The impact of long chain polyunsaturated fatty acids on food allergy and cardiovascular disease

Fish and no chips?

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Fish and no chips?

De invloed van lange keten meervoudig onverzadigde vetzuren op voedselallergie en cardiovasculaire ziekten

(met een samenvatting in het Nederlands)

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CHAPTER ONE

General introduction

Over the last years scientists have focused more and more on the relation between lifestyle, health and disease. Several food components are associated with beneficial health effects, while other dietary habits are associated with diseases such as allergies and cardiovascular disease (CVD). While conventional drugs act via the 'one target-one drug' concept, nutrition exerts smaller effects at multiple sites (1). Amongst others, polyunsaturated fatty acids (PUFA) are extensively studied in relation to health and disease.

The human diet contains a broad variety of fatty acids differing in chain length and amount of double bonds. PUFA contain two or more double bonds and are categorized in n-6 and n-3 PUFA according to the position of the first double bond near the methyl end (see review **Chapter Two**). The recommended daily consumption of fat is 30% of total calorie intake (approximately 65 g). The European Food Safety Authority (EFSA) recommends that 4.5% of total daily energy intake is derived from n-6 PUFA and 1% from n-3 PUFA (2). The last decades have seen a fall in the consumption of saturated fatty acids, whereas the intake of PUFA has increased. This shift in fatty acid intake mainly involved the increased use of vegetable oils and products such as margarine that are rich in n-6 PUFA, predominantly linoleic acid (LA; 18:2n-6) «Table 1». On the other hand the consumption of long chain n-3 PUFA (LCPUFA) from oily fish, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) consisting of 20 or more carbon atoms, has gradually decreased «Table 2» (3, 4). Humans evolved on a diet with a 1:1 ratio of n-6:n-3 PUFA. Nowadays this ratio is approaching 10-25:1 in westernized countries (4, 5).

Table 1 Typical fatty acid composition of vegetable oils (% of total fatty acids) (6)

	SFA	MUFA	PUFA	
			LA	ALA
Canola oil	6	62	22	10
Corn oil	13	28	58	1
Olive oil	17	72	10	1
Soybean oil	15	24	54	7
Sunflower oil	12	19	68	1

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic acid

The clinical health benefits from the use of n-3 LCPUFA from oily fish in the (secondary) prevention of CVD have been demonstrated clearly (see **Chapter Two**). A large part of the Western population has a high risk of developing CVD and it is one of the most important causes of death in developed countries (7, 8). N-3 LCPUFA research in CVD was inspired by the very low cardiovascular mortality rate among the Greenland Inuit (10-30% lower than in Denmark) which was associated with their diet (9, 10). Inuit consume large amounts of oily fish containing a high content of n-3 LCPUFA and therefore have high

plasma levels of EPA and DHA and low levels of n-6 PUFA (11, 12). Besides the low prevalence of CVD, Inuit also have a low prevalence of allergic diseases. 15-30% Of the Western population is affected by allergic disease. Atopic disease prevalence is low when a high n-3 LCPUFA content is found in sera (13, 14). Furthermore, a high intake of n-6 PUFA, especially LA, is associated with allergic sensitization and eczema (15, 16). However, it remains to be elucidated whether n-3 LCPUFA are capable of reducing the susceptibility to develop allergic disease.

Table 2 Typical fatty acid composition in selected fish (per 100 g edible portion, raw) (4)

	Total fat	SFA	MUFA	PUFA		
				Total	EPA	DHA
Anchovy	4.8	1.3	1.2	1.6	0.5	0.9
Cod	0.7	0.1	0.1	0.3	0.1	0.2
Haddock	0.7	0.1	0.1	0.2	0.1	0.1
Halibut	13.8	2.4	8.4	1.4	0.5	0.4
Herring (Pacific)	13.9	3.3	6.9	2.4	1.0	0.7
Mackerel	13.0	2.5	5.9	3.2	1.0	1.2
Salmon (Chinook)	10.4	2.5	4.5	2.1	0.8	0.6
Trout	3.4	0.6	1.0	1.2	0.1	0.4
Tuna (albacore)	4.9	1.2	1.2	1.8	0.3	1.0

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid

FOOD ALLERGY

With an estimated prevalence of 30% in children in Western societies, atopic disorders are an important public health problem (17). Atopic dermatitis and food allergy readily develop in the first year of life and in 30% of children with atopic dermatitis food allergy is the underlying cause (18, 19). Food allergy affects about 6% of young children (20) and the major food allergens are milk, egg, peanut, three nuts, shellfish, fish, wheat and soy (21). Symptoms include atopic dermatitis, gastro-intestinal and pulmonary distress and sometimes anaphylaxis. Children are more prone to develop an allergic reaction to food proteins, since their mucosal barrier and immune system is not yet fully developed and therefore less efficient (22).

Although also non-IgE mediated food allergic responses exist, often an IgE dependent type I hypersensitivity reaction is induced. This is characterized by T helper 2 (Th2) polarization of the immune response and results in acute (<1h) symptoms due to mast cell activation (see **Chapter Two**). The underlying etiology is still largely unclear. While there is a strong genetic factor contributing to food allergy, environmental factors may

contribute to the increased prevalence of allergic disease. This includes the hygiene hypothesis and dietary alterations (23). Besides a decline in breast-feeding and the early introduction of solid foods, major alterations in the diet have paralleled the increase in allergic disease (24-26). The latter includes, amongst others, the reduced consumption of n-3 LCPUFA as discussed above.

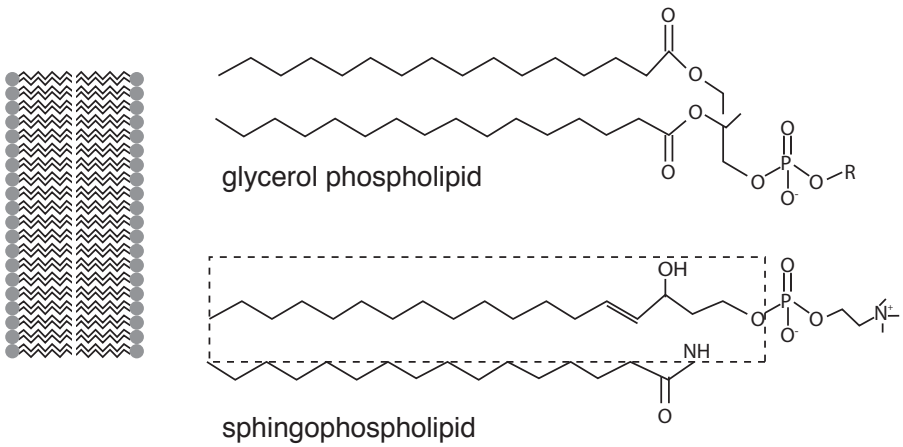

Figure 1 Lipid bilayer of the cell membrane containing phospholipids. Glycerol phospholipids consist of two hydrophobic fatty acid chains with varying chain length, double bond number and position and a polar phosphate-containing head group. The sphingophospholipid sphingomyelin is composed of a sphingoid base (indicated by dotted line), linked to a saturated or monounsaturated fatty acid and phosphocholine head group.

