal failure showed reduced oxidative stress after n-3 PUFA supplementation (Bouzidi et al. 2010). Furthermore, an *in vitro* study with human aortic endothelial cells also determined reduced oxidative stress after n-3 PUFA treatment (Richard et al. 2008). It is believed that n-3 PUFAs may reduce oxidative stress by elevating the expression and activity of antioxidative genes, such as glutathione (Joulain et al. 1994; Komatsu et al. 2003; Lemaitre et al. 1997; Leonardi et al. 2007) and α- and γ-tocopherol content (Véricel et al. 1999).

In addition, a pro-oxidative state is associated with inflammation, which is partly caused by ROS-mediated activating of the transcription factor nuclear factor kappa b (NFκB), which regulates the expression of several pro-inflammatory proteins and thus inflammatory processes. In this context, it is hypothesized that some anti-inflammatory effects of n-3 PUFAs are partly caused by local high inflammation. In centers of inflammation, the expression of oxidative enzymes, such as nicotinamid-adenin-dinukleotid-phosphat oxidase, myeloperoxidase, COX, and LOX, is elevated, leading to high ROS production which, in turn, results in the generation of a secondary oxidation product of n-3 PUFAs (Chaudhary et al. 2004, Mishra et al. 2004). Because of the fact that experiments with EPA and oxidized EPA (oxEPA) have shown an inhibition of leucocyte-endothel interactions (Chaudhary et al. 2004; Mishra et al. 2004) only by oxEPA, it is speculated that oxidized n-3 PUFA products especially are responsible for local anti-inflammatory effects. This connection might be one aspect why the anti-inflammatory effects of n-3 PUFAs could be observed *in vitro* (Calder 2003), but rarely *in vivo* (Calder 2006).

1.2.4 Molecular mechanisms of action

N-3 PUFAs excite several protective effects, but the molecular mechanisms are only rudimentarily understood. It is assumed that n-3 PUFAs act through several mechanisms,

including modifications in cell membrane composition and function, distinct eicosanoid production and gene expression modulation (**Figure 1.6**). The known mechanisms are described in detail in the following sections.

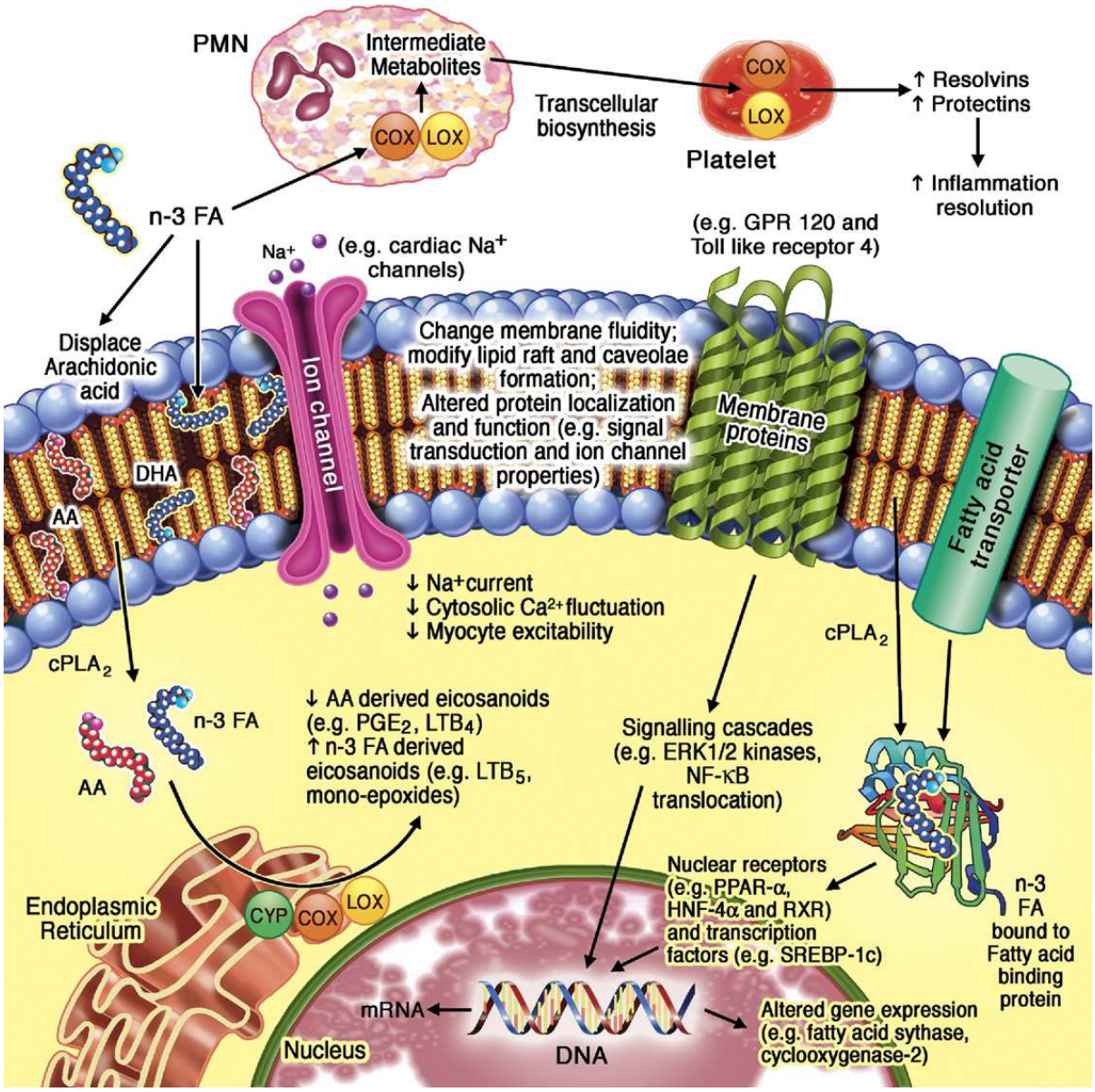

Figure 1.6: Molecular pathways affected by n-3 PUFAs (Mozzafarian and Wu 2011)

1.2.4.1 Modification of the cell membrane composition and consequences

It is believed that several effects are partly caused by the fact that n-3 PUFAs are integral parts of cell membrane PLs and cell surfaces (Drevon 1992; Simopoulus 1999). Basically, biological membranes consist of PLs and cholesterol, which are ordered into a lipid bilayer. In addition, several different proteins with important membrane functions, such as enzymes, receptors and transporters, are enclosed in the lipid bilayer. An increased intake of n-3 PUFAs results in a dose-dependent incorporation into membrane PLs (Blonk et al. 1990;

Harris et al. 1991), which is accompanied by displacement of n-6 PUFAs (such as AA). This change of the membrane composition is an initial trigger which could induce several further reactions, including not only changed membrane properties (fluidity and lipid raft structure), but also subsequently changed intracellular signaling and an altered pattern for the production of lipid mediators (Calder 2009).

An increased incorporation of n-3 PUFAs modifies the lipid protein composition of membrane microdomains. In this connection, lipid rafts play a key role within signaling transduction processes. Lipid rafts are cell membrane subdomains which are rich in sphingolipids, cholesterol, SFA-carrying PLs, and large amount of proteins, which are involved in signaling transduction (Pike 2003). Although n-3 PUFAs are preferably incorporated into non-raft structures, probably due to their low affinity to cholesterol, it is believed that n-3 PUFAs influence raft formation and function from outside of rafts (Calder 2012). Interestingly, n-3 PUFA incorporation into membranes reduces lipid rafts in T-cells by approximately 45% (Chapkin et al. 2008). Furthermore, it is believed that n-3 PUFAs could selectively displace acylated signaling proteins (Chapkin et al. 2008). A summary of experiments indicates that n-3 PUFAs modify the membrane lipid/protein compositions resulting in changed signaling transduction of a variety of signaling pathways, such as T-cell signaling (Fan et al. 2004; Kim et al. 2008a; Zeyda et al. 2003).

Besides changed signaling, receptors, transporters, ion channels, enzymes, and signaling molecules of receptors and proteins within the membrane are also modified by n-3 PUFA incorporation (Calder 2003). An example of the effects on receptors is the fact that the toll-like receptor 4 can be suppressed by n-3 PUFAs via dimer-disruption, which is associated with downstream signaling, resulting in repression of the transcription factor NFκB and thus reduced COX-2 expression and PGE_2 production (Lee et al. 2003). Among affected transporters within membranes, FA transport protein is induced by n-3 PUFAs via the activation of transcription factor peroxisome proliferator-activated receptors (PPARs) (Martin et al. 1997; Melton et al. 2011). Known ion channels which could be modified by n-3 PUFAs include, for example, the sodium channel, L-type calcium channel and potassium channel, and also ion pumps, such as the sodium–calcium exchanger (Richardson et al. 2011). The modulation of ion channels and ion pumps might be associated with the anti-arrhythmic effects of n-3 PUFAs in animals (McLennan 2001) and humans (Kumar et al. 2011). Membrane-bound enzymes such as phospholipase A2, which is responsible for the release of PUFAs from the membrane to generate substrates for the lipid mediator production, can be up-regulated by n-3 PUFAs (Grynberg et al. 1992). These released PUFAs can serve as precursors for the biosynthesis of lipid mediators (Calder 2010), which was described above

(see 1.2.3.2), and also as signaling molecules and ligands for transcription factors, modifying gene expression, which is explained in the next section.

1.2.4.2 Effects on gene expression

The regulation of gene expression is also discussed as a major part by which n-3 PUFAs excite their effects. In the last few decades, it was shown in *in vitro* studies that n-3 PUFAs could interact with several transcription factors, such as sterol regulatory element-binding protein (SREBP) 1 (Caputo et al. 2011), hepatic nuclear factor (HNF) 4α (López-Soldado et al. 2009), liver X receptors (LXRs) (Howell et al. 2009), retinoid X receptor (RXR) (Urquiza et al. 2000), farnesoid X receptor (FXR) (Zhao et al. 2004), carbohydrate response element binding proteins (ChREBP) (Nakamura et al. 2004), and PPARs (Oyekan 2011), resulting in expression changes of corresponding target genes (Clarke et al. 2002; Davidson 2006; Jump et al. 1999; Price et al. 2000; Vanden Heuvel 2009). In addition, several other transcription factors, such as NFκB, could be influenced indirectly, resulting in the regulation of a variety of pro-inflammatory and pro-atherogenic genes, including those encoding for IL-1, IL-6, IL-8, TNFα, E-selectin, VCAM-1, and COX-2 (Kang and Weylandt 2008; Massaro et al. 2008). As an example, the molecular mechanism of n-3 PUFAs on PPARs, SREBP-1 and NFκB are described in more detail.

PPARs can be activated by the binding of n-3 PUFAs and eicosanoids (Forman et al. 1997), and this activation controls the expression of genes which are involved in lipid/glucose metabolism and adipogenesis (Jump 2002). In more detail, PPARα activation results in an up-regulation of enzymes involved in the ß-oxidation, i.e. acyl-CoA oxidase, CYP4A2, mitochondrial carnitine palmitoyltransferase 1, and peroxisomal FA oxidase in FO fed rodents (Halvorsen et al. 2001; Ren et al. 1997). An increased ß-oxidation thus reduces the availability of FA for TG synthesis and reduces the TG level in the circulation (Lombardo and Chicco 2006). In addition, PPARα activation results in a regulation of genes of the lipoprotein metabolism. More precisely, PPARα activation induces a repression of apolipoprotein (Apo) CIII and Apo B which, in turn, results in an enhanced lipoprotein lipase (LPL)-mediated catabolism of VLDL and reduced VLDL production (Staels et al. 1998). Besides PPARα, activation of PPARγ results in hepatic down-regulation of lipogenic enzymes, such as acetyl-CoA carboxylase (ACC), FA synthase (FAS), S14 protein, stearyl-CoA desaturase-1 (SCD1), malic enzyme, ATP citrate lyase, and glycolytic enzymes (L-pyrovate kinase, Apo A1, Δ5 desaturase, Δ6 desaturase), which lead to a reduced hepatic de novo lipogenesis (Lombardo and Chicco 2006). This reduced lipogenesis results in decreased TG synthesis and VLDL secretion from the liver.

SREBPs are membrane-bound transcription factors and are found to modulate the transcription of genes encoding enzymes of synthesis and uptake, including ACC, FAS, SCD1, and LPL (Brown and Goldstein 1997). Experiments with FO fed mice showed that n-3 PUFA could repress SREBP-1, resulting in the down-regulation of cholesterogenic and lipogenic genes, such as LDL receptor (LDLR), 3-hydroxy-methyl glutaryl-CoA synthase, 3-hydroxy-methyl glutaryl-CoA reductase, ACC, and SCD1 (Kim et al. 1999).

NFκB is the key transcription factor in the regulation of inflammation and controls the expression of several pro-inflammatory genes, such as cytokines, CAMs, inducible NO synthase (iNOS), and COX-2. Animal studies have shown that FO supplementation decreases the activation of NFκB and the production of inflammatory cytokines, which could be also observed in human studies (Lombardo and Chicco 2006). It is believed that the inhibitory effect of n-3 PUFAs on NFκB activation is facilitated by two mechanisms. Firstly, activated PPARγ interacts with the p65 subunit of NFκB, preventing its translocation to the nucleus, or increases the synthesis of the inhibitor of κB (IκB) (Li et al. 2005; Storlien et al. 1996). The second possibility to diminish NFκB activation is via modified signaling pathways, where two pathways are currently described. Firstly, NFκB could be repressed by the down-regulation of protein kinase C within the mitogen-activated protein kinase (MAPK) pathway (Denys et al. 2005; Gorjao et al. 2007). Furthermore, DHA and probably EPA enhance the anti-inflammatory signaling of G-protein coupled surface receptor (GPR) 120, resulting in the maintenance of IκB and prevention of NFκB translocation to the nucleus (Calder 2012).

1.3 Study objectives

Although several approaches for explaining the molecular mechanism of n-3 PUFAs exist from *in vitro* studies, observations in humans could be evaluated by only a very few studies. Therefore, the aim of this study was to analyze gene expression changes after FO or CO supplementation of normo- and dyslipidemic male subjects and to find possible indications for the molecular mechanism of action described. The division of volunteers into normo- and dyslipidemic male subjects was performed for two reasons: Firstly, we wanted to consider the higher risk profile for developing CVD in dyslipidemic subjects; and secondly, to focus on the gene regulatory effects of n-3 PUFAs on lipid metabolism-related genes in regard to the TG-lowering effect of n-3 PUFAs, which was observed by many studies to be greater in these subjects (Hjerkinn et al. 2005; Marckmann and Gronbaek 1999).

Subjects of this study ingested either corn oil (CO) or FO capsules over twelve weeks and gene expression changes in whole blood samples were determined by microarray experiments and quantitative real-time polymerase chain reaction (qRT-PCR). The fundamental questions resulting from this study approach are as follows:

1. Is it possible to identify gene expression changes after n-3 PUFA supplementation by microarray experiments and/or qRT-PCR?

    1.1. If this is the case, one aim was to display gene expression patterns of normo- and dyslipidemic male subjects after supplementation.

2. Is human whole blood a suitable analysis material for gene expression changes after supplementation?

    2.1. Is it possible to present effects of the supplementation on inflammatory and lipid-related genes?

3. Is it possible to deduce some of the possible molecular mechanisms of n-3 PUFAs?

These questions are attended to within the following three chapters. In **chapter 2,** total data of microarray experiments of all groups were presented and greater influence of FO supplementation on pathways of the lipid metabolism, inflammation and CVD in comparison with CO supplementation was discovered. Furthermore, anti-inflammatory gene expression after FO supplementation was observed, particularly in dyslipidemic male subjects. Because of the fact that dyslipidemic male subjects may have increased oxidative stress as a result of elevated lipid parameters (Ohara et al. 1993; Rocha-Pereira et al. 2001), the effects of FO supplementation on oxidative processes were analyzed (**chapter 3**). In addition, the effects of FO supplementation on the lipid metabolism are well known. Therefore, analysis of lipid-related genes after FO supplementation was performed to investigate if n-3 PUFA effects on the lipid metabolism could be observed by gene expression changes in human blood (**chapter 4**).

2. Different gene expression profiles in normo- and dyslipidemic men after fish oil supplementation: Results from a randomized controlled trial

2.1 Introduction

Numerous epidemiologic and intervention studies have shown the beneficial effects of FO and its principal n-3 PUFAs, EPA and DHA, in the prevention of atherosclerosis and CVD (Amano et al. 2011; Sekikawa et al. 2008; Sudheendran et al. 2010). EPA and DHA are known to affect the lipid profile by reducing the elevated TG level and increasing the HDL-C level (Harris 1989; Musa-Veloso et al. 2010). Due to the effects on lipid levels, subjects with dyslipidemia, especially HTG, benefit from FO supplementation to reduce the cardiovascular risk (Balk et al. 2006; Skulas-Ray et al. 2008; Tremoli et al. 1994).

Beyond the positive effects on lipid levels, other cardioprotective effects of EPA and DHA are known involving anti-inflammation, modulation of cardiac ion channels, influence on membrane microdomains and downstream cell signalling pathways, anti-thrombotic and anti-arrhythmic effects, induction of haemodynamic changes, and improvement of endothelial function (Adkins and Kelley 2010; Cottin et al. 2011). The underlying molecular mechanisms by which EPA and DHA exert these beneficial effects on cardiovascular health are not completely understood. It is believed that many effects of n-3 PUFAs are mediated by interferences with signalling transduction pathways (Massaro et al. 2008).

N-3 PUFAs are known to affect a myriad of molecular pathways, including the regulation of gene expression (Mozaffarian and Wu 2011). This regulation can be driven by n-3 PUFAs directly, or by their secondary metabolites, for example, eicosanoids. It is known that n-3 PUFAs induce changes in the expression of several genes related to lipid and carbohydrate metabolism, cell differentiation and growth, cytokine, adhesion, and eicosanoid production, as well as oxidative and immune system processes (Jump 2008; Nakamura et al. 2004; Siddiqui et al. 2009). The inhibition of inflammatory signalling, observed in *in vitro* and animal studies, is viewed as one of the major mechanisms on how n-3 PUFAs may improve cardiovascular health (Massaro et al. 2011). However, the anti-inflammatory effect of n-3 PUFAs has not been completely confirmed in human subjects (Cottin et al. 2011).

Only a few human studies have investigated genome-wide expression changes after FO supplementation to identify specific metabolic pathways. Bouwens and co-workers analysed the effects of FO supplementation on whole genome expression changes and performed several pathway analyses which mainly showed a down-regulation of genes involved in

inflammatory and stress-related pathways (Bouwens et al. 2009). Another study monitored the expression changes of 588 genes after FO supplementation and discovered a regulative effect in several lymphocyte functions such as signalling, cell cycle, cytokine production, and apoptotic and stress response (Gorjão et al. 2006). To our knowledge, gene expression profiles in response to FO supplementation have not been investigated in dyslipidemic subjects so far. Connections between dyslipidemia, especially HTG and HDL-hypocholesterolemia, and chronic inflammation have already been uncovered (Esteve et al. 2005). The discovery of specific FO metabolic pathways which are influenced by FO in dyslipidemic states may provide insights into the anti-inflammatory and lipid-lowering effects of n-3 PUFAs. In addition, it is not known if normo- and dyslipidemic subjects display different gene expression profiles in response to FO supplementation. Accordingly, we conducted a nutrition study to identify the differences in gene expression profiles after FO supplementation between normo- and dyslipidemic subjects.

2.2 Material and Methods

A randomized, double-blind, controlled, parallel intervention study of three months duration was undertaken. This investigator-initiated study was designed and conducted according to the principles of the Good Clinical Practice (GCP) Guidelines laid down in the Declaration of Helsinki. The study was registered at ClinicalTrials.gov (ID: NCT01089231).

2.2.1 Subjects

The recruitment of subjects was performed by several advertisements and study placards in Hannover. One hundred and six subjects were pre-selected via telephone interviews according to the following exclusion criteria: Female; body-mass-index (BMI) > 35; smoker; intake of any corticosteroid, lipid-lowering or anti-inflammatory drugs; diagnosed chronic - cardiovascular or liver diseases; gastrointestinal disorders; blood coagulation disorders and intake of coagulation-inhibiting drugs (i.e. Marcumar); renal failure; periodic intake of laxatives; ingestion of supplements enriched with n-3 PUFAs, phytosterols, polyglucosamines, other lipid-binding ingredients, or daily eating of fatty fish; allergy to fish or FO; or participation in another clinical study < 30 days before the start of the study or at the same time. The pre-selected subjects were invited for a screening examination to collect fasting blood and determine serum lipid levels. Among these subjects, 20 normolipidemic men (TC < 200 mg/dl; LDL-C < 130 mg/dl; TG < 150 mg/dl) and 20 dyslipidemic men (TC > 200 mg/dl; LDL-C > 130 mg/dl; TG > 150 mg/ml), aged between 29 and 51 years, were enrolled in the study population (**Figure 2.1**). All subjects included gave their written informed consent to take part in the study, which was approved by the Freiburger ethics committee.

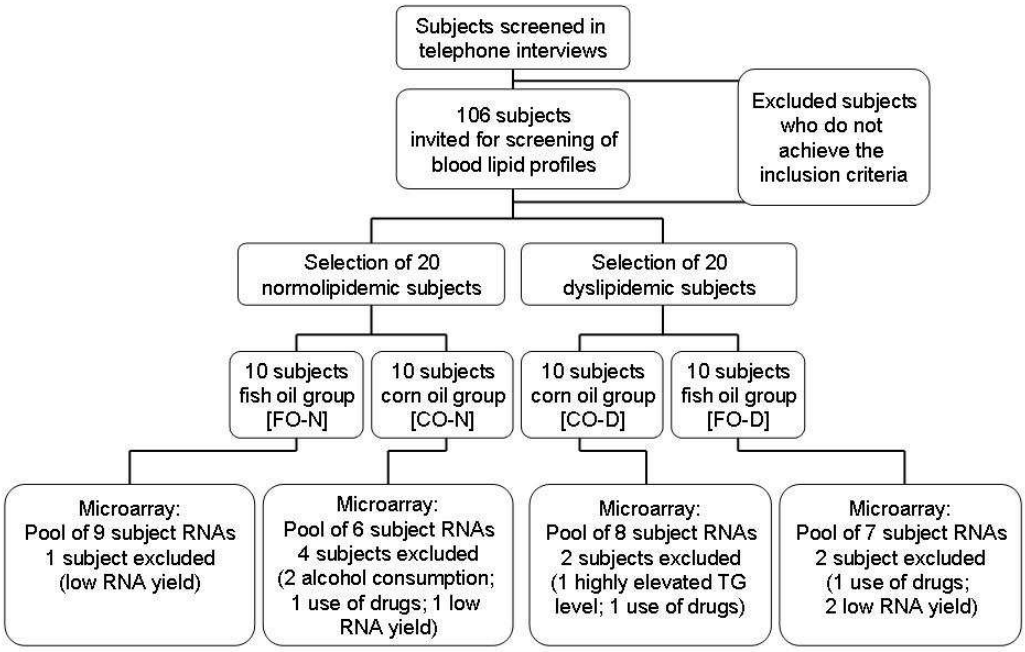

Figure 2.1: Flow chart of subject recruitment and sample selection for microarray analysis.

2.2.2 Study design

The 20 normolipidemic and the 20 dyslipidemic subjects were subdivided into two groups. Thus, a total of four groups with ten men per group passed through the study. The formation of groups was performed by stratified allocation according to each subject's age to realise a comparable mean age between the groups. The four study groups were randomly assigned to different study products by an uninvolved collaborator. Subjects ingested either six FO or six CO capsules per day for a period of twelve weeks. The daily n-3 PUFA intake via FO capsules was 2.7 g (1.14 g DHA and 1.56 g EPA). The predominant FA of the CO capsules was the n-6-PUFA linoleic acid (LA, 18:2n-6). Thus, the daily LA intake via CO capsules was 3.05 g LA. The subjects were instructed to ingest the capsules together with food, three in the morning and three in the evening, and to maintain their usual exercise and dietary habits throughout the intervention time. As an exception, on the first intervention day, all six capsules were ingested at the same time in the morning after a standardised breakfast. Fasting blood samples were collected by venepuncture during each visit. Additionally, participants completed a questionnaire to obtain information about changes in medication, diet (i.e. changes in weekly fish intake, preferred fish dishes or species, respectively) and lifestyle habits (i.e. physical activity), as well as the tolerability of the capsules.

