

The Effects of an Intake of Lean and Fatty Fish on PBMC Gene Expression in Healthy Subjects

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Johanne Kjellevik Ledang

Summary

The underlying molecular mechanisms of health effects related to high intake of fish are not completely understood. Fatty fish contain marine long-chain n-3 fatty acids which may modulate blood lipids and inflammatory markers. However, the effects of lean fish are poorly investigated. Peripheral blood mononuclear cells (PBMCs) are immune cells comprising primarily of lymphocytes and monocytes, which may be primed by the environment in the circulation, and are thus considered a good model system reflecting an *in vivo* inflammatory situation.

In the present master thesis, material from a previously randomized trial was analyzed. Healthy adults ($n = 30$) with the median age 24 (22-27) years and mean BMI 23.4 (22.1-24.4) kg/m² were allocated to receive 150 g of salmon, cod, blue mussel or potato daily for 15 days. Blood samples were collected at baseline and at the end of the study. Circulating inflammatory markers were investigated with enzyme linked immuno sorbant assay (ELISA). PBMC gene expression of 44 genes was analyzed with real-time quantitative polymerase chain reaction (RT-qPCR) with LDA-cards.

High density lipoprotein (HDL) -cholesterol was increased in the salmon group compared to the other groups. Triglycerides (TG) were decreased in the salmon, cod and blue mussel group compared to the potato group. Concentrations of the n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were increased in the salmon and blue mussel group, and DHA was increased in the cod group. Circulating inflammatory markers did not change between any of the groups, although high sensitivity C-reactive protein (hsCRP) was decreased within the blue mussel group. The PBMC gene expression of phospholipase A₂ group VIA (PLA2G4A) was increased in the cod and salmon group compared to the potato group. Furthermore, 12 genes had increased PBMC gene expression within the salmon group.

These findings are in line with some other studies which have investigated gene expression of inflammatory markers after intake of n-3 PUFAs in similar study populations. The physiological consequences of such an increase are not clarified.

List of abbreviations:

CVD	cardiovascular disease
CHD	coronary heart disease
CAD	coronary artery disease
MI	myocardial infarction
SMC	smooth muscle cell
PBMC	peripheral blood mononuclear cell
ICAM1	intracellular adhesion molecule 1
VCAM1	vascular cell-adhesion molecule 1
TLR	toll like receptor
TNF	tumor necrosis factor
TGF	transforming growth factor
MMP	matrix metalloprotease
APC	antigen presenting cell
PAMP	pathogen associated molecular pattern
DAMP	damage-associated molecular pattern molecules
PPR	pattern recognition receptor
MHC	major histocompatibility complex molecules
CTL	cytotoxic T-lymphocyte
CD	cluster of differentiation
ECM	extra cellular matrix
TF	transcription factors
NR	nuclear receptors
IL	interleukine
PPAR	peroxisome proliferator-activated receptors
NFkB	nuclear factor kappa B
CRP	C-reactive protein
hsCRP	high-sensitivity CRP
PLA2	phospholipase A2
TG	triglyceride
LDL	low density lipoprotein
HDL	high density lipoprotein
RT qPCT	real time quantitative polymerase chain reaction
qPCR	quantitative polymerase chain reaction
ELISA	enzyme linked immuno sorbant assay
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
ALA	α linoleic acid
ARA	arachidonic acid
LT	leukotriene
PG	prostaglandine

FA	fatty acid
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
PG	phospholipid
LF	lean fish
FF	fatty fish
n 3	omega 3 fatty acids
n 6	omega 6 fatty acids
AHA	American Heart Association
ROS	reactive oxygen species
MIQUE	Minimum information for publication of Quantitative Real Time PCR Experiments
MetS	metabolic syndrome
RCT	randomized controlled trial
MeHg	methyl mercury
PCB	polychloric biphenyls
Ig	immunoglobuline

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1 Background

1.1 Cardiovascular disease

1.1.1 Definitions and underlying mechanisms

Cardiovascular disease (CVD) is a collective term for all diseases in the heart and vessels, including coronary heart disease (CHD), stroke and congenital heart disease (1). Examples of diseases in the heart and circulation are myocardial infarction, stroke and hypertension (2). Myocardial infarction (MI) can be caused by reduced blood flow to the heart muscle due to narrow, stiff or clotted coronary arteries (3). A stroke is either hemorrhagic, caused by bleeding in a vessel walls, or ischemic due to a clot or thrombus in arteries preventing oxygenated blood from the heart to reach the brain (4).

Table 1: Different types of cardiovascular diseases, based on Cotran et al (5).

Cardiovascular Diseases		
<i>Coronary Heart Disease</i>	<i>Cerebrovascular Disease</i>	<i>Peripheral vascular disease</i>
Myocardial infarction	Stroke	Atherosclerosis
Congenital heart disease	- hemorrhagic	Hypertension
Heart failure	- ischemic	Occlusion of arteries or veins
Arrhythmias		- thrombosis
Endocarditis/infections		- emboli
Rheumatoid heart disease		Vasculitis
Neoplastic heart disease		Aneurisms
Angina		Dissections

Incidence

According to the Global Atlas on Cardiovascular Disease Prevention and Control published by World Health Organization in 2011, CVD is the leading cause of death worldwide, and responsible for 17.3 million deaths every year. Myocardial infarctions are responsible for 7.3 million of these, while stroke led to 6.2 million deaths (4). Even though the incidence of death caused by cardiovascular disease has declined in Norway since the 1970's, the Norwegian Health Institute calculated that approximately 320 000 individuals were treated for cardiovascular related conditions in hospitals in 2010, and it still is the major cause of death

in Norway (6, 7).

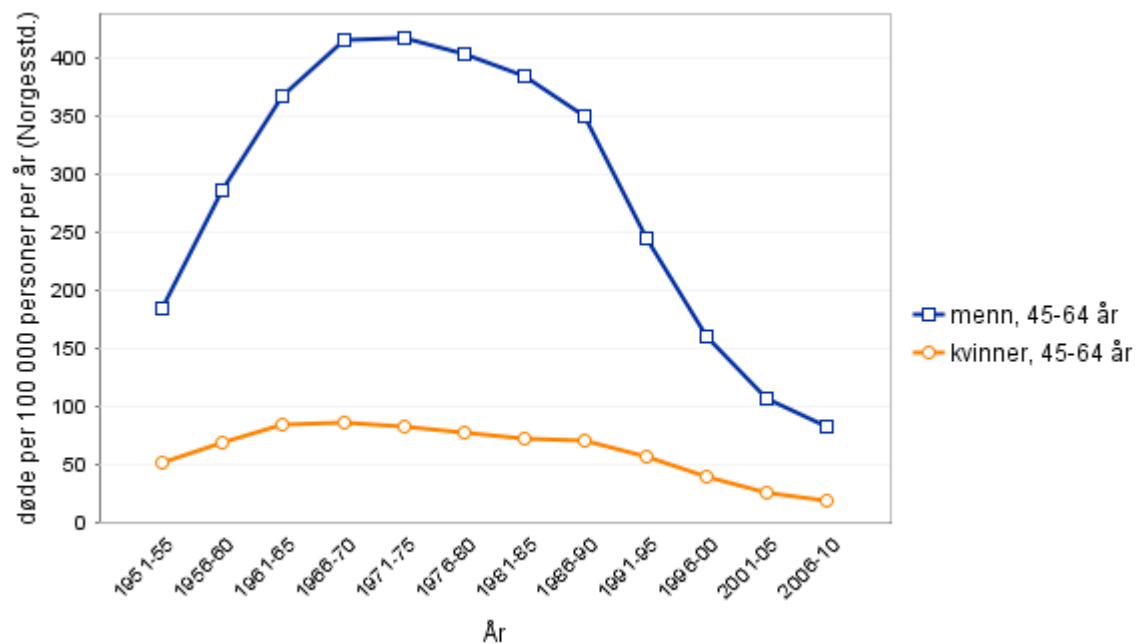


Figure 1: Mortality from myocardial infarction and other ischemic heart diseases in Norway, 1951 – 2010. Deaths per 100 000, standardized for age (6).

1.1.2 Risk factors for CVD

CVD occurs earlier in men than women, and the incidence increases with age for both sexes. Family history of CVD is another individual risk factor (7). African-Americans have a higher incidence than Caucasians in the UK, and Asians are more prone to stroke than CHD when compared to Caucasians (8). The Western style diet, a sedentary lifestyle and smoking are factors that correlate with increased CVD. Imbalance between caloric intake and caloric expenditure, excessive body fat and fatty acid composition of the diet, especially intake of trans fatty acids and saturated fatty acids (SFAs), are related to increased CVD (9).

Both genetic and environmental factors play a role in the development of CVD, thus some suggest that 70% of CVD can be prevented by means of dietary and lifestyle changes (10). WHO divides modifiable risk factors which play a role in the etiology of atherosclerosis into behavioral and metabolic risk factors (**table 2**). The behavioral ones are defined as life style related risk factors. The metabolic risk factors are often related to repercussions of life style. The WHO definition of an unhealthy diet which increases the risk of CVD consists of a low

intake of fruit, vegetables and fish as well as a high intake of saturated fat, trans-fat, cholesterol and salt. (4).

Table 2: Categories of risk factors for developing cardiovascular disease, based on the WHO-report Global Atlas on Cardiovascular Disease Prevention and Control (4)

Risk factors for CVD		
<i>Non-modifiable</i>	<i>Modifiable</i>	
	<i>Behavioral risk factors</i>	<i>Metabolic risk factors</i>
Sex (male > women)	Tobacco use	Hyperlipidemia
Ethnicity	Unhealthy diet	Hypertension
Age (increasing with age)	Physical inactivity	High blood sugar
Genetic inheritance (i.e FH)	Harmful use of alcohol	Overweight/obesity
Family history of CVD		

1.2 Fish and cardiovascular disease

In the search of dietary factors which might have a protective effect on the incidence of CVD there has been a focus on fish for decades. Populations with a high intake of fish show patterns of lower incidence of CVD (11). Some of the many observational studies which have aimed at investigating the relationship between the intake of fish and cardiovascular events will be described here. Randomized controlled trials (RCTs) have been carried out in a variety of populations and patient groups. These have mainly comprised of supplementation of n-3 polyunsaturated fatty acids (PUFAs), generating varying results. Nutritional aspects and current dietary guidelines regarding the intake of fish will also be presented below.

1.2.1 Observational and intervention trials

Fish as dietary component

In the Seven Countries Study, a lower incidence of coronary heart disease (CHD) in Greece and Japan than in Finland was demonstrated (10). An inverse relationship between the intake of fish and the risk of 25-year mortality from CHD was discovered. All though this association disappeared when confounding factors such as saturated fatty acids (SFAs) and smoking were adjusted for (12). This inverse association has later been described by several observational studies among healthy subjects. In a study among Swedes with no history of

CHD, the subjects consuming the most fish had the lowest incidence of CHD mortality during 14 years of follow up. Amounts of fish were not reported, but the fish-consumption was divided into low, medium or high (13). Though the Physicians Health Study did not find any associations between consumption of fish and the risk of CVD, they found that intake of fish was related to a decreased risk of all-cause mortality (14). Two studies which only included male participants concluded that an intake of fish reduced the risk of death from CVD. The intake was ≥ 35 g of fish daily, and once or twice a week, both compared to no intake of fish, respectively (15, 16). The same correlation has been seen among healthy women. In The Nurse's Health Study there was an inverse association between fish intake, n-3 fatty acids and CVD death (17). An Australian study with female subjects combined the intake of fish and n-3 PUFA, and found that those in the highest tertile (1.1 – 4.7 g/day) of dietary n-3 PUFAs had a reduced risk of inflammatory mortality when compared to the lowest tertile (0.0 – 0.73 g/day) (18). Analyses of healthy Swedish women revealed that the intake of ≥ 3 servings per week of lean fish compared to none or once a week, was inversely related to the incidence of stroke (19). The inverse relationship between intake of fish and CHD-mortality was found in a healthy elderly population (≥ 70 years) during 17 years of follow-up. Subjects were described as fish-consumers and non-fish consumers. Among the fish-consumers the mean intake of fish was 24 g/day. (20). Amongst diverse ethnicities in the Multi-Ethnic Study of Atherosclerosis (MESA) the inverse relationship between circulating marine n-3 PUFA and CVD incidence was also described. The authors suggest that an increased intake of marine derived n-3 PUFA from fish or supplementation may reduce the incidence of CVD (21).

There are studies which failed to find such an inverse correlation. A sub-analyses of the German study population in the European Prospective Investigation into Cancer and Nutrition (EPIC-study) found that the median intake of fish was 16.4 g daily. Participants had no history of MI or stroke, and during 8.1 years of follow-up, no relationship between consumption of fish and risk of MI or stroke was found (22).

Among CVD patients in The Diet and Re-infarction Trial (DART) 2033 subjects with myocardial infarction (MI) were allocated to three groups of different dietary advice. The advice was to eat fatty fish 2 – 3 times per week (equal to about 300 g) or consume 500 – 900 mg n-3 PUFA capsules daily, or to reduce the intake of fat along with an increase in PUFA, or

to increase the dietary fiber intake. The investigators found a 29% reduction in all-cause mortality in the fish/n-3 PUFA advice group during 2 years (23).

In summary, several observational studies describe an inverse relationship between intake of fish and CHD-mortality among healthy individuals (13, 16, 17, 20). Some have also found a reduced risk of stroke (19) and all-cause mortality (14), whereas others have found no correlation (22). A meta-analysis from 2004 investigated observational cohorts, and concluded that there is an inverse relationship between the intake of fish and the risk of cardiac death was present. The authors suggested that an increment of 20 g fish daily will reduce CHD death by 7%. (24). A meta-analysis published in 2012 pooled data on fish-consumption from 17 cohort-studies, and found that 1 to 4 servings of fish per week had a protective effect on cardiovascular mortality. It was further suggested that an increased intake of fish will reduce the risk of CHD mortality by 6% per 15 g/day (25). In a recent review Kromhout et al suggest that as primary prevention of CVD, intake of fish may protect against stroke (26). Others report that the most prominent associations between fish consumption and primary prevention of CVD have been found when there are large segments of the population which do not eat fish (27).

n-3 PUFA intervention trials

Several interventions have been conducted with n-3 PUFAs instead of fish. The majority of these studies have been designed as secondary prevention of CVD. Some have found promising effects from n-3 PUFA supplementation. The Italian GISSI-prevenzione trial included patients within 3 months of MI ($n = 11\ 324$). They received either 885 mg n-3 eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) daily and/or vitamin-E or placebo. After 3.5 years follow-up there was a 20% reduction in all-cause mortality in the n-3 PUFA group. Results further indicated a 30% reduction in coronary artery disease (CAD) death and a 45% reduction in sudden death. No effect could be ascribed to vitamin-E (28). Patients ($n = 7046$) fulfilling the American Heart Association (AHA) criteria for heart failure class II – IV were randomized to 1 g/dag of n-3 EPA/DHA or placebo in the Italian multi-center GISSI-HF trial. After a follow-up of 3.9 years a 9% reduction in all-cause mortality in the n-3 EPA/DHA group was revealed. The interpretation was that there is a small beneficial effect of n-3 PUFAs in patients with heart-failure (29).

Other studies have found little or no effect of n-3 PUFA supplementation in CVD-patients. In the Japanese placebo-controlled RCT JELIS participants ($n = 18\,645$) with hypercholesterolemia (total cholesterol >6.5 mmol/l) were assigned to 1800 mg n-3 EPA and statins (20 mg pravastatin or 10 mg simvastatin), or statin medication alone, with follow-up for 4.6 years. In terms of secondary prophylaxis, major coronary events were significantly reduced (19%) in the group receiving EPA and statins. In subjects with no history of CAD there was also a reduction, all though not significant (30). In the AlphaOmega RCT, MI survivors ($n = 4837$) were provided with different types of margarines for 4 months, alongside antihypertensive, antithrombotic and/or lipid-modifying therapy according to medical needs. The margarines consisted of 400 mg n-3 EPA/DHA daily and/or n-3 1.9g alpha-linoleic acid (ALA) or placebo. No reducing effects on major/deadly cardiovascular events attributable to n-3 were found, either between or within the groups (31). Another supplementation trial, Omega, enrolled German MI survivors ($n = 3851$). The infarction had occurred 3 – 14 days prior to enrollment, thus they were treated according to current guidelines regarding MI. Participants received either 1 g n-3 EPA/DHA or olive oil (serving as placebo) capsules daily for one year. The end points were mortality and nonfatal clinical events related to CVD. No reduction or effect applicable to n-3 was found (32). The SU.FUL.OM 3 RCT was a multicenter trial conducted in France. Subjects ($n = 2501$) with MI, angina or ischemic stroke were randomized to acquire the B-vitamins folate, B-6 and B-12 and/or 600 mg n-3 EPA/DHA daily, or placebo. The follow-up lasted for 4.7 years. End points were CVD related death, nonfatal MI or stroke. Concentration of B-vitamins and n-3 increased in plasma, but no reduction in end points were seen (33).

Intervention studies with n-3 PUFA supplementation as part of secondary prevention of CVD have generated varying results. Some have seen a reduced risk of all-cause mortality (28, 29). Others have failed to see prominent effects of the supplementation (30-33). A recent meta-analysis concluded that supplementation of n-3 PUFA in CVD patients does not prevent major coronary events, but reduces mortality. It further suggests that trials with supplementation of 2 – 4 g EPA/DHA daily might see greater impact on secondary prevention of CVD with longer follow-up periods (27). A state-of-the-art paper from 2011 concluded that the accordance between observational studies with clinical end points, RCTs that are appropriately powered and experimental trials is sufficient to determine that a modest n-3

PUFA consumption compared none reduces CHD mortality (34). Chowdhury et al further conclude that n-3 PUFA is related to reduced risk of coronary disease (35).

Meta-analyses and reviews

In a review from 2014 Kromhout et al summarize the cardio-metabolic health effects from both n-3 PUFA supplementation interventions and prospective cohort studies with dietary intake of fish. The writers conclude that the evidence of positive effects from n-3 PUFA intake on fatal CHD is strong, but that the evidence concerning other cardiovascular diseases and diabetes is less consistent (26). They also emphasize that the low absolute risk of CVD due to efficient drug-treatment might cause the weaker association between n-3 PUFAs and fatal CHD (26). Chowdhury et al included both observational and RCTs in their meta-analysis, and found an association between dietary intake of n-3 PUFAs and reduced risk of CVD. They underline that more data is needed among healthy subjects for a more complete understanding of the effects in such populations. Finally they conclude that there is not enough basis to support current cardiovascular guidelines which advocate a high intake of PUFAs, while the intake of total saturated fatty acids should be decreased (35).

1.2.2 Nutritional aspects of fish

Fatty fish is the primary source of marine n-3 polyunsaturated fatty acids (PUFAs) in the diet (36-38). The proteins in fish have a high bio-availability in humans, due to a low content of connective tissue (2% in marine fish species, 13% in red meat). Essential amino acids are present at high concentrations. Among these are valine, leucine, threonine, lysine and tryptophan. Another valuable amino acid which has been related to reduction of CVD is taurine (39). In blue mussels there is 510 mg taurine per 100 g wet weight, whereas cod and farmed salmon contain 120 and 94 mg/100 g, respectively. Even though the amounts depend on the fish species, fish contain vitamins A, D, B6 and B12. Increased vitamin D levels have been related to improved bone mass in women (40). Low vitamin D status is associated to an increase in BMI which may dispose for diabetes (41). Furthermore there is a low content of sodium alongside a high content of potassium (a ratio of 1:10). Fish is also an essential source of selenium and iodine in the diet (38).

Some fish species are also related to high concentrations of methyl mercury (MeHg), and data from The National Health and Nutrition Examination Survey (NHANES) revealed an association between the intake of fish and levels of MeHg (37). Other contaminants such as polychloric biphenyls (PCB) and inorganic arsenic are the reasons for advice on caution when choosing what fish to eat (42, 43). In addition to nutritional aspects, the burden on wild stock and concentration of toxins should be considered when publishing dietary guidelines. Thus investigators suggest a cost-benefit approach on recommendations regarding fish consumption (42, 43).

1.2.3 Current dietary guidelines

The US Dietary Guidelines recommend that Americans should consume 8 oz, equal to about 230 g, seafood each week, and AHA suggest that eating fish twice a week reduces the risk of developing CVD (44). The Norwegian Food Based Dietary Guidelines recommend a diet with an amount of fish equal to 2 - 3 dinner servings per week, in order to promote health. With a high consumption of fatty fish, the intake of heavy metals may exceed recommended concentrations. Hence the restrictions on the intake of fatty fish (200 g/week), while the consumption of lean fish species does not have this limitation (45). Consuming the recommended amount of fish will give rise to adequate levels of n-3 PUFAs, vitamin D, selenium and iodine and prevent an intake above upper tolerable limits of inorganic arsenic, dioxins, lead and cadmium and other harmful trace-elements which are present in fish (38, 45).

The third population-based dietary survey conducted in Norway between 2010 and 2011 (NORKOST 3) suggests that 31% of women and 39% of men meet the recommended amount of intake of fish. Among the oldest group in the survey (60 – 70 years) 42% consumed fish for dinner or lunch 3 times per week, while this was true for only 16% in the youngest responder group (18 – 29 years). Nevertheless 60% in the youngest group and 53% of the oldest responders did have fish for dinner or lunch once or twice a week. Out of the total energy intake, fish was responsible for 4E%, and 9E% of dietary polyunsaturated fat. While 40% of vitamin D was estimated to be generated from fish products (46). The Nordic Nutrition Recommendations from 2012 (NNR 2012) conclude that the intake of fish and seafood should be increased in the general population, and that 1E% should be obtained from

n-3 PUFAs. It is emphasized that there is no scientific evidence for that the use of supplementation can compensate for or replace a healthy diet (47).

1.3 Atherosclerosis as an inflammatory disease

The number one underlying mechanism causing CVD is atherosclerosis. Atherosclerosis is associated with ischemic and coronary heart disease, cerebrovascular disease and disease in the aorta and arteries. Furthermore it relates to hypertension and peripheral vascular disease (4). The development of atherosclerosis is a complex process, in which increasing amounts of oxidized low density lipoprotein (LDL) particles, smooth muscle cells, endothelial cells and different cellular components of the immune system accumulate in the intima layer of arteries (11, 48). Intimal thickening and lipid accumulation commence as fatty streaks which develop into fibrous plaques. The plaques may rupture and cause thrombosis (5). Mononuclear cells (lymphocytes and monocytes) promote adhesion and migration of additional inflammatory cells into the endothelium in the arteries during this process. Monocytes differentiate into macrophages. These cells engulf LDL-particles and release pro-inflammatory cytokines. Dyslipidemia and inflammation are accelerating components of the progression (48, 49).

1.3.1 Inflammation

Inflammation is defined as a complex reaction of the innate immune system, and can be initiated at the site of infection as a reaction to toxin exposure or cell injury in vascularized tissues (50). Acute inflammation is an immediate response with the cardinal signs of redness, swelling, heat, pain and loss of function. These cardinal signs occur due to vascular dilation. The dilation allows increased blood flow, extravasation of plasma proteins and fluid to surrounding tissue, and leukocyte migration to the site of injury. Chronic inflammation is described as one of prolonged duration, in which the attempts to repair damage occur simultaneously with tissue destruction (5). Thus, even though inflammation serves as a protective function which controls infection and promotes tissue repair, it can also cause tissue damage and disease (50). When regulated properly by negative feedback loops, inflammation is essential to maintain health. Pathological inflammation involves loss of regulatory processes or imbalances in the production of cell- or mediator components (51).