PUFA metabolites and allergy

PUFA are thought to function via several mechanisms (27). Dietary lipids incorporate in the phospholipids of the cell membrane «Figure 1» and thus can alter membrane fluidity and lipid raft composition of a large variety of cell types (28). Furthermore, PUFA may act on intra- and extracellular receptors and transcription factors including peroxisome proliferator-activated receptors (29), mitogen-activated protein kinases (30), Toll-like receptors (31) and G protein-coupled receptors (32). In addition, alterations in eicosanoid production may underlie the effects of PUFA. In contrast to n-3 LCPUFA, n-6 PUFA intake has increased during the last decennia. LA is a precursor for arachidonic acid (AA), which can be metabolized by cyclooxygenases and lipoxygenases into a range of eicosanoids «Figure 2». The AA metabolite prostaglandin (PG)E_2 may promote allergic sensitization by inhibiting the production of interferon γ but not interleukin (IL)-4, subsequently stimulating IgE synthesis (33, 34). The type of fatty acids consumed can influence the amount and type of eicosanoids produced. Dietary n-3 PUFA competitively replace AA in the cell membranes, therefore inhibiting AA conversion to PG and thromboxanes (TX) of the 2- and 4-series. While excessive availability of substrate for the generation of AA-derived

eicosanoids may result in allergic inflammation, EPA gives rise to PG and TX of the 3- and 5-series which are less pro-inflammatory. Furthermore, n-3 LCPUFA can be converted into anti-inflammatory resolvins (35). A more extensive overview of the mechanism of action of n-3 LCPUFA is provided in **Chapter Two**.

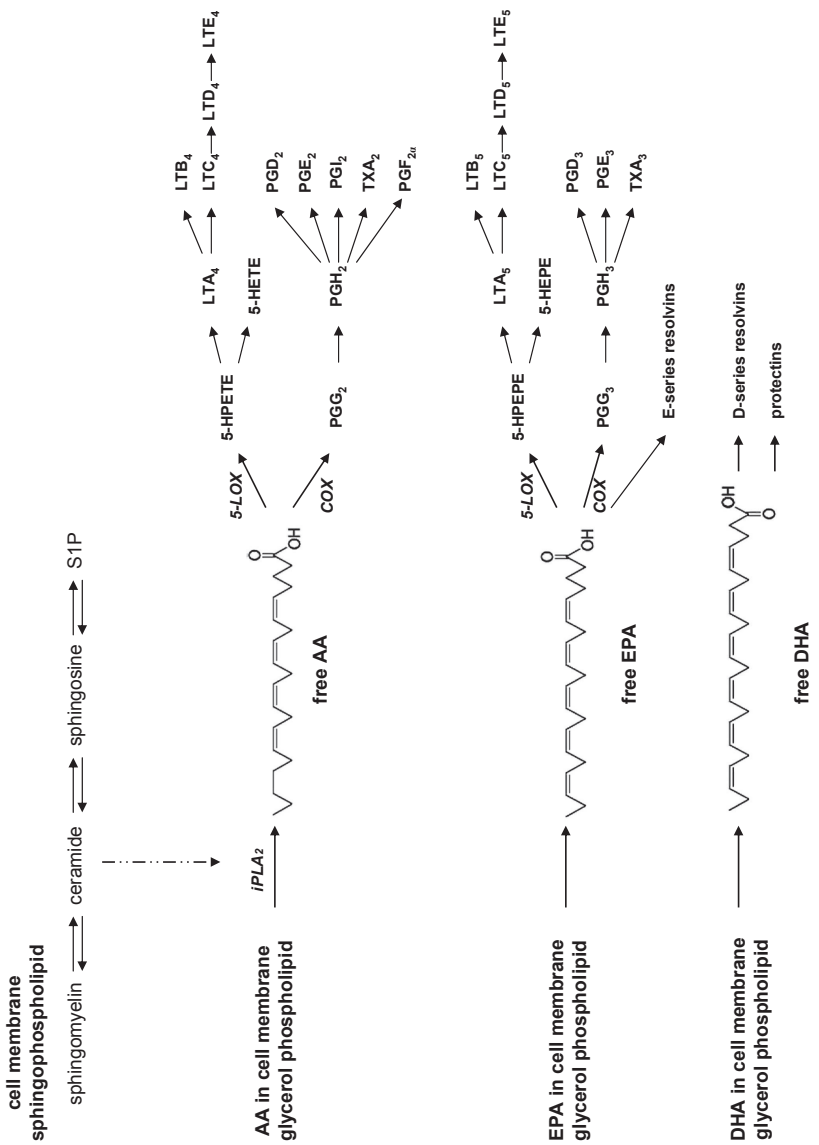

Figure 2 Simplified overview of the major biologically active metabolites (eicosanoids and resolvins) derived from arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the link with the sphingomyelin cycle. Calcium-independent phospholipase A_2 ($iPLA_2$) is involved in the release of AA from membrane glycerol phospholipids. Ceramide is one of the activators of $iPLA_2$. COX: cyclooxygenase HETE: hydroxyeicosatetraenoic acid; HPEPE: hydroperoxyeicosapentaenoic acid; HPETE: hydroperoxyeicosatetraenoic acid; LOX: lipoxygenase; LT: leukotriene; PG: prostaglandin; S1P: sphingosine-1-phosphate; TX: thromboxane.

Allergic sensitization in the gut associated lymphoid tissue

Although normally oral tolerance would be induced against harmless food proteins approximately 6% of young infants render a Th2 type immune response against typical food antigens (20). The immune response in allergy starts with sensitization. Sensitization to food allergens may include the inhalational route (36, 37) and the skin (38). However, the gastro-intestinal tract (GI-tract), dealing with a large range of food proteins daily, is very important in the recognition of food allergens. The gut-associated lymphoid tissue (GALT) is part of the intestinal immune system and consists of the Peyer's patches (PP) and mesenteric lymph nodes (MLN), which are involved in the induction phase of the immune response, and lymphocytes in the lamina propria (LP) and epithelium contributing to effector functions (39). Food allergens containing intact epitopes can be taken up in the intestine by specialized enterocytes, the microfold-cells (M cells), which reside in the epithelial layer that separates the PP from the gut lumen. Furthermore, allergens can cross the intestinal epithelium directly or are sampled from the lumen via dendritic cells (DC) in the LP that extend their dendrites or enter the LP via a disrupted epithelial layer (39-42). After uptake in the PP or LP, the allergens are processed by local DC functioning as allergen presenting cells (APC) «Figure 3». APC which reside in the PP or move from the PP or LP to the MLN, process the protein and subsequently present the peptide fragments via major histocompatibility complex class II (MHC-II) at their surface. Naive T cells in the PP or MLN recognize the allergen in the context of MHC-II (APC peptide-MHC-II complex) via the T cell receptor (39). In case of allergic sensitization for food components, immunity instead of tolerance is rendered against harmless proteins. The T cells develop into Th2 cells which secrete IL-4, IL-5 and IL-13 and leave the MLN to home back to the LP and enter the periphery (e.g. spleen) to exert their effector function. Th2 cells induce immunoglobulin (Ig) class-switching in B cells resulting in the production of allergen-specific IgE antibodies. Via the blood these antibodies attach to the surface of the effector cells (FcεRI receptors on mast cells) and upon second exposure to the allergen the surface-bound IgE is cross-linked (22, 43). Mast cells are activated and release inflammatory mediators like PG and histamine causing the immediate allergic reaction (44). Inflammatory mediator release also causes recruitment and activation of inflammatory cells inducing the late phase reaction of the allergic response (43).

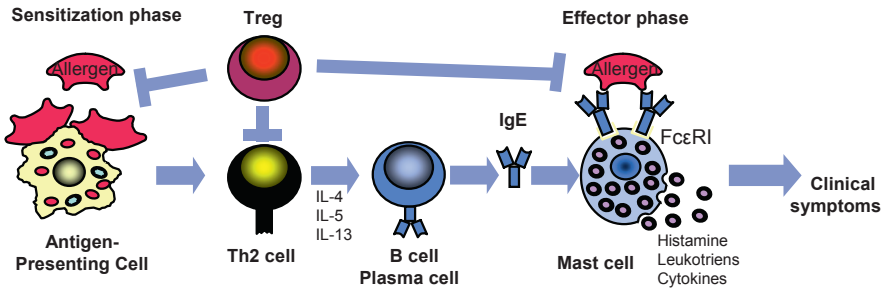

Figure 3 Schematic representation of an IgE-mediated allergic reaction to food allergens. During allergic sensitization the presentation of a food protein by dendritic cells results in the induction and activation of allergen-specific T helper 2 (Th2) cells instead of tolerance. Via the secretion of cytokines these Th2 cells stimulate B cells to produce allergen-specific IgE which binds to high affinity IgE receptors (FcεRI) present on mast cells and basophils. Re-exposure to the food allergen results in crosslinking of IgE bound to the effector cell (mast cell and basophil), which provokes degranulation and the release of mediators that induce clinical symptoms. Regulatory T cells (Treg) suppress this immune reaction.