2.2.3 Determination of fasting serum lipids and red blood cell membrane fatty acid composition

Fasting venous blood samples were collected in BD Vacutainer® Blood Collection Tubes (Becton Dickinson, Heidelberg, Germany). The plasma lipid levels were determined by an external contract laboratory (LADR, Hannover; Germany) at baseline (t_0), after one week (t_1) and after twelve weeks (t_{12}) of supplementation. Red blood cell (RBC) membrane FA composition including the omega-3 index, given as EPA and DHA, was analysed at t_0 and t_{12} according to the omega-3 index methodology (Harris and von Schacky 2004). Results are presented as a percentage of the total FAs identified after response factor correction. The coefficient of variation for EPA and DHA was 5%. Quality was assured according to DIN ISO 15189.

2.2.4 Statistical analysis of blood levels

Statistical analysis of clinical parameters was processed with SPSS software version 17 (SPSS Inc., Chicago, IL, USA). The results are based on per protocol population, defined as subjects completing all visits not infringing the study protocol, and are presented as mean ± SD (**Table 2.1**). Differences between t_0 and t_{12} were tested within the groups by t-test for dependent samples. Group differences were examined with a two-factor variance analysis (ANOVA) combined with a post hoc test of contrast (Scheffé). Additionally, differences between groups were analysed by covariance analysis (ANCOVA) using the corresponding baseline values as covariates to detect possible effects caused by diverse baseline values. Statistical significance was generally accepted at $p < 0.05$.

2.2.5 Microarray analyses

2.2.5.1 Sample collection

Fasting venous blood samples were collected in PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) at baseline (t_0), after one week (t_1), and after twelve weeks (t_{12}) of supplementation to analyse medium- and long-term effects of the FO and CO supplementation on gene expression regulation. For short-term effects, venous blood samples were collected four hours (t_{4h}) after the first intake of the capsules. The time point of four hours was selected in accordance with results achieved in a previous study where the strongest postprandial gene expression changes in peripheral blood mononuclear cells (PBMCs) of healthy men were observed after four and six hours (Bouwens et al. 2010). The whole blood samples collected were incubated for 24 hours in the PAXgene Blood RNA Tubes at room temperature.

2.2.5.2 Total RNA isolation from human whole blood and RNA purification

The total ribonucleic acid (RNA) was isolated with the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland), according to the manufacturer's recommended procedures. RNA yield was quantified by Nanodrop ND-1000 spectrophotometer (peQLab Biotechnologie GmbH, Erlangen, Germany) measurement. Total RNA was purified with the Globin Clear Kit (Ambion, Applied Biosystems, Darmstadt, Germany), according to the manufacturer's instructions. The reduction of highly abundant globin mRNA transcripts in whole blood samples is necessary to enable the detection of low-abundance transcripts (Debey et al. 2006). The purified RNA was quantified again, and the quality was measured with an Agilent 2100 Bioanalyzer using RNA 6000 Nano Chips and a RNA 6000 Nano Kit (Agilent Technologies, Böblingen, Germany).

2.2.5.3 Sample pooling

Equal amounts of purified RNA samples from each member of the respective group were pooled together. This was done for all different time points (t_0, t_{4h}, t_1, and t_{12}). Therefore, four pool-samples were generated by this process for each group, which results in a total of sixteen pool-samples for the microarray experiments. This approach was chosen to reduce biological inter-individual variability, which is frequent due to variations in relative proportions of specific blood cell subsets, gender, age, and disease state (Whitney et al. 2003).

2.2.5.4 cDNA synthesis and hybridisation

First-strand complementary desoxyribonucleic acid (cDNA) synthesis and tyramide signal amplification (TSA) were performed using Micromax™ TSA™ Labeling and Detection Kit (Perkin Elmer Life Sciences, Rodgau, Germany) with several protocol modifications. A total amount of 6 µg from every RNA pool, as well as random hexamer primer (Fermentas, St. Leon-Rot, Germany) and oligo dT primer (Roth, Karlsruhe, Germany), were used for the cDNA synthesis, which was facilitated by using Superskript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). The incubation time of two hours was split into two one hour incubations and additional Superskript III was added after first hour. Each RNA pool was synthesized into two different labelled cDNAs, fluorescein-labelled and biotin-labelled cDNA.

After labelling, the cDNA samples were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Furthermore, the cDNA samples were first vacuum-dried and then resolved in hybridization buffer (4 x SSPE; 2.5 x Denhardt's reagent; 30% formamid). After a final degradation step (3 minutes, 95°C),

one-tenth of top-block (Sigma-Aldrich, Steinheim, Germany) was added. Equal amounts of biotin-labelled cDNA and fluorescein-labelled cDNA were hybridized simultaneously in one experiment to human whole genome OneArray™ Microarrays (Phalanx Biotech Group; Belmont, CA, USA). Hybridizations were carried out overnight at 42℃ in hybridization chambers (Eppendorf AG, Hamburg, Germany).

After hybridization, unbound and non-specifically fixed cDNA was removed by stringent washing from the array. Specifically bound fluorescein- and biotin-labelled cDNA were sequentially detected with a series of conjugate reporter molecules according to the TSA process, ultimately with tyramide-Cy3 and tyramide-Cy5. Microarray experiments were performed for each study group in a loop design (**Figure 2.2**) to save microarrays and prevent dye-dependent variety effects (Kerr and Churchill 2001).

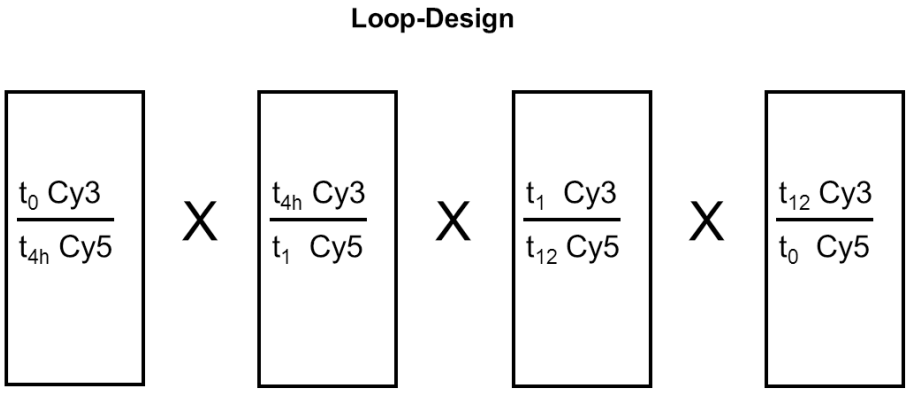

Figure 2.2: Loop-Design of microarray experiments.

2.2.5.5 Microarray data analysis

Microarrays were scanned several times with a 4000 B scanner (Axon Instruments, Inc., Union City, CA, USA) and images were quantified using GenePixPro 6.0 Software. The average pixel intensity within each spot was determined and a local background was computed for each spot. Net signal was determined by subtracting local background from the average intensity. Signals not consistently detectable (background corrected signal lower than two times background standard deviation) were excluded from further analysis. Following the primary analysis, data from different scans had to be summarized. The scans first had to be normalized by the sum of all corresponding spot-intensities due to different laser power and photomultiplier-tube settings. Afterwards, data from different scans for each individual spot could be averaged by the mean. The mean of the data for differently labelled targets for each gene on two microarrays was taken. It was assumed that the distribution of the pre-processed data was normal and hence, a standard two-state pooled-variance t-test

(1% and 5% probability of error) was applied in order to detect differentially expressed genes. Array data were submitted to Gene Expression Omnibus (GEO) which supports minimum information about a microarray experiment (MIAME) (Barrett et al. 2007). The accession number of the submitted dataset is GSE34898. Genes that were detected as differentially expressed between baseline and measurement time point (t_{4h}, t_1 or t_{12}) were subjected to pathway analysis by the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html).

2.3 Results

2.3.1 Subject characteristics

At baseline, no significant differences of the mean age, mean weight and the content of EPA and DHA in RBC membranes, as well as in the omega-3 index, were observed between all groups. However, comparison of both FO groups showed that the dyslipidemic FO group had a 4.8 kg/m² higher BMI, higher TC and TG level, lower HDL-C level, a higher LDL-C/HDL-C ratio, and a lower AA content in RBC membranes than the normolipidemic FO group (Table 2.1). Comparisons of both CO groups showed higher LDL-C and TC levels, as well as a higher LDL-C/HDL-C ratio, in the dyslipidemic CO group than in the normolipidemic CO group.

All 40 subjects (20 normolipidemic and 20 dyslipidemic men) completed the study. However, it was necessary to exclude the RNA samples of ten subjects from the microarray experiments and data analysis due to several reasons: Low RNA yield (four subjects), consumption of alcohol with effects on serum TG levels (two subjects), consumption of medication that lead to exclusion (three subjects), and highly elevated TG level (> 1000 mg/dl in one subject). Therefore, RNA pools were generated and data were analysed from nine (FO-N), six (CO-N), eight (CO-D), and seven (FO-D) subjects for each investigation time point (Figure 2.1).

2.3.2 Changes of blood lipids, fatty acid composition of red blood cell membranes and omega-3 index

The effects of the supplementation with either FO or CO in normo- and dyslipidemic subjects on serum lipids and FA composition of RBC membranes are shown in Table 2.1. Blood lipids showed only slight differences between t_0 and t_{12}. A significant increase in HDL-C levels was observed in the FO-D group, whereas a tendency for increased HDL-C levels was seen in the FO-N group. Furthermore, trends for an increased LDL-C and decreased TG level were observed in the FO-D group. Comparison of the t_{12} values between the FO and CO groups

with by ANCOVA using t_0 values as covariates, showed lower TG levels in the FO-N group than in the CO-N group and higher HDL-C levels, as well as a trend of lower LDL-C levels, in the FO-D group than in the CO-D group.

Several significant differences could be observed in the FA composition of RBC membranes and the omega-3 index between the groups. Within both FO groups, the percentage of EPA and DHA, as well as the omega-3 index in RBC membranes, significantly increased after twelve weeks of supplementation ($p < 0.001$). Additionally, the FO-N group showed a significant decrease of the percentage of AA and LA in RBC membranes. Within both CO groups, no statistical differences between t_0 and t_{12} values could be detected, except the increase of the percentage of EPA in RBC membranes in the CO-D group. Group comparisons of the t_{12} values between the FO and CO groups showed significantly higher percentages of EPA, DHA and omega-3 index, as well as lower percentages of AA in RBC membranes in both the FO-N and FO-D group in comparison to the respective CO groups.

2.3.3 Microarray analyses

2.3.3.1 Number of regulated genes

The total number of regulated genes of each study group was determined and compared between groups. Both dyslipidemic study groups showed a higher number of regulated genes than normolipidemic study groups, independent of the type of supplemented oil (**Figure 2.3**).

The differences in the total number of regulated genes between the two CO groups are small, whereas both FO groups showed substantial differences. A further determination of the direction of regulated genes in total within each group discovered a higher number of down-regulated genes in both dyslipidemic groups (CO-D and FO-D). In contrast, a higher number of up-regulated genes were observed in both normolipidemic groups (CO-N and FO-N). Another observation was made concerning the pattern of up- and down-regulated genes within each group depending on the time point of investigation. Whereas the pattern of up- and down-regulated genes was similar at the first two early time points (t_{4h} and t_1), the pattern changed into the opposite direction after twelve weeks of supplementation (t_{12}). This observation was made in all groups, except the FO-D group.

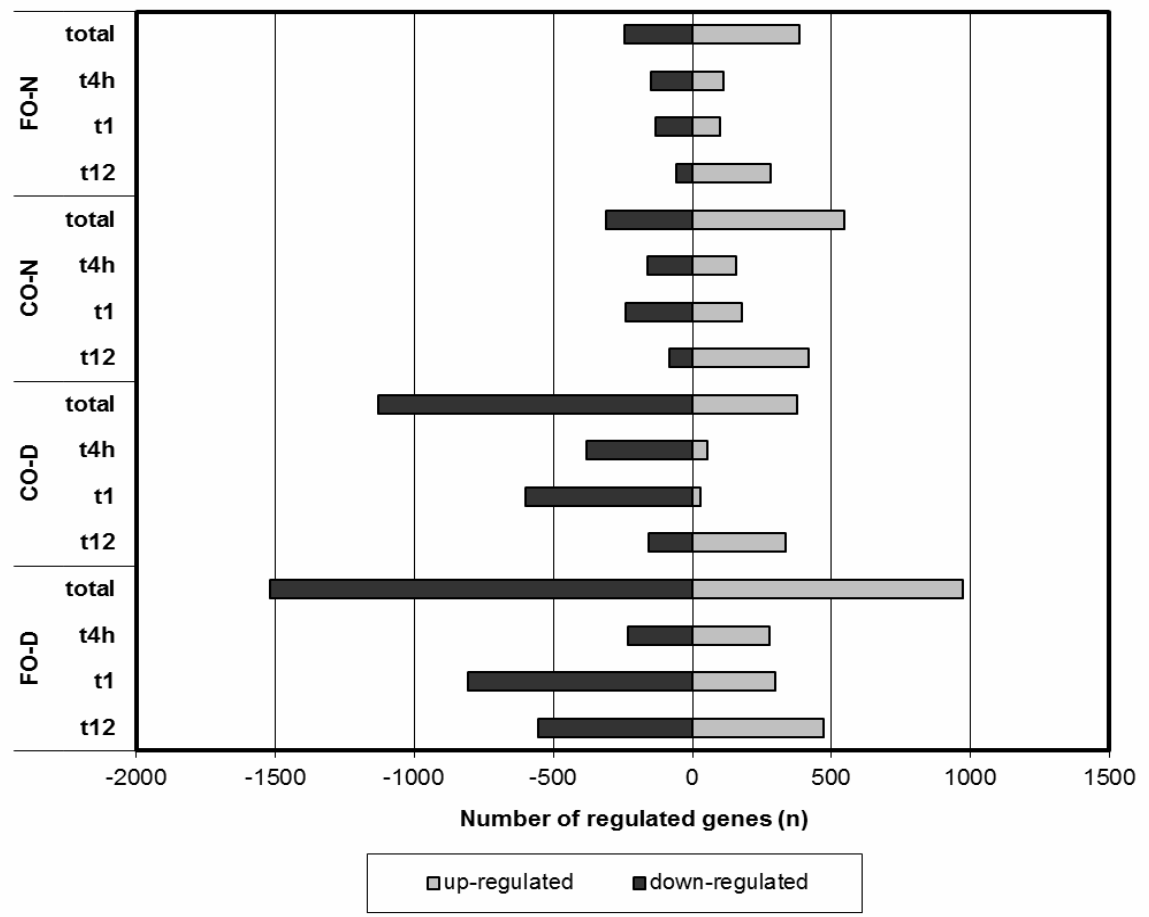

Figure 2.3: Number of regulated genes. Number of genes that were regulated in the normo-lipidemic fish oil group (FO-N), normolipidemic corn oil group (CO-N), dyslipidemic corn oil group (CO-D), and dyslipidemic fish oil group (FO-D) after four hours (t_{4h}), one week (t_1) and twelve weeks (t_{12}) of supple-mentation. Total regulated genes summarize all genes regulated during the different time points, without doubles, respectively.

Table 2.1: Anthropometric data, serum lipid levels and red blood cell membrane fatty acid composition of the four treatment groups (FO-N, CO-N, FO-D, CO-D) at baseline (t_0) and after supplementation with fish oil or corn oil over twelve weeks (t_{12}).

Parameters	FO-N (n = 10)		CO-N (n = 7)		CO-D (n = 8)		FO-D (n = 9)	
	t_0	t_{12}	t_0	t_{12}	t_0	t_{12}	t_0	t_{12}
Age [years]	37.50± 8.11		37.40± 8.30		41.80± 8.94		40.20± 8.64	
Body height [cm]	180.30± 6.29		181.60± 7.54		182.80± 7.02		180.40± 7.55	
Body weight [kg]	78.90± 15.25	78.51± 16.13	85.80± 10.90	84.79± 11.19	85.50± 14.80	84.53± 14.51	94.80± 13.20	94.06± 13.80
Body mass index [kg/m²]	24.20± 4.15 *c*	24.13± 4.50	25.90± 2.83	25.67± 2.78	25.40± 2.91	25.14± 2.86	29.00± 2.98 *c*	28.82± 3.25
TC [mg/dl]	184.10± 13.3 *c*	195.60± 26.10	194.00± 22.2 *d*	188.90± 21.50	261.00± 48.1 *d*	257.50± 60.30	262.20± 61.9 *c*	264.40± 49.20
TG [mg/dl]	82.40± 35.3 *c*	66.20± 16.7 *A*	155.60± 53.80	87.40± 14.8 *A*	175.00± 56.20	186.90± 49.60	322.40± 258.9 *c*	230.80± 147.0 *§_T*
HDL-C [mg/dl]	59.00± 10.4 *c*	65.20± 14.4 *§_T*	52.70± 12.70	52.60± 11.60	47.90± 10.02	47.40± 8.21 *B*	45.20± 6.50 *c*	50.80± 9.43 *§B*
LDL-C [mg/dl]	108.70± 12.8 *c_T*	117.20± 22.10	118.10± 25.1 *d*	118.60± 28.20	178.10± 45.4 *d*	172.60± 54.4 *B_T*	146.10± 5.31 *c_T*	167.40± 23.6 *§_T B_T*
LDL-C/HDL-C quotient	1.89± 0.4 *c*	1.88± 0.54	2.41± 0.89 d	2.41± 0.98	3.74± 0.68 *d*	3.60± 0.73	3.20± 0.58 *c*	3.24± 0.73
LA (18:2n-6)[%]*	0.25± 0.03	0.19± 0.03 *§*	0.23± 0.03	0.20± 0.05	0.22± 0.03	0.18± 0.05	0.23± 0.03	0.17± 0.03 *§*
AA (20:4n-6)[%]*	15.95± 0.81 *c*	13.00± 0.65 *§a*	15.74± 1.48	15.99± 1.74 *a*	14.94± 1.47	14.88± 1.22 *b*	12.82± 2.19 *c*	11.98± 1.61 *b*
EPA (22:5n-3) [%]*	2.31± 0.41	3.74± 0.51 *§a*	2.31± 0.22	2.44± 0.18 *a*	2.09± 0.41	2.39± 0.35 *§b*	2.00± 0.43	3.31± 0.26 *§b*
DHA (22:6n-3) [%]*	4.39± 0.68	6.87± 0.78 *§a*	4.16± 1.14	4.09± 0.94 *a*	4.28± 1.27	4.57± 1.10 *b*	3.90± 1.42	6.59± 0.75 *§b*
Omega-3-index [%]*	5.24± 0.70	10.70± 1.06 *§aA*	5.08± 1.41	4.78± 1.16 *aA*	5.27± 1.69	5.50± 1.57 *bB*	4.90± 1.80	9.84± 1.26 *§bB*

* percentage of total fatty acids
§ p < 0.05 (Changes between baseline and month three were evaluated within groups by Student's t test for dependent samples)
a p < 0.05 (Changes of means were evaluated between FO-N and CO-N by Student's t test)
b p < 0.05 (Changes of means were evaluated between FO-D and CO-D by Student's t test)
c p < 0.05 (Changes of means were evaluated between FO-N and FO-D by Student's t test)
d p < 0.05 (Changes of means were evaluated between CO-N and CO-D by Student's t test)
A p < 0.05 (Changes of means were evaluated between FO-N and CO-N group by ANCOVA using baseline as covariate)
B p < 0.05 (Changes of means were evaluated between FO-D and CO-D group by ANCOVA using baseline as covariate)
_T trend of significance (p < 0.1)

2.3.3.2 Pathway analyses

Pathway analysis of the regulated genes were performed for each group and time point. All pathways with regulated genes were attributed to processes according to the KEGG database (metabolism; genetic information processing, environmental information processing, cellular processes, organismal systems and human diseases) (http://www.genome.jp/kegg/pathway.html) to analyse the influence of the supplemented oil type on gene expression changes in different processes. Furthermore, the number of regulated genes in pathways belonging to different processes was determined for each group and compared as a percentage of total regulated genes between groups. Thus, the influence of the supplemented oil type on different processes could be investigated.

Table 2.2 shows processes where a higher number of genes in both FO groups were regulated at the different time points compared to the corresponding CO groups. It was noticeable that the lipid metabolism was regulated at all time points after FO supplementation. A greater number of processes were regulated in both FO groups after twelve weeks (t_{12}), including, for example, lipid metabolism, the immune system and CVD.

Further analyses concentrated on the long-term effects after twelve weeks of FO supplementation (t_{12}). Therefore, corresponding pathways and regulated genes from three exemplary processes that were dominantly regulated in both FO groups after twelve weeks (lipid metabolism, the immune system, CVD) were selected and listed for the FO-D group in **Table 2.3**. In some pathways of the lipid metabolism, genes were mainly down-regulated (FA synthesis,-metabolism and elongation, AA-, LA- and alpha linolenic acid metabolism), whereas in others pathways, genes were mainly up-regulated (glycerolipid and glycerophospholipid metabolism). The immune system-related pathway of complement and coagulation cascade, as well as the two pathways related to CVD, showed mainly down-regulated genes.

Generally, the majority of regulated genes in all groups were detected in pathways related to the immune system. It is known that FO supplementation provokes immune-modulatory and anti-inflammatory effects. Therefore, regulated genes related to inflammation and immune response, were individually selected for the FO-D group to show the direction of regulation (**Table 2.4**). This examination showed that a greater number of pro-inflammatory genes were down-regulated after twelve weeks of supplementation, while a higher number of immune response related genes were up-regulated. However, roughly the same number of anti-inflammatory genes was up- and down-regulated.

Table 2.2: Selected processes dominantly regulated in dys- and normolipidemic subjects in response to fish oil supplementation for four hours (t_{4h}), one week (t_1) and twelve weeks (t_{12}) compared to corn oil supplementation. The selection was performed as follows: The number of regulated genes within pathways was counted for each group. Pathways were assigned to corresponding processes and the number of regulated genes in these pathways was summed up. The resulting data represent the number of regulated genes in different processes for each group. These numbers were compared between groups and those processes were selected where both fish oil treatment groups (FO-D, FO-N) showed more regulated genes compared with the corn oil treatment groups (CO-D, CO-N).

t_{4h}	t_1	t_{12}
Cell Communication Cell Growth and Death Circulatory System Digestive System Endocrine System Excretory System Folding, Sorting and Degradation Immune System Lipid Metabolism Nervous System Signalling Molecules and Interaction	Carbohydrate Metabolism Cardiovascular Diseases Circulatory System Digestive System Energy Metabolism Folding, Sorting and Degradation Lipid Metabolism Signal Transduction Signalling Molecules and Interaction Xenobiotics Biodegradation and Metabolism	Amino Acid Metabolism Biosynthesis of Other Secondary Metabolites Cardiovascular Diseases Cell Growth and Death Endocrine System Energy Metabolism Excretory System Immune System Immune System Diseases Lipid Metabolism Metabolic Diseases Metabolism of Cofactors and Vitamins Metabolism of Other Amino Acids Nervous System Nucleotide Metabolism Sensory System

Table 2.3: Selected pathways and genes dominantly regulated in dyslipidemic subjects (FO-D) in response to fish oil supplementation for twelve weeks.