Low-grade chronic inflammation is associated with an increased risk of CVD, insulin resistance and type 2-diabetes (52). Tissue and vascular damage, intracellular lipid accumulation and increased insulin resistance occur, even though there is no overt pathology. Mononuclear cells, neutrophils and adipocytes are involved. Cytokines, reactive oxygen species (ROS) and eicosanoids are some of the mediators. Due to lack of pathologic manifestations, the condition rarely is treated with anti-inflammatory pharmaceuticals, thus nutrition is thought to have an influence. Some protective dietary components that have been suggested are fish and PUFAs, especially the marine n-3 PUFAs (52, 53).

1.3.2 A brief overview of inflammatory cells

Innate immunity is described as the first line of non-specific host defense. It further triggers and directs adaptive immune responses (54). Microbes express molecules which differ from mammalian cells, such as endotoxin and mannose residues, collectively termed pathogen-associated molecular patterns (PAMPs). The cells of innate immunity recognize PAMPs through pattern recognition receptors (PRRs), such as toll-like receptors, which promote autophagy or phagocytosis. Macrophages and granulocytes phagocytize foreign pathogens or injured cells, and secrete cytokines which attract other inflammatory cells. Injured host cells or tissues express damage-associated molecular pattern molecules (DAMPs), which are recognized and exposed to the same process of phagocytosis (55). Macrophages and dendritic cells are referred to as antigen presenting cells (APCs) and display parts of proteins from antigens on major histocompatibility complex molecules (MHCs) (50, 56).

The cells in adaptive immunity need to be presented to an antigen by APCs to be activated. Naïve T-lymphocytes circulate peripheral lymphoid organs. Upon antigen presentation, they are activated and differentiate into different subsets of effector and memory cells. CD8⁺ T-lymphocytes display MHCI, and are called cytotoxic T-lymphocytes (CTLs) as they recognize and kill infected cells. CD4⁺ T-lymphocytes express MHCII, and differentiate into T helper 1, T helper 2 or T helper-regulatory lymphocytes. They are referred to as helper lymphocytes since they secrete cytokines which activate macrophages and promote proliferation and differentiation of additional T- and B-lymphocytes. The CD4⁺ subset produces memory cells which reside in the peripheral lymphoid organs and recognize an antigen upon next encounter, promoting a more rapid immune response (50). B-lymphocytes

are able to recognize non-protein antigens and ingest them through endocytosis. Parts of the antigen are presented to CD4+ T-lymphocytes. CD40/CD40L linkage between B- and T-lymphocytes and secretion of cytokines from the T-cells will activate B-lymphocytes. They differentiate into plasma cells which secrete the different subsets of immunoglobulins (IgA, IgE, IgG and IgM) depending on cytokine exposure (50). Pro-inflammatory stimulation of CD40 leads to activation of NF- κ B pathway and transcription of inflammatory and pro-thrombotic mediators (57).

Table 3: A simplified overview of some of the immune cells, based on Abbas and Libbyl (50)

Immune cells	
Innate immunity	Adaptive immunity
Phagocytes	T-lymphocytes
- Monocytes/macrophages	- CD4+ helper cells
- Granulocytes	- CD8+ cytotoxic cells
Natural killer cells	B-lymphocytes
Antigen presenting cells	-Plasma cells
- Dendritic cells	- Immunoglobulins
- Macrophages	
Mast cells	

Cell-cell interactions are affected by cytokine secretion, and cytokine secretion is modulated through stimuli from the cells and surrounding environment (58). There is far more extensive knowledge on interactions between the cells of the innate and adaptive immunity, and their responses to cytokine exposure, which will not be presented here.

1.3.3 Inflammatory properties of atherosclerosis

From fatty streaks to plaques

Atherosclerosis is now recognized as a chronic inflammatory disease, as immune cells are involved in all stages of the development of an atheroma (2, 3, 5, 51, 59, 60). The atherosclerotic process constitutes of an imbalance in lipid metabolism and inflammatory responses. Endothelial dysfunction also plays an important role in the atherosclerotic development. (61). The dysfunction can be initiated by the lack of vasoactive substances such as nitric oxide (NO), maintained activation of endothelial cells or minor endothelial injuries caused by disturbed blood flow and shear stress on the vessel walls. This leads to impaired endothelial-dependent vasodilation (61). Elastin is reduced in the endothelial cells lining the vessels, which allows lipids and oxidized LDL particles to infiltrate the intima layer (60). Activation of endothelial cells leads to an increased expression and release of adhesion molecules, such as E-selectin and vascular cell-adhesion molecule 1 (VCAM-1) from endothelial cells and macrophages. Blood-borne inflammatory cells are then attracted to the site of perceived injury. Monocytes and lymphocytes infiltrate the vessel wall. The inflammatory response in the underlying intima makes the present cells produce cytokines, growth factors and macrophage colony-stimulating factor. These molecules make monocytes differentiate into macrophages. Innate immune cells express scavenger receptors and toll-like receptors-4 and -6 (TLR-4 and-6). TLRs bind to molecules with PAMPs, and the binding activates macrophages. The activated macrophages release NO and radical oxygen species (ROS) which harm surrounding tissue and cells (3). C-reactive protein (CRP) is an acute phase reactant produced by hepatocytes in the liver as a response to inflammatory cytokines. Leucocytes and smooth muscle cells (SMCs) secrete interleukine (IL) -6 in response to infection and trauma or atherosclerosis. CRP may facilitate monocyte infiltration to vessel walls and it binds to oxidized LDL-cholesterol (62).

Fatty streaks are the initial step of atherosclerosis and may develop into unstable plaques when lipids accumulate within the arterial wall. The plaque is prone to rupture (48). Fatty streaks predominantly consist of T-cells, monocytes and macrophages laden with lipids, forming foam cells and apoptotic cells (59). Activated CD4⁺ T-lymphocytes differentiate into Th1 effector cells which secrete cytokines. Cytokines promote further migration of

inflammatory cells to the atherosclerotic lesion (3). Pro-inflammatory cytokines such as interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α) and interleukin-1 (IL-1) are secreted from T-cells, mast-cells and dendritic cells, and cause further immune cell infiltration. The cytokine IFN- γ is macrophage stimulating and increases the efficiency of antigen presentation. Antigen presentation further promotes synthesis of TNF- α and IL-1 (3). Macrophage apoptosis occurs throughout the whole process, which leads to additional secretion of pro-inflammatory cytokines (60, 63). Antibody producing B-cells from the spleen and activation of Th2 T-cells have more anti-inflammatory properties, as they produce cytokines such as transforming growth factor β (TGF- β) and IL-10 which have anti-atherogenic effects (3). From CD4⁺ cells, the regulatory subset of T-cells (T-reg) can differentiate in presence of TGF- β and IL-10 (64). Thus the atherosclerotic process can be slowed down by the anti-inflammatory cytokines IL-10 and TGF β , as well as T-regulatory cells (60).

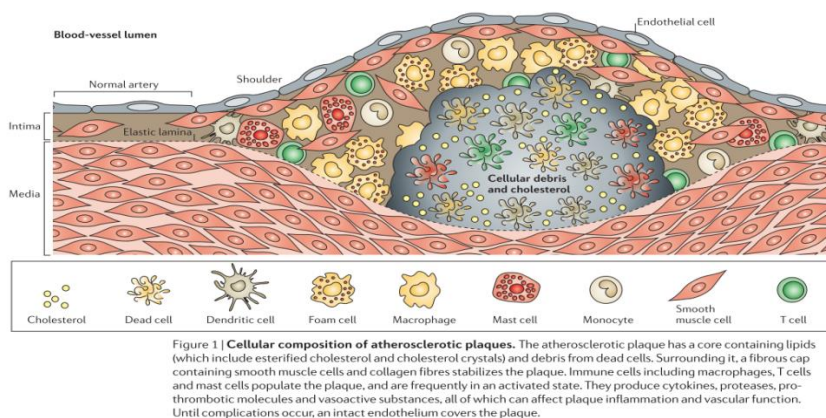


Figure 2: Cellular components of atherosclerotic plaques, reproduced with permission from Hansson GK et al 2006 (2).

Destabilization of atherosclerotic plaques

If the developing plaque is stable, it is characterized by a fibrous cap with smooth muscle cells (SMCs), collagen, elastin and extra cellular matrix (ECM). These components contribute to thickening of the cap. In cases of unstable plaques the cell content is highly inflammatory, thus macrophages and apoptosis of SMCs dominate. This enhances a large necrotic core and reduced amount of ECM components. Furthermore it stimulates secretion of cytokines and infiltration of additional inflammatory cells, as well as a weakening of the fibrous cap (60).

Neutrophil granulocytes and macrophages secrete matrix metalloproteases (MMPs) and cysteine proteases, which degrade the fibrous cap and weaken it (3, 60). An unstable plaque is more likely to rupture. The rupture of a plaque initiates the coagulation cascade because pro-thrombotic material, such as phospholipids, platelet adhesive molecules and tissue factor, is exposed to the blood. Parts of the plaque can act as a thrombus which prevents the circulation, ultimately leading to acute cardiovascular events such as myocardial infarction or stroke (3, 59, 60). In individuals at high risk of CVD an influenza vaccine was found to be protective of CVD through modulation of inflammatory mechanisms (65, 66).

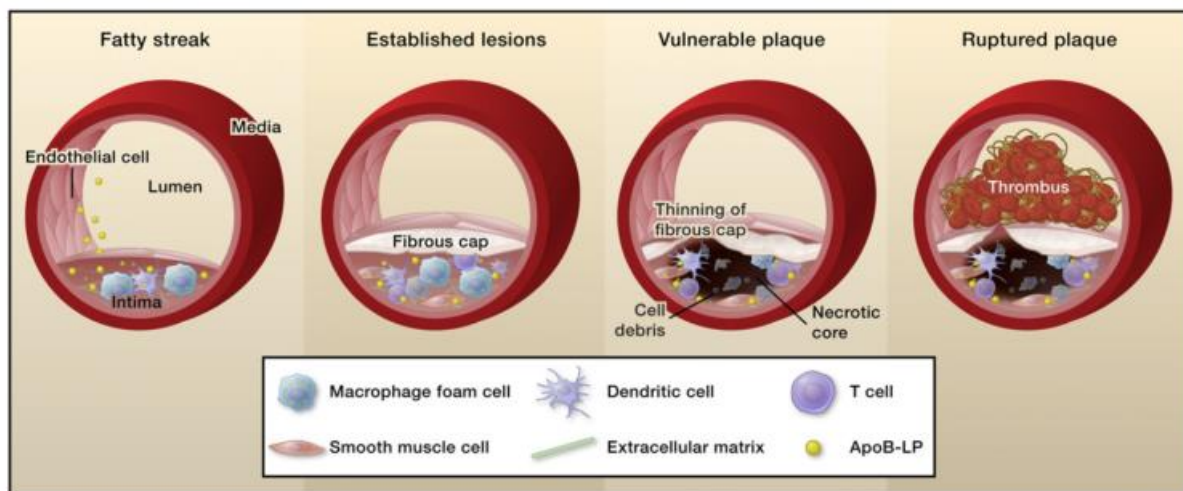


Figure 1. Progression of an Atherosclerotic Lesion

Early fatty streak lesions are characterized by the accumulation of apolipoprotein B-containing lipoproteins (apoB-LPs) in the subendothelial space, which incites the recruitment of dendritic cells and macrophages. As the atherosclerotic lesion progresses, smooth muscle and T cells also infiltrate the intima, and apoB-LP retention is amplified. Vulnerable plaques are characterized by the accumulation of apoptotic cells and defective phagocytic clearance (efferocytosis), resulting in the lipid-filled necrotic core. A thinning fibrous cap decreases lesion stability, making these atherosclerotic plaques susceptible to rupture and the formation of a thrombus.

Figure 3: Progression of an atherosclerotic plaque. Reproduced with permission from Moore et al, year 2011, doi: 10.106/j.cell.2011.04.005 (67).

Inflammatory markers as indicators of disease

Circulating levels of biomarkers may help detect early stages of atherosclerosis, prior to manifest CVD (62). Soluble CAM-molecules (such as ICAM1 and VCAM1) have been validated as predictors of inflammation and endothelial dysfunction, and may serve as biomarkers of CVD (26). High sensitivity C-reactive protein (hsCRP) is another biomarker which have received attention for its promising effects on predicting CVD, although one review suggests that the levels of circulating hsCRP should be interpreted in accordance with measurements of other biomarker and potential risk factors of CVD (62). The application of predictive and valid biomarkers is crucial when assessing health status. It needs to reflect the

inflammatory process and predict future development and/or prevention of disease (52). It should be noted that the expression and level of inflammatory markers can be modified by several factors, when designing studies and when interpreting subsequent results, as presented in **figure 4** (53).

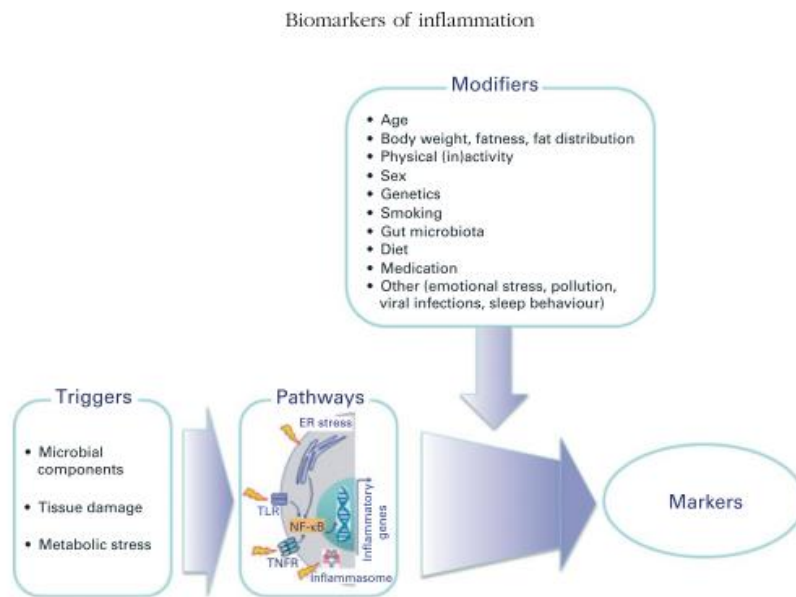


Figure 4: Biomarkers of inflammation, triggers, pathways and modifiers. Reproduced with permission from Calder PC et al, 2013 (52).

1.4 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) have two or more double bonds that are not saturated with hydrogen in their carbon chain (68). They all have a carboxyl group at one end and a methyl group at the opposing end, denoted as the n- or ω -end. The carbon number from the methyl group at which the first double bond is placed is part of the denotation. This decides whether a PUFA is part of the n-3 or n-6 family (68). The n-3 PUFAs in the human diet are predominantly obtained from fatty fish species such as salmon and mackerel, or from fish oil supplementation (69).

Mammalian cells lack the desaturase enzymes needed to convert oleic acid to linoleic acid (LA, C18:2 n-6) and alpha-linolenic acid (ALA, C18:3 n-3). Thus these are considered

essential fatty acids which need to be provided by the diet (70). LA (C18:2 n-6) and ALA (C18:3 n-3) are members of the n-6 and n-3 family, respectively. They can be converted by desaturation and elongation by the same enzymes, to either arachidonic acid (ARA, C20:4, n-6) or eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) (68).

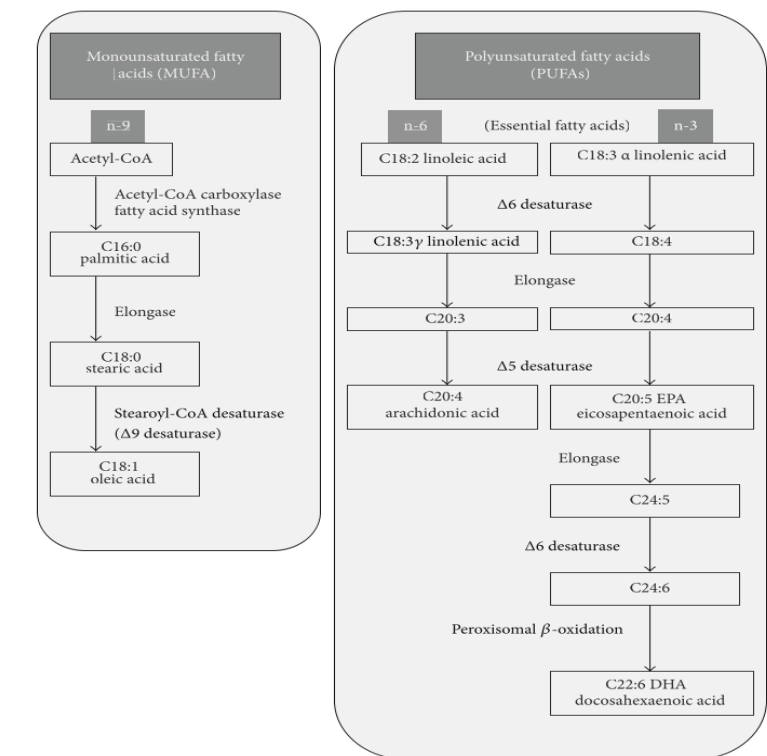


Figure 5: The metabolic pathways for elongation and desaturation of fatty acids. Reproduced with permission from Catala, 2013 (68).

1.4.1 Properties of n-3 PUFAs

PUFAs interact with G-protein coupled receptors (GPRs), transcription factors (TFs) and nuclear receptors (NRs), such as GPR-120, nuclear factor kappa B (NF-κB) and peroxisome proliferator-activated receptors (PPARs), respectively. These interactions modulate inflammatory responses and lipid metabolism (71). The NF-κB TF resides in the cytoplasm of the cell due to binding of protein inhibitor I-κB (I-κB). When I-κB is phosphorylated by cytokines, viruses or lipopolysaccharide (LPS) from bacteria, NF-κB is released and translocate to the nucleus. In the nucleus NF-κB modulates several genes involved in inflammation. NF-κB increases the expression of cytokines (e.g. IL-6, TNF-α), chemokines

(e.g. MCP-1) and inducible enzymes (e.g. iNOS, COX2, PLA2). NF- κ B furthermore may increase the expression of VCAM1 and ICAM1 (71). PPAR- α , - δ and - γ are NRs which forms heterodimers with retinoid X receptor (RXR). The heterodimer binds to response elements in the promoter region of target genes and modulates their expression (72). These target genes are involved in inflammation and lipid metabolism. The n-3 PUFAs EPA and DHA have high affinity for PPAR- α and - γ . Activation of PPAR- α and - γ can reduce NF- κ B binding activity. PPAR α may prevent VCAM1 expression in cytokine-affected smooth muscle cells. PPAR α also induces increased expression of genes involved in lipid oxidation, which may lead to reduced levels of TG in plasma and liver (72). PPAR- γ can prevent activated monocytes and macrophages from releasing cytokines, such as IL1 β , IL-6 and TNF α , to the circulation (71). n-3 PUFAs are ligands for GPR-120. Upon binding to n-3, GPR-120 can inhibit NF- κ B through activation of PPAR- γ (73). PUFAs precede eicosanoid production which serves as precursors for leukotrienes, thromboxanes and prostaglandins. PUFAs are incorporated into cell membranes and affect the cell membranes physiologic structure and function. n-3 PUFAs are also involved in cholesterol metabolism and are part of infant brain development (74). The composition of cell membranes and their concentration of either ARA or DHA and EPA affect functions and properties of the cell, such as membrane fluidity, production of cell signaling molecules and the substrates for eicosanoid production (75).

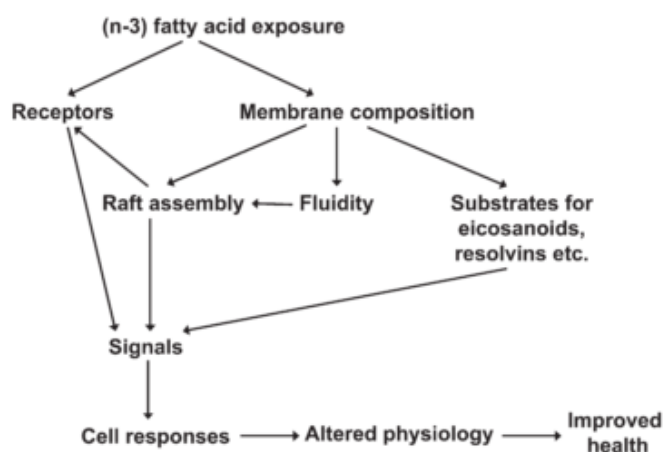


Figure 6: Cellular functions of *n*-3 PUFAs. Reproduced with permission from Calder PC 2012 (75).

The enzyme Phospholipase A₂ releases free fatty acids from cell membranes. Enzymatic pathways involving cyclooxygenase (COX) and lipoxygenase (LOX) produce downstream eicosanoids from the released fatty acids (76). Even though these eicosanoids resemble, the ones originating from EPA and DHA tend to exhibit less potent effects than the ones from ARA (77). The cells involved in the inflammatory responses usually have a high proportion of ARA in their membranes, thus their downstream eicosanoids predominantly have pro-inflammatory effects. If the intake of EPA and DHA is increased through diet or supplementation, incorporation into cell membranes will occur in a dose-response matter, partly at the expense of ARA. The metabolite production is altered as these fatty acids compete for the same downstream enzymes (77).

Table 4: Eicosanoids produced from different PUFA-precursors. Table based on Calder PC, 2011 (78).

Precursor	Eicosanoids			
	<i>Prostaglandines</i>	<i>Leukotrienes</i>	<i>Resolvins</i>	<i>Protectins</i>
ARA (C20:4, n-6)	2-series PGs	4-series LTs	-	-
EPA (C20:5, n-3)	3-series PGs	5-series LTs	E-series	-
DHA (C22:6, n-3)	-	-	D-series	D-series

The 2-series of prostaglandins and 4-series of leukotrienes have a high pro-inflammatory potential, while the 3-series and 5-series of prostaglandins and leukotrienes, respectively, have a low pro-inflammatory potential. Resolvins are produced from both EPA and DHA, while protectins originate from DHA. Both resolvins and protectins are considered anti-inflammatory (48, 78).

1.5 PBMC as model system

PBMCs are a central part of the immune system, and comprise of lymphocytes, monocytes and macrophages. As PBMCs are in contact with the arterial walls, they may serve as reporters of disease. These cells also affect the secretion of cytokines that mediate inflammation and adhesion of smooth muscle cells and endothelial cells, which narrow the lumen of arteries during the atherosclerotic process (79). They have been suggested as a promising agent for interpreting the sub-clinical state of CVD (80). Others suggest that alterations in the expression of circulating leukocytes may be used as biomarkers of inflammation (52).

2 Aims

The molecular effects of fish consumption on inflammatory markers have not yet been thoroughly investigated, and very little is known about the intake of fatty compared with lean fish on inflammatory markers.

The primary aim of this thesis was therefore to investigate the effect of intake of 150 g seafood for 15 days on inflammatory markers measured both at PBMC gene expression and in circulation.