Oral tolerance induction

In food allergic patients tolerance fails to develop or is broken, resulting in an allergic response (45). In the healthy situation, a hypersensitivity reaction to ingested harmless food allergens is prevented by oral tolerance induction suppressing the immune response. Tolerance is generated at the inductive sites of the GALT where proteins that escaped degradation in the GI-tract may be taken up (40, 42). Here immune cells must distinguish harmless food allergens from pathogens. Oral tolerance can be induced via anergy or clonal deletion of T cells leading to non-responsiveness for the allergen in cases of high allergen dose; while at lower doses of the allergen present, active suppression of the immune response via the induction of regulatory T cells (Treg) is involved (46).

Mucosal DC not encountering harmful microbes either ignore the allergen or promote regulatory responses. Several subclasses of DC with regulatory functions are present in the intestine and may contribute to oral tolerance induction (47, 48). These DC subsets suppress T cell responses by inducing the differentiation of naive T cells into Treg in PP and MLN (48-50). This includes the $CD11b^{+}CD8\alpha^{-}$ DC subset that may induce a tolerogenic response (47, 48, 51, 52). Furthermore, the tolerogenic subset of $CD103^{+}$ DC ($CD103^{+}CD11b^{+}CD8\alpha^{-}$) induces $FoxP3^{+}$ Treg via retinoic acid (24,32,60). In addition, $B220^{+}$ plasmacytoid DC (pDC) have been shown to induce the differentiation of $CD25^{+}$ Treg (53-55).

Regulatory cell types include transforming growth factor (TGF)-β producing Th3 cells, IL-10 producing Tr1 cell or $CD4^{+}CD25^{+}$ Treg expressing the transcription factor forkhead box P3 (FoxP3) (56-58). $CD4^{+}CD25^{+}FoxP3^{+}$ Treg either develop in the thymus (central, nTreg) or are induced in the periphery from naive precursors (iTreg) (56). They function directly via cell-cell contact or indirectly by secretion of suppressive cytokines such as IL-10 and

TGF-β that promote active suppression of effector T cells resulting in tolerance. Furthermore Treg may reduce the allergen presenting capacity of APC and target mast cell function «Figure 3» (56, 58). Improving the induction of oral tolerance, for example via the increase of iTreg to prevent or dampen initial sensitization to food allergens, is a strategy in the prevention of food allergy.

Cow's milk allergy

Currently, avoidance of protein ingestion is used to prevent early sensitization to food proteins in infants at risk of developing atopic disease. For cow's milk allergy (CMA) hydrolyzed infant milk formulas are used that contain small peptides that lack cross-linking capacity resulting in reduced allergenicity. CMA is the first food allergy in childhood and many other allergies develop secondary to CMA (59, 60). The prevalence of CMA ranges from 0.3-3.5% in the Western world (61, 62). The allergy resolves in 19% of the cases by the age of four and in 80% by 16 years of age (62, 63). However, these children are still predisposed to other food allergies and asthma (64, 65). Major protein classes in cow's milk are caseins (80%) and whey proteins (20%). Casein consists mainly of αS1, αS2, β, and κ casein and whey includes α-lactalbumin and β-lactoglobulin. Major allergens in children are β-lactoglobulin and αS1-casein (22, 66). Mostly, patients are allergic to more than one milk protein (59). The clinical symptoms have gastrointestinal, respiratory and cutanous manifestations and are divided into immediate symptoms (e.g. vomiting, angioedema, wheezing), late symptoms (e.g. atopic dermatitis, diarrhea and/or constipation, gastro-oesophagal reflux) and general disease symptoms (e.g. failure to thrive). Furthermore, anaphylactic shock may occur (60). In 60% of the cases CMA is IgE-mediated (67).

Peanut allergy

Another common immediate hypersensitivity reaction to food protein is peanut allergy. About 0.2-1.6% of the Western population is allergic to peanut (61, 62). Peanut allergy generally develops in childhood and is not easily outgrown. In 80% of the cases peanut allergy is persistent for life (68). Ara h 1 and Ara h 2 are considered major allergens since they are recognized by >90% of peanut-allergic patients (69, 70). Furthermore, Ara h 3 is recognized by 45% of the patients (71). Besides these three important allergens there are others that are recognized by a subpopulation of patients (72). Patients must strictly avoid ingestion of peanut since accidental ingestion of a very small amount can lead to serious symptoms, sometimes even involving life-threatening anaphylactic symptoms (73). However, complete avoidance is difficult due to the use of peanut in many products.

Mouse models for food allergy

Animal models are widely used to study allergic sensitization since processes involving the lymphoid organs, including intestinal lymphoid organs, cannot be studied in humans. In this thesis a well-established mouse model for orally induced, IgE-mediated allergy to whey was used to study the effect of n-3 LCPUFA on allergic sensitization and symptoms. Sensitization to cow's milk protein in children involves the oral route rather than systemic sensitization mostly used in mouse models. Therefore C3H/HeOuJ mice were exposed to whey by intragastric gavage for several times in the presence of the mucosal adjuvant cholera toxin (CT) (74). CT disrupts the integrity of the mucosal epithelium and CT-exposed DC prime naive T cells and drive them towards a Th2 polarizing phenotype (75, 76). The adjuvant activity of CT may be attributed to the enhanced allergen presentation and upregulation of various cell surface molecules such as co-stimulatory molecules and chemokine receptors in DC (77, 78). This results in the induction of whey-specific IgE and IgG1 and local intestinal and systemic mast cell opsonization and degranulation upon allergen challenge. The murine model mimics the type I hypersensitivity response in whey allergy in humans. Furthermore a mouse model for IgE-mediated peanut allergy was used to study the effect of n-3 LCPUFA in another food allergy model. The model was adapted from the model used by van Wijk *et al.* (79) and the same protocol was used as for whey. To the best of our knowledge, no studies have been described that determine the effect of PUFA in cow's milk or peanut allergic mice or other orally induced food allergy models.

CARDIOVASCULAR DISEASE

Globally CVD results in death of approximately 17.5 million people each year, which accounts for approximately 30% of deaths. CVD has become the leading cause of morbidity and mortality in developed countries, and surprisingly in some developing countries too (7, 80). Nowadays, a lot of attention is paid to the prevention of CVD. Primary prevention aims to avoid the development of CVD, whereas secondary prevention aims at reducing the incidence of new cardiovascular events and the increase of survival in patients with established disease.