	Pathway	Gene name	Gene symbol	Entrez_ID	Ratio t_{12} : t_0
Lipid metabolism	Fatty acid biosynthesis	acetyl-CoA carboxylase beta	ACACB	32	-4.67
	Fatty acid elongation in mitochondria	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	HADHA	3030	-3.70
		hydroxyacyl-CoA dehydrogenase	HADH	3033	-2.34
	Fatty acid metabolism	enoyl-CoA delta isomerase 2	ECI2	10455	-4.54
		enoyl-CoA delta isomerase 1	ECI1	1632	6.02
		hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	HADHA	3030	-3.70
		hydroxyacyl-CoA dehydrogenase	HADH	3033	-2.34
		acyl-CoA dehydrogenase, short/branched chain	ACADSB	36	-13.69
		aldehyde dehydrogenase 2 family (mitochondrial)	ALDH2	217	3.26
	Glycerolipid metabolism	1-acylglycerol-3-phosphate O-acyltransferase 6	AGPAT6	137964	2.67
		diacylglycerol kinase, alpha 80kDa	DGKA	1606	2.47
		diacylglycerol kinase, eta	DGKH	160851	-3.05
		diacylglycerol kinase, theta 110kDa	DGKQ	1609	3.40
		aldehyde dehydrogenase 2 family (mitochondrial)	ALDH2	217	3.26
		monoacylglycerol O-acyltransferase 3	MOGAT3	346606	-3.48
	Glycerophospholipid metabolism	1-acylglycerol-3-phosphate O-acyltransferase 6	AGPAT6	137964	2.67
		phospholipase B1	PLB1	151056	11.68
		diacylglycerol kinase, alpha 80kDa	DGKA	1606	2.47
		diacylglycerol kinase, eta	DGKH	160851	-3.05
		diacylglycerol kinase, theta 110kDa	DGKQ	1609	3.40
		phospholipase A2, group IIE	PLA2G2E	30814	2.51
		phosphatidylserine synthase 1	PTDSS1	9791	-2.84
	Arachidonic acid metabolism	phospholipase B1	PLB1	151056	11.68
		gamma-glutamyltransferase 5	GGT5	2687	7.98
		glutathione peroxidase 3 (plasma)	GPX3	2878	-2.15
		glutathione peroxidase 5	GPX5	2880	-2.41
		phospholipase A2, group IIE	PLA2G2E	30814	2.51
	Linoleic acid metabolism	phospholipase B1	PLB1	151056	11.68
		phospholipase A2, group IIE	PLA2G2E	30814	2.51
	Alpha-linolenic acid metabolism	phospholipase B1	PLB1	151056	11.68
		phospholipase A2, group IIE	PLA2G2E	30814	2.51
Immune system	Complement and coagulation cascades	complement component (3b/4b) receptor 1	CR1	1378	-3.37
		coagulation factor III (thromboplastin, tissue factor)	F3	2152	-4.23
		fibrinogen gamma chain	FGG	2266	-12.30
		serpin peptidase inhibitor, clade C (antithrombin), member 1	SERPINC1	462	3.23
Cardiovascular diseases	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	desmocollin 2	DSC2	1824	-2.50
		calcium channel, voltage-dependent, gamma subunit 5	CACNG5	27091	-9.92
		integrin, alpha 3	ITGA3	3675	-4.32
		transcription factor 7-like 1	TCF7L1	83439	-4.93
	Dilated cardiomyopathy	adenylate cyclase 7	ADCY7	113	-2.13
		calcium channel, voltage-dependent, gamma subunit 5	CACNG5	27091	-9.92
		integrin, alpha 3	ITGA3	3675	-4.32
		myosin, light chain 2, regulatory, cardiac, slow	MYL2	4633	2.68

Table 2.4: Selection of inflammation and immune response related genes regulated in dyslipidemic subjects after supplementation with fish oil for twelve weeks.

Gene name	Gene symbol	Entrez_ID	Ratio t_{12} : t_0
Pro-inflammatory genes			
Nitric oxide synthase, inducible	NOS2	4843	-4.77
C-C chemokine receptor type 3	CCR3	1232	-4.49
Tumour necrosis factor receptor superfamily member 18 Precursor	TNFRSF18	8784	-2.07
Interleukin-31 receptor A Precursor	IL31RA	133396	-4.74
72 kDa type IV collagenase Precursor	MMP2	4313	-6.16
Interleukin-8 Precursor	IL8	3576	-5.67
Tumour necrosis factor receptor superfamily member 5 Precursor	CD40	958	-4.72
Interleukin-2 receptor subunit beta Precursor	IL2RB	3560	-3.22
Interleukin-3 receptor subunit alpha Precursor	IL3RA	3563	-2.22
Stromelysin-1 Precursor	MMP3	4314	-2.17
C-C motif chemokine 5 Precursor	CCL5	6352	2.24
CD97 antigen Precursor	CD97	976	2.26
Prostaglandin D2 receptor	PTGDR	5729	4.47
Integrin alpha-L Precursor	ITGAL	3683	5.07
Arachidonate 5-lipoxygenase-activating protein (FLAP)	ALOX5AP	241	6.97
Anti-inflammatory genes			
C-X-C motif chemokine 11 Precursor	CXCL11	6373	-2.98
NF-kappa-B-repressing factor	NKRF	55922	-82.11
NF-kappa-B inhibitor-like protein 2	NFKBIL2	4796	-3.30
Cytokine receptor-like factor 1 Precursor	CRLF1	9244	-3.07
Lipopolysaccharide-induced tumour necrosis factor-alpha factor	LITAF	9516	3.06
Interferon regulatory factor 1	IRF1	3659	3.16
Interferon-alpha/beta receptor alpha chain Precursor	IFNAR1	3454	5.13
Suppressor of cytokine signalling 6	SOCS6	9306	6.01
Immune response related genes			
Suppressor of cytokine signalling 2	SOCS2	8835	-6.05
Disintegrin and metalloproteinase domain-containing protein 10 Precursor	ADAM10	102	-5.23
A disintegrin and metalloproteinase with thrombospondin motifs 10 Precursor	ADAMTS10	81794	-3.14
Nitric oxide synthase, endothelial	NOS3	4846	-2.55
SL cytokine Precursor	FLT3LG	2323	-2.46
Prostaglandin E2 receptor EP3 subtype	PTGER3	5733	-2.21
CD99 antigen-like protein 2 Precursor	CD99L2	83692	2.18
Cytokine receptor common gamma chain Precursor	IL2RG	3561	2.85
Monocyte differentiation antigen CD14 Precursor	CD14	929	2.88
Chemokine-like factor (C32)	CKLF	51192	11.73
T-cell surface glycoprotein CD3 delta chain Precursor	CD3D	915	7.65
Tumour necrosis factor receptor superfamily member 1B Precursor	TNFRSF1B	7133	2.51
B-lymphocyte antigen CD20	MS4A1	931	2.12
Interleukin-31 receptor A Precursor	IL31RA	133396	5.72
Intercellular adhesion molecule 3 Precursor	ICAM3	3385	5.85
CD320 antigen Precursor	CD320	51293	8.17
CD2 antigen cytoplasmic tail-binding protein 2	CD2BP2	10421	8.77
T-cell antigen CD7 Precursor	CD7	924	2.30
C-C chemokine receptor-like 2 (Putative MCP-1 chemokine receptor)	CCRL2	9034	17.55

2.4 Discussion

It is known from numerous *in vitro* and *in vivo* studies that FO and its general n-3 PUFAs, EPA and DHA, regulate the expression of genes, but human intervention studies investigating the effect of FO on whole genome gene expression are lacking. Our study aimed to investigate two main objectives: The first intention was to investigate the effects of FO supplementation on gene expression changes in dyslipidemic subjects in comparison to normolipidemic subjects. The second objective was to compare the effects of FO with CO, which is used as placebo in many intervention studies, investigating the physiological effects of FO. To the best of our knowledge, this is the first randomized intervention study disclosing gene expression profiles on the whole genome level under these specific conditions. In general, we anticipated regulative effects of FO on the expression of genes associated with the immune system and the lipid metabolism, especially in dyslipidemic subjects.

2.4.1 Serum lipid and omega-3 index levels

As expected, the FO supplementation of dyslipidemic subjects was followed by an increase in HDL-C levels and a decrease in TG levels after twelve weeks, although not significant due to the low group size. There was little or no effect on the lipid levels in normolipidemic subjects after FO or CO supplementation. The omega-3 index increased significantly in both FO treatment groups from levels ~ 5% to levels of ~10% after twelve weeks of supplementation, which is a shift from an unfavourable area in view of the cardiovascular risk to optimal levels of 8% or greater (Harris 2010). Moreover, the omega-3 index increased in all subjects of the two FO groups, indicating an efficient uptake and subsequent incorporation of EPA and DHA in tissue membranes. It can, therefore, be assumed that changes observed in gene expression in the FO supplementation groups are the result of the treatment.

2.4.2 Normolipidemic vs. dyslipidemic subjects

Gene expression profiles of normo- and dyslipidemic subjects differed substantially after FO supplementation. In the dyslipidemic study group, considerably more genes were regulated after FO supplementation, whereas genes were mainly down-regulated. Although the reason for this finding cannot be clarified here, it might be partly explained by the interrelation between inflammation and dyslipidemia (Esteve et al. 2005). Several studies have demonstrated that a dyslipidemic – in particular hypertriglyceridemic – state is accompanied by an induction of pro-inflammatory pathways (Jonkers et al. 2002; Lundman et al. 2003), while n-3 PUFAs are known to suppress these pathways (Adkins and Kelley 2010). Indeed, a number of pro-inflammatory genes were down-regulated in dyslipidemic subjects after FO supplementation, including members of the TNFα pathway. However, besides the regulated

genes involved in inflammatory pathways, it is apparent that a large number of other genes were regulated in dyslipidemic subjects in response to FO treatment, suggesting a strong regulative effect of FO in people with dyslipidemia. It needs to be clarified in future studies if some of these genes are associated with the known beneficial effects FO or n-3 PUFAs in dyslipidemia.

In contrast to dyslipidemic subjects, a higher number of genes were up-regulated in normolipidemic subjects in response to FO supplementation. This finding corresponds with the outcomes of two intervention studies investigating the effect of FO supplementation on whole genome expression in healthy subjects (Bouwens et al. 2009; Gorjão et al. 2006). The study of Gorjão and co-workers was conducted with ten men aged between 25 and 45 years supplemented with 1.62 g DHA and 0.78 g EPA per day over a period of eight weeks. The design of this study is comparable with our study, except for the sample material used for RNA isolation (fractionated lymphocytes vs. whole blood). Different RNA sources could yield different expression patterns, and cell fractioning itself could alter the gene expression pattern (Vartanian et al. 2009). Nevertheless, both studies provided similar results. Gorjão and co-workers discovered 71 up- and 6 down-regulated genes by macroarray analysis. Our normolipidemic FO group showed 627 total regulated genes, 383 of which were up-regulated and 244 down-regulated. Therefore, both studies indicate that genes in normolipidemic subjects are mainly up-regulated after FO supplementation. In the study by Bouwens and colleagues, a total of 111 men and woman aged between 66 and 80 years were supplemented with 1.09 g EPA and 0.85 g DHA per day over 26 weeks. There were great differences in the study design compared to our study with regard to gender (men and woman vs. men), subject age (older vs. middle aged), daily n-3 PUFAs intake (1.94 g vs. 2.7 g/d EPA+DHA), and sample material used for RNA isolation (PBMCs vs. whole blood), which makes it difficult to compare the results. However, the number of total regulated genes in both studies was in the same order of magnitude. Bouwens and co-workers identified a total of 1040 regulated genes, with 537 up-regulated and 503 down-regulated genes.

2.4.3 Fish oil vs. corn oil

CO serves as placebo in many FO supplementation studies based on the assumption that CO is inert without biological effects. However, CO is rich in the n-6 PUFA LA, a precursor for AA. Thus, CO similarly contains FAs, capable of directly regulating gene expression or serving as precursors for other bioactive lipid mediators which regulate gene expression. It was, therefore, our intention to enlighten the possible regulative effects of CO on gene expression. The administration of six CO capsules provided three grams of LA per day, which is less than a quarter of the usual dietary intake of LA, and considerably less than a

high consumption of vegetable oil. In view of this small amount and the low conversion rate of LA to AA, we assumed that the effect of LA on gene expression would be infinitesimal. Surprisingly, our data revealed that the administration of even small amounts of LA caused changes in gene expression patterns. However, during the evaluation of the study data, we focussed on the specific effects of FO on gene expression.

Our data demonstrated substantial differences in gene expression regulation between FO and CO supplementation. Pathway analysis showed that considerably more pathways were dominantly regulated in the FO groups compared to the CO groups (in both normolipidemic and dyslipidemic subjects). Additionally, significantly more genes in these pathways and metabolisms were shown to be regulated in dyslipidemic subjects after FO supplementation compared to normolipidemic subjects, suggesting a more pronounced regulative potential of FO in dyslipidemic subjects. Metabolisms that were dominantly affected by FO include, for example, the immune system, lipid metabolism and CVD.

2.4.3.1 Immune system metabolism

Both FO and CO administration resulted in the regulation of genes associated with the immune system and infectious diseases, which is expected in view of the RNA source used. RNA was isolated from whole blood, including leukocytes, which are mainly involved in the immune system and pathogen defence. Therefore, it is not surprising that a great number of regulated genes are involved in immune system related pathways. However, comparing the effect of FO and CO supplementation on the expression of immune system related genes, it appears that FO regulates significantly more genes in specific pathways compared to CO, indicating the immune-modulatory capability of FO and its bioactive FAs, EPA and DHA. Generally, EPA and DHA have anti-inflammatory properties by increasing the production of potent bioactive lipid mediators (protectins and resolvins), and inhibiting the formation of n-6 FA-derived pro-inflammatory eicosanoids (i.e. PGE2 and LTB4) (Kang and Weylandt 2008). Together, these effects directly or indirectly suppress the activity of nuclear transcription factors, such as NFκB, which controls the expression of a variety of pro-inflammatory and pro-atherogenic genes, including those encoding for IL-1, IL-6, IL-8, tumour necrosis factor alpha (TNFα), E-selectin, VCAM-1, and COX-2 (Kang and Weylandt 2008; Massaro et al. 2008).

With a few exceptions, our data revealed that none of the key regulators mentioned above were regulated after FO supplementation, either in dyslipidemic or in normolipidemic subjects (IL-8 was down-regulated after one week and twelve weeks in dyslipidemic FO-supplemented subjects, as well as IL-1 receptor, type II after one week). However, a number of inflammation and immune response related genes were regulated after twelve weeks of

FO supplementation, especially in dyslipidemic subjects. The ratios demonstrate that more pro-inflammatory genes were down-regulated than up-regulated, whereas the rate of up- and down-regulation was balanced for anti-inflammatory genes. Moreover, pathway analyses showed that genes of the complement and coagulation cascades (complement component [3b/4b] receptor 1, coagulation factor III [thromboplastin, tissue factor], fibrinogen gamma chain) were mainly down-regulated in dyslipidemic subjects supplemented with FO. Since the blood coagulation cascade is evolutionarily closely related to the innate immune response, its pathways are assigned to the immune system. The coagulation pathway is essential for clot formation and the prevention of excessive bleeding. A dysregulation of the cascade activities can result in clinical manifestations of several diseases with critical thrombotic and/or inflammatory complications (Oikonomopoulou et al. 2012). Our results underline the well-known effect of FO to diminish the coagulant activity by the lowering of several coagulation factors and by reducing the capability of plasma to support thrombin generation, especially in patients with hyperlipidemia (Vanschoonbeek et al. 2004).

2.4.3.2 Lipid metabolism

As expected, pathway analyses revealed that the effect of FO supplementation on the expression of genes involved in lipid metabolism was more pronounced compared to CO. Similarly, the regulatory effect of FO supplementation was markedly stronger among dyslipidemic subjects compared to normolipidemic subjects relating to the number of regulated genes. The liver plays a central role in lipid metabolism and n-3 PUFAs have been shown to regulate hepatic gene expression by targeting several transcriptional regulatory networks (Adkins and Kelley 2010): For example, n-3 PUFAs regulate several inflammation molecules, including serum amyloid A, TNFα and IL-6 (Tai and Ding 2010). These inflammation mediators modulate the expression of many lipid metabolism-related genes, for example, by suppressing the expression of perilipin, SREBP-1 and LPL. Together, these regulatory pathways result in induced lipolysis and reduced lipogenesis (Tai and Ding 2010). Besides the down-regulation of FA synthesis gene expression, n-3 PUFAs up-regulate gene expression involved in FA oxidation, which is triggered by an activation of the transcription factor PPARα (Adkins and Kelley 2010).

Again, none of the lipid metabolism-related genes mentioned above were regulated in the present study after 12 weeks of supplementation, probably because the compartment examined (whole blood) is inappropriate to reflect the transcriptional profile of the liver. However, FO supplementation provoked a regulation of several lipid metabolism-associated pathways in dyslipidemic subjects in contrast to normolipidemic subjects, where only a few genes were regulated. This finding emphasises the regulating effect of n-3 PUFAs on the

lipid metabolism in dyslipidemic conditions. Genes, for example, in pathways related to FA metabolism and FA elongation in mitochondria, including genes coding several enzymes, were mainly down-regulated in FO supplemented dyslipidemic subjects. These enzymes (several dehydrogenases and an isomerase) are mainly promoters of mitochondrial FA oxidation. This down-regulation is in contrast to studies, which showed an increased peroxisomal FA oxidation in rats in response to FO administration, while the effect of FO on mitochondrial ß-oxidation was inconsistent (Ide et al. 2000). The observed reduced FA oxidation after 12 weeks of FO treatment in this study may be the result of increasing levels of oxidised n-3 PUFAs, which are prone to oxidation. Subsequently, the resulting oxidative stress may lead to an induction of antioxidative mechanisms, which in turn reduce oxidation. The finding that PPARα was up-regulated one week after FO capsule ingestion in dyslipidemic subjects (data not shown) support this hypothesis.

We observed a down-regulation of the gene coding for acetyl-CoA carboxylase beta, which is one of the key enzymes in FA biosynthesis, indicating a reduced lipogenesis. This finding may partly explain the reduced TG levels observed in this study. In the same group, several genes in pathways involved in FA metabolism were regulated. Interestingly, genes coding for phospholipase A2, group IIE (PLA2G2E), a member of the secreted phospholipase A2 [sPLA2] family) and phospholipase B1 (PLB1) were up-regulated. The regulatory functions of PLA2G2E have not been completely uncovered. It is known that PLA2G2E catalyzes the hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides in membranes and could promote inflammation, since the release of AA is the first step in the AA breakdown pathway, which can be metabolized to several inflammatory and thrombogenic eicosanoids (PGs and LTs) by the activity of COX and LOX (Murakami et al. 2002). The up-regulation of PLA2G2E observed could also point to an intensified exchange of membrane bound AA in favour of EPA and DHA, which is indeed reflected in the decrease in AA levels and concomitant increase in EPA and DHA levels in RBC membranes observed.

In addition, an up-regulation of several genes related to glycerolipid metabolism was observed in FO supplemented dyslipidemic subjects. Glycerolipids are essential components of membranes and an up-regulation of the glycerolipid metabolism indicates remodelling activities of the membrane. Finally, several genes of the glycerophospholipid metabolism were up-regulated in FO supplemented dyslipidemic subjects. Glycerophospholipids, also referred to as PLs, are key components of the lipid bilayer of biological membranes and constitute the binding site for EPA and DHA, which are integrated in the membrane. An up-regulation of the glycerophospholipid metabolism results in an increased de novo PL biosynthesis, enabling the incorporation of EPA and DHA into the membrane (Wilkinson et al. 1996).

2.4.3.3 CVD metabolism

Pathway analysis showed that FO supplementation induced the regulation of pathways involved in specific CVD related metabolisms, especially among dyslipidemic subjects. Several genes from pathways involved in arrhythmogenic right ventricular cardiomyopathy (ARVC) and dilated cardiomyopathy (DCM) were mainly down-regulated. ARVC and DCM belong to cardiomyopathies, a group of diseases that primarily affect the myocardium. While ARVC is characterized by a fibro-fatty replacement of right ventricle myocardium, DCM is a myocardial disease with dilated left ventricle myocardium impairing the systolic pump function of the heart. ARVC and DCM may cause ventricular tachyarrhythmias, blood clots or sudden death. Although the aetiopathogenesis, including the role of dyslipidemia in ARVC and DCM, is largely unknown (Skwarek et al. 2008), inflammatory processes are likely to be involved (Campian et al. 2010; Cihakova and Rose 2008). The down-regulating effect of FO on genes involved in ARVC and DCM pathways observed may be the result of diverse regulatory effects on lipid metabolism and anti-inflammatory processes. However, the effect of n-3 PUFA supplementation on the pathogenesis of ARVC and DCM in humans is unknown. An animal study showed that FO supplementation reduces arrhythmia in boxers (*Canis lupus)* with ARVC (Smith et al. 2007). In a recent intervention study with non-ischemic DCM patients, it was shown that n-3 PUFA treatment increased left ventricle systolic function and functional capacity (Nodari et al. 2011).

2.4.4 Strengths and Limitations

Strengths: The methodological approach of this study was carefully elaborated. The use of whole blood for RNA isolation is advantageous in view of the easy sample collection and the prevention of altered gene expression patterns which emerge during cell fractionation steps (Vartanian et al. 2009). In addition, the pooling of RNA samples reduces inter-individual variation, enabling one to focus on the characteristics of a population in contrast to an individual level (Kendziorski et al. 2005). Moreover, only men were enrolled in the study population. Women are subjected to several hormonal changes which involve individual gene expression changes and hamper the attribution of observed effects to treatment.

Limitations: The study has a number of potential limitations, for example, the small sample size. Moreover, nine subject samples had to be excluded from microarray analysis. To minimize the already high effort for the participants, it was desisted from obtaining multiple baseline samples, which is recommended due to heavy fluctuations in lipid – especially TG – levels. The effect of FO on gene expression was compared to CO, which is often used in n-3 PUFA supplementation studies as a placebo control. It would have been instructive to

examine the gene expression of a third untreated study group. However, this additional expenditure would have gone beyond the scope of our study.

2.4.5 Conclusion

This is the first study showing significant differences in gene expression profiles between normo- and dyslipidemic subjects after FO supplementation. Dyslipidemic subjects presented substantially more regulated genes and pathways, which were mainly down-regulated. Several of the pathways that were especially regulated in dyslipidemic subjects in response to FO supplementation are related to the immune system, inflammation, lipid metabolism, and CVD. In particular, several genes involved in FA metabolism were down-regulated, emphasising the regulating effect of n-3 PUFA on the lipid metabolism in dyslipidemic conditions. Further studies combining genetic with physiological endpoints need to clarify the mechanisms by which n-3 PUFAs trigger gene regulation and affect various regulatory networks. The disentanglement of such interferences may also explain the beneficial effects of n-3 PUFAs on dyslipidemia, atherosclerosis and CVD in many experimental models and clinical conditions.

3. Transcriptome-based identification of antioxidative gene expression after fish oil supplementation in normo- and dyslipidemic men

3.1 Introduction

CVD is the leading cause of morbidity and mortality in Europe, and frequently appears in subjects with disorders of lipid metabolism. Evidence of an association between dyslipidemia and increased oxidative stress (Ohara et al. 1993; Rocha-Pereira et al. 2001), as well as between increased oxidative stress and the pathogenesis of CVD, are given by many studies (Jung et al. 2004; Knight 1995; Landmesser et al. 2000; Madamanchi et al. 2005; Wang et al. 1998). These associations indicate that dyslipidemia increases oxidative stress, and thus promotes the pathogenesis of CVD.

Enhanced oxidative stress results from either an overproduction of ROS or a decreased antioxidative defence system. The most important ROS producers are nicotinamide adenine dinucleotide phosphate oxidase (Griendling et al. 2000), xanthine oxidase (Spiekermann et al. 2003), uncoupled endothelial nitric oxide synthase (Alp and Channon 2004), and enzymes of the AA metabolism and the mitochondria (Mueller et al. 2005). The consequences of an increased ROS production in CVD are vascular cell dysfunction (Kondo et al. 2009), increased growth of the myocard, apoptosis (Tsutsui et al. 2011), and cardiac remodelling via activation of MMPs (Kameda et al. 2003).

The human body possess enzymatic and non-enzymatic strategies to compensate oxidative damage and protect itself against such cytotoxic effects. Important antioxidative enzymes include catalase (CAT), superoxide dismutase (SOD), heme oxygenase (HMOX), and glutathione peroxidase (GPX). Non-enzymatic antioxidants, such as glutathione, ascorbate and α-tocopherol, are also important regulators of the oxidative status.

In the last few decades, numerous observational and intervention studies have shown the beneficial effects of FO and its principal n-3 PUFAs, EPA and DHA, in the prevention of atherosclerosis and CVD (Amano et al. 2011; Sekikawa et al. 2008; Sudheendran et al. 2010). Beyond the beneficial effects of n-3 PUFAs on the lipid profile (Harris 1996, 1997; Musa-Veloso et al. 2010), especially in subjects with HTG (Balk et al. 2006; Skulas-Ray et al. 2008; Tremoli et al.1994), n-3 PUFAs appear to increase antioxidative capacity and thus reduce oxidative stress (Bouzidi et al. 2010; Richard et al. 2008). However, the effects of n-3 PUFAs on oxidative stress have not been studied in detail and some existing results are inconsistent. Investigations in patients with chronic renal failure showed reduced oxidative stress after n-3 PUFA supplementation (Bouzidi et al. 2010). Furthermore, *in vitro* studies

with human aortic endothelial and human hepatocellular carcinoma (HepG2) cells also determined reduced oxidative stress after n-3 PUFA treatment (Richard et al. 2008). However, an indication of increased oxidative stress in healthy judo athletes after n-3 PUFA supplementation was observed (Filaire et al. 2010).