3 Materials

Equipment	Producer	Country
NUNC Cryotubes, 1.8 mL (PN7325568)	Thermo Scientific	Waltham, MA, USA
Megablocks, 96 wells (PN 821972002)	Sarstedt	Numbrecht, Germany
Microtiter plates, 96 wells (PN 7325568)	VWR International LLC	West Chester, PA, USA
CPT tubes (reorder No 362761)	Beckton, Dickinson and Company	Franklin Lakes, NJ, USA
Sterile Pasteur pipettes (PN 6121682)	VWR International LLC	West Chester PA, USA
Falcon Serological pipette, 10 ml (PN 357551)	Beckton, Dickinson and Company	Franklin Lakes, NJ, USA
Pipeteboy Acu	IBS Integrated	Lewisberry, PA, USA
Falcon Tubes, 50 ml (PN 352070)	Thermo Scientific	Waltham, MA, USA
Falcon Tubes, 15 ml (PN 352099)	Thermo Scientific	Waltham, MA, USA
Axygen Sterile Eppendorf tubes (PN MCT 400-C-S)	Corning	Amsterdam, The Netherlands
Rneasy Mini Spin Column (PN 1011708)	Qiagen	Hilden, Germany
Collection tubes (PN 1017981)	Qiagen	Hilden, Germany
Finnpipette Novus Single- Channel Pipettors, 1 - 10µl (PN 46200100), 10 - 100 µl (PN 46200400)	Thermo Scientific	Waltham, MA, USA
Finnpipette Novus Multi- Channel Pipettors, 1 - 10µl (PN 46300100), 10 - 100 µl (PN 46200400)	Thermo Scientific	Waltham, MA, USA
Impact Equalizer Matrix 8-channel pipetter (PN 2032)	Thermo Scientific	Waltham, MA, USA
Pipette Tips	Biotix	San Diego, USA
Pipette Filtertips	Biotix	San Diego, USA
Strips (PN AB-0266)	Thermo Scientific	Waltham, MA, USA
Lids for strips (PN AB -0784)	Thermo Scientific	Waltham, MA, USA
96 well plate (PN AB-0900)	Thermo Scientific	Waltham, MA, USA
MicroAmp® 96-Well Reaction Plate (PN N8010560)	Applied Biosystems	Foster City, CA, USA
MicroAmp® Optical Adhesive Film (PN 4311971)	Applied Biosystems	Foster City, CA, USA
TaqMan® Low Density Array Cards (PN 4342265)	Applied Biosystems	Foster City, CA, USA

Chemicals	Producer	Country
PBS (PN D8537)	Sigma-Aldrich	St. Louis, MO, USA
BSA (KA 41-001)	The Cell Culture Company	St. Louis, MO, USA
Stop Solution (H ₂ SO ₄) (PN 30149.291)	VWR International LLC	West Chester PA, USA
TRIzol® Reagent	Invitrogen	Carlsbad, CA USA
Nuclease free H ₂ O (PN AM9938)	Ambion, Inc.	Austin, TX, USA
100% ethanol (PN 601557)	Arcus Kjemi	Oslo, Norway
RNase Zap (Cat No 9780)	Ambion, Inc.	Austin, TX, USA
TaqMan® Gene Expression Master Mix (PN 4369016)	Applied Biosystems	Foster City, CA, USA
TaqMan® Gene Expression Assays 20X (PN 4335626)	Applied Biosystems	Foster City, CA, USA

Kits	Producer	Country
DuoSet VCAM1 Kit (Cat no DY 809)	R&D Systems Europe, Ltd	Abingdon OX, UK
Quantikine VCAM1 Kit (Cat no DCRP0)	R&D Systems Europe, Ltd	Abingdon OX, UK
Quantikine ICAM1 Kit (Cat no DVC00)	R&D Systems Europe, Ltd	Abingdon OX, UK
Quantikine hsCRP Kit (Cat no DCD540)	R&D Systems Europe, Ltd	Abingdon OX, UK
RNeasy MiniKit (Cat No 74104)	Qiagen	Hilden, Germany
RNA 6000 NanoKit (Reorder No 5067-1511)	Agilent Technologies Inc.	Waldbronn, Germany
High Capacity RNA-to-cDNA Kit (Sku# 4387406)	Agilent Technologies Inc.	Waldbronn, Germany

Instruments	Producer	Country
Lab Dancer (Art no 444-0004)	VWR International LLC	PA, USA
Spectrafuge™ Mini Complete (PN C1301 - 230V)	Labnet International	Edison, NJ, USA
MPS 1000 Mini PCR Plate Spinner (PN C1000 - 230EU)	Labnet International	Edison, NJ, USA
Heraeus Labfuge 400	Thermo Scientific	Waltham, MA, USA
Heraeus Biofuge Fresco	Thermo Scientific	Waltham, MA, USA
Eppendorf centrifuge 5424	Eppendorf	Hamburg, Germany
Heraeus Multifuge 3S+	Thermo Scientific	Waltham, MA, USA
Thermo Well Wash 4MK2, VWR (PN 5160770)	Thermo Scientific	Waltham, MA, USA
EIA Thermo Multiscan EX	Thermo Scientific	Waltham, MA, USA
NanoDrop Spectrophotometre (ND)-1000	Saveen & Werner	Malmö, Sweden
Agilent 2100 Bioanalyzer	Agilent Technologies Inc.	Waldbronn, Germany
NanoChip priming station (PN 5065-4401)	Agilent Technologies Inc.	Waldbronn, Germany
IKA vortexer (MS2-S8)	Agilent Technologies Inc.	Waldbronn, Germany
Veriti 96 well Thermal Cycler	Applied Biosystems	Foster City, CA, USA
7900 HT Fast Real-Time PCR System	Applied Biosystems	Foster City, CA, USA

Software	Producer	Country
Genlite Software, v 3.03	Genesis Labsystems	UK
NanoDrop ND-1000, v 3.8.1	Thermo Scientific	Waltham, MA, USA
Agilent 2100 Expert Software	Agilent Technologies Inc.	Waldbronn, Germany
SDS software, v 2.4	Applied Biosystems	Foster City, CA, USA
RQ Manager software, v 1.2.1	Applied Biosystems	Foster City, CA, USA
GraphPad Prism 5.0	GraphPad Software Inc.	La Jolla, CA, USA
SPSS statistics software, v 20	IBM	Armonk, NY, USA
Microsoft Office XP 2010	Microsoft Corporation	Redmont, WA, USA
EndNote version 7.0		

4 Subjects and methods

4.1 Study design

The original randomized controlled parallel group study lasted for 37 days. It consisted of a 15 days semi-controlled diet period, two run-in periods of 7 days, and two periods with a strictly controlled diet each lasting for 3 days. This design is reported in detail elsewhere (81, 82). In this master thesis, all analyses are executed on material from the semi-controlled diet period lasting for 15 consecutive days. Four different types of test meals were served at Akershus University College (**figure7**). The meals were similar, differing only on whether they consisted of 150 g of seafood or potato. All participants were encouraged to eat all the food served and to maintain their physical activity routines during the intervention period (81).

From five weeks prior to day 0 throughout the intervention period, participants were to avoid intake of cod liver oil (a food supplement commonly used in Norway). They were also instructed not to use other dietary supplements and to avoid additional seafood than what they were served in the study. The study was approved by the National Committee for Research Ethics and was carried out in accordance with The Code of Ethics of the World Medical Association. All participants gave their written and informed consent (82).

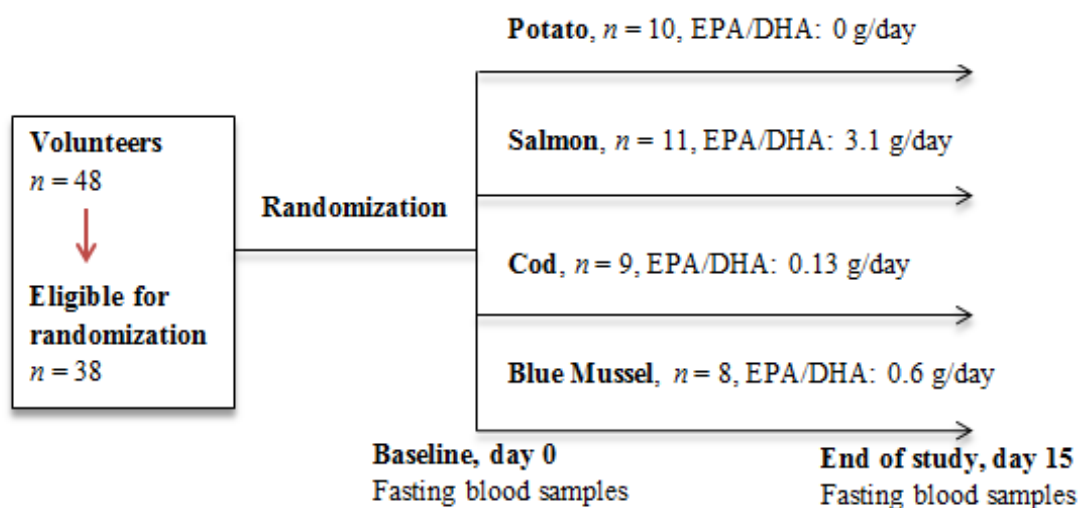


Figure 7: Study design of the parallel intervention trial

4.1.1 Participants

From 48 potential participants, 28 women and 10 men eligible for randomization were recruited from Akershus University College, Norway, in March 2006. Exclusion criteria were C-reactive protein >10 mmol/L, smoking, pregnancy, lactation or the use of other medical drugs than contraceptives. Subjects with a seafood consumption higher than what is recommended in the Norwegian Food Based Dietary Guidelines (>three servings per week) were also excluded. The participants were randomized into four different intervention groups, consuming 150 g of either cod (*Gardus morhua*) (n=9), farmed salmon (*Salmo salar*) (n=11), blue mussel (*Mytilus edulis*) (n=8) or potato (control) (n=10) daily (81). Though participants were randomized, they were not stratified for gender, thus all the participants in the salmon group were women by chance (**figure 2**) (83). All participants were compliant with the protocol throughout the study. Compliance was estimated from the amount of leftovers from the trial-food, and was calculated to be between 93 and 100 % during the intervention (81).

4.1.2 Test meals

Other than the test meals, food items and drinks were consumed ad libitum, but within the food restrictions described. Test meals were served as lunch at the University College, except for on the weekends when participants were equipped with lunchboxes. In addition a final test meal was served as breakfast at the University College on day 15. The menu consisted of both warm and cold dishes that were identical except for the 150 g seafood/potato. A homogenous mixture of cod or salmon fillets was prepared as fish puddings and cut into cubes. Potatoes were cooked and cut into cubes. The blue mussels were steamed for 10 minutes, removed from their shells, and stored in a freezer at -20°C until serving (81, 82). The blue mussels were purchased from Safjord Shellfish (Varaneset, Norway), and examined for algal toxins by the Norwegian Food Safety Authority. Their estimated n-3 content was 0.6 g per meal (84). The cod was bought from Ural Nor Fish AS (Moldtustranda, Norway), and the salmon was obtained from Coast Seafood AS (Måløy, Norway). The n-3 content of the fish was analyzed by Nifes, and the EPA/DHA content in one meal was 3.1 g and 0.13 g in the salmon and cod, respectively (83). The cod and the salmon were already cleaned and filleted when purchased (83).

4.1.3 Blood samples and biochemical analyses

Blood samples were collected from fasting subjects at baseline and at the end of the study.

Plasma obtained from EDTA tubes were kept at room temperature (0-30min) and centrifuged at 1300 g for 10min. All plasma samples were kept frozen (-70° C) until analysis (82). For determination of total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides, Først Medical Laboratory, Norway performed routine laboratory analyses (83).

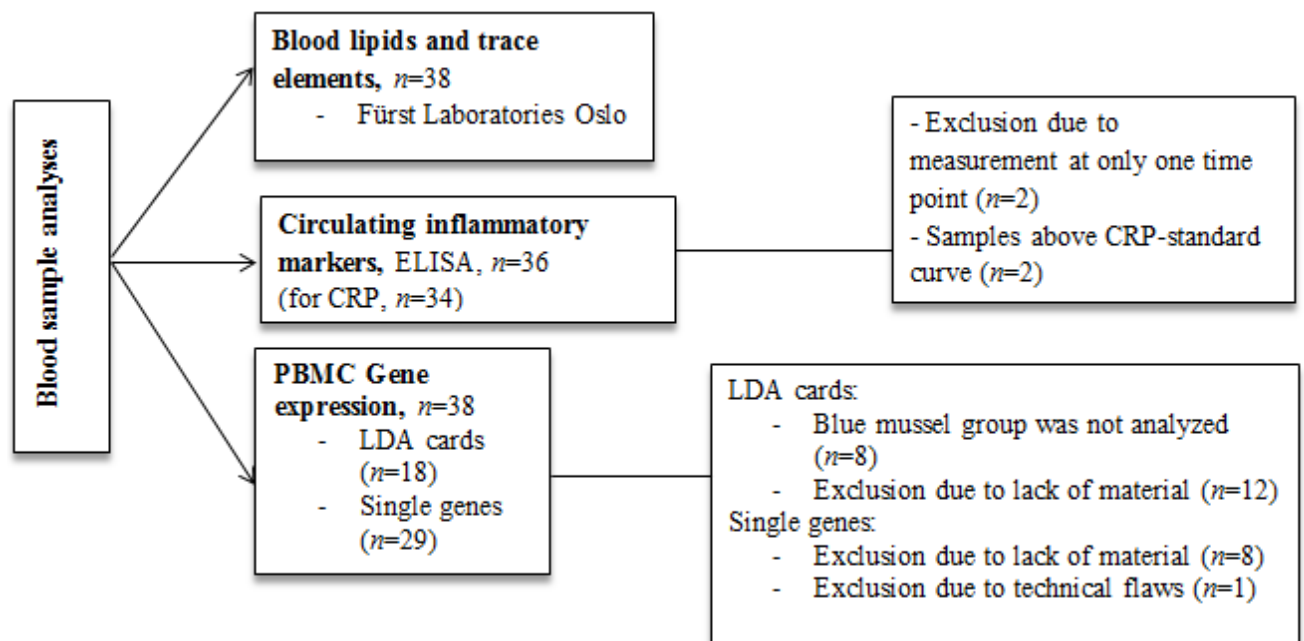


Figure 8: Blood sample treatment and analyses.

4.2 Laboratory analyses

4.2.1 Circulating inflammatory markers

ELISA

Enzyme Linked Immuno Sorbent Assay (ELISA) is a well-recognized method used to quantify the concentration of circulating proteins (85). Its principle is built upon the reaction between antibody and antigen, a so-called sandwich reaction, wherein antibody, antigen and antibody bind to each-other in relation to the concentration of a certain antigen in a sample. An enzyme/substrate binding to the second antibody will cause development of color corresponding to the concentration of the antibody, and can be read off and translated (86). In the Quantikine Kits from R&D Systems, microtiter plates are pre-coated with antibodies that react with the added analyte (87).

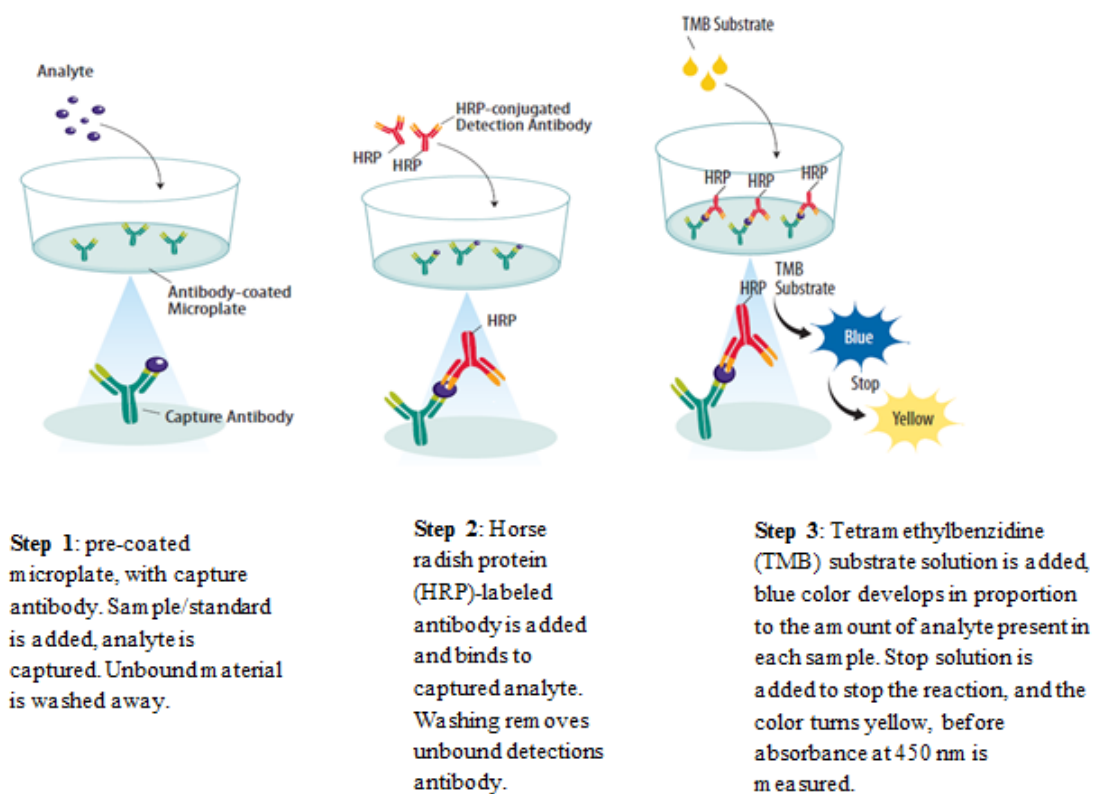


Figure 9: Steps in the ELISA QuantiKine procedure. Reproduced from ELISA Reference Guide and Catalog, with rights from R&D Systems (86).

Intra-assay precision

In order to validate pipetting performance and accuracy, intra-assay precision was measured, and CV% <10 was considered well (88). One plate with VCAM1 Duo Set standards was examined (Cat no DY 809, R&D Systems Europe, Ltd, Abingdon OX14 3NB, UK).

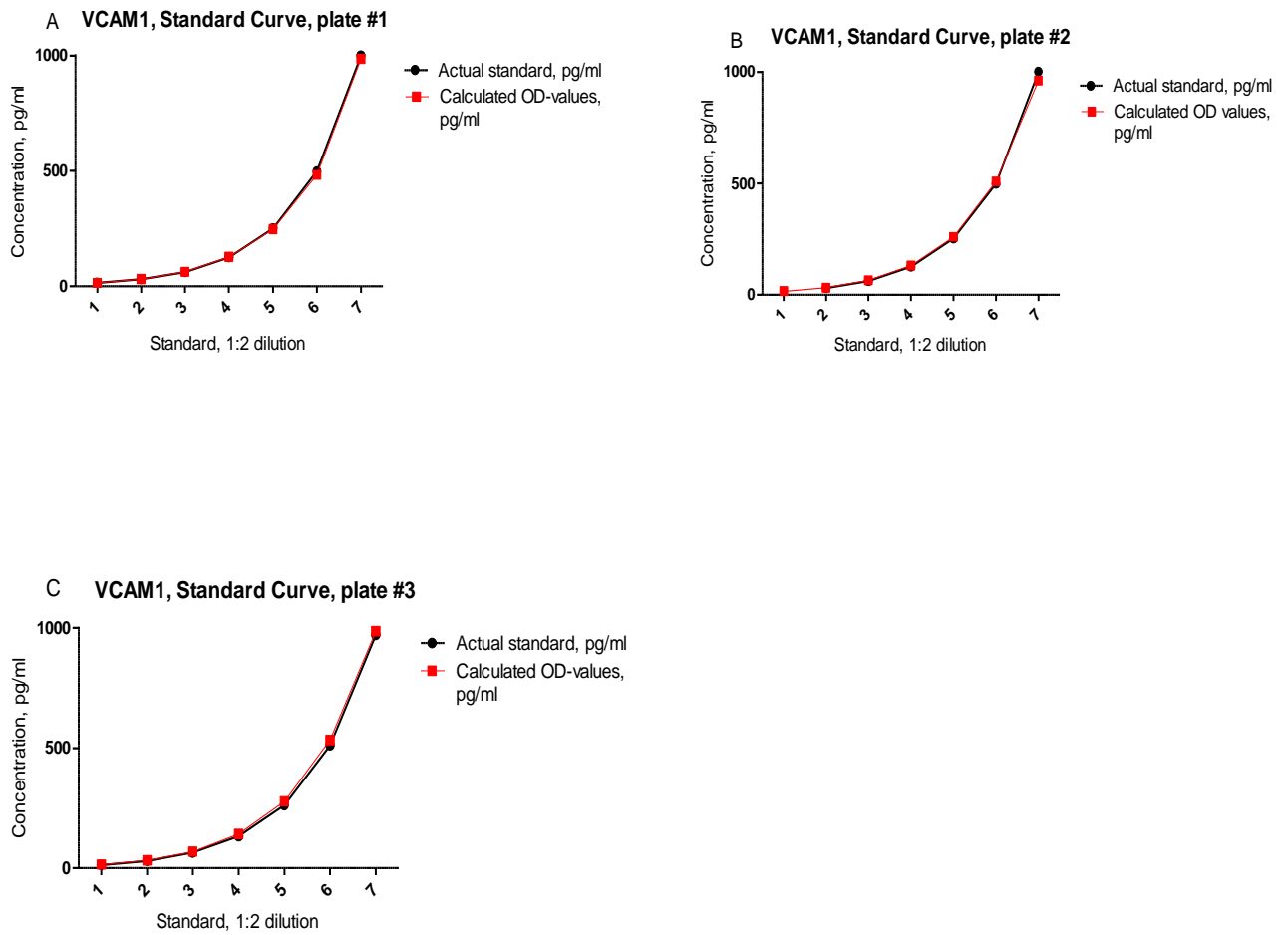
Table 5: Intra-assay precision to validate pipetting

Intra assay precision, VCAM1, DuoSet, R&D Systems
Replicates, n = 10

Standard, pg/ml	Mean (SD), pg/ml	CV%
1000.3	986,4 (32,7)	3,3
498.6	482,5 (13,3)	2,7
251.8	248,6 (7,6)	3,1
125.8	127,5 (4,1)	3,2
61.3	63,6 (1,9)	2,9
31.0	31,5 (1,2)	3,6
15.5	14,9 (1,5)	8,1

Standard curves

For further validation of pipetting three plates with 2:1 dilution of VCAM1 DuoSet standards were prepared (Cat No DY 809 R&D Systems Europe, OX, UK). Standard curves were compared with the mean of 10 samples for each standard in the 2:1 dilution. Mean concentration for the dilutions in the standard curves from the 3 plates were as follows: 990.4 pg/ml – 502.4 pg/ml – 255.6 pg/ml – 128.4 pg/ml – 62.5 pg/ml – 30.7 pg/ml – 14.6 pg/ml. Results are displayed in **figures 10 A – C**.



Figures 10 A – C: Standard curves from 3 plates with DuoSet VCAM1 standards. Black line indicates the actual measured standard (pg/ml), red line indicates the calculated OD-values in each of the three plates (pg/ml).

Protocol

Preparations

Plasma EDTA samples were aliquot and added to six mega-blocks (PN 821972002, Sarstedt, Numbrecht, Germany) for ELISA Quantikine human sVCAM1/CD106, human sICAM1/CD54 and human hsCRP (R&D Systems Europe, OX, UK). Contents and product numbers from all kits are listed in **table 7**. Samples were diluted in mega-blocks according to each kit protocol, with pertaining Calibrator Diluent. Reagents were diluted and prepared as protocols instructed. Automatic washer (Thermo Well Wash 4MK2 VWR, PN 5160770, Thermo Scientific, Waltham, MA, USA) was used to wash the plates with wash buffer, made from 20 ml Wash Buffer Concentrate (PN 895003, R&D Systems Europe, OX, UK) and 480 ml MilliQ distilled water. For preparation of 100 ml Calibrator Diluents RD5P (1X), 20 ml RD5P Concentrate was added to 80 ml MilliQ. The Calibrator Diluent RD5-7 was not diluted. Standards were prepared in a 1:2 series with concentrations presented in **table 6**.