N-3 PUFA in the prevention of CVD

Epidemiological studies have indicated the contribution of dietary fatty acids in the primary prevention of CVD. Fish consuming populations (e.g. Inuit and Japanese) have low prevalence of inflammatory diseases and are protected against cardiovascular morbidity and mortality (81, 82). Furthermore, there are several large prospective observational studies demonstrating a negative association between fish consumption and cardiovascular and/or overall mortality (83-87). However, also studies with no significant re-

lationship exist (88, 89). A case control study in subjects without cardiovascular history consuming one fish meal a week showed a 50% reduction in the risk of primary cardiac arrest as compared to non-fish eaters (90, 91). Meta-analysis showed that the cardio-protective effect of fish consumption is particularly prevalent in high-risk populations (92). Secondary intervention studies in survivors of a cardiovascular incident support the heart health promoting effect of fish (oil) consumption. The Diet and Reinfarction Trial (DART) showed a 29% lower mortality risk in consumers of 300 g fish per week for 2 years compared to non consumers (93). Comparable results were found in a smaller placebo-controlled study. Total cardiac events, non-fatal infarctions and cardiac deaths were reduced after fish oil treatment (94). The GISSI study, including patients hospitalized for myocardial infarction in the last 3 months confirmed these findings. 850 Mg ethyl ester of EPA-DHA/day reduced the cardiovascular mortality rate by 30% compared to placebo. In addition, the rate of sudden death was reduced by 45% (95). Also blood lipids involved in increasing blood pressure (BP) and the development of CVD benefit from n-3 LCPUFA supplementation (96, 97). Hence, n-3 LCPUFA can clearly reduce the risk of CVD. However, the mechanism of action still needs to be further elucidated.

Risk factors in CVD

The most important contributor to the increasing prevalence of CVD is atherosclerosis. Atherosclerosis involves a complex chronic inflammatory process accompanied by accumulation of lipids and fibrous elements in the large arteries (7).

Cardiovascular risk factors contributing to the process of atherosclerosis include hypertension and dyslipidemia. Especially elevated plasma concentrations of low densitiy lipoprotein (LDL) can initiate inflammatory processes (98). Vascular inflammation is frequently involved in the early stage of CVD. It can lead to upregulation of endothelial cell adhesion molecule (CAM) expression and pro-inflammatory cytokines. This is followed by the adherence and transmigration of leukocytes through the endothelium, endothelial dysfunction and tissue injury. This can result in arterial atherosclerosis since it triggers a continuous circle of damage and inflammation, leading to accumulation of a diversity of cells, including macrophage-derived foam cells and leukocytes, which can form a plaque (7, 99). Plaques may occlude the flow of oxygen-rich blood to the organs and other parts of the body and under certain conditions may rupture. This can lead to serious problems including a heart attack, stroke and death due to disruption of the blood flow caused by the obstruction of blood vessels.

Hypertension

Hypertension is one of the major risk factors for cardiovascular disease, affecting 30% of the population worldwide (100). Hypertension promotes atherosclerosis and induces left ventricular hypertrophy contributing to coronary heart disease and heart failure (101). In most cases (95%) patients suffer from essential hypertension, with the pathogenesis

being poorly understood. Hypertension is classified as a systolic BP >140 mmHg and diastolic BP >90 mmHg. Besides a genetic background, obesity, dyslipidemia, low physical activity, smoking and high alcohol or salt consumption are risk factors for hypertension. Treatment of high BP involves the use of antihypertensive drugs including angiotensin signaling inhibitors, β-adrenergic receptor antagonists, calcium-channel inhibitors and diuretics. Next to lowering BP the restoration of endothelial function is important to prevent organ damage.

Endothelial activation

Endothelial cells (EC) form a continuous single-cell lining at the luminal side of blood vessels and therefore constitute the interface between the blood and the extravascular matrix (tissues and organs). Upon activation (e.g. abnormal shear stress, hypoxia, inflammatory cytokines, leukocyte activation) the endothelium expresses selectins, required for adhesion of leukocytes to the vessel's luminal wall. Integrins on lymphocytes become activated and bind to endothelial intercellular adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM) permitting firmer adhesion and diapedesis (102-106). Adhesion molecule expression by EC contributes together with the secretion of pro-inflammatory cytokines such as tumor necrosis factor-α, IL-6, growth factors granulocyte colony-stimulating factor and TGF-β and chemokines such as IL-8 and RANTES (regulated on activation, normal T cell expressed and secreted) the adherence of leukocytes to the endothelium (98, 107-113) which is involved in the early phases of atherosclerosis (99). *In vitro* studies have shown that n-3 LCPUFA suppress EC inflammatory responses which have been suggested to contribute to CVD prevention (111, 114-116).

Sphingolipids in blood pressure regulation

The structure of the blood vessel wall has a major influence on the regulation of BP. The endothelium translates changes in the bloodstream into chemical processes to which the underlying vascular smooth muscle cell layer responds resulting in altered tension and diameter of the vessel wall (117). Vascular tone is exerted by the secretion of endothelium-derived relaxing factors (e.g. nitric oxide and prostacyclin) and endothelium-derived contractile factors (e.g. thromboxane A_2 (TXA_2) and endothelin-1) (118). Endothelial dysfunction, associated with hypertension, is accompanied by reduced relaxation, enhanced contraction, vascular remodeling and inflammation (119, 120).

Sphingolipids play an important role in endothelial functioning and vascular tone (118). Sphingolipids consist of a sphingoid base, often linked via amide bonds to varying fatty acids (121). Sphingomyelin «Figure 1» is a membrane phospholipid that serves as a substrate for sphingomyelinases for the production of ceramide «Figure 2» (122). Alterations in sphingolipid biology, especially increased membrane levels of ceramide, are associated with hypertension. In normotensive rats the concentrations of sphingomyelin, ceramide, sphingosine and sphingosine-1-phosphate are at equilibrium in both plasma

and the vessel wall. However, basal ceramide levels are elevated in both spontaneously hypertensive rats (SHR) and humans with hypertension (123). The SHR model is considered as the best animal model resembling human essential hypertension. Due to multiple genetical defects the BP rises progressively over time in SHR in association with endothelial dysfunction. Increased basal ceramide levels may underlie this process and stimulate the activation of calcium-independent phospholipase A_2 which releases AA from the cell membrane «Figure 2». The n-6 LCPUFA AA serves as a substrate for cyclooxygenase-1 and thromboxane synthase involved in TXA_2 synthesis (124, 125). In SHR the expression of these enzymes is elevated. An increase in TXA_2 release in blood vessels of SHR leads to G-protein coupled thromboxane/prostanoid receptor activation on vascular smooth muscle cells resulting in contraction (123). N-6 LCPUFA AA is a precursor for TXA_2 which may contribute to vasoconstriction. By contrast, fish oil has been shown to reduce hypertension, however the mechanism by which it exerts its effects remains to be unraveled (126-128).

SCOPE OF THIS THESIS

This thesis aims to create more insight into the efficacy and mechanism of action of n-3 LCPUFA, which act on the interface between pharmacology and nutrition in the prevention of allergic and cardiovascular disease. Effects of PUFA were tested *in vitro* on human mast cells. Dietary intervention studies, using fish oil as source of n-3 LCPUFA compared to soybean oil rich in n-6 PUFA, were performed in the mouse model for orally induced cow's milk or peanut allergy. The effect of PUFA on sensitization and/or oral tolerance induction by orally administered whey or peanut protein was studied. In the SHR model of essential hypertension the effects of n-3 LCPUFA on BP and endothelial function was studied. Finally, human umbilical vein endothelial cells (HUVEC) from women who consumed a diet rich in salmon or their habitual diet low in n-3 LCPUFA during pregnancy were stimulated *ex vivo* to study the effect of dietary intervention on endothelial activation.