The underlying molecular mechanisms by which EPA and DHA influence oxidative stress are not completely understood. Changes in expression levels of antioxidative genes in response to FO supplementation have not been investigated in dyslipidemic subjects so far. In regard to the fact that dyslipidemia increases oxidative stress and that dyslipidemic subjects possess a pro-oxidant status (Araujo et al. 1995; Hiramatsu and Arimori 1988), we hypothesised that n-3 PUFAs show their potential antioxidative effects especially in dyslipidemic subjects. Therefore, this intervention trial aimed to investigate the expression changes of oxidative stress-related genes in normo- and dyslipidemic subjects after FO supplementation to gain information about the potential antioxidant effects of n-3 PUFAs.

3.2 Material and Methods

This study focused on changes in oxidative stress-related genes as part of a trial investigating the effects of FO supplementation on whole-genome gene expression profiles in normo- and dyslipidemic men. The randomized, controlled, parallel intervention study was conducted at the Institute of Food Science and Human Nutrition at the Leibniz University of Hannover in Germany, and was designed and performed according to the principles of the GCP Guidelines laid down in the Declaration of Helsinki. It was approved by the Freiburg International Ethical Commission and registered at ClinicalTrials.gov (ID: NCT01089231).

3.2.1 Subjects

Normo- and dyslipidemic men were recruited by several advertisements and study placards in Hannover. The suitability of volunteers was checked in telephone interviews and by diet, lifestyle and disease questionnaires. Exclusion criteria comprised smoking; body mass index > 35; intake of any corticosteroids, lipid-lowering or anti-inflammatory drugs; diagnosed chronically cardiovascular or liver diseases; gastrointestinal disorders; blood coagulation disorders and intake of coagulation-inhibiting drugs; renal failure; periodic intake of laxatives; ingestion of supplements enriched with n-3 PUFAs, phytosterols, polyglucosamines, other lipid-binding ingredients or daily eating of fatty fish; allergy to fish or FO; and participation in another clinical study < 30 days before the start of the study or at the same time. Selected subjects were invited for a screening examination to collect fasting blood and determine serum lipid levels. Among these subjects, ten normolipidemic (TC < 200 mg/dl; LDL-C < 130 mg/dl; TG < 150 mg/dl) and ten dyslipidemic (TC > 200 mg/dl; LDL-C > 130 mg/dl;

TG > 150 mg/ml) men, aged between 29 and 51 years, were enrolled in the study population. All participants included gave their written informed consent to take part in the study. The study protocol was approved by the Freiburger ethics committee.

3.2.2 Study design

The normo- and dyslipidemic subjects each ingested six FO capsules per day for a period of twelve weeks. The daily n-3 PUFA intake for each subject via FO capsules was 2.7 g (1.14 g DHA and 1.56 g EPA). The subjects were instructed to take the capsules together with food, three in the morning and three in the evening, and to maintain their usual exercise and dietary habits throughout the intervention time. As an exception, on the first intervention day, all six capsules were ingested at the same time in the morning after a standardised breakfast. Additionally, participants completed a questionnaire to obtain information about changes in medication, diet (i.e. changes in weekly fish intake, preferred fish dishes or species, respectively) and lifestyle habits (i.e. physical activity), as well as the tolerability of the capsules.

3.2.3 Determination of red blood cell membrane fatty acid composition

Fasting venous blood samples were collected into BD Vacutainer® Blood Collection Tubes (Becton Dickinson, Heidelberg, Germany). RBC membrane FA composition, including the omega-3 index (EPA+DHA levels in RBC membranes), was analysed at baseline (t_0) and after twelve weeks of supplementation with FO (t_{12}), according to the omega-3 index methodology (Harris and Schacky von 2004). Accordingly, RBCs were first transesterificated, resulting in a generation of FA methyl esters, followed by gas chromatography analysis using a GC2010 Gas Chromatograph (Shimadzu, Freising, Germany) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA) using hydrogen as carrier gas. Identification of FAs was enabled by comparison with a standard mixture of FAs characteristic for RBCs. The results are presented as a percentage of the total identified FAs after response factor correction. The coefficient of variation for EPA and DHA was 5%. Quality was assured according to DIN ISO 15189.

3.2.4 Gene expression analyses

3.2.4.1 Sample collection

Fasting venous blood samples were collected in PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) at baseline (t_0), after one week (t_1) and after twelve weeks (t_{12}) of supplementation to analyse medium- and long-term effects of the FO supplementation on gene expression regulation. For short-term effects, venous blood samples were collected

four hours (t_{4h}) after the first intake of the capsules. The whole blood samples were collected and incubated for 24 hours in the PAXgene Blood RNA Tubes at room temperature. Whole blood samples were used for RNA isolation and examination of gene expression, because cell fractioning steps such as lymphocyte isolation could alter the gene expression pattern (Vartanian et al. 2009).

3.2.4.2 Total RNA isolation from human whole blood, RNA purification and sample pooling

The total RNA was isolated with the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland), according to the manufacturer's recommended procedures. The RNA yield was quantified by Nanodrop ND-1000 spectrophotometer (peQLab Biotechnologie GmbH, Erlangen, Germany) measurement. The total RNA was purified with the Globin Clear Kit (Ambion, Applied Biosystems, Darmstadt, Germany), according to the manufacturer's instructions. The reduction of highly abundant globin mRNA transcripts in whole blood samples is necessary to enable the detection of low-abundance transcripts (Debey et al. 2006). The purified RNA was quantified again, and the quality was measured with an Agilent 2100 Bioanalyzer using RNA 6000 Nano Chips and a RNA 6000 Nano Kit (Agilent Technologies, Böblingen, Germany).

Equal amounts of purified RNA samples from each member of the respective group were pooled together. This was done for all different time points (t_0, t_{4h}, t_1, and t_{12}). Therefore, four pool samples were generated by this process for each group. The approach of sample pooling was chosen to reduce biological inter-individual variability, which is frequent due to variations in the relative proportions of specific blood cell subsets, gender, age, and disease state (Whitney et al. 2003).

3.2.4.3 Microarray analysis (cDNA synthesis, hybridisation and data analysis)

First-strand cDNA synthesis and TSA was performed using the Micromax TSA Labelling and Detection Kit (Perkin Elmer Life Sciences, Rodgau, Germany) with several protocol modifications. A total amount of 6 µg from every RNA pool, as well as random hexamer primer (Fermentas, St. Leon-Rot, Germany) and oligo(dT) primer (Roth, Karlsruhe, Germany), were used for the cDNA synthesis, which was facilitated by using Superskript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). The incubation time of two hours was split into two one-hour incubations and additional Superskript III was added after the first hour. Each RNA pool was synthesized into two differently labelled cDNAs, fluorescein-labelled and biotin-labelled cDNA.

After labelling, the cDNA samples were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Furthermore, the cDNA samples were first vacuum-dried and then resolved in hybridization buffer (4 x SSPE; 2.5 x Denhardt's reagent; 30% formamid). After a final degradation step (3 minutes, 95°C), one-tenth of top-block (Sigma-Aldrich, Steinheim, Germany) was added. Equal amounts of biotin-labelled and fluorescein-labelled cDNA were hybridized simultaneously in one experiment to human whole genome OneArray™ Microarrays (Phalanx Biotech Group; Belmont, CA, USA). Hybridizations were carried out overnight at 42°C in hybridization chambers (Eppendorf AG, Hamburg, Germany). After hybridization, unbound and non-specific fixed cDNA was removed by stringent washing from the array. Specifically bound fluorescein- and biotin-labelled cDNA were sequentially detected with a series of conjugate reporter molecules according to the TSA process, ultimately with tyramide-Cy3 and tyramide-Cy5. Microarray experiments were performed for each study group in a loop design to prevent dye-dependent variety effects (Kerr and Churchill 2001).

The array data were submitted to GEO (Barrett et al. 2011), which supports MIAME (Brazma et al. 2001). The accession number of the submitted dataset is GSE34898. Genes that were detected as differentially expressed between baseline and time point t_{4h}, t_1 or t_{12} were subjected to pathway analysis using the KEGG database and GenMAPP (Dahlquist et al. 2002).

3.2.4.4 Quantitative real-time polymerase chain reaction and data analysis

In order to quantify the expression levels of selected genes, equal amounts of cDNA were synthesized using 2 µg of purified RNA and M-MLV reverse transcriptase (Promega, Mannheim, Germany), as well as random hexamer (Fermentas, St. Leon-Rot, Germany) and oligo(dT) primers (Carl Roth; Karlsruhe, Germany). Synthesized cDNA was diluted 1:20 with nuclease-free water and used for the qRT-PCR together with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, Ca, USA) and 5 pmol of both forward and reverse primers. The sequences for target and reference genes were retrieved from GenBank and applied primers were manually designed with the Primer-Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information, which is based on the program Primer3 (Rozen and Skaletsky 2000). The primer sequences used are listed in **Table 3.1**. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein S2 (RPS2) were identified as the most stable reference genes by the freely available algorithm geNorm version 3.5.

Table 3.1: Sequences of primer pairs of catalase (CAT), heme oxygenase 2 (HMOX2), cytochrome P450 enzyme 1A2 (CYP1A2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein S2 (RPS2) for quantitative real-time polymerase chain reaction

	Gene symbol	RefSeq_ID	Sequences (5´-> 3´)	
Target genes	CAT	NM_001752.2	forward	CTGACACTCACCGCCATCGCC
			reverse	TGTCCTGCATGCACATCGGGC
	HMOX2	NM_001127204.1 NM_001127205.1 NM_001127206.1 NM_002134.3	forward	GCAGCAAGAACCACACCCAGCA
			reverse	TGGGTGTTTTCTGCCCGGTCG
	CYP1A2	NM_000761.3	forward	AGCGCCGGTGTATCGGGGAAG
			reverse	TCAGTTGATGGAGAAGCGCAGCCG
Reference genes	GAPDH	NM_002046.3	forward	AAGGTGGTGAAGCAGGCGTCG
			reverse	AATGCCAGCCCCAGCGTCAAAG
	RPS2	NM_002952.3	forward	GCAACTTCGCCAAGGCCACCTT
			reverse	TGGGTCTTGACGAGGTGGTCAGT

3.2.5 Statistics

Statistical analysis of blood lipids and RBC membrane FAs were processed with SPSS software version 17 (SPSS Inc., Chicago, IL, USA). The results are based on per protocol population, defined as subjects completing all visits not infringing the study protocol, and are presented as mean ± SD. Differences between baseline blood lipid values of both groups were tested by t-test. Differences of FAs in RBC membranes between t_0 and t_{12} were tested within groups by t-test for dependent samples. Statistical significance was accepted generally at $p < 0.05$.

For statistical analysis of the microarray data, arrays were scanned with a 4000 B scanner (Axon Instruments, Union City, CA, USA) and images were quantified using GenePixPro 6.0 software. The average pixel intensity within each spot was determined and a local background was computed for each spot. The net signal was determined by subtracting local background from the average intensity. Signals not consistently detectable (background corrected signal lower than two times background standard deviation) were excluded from further analysis. Following the primary analysis, data from different scans had to be summarized. The scans first had to be normalized by the sum of all corresponding spot-intensities due to different laser power and photomultiplier-tube settings. Afterwards, data from different scans for each individual spot could be averaged by the mean. The mean of the data for differently labelled targets for each gene on two microarrays was taken. It was assumed that the distribution of the preprocessed data was normal, and hence, a standard

two-state pooled-variance t-test (1% and 5% probability of error) was applied in order to detect differentially expressed genes.

Statistical analysis of expression ratios of genes, which were quantified by qRT-PCR were calculated with the Gene Expression Macro tool (Bio-Rad), which is based on the algorithm of geNorm (Vandesompele et al. 2002). First, normalization factors were calculated from the geometric mean of the reference genes GAPDH and RPS2. Furthermore, the baseline values of the normolipidemic group were defined as control values so that relative expression values could be calculated. Therefore, the baseline samples of the normolipidemic group are given a value of 1. By the reason that the Gene Expression Macro tool offers no statistics, differences between baseline and endpoint (t_{12}) Ct values were tested by a paired t-test using the statistical package R version 2.15.0.

3.3 Results

3.3.1 Subject characteristics

All 20 subjects (ten normolipidemic and ten dyslipidemic men) completed the study. No significant differences of the mean age and mean weight were observed between both study groups at baseline. The dyslipidemic subjects presented a 4.47 kg/m² higher BMI, higher TC and TG level as well as a higher LDL-C/HDL-C quotient than the normolipidemic subjects (**Table 3.2**). Subjects of the dyslipidemic group can be chracterised as pre-obese (BMI25-30), which is, among others, an underlying cause for dyslipidemia. The BMI was not changed by dietary intervention in either study groups (data not shown).

Table 3.2: Subjects characteristics of normo- and dyslipidemic men at baseline (t_0).

Parameters	Normolipidemic (n=9)	Dyslipidemic (n=7)
Age [years]	36.56 ± 8.00	41.43 ± 6.63
Height [cm]	180.61 ± 6.56	180.28 ± 8.62
Weight [kg]	77.41 ± 15.44	91.84 ± 12.83
Body mass index (kg/m²]	23.66 ± 3.97 [a]	28.13 ± 1.99 [a]
Total cholesterol [mg/dl]	183.33 ± 13.88 [a]	272.86 ± 67.1 [a]
Triacylglycerol [mg/dl]	82.22 ± 37.42 [a]	362.00 ± 284.62 [a]
High density lipoprotein [mg/dl]	58.67 ± 10.92	45.86 ± 6.15
Low density lipoprotein cholesterol [mg/dl]	108.33 ± 13.54	146.60 ± 6.43
LDL-C/HDL-C quotient	1.90 ± 0.37 [a]	3.10 ± 0.47 [a]

[a] t_0 values of normolipidemic subjects vs. t_0 values of dyslipidemic subjects were tested by student's t-test; $p < 0.05$

3.3.2 Fatty acid composition of red blood cell membranes and omega-3 index

No significant differences of EPA or DHA levels in RBC membranes or in the omega-3 index were observed between the study groups at baseline (**Table 3.3**). However, dyslipidemic subjects presented lower AA levels in RBC membranes than normolipidemic subjects at baseline. The percentage of EPA and DHA, and the omega-3 index in RBC membranes

significantly increased within both study groups after twelve weeks of supplementation. Additionally, the normolipidemic group showed a significant decrease of the percentage of AA in RBC membranes.

Table 3.3: Red blood cell membrane fatty acid composition of normo- and dyslipidemic men at baseline (t_0) and after supplementation with fish oil over twelve weeks (t_{12}).

	Normolipidemic (n = 9)		Dyslipidemic (n = 7)	
Fatty acid [%]*	**t_0**	**t_{12}**	**t_0**	**t_{12}**
C20:4n-6 (AA)	16.04 ± 0.80 [a]	13.09 ± 0.63 [#]	12.71 ± 2.52 [a]	11.56 ± 1.58
C20:5n-3 (EPA)	0.85 ± 0.20	3.85 ± 0.64 [#]	1.02 ± 0.43	3.46 ± 0.68 [#]
C22:6n-3 (DHA)	4.47 ± 0.67	6.92 ± 0.81 [#]	3.85 ± 1.45	6.46 ± 0.77 [#]
SFA	36.42 ± 1.46	39.24 ± 1.25 [#]	36.74 ± 1.78	39.24 ± 1.74
MUFA	17.98 ± 1.64 [a]	17.20 ± 1.02 [b]	22.15 ± 3.44 [a]	19.03 ± 1.18 [b#]
PUFA	44.59 ± 2.37 [a]	42.98 ± 1.16 [#]	40.13 ± 2.90 [a]	41.11 ± 1.71
n-3 PUFA	7.90 ± 0.94	14.71 ± 1.45 [#]	7.33 ± 2.04	13.53 ± 1.49 [#]
n-6 PUFA	36.68 ± 2.09 [a]	28.27 ± 1.39 [#]	32.80 ± 2.04 [a]	27.58 ± 1.86 [#]
Omega-3 index	5.32 ± 0.74	10.77 ± 1.10 [#]	4.87 ± 1.83	9.92 ± 1.34 [#]

* percentage of total fatty acids

[a] t_0 values of normolipidemic subjects vs. t_0 values of dyslipidemic subjects were tested by student's t-test; $p < 0.05$

[b] t_{12} values of normolipidemic subjects vs. t_{12} values of dyslipidemic subjects were tested by student's t-test; $p < 0.05$

[#] t_0 values vs. t_{12} values were tested by student's t-test for dependent samples; $p < 0.05$

3.3.3 Regulation of gene expression by n-3 PUFA supplementation

It was necessary to exclude the RNA samples of one normo- and three dyslipidemic subjects from the microarray experiments and following data analysis due to several reasons: Low RNA yield (three subjects) and consumption of medication that led to exclusion (one subject). Therefore, RNA pools were generated and data was analysed from nine normolipidemic and seven dyslipidemic subjects for each investigation time point.

Microarray experiments showed that several genes related to different oxidative processes were regulated. These genes are listed with the respective regulation ratio for each time point in **Table 3.4**. Several enzymes of the glutathione metabolism are regulated after FO supplementation, particularly in dyslipidemic subjects. While genes related to the glutathione synthesis were similarly up- and down-regulated during the first two time points (t_{4h} and t_1), these genes were mainly up-regulated after twelve weeks of FO supplementation. Two different glutathione transferases (GST) and glutathione reductase (GR) were up-regulated, whereas glutathione peroxidases were down-regulated in both normo- and dyslipidemic subjects. MMPs were down-regulated in both normolipidemic (MMP25) and dyslipidemic subjects (MMP2, MMP3) after twelve weeks of supplementation. Furthermore, CYP enzymes were mainly down-regulated after twelve weeks of supplementation, especially in

dyslipidemic subjects. Additionally, some antioxidative enzymes, such SOD3, CAT and HMOX2, were up-regulated after twelve weeks of supplementation in dyslipidemic subjects. Moreover, pathway analysis discovered several regulated genes within stress-activated signalling pathways such as MAPK signalling pathway, NFκB pathway and oxidative stress pathway (see **appendix 7.1**).

Several genes were selected for analyses of gene expression ratios by qRT-PCR, including the two antioxidative enzymes, CAT and HMOX2, and CYP1A2 (**Figure 3.1**), a member of the CYP family known to be involved in epoxidation of EPA and DHA (Fer et al. 2008). The expression of HMOX2 was significantly up-regulated after FO supplementation over a period of twelve weeks in both normo- and dyslipidemic subjects ($p = 0.02$ and $p = 0.04$). The expression of CAT was also up-regulated, but reached significance only in normolipidemic subjects ($p = 0.002$). The expression of CYP1A2 was significantly up-regulated only in dyslipidemic subjects ($p = 0.04$). The qRT-PCR results mainly confirm the microarray results observed, whereupon differences in the strength of expression occur.

Table 3.4: Ratios of differentially expressed genes related to oxidative processes

Regulated genes	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$
Gluthatione metabolism									
Glutathione peroxidase 1	GPX1	2876	NM_000581.2 NM_201397.1	-	-	-	-	-	-2.48 [3]
Glutathione S-transferase Mu 3	GSTM3	2947	NM_000849.4	-2.30 [1]	4.12 [2]	2.47 [2]	-	-	-
Glutathione synthetase	GSS	2937	NM_000178.2	-7.97 [1]	-	-	-	-	-
Phospholipid hydroperoxide glutathione peroxidase, mitochondrial	GPX4	2879	NM_001039848.1 NM_001039847.1 NM_002085.3	-	-3.74 [1]	-	-	-	-
Glutathione S-transferase P	GSTP1	2950	NM_000852.3	-	-2.00 [2]	-	-	-	-
Gamma-glutamyltransferase 5	GGTLA1	2687	NM_001099782.1 NM_001099781.1 NM_004121.2	3.44 [1]	14.90 [1]	7.98 [1]	-	-	-
Glutathione peroxidase 3 (plasma)	GPX3	2878	NM_002084.3	-	-	-2.15 [1]	-	-	-
Glutathione reductase	GSR	2936	NM_000637.2	-	-	2.38 [3]	-	-	-
Matrix metalloproteinases									
Matrix metalloproteinase-25	MMP25	64386	NM_022468.4	-	-	-	2.14 [2]	-	-2.30 [1]
Metalloproteinase inhibitor 2	TIMP2	7077	NM_003255.4	-	-4.18 [1]	-2.29 [1]	-	-	-
stromelysin-1	MMP3	4314	NM_002422.3	-	-	-2.17 [1]	-	-	-
72 kDa type IV collagenase	MMP2	4313	NM_001127891.1 NM_004530.4	-	-3.91 [1]	-6.16 [1]	-	-	-
Cytochrom P450 enzymes									
Cytochrome P450 1A2	CYP1A2	1544	NM_000761.3	-	-	-	-	-	-7.74 [2]
Cytochrome P450 2A7	CYP2A7	1548	NM_000762.5	-	-2.46 [1]	-		2.42 [1]	2.70 [1]
Cytochrome P450 4X1	CYP4X1	260293	NM_178033.1	-	-5.66 [1]	-11.35 [1]	-	-	-
Cytochrome P450 26A1	CYP26A1	1592	NM_057157.2 NM_000783.3	2.87 [1]	-	-	-	-	-
Cytochrome P450 2B6	CYP2B6	1555	NM_000767.4	2.60 [1]	-	-	-	-	-
Cytochrome P450 4F12	CYP4F12	66002	NM_023944.3	2.46 [1]	-	-	-	-	-
Cholesterol side-chain cleavage enzyme, mitochondrial	CYP11A1	1583	NM_001099773.1 NM_000781.2	-2.30 [1]	-2.78 [1]	-2.79 [1]	-	-	-
Cytochrome P450 26B1	CYP26B1	56603	NM_019885.2	-3.11 [1]	-	-	-	-	-
Cytochrome P450 2C19	CYP2C19	1557	NM_000769.1	-7.48 [1]	-	-	-	-	-
Steroid 17-alpha-hydroxylase/17,20 lyase	CYP17A1	1586	NM_000102.3	-7.96 [1]	-	-4.19 [1]	-	-	-
Cytochrome P450 2J2	CYP2J2	1573	NM_000775.2	-	3.54 [1]	-	-	-	-
Cytochrome P450 27C1	CYP27C1	339761	NM_001001665.3	-	-4.46 [1]	-3.41 [1]	-	-	-
Cytochrome P450 2A13	CYP2A13	1553	NM_000766.3	-	-4.49 [1]	-8.90 [1]	-	-	-

Regulated genes	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$
Cytochrom P450 enzymes (continued)									
Cytochrome P450 2J2	CYP2J2	1573	NM_000775.2	-	3.54 [1]	-	-	-	-
Cytochrome P450 27C1	CYP27C1	339761	NM_001001665.3	-	-4.46 [1]	-3.41 [1]	-	-	-
Cytochrome P450 2A13	CYP2A13	1553	NM_000766.3	-	-4.49 [1]	-8.90 [1]	-	-	-
Others									
Extracellular superoxide dismutase [Cu-Zn]	SOD3	6649	NM_003102.2	-	-	4.70 [3]	-	-	2.91 [3]
Catalase	CAT	847	NM_001752.2	-	13.15 [1]	8.90 [1]	-	-	-
Heme oxygenase 1	HMOX1	3162	NM_002133.1	-	-8.57 [1]	-17.52 [1]	-	-	-
Heme oxygenase 2	HMOX2	3163	NM_001127204.1 NM_001127205.1 NM_001127206.1 NM_002134.3	-	11.44 [1]	7.84 [1]	-	-	-
Epoxide hydrolase 1	EPHX1	2052	NM_000120.3 NM_001136018.2	-	-	3.14 [2]	-2.24 [2]	-	2.22 [2]
Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	241	NM_001629.2	-	-	6.97 [1]	-	-	-
Nitric oxide synthase, endothelial	NOS3	4846	NM_000603.4	-	-4.22 [1]	-2.55 [1]	-	-	-
Nitric oxide synthase, inducible	NOS2	4843	NM_000625.4	-	-8.11 [1]	-4.77 [1]	-	-	-
Nitric oxide synthase-interacting protein	NOSIP	51070	NM_015953.3	-	-	-	-	-	3.64 [1]
NADPH oxidase 1	NOX1	27035	NM_013955.2 NM_007052.4	-	-	2.06 [1]	-	-	-

Expression ratios were displayed for genes which were differentially expressed after four hours (t_{4h}), one week (t_1) and twelve weeks (t_{12}) of fish oil supplementation in normolipidemic and dyslipidemic men.