Table 6: Concentrations of standards in the different QuantiKine kits

Protein	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
sVCAM1, ng/ml	5.00	2.50	1.250	0.625	0.313	0.156	None
sICAM1, ng/ml	50.00	25.00	12.50	6.25	3.13	1.56	None
hsCRP, ng/ml	50.00	25.00	12.50	6.25	3.13	1.56	0.78

Execution

Conjugate Solutions were ready-made and added to each well on the plates for the sICAM1 and sVCAM1 kits. For the CRP-kit, 100µl of Assay Diluent RD1F (PN 895041, R&D Systems Europe, Ltd, OX, UK) was added as the first solution. Samples, standards and blanks were added in duplicates to each plate according to plate set-up. After incubation in room temperature plates were washed x 4. For the hsCRP kit, hsCRP Conjugate was then added and plates were incubated again, before a second wash x 4. Substrate Solution was prepared by mixing Color Reagents A and B within 15 minutes of use for all kits, and added to all wells. After incubation at room temperature in the dark, Stop Solution (H₂SO₄) was added, before the plates were read off with EIA Thermo Multiscan EX. Computer was set with Genlite Program, wavelengths at 450 and 540 nm, log-by-log scale. OD-values were set in relation to the standard curves, and dilutions from each kit were registered for all plates. Values above or below highest or lowest standard were taken out of the result file.

Table 7: Contents, dilutions, volumes and particle numbers (PN) in the different QuantiKine kits

Protein	sVCAM1	sICAM1	hsCRP
Catalog number	DCRP00	DVC00	DCD540
Plate, PN	892717	893602	893167
Dilution of plasma	1:20	1:20	1:100
Calibrator Diluent (PN)	RD5P (1X) (895151)	RD5-7 (895045)	RD5P (1X) (895151)
µl sample/std/blank (PN, std)	100 (893604)	100 (893604)	50 (893169)
No of incubations (time)	2 (1.5h + 20 min)	2 (1.5h + 30 min)	3 (2h x 2 + 30 min)
µl Conjugate solution (PN)	100 (892718)	100 (893603)	200 (893168)
µl Substrate solution*	100	200	200
µl Stop solution **	50	50	50

µl is the volume added to each well

* Substrate solutions were the same for all kits: made by mixing equal volumes of color reagent A (PN 895000) and color reagent B (PN 895001)

** Stop solutions were the same for all kits (PN 895032)

4.2.2 Gene expression analyses

Isolation of PBMCs

For collection and isolation of PBMCs, venous blood (8 ml) was drawn into BD vacutainer Cell Preparation Tubes (CPT) containing the anti-coagulant sodium heparin and a separation medium consisting of thixotrope gel and FICOLLTM HypaqueTM fluid. The separation medium with a gradient for density causes cells with higher density (granulocytes and erythrocytes) to appear below the gel-layer, preventing them to lyse or interfere with other cell fractions (89). PBMCs and platelets have a lower density, and form a whitish layer above the gel-membrane, just beneath the plasma layer, and were removed for use in further analyses (**figure 11**) (90).

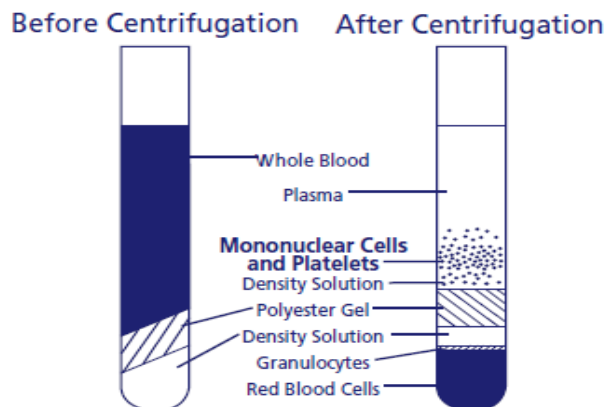


Figure 11: CPTs before and after centrifugation. Reproduced with the rights from Becton, Dickinson and Company, from CPT protocol ref (90).

Protocol:

Venous blood was collected in CPTs (reorder No 362761, Beckton, Dickinson and Company, Franklin Lakes, NJ, USA), according to the producer's protocol, including inverting tubes 8 to 10 times immediately after blood collection and prior to centrifugation for 25 minutes at 2900 rpm/1636 g without brake, within two hours of blood collection (90).

PBMCs were transferred to a 15 ml Falcon tube with a sterile Pasteur Pipette (PN 6121682, VWR International LLC, West Chester, PA, USA). To accomplish removal of platelets 10 ml phosphate buffered saline (PBS) (PN D8537, Sigma-Aldrich, St. Louis, MO, USA) was added, tubes were inverted 2-3 times, followed by centrifugation for 10 minutes at 1300 rpm/300 g with brake. Supernatant was removed, and the washing procedure was repeated. After removing the supernatant the pellet was dissolved 500-100 μ l PBS, and transferred to an Eppendorf-tube, which was centrifuged at 13000 rpm for 3 minutes at 4° C. The supernatant was removed, and pellet stored dry at -80° C.

Isolation of RNA

For further down-stream gene expression analyses, total RNA has to be isolated from the PBMCs. Firstly cells need to be lysed and homogenized, for which there are several applicable methods. One of them is adding TRIzol® Reagent (PN 15596026 Invitrogen, Carlsbad, CA, USA) and chloroform to each sample, separating the sample into three phases:

an aqueous phase with RNA, an interphase with DNA and a red organic phase with proteins and lipids. The RNA is precipitated from the aqueous phase with isopropanol, and transferred to a new tube (91). For the gene expression analyses, lyses and homogenization of cells was followed by use of RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA was transferred to a spin column and treated according to further instructions in the RNeasy Mini Kit protocol (83). Due to toxic and carcinogenic properties of the TRIzol® Reagent, its usage can be replaced by buffer RLT with DTT for lysis and homogenization of the cells, which was the method applied when I performed this procedure. Hence, the RNeasy Mini Kit protocol is described in detail below. The TRIzol method was used in the original study design. During laboratory training was taught the RNeasy Qiagen protocol.

The RNeasy Mini Kit (Cat No 74104, Qiagen, Hilden, Germany) is a well-established technology for RNA purification, wherein mini spin columns with a silica based membrane can bind up to 100 µg nucleic acids (RNA) longer than 200 base pairs/nucleotides (92). While mRNA usually has this characteristic and binds to the membrane, other fractions of RNA (such as rRNA and tRNA) are shorter, and will not bind (92). As mentioned, the cells have to be lysed and RNA has to be stabilized in order to isolate RNA. The buffer RLT lyses the cells, and due to its high concentration of guanidine isothiocyanate, it also promotes binding to the silica-membrane. To eliminate RNases released during lysis, dithiothreitol (DTT) was added in the RLT buffer. As these solutions are considered hazardous, all work was executed in the hood. Ethanol homogenizes the solution and ensures favorable binding conditions. DNase mix is added to degrade genomic DNA. Wash buffers RW1 and RPE eliminate contaminants (92).

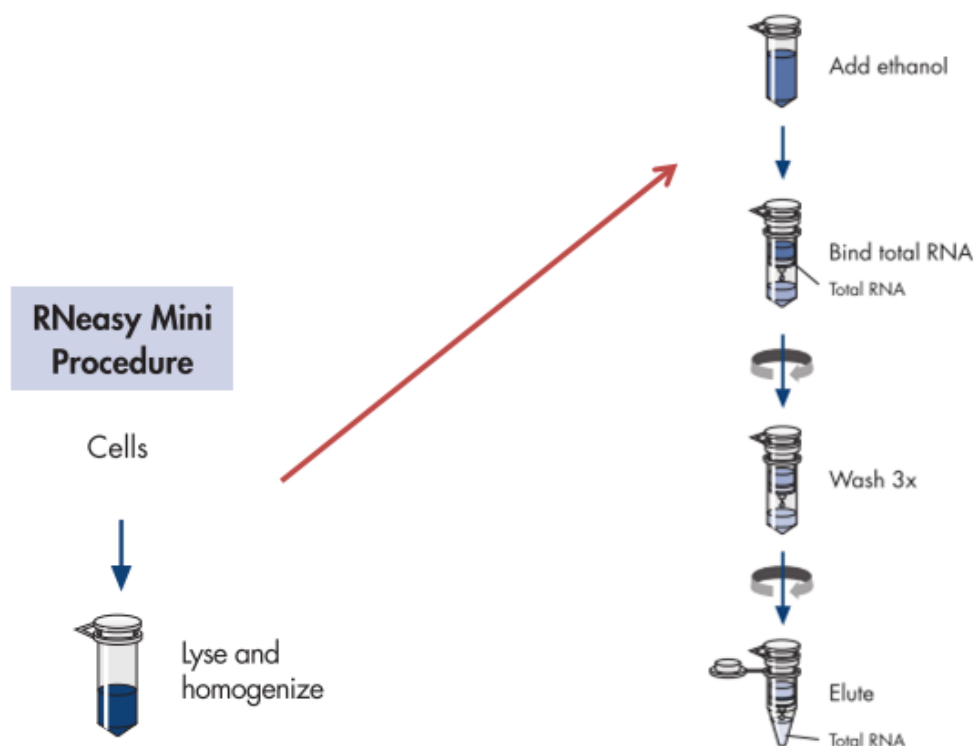


Figure 12: Principles for RNA isolation in the RNeasy Mini Kit. Reproduced from Qiagen RNeasy MiniKit protocol (92).

Protocol:

Preparations on the bench included making RLT buffer with DTT, 70% ethanol and DNase mix. DTT comes as a powder, which is added to 800 μL RNase free H_2O , making 1 mL. For 45 mL RLT buffer 900 μL DTT mixture is needed. 70% ethanol was made by adding 3 mL RNase free water to 7 mL absolute ethanol. DNase mix 1:7 was made from DNase 1 stock (10 μL per sample) and RDD buffer (70 μL per sample).

The PBMC pellets were added 600 μL RLT buffer with DTT and vortexed for 1 minute, one at the time. Ethanol was added to the sample material and the volume was transferred to the column. After the first wash with RW1 buffer, DNase mix was pipetted on to the membrane of the column. Contaminants were further removed by one wash with buffer RW1 and two washes with buffer RPE. RNA was eluted with 30 μL RNase free water. Each sample was aliquot into three marked Eppendorf tubes, containing 4 μL , 11 μL and 15 μL RNA respectively. Tubes were kept in a freezer at -80°C .

Measurement of RNA quality and integrity

Nano Drop

It is necessary to be familiar with the RNA concentration in each sample, as well as making sure that the samples are clean enough for further analyses (93). Nucleotides, RNA, single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) absorb ultra violet (UV) light at the wavelength of 260 nm, while molecules such as phenols and proteins absorb UV-light at 280 nm. The 260/280 ratio reflects the purity of DNA and RNA. A ratio around 2 is generally accepted as a pure RNA-sample, while a higher ratio indicates the presence of proteins. More light absorbed at 260 nm is correlated to a higher concentration of RNA in the sample (94). To evaluate RNA concentration and purity NanoDrop-1000 Spectrophotometer was used.

Protocol:

The program ND-1000 was set to Nucleic Acids, with assay RNA-40. From each sample 1.5 µl RNA was placed on the optical arm of NanoDrop Spectrophotometer (Saveen&Werner, Malmö, Sweden). A second fiber optic cable was placed on top of the sample drop. Light with the wavelengths of 230, 260 and 280 nm was sent through the sample, and the absorbance measured.

Bio Analyzer

RNA is quickly disrupted by RNase enzymes, thus the degradation degree must be evaluated for all samples (95). To assess RNA integrity, Agilent 2100 Bioanalyzer offers an automated and standardized report on RNA integrity number (RIN-value) based on total RNA or mRNA fragment sizes. The RIN-scale ranges from 0 to 10, a low number indicates ribosome degradation during isolation and purification steps. The pictures in **figure 13** demonstrate RIN-values at 10 and 5 (96). Small amounts of RNA from each sample are separated in channels on Agilent RNA 6000 Nanochip from the Agilent RNA 6000 NanoKit. On the basis of molecular weight the fragments are detected by laser-induced fluorescence. Results are visualized as an electropherogram, where the measured fluorescence correlates with the amount of RNA of a given size, shown as a graph with peaks at 28S and 18S (ribosomal RNA). **Figure 14** is a picture of intact rRNA. A ratio of 28S:18S >2 is considered good (95).

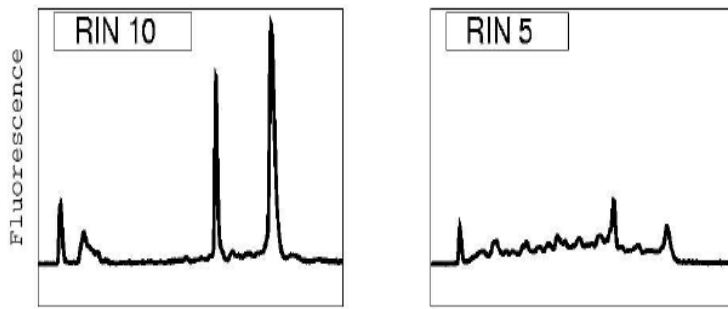


Figure 13: RIN-values at 10 and 5, from RNA samples which are not degraded and degraded, respectively. Reproduced from Schroeder et al, 2006 (95).

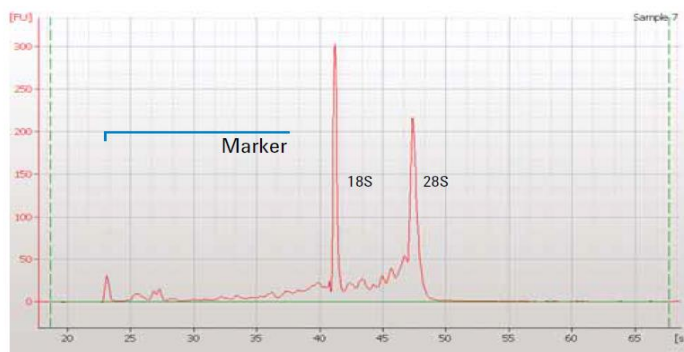


Figure 14: Peaks at 18S and 28S when rRNA fragments are intact. Reproduced from Agilent BioAnalyzer protocol (97).

Protocol:

The Agilent RNA 6000 NanoKit (Reorder No 5067-1511, Agilent Technologies Inc., Waldbronn, Germany) was taken out of the fridge 30 minutes prior to use. The reagents in the kit are sensitive to light, so they needed to be kept inside the box when they were not used. The ladder was taken out of the freezer and placed on a heating block together with the samples for denaturation, at 70 °C for 2 minutes. Gel matrix (550 µl) was filtered in a spin column by centrifugation for 10 minutes at 4000 rpm. To make the gel dye mix 1 µl dye and 65 µl of the filtered gel matrix were transferred to an Eppendorf-tube which was centrifuged for 10 minutes at 14 000 rpm. The Nano Chip was placed in the priming station (Reorder No 5065-4401, Agilent Technologies Inc., Waldbronn, Germany). Gel dye mix was added to all three wells marked with a G. Then 5 µl marker was added to all sample wells, before 1 µl ladder was added to the ladder well. Finally 1 µl from each sample was added to the sample wells. If there were any empty wells, 1 µl marker would replace the sample volume. The chip

was then vortexed in an IKA vortexer (MS2-S8, Agilent Technologies Inc., Waldbronn, Germany) at 2500 rpm for 1 minute and placed in the Agilent 2100 Bioanalyzer (PN, Agilent Technologies Inc., Waldbronn, Germany) within 5 minutes, after electrodes on Agilent 2100 Bioanalyzer were cleansed with RNase free water and RNase Zap (Cat No 9780, Ambion, Inc., Austin, TX, USA). Agilent 2100 Expert Software with assay RNA Eukaryot Nano Series II was used for reading off results, and showed RIN-values between >8.7 (83).

cDNA synthesis

By reversely transcribing RNA to cDNA, a single strand of cDNA is synthesized. This strand serves as template for further amplification (**figure 15**). A reverse transcriptase enzyme and primers are necessary to ensure transcription of all fragments of RNA (98).

To achieve similar concentration of cDNA in all samples for the quantitative polymerase chain reaction/qPCR-run, varying concentrations of RNA were adjusted for in the cDNA synthesis. Volumes of RNA and RNase free H₂O needed for each sample were calculated in advance. A No RT sample with buffers, dNTPs, RNA and no reverse transcriptase enzyme was included in the set-up. The No RT sample is included in order to validate the amount of genomic DNA present in the RNA samples (99).

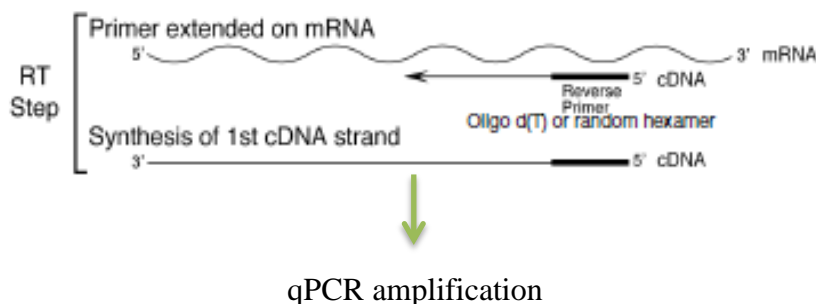


Figure 15: Synthesis of first strand of cDNA. Reproduced from Life Technologies, Applied Biosystems, Real-Time PCR Chemistry Guide (100).

Protocol:

In the High Capacity RNA-to-cDNATM Kit (Sku# 4387406, Applied Biosystems, Foster City, CA, USA), Multi ScribeTM MuLV is acting as the reverse transcriptase enzyme in the Enzyme Mix. In addition to cNTPs, the RT Buffer Mix contains two separate kinds of primers: random octamers and oligo-d(T)₁₆. Random octamers anneal to random complementary sites

on target RNA, ensuring that all fragments are transcribed. Oligo-d(T)16 primer hybridizes to the poly-A tale of eukaryotic mRNA (101).

Samples and solutions from the kit were thawed on ice. Reaction mix was made according to producer's protocol (RT buffer mix 10 μ l + Enzyme mix 1 μ l), 11 μ l for each sample. Each sample was added a corresponding volume of water, lastly sample volumes were added. Lids were placed on top of the plate, before spinning samples down and running the cDNA synthesis on Veriti 96 well Thermal Cycler (PN 43755786, Applied Biosystems, Foster City, CA, USA). The following conditions were set: 37 °C for 60 minutes, 95 °C for 5 minutes, 4 °C hold.

In the LDA-card analyses a RNA concentration of 500 ng/ μ l was used. Due to limited material available, RNA concentration for the single gene analyses was 300 ng/ μ l. This resulted in cDNA amounts of 25 ng/ μ l and 15 ng/ μ l for LDA card analyses and single gene assay analyses, respectively.

For the single genes assay analyses, cDNA samples were diluted 1:5 in RNase free water, to ensure better efficiency in the qPCR reaction by making any inhibitors present inefficient (99). Undiluted cDNA and RNase free water were added to a 96 well plate with a multichannel pipette. Samples were mixed 10 times and spun down, and kept in a freezer at -20 °C if needed. For the LDA-cards cDNA was not diluted until the samples were loaded to the card with Master mix and RNase free water.

Real-time polymerase chain reaction

The real-time quantitative polymerase chain reaction (RT-qPCR) is one of the most powerful and sensitive gene analysis techniques available (102). The method quantifies RNA, as the amplification plot of an amplicon corresponds to the expression level of a certain gene present in a sample. The first step is to replicate the complementary strand of the single stranded cDNA from the cDNA synthesis using a DNA polymerase enzyme forming a double stranded DNA (**figure 16**) (100). When the synthesis of the single stranded cDNA is part of the procedure, the method is referred to as RT-qPCR. When referring only to the amplification step, the method is denoted qPCR (93).

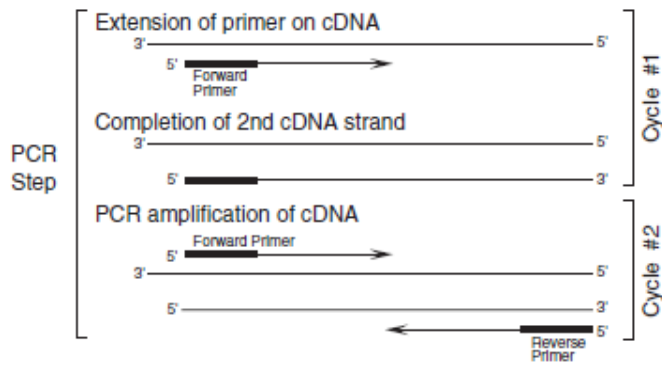
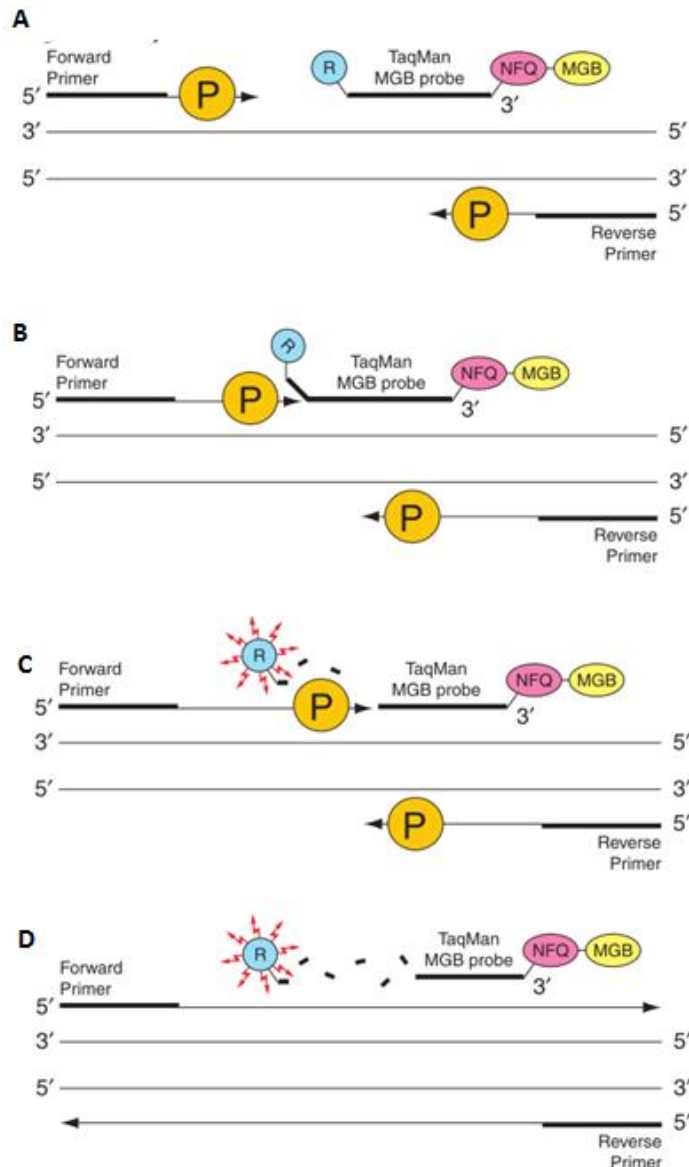


Figure 16: qPCR step, synthesis of complementary strand of cDNA. Reproduced from Life Technologies, Applied Biosystems, TaqMan Gene Expression Master Mix Protocol (103)

Forward and reverse primers anneal to the DNA strands downstream of the target sequence, the polymerase enzyme and the probe, and are extended by the DNA polymerase and dNTPs (**figure 17 A - D**). The probe is chemically marked and attaches to the target gene (upstream of the polymerase enzyme). It contains a quencher and a reporter dye on the 3' and 5' end respectively. As long as the probe is intact, the fluorescence from the reporter is quenched by the quencher. The probe is degraded by the polymerase enzyme when the strand is completed and amplified, causing the reporter to release light as it is no longer quenched (99).