The first chapter of this thesis describes the scientific background for the studies performed. This is further described in **Chapter Two**, providing a more in-depth review on the mechanistic basis of the preventive role of n-3 LCPUFA in allergic disease and CVD. In **Chapter Three** it was demonstrated that increasing the dietary intake of soybean oil, rich in n-6 PUFA, abrogates oral tolerance induction generated by partial whey hydrolysate and enhances allergic symptoms as assessed in a mouse model for orally induced cow's milk allergy. **Chapter Four** describes the effects of n-6 LCPUFA AA versus n-3 LCPUFA EPA and DHA on mast cell degranulation, phenotype and signaling *in vitro*. These data suggest n-3 LCPUFA to decrease secretion of Th2 type mediators involved in allergy most

effective, via suppression of reactive oxygen species. The effect of partial substitution of dietary n-6 PUFA by n-3 LCPUFA from fish oil on the *in vivo* process of sensitization was studied in **Chapter Five**. In this chapter is described that n-3 LCPUFA in mice largely prevent allergic sensitization to the cow's milk protein whey in association with an increased frequency of intestinal and systemic Treg. The involvement of $CD25^+$ Treg in whey-allergy prevention by n-3 LCPUFA was further studied, as described in **Chapter Six**. Adoptive transfer of splenocytes from protected donor mice confirmed that $CD25^+$ Treg from the donor mice are involved in whey-allergy prevention in recipient mice. **Chapter Seven** continues with the comparison of dietary EPA versus DHA. In this chapter the increased efficacy of DHA over EPA in suppressing allergic symptoms is described for both whey and peanut allergic mice. **Chapter Eight** shows that n-3 LCPUFA in SHR reduce BP and improve endothelial function. A diet rich in DHA lowers membrane AA content, probably contributing to the reduced ceramide-mediated contractions. In addition, enhanced endothelium-dependent relaxation to metacholine and reduced production of contractile TXA_2 and IL-10 were demonstrated *ex vivo*. The data in **Chapter Nine** suggest a role for n-3 LCPUFA in improving endothelial function in human, using samples of the first dietary intervention study providing fish to pregnant woman (Salmon in Pregnancy Study). We describe that fish consumption by pregnant women dampens *ex vivo* EC activation as demonstrated by reduced ICAM-1 expression on LPS stimulated HUVEC. This implicates that dietary fish (oil) could suppress cardiovascular inflammation in humans. Finally, the results are summarized, discussed and concluded in **Chapter Ten**.

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CHAPTER TWO

Long chain n-3 polyunsaturated fatty acids in the prevention of allergic and cardiovascular disease

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ABSTRACT

The diet is considered to have a major impact on human health. Dietary lipids including long chain polyunsaturated fatty acids (LCPUFA) possess potent immunomodulatory activities. Over the last decades the incidence of inflammatory disorders including allergic and cardiovascular diseases (CVD) has been rising. This phenomenon is associated with deficiencies in n-3 LCPUFA, found in fatty fish, and increased content of n-6 LCPUFA in the Western diet. LCPUFA act via different mechanisms including membrane fluidity, raft composition, lipid mediator formation, signaling pathways and transmembrane receptors. N-3 LCPUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can reduce the development of allergic disease by affecting both the innate and adaptive immune system involved in the initiation and persistence of allergic disease. Fish oil has been shown to be effective in the primary prevention of allergic disease in infants at risk when supplemented during pregnancy and lactation. Subtle effects of n-3 LCPUFA on the outcome of the immune response may underlie these protective effects. This review describes the currently reported effects of LCPUFA on dendritic cells, T/B cells and mast cells. Also CVD are positively affected by n-3 LCPUFA. Populations consuming high amounts of oily fish are protected against CVD. Moreover n-3 LCPUFA are effective in the secondary prevention of cardiovascular events. Amongst other effects, EPA and DHA have been shown to suppress endothelial cell activation hereby reducing adhesion molecule expression and endothelial cell – leukocyte interactions. This review describes the mechanistic basis of the preventive role for n-3 LCPUFA in allergic disease and CVD.

SOURCE AND CONVERSION OF DIETARY POLYUNSATURATED FATTY ACIDS

The lipid composition of the cell membrane determines cell structure and function for a large part. Dietary lipids incorporate in the phospholipids of the cell membrane and can alter membrane structure and function in a large variety of cell types (1). Lipids are hydrophobic structures, rich in nonpolar hydrocarbon regions and relatively few polar groups. Lipids play a key role in energy storage, membrane structure or have specific biological functions. One class of lipids are the fatty acids, which are generally bound to a glycerol backbone and stored as triacylglycerols (or triglycerides) in animal fats and vegetable oils as well as phospholipids found in the lipid bilayer of cell membranes (2, 3). These amphipathic phospholipids consist of two hydrophobic fatty acid chains and a polar phosphate-containing head group «Figure 1A».

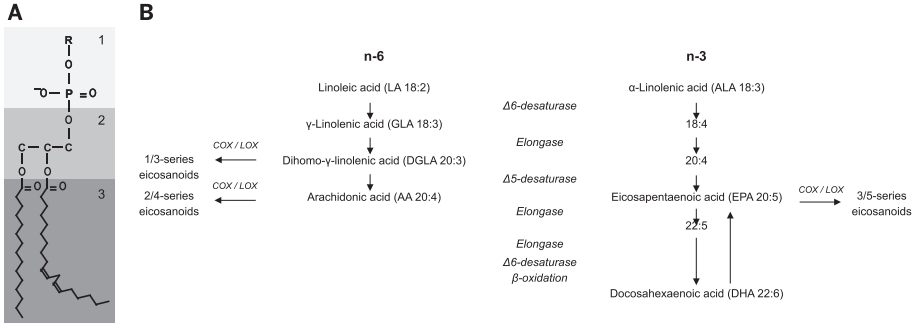


Figure 1 (A) Phospholipids are the principal components of the cell membrane. Glycerol phospholipids are most common and are composed of a polar phosphate group (1) bound to glycerol (2). R is a small polar head group such as choline. Also bound to glycerol are two fatty acids (3) that may differ from each other. (B) The metabolic conversion pathways and eicosanoid synthesis from PUFA of the n-6 and n-3 series.

Fatty acids are composed of an unbranched hydrocarbon chain with a carboxyl group at the end and contain a variable, usually even, number of carbon atoms. The most common range is from 12 to 20 carbons per chain, with 16- and 18-carbon fatty acids particularly common. The aliphatic chain is either saturated or unsaturated, depending on the absence or presence of double bonds. Polyunsaturated fatty acids (PUFA) form a subgroup within the group of fatty acids and are referred to as polyunsaturated when containing two or more double bonds (2, 3). Double bonds can have either a *cis* or *trans* configuration. Most often unsaturated fatty acids contain *cis* double bonds which may result in bending of the structure, preventing tight packing. By contrast *trans* double bonds do not cause this change in shape and are often the result of human processing (*trans* fats) (3). Chain length and degree of unsaturation of a fatty acid determines its

melting point. Most plant triacylglycerols are liquid at room temperature because the fatty acids of these vegetable oils are predominantly unsaturated.

PUFA are categorized into n-6 or n-3 fatty acids according to the position of the double bond nearest to the methyl end «Figure 1B». Linoleic acid, consisting of 18 carbon atoms and two double bonds as is referred to in its notation (LA 18:2n-6), and α-linolenic acid (ALA 18:3n-3) are essential fatty acids and found in plants only, they are precursors for long chain (LC)PUFA consisting of 20 or more carbon atoms. LA is found in vegetable oils such as corn, sunflower and soybean oil. ALA can be found in green leafy vegetables, walnuts and rapeseed and flaxeed (linseed) oil (2-4). These PUFA can be metabolized in the body into other n-6 or n-3 fatty acids by elongation of the acyl chain by elongases and insertion of extra double bonds by desaturases. Via several steps LA can be converted into arachidonic acid (AA 20:4n-6) whereas ALA can be converted into eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3) (2, 3, 5, 6). AA can be found in meat whereas oily fish is a rich source of n-3 LCPUFA EPA and DHA. Salmon, fresh tuna, mackerel, herring, pilchards and sardines are examples of fatty fish. Depending upon the type of fish the average content of EPA plus DHA for an oily fish meal varies between 1 and 3.5 g (2, 3, 7).