- no regulation

[1] slightly significant regulation; $p = 0.05$

[2] significant regulation regulation; $p < 0.05$

[3] highly significant regulation; $p < 0.01$

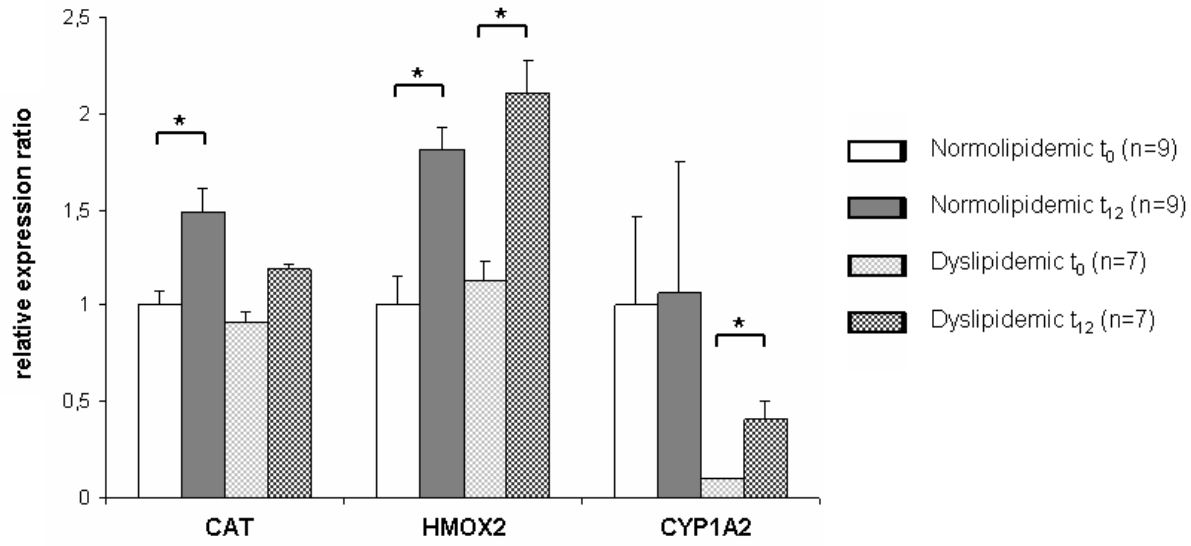

Figure 3.1: Transcript levels of catalase (CAT), heme oxygenase 2 (HMOX2) and dytochrome P450 enzyme 1A2 (CYP1A2) in normo- and dyslipidemic men. Transcript levels of CAT, HMOX2 and CYP1A2 were determined by qRT-PCR in normo- and dyslipidemic men before (t_0) and after twelve weeks (t_{12}) of fish oil supplementation. Pooled group samples were used in triplicates. Triplicates were averaged and corrected by two reference genes, GAPDH and RPS2. Corrected expressions were compared with baseline gene expression of normolipidemic and relative expression changes are displayed. Differences between baseline and endpoint (t_{12}) Ct values were tested by a paired t-test and differences between groups at each time point were tested by unpaired t-test.

* $p < 0.05$

3.4 Discussion

To the best of our knowledge, this is the first intervention study disclosing gene expression changes in normo- and dyslipidemic subjects after FO supplementation. We identified several genes involved in oxidative processes, which were regulated by FO. The expression of antioxidative enzymes was up-regulated particularly in dyslipidemic subjects, while the expression of pro-oxidative or tissue damage-related enzymes was down-regulated. We suggest that n-3 PUFAs may have an antioxidative potential.

Antioxidative effects could be facilitated by either a reduced production of ROS or an increased production of antioxidative enzymes. Several human studies and *in vitro* experiments showed reduced superoxide or ROS production by monocytes and neutrophils after n-3 PUFA administration (Hill et al. 2007; Luostarinen and Saldeen 1996; Zhang et al. 2002). Additionally, negative correlations between ROS production and n-3 PUFA membrane content in healthy (Hill et al. 2007) and dyslipidemic subjects (Luostarinen and Saldeen

1996) were observed. On the other hand, positive correlations between the n-3 PUFA membrane content and the activity of antioxidative enzymes could be investigated in fibroblasts cell cultures (Benito et al. 1997) and in type 2 diabetes patients (Smaoui et al. 2006). In the present study, the supplementation of normo- and dyslipidemic subjects with FO resulted in decreasing AA levels in RBC membranes in favour of EPA and DHA, whose levels increased considerably. Accordingly, the increase of EPA and DHA levels observed in RBC membranes together with an increased expression ratio of the antioxidative enzymes CAT and HMOX2 are in agreement with the findings of Benito (Benito et al. 1997) and Smaoui (Smaoui et al. 2006). Moreover, the replacement of AA, which is an important ROS producer, in biological membranes may partly explain the antioxidative properties of n-3 PUFAs. On the other hand, the incorporation of EPA and DHA in RBC membranes in response to long-term administration results in increased induced lipid peroxidation (Palozza et al. 1996). In this context, an activation of antioxidative gene expression in response to n-3 PUFA supplementation might be a reaction of the defence system to lower lipid peroxidation. Complementary analysis such oxidative damage assay or the determination of oxidative stress markers combined with expression changes of anti- and pro-oxidant genes should be used to indentify global antioxidative effects.

Nevertheless, an increased expression of HMOX2 and CAT in normo- and dyslipidemic subjects may indicate some antioxidative effects of n-3 PUFAs. To our knowledge, this is the first study at all showing a regulation of HMOX2 expression after n-3 PUFA supplementation in humans. HMOXs are antioxidative enzymes which catabolise heme to biliverdin and carbon monoxide. The two existing HMOXs 1 and 2 differ in their activity. HMOX2 is constitutively expressed, whereas HMOX1 is inducible, i.e. by cellular stress (Abraham and Kappas 2008). HMOX2 was identified as part of the large-conductance calcium and voltage-activated potassium (BK(Ca)) channel complex and could enhance its activity, while knockdown of HMOX2 expression reduced channel activity (Williams et al. 2004). BK(Ca) channels could influence the cell membrane potential and, therefore, play an important role in many physiological functions, including oxygen-sensing, neuronal excitability, vascular tone regulation, and neurotransmitter release (Hou et al. 2009; Salkoff et al. 2006). However, a possible clinical relevance of an increased HMOX2 expression after FO supplementation has to be clarified in further studies.

CAT is an effective antioxidative enzyme (Goyal and Basak 2010) known to compensate hydrogenperoxide (H_2O_2) (Gaetani et al. 1996; Mueller et al. 1997), i.e. in the centre of inflammation (Agar et al. 1986; Halliwell and Gutteridge 1984). In this study, expression ratios of the microarray experiments showed an increased expression of CAT in dyslipidemic subjects, whereas qRT-PCR showed an increased expression in both study groups, reaching

statistical significance only in normolipidemic subjects. These differences are also known from several other gene expression studies and are mainly explained by the greater sensitivity of the qRT-PCR (Rajeevan et al. 2001; Toft et al. 2008). The increased expression of CAT in normolipidemic subjects is in contrast to studies with healthy volunteers, which mostly showed no effects on CAT activity after FO supplementation (Jenkinson et al. 1999; Poprzecki et al. 2009). Results from animal studies, however, indicated an increased CAT activity after treatment with n-3 PUFA (Iraz et al. 2005; Venkatraman et al. 1998). Human studies analysing the effects of n-3 PUFAs on the activity or expression of CAT in dyslipidemic subjects are very limited. In accordance with our results, Bouzidi and coworkers (Bouzidi et al. 2010) reported an increased CAT activity in patients with dyslipidemia and chronic renal failure after n-3 PUFA supplementation, assuming a greater protection against oxidative stress and prevention of vascular complications. Similarly, an animal study with hypercholesterolemic rats also observed increased CAT activity after DHA feeding. Taken together, these findings suggest that long-term supplementation with n-3 PUFAs results in an enhanced capacity to detoxify H_2O_2 and might induce adaptive changes in the antioxidative defence system (Hossain et al. 1999).

Glutathione is an important antioxidant which could be readily oxidized non-enzymatically to glutathione disulfide (Wu et al. 2004). Most studies analysing the effects of n-3 PUFA supplementation on the activity of glutathione metabolism related enzymes, such as GPX, gamma-glutamylcysteine synthetase (gamma-GCL), GST, and GR, in healthy and dyslipidemic subjects showed increased activities of these enzymes (Arab et al. 2006; Bellisola et al. 1992; Olivieri et al. 1988). In our study, the expression of GST and GR was increased in dyslipidemic subjects, while the expression of GPX was decreased in both normo- and dyslipidemic subjects. The increased expression of GST and GR is an indication of an increased glutathione synthesis and, therefore, an increased antioxidative defence status. GPX is recognized as an antioxidative enzyme which oxidizes glutathione to reduce and detoxify H_2O_2. Consequently, this enzyme is required when H_2O_2 levels rise in phases of oxidative stress (Felice et al. 2010; Matsunami et al. 2009). Therefore, a decreased expression of GPX – observed in this study – could be an indicator of decreased oxidative stress. However, the results in the literature are inconsistent. Mabile and co-workers could not observe a change in the GPX activity in healthy and hypertriglyceridemic subjects (Mabile et al. 2001), while other studies reported a stimulated GPX activity after n-3 PUFA supplementation in healthy (Bellisola et al.1992) and hyperlipidemic subjects (Olivieri et al. 1988). Furthermore, it was shown that DHA increased the activity of GST, gammaGCL and GR, as well as the mRNA expression of gamma-GCL and GR (Arab et al. 2006), in human fibroblasts, which is in agreement with our results.

CYP enzymes catalyze the oxidation of xenobiotic substances, such as pharmaceuticals, but also metabolize many endogenous substances, such as lipids and steroidal hormones. Besides COX and LOX, CYPs are also involved in the metabolism of PUFAs to form numerous different oxidized FA metabolites, also named oxylipines. The CYP isoforms of families 1 to 3 are mainly epoxygenases, and CYP isoforms from family 4 are mainly ω-hydroxylases (Lucas et al. 2010). In this study, several CYPs, mostly isoforms of family 2, were regulated after FO supplementation. The oxidation of EPA and DHA by epoxygenases could produce epoxy-derivates (Fer et al. 2006) and highly anti-inflammatory resolvins and protectins (Stables and Gilroy 2011). Generated EPA and DHA epoxides are effective dilators of coronary arterioles, facilitated by the activation of calcium-activated potassium channels (Lauterbach et al. 2002; Ye et al. 2002). The qRT-PCR showed that CYP1A2, which is one of the most efficient CYPs for the epoxidation of EPA and DHA in human liver microsomes (Fer et al. 2008), was up-regulated in dyslipidemic subjects after FO supplementation, suggesting the formation of specific EPA and DHA epoxides. Expression ratios of the microarray experiments showed decreased expression of CYP1A2 in normolipidemic subjects, which was, however, not confirmed by qRT-PCR. According to qRT-PCR experiments, the expression of CYP1A2 in normolipidemic subjects was not affected by FO treatment. Both results are in contrast to microarray experiments, where CYP1A2 was unregulated in dyslipidemic subjects and down-regulated in normolipidemic subjects. In view of the higher accurancy of qRT-PCR, it is suggested that the microarray results fpr CYP1A2 was false positive for normolipidemic subjects, while the microarray technique was insensitive to analyse the up-regulation of CYP1A2 in dyslipidemic subjects, which was generally much weaker. Interestingly, human liver microsomes, which were incubated with EPA and DHA (200 µM) showed a decreased CYP1A2 activity (Yao et al. 2006). Although the results are contradictory, it has been repeatedly shown that n-3 PUFAs could induce the expression or activity of CYP enzymes, resulting in the formation of EPA and DHA metabolites (Arnold et al. 2010; Fer et al. 2006, 2008; Lucas et al. 2010; Yao et al. 2006). The complex formation of n-3 PUFA metabolites by CYPs has not been investigated systematically so far; however, it is likely that the formation of these metabolites may explain numerous of the anti-inflammatory and cardioprotective effects of n-3 PUFAs (Calder 2009).

MMPs are zinc-based proteases and could cleave macromolecules of the extracellular matrix, i.e. collagens, as well as non-ECM molecules, such as growth factors, cytokines and their receptors (Shiomi et al. 2010). ROS could induce the activity of MMPs (Lim et al. 2010), which could result in tissue remodelling processes (Clee 2010) and promote the pathogenesis of several CVDs (Spinale et al. 2000; Tyagi et al. 1996). In this study, MMP2 and MMP3 in dyslipidemic subjects and MMP25 in normolipidemic subjects were down-regulated after FO supplementation. In accordance with our results, several other authors

have shown decreased MMP2 and/or MMP9 expression or activity by n-3 PUFA in dyslipidemic subjects (Derosa et al. 2009) and human cell cultures (Delbosc et al. 2008; Kim et al. 2008b). However, no changes in MMP9 activity were detected after FO supplementation in patients with CHD (Furenes et al. 2008). Similarly, another study observed a slight increase of the MMP2 activity in hypertriglyceridemic men after FO supplementation (Kelley et al. 2009). Further studies are needed to clarify these discrepancies and the function of n-3 PUFAs in the regulation of MMPs with regard to potential cardioprotective effects.

3.4.1 Strengths and Limitations

The methodological approach of this study was carefully elaborated. The use of whole blood for RNA isolation is advantageous in view of the easy sample collection and the prevention of altered gene expression patterns which emerge during cell fractionation steps (Vartanian et al. 2009). In addition, the pooling of RNA samples reduces inter-individual variation, enabling one to focus on the characteristics of a population in contrast to an individual level (Kendziorski et al. 2005). However, the approach of sample pooling also provide several limitations, primarily the reduction of statistical power. Finally, oxidative damage and oxidative stress markers were not analysed in this study, which complicates the evaluation of the antioxidative effects.

3.4.2 Conclusions

In conclusion, this study showed indications of the antioxidative potential of n-3 PUFAs, especially in dyslipidemic subjects. FO supplementation resulted in an increased expression of glutathione synthesis-related genes, an up-regulation of antioxidative enzymes, such as CAT and HMOX2, and a reduced expression of MMPs and several CYPs. Interestingly, CYP1A2 was up-regulated in dyslipidemic subjects, suggesting an increased formation of n-3 epoxides. Taken together, these results indicate that n-3 PUFAs may have numerous different possibilities to reduce oxidative stress. It appears that n-3 PUFAs not only up-regulates antioxidative enzymes, but rather induces a specific interplay of differential regulations to generate an optimal balance of the oxidative status. Although the molecular mechanisms by which n-3 PUFAs mediate potential antioxidative effects could not clarified here, we hypothesise an involvement of PPARs. *In vitro* studies with human hepatocytes and pancreatic ß-cells have demonstrated an activation of PPARα or -γ by n-3 PUFAs, which resulted in an increased expression of CAT, as well as antioxidative effects (Chung et al. 2011; Li et al. 2012). Beside CAT, HMOX-1 has also been demonstrated as a target gene of PPAR (Krönke et al. 2007). Moreover, an increased expression of antioxidative genes could result in reduced oxidative stress, which further influences stress-activated pathways (MAPK

and NFκB pathway) as well as other stress-related genes such as MMPs. However, studies analysing the expression of antioxidative enzymes, oxidative signalling processes and metabolic outcomes are needed to clarify the exact role of n-3 PUFAs within the antioxidative defence system.

4. Lipid-related gene expression in normo- and dyslipidemic men after fish oil supplementation

4.1 Introduction

FO and its principal n-3 PUFAs, EPA and DHA have shown beneficial effects on the lipid profile in numerous interventional studies (Harris 1996, 1997; Musa-Veloso et al. 2010). Primarily, n-3 PUFAs lower TG levels, especially in subjects with HTG (Balk et al. 2006; Skulas-Ray et al. 2008; Tremoli et al. 1994). The TG-lowering effect of n-3 PUFAs is more pronounced at higher baseline TG levels (Musa-Veloso et al. 2010) and appears to be dose-dependent (Harris 1996; Musa-Veloso et al. 2010). The recommended daily intake of n-3 PUFAs for TG lowering in hypertriglyceridemic subjects ranges from 2 to 5 g/d; amounts which could only be reached by supplementation (Kris-Etherton et al. 2002; Park and Harris 2003). However, moderate n-3 PUFA doses (1.68 mg/d) are similarly efficient at reducing elevated TG levels in subjects with mild HTG (Schuchardt et al. 2011).

Numerous mechanisms have been proposed as contributors to the TG-lowering effect of n-3 PUFAs, for example, by reducing very VLDL-TG synthesis and secretion from the liver, or by enhancing the TG clearance of circulating VLDL and chylomicron particles (Shearer et al. 2012). Beyond TG lowering, n-3 PUFAs additionally affect the HDL-C metabolism by elevating the cholesterol-rich HDL2 subtype and reducing the TG-rich HDL3 subtype (Mori et al. 1999, 2000). Beside these beneficial effects, studies have repeatedly shown that n-3 PUFAs increase LDL-C levels, which may result from the conversion of VLDL to LDL-C (Lu et al. 1999).

The molecular mechanisms by which n-3 PUFAs modify the lipid metabolism are not completely clarified. The regulation of gene expression is believed to be a key mechanism of how n-3 PUFAs mediate their functions. Specifically, n-3 PUFAs can modulate the activity of several transcription factors, such as SREBP1 (Caputo et al. 2011), HNF4α (López-Soldado et al. 2009), LXRs (Howell et al. 2009), RXR (Urquiza et al. 2000), FXR (Zhao et al. 2004), and PPARs (Oyekan 2011), resulting in an altered expression of corresponding target genes (Clarke et al. 2002; Davidson 2006; Jump et al. 1999; Price et al. 2000; Vanden Heuvel 2009). Although it is known that these genes, or rather their products, play eminent roles in the regulation of the lipid metabolism, the influence of n-3 PUFAs on a number of additional lipid metabolism-related genes and involved pathways remain to be discovered. Unravelling these connections may contribute to the understanding of the molecular mechanisms explaining the physiological functions of n-3 PUFAs.

The approach of this interventional trial was to monitor gene expression changes in normo- and dyslipidemic male subjects after n-3 PUFA supplementation using whole blood samples. With a focus on lipid metabolism-related genes, we aimed to not only identify genes and associated pathways that confirm already known mechanisms, but also to point out alternative mechanisms of how n-3 PUFAs affect lipid metabolism.

4.2 Material and methods

This controlled, parallel group intervention study was conducted at the Institute of Food Science and Human Nutrition, Leibniz University of Hannover, Germany, and performed with respect to GCP Guidelines. The approval of the Freiburg Ethics Commission International was received. The clinical investigation was registered at ClinicalTrials.gov with the identification number NCT01089231.

4.2.1 Subjects

Normo- and dyslipidemic men were recruited by several advertisements and study placards in the area of Hannover. The suitability of volunteers was checked in telephone interviews and by an admission questionnaire on diet, lifestyle and diseases. Exclusion criteria were defined as: Smoking; BMI> 35 kg/m²; intake of any corticosteroids, lipid-lowering or anti-inflammatory drugs; chronic cardiovascular or liver diseases; gastrointestinal disorders; blood coagulation disorders and intake of coagulation-inhibiting drugs; renal failure; periodic intake of laxatives; regular use of dietary supplements containing n-3 PUFAs, phytosterols, polyglucosamines, and other lipid-binding ingredients or daily eating of fatty fish; allergy to fish or FO; and participation in another clinical study < 30 days before the study start or at the same time. Selected subjects were invited to a screening examination where serum lipid levels were determined. Among these subjects, ten normolipidemic (TC < 200 mg/dl; LDL-C < 130 mg/dl; TG < 150 mg/dl) and ten dyslipidemic (TC > 200 mg/dl; LDL-C > 130 mg/dl; TG > 150 mg/ml) men, aged between 29 and 51 years, were enrolled in the study population. Written informed consent was obtained from all participants.

4.2.2 Study design

Subjects ingested six FO capsules per day for a period of twelve weeks. The daily n-3 PUFA intake was 2.7 g (1.14 g DHA and 1.56 g EPA). The subjects were instructed to take three in the morning and three in the evening together with food. Usual exercise and dietary habits should be maintained throughout the intervention time. As an exception, all six capsules were ingested at the same time in the morning after a standardised breakfast on the first intervention day. Additionally, participants completed a questionnaire to obtain information

about changes in medication, diet (i.e. changes in weekly fish intake, preferred fish dishes or species, respectively) and lifestyle habits (i.e. physical activity), as well as the tolerability of the capsules.

4.2.3 Determination of fasting serum lipids and statistics

Fasting venous blood samples were collected into BD Vacutainer® Blood Collection Tubes (Becton Dickinson, Heidelberg, Germany) at baseline (t_0) and after twelve weeks (t_{12}) of supplementation. The plasma lipid levels were determined by specific enzymatic colour reactions from an external contract laboratory (LADR, Hannover, Germany). Statistical analysis was processed with SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical analyses were based on per protocol population, defined as subjects completing all visits not infringing the study protocol. The results are presented as mean ± SD (**Table 4.2**). Differences between t_0 and t_{12} were tested within groups by paired t-test and differences between groups were examined by t-test. P-values < 0.05 were interpreted as statistically significant.

4.2.4 Gene expression analyses

4.2.4.1 Sample collection

Fasting venous blood samples were collected in PAXgene Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) at baseline (t_0), after one week (t_1) and after twelve weeks (t_{12}) of supplementation to analyse the medium- and long-term effects of the FO supplementation on gene expression regulation. Venous blood samples were collected four hours (t_{4h}) after the first intake of the capsules for the short-term effects. The whole blood samples collected were incubated for 24 hours in the PAXgene Blood RNA Tubes at room temperature.

4.2.4.2 Total RNA isolation from human whole blood, RNA purification and sample pooling and microarray analysis (cDNA synthesis, hybridisation and data analysis)

Detailed description of RNA isolation, RNA purification, sample pooling and microarray experiment procedures such cDNA synthesis, hybridisation and data analysis have been already published by Schmidt et al. (2012).

4.2.4.3 Quantitative real-time polymerase chain reaction and data analysis

In order to quantify the expression levels of selected genes, equal amounts of cDNA were synthesized using 2.0 µg of purified RNA and M-MLV reverse transcriptase (Promega,

Mannheim, Germany), as well as random hexamer (Fermentas, St. Leon-Rot, Germany) and oligo(dT) primers (Carl Roth, Karlsruhe, Germany). Synthesized cDNA was diluted 1:20 with nuclease-free water and used for the qRT-PCR together with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, Ca, USA) and 5 pmol of both forward and reverse primers. The sequences for target and reference genes were retrieved from GenBank and the primers applied were manually designed with the Primer-BLAST of the National Centre for Biotechnology Information, which is based on the program Primer3 (Rozen and Skaletsky 2000). The primer sequences used are listed in **Table 4.1**. GAPDH and RPS2 were identified as the most stable reference genes by the freely available algorithm geNorm version 3.5. The Gene Expression Macro tool (Bio-Rad), which is based on the algorithm of geNorm (Vandesompele et al. 2002), was used to calculate relative expression values. Normalization factors for this are calculated from the geometric mean of the reference genes GAPDH and RPS2. Furthermore, the baseline values of the normolipidemic group were defined as control values so that relative expression values could be calculated. Therefore, the baseline samples of the normolipidemic group are given a value of 1. Because the Gene Expression Macro tool offers no statistics, differences between baseline and endpoint (t_{12}) Ct values were tested by a paired t-test, and differences between groups at each time point were tested by unpaired t-test using the statistical package R version 2.15.0

Table 4.1: Sequences of primer pairs of apolipoproetin CII (Apo CII), low-density lipoprotein receptor (LDLR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein S2 (RPS2) for quantitative real-time polymerase chain reaction

	Gene symbol	**RefSeq_ID**	**Sequences (5´-> 3´)**	
Target genes	Apo C II	NM_000483.3	forward	GCTCCCCCTTCCCAGTAGCTCT
			reverse	TTCACTGCTTTATTCCCATGGACCC
	LDLR	NM_000527.3	forward	GGGGCCCTGTGTAGGGGGTT
			reverse	AAAGTGACACCCATCTCCCAGAAGC
Reference genes	GAPDH	NM_002046.3	forward	AAGGTGGTGAAGCAGGCGTCG
			reverse	AATGCCAGCCCCAGCGTCAAAG
	RPS2	NM_002952.3	forward	GCAACTTCGCCAAGGCCACCTT
			reverse	TGGGTCTTGACGAGGTGGTCAGT

4.3 Results

4.3.1 Subject characteristics

All subjects completed the study. One normo- and three dyslipidemic subjects had to be excluded from the analyses because of low RNA yield (n = 3) and consumption of medication that led to exclusion (n = 1). Thus, data were available from nine normolipidemic and seven dyslipidemic subjects for each investigation time point.