Figures 17A - D: Demonstrating the Chemistry of the TaqMan Probes. A: Polymerization, B: Strand displacement, C: Cleavage, D: Completion of polymerization. Reproduced from Life Technologies, Applied Biosystems, TaqMan Gene Expression Assays protocol (104).

Several temperature cycles cause the reaction to occur, firstly the temperature is raised to melt the bindings between the double strands (referred to as denaturation), followed lowering of the temperature to allow the primers to anneal to the strands, before elongation takes place in which the polymerase extends the primers. The amount of product produced is reflected by the fluorescence signal (105). The Applied Biosystems 7900 HT Fast Real-Time PCR System instrument has the standard settings of 50°C for 2 minutes, 95°C for 10 minutes and 15 seconds, and 60°C for 1 minute. These temperature cycles are repeated 40 times (100).

A PCR run consists of three phases (**figure 18**): an exponential phase in which exact doubling of product occurs at every cycle, as all reagents are fresh and available. In the linear phase the reaction starts to slow down, as the product of the reaction is no longer doubled. The plateau phase is where the reaction ends. Quantitation of the product occurs in the exponential phase, as the SDS 2.4 software calculates the threshold level: the level where the reaction reaches a fluorescent intensity above background. Cycle threshold (Ct) is the cycle number at which the sample reaches this threshold (102).

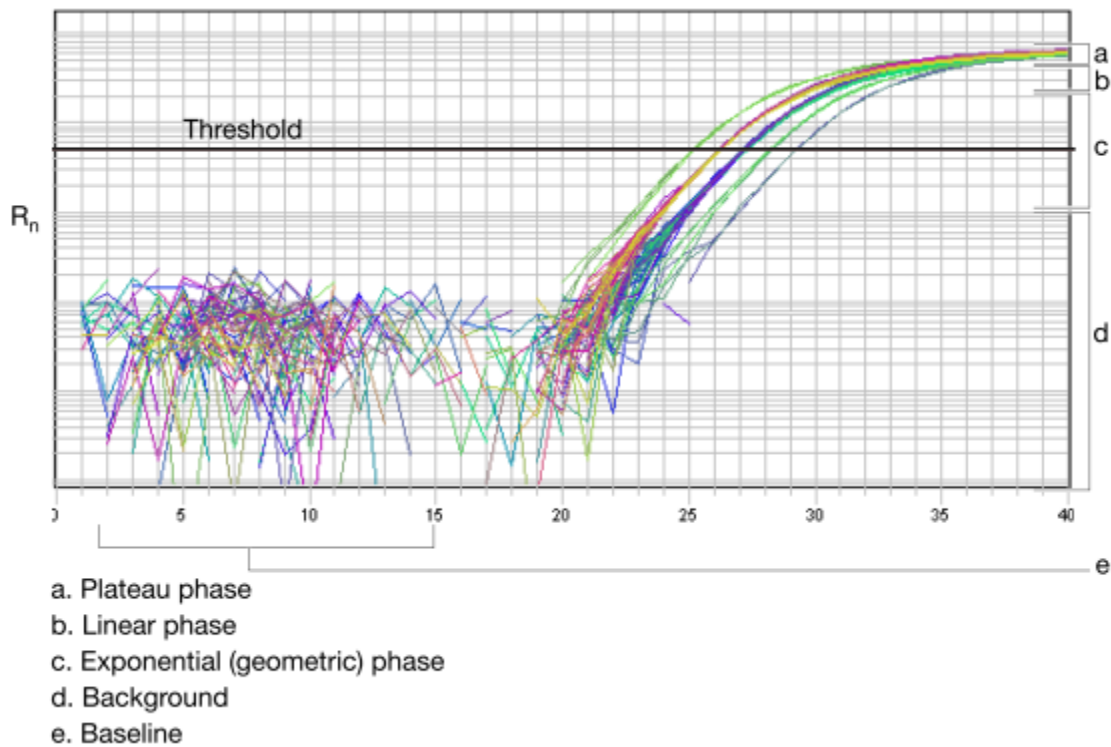
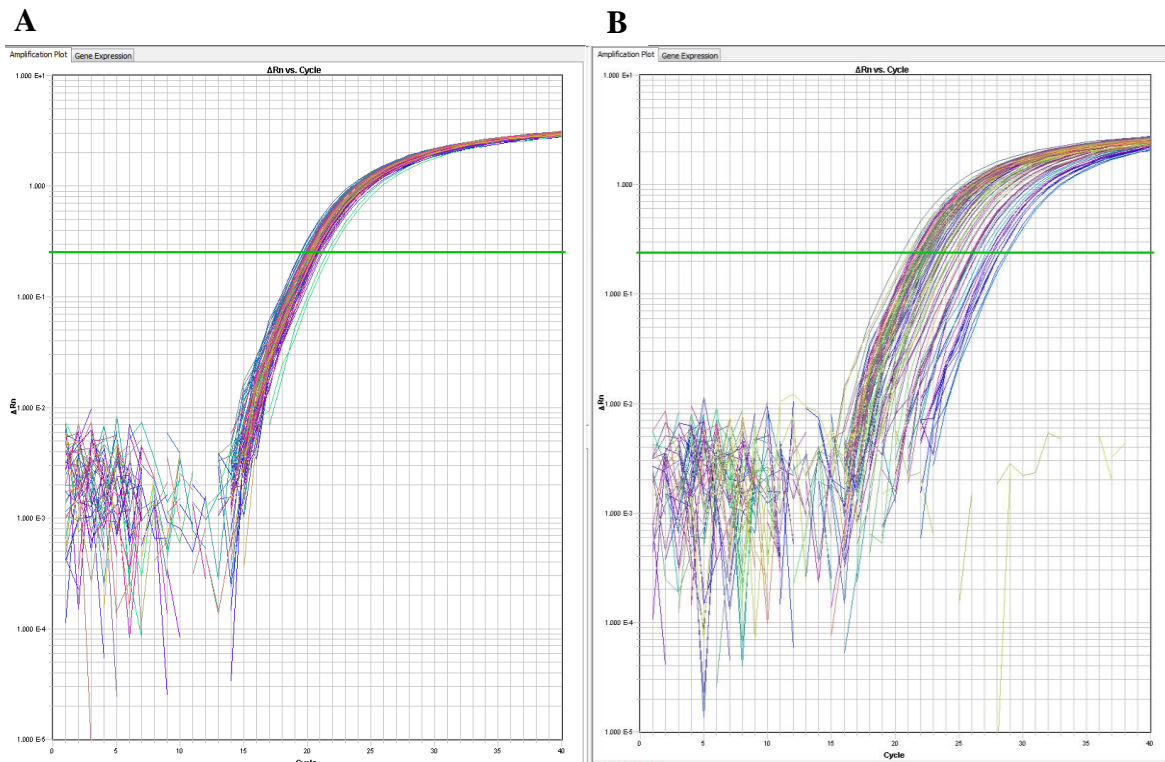


Figure 18: A typical amplification plot. Reproduced from Life Technologies, Applied Biosystems, TaqMan Gene Expression Master Mix Protocol (103).

Results from the qPCR runs were evaluated on the basis of the comparative Ct method ($\Delta\Delta Ct$ method). The target gene expression is related to the expression of an unregulated reference gene at baseline and at the end of the study. To further assess the possible change in expression during the intervention, the relative expression level of the target gene at one time point (end point) is compared to the relative expression level of the same target gene at a different time point (baseline), giving rise to the equation:

$$\Delta\Delta Ct = (Ct^{\text{target}} - Ct^{\text{reference}})_{\text{end point}} - (Ct^{\text{target}} - Ct^{\text{reference}})_{\text{baseline}} \quad (99)$$

In the relative quantitation experimental design, a main issue is the utilization of a valid reference gene, which is applied for normalization of gene expression. It is applied in order to adjust for different amount of input RNA in the different samples (106). The reference gene needs to be expressed at the same level for all study samples regardless of intervention or treatment (99).



Figures 19 A and B: Amplification plots with a stable and a regulated reference gene, A and B respectively.

TaqMan Low Density Array Cards

With TaqMan® Low Density Array Cards (LDA cards), multiple samples are screened against custom-selected gene-panels, as TaqMan Array Cards' 384 wells are pre-loaded with TaqMan® Gene Expression Assays (PN 4335626, Applied Biosystems, Foster City, CA, USA) (107). The assays include two unlabeled primers and one FAM-dye labeled probe for each gene (100). One card can be pre-designed with 44 target genes and 4 reference genes for 8 samples in single replicates. (108). **Appendix 2** demonstrates the genes selected for the cards. This method retains the qPCR sensitivity, but allows simultaneous quantification of large numbers of target genes, and hereby offers a higher throughput for quantitative gene expression profiling (109).

Protocol:

Undiluted cDNA samples with 500ng/μl were thawed on ice. 55 μl TaqMan® Gene Expression Master Mix (PN 4369016, Applied Biosystems, Foster City, CA, USA), 11μl cDNA and 44 μl RNase free water were prepared for each sample in separate Eppendorf tubes. Together there was 110 μl for each sample in the LDA card (PN 4342265, Applied Biosystems, Foster City, CA, USA), which included 10% extra volume due to loss of material while pipetting. The TaqMan® Gene Expression Master Mix (PN 4369016, Applied Biosystems, Foster City, CA, USA) contains the heat-stable AmpliTaq Gold® DNA Polymerase (100). After being gently mixed and spun down, 100 μl from each sample was applied to one of the eight ports on the card. The probes in the assays on the LDA card were TaqMan® minor groove binder (MGB) probes which contain reporter FAM™ dye on the 5' end and a non-fluorescent quencher (NFQ) at the 3' end (100). The LDA cards were centrifuged for 2 x 1 minute at 1200 rpm, further they were sealed with Low Density Array Sealer with the handle being moved upwards. The ports and edge were cut off with scissors, and cards were run using an Applied Biosystems 7900 HT Fast Real-Time PCR Relative Quantitation instrument with a TaqMan® Array Fluidic Thermal Cycling Block (107). The SDS 2.3 software was set to $\Delta\Delta C_t$ Assay and 384 Wells TaqMan® Low Density Assay. The CD with the set-up of the selected genes on the LDA card was imported to the computer. Samples were named and the file saved before run was started. Results were analyzed in RQ Manager 1.2.1 (Applied Biosystems, Foster City, CA, USA).

Single genes**Protocol:**

All work with samples and solutions was executed on ice. RNase free water and TaqMan® Gene Expression Assays 20X (PN 4335626, Applied Biosystems) with 18μM primer and 5 μM MGB probe was added to TaqMan® Master Mix (PN 4369016, Applied Biosystems): RNase free water (6 μl/well), primer and probe (1μl/well) and TaqMan® Master Mix (10 μl/well) was prepared for each gene. The TaqMan® Gene Expression Master Mix was similar as for the LDA-card analyses, with the polymerase enzyme AmpliTaq Gold® DNA polymerase. **Appendix 3** shows the primers selected for the single gene analyses.

An automatic pipette was used to aliquot 17 μ l Master Mix in the required amount of wells on the optical 96 well qPCR plate (PN N8010560, Applied Biosystems, Foster City, CA, USA). Diluted cDNA samples were applied in triplicates for the single genes (3 μ l/well). Total volume per well was 20 μ l. One blank sample was applied to all plates, wherein 3 μ l RNase free water replaced sample volume. The optical plate was carefully tapped after being sealed with adhesive film (to obtain mixing of reagents and sample), then centrifuged, and placed in the Applied Biosystems 7900 HT Fast Real-Time PCR System instrument. SDS 2.3 (Applied Biosystems, Foster City, CA, USA) software was set with assay $\Delta\Delta$ Ct method and 96 well clear plate container. Samples were named, detector added and volume set to 20 μ l before the run was started. Results were analyzed in RQ Manager 1.2.1. (Applied Biosystems, Foster City, CA, USA).

4.3 Statistical analyses

For statistical analyses of the results, the IMB SPSS Statistics software (version 20.0, SPSS Inc., Chicago, IL; USA) was used. Non-parametric tests were preferably applied (except for the variables total-cholesterol and LDL-cholesterol), due to a small sample size causing difficulties in assessing whether the variables were normally distributed regardless of normal-distributed histograms.

To assess the differences between groups, Kruskal Wallis or ANOVA One-Way were used. When the Kruskal Wallis test revealed a p-value ≤ 0.05 , pair wise comparisons were completed with Mann-Whitney U test, in order to distinguish where the differences could be ascribed to. Firstly, baseline differences between the groups were assessed for all variables. In order to investigate different changes between the groups during the intervention, the delta-variable for all measurements was used in the statistics. The delta-variable was estimated in the same way for lipid parameters in blood, EPA and DHA in plasma phospholipids and circulating inflammatory markers: variable $X_{\text{end of study}} - \text{variable } X_{\text{baseline}}$. Lastly, Kruskal Wallis or ANOVA One-Way was applied for end of study measurements. For the PBMC gene expression analyses, Δ Ct-values for each group were used in the statistics to identify differences at baseline and end of study. When estimating change during the intervention, $\Delta\Delta$ Ct-values for each group were compared in the analyses. For the LDA card analyses, comparisons were done for three groups.

The within group changes of lipid parameters in blood, n-3 PUFAs in plasma phospholipids, circulating markers and gene expression were estimated with Wilcoxon or Related samples t-test for baseline and end of study measurements. The ΔC_t -values for gene expression at baseline and end of study were used in the statistics. Significance level was set at $p \leq 0.05$.

For correlation analyses, Spearman's rho was used to interpret the data, as it was not normally distributed for the parameters in question. Significance level was set at $p \leq 0.05$.

For calculations and visualization of results the software programs SDS 2.3 (Applied Biosystems, Foster City, CA; USA), RQ Manager 1.2.1 (Applied Biosystems, Foster City, CA; USA) and GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA; USA) were used.

5 Results

5.1 Lipids and n-3 PUFA parameters

Baseline characteristics for all participants are presented in **table 8**. The study population had the median age 24 (22-27) years, and median BMI 23.4 (22.1-24.4) kg/m². All cholesterol values and triglycerides (TG) were within national recommendations for primary prevention of CVD (7). There were no differences between the intervention groups at baseline.

Table 8: Baseline characteristics of the study population

Parameter	Total population (n=38)
Male/female, n (%)	10/28 (26/74)
Age, years	24 (22 - 27)
BMI, kg/m ²	23.4 (22.1 - 24.4)
Total intake of seafood, g/week*	43.2 (29.0 - 52.2)
Total cholesterol, mmol/L	4.51 (0.89)
LDL-cholesterol, mmol/L	2.63 (0.82)
HDL-cholesterol, mmol/L	1.70 (1.48 - 1.90)
Triglycerides, mmol/L	0.87 (0.64 - 1.31)

Data are presented as Mean (SD) or Median (25th - 75th percentiles)

* Recorded by an FFQ: the intake of food one year prior to intervention

Changes in blood lipids and plasma phospholipids (PL) of n-3 PUFAs (EPA and DHA) are shown in **table 9**. The change in HDL-cholesterol was significantly different between the groups ($p = 0.04$). HDL-cholesterol increased with 5.2% in the salmon group. In the potato group there was a 6% decrease. In the cod and blue mussel group there were no changes. Change in triglycerides (TG) were significantly different between the groups ($p = 0.01$). TG increased with 3.4% in the potato group. In the cod, blue mussel and salmon group there was a 10.2%, 16.7% and 20.9% decrease, respectively.

There were significantly different changes between the groups in plasma PL for EPA and DHA. The largest increase in plasma levels of EPA and DHA was seen in the salmon group. The potato group had no significant changes. The salmon group had a higher increase in EPA concentrations than the other groups, as well in DHA compared to the potato group. The blue mussel group had larger increase than the potato and cod group in both EPA and DHA. The cod group had a significant increase in DHA compared to the potato group.

Table 9: Lipids and n-3 PUFAs EPA and DHA at baseline and at the end of study

Parameter	Treatment	n	Baseline	End of study	P ¹	P ²	P ³	P ⁴	P ⁵
Tot-choL, mmol/L	Control/potato	10	4.22 (0.82)	4.32 (0.89)	1.00	0.79	NS	0.88	NS
	Cod	9	4.64 (0.96)	4.73 (0.60)					
	Salmon	11	4.62 (0.88)	4.59 (1.09)					
	Blue Mussel	8	4.60 (0.99)	4.53 (0.91)					
LDL-choL, mmol/L	Control/potato	10	2.39 (0.83)	2.47 (0.88)	0.45	0.68	NS	0.80	NS
	Cod	9	2.72 (0.96)	2.72 (0.78)					
	Salmon	11	2.54 (0.50)	2.46 (0.72)					
	Blue Mussel	8	2.99 (0.96)	2.89 (0.92)					
HDL-choL, mmol/L	Control/potato	10	1.65 (1.38 - 1.93)	1.55 (1.40 - 1.83)	0.20	0.02	<0.05^{a, b}	0.04	<0.05^a
	Cod	9	1.80 (1.60 - 2.10)	1.80 (1.60 - 2.10)					
	Salmon	11	1.90 (1.50 - 2.20)	2.00 (1.60 - 2.60)					
	Blue Mussel	8	1.50 (1.23 - 1.85)	1.50 (1.40 - 1.68)					
TG, mmol/L	Control/potato	10	1.17 (0.53 - 1.35)	1.21 (0.57 - 1.77)	0.99	0.29	NS	0.01	<0.05^c
	Cod	9	0.85 (0.73 - 1.28)	0.77 (0.57 - 0.92)					
	Salmon	11	0.86 (0.56 - 1.64)	0.68 (0.49 - 0.84)					
	Blue Mussel	8	0.90 (0.81 - 1.34)	0.75 (0.61 - 1.43)					
EPA, mg/ml	Control/potato	9	0.006 (0.00 - 0.009)	0.008 (0.007 - 0.009)	0.44	<0.001	<0.05 ^{c, f}	<0.001	<0.05 ^c
	Cod	8	0.007 (0.003 - 0.009)	0.012 (0.008 - 0.013)					
	Salmon	11	0.009 (0.006 - 0.01)	0.057 (0.043 - 0.073)					
	Blue Mussel	8	0.007 (0.00 - 0.008)	0.034 (0.026 - 0.038)					
DHA, mg/ml	Control/potato	9	0.052 (0.046 - 0.063)	0.053 (0.05 - 0.058)	0.17	<0.001	<0.05 ^{d, e}	<0.001	<0.05 ^{d, f, g}
	Cod	8	0.052 (0.042 - 0.066)	0.066 (0.061 - 0.07)					
	Salmon	11	0.078 (0.054 - 0.089)	0.092 (0.078 - 0.108)					
	Blue Mussel	8	0.053 (0.044 - 0.070)	0.082 (0.068 - 0.090)					

Data are given as mean (SD) or median (25th – 75th percentile), NS indicates not significant

P¹: ANOVA or Kruskal Wallis test for differences between the groups at baseline

P²: ANOVA or Kruskal Wallis test for differences between groups at end point

P³: Mann Whitney test, pair wise comparisons at end point

P⁴: Kruskal Wallis test for different changes between groups

P⁵: Mann Whitney test, pair wise comparisons for change

^a indicates P ≤ 0.039, when potato and blue mussel were compared to salmon

^b indicates P = 0.039, when cod and blue mussel were compared

^c indicates P ≤ 0.001 when cod, potato and blue mussel were compared to salmon

^d indicates P ≤ 0.05 when cod and potato were compared to blue mussel

^e indicates P ≤ 0.001 when cod and potato were compared to salmon

^f indicates P ≤ 0.03 when cod and potato were compared

^g indicates P = 0.001 when salmon and potato were compared

5.3 Circulating inflammatory markers

Table 10 demonstrates the concentrations of circulating inflammatory markers at baseline and end of study for all groups. At baseline there were no differences in circulating markers between the groups for VCAM1 and CRP. ICAM1 concentrations differed at baseline due to a lower level in the potato group. Within the blue mussel group there was a decrease in CRP concentration during the intervention ($p = 0.02$) (**figure 20**). Though there was a decrease in the cod group as well, the IQR was wide (0.16 – 1.12), and the effect was not significant.

Table 10: Circulating inflammatory markers at baseline and end point

Protein	Treatment	n	Baseline	End of study	P ¹	P ²	P ³	P ⁴
VCAM1, µg/mL	Control/potato	10	0.69 (0.58 - 0.82)	0.70 (0.59 - 0.84)	0.47	0.60	0.24	0.29
	Cod	8	0.67 (0.59 - 0.71)	0.66 (0.57 - 0.71)				0.26
	Salmon	11	0.74 (0.69 - 0.87)	0.73 (0.58 - 0.85)				0.21
	Blue Mussel	7	0.66 (0.55 - 0.80)	0.61 (0.59 - 0.72)				0.24
ICAM1, µg/mL	Control/potato	10	0.16 (0.11 - 0.18)	0.16 (0.11 - 0.19)	0.03 ^a	0.14	0.10	0.24
	Cod	8	0.19 (0.18 - 0.21)	0.18 (0.18 - 0.20)				0.16
	Salmon	11	0.21 (0.17 - 0.25)	0.18 (0.17 - 0.21)				0.09
	Blue Mussel	7	0.20 (0.18 - 0.24)	0.19 (0.18 - 0.23)				0.24
CRP, µg/mL	Control/potato	10	0.92 (0.24 - 1.63)	0.86 (0.43 - 1.52)	0.37	0.44	0.11	0.96
	Cod	7	0.60 (0.15 - 0.82)	0.24 (0.16 - 1.12)				1.00
	Salmon	10	0.85 (0.37 - 1.39)	0.86 (0.65 - 2.65)				0.09
	Blue Mussel	7	1.79 (0.67 - 2.11)	1.06 (0.17 - 2.06)				0.02

P¹: Kruskal Wallis for baseline differences

P²: Kruskal Wallis for differences at end of study

P³: Kruskal Wallis for change

P⁴: Wilcoxon for change from baseline to end of study

^a MW $P \leq 0.03$, potato compared to the other groups

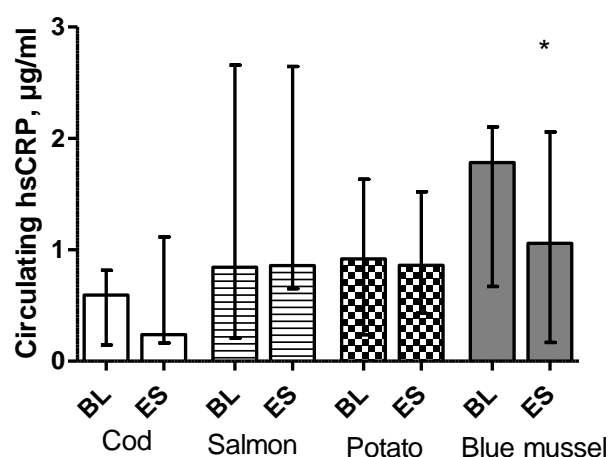


Figure 20: Circulating levels of hsCRP, µg/ml, at baseline (BL) and end of study (ES). $n = 7$ in the cod group, $n = 10$ in the salmon group, $n = 10$ in the potato group, $n = 7$ in the blue mussel group. * indicates a significant reduction within the blue mussel group ($p = 0.02$). Values are presented as median and 25th – 75th percentile represented by the vertical bars.

5.4 PBMC gene expression

Inflammatory responses are complex as the mediators exert both pro- and anti-inflammatory effects, thus investigators suggest that several end points and tissues involved in inflammation should be analyzed in intervention trials (110). Gene expression was further analyzed. All gene expression values (Δ Ct-values) are presented in **appendix 1**.