Besides oily fish, products rich in ALA also can add to the n-3 LCPUFA status due to conversion of ALA into n-3 LCPUFA in the body. However, the proportion of ALA converted into EPA is low and conversion to DHA is even less efficient. There is heterogeneity in the findings; highest estimated fractional conversion is 4% (8), while most other studies have reported lower estimates of conversion (0.05% or less), see Burdge *et al.* 2006 for an overview (6). Nevertheless, an increase in the ALA intake is demonstrated to increase proportions of EPA in plasma and cell lipids, though not of DHA (9-11). ALA has been shown to be less potent than n-3 LCPUFA in providing health benefits so ALA requires high intake. The best strategy to increase the n-3 LCPUFA content is to eat oily fish on a regular basis.

MECHANISM OF ACTION OF LCPUFA

Among all lipids in particular LCPUFA are widely held to act on physiological functions via several cell mechanisms. Regarding the immune status, in general n-6 LCPUFA are considered as being pro-inflammatory and n-3 LCPUFA as being protective against inflammation. N-6 LCPUFA AA is a precursor for inflammatory type 2-series prostanoids and the 4-series leukotriens since it serves as a substrate for cycooxygenases (COX) and lipoxygenases (LOX). By contrast n-3 LCPUFA EPA is converted by the same enzyme families into 3- and 5-series eicosanoids, known to be less biologically active or even protective against inflammation. In addition, many of the anti-inflammatory effects of n-3 LCPUFA are exerted at the level of altered gene expression through modification of transcription

factor activity. For example enzymes responsible for producing inflammatory eicosanoids (e.g. COX-2 and 5-LOX), inflammatory cytokines and adhesion molecules are regulated on gene expression level by LCPUFA. This can be achieved either by direct interaction with ligand-binding transcription factors or via membrane or cytoplasmic signaling pathways finally altering transcription factor activation (1). Peroxisome proliferator-activated receptors (PPAR), nuclear factor kappa beta (NF-κB) and mitogen activated protein kinase (MAPK) are examples of transcription factors being regulated by PUFA. Recently it was discovered that G protein-coupled receptor 120 (GPR120) is a transmembrane DHA receptor and moreover LCPUFA can affect the Toll-like receptor (TLR) signaling pathway which involves NF-κB. Hence, LCPUFA may act on intra- and extracellular receptors which may activate the cell and result in the expression or suppression of genes encoding for proteins including cytokines.

LCPUFA enhance membrane fluidity

The fluid mosaic model is frequently used to describe the structure of cell membranes. The cell membrane consists of an asymmetric lipid bilayer with functional lipids such as cholesterol and proteins embedded; these can migrate within the membrane. The fatty acid composition of the cell is mostly cell specific but may change for example over time or due to the diet (12, 13). Membrane composition strongly affects membrane fluidity. An increase in the content of unsaturated fatty acids like LCPUFA in membrane phospholipids is accompanied by an increase in membrane fluidity so a decrease in microviscosity (13, 14). More fluidic membranes can give rise to changes in cell function since in a more fluid membrane protein-protein and protein-lipid interaction can take place more easily.

LCPUFA alter lipid raft composition

Lipid rafts are highly organized regions in the cell membrane that differ in composition between various cell types. Rafts are relatively rigid as compared to their surrounding and are rich in cholesterol and sphingolipids with long saturated fatty acyl chains. Many signaling proteins including receptors, enzymes and substrates are concentrated in these areas in order to facilitate efficient intracellular signaling in response to extracellular stimuli. This occurs as a result of clustering of different lipid rafts rich in signaling proteins (15, 16). Because of the kinked structure of LCPUFA by incorporation of LCPUFA affects raft organization and interactions between lipids and proteins. This is not compatible with the highly organized lipid raft structure and will cause displacement of proteins, and by increasing membrane fluidity affect lipid raft clustering, hereby suppressing signal transduction (17).

LCPUFA as precursors of lipid mediators

Eicosanoids

20-Carbon n-6 LCPUFA AA and dihomo-γ-linolenic acid (DGLA) and n-3 LCPUFA EPA are precursors for eicosanoids. Eicosanoids derived from AA have the most potent biological functions. Many cell types liberate AA from membrane phospholipids upon stimulation. PUFA are liberated from membrane phosphatidylcholine or phosphatidylinositol-4, 5-bisphosphate (PIP_2) by cytosolic phospholipase A_2 (PLA_2) or by actions of phospholipase C (PLC) and a diacylglycerol (DAG) lipase respectively (18-21). After release from the cell membrane AA can be metabolized by COX enzymes resulting in the formation of inflammatory prostaglandins (PG) and thromboxanes (TX) of the 2-series (e.g. PGD_2) or by 5-, 12-, and 15-LOX which yield inflammatory leukotriens (LT) of the 4-series, lipoxins (LX), hydroperoxyeicosatetraenoic acids (HPETE) and hydroxyeicosatetraenoic acids (HETE). Major biologically active AA-metabolites are PGD_2, PGE_2, PGI_2, PGF_{2a} and TXA_2, supporting the inflammatory cascade. It is cell type dependent which metabolite is formed predominantly. Half life of these mediators is very short (only seconds) and they act locally on cell surface receptors (22). Furthermore non-esterified PUFA can be a substrate for cytochrome P450 monooxygenases (CYP). CYP-mediated oxidation of AA results in a variety of eicosanoids such as epoxides known to contribute to cell activation. N-3 LCPUFA are converted by CYP especially when little COX or LOX activity is present (23).

N-3 LCPUFA EPA and DHA competitively inhibit AA metabolism because incorporation of EPA and DHA in the cell membrane phospholipids occurs partly at the expense of AA. Subsequently, less substrate will be available for synthesis of AA-derived eicosanoids (14, 24). Conversion of EPA is resulting in mediators of the 3-series of prostaglandins and tromboxanes via COX and the 5-series of leukotriens via 5-LOX, which are believed to be less pro-inflammatory than the AA-derived metabolites (18, 21). Indeed in humans fish oil supplementation has been shown to reduce production of PGE_2, TXB_2, LTB_4, 5-HETE and LTE_4 by inflammatory cells while resulting in increased production of LTB_5, LTE_5 and 5-HEPE (25, 26). EPA and DHA are poor COX and LOX substrates compared to AA and inhibit COX activity (18, 27, 28). Also gene expression of other proteins involved in AA-metabolism can be regulated by n-3 LCPUFA (29).

Resolvins

N-3 LCPUFA in the cell membrane give rise to other anti-inflammatory mediators as well. These are called EPA and DHA-derived resolvins (resolution phase interaction products) (30). Resolvins are anti-inflammatory lipid mediators derived from EPA and DHA via COX-2 activity. Resolvins of the E-series such as Resolvin E1 (RvE1) are derived from EPA, while resolvins from the D-series are products of DHA (31, 32). RvE1 receptor activation can lead to potent anti-inflammatory effects. In a mouse model for peritonitis administration of EPA and COX inhibitor aspirin resulted in the generation of RvE1 in peritoneal exudates presumably via 5-LOX and aspirin-acetylated COX-2 as well as inhibition of leukocyte

infiltration. Administration of synthetic RvE1 gave similar results. Furthermore, synthetic RvE1 was shown to protect against the development of colitis in mice via reduction of leukocyte infiltration and pro-inflammatory gene expression (33). In addition, RvE1 reduced IL-12 production and migration of mouse spleen dendritic cells activated with pathogen extract (34). Moreover, DHA conversion via COX-2 gives rise to immunoregulatory docosatrienes and neuroprotective neuroprotectins (35, 36).