Age and mean weight at baseline did not show any differences between either group. However, the dyslipidemic subjects had a significantly higher BMI than the normolipidemic subjects (28.13 kg/m² vs. 23.66 kg/m², respectively). Subjects of the dyslipidemic group can be characterised as pre-obese (BMI 25-30), which is, among others, an underlying cause for dyslipidemia. Nevertheless, the BMI was not changed by dietary intervention in either of the study groups.

4.3.2 Changes of blood lipids

According to the inclusion criteria, dyslipidemic subjects had significantly higher TC and TG levels as well as a higher LDL-C/HDL-C ratio at baseline (Table 4.2). After twelve weeks of supplementation with FO, the group differences in TC and TG levels and the LDL-C/HDL-C ratio remained unaffected and similar to differences observed at baseline. TG levels decreased both in normolipidemic (-19.2 mg/dl; -23.34%) and dyslipidemic subjects (-99.86 mg/dl, -27.59%). The difference between both groups was not statistically significant. The LDL-C level increased significantly after FO supplementation in dyslipidemic subjects (29.6 mg/dl; 20.19%), whereas the effect in normolipidemic subjects was marginal (4.0 mg/dl; 3.69%). Furthermore, FO supplementation resulted in a significant increase in the HDL-C levels in dyslipidemic subjects (6.28 mg/dl; 13.69%), whereas the HDL-C increase in normolipidemic subjects (7.0 mg/dl; 11.93%) showed a tendency towards statistical significance.

Table 4.2: Serum lipid levels of the normo- and dyslipidemic men at baseline (t_0) and after supplementation with fish oil over twelve weeks (t_{12})

Parameters	Normolipidemic (n = 9)		Dyslipidemic (n = 7)	
	t_0	t_{12}	t_0	t_{12}
TC [mg/dl]	183.33 ± 13.88 [a]	190.56 ± 21.88 [b]	272.86 ± 67.17 [a]	278.40 ± 45.21 [b]
TG [mg/dl]	82.22 ± 37.42 [a]	63.00 ± 14.09 [b]	362.00 ± 284.62 [a]	262.14 ± 153.52 [b]
HDL-C [mg/dl]	58.67 ± 10.92	65.67 ± 15.23 [c_T]	45.86 ± 6.15	52.14 ± 9.84 [c]
LDL-C [mg/dl]	108.33 ± 13.54	112.33 ± 16.88 [b]	146.60 ± 6.43	176.20 ± 20.56 [b c]
LDL-C/HDL-C quotient	1.90 ± 0.37 [a]	1.80 ± 0.51 [b]	3.10 ± 0.47 [a]	3.28 ± 0.89 [b]

[a] $p < 0.05$ (Changes of means at baseline were evaluated between normolipidemic and dyslipidemic subjects by Student's t-test)

[b] $p < 0.05$ (Changes of means after twelve weeks of supplementation were evaluated between normolipidemic and dyslipidemic subjects by Student's t-test)

[c] $p < 0.05$ (Changes between t_0 and t_{12} were evaluated within groups by Student's t-test for dependent samples)

[_T] $p < 0.1$ (trend of significance)

4.3.3 Gene expression changes of lipid-related genes after n-3 PUFA supplementation

Microarray experiments revealed a transcriptional regulation of several transcription factors after FO supplementation, including PPAR alpha (PPARα), RXR alpha (RXRα), RXR gamma (RXRγ), HNF6, and HNF1ß (**Table 4.3**). While some transcription factors were similarly regulated in normolipidemic men, the expression was distinctly more strongly regulated in dyslipidemic men. Additionally, several genes related to TG synthesis and HDL-C and cholesterol metabolism were regulated in dyslipidemic men (Table 3). More precisely, the PPAR target gene phospholipid transfer protein (PLTP), as well as the ATP-binding cassette sub-family G member 5 (ABCG5) were up-regulated, while 2-acylglycerol O-acyltransferase 3 (MOGAT3), MOGAT2, diacylglycerol O-acyltransferase 1 (DGAT1), and sterol O-acyltransferase 1 (SOAT1) were down-regulated after FO supplementation in dyslipidemic men.

Gene expression changes of Apo CII and LDLR were quantified by qRT-PCR (**Figure 4.1**). The expression of Apo CII in dyslipidemic subjects at baseline was three times higher compared to normolipidemic subjects ($p = 0.05$). After twelve weeks of FO supplementation, the expression of Apo CII was slightly up-regulated in normolipidemic subjects, and significantly down-regulated in dyslipidemic subjects (-57.2%; $p = 0.04$). The expression of LDLR in normolipidemic subjects at baseline was twice as high compared to dyslipidemic subjects ($p = 0.008$). After twelve weeks of FO supplementation, no changes in LDLR expression were observed in normolipidemic subjects, while dyslipidemic subjects showed a significant down-regulation in LDLR expression ($p = 0.02$).

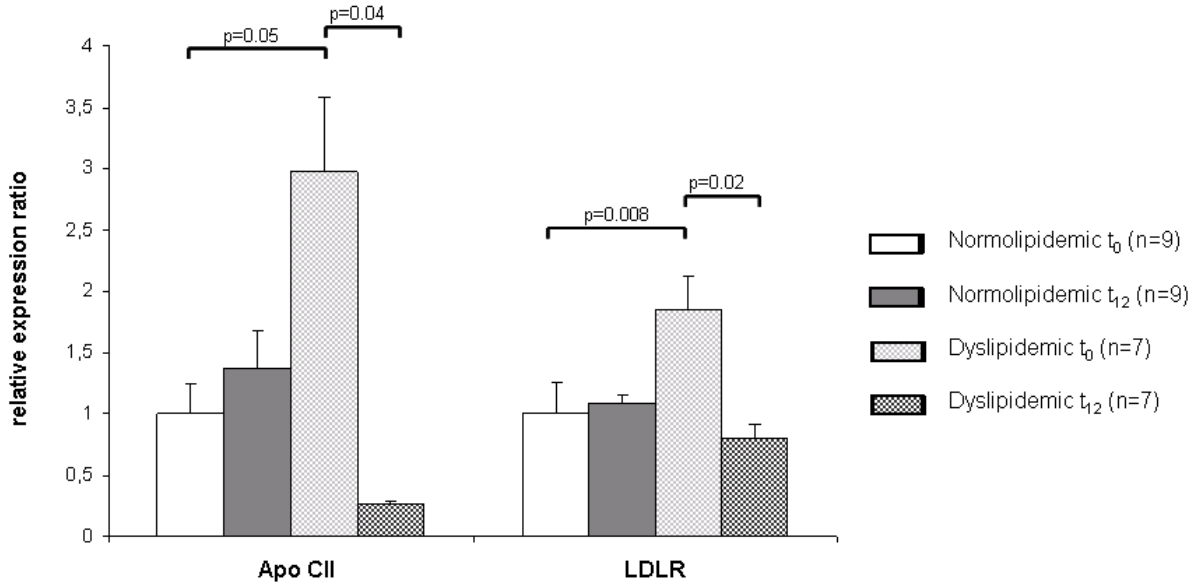

Figure 4.1: Transcript levels of apolipoprotein CII (Apo CII) and low-density lipoprotein receptor (LDLR) in normolipidemic and dyslipidemic men. Transcript levels of Apo CII and LDLR was determined by qRT-PCR in normo- and dyslipidemic men before (t_0) and after twelve weeks (t_{12}) of fish oil supplementation. Pooled group samples were used in triplicates. Triplicates were averaged and corrected by two reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal proteine S2 (RPS2). Corrected expressions were compared with baseline gene expression of normolipidemic subjects and relative expression changes are displayed.

Table 4.3: Expression ratios of lipid metabolism-related genes. Expression ratios were displayed for genes which were differentially expressed after four hours (t_{4h}), one week (t_1) and twelve weeks (t_{12}) of fish oil supplementation in normolipidemic and dyslipidemic men.

Gene	Gene symbol	Entrez_ID	RefSeq_ID	dyslipidemic			normolipidemic			Function
				Ratio t_{4h}:t_0	Ratio t_1:t_0	Ratio t_{12}:t_0	Ratio t_{4h}:t_0	Ratio t_1:t_0	Ratio t_{12}:t_0	
Transcription factors										
Peroxisome proliferator-activated receptor alpha	PPARA	5465	NM_001001928.2 NM_005036.4	-8.19[1]	2.72[2]	-	-	-	-	Regulation of the lipid metabolism
Retinoic X receptor RXR-alpha	RXRA	6256	NM_002957.4	-	-4.50[1]	-	3.14[1]	3.79[1]		
Retinoic acid receptor RXR-gamma	RXRG	6258	NM_006917.3	3.16[3]	4.79[3]	4.11[3]	-3.19[2]	-	-2.03[2]	
Hepatocyte nuclear factor 6	HNF6	3175	NM_004498.1	-2.27[1]	-4.89[1]	-3.43[1]	-	-	-	
Hepatocyte nuclear factor 1-beta	HNF1B	6928	NM_000458.2	-	-3.64[1]	-2.56[1]	-	-	-	
Triacylglycerol synthesis										
2-acylglycerol O-acyltransferase 3	MOGAT3	346606	NM_178176.2	-	-27.07[1]	-3.48[1]	-	-	-	TG synthesis
2-acylglycerol O-acyltransferase 2	MOGAT2	80168	NM_025098.2	-3.08[1]	-	-	-	-	-	
Diacylglycerol O-acyltransferase 1	DGAT1	8694	NM_012079.4	-	-2.67[1]	-	-	-	-	
HDL-C metabolism										
Phospholipid transfer protein	PLTP	5360	NM_006227.2 NM_182676.1	-	3.30[1]	4.15[1]	-	-	-	Modify HDL-C particles size
Cholesterol metabolism										
ATP-binding cassette sub-family G member 5	ABCG5	64240	NM_022436.2	-	4.06[1]	5.34[1]	-	-	-	Cholesterol efflux
sterol O-acyltransferase 1	SOAT1	6646	NM_003101.4	-	-2.45[1]	-2.37[2]	-	-	-	Cholesterol synthesis

- no regulation
[1] $p = 0.05$
[2] $p < 0.05$
[3] $p < 0.01$

4.4 Discussion

The effects of n-3 PUFAs on lipid levels in dyslipidemic conditions are well-known, however, only a few human studies investigated the underlying molecular mechanisms of gene expression levels in humans. Therefore, we performed a twelve-week long n-3 PUFA supplementation trial with normo- and dyslipidemic male subjects aiming to identify possible molecular pathways by which n-3 PUFAs influence lipid metabolism. Although the lipid metabolism is mainly located in the liver, several key regulators of the TG, HDL-C and LDL-C metabolism were found to be regulated on a transcriptional level in whole blood cells.

4.4.1 TG metabolism

Supplementation of normo- and dyslipidemic subjects with n-3 PUFAs resulted in a decrease of TG levels by 23 and 28%, which is comparable to other studies (Shearer et al. 2012). Several genes which are likely to be involved in TG lowering were observed to be regulated (**Figure 4.2**). While PPARα was up-regulated in dyslipidemic men after one week of n-3 PUFA supplementation, the expressional regulation of PPAR heterodimer partner RXRα and RXRγ was different between the two receptors as well as between dys- and normolipidemic subjects. The fact that the exact roles of RXR isotypes are not clearly defined further hampers the interpretation of our findings. However, linkage analysis unveiled an association of RXRγ with TG and cholesterol levels (Knoblauch et al. 1999; Pei et al. 2000). The continuous up-regulation of RXRγ in dyslipidemic subjects might suggest a role of RXRγ together with PPARα in the lipid metabolism. Consistent with our findings, it is also known from other studies that EPA, DHA and/or their oxidized metabolites transactivate PPARs and RXRs (Forman et al. 1997; Kliewer et al. 1997; Lengqvist et al. 2004; Urquiza et al. 2000), resulting in a repression of Apo CIII and Apo B, which, in turn, results in an enhanced LPL-mediated catabolism of VLDL and reduced VLDL production (Staels et al. 1998). Currently, this metabolic pathway is believed to be the main mechanism by which n-3 PUFAs reduce TG levels (Shearer et al. 2012). Expression changes of genes coding for Apo CIII and Apo B were not observed in this study, possibly because these factors are not expressed in blood cells but in other organs and tissues (Jong et al. 1999; Knott et al. 1985; Wang and Eckel 2009). *In vivo* studies are required to evaluate this hypothetical molecular pathway.

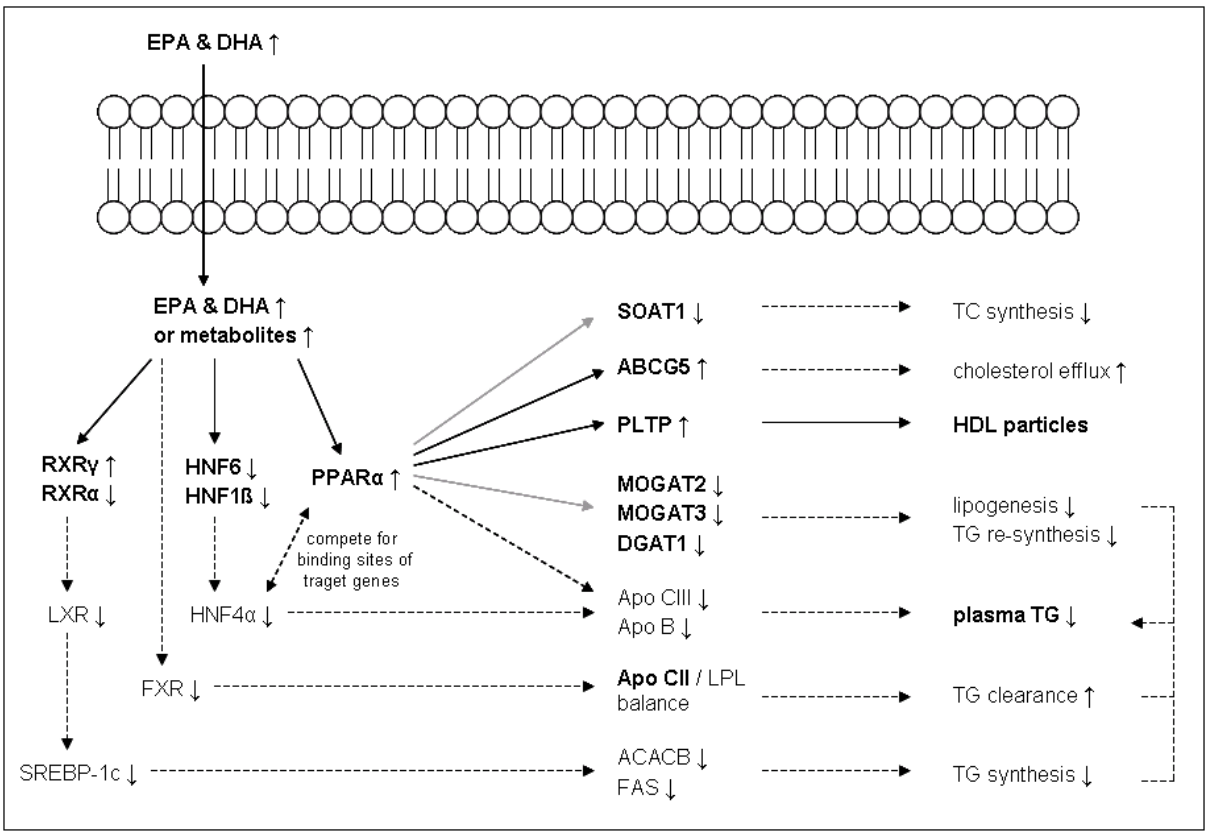

Figure 4.2: Regulatory effects of eicosapentaenoic acid and docosahexaenoic acid on lipid metabolism related genes. The Figure presented is based on the analysis of gene expression changes after fish oil supplementation in dyslipidemic male subjects. While bold text and black and grey arrows present findings discovered in this thesis, grey arrows symbolize that until now it has not been clarified if genes are target genes of PPARα. In addition, broken arrows symbolize further possible mechanisms of action based on findings from the literature.

Two other transcription factors besides PPARα were regulated after n-3 PUFA supplementation. HNF6 and HNF1ß were down-regulated in dyslipidemic subjects at nearly all time-points. HNF6 is associated with several regulatory pathways influencing glucose metabolism, cholesterol metabolism, bile acid biosynthesis, as well as the synthesis and transport of serum carrier proteins (Wang and Holterman 2012). Synergisms between HNF6 and HNF1α or between HNF1β and GATA6 could increase the activity of the HNF4α promoter (Hatzis and Talianidis 2001), which, in turn, increases VLDL secretion. Since Apo CIII and Apo B are target genes of HNF4α (Ladias et al. 1992), a reduced HNF4α expression, mediated by a down-regulation of HNF6 and HNF1ß, may reduce the VLDL secretion; the main TG-lowering effect of n-3 PUFAs. However, expression changes of HNF4α were not observed after FO supplementation in our experiments, most likely because HNF4α is expressed in liver, kidney, intestine, and pancreas (Sladek 1994). Nevertheless, a robust suppression of the transcriptional HNF4α activity by n-3 PUFAs has been shown *in*

vitro (Hertz et al. 1998). Furthermore, it was found that n-3 PUFAs reduce the expression of HNF4α in rat hepatocytes resulting in a decreased expression of Apo B and microsomal TG transfer protein, which suggests a reduced VLDL secretion (López-Soldado et al. 2009). Taken together, these findings suggest that this molecular pathway is a possible mechanism explaining the TG-lowering effect of n-3 PUFAs. Moreover, it is believed that PPARα and HNF4α compete for the binding site of the Apo CIII promoter (Hertz et al. 1995). While PPARα binding reduces Apo CIII expression, HNF4α binding increases Apo CIII expression. Further studies should investigate the potential of n-3 PUFAs to activate PPARα and inhibit HNF4α.

In addition to transcription factors, the expression of several other TG metabolism-associated genes was regulated after n-3 PUFA supplementation. Apo CII acts as a cofactor for LPL, which hydrolyses TGs in chylomicrons and VLDL particles to glycerine and free FAs. Therefore, constant Apo CII levels are crucial for LPL activation (Kei et al. 2012), ensuring efficient lipolysis of TG-rich lipoproteins. Studies have shown that dyslipidemic subjects without genetic disorders present higher Apo CII levels (7.0 mg/dl) than normolipidemic subjects (3.0 mg/dl) (Liu et al. 2009; Sakurabayashi et al. 2001), which results in a disturbance of the Apo CII/LPL balance. Dyslipidemic subjects of our study consistently exhibited higher Apo CII mRNA expression levels than normolipidemic subjects at baseline. After FO treatment, Apo CII expression was down-regulated in dyslipidemic men suggesting a re-establishment of the Apo CII/LPL balance and an increased LPL-mediated TG clearance. In agreement, Zhang et al. (2012) observed reduced plasma Apo CII levels in Chinese woman after increased fish intake (80 g fatty fish five times per week) over eight weeks.

Finally, the mRNA expression of three genes involved in TG synthesis, MOGAT3, MOGAT2 and DGAT1, were down-regulated in dyslipidemic men after n-3 PUFA supplementation. MOGATs and DGATs are key enzymes of the monoacylglycerol (MAG) pathway (Hall et al. 2012), which is primarily responsible for the re-synthesis of TGs from FAs and 2-MAG in enterocytes (Cheng et al. 2003; Hiramine and Tanabe 2011; Yen and Farese 2003). Besides the intestine, MOGAT2 and MOGAT3 are similarly expressed in the liver (Hall et al. 2012; Yen and Farese 2003), whereas DGAT1 is expressed ubiquitously (Cases et al. 1998). To the best of our knowledge, an effect of n-3 PUFAs on the expression of MOGAT2 and MOGAT3 has not yet been described. Knockdown of MOGAT3 by siRNA in liver-derived cells was followed by a reduced enzyme activity (Hall et al. 2012). A reduced enzyme activity was accompanied by reduced intestinal dietary fat absorption in mice (Yen et al. 2009). It is believed that MOGAT3 might be a potential therapeutic drug target for treating metabolic abnormalities, such as dyslipidemia (Hall et al. 2012). Similarly, MOGAT2 knockout mice

were also protected against diet-induced obesity and hypercholesterolemia (Yen et al. 2009), indicating that MOGAT2 might be a potential drug target too. Similarly, the existing data describing an effect of n-3 PUFAs on DGAT1 are rare. Two studies reported a reduced DGAT activity in cultured rat hepatocytes in response to EPA treatment, resulting in reduced TG synthesis and secretion (Berge et al. 1999; Rustan et al. 1988), whereas an activation of PPARα was suggested as the initial regulator (Berge et al. 1999). Similarly to MOGAT2, DGAT1 deficient mice are also resistant to diet-induced obesity and show reduced TG levels in the liver (Smith et al. 2000; Buhman et al. 2002). It was also shown that DGAT1 knockout mice accumulated lipid droplets in the cytoplasm of enterocytes when fed a chronically high-fat diet. We assume that the down-regulation of MOGAT2, MOGAT3 and DGAT1, observed in whole blood cells after FO supplementation, will similarly take place in enterocytes or hepatocytes, resulting in a reduced re-synthesis of TG and thus TG levels in circulation.

4.4.2 HDL cholesterol metabolism

As expected, FO supplementation increased HDL-C levels in both normo- and dyslipidemic subjects. The underlying HDL-C level raising mechanisms are not completely understood. Another target gene of PPARα that might be involved in raising HDL-C levels is PLTP (Bouly et al. 2001; Lemay and Hwang 2006; Tu and Albers 1999), which was up-regulated in dyslipidemic subjects (Figure 4.2). PLTP can modulate HDL-C size and composition (Jauhiainen et al. 1993; Settasatian et al. 2001) by the transfer of PLs from TG-rich lipoproteins to HDL-C particles (Tall et al. 1985). However, in contrast to our results, incubation of Hep G2 cells with EPA, DHA or AA resulted in a decreased expression of PLTP (Kuang et al. 2012). In the same way, results from animal trials were inconsistent. Both PLTP deficiency and PLTP overexpression resulted in a significant reduction of HDL-C levels in the circulation (Yazdanyar et al. 2011). Further studies need to clarify the influence of n-3 PUFAs on the expression of PLTP and the resulting HDL-C levels.

4.4.3 Total cholesterol and LDL cholesterol metabolism

A well-known effect of FO supplementation is a slight increase of LDL-C levels after n-3 PUFA supplementation (Balk et al. 2006), which is most likely the result of an increased conversion of VLDL-C to LDL-C (Lu et al. 1999). Although we observed a considerable increase in LDL-C levels in dyslipidemic subjects, the expression of several regulated genes in the same subjects indicate not only cholesterol-lowering, but also an enhancing effect (Figure 4.2). LDLR, which is expressed in nearly all cells, but predominantly in liver cells, transports cholesterol-rich lipoprotein particles, preferably LDL-C, via endocytosis into the cell, resulting in LDL-C clearance (Brown and Goldstein 1986). Even though high cholesterol concentrations inhibit the LDLR expression (Goldstein and Brown 2001), LDLR expression

values in dyslipidemic subjects were two times higher compared to normolipidemic subjects. After FO supplementation, the LDLR expression was down-regulated in dyslipidemic subjects, which is in agreement with other studies (Dawson et al. 2011). The reduced LDLR expression suggests a diminished LDL-C clearance and could be another mechanism explaining the n-3 PUFA-induced rise in LDL-C levels.

In contrast, the regulation of two other factors points to a cholesterol-lowering effect. The half-transporters ABCG5 and ABCG8 play a pivotal role in the regulation of dietary cholesterol transport into the intestinal and biliary lumen for faecal excretion (Yu et al. 2002). Consequently, these transporters are strongly expressed in the liver, while lower levels are found in the small intestine and colon (Kidambi and Patel 2008). The up-regulation of ABCG5 in dyslipidemic subjects might induce a pathway that results in increased cholesterol efflux. In agreement with our results, two animal studies revealed an increased hepatic ABCG5 and ABCG8 expression in mice after FO feeding (Kamisako et al. 2012; Nishimoto et al. 2009). The regulation might be triggered by PPARα, since PPARα agonists caused an up-regulation of ABCG5 expression in hypercholesterolemic subjects (Roglans et al. 2004).

SOAT1, also referred to as acyl-Coenzyme A cholesterol acyltransferase 1 (ACAT), is a membrane-bound protein that utilizes long-chain fatty acyl-CoA and cholesterol as substrates to form cholesteryl esters (Chang et al. 2009). SOATs play important roles in cellular cholesterol homeostasis in various tissues, and SOAT inhibitors have been focused as drug targets for atherosclerosis and for Alzheimer's disease (Chang et al. 2009). The down-regulation of SOAT1 observed in dyslipidemic subjects after FO supplementation similarly suggests a cholesterol-lowering effect. Accordingly, a down-regulation of SOAT1 expression after n-3 PUFA treatment has been observed in human breast cancer cells (Hammamieh et al. 2007). Further studies need to clarify the exact role of n-3 PUFAs in the regulation of these factors and the resulting effects on cholesterol metabolism.