At baseline there were eight genes (CD40, CD36, toll like receptor-2 (TLR2), TLR4, cathepsin S (CTSS), phospholipid transfer protein (PLTP), sequestome 1 (SQSTM1) and acyl-Coenzyme A oxidase 1 (ACOX)) with different expression across the groups. The expression level of CD40 was similar in the salmon and potato group, while the cod group had higher expression. For the seven remaining genes the salmon group had lower gene expression levels at baseline compared to the other groups.

The change in gene expression from baseline to end of study was different between the groups for the phospholipase A₂, group IVA (PLA2G4A) gene ($p = 0.03$) (**figure 21**). The salmon and potato group ($p = 0.019$) and the cod and potato group ($p = 0.046$) were significantly different. While the expression decreased in the potato group, it increased in both the salmon and cod group (Δ Ct-values are presented in **appendix 1**). When males were

taken out of the result file, the significance between the groups disappeared ($p = 0.189$), data not shown.

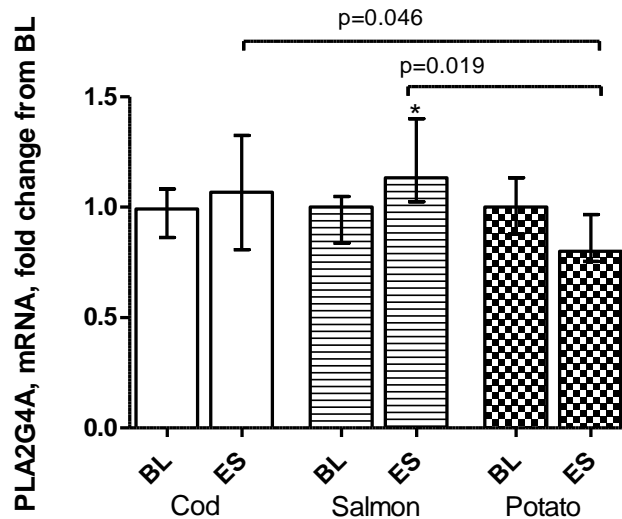
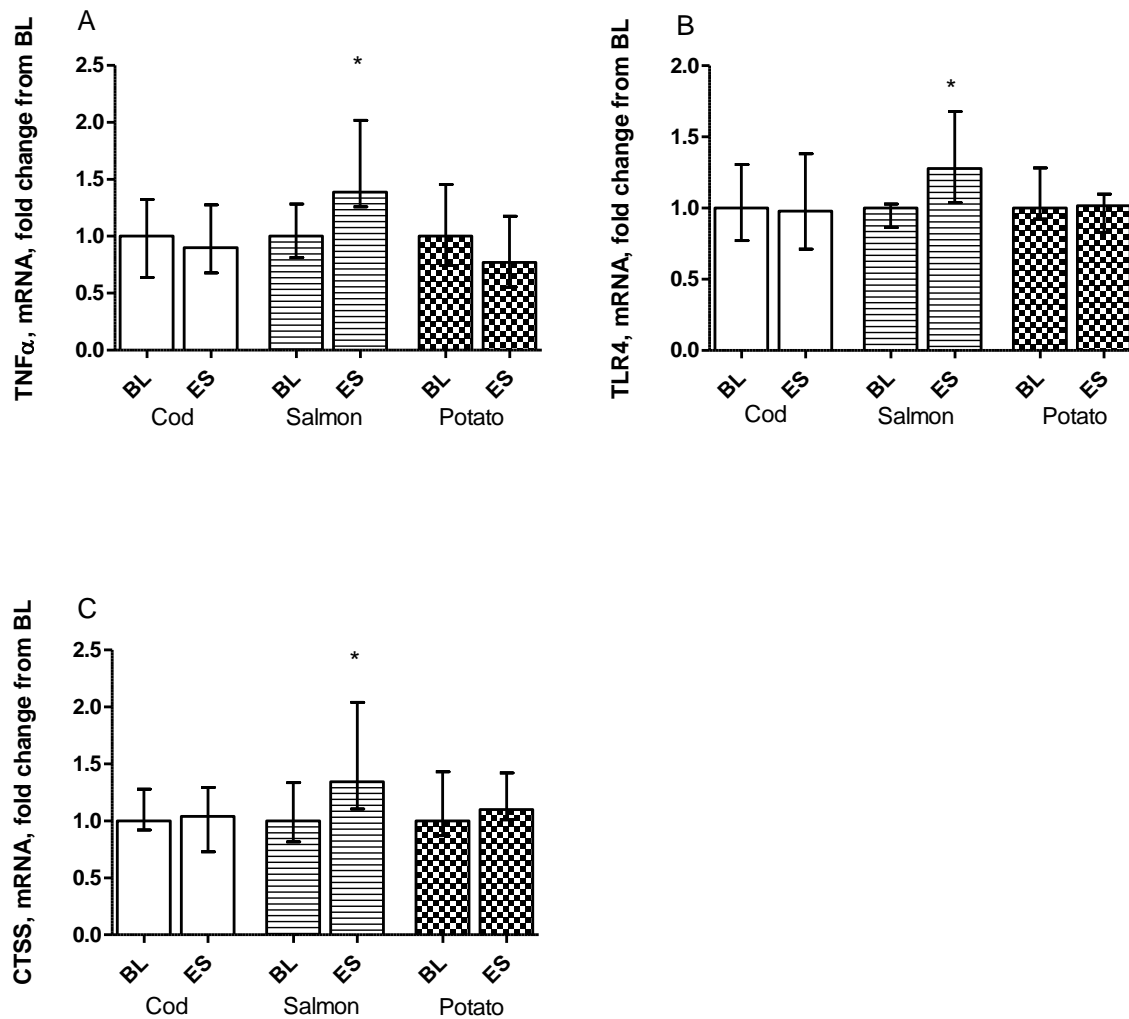
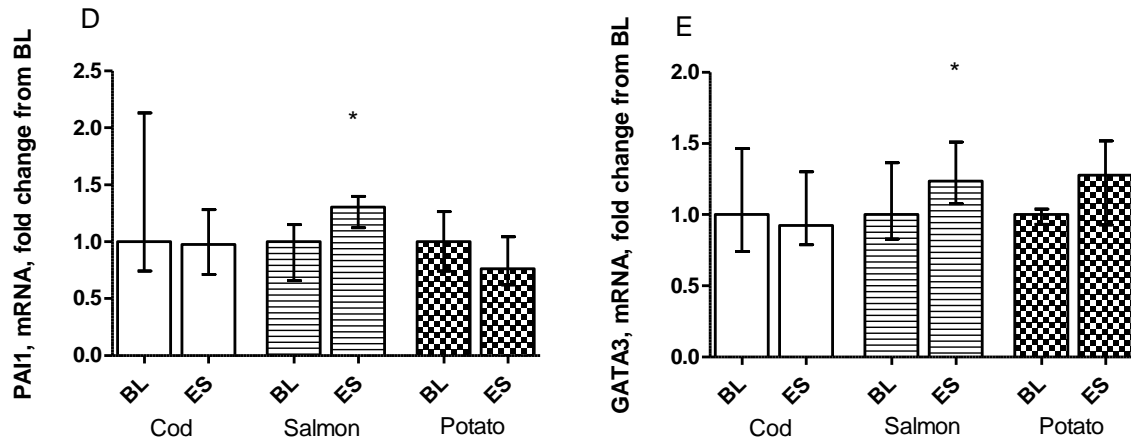


Figure 21: mRNA levels of phospholipase A₂, group IVA (PLA2G4A) in PBMC at baseline (BL) and end of study (ES). $n = 6$ in the cod group, $n = 5$ in the salmon group, $n = 7$ in the potato group. The fold change of mRNA of the target gene was related to the reference gene TATA binding box (TBP) and was increased in the cod and salmon group compared to the potato group. The mRNA data are presented relative to baseline mRNA levels. Values are presented as median and 25th -75 th percentile, represented by the vertical bars. * indicates a significant change within the salmon group ($p = 0.04$). All Ct-values are presented in **Appendix 1**.

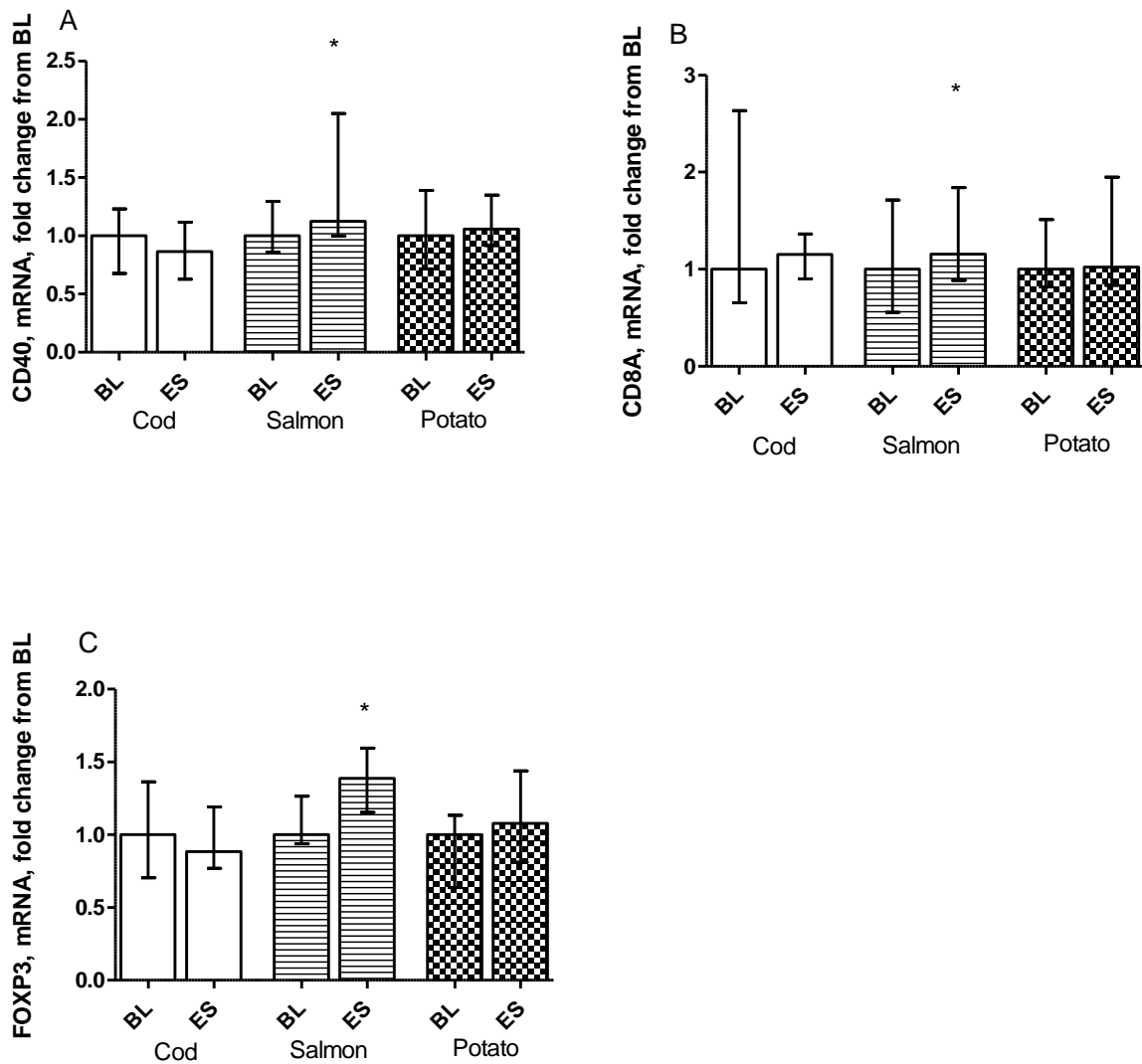
In the salmon group the PBMC gene expression of thirteen genes increased during the intervention period (**figure 22 A – E, figure 23 A - C, figure 24 A - D**). Among these were PLA2G4A and three of the genes with different expression at baseline (CD40, TLR4 and CTSS). Expression was increased in all three groups for cathepsin S (CTSS) and hormone sensitive lipase (LIPE). While for serpin peptidase inhibitor, clade E member1 (PAI-1/SERPINE1), CD40, GATA binding protein 3 (GATA3), tumor necrosis factor- α (TNF), forkhead box P3 (FOXP3), peroxisome proliferator-activated receptors- δ (PPARD) and uncoupling protein 2 (UCP2) the expression was reduced in the cod group. For peroxisome proliferator-activated receptors- γ (PPARG1CB) and CD8A both the cod and salmon group an increased expression was observed, while the potato group stayed stable. See **appendix 1** for Δ Ct-values. As there were only females in the salmon group, analyses were conducted with

males taken out of the statistical file. In the cod group the expression of TLR6 was significantly decreased within the cod group ($p = 0.012$, data not shown), while the expression of tissue plasminogen activator (PLAT) was significantly increased ($p = 0.023$, data not shown). In the potato group there were no changes.

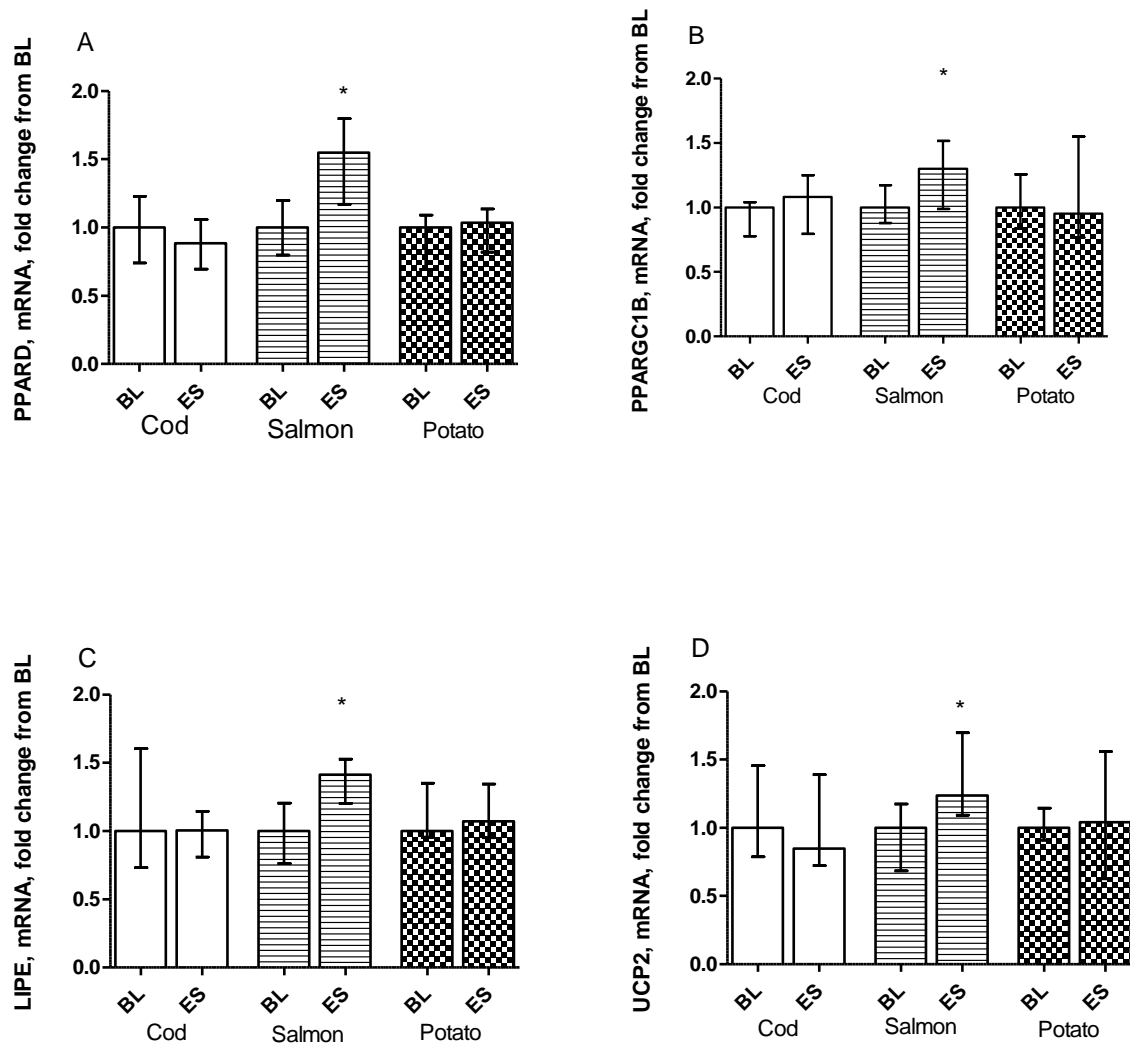




Figures 22 A – E: mRNA levels of tumor necrosis factor- α (TNF), toll like receptor-4 (TLR4), cathepsin S (CTSS), serpin peptidase inhibitor, clade E member1 (PAI-1/SERPINE1) and GATA binding protein 3 (GATA3) in PBMC at baseline (BL) and end of study (ES). $n = 6$ in the cod group, $n = 5$ in the salmon group, $n = 7$ in the potato group. The fold change of mRNA of the target gene was related to the reference gene TATA binding box (TBP). The mRNA data are presented relative to baseline mRNA levels. Values are presented as median and 25th -75 th percentile, represented by the vertical bars. * indicates a significant change within the salmon group ($p = 0.04$). All Ct-values are presented in **Appendix 1**.



Figures 23 A – C: mRNA levels of CD40, CD8A and forkhead box P3 (FOXP3) in PBMC at baseline (BL) and end of study (ES). $n = 6$ in the cod group, $n = 5$ in the salmon group, $n = 7$ in the potato group. The fold change of mRNA of the target gene was related to the reference gene TATA binding box (TBP). The mRNA data are presented relative to baseline mRNA levels. Values are presented as median and 25th -75 th percentile, represented by the vertical bars. * indicates a significant change within the salmon group ($p = 0.04$). All Ct-values are presented in **Appendix 1**.



Figures 24 A – D: mRNA levels of peroxisome proliferator-activated receptors- δ (PPARD), peroxisome proliferator-activated receptors- γ (PPARG1CB), hormone sensitive lipase (LIPE) and uncoupling protein 2 (UCP2) in PBMC at baseline (BL) and end of study (ES). $n = 6$ in the cod group, $n = 5$ in the salmon group, $n = 7$ in the potato group. The fold change of mRNA of the target gene was related to the reference gene TATA binding box (TBP). The mRNA data are presented relative to baseline mRNA levels. Values are presented as median and 25th -75 th percentile, represented by the vertical bars. * indicates a significant change within the salmon group ($p = 0.04$). All Ct-values are presented in **Appendix 1**.

5.5 Correlations

Correlations between PBMC gene expression and concentrations of EPA and DHA were examined. The change in PLA2G4A correlated with the change in DHA, $R = 0.52$ ($p = 0.03$). The effect disappeared when only the salmon group was investigated. The change in hormone sensitive lipase (LIPE) correlated with the change in EPA, $R = 0.66$ ($p = 0.004$). In the salmon group the change in PBMC gene expression of PLA2G4A and cathepsin S (CTSS) correlated with the change in EPA, $R = -0.90$ ($p = 0.04$), for both genes. None of the other up-regulated genes within the salmon group correlated with EPA or DHA.

6 Discussion

6.1 Discussion of methods

6.1.1 Study design

The participants were randomized but not stratified by gender, thus all participants in the salmon group were women by chance. This could affect the findings as women are more responsive to n-3 PUFAs than men (111). Participants were supplied with intervention meals, also on the week-ends, and compliance was estimated as good. This was based on the leftovers from intervention meals. The change of n-3 PUFA in PL in plasma confirms the adherence for the majority of the participants. The mean dietary intake prior to the intervention was estimated by an FFQ. Nevertheless we do not hold data on composition of the diet for the participants, or how this plausibly was changed during the intervention period. The E% from fat and potential change in E% from fat as well as the ratio of saturated and unsaturated fat could have been of interest. Potato was allocated as control diet. Perhaps a more appropriate dietary component would resemble the protein content in fish. This was not considered when the study was originally designed, as it was meant to measure the content of arsenic metabolites in blood and urine after intake of seafood, and not gene expression of PBMC.

6.1.2 Laboratory analyses

ELISA is a well validated method applied for measurements of circulating proteins (85). Intra-assay precision and standard curve measurements were conducted before experiments with sample material in order to ensure proper pipetting skills (88). The RT-qPCR method is considered a very sensitive analysis for mRNA concentrations (102). Its reliability presumes proper use of an unregulated reference gene, which will be closer elucidated below.

6.1.3 Methodological challenges

Interpretation of results – PBMC gene expression, single genes

The importance of finding a good reference gene has been mentioned. It is used to adjust results for varying amounts of input RNA (106). According to the Minimum information for publication of Quantitative Real Time PCR Experiments (MIQE) Guidelines the utility of a reference gene should be validated for the sample material in question for each experiment before it is applied as an active reference (93). The stability of the gene expression of a reference gene depends on intervention treatment and pathologic state, and there is also varying expression among cells and tissues (112, 113). The main challenge for the single gene analyses was to find an unregulated reference gene. Some investigators apply two reference genes, calculate their geometric mean, and relate target genes to this mean (108, 114).

According to the manufacturers protocol (Applied Biosystems), one reference gene can be used if the gene has a stable Ct-range regardless of experiment protocol. When interpreting the amplification plots of the reference genes that were tested (peptidylprolyl isomerase A (PPIA), TATA binding protein (TBP) and actin β (ACTB)), all displayed wide ranges of Ct-values. **Figures 25 – 27** demonstrate amplification plots of the reference genes from the RQ 1.2.1 – software for the single genes.

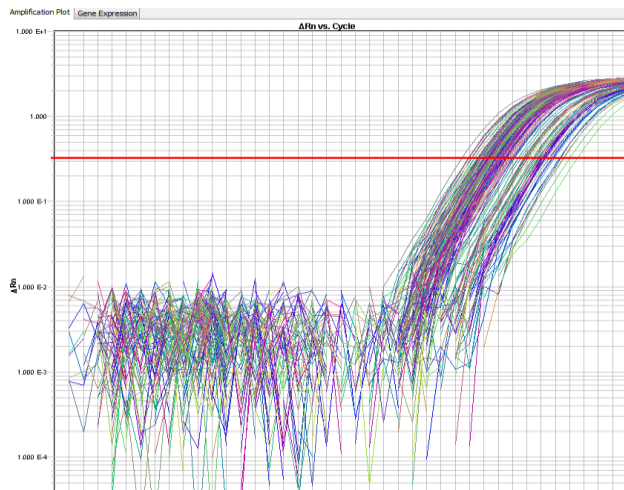


Figure 25: Amplification plot from single genes analyses. Reference gene TATA binding protein (TBP), with Ct-range from 28.9 to 35.5

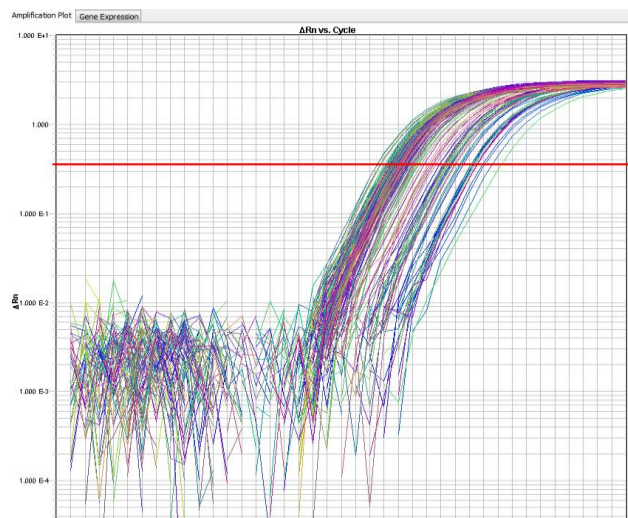


Figure 26: Amplification plot from single genes analyses. Reference gene peptidylprolyl isomerase A (PPIA), with Ct-range from 22.5 to 29.9

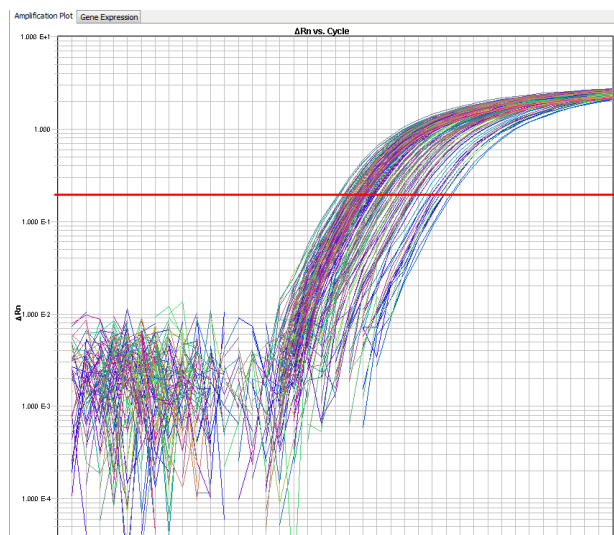


Figure 27: Amplification plot from single genes analyses. Reference gene actin β (ACTB), with Ct-range from 20.1 to 27.8

It was decided that these results were not valid for analyses. Instead all statistical analyses were executed with results from the previously run LDA-cards. In comparison, the amplification plot for the reference gene TBP from the LDA card analyses is presented in **figure 28**, demonstrating a stable Ct-range from 26 to 28. For single genes analyses, samples were added in triplicates. For LDA-card analyses there is one replicate for each sample.