LCPUFA alter cell signaling pathways

Fatty acids may have other functions in signal transduction than discussed above. Many phospholipids are involved in signaling pathways and changes in the type of fatty acid present may alter activity and function in signal transduction (19). It has been shown that the fatty acid composition of DAG may affect the activity of protein kinase C (PKC) (37). Furthermore, PKC was less active in the presence of phosphatidylserine rich in LCPUFA as compared to a phosphatidylserine low in LCPUFA content (38). However, not only the fatty acid composition of phospholipids involved in intracellular signaling is important; fatty acids can have direct effects on signal transduction pathways as well. Expression and activity of protein kinases can be altered by different fatty acids; this has been most extensively studied for PKC and was shown to be reduced by EPA and DHA. Also fish oil supplementation in mice reduced spleen lymphocyte PKC activity (39). Furthermore, cytosolic calcium concentrations are regulated by PUFA. LA, AA, ALA, EPA and DHA have shown to induce calcium release from the endoplasmic reticulum (ER) and block the calcium influx via receptor-mediated calcium channels in T cells (40, 41). In addition, they can enhance receptor-mediated calcium efflux (42). Due to these kinds of downstream changes the activity of transcription factors such as NF-κB can be suppressed too.

N-3 LCPUFA may suppress generation of reactive oxygen species

A variety of tissues and cells produce reactive oxygen species (ROS). Superoxide is the main relevant ROS since it is the precursor of most other ROS. Studies supplementing animals with fish oil are inconclusive about the effect of n-3 LCPUFA on ROS production in macrophages, monocytes and neutrophils (see (19) for an overview) which might be caused by differences in study setup such as cell type analyzed, diet or stimulus used. However, in most studies dietary n-3 LCPUFA reduce superoxide and/or hydrogen peroxide production (43-45). Furthermore it has been reported that EPA (+DHA) supplementation to healthy human volunteers can lower superoxide production in blood neutrophils and monocytes (46, 47). Many enzymes including NADPH oxidase are involved in ROS formation and different eicosanoids, cytokines and PKC control several of these enzymes (18, 48). N-3 LCPUFA might suppress the production of ROS by affecting these molecules. This was shown by Wong *et al.* reporting that DHA inhibited NADPH oxidase and ROS generation in RAW264.7 cells while saturated lauric acid activated both.

N-3 LCPUFA suppress NF-κB and MAPK signaling

The NF-κB signal transduction cascade is important in the generation of inflammatory responses. It induces the transcription of a range of inflammatory genes including COX-2, E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor-α (TNF-α), interleukin (IL) 1β, IL-6 and inducible nitric oxide synthase (iNOS) (49, 50). N-3 LCPUFA reduce NF-κB activity, although the exact mechanism is not clear. EPA or fish oil incubation of human monocytes was found to reduce lipopolysaccharide (LPS)-induced activation of NF-κB, whereas AA did not (51-54). Zhao *et al.* showed that this was in association with a reduction in phosphorylation of inhibitor of κB (IκB), preventing dissociation of the NF-κB complex and consequent nuclear translocation and gene transcription. This suggests an effect of n-3 LCPUFA upstream in the NF-κB pathway (54). In addition, TNF-α induced IκB degradation can be eradicated by EPA incubation in a pancreatic cell line (55). N-3 LCPUFA can suppress activation of other signaling cascades as well, which results in the activation of various transcription factors. Lo *et al.* reported that incubation of murine macrophages with EPA reduced LPS-induced phosphorylation and activation of MAPK (56). So far it is not exactly known how n-3 LCPUFA can diminish MAPK signaling, both PKC-dependent and independent mechanisms are involved. Furthermore they seem to act upstream and downstream of MAPK kinase (MAPKK) (57, 58).

PUFA as ligand for the nuclear receptor PPAR

Besides suppression of signaling pathways that converge on transcription factor activation, fatty acids appear to enhance the activity of the transcription factor family PPAR directly. PPAR-α is important in lipid metabolism and is found mostly in liver. PPAR-β/δ is found in many tissues, but its role is still unclear while PPAR-γ is expressed mainly in adipose tissue and has been shown to influence adipocyte differentiation, glucose metabolism and macrophage development and function (59). PPAR are also found in inflammatory cells and certain fatty acids and eicosanoids can act as their ligands (60, 61). To regulate gene expression PPAR dimerize with another nuclear receptor, the retinoic-X-receptor (RXR) that among others is activated by DHA (62). Activation of PPAR by ligand binding (e.g. fatty acids from the diet) promotes alterations in gene transcription of genes containing PPAR response elements (63). LA, ALA, AA, EPA and DHA have been shown to be efficient activators of PPAR-α and –δ, and to a much lesser extent PPAR-γ (64). Kleiwer *et al.* reported that PPAR-α and -γ were activated by LA, ALA and AA (63). Activators of both PPAR-α en -γ inhibited activation of inflammatory genes including TNF-α, IL-1β, IL-6, IL-8, COX-2, iNOS and VCAM-1 (61, 65-69). The anti-inflammatory effects of PPAR might be due to stimulation of inflammatory eicosanoid breakdown via induction of peroxisomal β-oxidation and/or the suppression of activation of other transcription factors. For example, PPAR-γ activation by n-3 LCPUFA may prevent NF-κB activation (68). Nuclear receptors generally lack specificity for n-3 LCPUFA; therefore they are not sufficient in explaining the effects of n-3 LCPUFA on the immune system (1).

Transmembrane receptors for LCPUFA

N-3 LCPUFA as ligand for GPR120

GPR120 has been discovered recently as a n-3 LCPUFA receptor. Stimulation of GPR120 by long-chain fatty acids enhanced intracellular calcium and MAPK extracellular signal-regulated kinase (ERK) (p44/42) activation in HEK 293 cells, while reducing IκB kinase (IκK) β and c-Jun N-terminal kinase (JNK) phosphorylation, IκB degradation and TNF-α and IL-6 secretion in RAW264.7 cells (70, 71). GPR120 is highly expressed in macrophages, monocytic cells and adipose tissue and EPA and DHA have potent anti-inflammatory effects in these cell types. DHA showed comparable reduction of the LPS-induced inflammatory response as the synthetic GPR120 agonist GW9508. Also suppression of TNF-α induced inflammation was shown. β-Arrestin2 (β-Arr2) associates with GPR120 leading to internalization of this complex and β-arr2 then binds to TAK1 binding protein 1 (TAB1), resulting in reduced TAK1 activation. Downstream of TAK1 are the IKKβ/NF-κB and JNK pathway which explains the suppression of both LPS and TNF-α signaling after GPR120 activation. Although GPR120 is a Gαq/11 G protein coupled receptor, these effects were independent of Gαq/11. In addition, macrophage-mediated tissue inflammation could be reduced in obese mouse fed a n-3 LCPUFA diet via GPR120 signaling (71).

PUFA suppress TLR activation

In addition to GPR120, n-3 LCPUFA can signal via TLR. Saturated fatty acids are - as an integral part of the lipid A tail of LPS from Gram-negative bacteria - essential for the biological activities of this TLR4 ligand. Removal of acylated saturated fatty acids from lipid A results in complete loss of endotoxic activity (72, 73). Furthermore, free fatty acids modulate TLR activation. Saturated fatty acids activate TLR4 inducing the expression of COX-2, iNOS and IL-1α via NF-κB signaling in murine monocytic RAW264.7 cells, while unsaturated fatty acids including LA, AA, EPA and DHA inhibit the induced expression of these inflammatory markers involving reduced degradation of IκB (73). These effects involve lipid raft association. Saturated lauric acid has shown to induce receptor dimerization and recruitment of TLR4 into lipid rafts as well as downstream signaling pathways and gene expression. DHA on the other hand reduced LPS- and lauric acid-induced dimerization and recruitment of TLR4 into lipid rafts. This process occurred in a ROS-dependent manner (72). In addition, lauric acid potentiates while DHA reduces lipopeptide-induced TLR2 activation (72, 74).