4.4.4 Limitations

The study has a number of potential limitations. As has already been pointed out, it is critical to investigate the regulative effect of n-3 PUFAs on the expression of lipid metabolism-associated genes in whole blood cells instead of liver cells. However, the taking of biopsy samples from the liver in this study was precluded for ethical and medical reasons. We similarly abstained from isolating lymphocytes or PBMCs, an approach which was chosen by two other groups investigating a similar question (Bouwens et al. 2009; Gorjão et al. 2006), since cell fractioning steps involve stress-induced alterations in gene expression profiles (Vartanian et al. 2009). However, many lipid metabolism-associated genes are also expressed in nucleated blood cells: For example, PPARα is expressed in monocytes,

macrophages and lymphocytes (Chinetti et al. 1998; Mandard et al. 2004), while HNF6 and DGAT were found to be expressed in PBMCs (Cases et al. 1998; Zhao et al. 2007).

4.4.5 Conclusion

This pilot study suggests molecular pathways on how n-3 PUFAs affect lipid metabolism. Although the study is limited by the usage of whole blood cells obviating strong conclusions about lipid metabolism, several lipid metabolism-associated genes were shown to be regulated on a transcriptional level in dyslipidemic subjects, including transcription factors PPARα, RXRα, RXRγ, HNF6, and HNF1ß, as well as other lipid regulators, MOGAT2, MOGAT3, DGAT1, Apo CII, PLTP, LDLR, ABCG5, and SOAT1. We assume that this transcriptional regulation will equally take place in cells of the liver or other tissues. Given this assumption, n-3 PUFAs activate several transcription factors resulting in the regulation of numerous target genes which, in turn, affect multiple lipid regulators. Accordingly, the results give indications for (1) decreased TG levels as a result of an enhanced VLDL catabolism and reduced VLDL production, as well as a decreased TG re-synthesis in enterocytes and hepatocytes, while (2) increased HDL-C levels may be the result of an increased transfer of PLs from TG-rich lipoproteins to HDL-C particles. Finally, (3) LDL-C levels may be influenced in both directions: A decreased LDL-C clearance may result in rising LDL-C levels, on the one hand, while an increased cholesterol efflux and a reduced cholesteryl ester synthesis result in decreasing LDL-C levels, on the other hand. Future studies combining gene expression, metabolic markers and clinical end points need to clarify the significance of the hypothesized molecular mechanisms.

5. General discussion

The work presented here was aimed at investigating the effect of FO or CO supplementation on gene expression changes in whole blood samples from normo- and dyslipidemic male subjects to find possible indications for the molecular mechanism of action described. In addition, the suitability of whole blood samples for investigating gene expression changes after supplementation was tested to clarify if supplemental effects of n-3 PUFAs on inflammatory and lipid-related genes could be observed in this kind of sample material. For these purposes, a double-blind, placebo-controlled, monocentric human interventional trial of twelve weeks duration was undertaken with normo- and dyslipidemic men. The subjects of this study ingested either CO or FO capsules and gene expression changes were determined by microarray experiments and qRT-PCR. The results of the interventional trial conducted were divided into three parts with different main focuses and are presented within this thesis (chapters 2, 3 and 4). Each chapter contains an individual discussion related to its main content. In this section, the results are discussed in the context of the suitability of the methods applied, as well as of the effects of n-3 PUFAs on gene expression changes detected in humans. In addition, future perspectives for further research topics based on the results from this thesis are given.

5.1 Methodological aspects

5.1.1 Suitability of transcriptomics

Transcriptomics – the study of transcribed sequences – enable the measurement of the expression level of messenger ribonucleic acids (mRNAs) in a given cell population. The usage of high-throughput techniques such as DNA microarrays, permit the analysis of expression changes of thousands of genes simultaneously. Determination of whole genome gene expression changes by microarray experiments in this proof of concept study facilitated it becoming an overview of complex regulatory networks and revealed several already known gene expression changes after FO supplementation, but also showed many regulated genes which might be part of potential new regulatory networks of n-3 PUFAs (see 5.2). The result of a microarray experiment is a large list of differentially expressed genes. These gene lists are hard to interpret in a biological context (Morine et al. 2011) and bioinformatics tools are indispensable for analyzing and interpreting such large amounts of data (Goodman 2002). Pathway analyses are the most applied tool for the interpretation of differentially expressed gene lists. Although this tool is easy to execute, technical limitations, such as inherent redundancy among pathways and interconnectedness between one pathway and the next, are the reason for the limited biological interpretation of high-throughput datasets (Morine et al. 2011). However, the results of microarray experiments could give an initial indication and

thus afford a good opportunity to investigate the effects of nutrition components on human health. In addition, qRT-PCR analysis was performed in this study to validate microarray results and to quantificate the transcript levels of target genes sensitively. In conclusion, the combination of both of these transcriptomic tools, microarray and qRT-PCR, is very suitable for producing high quality data and to investigate nutrient-induced gene expression changes in humans.

5.1.2 Suitability of blood samples for gene expression analysis

Analyzing gene expression changes in humans presents some difficulties due to methodical limitations. Firstly, human nutrition studies are mostly influenced by side-effects, unless volunteers were admitted to a hospital or casern and physical activity and food intake is constantly controlled. The second limitation is the availability of sample material. Most nutritional studies use blood samples, because blood can be easily collected by minimal invasiveness under low pain conditions. Some studies also use samples which were collected during surgery, and others take cell biopsies and cultivated these cells. However, both of these methods do not reflect a normal physiological state and, in both cases, tissue and cells are under the influence of anesthesia or narcotic drugs, as well as damaging procedures, which may alter gene expression patterns. Isolated and cultured cells additionally present only one small unit of the whole body, which limits the observations to this small unit without knowing if this reaction would also occur in the complex body. Due to ethical reasons, blood samples were used in this study for analyzing whole genome expression changes after supplementation with FO or CO in normo- and dyslipidemic male subjects.

Whole blood cells could be divided into three main cell subtypes: erythrocytes, thrombocytes and leucocytes (monocytes, lymphocytes and granulocytes). Leucocytes are a main component of the cellular immune system, facilitating complex processes of the immune defense against pathogens and toxins, such as recognition of pathogen structures, production of antibodies and destruction of pathogens by phagocytosis. Therefore, whole blood – containing immune cells – is a good choice to analyze the immune-modulatory effects of n-3 PUFAs. As has already been mentioned in chapter 4, it is critical to investigate the regulative effect of n-3 PUFAs on the expression of lipid metabolism-associated genes in whole blood cells instead of liver cells. However, many lipid metabolism-associated genes are also expressed in nucleated blood cells, such as PPARα (Chinetti et al. 1998; Mandard et al. 2004), HNF6 and DGAT (Cases et al. 1998; Zhao et al. 2007). The results of this study indicate that whole blood cells have the potential to reflect the regulatory effects of n-3

PUFAs in other body compartments and thus provide a good opportunity to analyze the effects of n-3 PUFAs by a minimum invasive method.

5.2 Effects of n-3 PUFAs on gene expression

N-3 PUFAs can modify gene expression through several mechanisms (see 1.2.4), of which the modification of transcription factors plays a major part in this regulation. Gene expression changes of specific transcription factors and corresponding target genes, and expression changes of genes related to the lipid metabolism, inflammation and oxidative metabolism are observed by this study. In the following section, firstly, the main study findings are summarized. Afterwards, the role of n-3 PUFAs in the regulation of gene expression within the lipid metabolism, inflammation and oxidative metabolism are described in detail.

5.2.1 Summarized evaluation of main study findings

The objective of this thesis was to analyze the influence of an FO or CO supplementation on whole genome gene expression patterns of whole blood samples derived from normo- and dyslipidemic male subjects and to propose possible molecular mechanism of action. This study approach permits, on the one hand, a comparison of gene expression changes between dyslipidemic and normolipidemic male subjects and, on the other hand, the comparison between the effects of FO and CO. Regarding the second, comparison between FO and CO supplementation, pathway analyses showed a higher number of influenced pathways after FO than after CO supplementation, including pathways of the immune system, inflammation and lipid metabolism. In the following sections, firstly, differences in gene expression changes after supplementation with FO or CO in normo- and dyslipidemic male subjects were discussed. Afterwards, all gene expression changes observed of transcription factors and genes after FO supplementation were taken together to discover the role of n-3 PUFAs in the transcriptional regulation of the lipid metabolism, inflammation and oxidative metabolism, as well as other potential cardioprotective effects.

5.2.1.1 Normo- vs. dyslipidemic male subjects

The analysis of whole genome microarray data (chapter 2) revealed that normo- and dyslipidemic male subjects feature different expression profiles after supplementation, which is characterized by the direction of gene regulation (normolipidemic male subjects showed more up-regulated genes and dyslipidemic male subjects showed more down-regulated genes) and by the extent of gene regulatory effect of n-3 PUFAs (higher number of regulated genes and thus more pronounced effects in dyslipidemic male subjects). In this connection, the more pronounced gene regulatory effect of n-3 PUFAs in dyslipidemic male subjects

might be in response to dyslipidemia, a metabolic state, which is often associated with a low grade of inflammation, being present (Esteve et al. 2005). It is believed that subjects with inflammatory conditions are more sensitive to the immune-modulatory effects of n-3 PUFAs than healthy subjects, because they have a disease-dependent higher turnover rate of immune cells (Sjiben and Calder 2007). A higher number of immune cells possess a higher capacity to produce eicosanoids and other metabolites, which could influence inflammation and serve as ligands for transcription factors (Sjiben and Calder 2007). In addition, n-3 PUFA supplementation might influence the count of immune cells, especially granulocytes and, therefore, leucocyte numbers, resulting in anti-inflammatory effects by reduced inflammatory cell numbers and responses (Kelley et al. 1998, Kelley et al. 1999). Furthermore, subjects with inflammatory conditions feature a lower buffering capacity than healthy subjects (Sjiben and Calder 2007) and inflammation could result in an increased production of oxidized metabolites of n-3 PUFAs (see 1.2.3.3). This suggests that local anti-inflammatory effects are perhaps greater than systemic effects in healthy subjects, which might explain why n-3 PUFA supplementation in healthy subjects mostly showed no immune-modulatory effects.

5.2.1.2 Effects of n-3 PUFA supplementation on the lipid metabolism

Interventional studies have shown that n-3 PUFAs lower TG levels and elevate HDL-C levels (Harris 1996, 1997; Musa-Veloso et al. 2010). In this study, FO supplementation resulted in a TG decrease by ~25% as well as an HDL-C increase by ~12% in both normo- and dyslipidemic male subjects. It is believed that the TG-lowering effect of n-3 PUFAs results from reduced VLDL-TG synthesis and secretion from the liver, and enhanced TG clearance (Shearer et al. 2012). The HDL-C increase might be the cause of the modification of HDL-C subtypes (Mori et al. 1999, 2000). However, molecular mechanisms by which n-3 PUFAs modify the lipid metabolism are not completely clarified. The results of this study showed that EPA and DHA may exert their effects on lipid metabolism-related genes via direct effects on the transcription factors, such as PPARα, RXRs and HNFs (**Figure 5.1**). The down-regulation of HNF1ß and HNF6, which could both activate HNF4α together with HNF1α or GATA6 (Hatzis and Talianidis 2001), might reduce the promotor activity of HNF4α, resulting in repressed expression of Apo CIII and Apo B which, in turn, reduces the VLDL secretion and TG level in the circulation (Ladias et al. 1992). In addition, the activation of PPARα causes several downstream processes, resulting in modified cholesterol and TG metabolism. PPARα activation could increase the expression of ABCG5 (Roglans et al. 2004), leading to increased cholesterol efflux (Yu et al. 2002) and may reduce the expression of SOAT1, resulting in decreased cholesterol synthesis (Chang et al. 2009). Regarding the influence of HNF4α on Apo CIII and Apo B expression described above, PPARα compete for binding sites for these target genes and this resulted in an inverse regulation (Hertz et al. 1995).

Therefore, activation of PPARα could result in a repression of the expression of Apo CIII, leading to a decreased VLDL secretion and TG level. Furthermore, PLTP is a target gene of PPARα, which could modify the HDL particle size and might be a reason for the HDL increase observed. Besides these named transcription factors, several genes which are related to the TG metabolism were regulated after FO supplementation. While genes of the TG re-synthesis were down-regulated (MOGAT2, MOGAT3, DGAT1), the level of Apo CII was modified, suggesting the generation of an optimal balance to LPL. Apo CII could be increased by the transcription factor FXR, which was regulated by n-3 PUFAs in liver cells (Adkins and Kelley 2010). In addition, the down-regulation of RXRα could be an indication for a reduced formation of the RXRα/LXRα and, in turn, reduced activity of SREBP-1c (Adkins and Kelley 2010), which leads to a decreased expression of FAS and acetyl CoA carboxylase ß (ACACB). These named factors are indications of a reduced lipogenesis, TG synthesis and increased TG clearance. Taken together, the results of this study with the use of whole blood samples indicate that effects of EPA and DHA on the lipid metabolism in humans are mainly exerted via activation of PPARα.

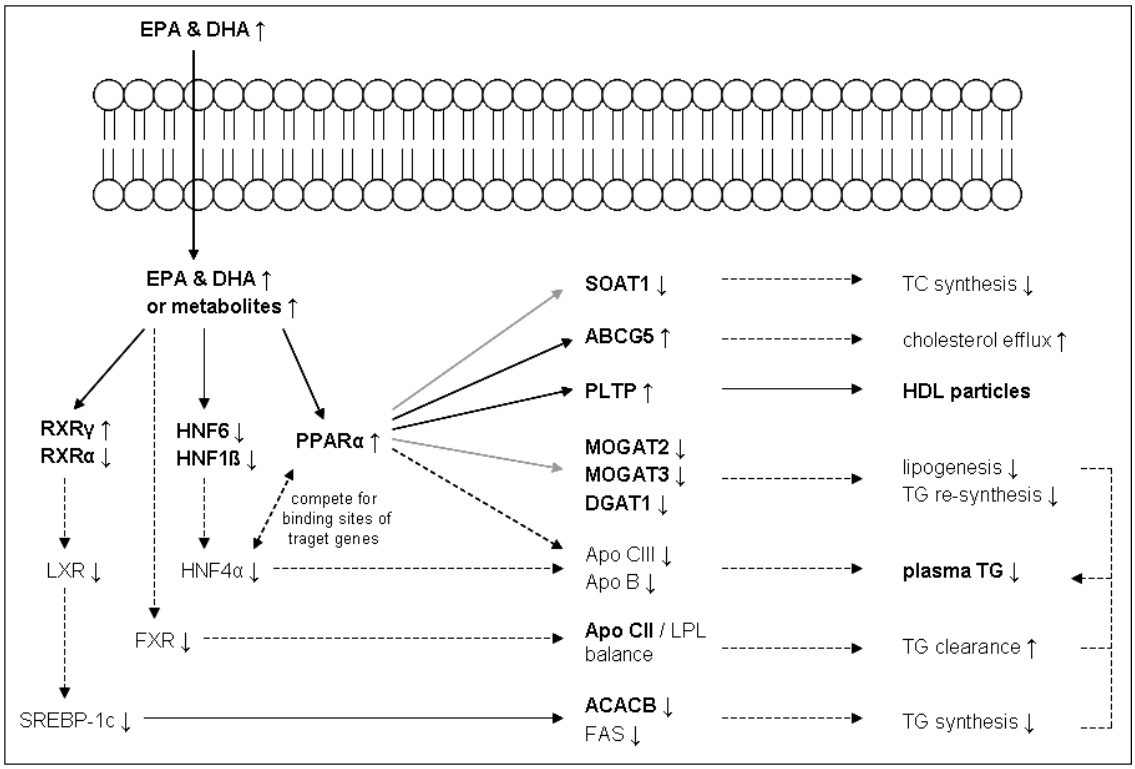

Figure 5.1: Summarized regulatory effects of eicosapentaenoic acid and docosahexaenoic acid on lipid metabolism. The Figure presented is based on the analysis of gene expression changes after fish oil supplementation in dyslipidemic male subjects. While bold text and black and grey arrows present findings discovered in this thesis, grey arrows symbolize that until now it has not been clarified if genes are target genes of PPARα. In addition, broken arrows symbolize further possible mechanisms of action based on findings from the literature.

5.2.1.3 Effects of n-3 PUFA supplementation on inflammation and oxidative metabolism

In addition to the lipid profile improving effects, n-3 PUFAs have shown anti-inflammatory properties *in vitro* and partly *in vivo* (Calder 2003, 2006). All observations within this study, which are related to the anti-inflammatory as well as to antioxidative effects of EPA and DHA, are displayed in **Figure 5.2**. Similar to the regulatory effects within the lipid metabolism, PPARα activation also plays an important role in the effects on inflammation and oxidative processes. It is known that PPARα activation induces the expression of genes related to FA oxidation (Mozaffarian and Wu 2011; Shearer et al. 2012). Although this could not be observed in this study, indication for an increased production of oxidized metabolites of EPA and DHA was detected (CYP1A2 was up-regulated in dyslipidemic male subjects). An increased FA oxidation could increase oxidative stress (Harats et al. 1991; Meydani et al. 1991) and may induce the body's defense system to generate antioxidative enzymes for the prevention of cellular damage. In this case, the up-regulation of CAT and HMOX2 discovered gives a hint of such antioxidative induced processes. In addition, PPARα could activate CAT directly, which is an additional indication that PPARα activation results simultaneously in increased FA oxidation and increased antioxidative gene expression. The increased expression of CAT and HMOX2, as well as the down-regulation of FA oxidation-related genes (HADHA, HADH; ECI2) after FO supplementation, suggests that oxidative stress and ROS production might be reduced. Regarding the fact that ROS induce MMP expression, reduced ROS concentration may lead to decreased MMP expression, which could be observed in this study and may prevent plaque rupture during atherosclerosis. Furthermore, reduced oxidative stress could influence stress-activated pathways, such as NFκB, and the MAPK pathway, which was also influenced by FO supplementation in this study. These pathways regulate several inflammatory genes, of which, for example, IL-8 was seen to be down-regulated in this study. This last connection could also be induced by reduced AA concentration in membranes, because AA is an important ROS producer. The membrane content of AA decreased, while the content of EPA and DHA increased after FO supplementation. In addition, the up-regulation of PLA2GE2 and PLB1, as well as several up-regulated genes within the glycerolipid- and glycerophospholipid metabolism, indicates an exchange of membrane bound AA and membrane remodeling processes. In conclusion, this study shows several indications that the anti-inflammatory effects of EPA and DHA might be exerted via antioxidative effects by PPARα activation.

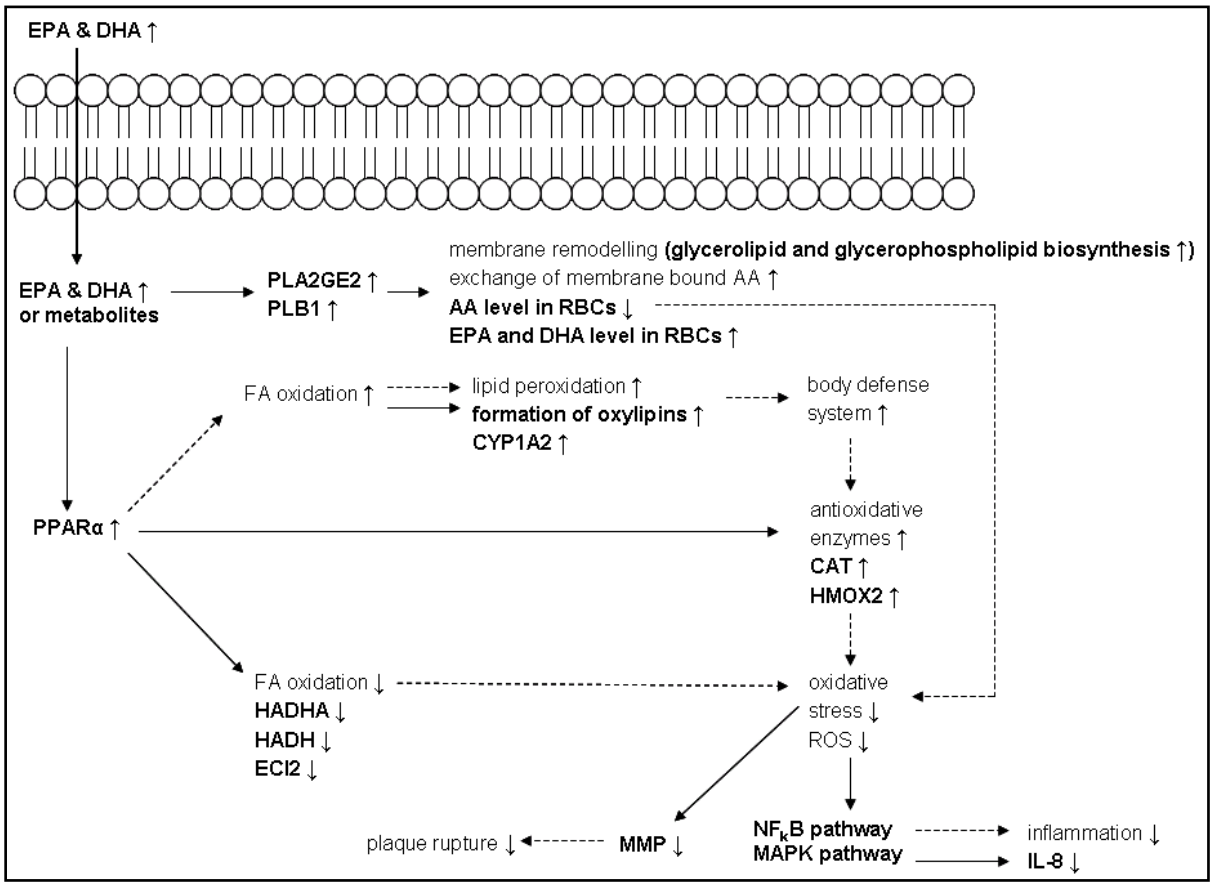

Figure 5.2: Summarized regulatory effects of eicosapentaenoic acid and docosahexaenoic acid on inflammation and oxidative processes. The Figure presented is based on the analysis of gene expression changes after fish oil supplementation in dyslipidemic male subjects. While bold text and black arrows present findings discovered in this thesis, broken arrows symbolize further possible mechanisms of action based on findings from the literature.

5.2.1.4 Effects of n-3 PUFA supplementation on other potential cardioprotective effects

Beside regulated genes, which are related to the lipid metabolism, inflammation and oxidative metabolism, numerous other genes which might be associated with the cardioprotective effects of n-3 PUFAs were seen to be regulated after FO supplementation. Several cardioprotective effects of EPA and DHA are described in the literature, such as inhibition of inflammation, monocyte infiltration, NFκB activation, platelet aggregation, vasoconstriction, and arrhythmia, as well as stimulation of pro-resolving mediators, stabilization of atherosclerotic plaques, TG lowering, and changes in membrane lipid composition (Adkins and Kelley 2010). Some indications for these named cardioprotective effects could also be observed in this study. These include the improvement of the inhibition

of platelet aggregation, the modulation of ion channels, the stabilization of potential atherosclerotic plaque, and anti-inflammatory properties.

- Indications for the inhibition of platelet aggregation by EPA and DHA were discovered by pathway analyses of differential regulated genes of dyslipidemic male subjects. While blood coagulation supporting genes such as complement component (3b/4b) receptor 1, coagulation factor III (thromboplastin, tissue factor) and fibrinogen gamma chain were down-regulated, and anti-thrombin (serpin peptidase inhibitor, clade C member 1) was up-regulated. N-3 PUFAs could reduce platelet aggregation (Harris et al. 2008), which prevents critical thrombotic and/or inflammatory complications during several diseases (Oikonomopoulou et al. 2012; Vanschoonbeek et al. 2004), indicating that n-3 PUFAs posses anti-thrombotic properties.

- Modulation of ion channels is an important approach by which n-3 PUFAs may exert their anti-arrhythmic effects. In this study, HMOX2 was up-regulated after FO supplementation in both normo- and dyslipidemic male subjects. The fact that HMOX2 was identified as part of the BK(Ca) channel complex (Williams et al. 2004) suggests that n-3 PUFAs may influence cell membrane potential, resulting, for example, in oxygen-sensing, neuronal excitability, vascular tone regulation, and neurotransmitter release (Hou et al. 2009; Salkoff et al. 2006).