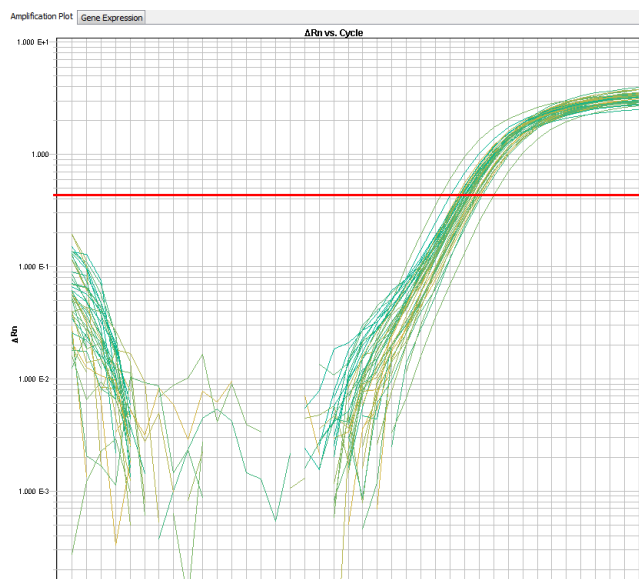


Figure 28: Amplification plot from the LDA card analyses. TATA binding protein (TBP) was used as reference gene for all target genes. Ct-values ranged from 26.3 to 29.9, but the majority was between 27 and 28.

6.1.4 Sample material and challenges during analyses

For statistical analyses of results, change between and within the groups was of primary interest. Thus, subjects with measurements at only one time point were excluded from the analyses. For the n-3 PUFA results, two subjects had measurements at only one time point. One was in the cod group and the second in the potato group. For analyses of the ELISA results, two subjects had measurements at one time point only. One was in the cod group and the second in the blue mussel group. Furthermore, two samples showed concentrations above the CRP-standard curve, and these subjects were taken out of the result file. These samples were in the cod and salmon groups. This is the reason for four excluded subjects for the hsCRP analysis. For the change in gene expression, the same principle was applied, hence the exclusion of 12 subjects from the LDA-card analyses. Three were in the cod group, six in the salmon group and three in the potato group. Wells with the error messages BPR (bad passive reference) or NAW (none amplified wells) were not used in further analyses. This occurred to 18 of the wells in the LDA-card set-up. Furthermore, the blue mussel group was not analyzed in the LDA-card set-up. These are the reasons for a varying n for the different methods in this thesis. The low n for gene expression analyses reduces the statistical power of the results. Nevertheless, these findings may contribute to the knowledge concerning the intake of fish and effects on PBMC in healthy subjects.

In the single genes set up 8 subjects were excluded due to measurements at only one time point. There was one well with BPR and NAW which was also excluded. The blue mussel group was included in these analyses, thus the *n* was higher. Unfortunately the results could not be further investigated due to the problems with the reference gene described above.

6.2 Discussion of results

6.2.1 Blood lipid parameters

The changes in HDL-cholesterol and TG were significantly different between the groups. HDL-cholesterol increased in the salmon group and was reduced in the potato group. TG was increased in the potato group while there was a decrease in all other groups. EPA and DHA were increased in plasma PL in the salmon and blue mussel group, while DHA was increased in the cod group. There were no changes in plasma PL of EPA or DHA in the potato group.

The reduced TG-level and increased EPA/DHA concentrations in the blue mussel group have not previously been reported, as the blue mussel group or lipid parameters were not included in previous publications by Telle-Hansen et al and Molin et al (81-83), respectively. An intake of fatty fish and/or fish oils may stimulate fatty acid oxidation through activation of the nuclear receptors PPARs, which favors a decrease in TG-levels (71, 115). Thus the increased EPA/DHA in the salmon, blue mussel and cod group yielded reductions of TG in the corresponding intervention groups. The reduction of TG seen in these groups could be related to the increased intake and subsequent plasma concentrations of EPA and DHA (116).

6.2.2 Circulating inflammatory markers

There were no differences between the groups in circulating biomarkers at the end of the study, although hsCRP was reduced within the blue mussel group. This group also had the highest concentrations at baseline (**figure 24**). One of the most commonly measured circulating biomarkers in relation to inflammation is hsCRP, alongside IL-6 and TNF- α (117). Among healthy individuals there are varying results concerning intake of fish or n-3 PUFAs and subsequent changes in circulating levels of hsCRP, ICAM1 and VCAM1 (118).

Several observational studies have found an inverse relationship between the intake of n-3 PUFAs from fish or fish oil supplementation and this biomarker (117, 119-122). Even so, a recent review concluded that circulating hsCRP should not be applied as an individual biomarker or target of treatment for CVD, as several factors interact with baseline levels of circulating hsCRP (62).

Our results are not in agreement with a dietary intervention study by Tsitouras et al including 12 healthy men and women aged 60 to 75 years. They all first consumed a control diet for 6 week, followed by a so-called omega-3 diet with 30E% from polyunsaturated fat for 8 weeks. During this 8 week period they were supplied with 720 g fatty fish every week, in addition to 15 ml of sardine oil contributing with 4 – 5 g EPA/DHA daily. A significant reduction in circulating hsCRP was observed during the omega-3 diet period compared to the control-diet period (123). The reason for the discrepancy in the results from our study may be the duration of the intervention, which was markedly longer, along with higher amounts of EPA/DHA supplementation. In a study by Raatz et al, a group of healthy subjects ($n = 19$) between 40 and 65 years were allocated to different amounts of salmon, 90 g, 180 g and 270 g every week for 4 weeks. This daily contributed with 307, 614 and 923 mg EPA/DHA, respectively. Levels of plasma hsCRP were not reduced within or between any of the groups (44). The amount of EPA/DHA supplementation were lower in the intervention by Raatz et al than in the study by Tsitouras et al, as well as in the present study.

There are several interventions with measurements of hsCRP which have been conducted with n-3 PUFA supplementation. Some have found opposing results from ours. Bloomer et al included trained males ($n = 15$) and found decreased resting levels of hsCRP after 6 weeks of 4.3 g EPA/DHA daily (124). Ciubotaru et al conducted an intervention among postmenopausal women ($n = 30$) who received either 1.3g or 2.56 g EPA/DHA daily for 5 weeks. Circulating levels of hsCRP were reduced (125). In a review from Myhrstad et al from 2011, five out of the six supplementation studies where hsCRP levels were measured did not see any changes (118). Rangel-Huerta published a review in 2012 with results from RCTs which had investigated the effect of EPA/DHA supplementation on circulating inflammatory markers. Four out of the five studies which measured hsCRP did not find a reduction (119). Together the two reviews included seven RCTs among healthy subjects. The majority of these studies did not result in reduced levels of circulating hsCRP (118, 119). Likewise, an

intervention allocating healthy subjects ($n = 54$) with the mean age of 27 to 1.6 g EPA/DHA, oxidized 1.6 g EPA/DHA or sunflower oil daily for 7 weeks did not see any differences within or between the groups in circulating hsCRP (126).

A common characteristic of the interventions without reducing effect on circulating hsCRP in healthy subjects is that most of them included relatively young participants (≤ 60 years in five of them). Some have also used quite low supplementation doses (between 0.86 and 2.4 g EPA/DHA). Features of the interventions which have obtained an effect are that they have lasted longer or that they have included older subjects than the one presented here. According to Li et al, longer duration of interventions is related to increased effects on circulating inflammatory markers. Furthermore they describe a negative linear relationship between increasing age and n-3 PUFA efficiency. In both supplementation and diet trials with effect, the participants have been older than in the present study. hsCRP increases with age, and a higher baseline level of circulating hsCRP is related to improved reduction(127). The low concentration and stable levels of hsCRP observed in the present study population may be related to that the participants were young and healthy, and that the intervention period was quite short.

ICAM1 and VCAM1

Increased circulating levels of the adhesion molecules ICAM1 and VCAM1 are related to endothelial dysfunction (61). Measurements of their circulating concentration estimate endothelial activation (11). Among the participants in the potato group, there was a lower baseline level of circulating ICAM1, but no changes were seen between or within the groups during the intervention.

van Bussel et al conducted an observational study ($n = 301$) and found an inverse relationship between markers of endothelial dysfunction and each 100 g increment of fish per week among healthy adults (128). They further separated fatty and lean fish species, and found no difference between them, thus the authors conclude that the inverse relationship between intake of fish and plasma concentrations of markers of endothelial dysfunction is also related to lean fish without EPA/DHA (128). Paulo et al combined fish as a dietary component and n-3 PUFA, and found reduced level of circulating ICAM1, in difference to the present study. The intervention period in the study by Paulo et al lasted longer than the present one. Healthy

subjects ($n = 275$) between 20 and 40 years were allocated to four different intervention groups: three servings of cod (0.3 g EPA/DHA per portion) or salmon (3 g EPA/DHA per portion) every week for 8 weeks, supplementations of EPA/DHA 1.4 g/day or placebo. There was a reduction in circulating ICAM1 levels only within the cod group. The authors related the reduction to other nutrients in cod, such as the amino acid taurine (129). Taurine has previously been related to preventive effects on CVD (130). In the EPA/DHA supplementation group and salmon group levels of circulating VCAM1 were increased from baseline to the end of the study. The authors suggest that high doses of EPA/DHA might exert pro-inflammatory effects, and emphasize the importance of deciding whether an upper n-3 PUFA limit exists (129). Some have suggested that VCAM1 and ICAM1 may require different sensitivity to n-3 PUFAs (119).

Among supplementation trials with n-3 PUFA, there are contrary findings from ours. Two studies in the previously mentioned review by Rangel-Huerta et al found reduced levels of circulating ICAM1 or VCAM1 (119). In the review from Myhrstad et al two additional studies found a reduction of circulating ICAM1 or VCAM1 (118). These studies had longer intervention periods (8 to 12 weeks) than the present study. They furthermore included subject who were older than the present study population. It has been hypothesized that age and presence of disease and/or low-grade inflammation promotes the n-3 PUFA reducing properties on concentrations of circulating adhesion molecules (119, 129). In a review summarizing results among subjects with ≥ 1 factor of MetS, the authors state that n-3 PUFA have been shown to reduce inflammatory markers in plasma, but as these interventions often are combined with weight loss it is a challenge to isolate the effect of n-3 PUFA from other factors which also ameliorate inflammatory markers (110). In patients with heart-failure, a 3 month intervention with 2 g EPA/DHA daily reduced plasma levels of ICAM1 (131).

In line with the findings herein, Pot et al failed to see a reduction in circulating levels of ICAM1 or VCAM1 during their intervention which lasted for 12 weeks and included healthy elderly (132). Even though Pot et al included elderly subjects, their supplementation doses were quite low (1.26 g EPA/DHA daily), which could explain the lack of reduction. Robinson et al conclude that the varying length of interventions and the discrepancy in amounts of n-3 PUFA supplementation and/or diet supplied have contributed to the varying results yielded from intervention studies. They underline that currently there is not a specific optimal amount

of EPA/DHA which is recognized to obtain favorable effects on inflammatory mediators, but hold that there seems to be an association between the intake of n-3 PUFA and reduction of circulating inflammatory markers (110). Myhrstad et al suggest that plasma concentrations of inflammatory markers cannot identify/report the local changes in the arterial wall, and propose analysis of PBMC gene expression as a candidate measure to closer investigate possible changes of expression of the markers involved in the early stages of atherosclerosis (118).

6.2.3 PBMC Gene expression

Group IV A phospholipase A₂

There was one gene which had different PBMC gene expression in the cod and salmon group than in the potato group, namely phospholipase A₂ group IVA (PLA2G4A). The PBMC gene expression was increased in the salmon and cod groups and reduced in the potato group (**figure 25**). The phospholipase enzymes constitute a superfamily of 15 groups, the phospholipase A₂ (PLA₂) is mainly divided into three categories: sPLA₂ which is secretory, cPLA₂ in the cytosol, and iPLA₂ which is independent of calcium. (133). PLA2G4A is widely expressed in mammalian cells, and has specificity for phospholipids containing arachidonic acid or EPA (76, 134). Others have suggested that it has a specificity for releasing DHA from cell membranes (135). Its activities are complex as they are related to both inhibition and promotion of disease. It is suggested by some as a target of pharmacological treatment (136).

In animal models the properties of PLA2G4A have been closer investigated than in humans. Cytosolic PLA₂ has been related to pathologic states of the brain, and cPLA₂ deficient mice with Alzheimers Disease (AD) showed reduced mRNA levels of cyclooxygenase-2 (COX2) pathways. There were no changes in COX1, lipooxygenase-5 or cytochrome 450 pathways. This led to the conclusion that cPLA₂ could primarily be related to COX2 in prostaglandin synthesis. Furthermore, reduced levels of PLA2 seem to prevent cognitive deficit in AD. Transgenic mice with reduction of cPLA₂ and blockage of ARA had improvement in cognitive deficit compared to those without reduced PLA₂-levels. (137). In an animal model of multiple sclerosis (MS), increased levels of cPLA2 seemed to play a role in the onset of MS (138).

In the atherosclerotic process, the role of PLA2G4A is not fully elucidated, as effects induced by phospholipases seem to be both pro-inflammatory and protective (139). Investigators have found that a single nucleotide polymorphism (SNP) (A→G) of PLA2G4A reduced the risk of MI in subjects who were under cardiac evaluation (140). They further investigated the interaction of the SNP (A→G) and a high dietary intake of n-6 PUFA, as such an intake is related to a higher risk of CVD. Subjects with the SNP (A→G) and an intake of n-6 PUFA above median had a lower risk of MI. Subjects with the SNP (A→G) had lower expression of PLA2G4A in HEAC cells investigated by microarray. The authors suggest that the SNP (A→G) could exert protective effects of MI in subjects with a high intake of n-6 PUFA. They did not find the same interaction with n-3 PUFA (76). In the present study the expression of PLA2G4A was increased after n-3 PUFA intake, thus the SNP (A→G) did not exert effects after intake of n-3 PUFA, in line with findings from Hartiala et al (76).

Differing from the present study, Weaver et al did not see any changes in mRNA of PLA2G4A after supplementation of 775 mg EPA daily for 6 weeks in healthy subjects (141). The intake of EPA and DHA was higher in the present study. Rudkowska et al investigated different responder patterns to n-3 PUFA. They found that after supplementation of 3 g EPA/DHA daily for 6 weeks among healthy subjects ($n = 30$), the responsiveness to n-3 PUFA differed. Non-responders were defined as those who without reduction in TG-levels after the n-3 PUFA supplementation. Among the non-responders there was a significant increase in EPA as well as a down-regulation of PLA2G4A (135). Correlation analyses in our study population showed that the subjects with the largest increase in DHA had higher expression of PLA2G4A. The change of EPA within the salmon group was negatively correlated with change in PLA2G4A. Our correlation analysis also revealed that the change of EPA in plasma PL varied among the participants, which might be related to a fluctuating responsiveness as described by Rudkowska et al (135). In the present study population it seems that EPA and DHA may have opposing effects on PLA2G4A gene expression in PBMC. The daily dosage of EPA/DHA in the present study design was approximately the same as the supplementation distributed in the intervention by Rudkowska et al. EPA and DHA were increased in plasma PL in the salmon group, while the cod group had increased levels of DHA. The observed increased expression of PLA2G4A could be related to a higher amount of n-3 PUFA in cell membranes readily to be released (142). Furthermore, it is

recognized that an increase of EPA/DHA in cell membranes promote a shift towards production of less potent down-stream eicosanoids than the ones originating from ARA (77).

PBMC gene expression within the salmon group

The PBMC gene expression was increased for 12 genes within the salmon group. Toll like receptor-4 (TLR4), tumor necrosis factor α (TNF), cathepsin S (CTSS), serpin peptidase inhibitor, clade E member1 (PAI1/SERPINE1), GATA binding protein 3 (GATA3), forkhead box P3 (FOXP3), CD40 and CD8A are traits of immunity and inflammation, while peroxisome proliferator-activated receptors- δ (PPARD), peroxisome proliferator-activated receptors- γ (PPARG1CB), uncoupling protein 2 (UCP2) and hormone sensitive lipase (LIPE) are involved in lipid metabolism.

Inflammatory markers

Some of the inflammatory markers with increased expression are related to up-regulation of the transcription factor NF- κ B, such as TLR4 and CD40 (57, 143). Activation of NF- κ B in turn leads to increased secretion of pro-inflammatory cytokines such as TNF- α (144), which also had increased PBMC gene expression in the salmon group. The number of studies on fish consumption and effects on human gene expression is limited, and TNF- α is the only inflammatory marker in common between other studies and the present (except PLA2G4A, which has already been mentioned). de Mello et al carried out an intervention which got different results from the present study. Patients with established CHD were included ($n = 27$), and were allocated to an intake of 5 portions of lean fish, fatty fish or a control diet for 8 weeks. There were no changes in PBMC gene expression of IL-1 β , TNF- α , chemokine C-C motif ligand (CCL)-2, CCL-5, ICAM1 and soluble selectins-E and -P between the groups. The change of AA:EPA ratio in cholesterol esters and PL fractions correlated with the change in mRNA expression of IL1 β in the fatty fish group. There was also a reduced amount of total saturated fatty acids in cholesterol esters in the lean fish group. The reduction correlated with a decrease in ICAM1 expression. The results were interpreted to the direction that fatty fish could reduce expression of inflammatory markers and that lean fish might have promising effects on endothelial function (145). In the present study population, the change of EPA was negatively correlated with the change of cathepsin S (CTSS) expression in PBMC within the salmon group. PBMC gene expression for the other inflammatory markers did not correlate

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with EPA or DHA. de Mello et al argue that had their intervention lasted longer, a higher amount of DHA could be incorporated into the cell membranes of PBMCs, which again could have had a greater impact on gene expression (145).

More interventions have been completed with supplementation of n-3 PUFA. Weaver et al included a similar population as the present, and found opposing results from us. Healthy subjects ($n = 27$) were allocated to 775 mg EPA and 831 mg borage oil (GLA) daily for 4 weeks, and the authors found a reduced expression of PI3K γ along with several interleukines (IL-10, -23 and -17). There was no change in the expression of Akt and NF- κ B (141). It is noteworthy that there was no control group described in this study. Some RCTs have found results in line Weaver et al. Bouwens et al included healthy elderly subjects ($n = 352$) who received 1.8 g EPA/DHA, 0.4 g EPA/DHA or 4.0 g high-oleic acid sunflower oil daily for 26 weeks. The PBMC gene expression of several genes involved in the NF- κ B pathway, lipid metabolism and eicosanoid production was decreased within the high EPA/DHA group. Genes with different expression between the groups were scavenger receptor CD36, adipose differentiation protein (ADFP) and leukotriene A4 hydrolase (LTA4H) (146). Cawood et al included patients awaiting carotid endarterectomy ($n = 100$). They received 1.55 g oleic acid serving as placebo or 1.49 g EPA and DHA daily for 21 days prior to surgery. The mRNA expression of matrix metalloproteases-7 -9 and -12, IL-6 and ICAM1 were significantly reduced in plaques in the n-3 PUFA group. These patients also had fewer foam cells and T-cells, as well as more stable plaques than the patients receiving placebo (147). In subjects ($n = 23$) with insulin resistance, Spencer et al found that the intake of 4g fish oil daily for 12 weeks reduced the expression of macrophage attractant protein-1 (MCP1) in adipose tissue macrophages (148). The interventions which have seen a reduced gene expression in inflammatory cells had longer duration than the one presented herein. In the studies from Bouwens et al and Cawood et al the number of participants was markedly higher. Bouwens et al further argue that as the participants were defined as elderly, they may have had a more pro-inflammatory and pro-atherogenic gene expression profile, which make the results less transmittable to the general population (146). This could also be applicable for the participants with CVD in the RCT from Cawood et al and the subjects with insulin resistance in the study by Spencer et al. Some suggest that hsCRP needs to be elevated in order to see changes in PBMC expression of inflammatory markers (149). The dosage in the study from Spencer et al was also higher than in the present study.

There are interventions with findings similar to ours, and these interventions were conducted among younger healthy adults. Myhrstad et al included female participants ($n = 14$) who were served cakes enriched with EPA/DHA (5 E %) from cod liver oil, ALA (5 E %) from linseed oil or coconut oil (5 E %) serving as control. Postprandial effects on PBMC gene expression were then investigated. After intake of the cake enriched with EPA and DHA there was an increased expression of IL-8 after 6 hours compared to the ALA group and compared to baseline (150). The increased expression of IL-8 was confirmed in EPA-stimulated cells *ex vivo*, while there were no changes in circulating markers. The writers suggest that ALA has a more neutral effect on gene expression in PBMC, and as an increase in PPAR- γ was observed in the EPA stimulated cells they further hypothesize that the longer unsaturated fatty acids exert stronger effects on PPARs and TLRs in PBMC (150). This could be transferable to the results presented here. Gray et al conducted an intervention among young males ($n = 16$), and had an endurance test at baseline and after the supplementation with fish oil, which consisted of an intake of 3g EPA/DHA daily for 6 weeks. The PBMC gene expression of IL-2 was increased compared to the control group receiving olive oil capsules, and they also reported an increase in NK-cell activity. The writers claim that there is little known about the physiological effects of such an increase, but hypothesize that it may promote the antiviral defense, and in that sense protect against upper airway infections among athletes/trained subjects (151).

Lipid metabolism genes

The PBMC gene expression of PPAR γ , PPAR δ , UCP2 and LIPE was increased within the salmon group. The change in EPA correlated with the change in LIPE for the whole group, but the association disappeared when only the salmon group was investigated. There were no other correlations between up-regulated lipid metabolism genes and EPA or DHA. Myhrstad et al found increased PPAR γ in EPA stimulated cells (150). This is line with our findings. Fatty acids and their oxidized metabolites are ligands for PPARs (152). A study by Rudkowska et al revealed that responders and non-responders to n-3 PUFA supplementation had different patterns of expression of genes involved in lipid metabolism, including ARA-metabolism (135). Rudkowska et al further described different responsiveness of n-3 PUFA in effects on the PPAR- α pathway. The responders had increased expression of PPAR- α , while it was unchanged for the non-responders (153).