- The ability to stabilize atherosclerotic plaque is another aspect by which n-3 PUFAs may prevent CVD. Increased expression of MMPs could result in tissue remodeling processes (Clee 2010) and promote the pathogenesis of several CVDs (Spinale et al. 2000; Tyagi et al. 1996). In this study, different MMPs were down-regulated after FO supplementation in normo- and dyslipidemic male subjects, indicating the potential cardioprotective effects of n-3 PUFAs by the prevention of plaque rupture.

- Moreover, several genes of the ARVC and dilated DCM pathways were down-regulated in dyslipidemic men after FO supplementation. This might be the result of diverse regulatory effects on lipid metabolism and anti-inflammatory processes. However, the effect of n-3 PUFA supplementation on the pathogenesis of ARVC and DCM in humans is unknown and further studies are needed to analyze the protective function of n-3 PUFAs in this group of patients.

5.3 Conclusion

In conclusion, this proof of concept study showed significant differences in gene expression profiles between normo- and dyslipidemic men after FO supplementation. Dyslipidemic men presented substantially more regulated genes and pathways, of which several are related to the immune system, inflammation, lipid metabolism, and CVD. Several lipid metabolism-associated transcription factors and genes were shown to be regulated on a transcriptional level in the blood samples analyzed, and it is assumed that this transcriptional regulation will equally take place in the cells of the liver or other tissues. Additionally, the study showed that n-3 PUFAs activate and repress several transcription factors resulting in the regulation of numerous target genes. In this case, lipid metabolism-related transcription factors, such as the hepatic nuclear factor (HNF) 6 and HNF1ß, which could affect HNF4α, an additional important regulator of the lipid metabolism, were repressed by n-3 PUFA supplementation. Otherwise, peroxisome proliferator-activated receptor (PPAR) α was activated by n-3 PUFA supplementation. The regulation of named transcription factors may induce expression changes of target genes resulting in TG lowering, which could be observed in normo- and dyslipidemic male subjects. Moreover, the activation of PPARα appears to play a significant role in various other cardioprotective effects of n-3 PUFAs. In this case, antioxidative genes were up-regulated and pro-inflammatory genes were down-regulated after n-3 PUFA supplementation, suggesting that n-3 PUFA may induce anti-inflammatory and antioxidative effects via PPARα activation.

Although this study revealed several indications of possible molecular mechanisms of action in what extent n-3 PUFAs mediate their cardioprotective effects, further studies analyzing a combination of gene expression, metabolic markers and clinical end-points are needed to clarify the mechanisms by which n-3 PUFAs trigger gene regulation and affect various regulatory networks.

5.4 Perspectives

With regard to lipid metabolism-related effects, several open questions arose from this study. Firstly, the role of PLTP in the regulation of the HDL level is not clarified and inconsistent results were observed in literature and this study. While the incubation of Hep G2 cells with EPA, DHA or AA resulted in a decreased expression of PLTP (Kuang et al. 2012), this study showed an up-regulation of PLTP after FO supplementation in dyslipidemic men. However, both PLTP deficiency and PLTP over-expression resulted in a significant reduction of HDL-C levels in the circulation (Yazdanyar et al. 2011). Therefore, the influence of n-3 PUFAs on the expression of PLTP and the resulting HDL-C levels has to be analyzed and the importance of protein concentration of PLTP for the modification of HDL particles has to be investigated. In addition, the relevance of the up-regulation observed of the half-transporter ABCG5, which plays a pivotal role in the regulation of dietary cholesterol transport into the intestinal and biliary lumen for fecal excretion (Yu et al. 2002), for increased cholesterol efflux should be determined. Moreover, the potential involvement of PPARα in the regulation of ABCG5 should be investigated in more intensity. Furthermore, *in vitro* studies enlightened that PPARα compete for binding sites of target genes with HNF4α, but until now, this has not been shown *in vivo* and might be a further study approach for analyzing the TG-lowering effects of n-3 PUFAs. Last but not least, existing literature as well as this study showed that an optimal balance of Apo CII and LPL might be essential for efficient TG clearance. Unfortunately, only a limited number of cross-section studies showing Apo CII and LPL levels in different populations or subjects with different diseases have been undertaken so far. The determination of Apo CII, LPL and their relationship to each other may reveal essential knowledge about the modification of TG clearance by n-3 PUFAs.

In addition, an important question, which arises from this and also from other studies is, why anti-inflammatory effects could be observed *in vitro*, but rarely *in vivo* (Calder 2003, 2006). *In vitro* studies showed a down-regulation of IL-1, IL-6, TNFα, COX-2, and several CAMs by n-3 PUFAs (Calder 2003), but these genes were not seen to be regulated in this study. One possibility for this phenomenon could be differences in the concentration of free n-3 PUFAs: While the concentration of n-3 PUFAs in cultured cells was ≥ 50 µM (Galli and Calder 2009), intracellular concentrations of free n-3 PUFAs *in vivo* is less than 10 µM (Jump 2002). In addition, it might be possible that the decreased expression of these genes is linked to areas of high inflammation (see 1.2.3.3) and that, in turn, anti-inflammatory effects could be observed mainly locally instead in a systemic reaction in the whole body. Therefore, an intervention study with n-3 PUFAs analyzing gene expression changes in centers of inflammation compared with changes within the peripheries would be interesting to clarify this hypothesis.

Following studies investigating the initial cause of the antioxidative properties of n-3 PUFAs would help us to understand possible complex regulator networks induced by n-3 PUFAs. It has to be clarified if the antioxidative potential of n-3 PUFAs is caused only by n-3 PUFA-mediated increased oxidative stress with initiated body defense reaction to reduce oxidative damage, or is it also caused by the direct increased expression of antioxidative enzymes, such as the regulation of CAT via PPARα activation. Hence, studies analyzing the expression of antioxidative enzymes, oxidative signaling processes and metabolic outcomes should be undertaken to investigate this question. Furthermore, it would be interesting to know if other antioxidative genes, such as GPX, GR, GST, and HMOX2, are also target genes of PPARα, which may reveal that n-3 PUFAs induce both antioxidative genes expression directly and indirectly via an increase of the body's defense system induced by elevated PUFA-mediated FA oxidation. Moreover, analyzing further influences on stress-activated pathways (MAPK and NFκB pathways), as well as other stress-related genes such as MMPs, may reveal additional properties of n-3 PUFAs.

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7. Appendix

7.1 Regulated genes within stress-activated pathways

Genes, which were regulated after four hours (t_{4h}), one week (t_1) and twelve weeks (t_{12}) of fish oil supplementation in normolipidemic and dyslipidemic men were submitted to pathway analyses according to the KEGG database as well as performed with GenMAPP. Expression ratios of regulated genes within mitogen-activated protein kinase (MAPK) signalling pathway, nuclear factor kappa b (NFκB) pathway and oxidative stress pathway were displayed.

Gene name	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $_{t1} : t_0$	Ratio $t_{12} : t_0$
Mitogen-activated protein kinase (MAPK) signaling pathway									
Arrestin beta-1	ARRB1	408	NM_004041.3 NM_020251.2	-	-	8.60[1]	-	-	-2.12[2]
Arrestin beta-2	ARRB2	409	NM_199004.1 NM_004313.3	2.13[2]	3.86[3]	2.58[2]	-	-2.37[2]	-
V-raf murine sarcoma viral oncogene homolog B1	BRAF	673	NM_004333.4	-2.16[1]	-2.93[1]	-3.43[1]	-	-	-
Monocyte differentiation antigen CD14 Precursor	CD14	929	NM_000591.2 NM_001040021.1	4.82[3]	-	2.88[2]	-	-	-
Dual specificity phosphatase Cdc25B	CDC25B	994	NM_021873.2 NM_021872.2 NM_004358.3	-2.07[1]	-2.66[1]	-	-	-	2.83[1]
Cell division cycle 42	CDC42	998	NM_001791.3 NM_001039802.1	-	-	-	-	-	14.42[1]
Dual specificity protein phosphatase 1	DUSP1	1843	NM_004417.2	-2.071	-9.67[1]	-	-2.39[2]	-2.53[1]	-
Dual specificity protein phosphatase 8	DUSP8	1850	NM_004420.2	-	-4.37[1]	-3.09[1]	-	-	-
ETS domain-containing protein Elk-4	ELK4	2005	NM_021795.2 NM_001973.2	-	-	2.38[2]	-	-	-
Fibroblast growth factor 3	FGF3	2248	NM_005247.2	-	-4.42[1]	-16.68[1]	-	-	-
Fibroblast growth factor receptor 4 Precursor	FGFR4	2264	NM_022963.2 NM_213647.1 NM_002011.3	-	-	2.08[1]	-	-	-
MAP kinase-interacting serine/threonine-protein kinase 2	MKNK2	2872	NM_017572.3 NM_199054.2	-	-	2.25[2]	-	-	-2.04[1]
Growth factor receptor-bound protein 2	GRB2	2885	NM_203506.2 NM_002086.4	-	-2.94[2]	-	-	2.36[3]	-
Heat shock 70 kDa protein 6	HSPA6	3310	NM_002155.3	-	-	-	2.41[1]	-	-

Gene name	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$
Mitogen-activated protein kinase (MAPK) signaling pathway [continuation]									
Heat shock 70kDa protein 8	HSPA8	3312	NM_153201.1 NM_006597.3	-	-3.04[1]	-	-	2.63[1]	4.80[1]
Heat shock protein beta-1	HSPB1	3315	NM_001540.3	-	-2.29[1]	2.29[2]	-	-	-
Transcription factor jun-D	JUND	3727	NM_005354.4	-	2.14[1]	8.66[1]	-	-	-
GTPase KRas Precursor	KRAS	3845	NM_033360.2 NM_004985.3	-	-2.86[1]	-3.97[1]	-	-	-
Protein max	MAX	4149	NM_145113.1 NM_145112.1 NM_002382.3	5.49[1]	-	5.05[1]	-	-2.62[1]	-
Mitogen-activated protein kinase kinase kinase 3	MAP3K3	4215	NM_203351.1 NM_002401.3	-	-3.53[1]	-5.15[1]	-	-	-
Mitogen-activated protein kinase kinase kinase 5	MAP3K5	4217	NM_005923.3	-	-	-3.65[1]	-	-	-
Mitogen-activated protein kinase kinase kinase 11	MAP3K11	4296	NM_002419.3	-	2.71[1]	-	-	-	-
High affinity nerve growth factor receptor Precursor	NTRK1	4914	NM_001012331.1 NM_002529.3 NM_001007792.1	4.93[1]	-	-	-	-	-
cAMP-dependent protein kinase catalytic subunit alpha	PRKACA	5566	NM_207518.1 NM_002730.3	-	-5.55[1]	-	-	-	-
Protein kinase C alpha type	PRKCA	5578	NM_002737.2	-4.94[1]	-	-2.60[1]	-	-	-
Protein kinase C beta type	PRKCB	5579	NM_002738.6	-	12.47[1]	23.65[1]	-	-	-
Mitogen-activated protein kinase 8	MAPK8	5599	NM_139049.1 NM_139047.1 NM_139046.1 NM_002750.2	-	-2.90[1]	-2.52[1]	-	-	-
Dual specificity mitogen-activated protein kinase kinase 3	MAP2K3	5606	NM_002756.3 NM_145109.2	-	-6.68[1]	-	-2.34[2]	-	-
Tyrosine-protein phosphatase non-receptor type 7	PTPN7	5778	NM_080588.1 NM_002832.2	3.51[1]	-	-	-	-	-
Ras-related C3 botulinum toxin substrate 2	RAC2	5880	NM_002872.3	2.01[1]	-	-	-2.58[2]	-2.76[1]	-
Mitogen-activated protein kinase kinase kinase kinase 2	MAP4K2	5871	NM_004579.2	-	-8.82[1]	-	-	-	2.78[1]

Gene name	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$
Mitogen-activated protein kinase (MAPK) signaling pathway [continuation]									
Ras GTPase-activating protein 1	RASA1	5921	NM_002890.1 NM_022650.1	-	14.46[1]	16.67[1]	-	-	-
Ribosomal protein S6 kinase alpha-1	RPS6KA1	6195	NM_002953.3 NM_001006665.1	-	-5.52[1]	2.99[2]	-	-	2.32[3]
Dual specificity mitogen-activated protein kinase kinase 4	MAP2K4	6416	NM_003010.2	-2.35[1]	-	-2.72[1]	-	-	-
TGF-beta receptor type-2 Precursor	TGFBR2	7048	NM_003242.5 NM_001024847.2	8.64[1]	-	5.95[1]	-3.10[1]	-2.36[1]	-
Interleukin-1 receptor type II	IL1R2	7850	NM_173343.1 NM_004633.3	-	-5.50[1]	-	-	-	-
MAP kinase-activated protein kinase 5	MAPKAPK5	8550	NM_139078.1 NM_003668.2	-	-9.40[1]	-6.52[1]	-	-	-
Tumor necrosis factor receptor superfamily member 1A	TNFRSF1A	7132	NM_001065.2	-	-10.73[1]	-	-2.74[2]	-3.62[1]	-
Ribosomal protein S6 kinase alpha-4	RPS6KA4	8986	NM_003942.2 NM_001006944.1	-4.00[1]	-4.65[1]	-3.42[1]	-	-	-
Mitogen-activated protein kinase kinase kinase 6	MAP3K6	9064	NM_004672.3	-	-	-	-	-	2.34[1]
Serine/threonine-protein kinase TAO2	TAOK2	9344	NM_016151.2	-	-	3.04[1]	-	2.16[1]	-
Mitogen-activated protein kinase kinase kinase kinase 4	MAP4K4	9448	NM_145687.2 NM_004834.3 NM_145686.2	-	-	-6.73[2]	-	-	-
Rap guanine nucleotide exchange factor 2	RAPGEF2	9693	NM_014247.2	-	-2.73[1]	-	-	-	-
Mitogen-activated protein kinase kinase kinase 7-interacting protein 1	TAB1	10454	NM_006116.2	4.41[1]	16.23[1]	6.79[1]	-	-	-
Calcium-binding protein p22	CHP	11261	NM_007236.4	5.03[3]	-	-2.58[1]	-	-	-
Mitogen-activated protein kinase kinase kinase 7-interacting protein 2	TAB2	23118	NM_015093.3	-	-3.91[1]	-9.24[1]	-	-	-
Voltage-dependent calcium channel gamma-5 subunit	CACNG5	27091	NM_014404.1	-	-5.04[1]	-9.92[1]	-	-	-

Gene name	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$
Mitogen-activated protein kinase (MAPK) signaling pathway [continuation]									
Group IIE secretory phospholipase A2	PLA2G2E	30814	NM_014589.1	-	-	2.51[1]	-2.01[1]	-	-
Voltage-dependent calcium channel gamma-6 subunit	CACNG6	59285	NM_031897.2 NM_145815.1 NM_145814.1	-	-	-	-	2.92[1]	5.89[1]
Calcineurin B homologous protein 2	CHP2	63928	NM_022097.2	-	-5.75[1]	-5.31[1]	-	-	-
Protein kinase C eta type	PRKCH	5583	NM_006255.3	-	-2.91[1]	-2.36[1]	-	-	-
Mitogen-activated protein kinase 4	MAPK4	5596	NM_002747.3	-	-13.56[1]	-2.92[1]	-	-	-
Member of RAS oncogene family	RAPIB	5908	NM_001010942.2 NM_001251917.1 NM_001251918.1 NM_001251921.1 NM_001251922.1 NM_015646.5	-	4.15[1]	2.05[1]	-	-	-
Dual specificity mitogen-activated protein kinase kinase 6	MAP2K6	5608	NM_002758.3	-	-3.25[1]	-2.89[1]	-	-	-
Protein kinase C zeta type	PRKCZ	5590	NM_001033581.1 NM_001033582.1 NM_001242874.1 NM_002744.4	-2.65[1]	-	-	-	-	-
Caspase-9	CASP9	842	NM_032996.1 NM_001229.2	3.69[1]	-	-	-	-	-
Caspase-1	CASP1	834	NM_033293.2 NM_033292.2 NM_001223.3	2.12[1]	-	-	-	-	-
ECSIT homolog (Drosophila)	ECSIT	51295	NM_001142464.2 NM_001142465.2 NM_001243204.1 NM_016581.4	-	8.23[1]	8.72[1]	-	-	-
Receptor-interacting serine/threonine-protein kinase 1	RIPK1	8737	NM_003804.3	-	3.45[1]	2.20[2]	-	-	-

Gene name	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$
Nuclear factor kappa b pathway									
Alkaline phosphatase, tissue-nonspecific isozyme	ALPL	249	NM_000478.4 NM_001127501.2 NM_001177520.1	-	-3.47[1]	2.03[2]	-	2.42[1]	2.81[1]
Tumor necrosis factor receptor superfamily member 8	TNFRSF8	943	NM_152942.2 NM_001243.3	7.79[1]	-	-	-	-	-
Tumor necrosis factor receptor superfamily member 1A	TNFRSF1A	7132	NM_001065.2	-	-10.73[1]	-	-2.74[2]	-3.62[1]	-
Protein kinase C zeta type	PRKCZ	5590	NM_001033581.1 NM_001033582.1 NM_001242874.1 NM_002744.4	-2.65[1]	-	-	-	-	-
cAMP-dependent protein kinase catalytic subunit alpha	PRKACA	5566	NM_207518.1 NM_002730.3	-	-5.55[1]	-	-	-	-
Tumor necrosis factor receptor superfamily member 1B	TNFRSF1B	7133	NM_001066.2	-	-	2.51[2]	-2.66[2]	-	-
Receptor-interacting serine/threonine-protein kinase 1	RIPK1	8737	NM_003804.3	-	3.45[1]	2.20[2]	-	-	-
Kinectin	KTN1	3895	NM_182926.2	-5.37[1]	-	-	-	-	-
DNA replication licensing factor MCM5	MCM5	4174	NM_006739.3	-4.07[1]	-	-	-	-	-
Guanine nucleotide-binding protein subunit beta-2-like 1	GNB2L1	10399	NM_006098.4	9.05[3]	6.06[3]	14.73[3]	-	-2.76[2]	-
BAG family molecular chaperone regulator 4	BAG4	9530	NM_004874.2	-	6.19[1]	4.97[1]	-	-	-
Ribosomal protein L4	RPL4	6124	NM_000968.2	-	-3.85[1]	2.91[2]	-	-2.59[2]	-
Ribosomal protein S11	RPS11	6205	NM_001015.3	-	-4.94[2]	2.10[2]	-	-	2.20[3]
Ribosomal protein L30	RPL30	6156	NM_000989.2	-	-	3.34[1]	-	-	2.73[2]
Mitogen-activated protein kinase kinase kinase 3	MAP3K3	4215	NM_203351.1 NM_002401.3	-	-3.53[1]	-5.15[1]	-	-	-
Ras GTPase-activating protein-binding protein 2	G3BP2	9908	NM_012297.4 NM_203505.2 NM_203504.2	-2.93[1]	-3.23[1]	-5.21[1]	-	-	-

Gene name	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$
Nuclear factor kappa b pathway [continuation]									
14-3-3 protein eta	YWHAH	7533	NM_003405.3	-	-9.33[1]	-	-	-	-
Heat shock protein beta-1	HSPB1	3315	NM_001540.3	-	-2.29[1]	2.29[2]	-	-	-
Mitogen-activated protein kinase kinase kinase 7-interacting protein 1	MAP3K7IP1	10454	NM_006116.2	4.41[1]	16.23[1]	6.79[1]	-	-	-
Mitogen-activated protein kinase kinase kinase 7-interacting protein 1	MAP3K71P2	23118	NM_015093.3	-	-3.91[1]	-9.24[1]	-	-	-
Casein kinase 2, beta polypeptide	CSNK2B	1460	NM_001320.5	-	-	2.81[2]	-2.11[2]	-	-
14-3-3 protein gamma	YWHAG	7532	NM_012479.3	-	-3.29[1]	-2.41[1]	-	-	-
Histone deacetylase 6	HDAC6	10013	NM_006044.2	-	5.46[3]	6.89[3]	-	-	-
SWI/SNF complex subunit SMARCC2	SMARCC2	6601	NM_001130420.1 NM_003075.3 NM_139067.2	-	-7.44[1]	-2.60[2]	-	-	2.54[1]
DNA-directed RNA polymerases I and III subunit RPAC1	POLR1C	9533	NM_004875.2 NM_203290.1	-	-14.84[1]	-5.72[1]	-	-	-
5-azacytidine-induced protein 2	AZI2	64343	NM_022461.3	-	-2.15[1]	-5.29[1]	-	-	-
Mitogen-activated protein kinase kinase kinase 7-interacting protein 2	TXLNA	200081	NM_175852.3	-	5.06[1]	3.45[1]	-	-	-
oxidative stress pathway									
Glutathione peroxidase 1	GPX1	2876	NM_000581.2 NM_201397.1	-	-	-	-	-	-2.48[3]
Extracellular superoxide dismutase [Cu-Zn]	SOD3	6649	NM_003102.2	-	-	4.07[3]	-	-	2.91[3]
Nuclear factor 1 X-type	NFIX	4784	NM_002501.2	-	-3.50[1]	3.04[2]	-2.57[1]	-	-
Superoxide dismutase 2, mitochondrial	SOD2	6648	NM_000636.2	-	-	-	2.68[1]	-	-
Glutathione reductase	GSR	2936	NM_000637.2	-	-	2.38[3]	-	-	-2.54[2]
Transcription factor Sp1	SP1	6667	NM_001251825.1 NM_003109.1 NM_138473.2	3.91[1]	-	-	-	-	-
Cytochrome b-245 light chain	CYBA	1535	NM_000101.2	-	-3.77[1]	2.61[2]	-	-	-

Gene name	Gene symbol	Entrez_ID	RefSeq_ID	Dyslipidemic			Normolipidemic		
				Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$	Ratio $t_{4h} : t_0$	Ratio $t_{t1} : t_0$	Ratio $t_{12} : t_0$
oxidative stress pathway [continuation]									
Heme oxygenase 1	HMOX1	3162	NM_002133.1	-	-8.57[1]	-17.52[1]	-	-	-
Glutathione peroxidase 3	GPX3	2878	NM_002084.3	-	-	-2.15[1]	-	-	-
NAD(P)H dehydrogenase [quinone] 1	NQO1	1728	NM_001025434.1 NM_001025433.1 NM_000903.2	-	4.66[1]	-	-	-	-
Catalase	CAT	847	NM_001752.2	-	13.15[1]	8.90[1]	-	-	-

- no regulation
1 slightly significant regulation; p = 0.05
2 significant regulation; p < 0.05
3 highly significant regulation; p < 0.01

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Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel:

„Effects of omega-3 polyunsaturated fatty acids on gene expression profiles of normo- and dyslipidemic men."

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, 15.08.2012

_____

Simone Bianca Schmidt

Lebenslauf

Persönliche Daten

Name	Simone Bianca Schmidt
Geburtsdatum/-ort	07.09.1984 in Hannover
Familienstand	ledig

Studium

seit 03/2009	Promotionsstudium am Institut für Lebensmittelwissenschaft und Humanernährung der Leibniz Universität Hannover
10/2008	Studium Gartenbauwissenschaften (Master of Science), Leibniz Universität Hannover Masterarbeit: Untersuchung der genetischen Diversität von *Diplocarpon rosae* und deren Bedeutung für die Resistenzzüchtung an Rosen
10/2006	Studium Gartenbauwissenschaften (Bachelor of Science), Leibniz Universität Hannover Bachelorarbeit: Energiepflanzen – eine Alternative für die Gewächshausbeheizung?

Schulabschluss

07/2003	Allgemeine Hochschulreife, St. Ursula Schule Hannover

Erweiternd zum Studium

08/2006 - 09/2006	Julius Kühn-Institut - Bundesforschungsinstitut für Kulturpflanzen (ehemals Biologische Bundesanstalt), Institut für Pflanzenschutz im Ackerbau und Grünland in Braunschweig Untersuchungsschwerpunkt: Fusarium-Resistenz
03/2005	Gärtnerei Tangermann, Nordstemmen Aufgabengebiet: Aufzuchtbedingungen von Zierpflanzen
09/2003	Baumschulbetrieb Turnau, Pattensen Aufgabengebiet: Grundlagen des Gehölzschnittes und der Veredlung (speziell Okulation)

Sprachkenntnisse

Englisch	Fließend in Wort und Schrift
Französisch	Grundkenntnisse
Latein	Grundkenntnisse