Inter-individual differences in gene expression determine the inflammatory responsiveness to interventions. Thus effectiveness of diet or supplementation may vary between individuals independently of dosage and intervention (117). Genes are expressed differently depending on sex, age, BMI and the presence of the different leukocyte populations in the organism and/or circulation (154). In an intervention study participants were allocated to 0, 1, 2 or 4 servings of oily fish every week for 12 months, and stratified for age and sex. Results demonstrated that females had higher levels of EPA than men, and that the change during the intervention decreased with increasing age (111). It is postulated that varying response to dietary intake of n-3 PUFAs among men and women is due to hormonal levels, as estrogen enhances the n-3 effects on gene expression, while testosterone inhibits it (155). Rudkowska et al identified different responsiveness of n-3 PUFA among women and men. Men had changes in NF- κ B genes, while women exhibited changes in Nrf2 pathway (153). When the males were taken out of this result file, the significance disappeared for the change in PLA2G4A expression between the groups. Furthermore, within group tests revealed significantly increased PBMC expression within the cod group, TLR6 ($p = 0.01$) and tissue plasminogen activator (PLAT) ($p = 0.02$). As the number of participants with measurements for change of gene expression in the salmon group was only five and they were all female, these findings could also be attributable to the inter-individual differences in gene expression, or related to the small n .

The results in the present thesis are somewhat contradictory to the understanding that fish and n-3 PUFA supplementation reduce inflammation, although other studies have found the similar tendency of increased PBMC gene expression of inflammatory markers following intake of n-3 PUFAs among healthy young adults (150, 151). In elderly or CVD patients n-3 PUFA supplementation have generated no effect or an anti-inflammatory effect (145-147). In any case it would be hard to generalize and draw any firm conclusions on this basis, considering the low number of participants and the possibility of inter-individual responses having an actuating effect in such a small group. Furthermore, the physiological effects of an increased PBMC gene expression described herein are not completely understood. Thus more research is needed on gene expression level among this group.

6.3 Limitations and strengths of the study

A limitation of the study is its low number of participants for the LDA-card analyses. In the salmon group all participants were female, due to lack of stratification for gender. Even though data on dietary intake was reported with an FFQ, the dietary composition prior to and during the intervention was not known. Thus, possible changes of dietary habits attributable to the intervention were not obtainable. Furthermore, the energy contributions from fat, protein and carbohydrates were not estimated for the participants. It is not possible to determine whether the changes seen in blood lipids and PBMC gene expression could be related to other factors than the intake of fish due to the lack of knowledge on how the intervention otherwise changed the diet in the study population. As mentioned by Myhrstad et al, subjects who participate in dietary interventions are likely to alter other aspects of their everyday lives. The other bioactive components in fish could exert unmeasured effects (118). Potato in itself holds a larger amount of carbohydrates than fish, whereas fish constitutes of highly bio-available proteins (38). The intake of salmon during the 15 day intervention far exceeds the dietary guidelines from health authorities, who recommend an intake of no more than 200 g of fatty fish per week due to contaminants that are transferred and stored in adipose tissue in humans (45).

Strengths of the study are that participants were randomized, thus the unfortunate gender distribution occurred by chance. Furthermore, participants were equipped with lunch boxes to reassure good compliance, which was estimated as high. The use of the dietary component fish rather than supplementation of n-3 PUFA is another strength, as some suggest that the incorporation of n-3 PUFAs from fish seemingly is more efficient than from supplementation (156). Fish also contain other nutrients which may promote health (118). A recent review proclaims that a change in the overall dietary patterns are of more importance than the focus on n-3 PUFA supplementations, and concludes that a balanced intake of PUFA can be obtained by diet instead of use of supplements (157). The measures of both PBMC gene expression and circulating markers is also a strength, as PBMC gene expression offers a picture of the local changes in the arterial wall due to their sensitivity towards changes in their environment, and as the concentration of circulating markers may originate from several cells and tissues, such as adipose tissue or muscle, in addition to the generally low level of circulating inflammatory markers in healthy humans which can cause difficulties in detecting

changes (150). Furthermore the high number of genes that were analyzed with the LDA-cards contributed to a more complete impression of inflammatory traits as there is an interplay between the different components in the immune system (132). Compliance was estimated as high, physical activity levels and weight were maintained during the intervention period (83).

7 Conclusion

The molecular effects of fish consumption on inflammatory markers have not yet been thoroughly investigated, and very little is known about the intake of fatty compared with lean fish on inflammatory markers. The present thesis yielded the following findings:

- HDL-cholesterol was increased in the salmon group and decreased in the potato group, while TG-levels were reduced in the salmon, cod and blue mussel group. TG-levels were increased in the potato group.
- The plasma PL levels of EPA and DHA were increased in the salmon and blue mussel group, and DHA was increased in the cod group. There were no changes in the potato group.
- There were no changes between the groups in the concentration of circulating inflammatory markers. Nevertheless, hsCRP was decreased within the blue mussel group.
- The PBMC gene expression of PLA2G4A was increased in the groups who consumed salmon and cod compared to those who consumed potato.
- Within the salmon group, the PBMC gene expression of 12 genes was increased. Four of these are involved in lipid metabolism, whereas the rest are inflammatory markers of the innate and adaptive immune system.

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Appendices/supplementary

Appendix 1: Δ Ct values at baseline and end of study for all genes in the intervention groups

Inflammatory genes	Treatment	n	Baseline	End of study	P ¹	P ²
CPT	Control/potato	6	3.78 (3.09 - 4.66)	3.22 (2.19 - 6.63)	0.92	0.91
	Cod	6	3.22 (2.58 - 6.49)	3.16 (2.86 - 4.35)	0.92	
	Salmon	5	3.64 (2.77 - 4.32)	3.40 (3.00 - 4.38)	0.50	
CXCL	Control/potato	7	1.72 (1.32 - 1.94)	1.47 (0.99 - 1.53)	0.50	0.83
	Cod	6	1.47 (1.14 - 2.33)	1.26 (1.03 - 1.90)	0.92	
	Salmon	5	1.09 (0.88 - 1.34)	1.07 (0.91 - 1.51)	0.89	
IL18	Control/potato	6	0.13 (0.10 - 0.18)	0.21 (0.13 - 0.24)	0.17	0.70
	Cod	6	0.17 (0.10 - 0.27)	0.21 (0.12 - 0.24)	0.35	
	Salmon	5	0.11 (0.07 - 0.13)	0.12 (0.06 - 0.16)	0.50	
IL1B	Control/potato	6	0.32 (0.24 - 0.56)	0.26 (0.18 - 0.68)	0.75	0.68
	Cod	6	0.26 (0.18 - 0.41)	0.41 (0.22 - 0.61)	0.46	
	Salmon	5	0.31 (0.25 - 0.33)	0.32 (0.22 - 0.53)	0.50	
IL8	Control/potato	7	0.06 (0.03 - 0.23)	0.06 (0.04 - 0.24)	0.40	0.83
	Cod	6	0.12 (0.06 - 0.22)	0.18 (0.11 - 0.99)	0.25	
	Salmon	5	0.02 (0.02 - 0.11)	0.11 (0.05 - 0.22)	0.23	
SERPINE	Control/potato	7	0.23 (0.17 - 0.29)	0.17 (0.14 - 0.24)	0.50	0.31
	Cod	6	0.21 (0.15 - 0.44)	0.20 (0.15 - 0.26)	0.35	
	Salmon	5	0.21 (0.14 - 0.24)	0.28 (0.24 - 0.30)	0.04	
TFPI	Control/potato	7	0.42 (0.36 - 0.69)	0.50 (0.32 - 0.57)	0.50	0.47
	Cod	6	0.56 (0.38 - 1.00)	0.72 (0.40 - 0.78)	0.92	
	Salmon	5	0.39 (0.30 - 0.45)	0.52 (0.29 - 0.57)	0.23	
PLAT	Control/potato	6	0.011 (0.008 - 0.013)	0.010 (0.007 - 0.014)	0.92	0.18
	Cod	6	0.008 (0.005 - 0.010)	0.015 (0.005 - 0.019)	0.17	
	Salmon	4	0.012 (0.006 - 0.021)	0.024 (0.015 - 0.035)	0.07	
CD40 *	Control/potato	7	0.49 (0.35 - 0.67)	0.51 (0.45 - 0.66)	0.61	0.22
	Cod	6	0.81 (0.55 - 0.99)	0.70 (0.51 - 0.90)	0.75	
	Salmon	5	0.48 (0.41 - 0.62)	0.54 (0.48 - 0.98)	0.04	
CD40L	Control/potato	7	1.65 (1.20 - 1.72)	1.69 (1.18 - 2.23)	0.18	0.51
	Cod	6	1.73 (1.56 - 2.99)	1.93 (1.47 - 2.30)	0.60	
	Salmon	5	1.86 (1.54 - 3.01)	2.27 (2.08 - 3.11)	0.23	
CD36 *	Control/potato	6	14.71 (9.44 - 16.48)	14.02 (9.13 - 16.25)	0.75	0.39
	Cod	6	14.55 (11.21 - 21.53)	15.27 (12.40 - 17.90)	0.92	
	Salmon	5	8.87 (8.47 - 9.46)	12.23 (8.27 - 15.74)	0.23	
CD8A	Control/potato	7	16.61 (13.62 - 25.16)	17.01 (13.89 - 32.39)	0.24	0.75
	Cod	6	17.73 (11.59 - 46.70)	20.46 (15.99 - 24.16)	0.75	
	Salmon	5	16.34 (9.10 - 28.02)	18.91 (14.48 - 30.11)	0.04	
CD3E	Control/potato	7	32.01 (18.59 - 39.83)	28.37 (22.17 - 37.40)	1.00	0.51
	Cod	6	28.01 (25.19 - 63.42)	30.39 (26.10 - 39.08)	0.46	
	Salmon	5	29.94 (24.56 - 41.14)	34.79 (30.99 - 45.60)	0.14	

Continued from Appendix 1 , Δ Ct values at baseline and end of study, for the intervention groups						
GATA	Control/potato	7	3.99 (3.72 - 4.15)	5.10 (3.70 - 6.06)	0.50	0.849
	Cod	6	4.52 (3.35 - 6.62)	4.18 (3.57 - 5.88)	0.92	
	Salmon	5	4.43 (3.66 - 6.05)	5.48 (4.78 - 6.69)	0.04	
PTGS	Control/potato	7	0.16 (0.11 - 0.19)	0.12 (0.11 - 0.23)	0.74	0.31
	Cod	6	0.10 (0.09 - 0.20)	0.25 (0.11 - 0.32)	0.17	
	Salmon	5	0.08 (0.07 - 0.16)	0.09 (0.08 - 0.18)	0.89	
TLR2 *	Control/potato	7	2.33 (1.43 - 2.86)	2.27 (1.48 - 2.53)	1.00	0.26
	Cod	6	2.98 (2.53 - 3.81)	3.30 (1.76 - 5.18)	0.60	
	Salmon	5	1.62 (1.04 - 1.79)	2.04 (1.60 - 3.05)	0.08	
TLR4 *	Control/potato	7	2.29 (2.12 - 2.93)	2.33 (1.90 - 2.52)	1.00	0.18
	Cod	6	2.59 (2.00 - 3.38)	2.54 (1.85 - 3.58)	0.60	
	Salmon	5	1.73 (1.49 - 1.77)	2.21 (1.79 - 2.89)	0.04	
TLR6	Control/potato	7	0.32 (0.26 - 0.40)	0.25 (0.18 - 0.34)	0.13	0.77
	Cod	6	0.31 (0.22 - 0.36)	0.26 (0.19 - 0.36)	0.08	
	Salmon	4	0.22 (0.19 - 0.37)	0.19 (0.13 - 0.23)	0.47	
CTSS *	Control/potato	7	69.75 (60.88 - 99.78)	76.85 (70.62 - 99.23)	0.50	0.41
	Cod	6	95.06 (87.40 - 121.65)	99.00 (69.39 - 123.03)	0.92	
	Salmon	5	54.54 (44.49 - 72.86)	73.29 (60.25 - 111.27)	0.04	
PLTP *	Control/potato	7	0.13 (0.11 - 0.19)	0.13 (0.09 - 0.17)	0.87	0.65
	Cod	6	0.15 (0.10 - 0.21)	0.12 (0.11 - 0.25)	0.75	
	Salmon	5	0.08 (0.05 - 0.09)	0.09 (0.08 - 0.09)	0.14	
NAMPT	Control/potato	7	2.73 (2.32 - 3.22)	2.47 (2.04 - 2.72)	0.18	0.80
	Cod	6	2.69 (2.22 - 3.19)	2.70 (2.27 - 3.38)	0.92	
	Salmon	5	2.23 (1.60 - 2.84)	2.05 (1.67 - 2.73)	0.89	
NR1H3	Control/potato	7	0.22 (0.19 - 0.26)	0.22 (0.18 - 0.26)	1.00	0.81
	Cod	6	0.22 (0.19 - 0.31)	0.23 (0.21 - 0.30)	0.60	
	Salmon	5	0.21 (0.17 - 0.21)	0.20 (0.16 - 0.22)	0.69	
TBX21	Control/potato	7	8.08 (7.18 - 9.69)	7.88 (5.41 - 11.23)	0.74	0.67
	Cod	6	7.00 (6.08 - 11.27)	6.05 (5.41 - 11.98)	0.75	
	Salmon	5	9.27 (3.03 - 11.45)	7.78 (4.49 - 12.34)	0.50	
SQSTM1 *	Control/potato	7	4.77 (3.99 - 5.50)	5.15 (4.10 - 5.72)	0.61	0.67
	Cod	6	5.34 (4.39 - 7.64)	5.51 (3.97 - 6.38)	0.75	
	Salmon	5	3.98 (3.60 - 4.88)	5.90 (3.96 - 6.60)	0.23	
TGF2	Control/potato	7	0.05 (0.02 - 0.06)	0.06 (0.02 - 0.06)	0.50	0.38
	Cod	5	0.03 (0.02 - 0.07)	0.04 (0.03 - 0.06)	0.89	
	Salmon	5	0.03 (0.02 - 0.05)	0.02 (0.01 - 0.06)	0.89	
TNF	Control/potato	7	0.99 (0.73 - 1.43)	0.76 (0.55 - 1.16)	0.40	0.06
	Cod	6	0.71 (0.46 - 0.96)	0.65 (0.49 - 0.92)	0.75	
	Salmon	5	0.71 (0.58 - 0.91)	0.99 (0.90 - 1.41)	0.04	
IFNG	Control/potato	7	0.25 (0.14 - 0.67)	0.30 (0.19 - 0.44)	0.50	0.44
	Cod	6	0.14 (0.11 - 0.31)	0.16 (0.09 - 0.20)	0.46	
	Salmon	5	0.20 (0.12 - 0.33)	0.17 (0.14 - 0.25)	0.50	
FOXP3	Control/potato	7	0.37 (0.24 - 0.42)	0.40 (0.30 - 0.54)	0.61	0.35
	Cod	6	0.42 (0.30 - 0.57)	0.37 (0.32 - 0.50)	0.75	
	Salmon	5	0.39 (0.36 - 0.49)	0.54 (0.45 - 0.62)	0.04	

Continued from Appendix 1 , Δ Ct values at baseline and end of study, all intervention groups						
PLA2G7	Control/potato	7	0.24 (0.08 - 0.35)	0.16 (0.10 - 0.32)	0.50	0.89
	Cod	5	0.26 (0.14 - 0.33)	0.25 (0.16 - 0.31)	0.89	
	Salmon	4	0.19 (0.15 - 0.24)	0.20 (0.13 - 0.34)	0.35	
PLA2G4A	Control/potato	7	0.32 (0.28 - 0.36)	0.26 (0.24 - 0.31)	0.06	0.03^{a,b}
	Cod	6	0.32 (0.28 - 0.35)	0.34 (0.26 - 0.43)	0.35	
	Salmon	5	0.25 (0.21 - 0.27)	0.29 (0.26 - 0.36)	0.04	
Lipid metabolism genes	Treatment	n	Baseline	End of study	P ¹	P ²
LIPE	Control/potato	7	0.42 (0.40 - 0.57)	0.45 (0.40 - 0.57)	0.74	0.17
	Cod	5	0.49 (0.36 - 0.79)	0.50 (0.40 - 0.56)	0.89	
	Salmon	5	0.45 (0.34 - 0.54)	0.64 (0.54 - 0.69)	0.04	
BAG3	Control/potato	6	0.49 (0.32 - 0.65)	0.44 (0.34 - 0.59)	0.92	0.15
	Cod	6	0.54 (0.42 - 0.80)	0.44 (0.37 - 0.53)	0.35	
	Salmon	5	0.46 (0.31 - 0.54)	0.67 (0.48 - 0.87)	0.14	
ABCA1	Control/potato	6	0.24 (0.16 - 0.27)	0.19 (0.11 - 0.28)	0.75	0.24
	Cod	6	0.15 (0.09 - 0.21)	0.19 (0.15 - 0.23)	0.17	
	Salmon	3	0.16 (0.15 - 0.26)	0.29 (0.20 -)	0.11	
ABCG1	Control/potato	7	1.05 (0.56 - 1.19)	0.88 (0.68 - 1.42)	0.61	0.39
	Cod	6	0.94 (0.76 - 1.34)	0.92 (0.77 - 0.96)	0.46	
	Salmon	5	0.68 (0.43 - 1.21)	0.91 (0.80 - 1.22)	0.35	
ACOX *	Control/potato	5	1.51 (1.22 - 1.96)	1.49 (1.24 - 2.14)	0.50	0.35
	Cod	6	1.30 (1.01 - 2.20)	1.51 (1.27 - 1.86)	0.92	
	Salmon	4	0.92 (0.82 - 1.02)	1.33 (1.23 - 1.66)	0.07	
PPARD	Control/potato	7	1.62 (1.12 - 1.77)	1.68 (1.33 - 1.84)	0.74	0.17
	Cod	6	1.95 (1.44 - 2.39)	1.72 (1.36 - 2.06)	0.60	
	Salmon	5	1.63 (1.30 - 1.95)	2.53 (1.91 - 2.93)	0.04	
PPARGC1	Control/potato	7	0.68 (0.57 - 0.86)	0.65 (0.52 - 1.06)	0.31	0.61
	Cod	6	0.76 (0.59 - 0.79)	0.82 (0.61 - 0.95)	0.17	
	Salmon	5	0.59 (0.52 - 0.69)	0.77 (0.58 - 0.90)	0.04	
SCD	Control/potato	7	0.23 (0.21 - 0.28)	0.24 (0.16 - 0.34)	0.87	0.58
	Cod	6	0.33 (0.19 - 0.48)	0.26 (0.21 - 0.37)	0.75	
	Salmon	5	0.18 (0.12 - 0.27)	0.22 (0.19 - 0.27)	0.50	
SREBP1F	Control/potato	7	1.09 (0.93 - 1.25)	1.27 (0.66 - 1.35)	0.87	0.24
	Cod	6	1.33 (0.82 - 1.77)	0.95 (0.87 - 1.21)	0.25	
	Salmon	5	1.09 (0.86 - 1.30)	1.41 (1.17 - 1.56)	0.23	
UCP2	Control/potato	7	31.65 (28.82 - 36.18)	32.98 (19.88 - 49.41)	0.74	0.27
	Cod	6	33.42 (26.33 - 48.66)	28.33 (24.14 - 46.42)	0.75	
	Salmon	5	27.27 (18.67 - 32.08)	33.73 (29.81 - 46.28)	0.04	

Data are given as median (IQR)

P¹ Wilcoxon test for expression at baseline vs end point

P² Kruskal Wallis for change of expression, between all groups

* Indicates differences at baseline

Bold indicates significant findings

^a P=0.046 for cod compared to potato, Mann Whitney

^b P=0.019 for samlon compared to potato, Mann Whitney

Appendix 2: Primers for the LDA-cards analyses

LDA cards		
Gene name	Full name	Hs number
ABCA1	ATP-binding cassette, sub-family A	Hs01059118_m1
ABCG1	ATP-binding cassette, sub-family G	Hs01555189_m1
ACOX1	acyl-Coenzyme A oxidase	Hs00244515_m1
CD36	thrombospondin receptor	Hs00169627_m1
CPT1A	carnitine palmitoyltransferase 1A	Hs00912681_m1
CXCL16	chemokine (C-X-C motif) ligand 16	Hs00222859_m1
IL18	interleukin 18	Hs01038788_m1
IL1B	interleukin 1 β	Hs00174097_m1
IL8	interleukin 8	Hs99999034_m1
PLA2G4A	phospholipase A2, group IV	Hs00233352_m1
CD8A	CD8a molecule	Hs00233520_m1
CD3E	CD3e molecule, ϵ	Hs01062241_m1
FOXP3	forkhead box P3	Hs01085834_m1
NAMPT	nicotinamide phosphoribosyltransferase	Hs00237184_m1
PLTP	phospholipid transfer protein	Hs01067287_m1
PPARD	peroxisome proliferator-activated receptor δ	Hs00602622_m1
PPARGC1B	peroxisome proliferator-activated receptor γ , coactivator 1 β	Hs00370186_m1
SCD	stearyl-CoA deaturase	Hs01682761_m1
SREBF1	sterol regulatory element binding transcription factor 1	Hs01088691_m1
TNF	tumor necrosis factor 1	Hs00174128_m1
UCP2	uncoupling protein 2	Hs01075225_m1
IFNG	interferon γ	Hs00989291_m1
TLR4	toll-like receptor 4	Hs00152939_m1
TLR6	toll-like receptor 6	Hs00271977_s1
PLA2G7/Lp-PLA2	phospholipase A2, group VII	Hs00173726_m1
NR1H3	nuclear receptor subfamily 1, group H, member 3	Hs00172885_m1
LIPE	hormone sensitive lipase	Hs00943410_m1
PLAT (t-PA)	tissue plasminogen activator	Hs00263492_m1
CD40/TNFRSF5	CD40 molecule, TNF receptor superfamily member 5	Hs99999100_s1
CD40LG/TNFSF5	CD40 ligand	Hs00163934_m1
CTSS	cathepsin S	Hs00175403_m1
TGFB2	transforming growth factor, β 2	Hs00234244_m1
TBX21	T-box 21	Hs00203436_m1
GATA3	GATA binding protein 3	Hs00231122_m1
TLR2	toll-like receptor 2	Hs01014511_m1

Continued from Appendix 2: Primers for LDA-card analyses		
PTGS2	prostaglandin-endoperoxide synthase 2	Hs00153133_m1
BAG3	BCL2-associated athanogene 3	Hs00188713_m1
SQSTM1	sequestosome 1	Hs00177654_m1
SERPINE1/PAI-1	serpine peptidase inhibitor, clade E member 1	Hs01126607_g1
TFPI	tissue factor pathway inhibitor	Hs00196731_m1
Reference genes		
TBP*	TATA-binding protein	Hs00427620_m1
GUSB	glucuronidase β	Hs99999908_m1
PPIA	peptidyl prolyl isomerase A	Hs99999904_m1

* Ct values from the target genes were related to this reference gene

Appendix 3: Primers for the single gene analyses

Single genes		
Gene name	Full name	Hs number
MMP9	matrix metalloproteinase 9	Hs00234579_m1
TIMP1	metallo peptidase inhibitor 1	Hs00171558_m1
IL-6	interleukin 6	Hs00985639_m1
Reference genes		
ACTB	actin β	Hs99999903_m1
TBP	TATA-binding protein	Hs00427620_m1
PPIA	peptidyl prolyl isomerase A	Hs99999904_m1