ENHANCED N-6 OVER N-3 LCPUFA INTAKE IS ASSOCIATED WITH INCREASED INCIDENCE OF ALLERGIC AND CARDIOVASCULAR DISEASE

Epidemiological data provide strong evidence of a steady increase in incidence and prevalence of many allergic and inflammatory diseases over the last decades in the Western world. The diseases which incidences are rising since the 1950s includes allergic rhinitis, asthma, atopic dermatitis, food allergies, cardiovascular disease (CVD), inflammatory bowel disease, and autoimmune diseases like diabetes mellitus type 1 (1, 75-78). For example between 1979 and 1991 the prevalence of asthma, hay fever and atopic dermatitis doubled in Swedish school children (79). These changes cannot solely be explained by genetics (1). The modern diet differs largely from more traditional diets. Amongst other changes in the diet, in the Western countries the consumption of n-3 PUFA has gradually decreased. On the other hand the diet has excessive amounts of n-6 PUFA, mainly LA, from increased consumption of plant oils and products such as margarines. Historically the dietary ratio of n-6 to n-3 fatty acids approached 1/1 and Inuits consume a diet with a ratio of roughly 1/2.5 (76, 80, 81). Populations consuming high levels of n-3 LCPUFA from seafood such as Inuits and Japanese have low prevalence of CVD, psoriasis, asthma, allergic sensitization towards inhalant allergens and rheumatoid arthritis (82-84). Besides living condition, allergen exposure and genetics the diet high in n-3 LCPUFA is likely to positively affect disease prevalence. However, in current Western diets this ratio is 10/1 up to 25/1 (76, 80, 81). This suggests an insufficient n-3 LCPUFA intake and as a consequence human inflammatory cells and structural cells like blood vessel endothelial cells contain high proportions of AA and low proportions of n-3 LCPUFA. Currently, the mean ratio for immune cells is about 20% AA, 1% EPA and 2.5% DHA (85). The exact proportion of AA in these cells varies according to the cell type and lipid fraction used.

Dietary supplementation with n-3 LCPUFA increases membrane EPA and DHA content

Increased exposure to n-3 LCPUFA changes the fatty acid composition of cells in a dose-dependent way. It has been shown *in vitro* that EPA and DHA fairly easily incorporate into mammalian cells (14, 24). Also in animal studies, feeding fish oil to rodents confirms an increased EPA and DHA content in various cell types, while AA content is reduced (86, 87). In humans consumption of n-3 LCPUFA increases the content of these fatty acids in plasma lipids and many different cell types including cord blood cells, platelets, erythrocytes, leukocytes, endothelial cells and cardiac cells (88-91). Incorporation of EPA and DHA in human cells occurs in a dose dependent manner. Several studies show that supplementation between 1 and 6.5 g EPA plus DHA per day results in (near) linear relationships between EPA and DHA intake and content of plasma and platelet phospholipids and neutrophils (92, 93). However, incorporation into the different cell types occurs at

different rates and with different efficiencies (94). After four weeks of supplementation with EPA and DHA incorporation of these fatty acids reaches its maximum for serum cholesteryl esters and mononuclear cells, whereas for erythrocytes this takes between 2 and 6 months in humans (90, 94). If fish oil supplementation is abandoned EPA levels return to basal levels in mononuclear cells and erythrocytes within 8 weeks, while DHA remains elevated for a longer period of time. Erythrocyte DHA content is not even reduced to baseline levels 20 weeks after ending supplementation (89, 90, 95). Current intake of n-3 LCPUFA in Western countries is low, for the UK < 0.25 g/day is consumed which is probably representative for other Western countries (96). Recommendations vary in general between 0.45 – 1.2 g n-3 LCPUFA/day in adults (96, 97).

The recognition that n-3 LCPUFA exert anti-inflammatory effects has led to the insight that supplementation with n-3 LCPUFA may be of clinical benefit in the prevention or treatment of allergic or inflammatory diseases. In this review we will highlight the effects of n-3 LCPUFA in the prevention of allergic disease and cardiovascular inflammation. For allergic disease both the innate and adaptive immune response will be discussed. In view of the steadily increasing prevalence of CVD we chose to discuss this type of inflammation too and describe the effect of n-3 LCPUFA on a structural cell type, the endothelial cell (EC).

N-3 LCPUFA IN THE PREVENTION OF ALLERGIC DISEASE

The incidence of allergic disease has been rising steadily in the Western world during the last decades and affects at least 15% of the infant population and 7% of adults is affected with asthma being only one of the major allergic diseases (98-100). Alterations in dietary composition may have impact on the susceptibility to develop allergic disease which can already be established during gestation or early infancy. Studies in both humans and animals have shown that environmental influences (including diet) during early life have profound and long-lasting effects on human health (101-103). LCPUFA might exert positive effects on the unborn child, since they are important for the developing fetus. It requires fatty acids to build cell membranes and maintain their fluidity and conformation. Large amounts of PUFA (especially LCPUFA AA and DHA) need to be transferred to the fetus. A deficiency in LCPUFA is directly linked to poor fetal development, and consequently to defects (104). Kabesch *et al.* have reported that in some European countries one in three children shows allergic sensitization and one in ten suffers from asthma (105). Many factors, including dietary deficiencies in antioxidants and fibers as well as increased total fat intake have been incriminated to contribute to the increase in allergic manifestations. However, also the increased intake of n-6 over n-3 PUFA may have impact on the allergic outcome (81). Indeed, n-6 PUFA have been shown to increase the susceptibility of allergic

sensitization since these are precursors for PGE_2 (77, 106). Epidemiological evidence by Hodge *et al.* showed a protective effect of n-3 LCPUFA (106). Furthermore, in 22 children with asthma and/or allergic dermatitis aged 12-15 years and 23 non-atopic age-matched controls was observed that n-3 LCPUFA concentrations were reduced in serum of allergic adolescents in comparison to the control group. In addition, the ratio of n-6/n-3 LCPUFA was higher in the allergic group (107).

N-3 LCPUFA supplementation during pregnancy and lactation reduce allergic incidence and symptoms in offspring

Pregnancy studies

Allergic diseases are determined in early life, or even *in utero;* therefore prevention should preferably start very early in life. Supply of n-3 LCPUFA to the fetus must come from maternal sources (placenta and breast milk) which make the maternal LCPUFA status important. The maternal plasma phospholipid DHA concentration determines DHA supply to the fetus. Increased consumption of DHA during pregnancy or lactation increases the DHA content of maternal and cord plasma and breast milk (108, 109). Regular consumption of oily fish has been shown to result in higher maternal erythrocyte DHA content (110, 111). Possible protective effects of early n-3 LCPUFA exposure have been examined in supplementation studies with fish oil.

Recent studies report the effects of fish oil supplementation during pregnancy for the primary prevention of allergic disease. In a randomized placebo-controlled trial, women at risk of giving birth to a child at high risk of developing allergic disease were supplemented with 1.6 g EPA and 1.1 g DHA or placebo (soy oil) from week 25 of gestation to on average 3-4 months of lactation. 117 Children were followed on the risk of developing allergic sensitization and disease during the first year of life. The accumulative incidence of food allergy during the first year of life was reduced by 85% in the n-3 LCPUFA group compared to the placebo group as determined by a reaction to egg or milk in presence of detectable IgE antibodies or a positive skin prick test towards these antigens. Furthermore, IgE associated eczema was reduced by more than 60% in the 12 month follow up period. This study indicated that maternal n-3 LCPUFA supplementation during late pregnancy and lactation reduced the risk of food allergy and IgE-associated eczema during the first year of life in children at risk of allergic disease (112). Moreover, Furujehlm *et al.* have shown a reduced incidence of IgE-associated disease after n-3 LCPUFA supplementation at 2 year of age too. This was related to EPA and DHA proportions in plasma phospholipids of mother and child (113). Dunstan *et al.* supplemented 83 pregnant woman whose offspring were at risk of developing allergic disease in a double blind, randomized, placebo-controlled trial with fish oil (3.7 g n-3 LCPUFA (1.1 g EPA + 2.2 g DHA) per day) or control capsules (olive oil) from week 20 of pregnancy until delivery (114, 115). Umbilical cord plasma IL-13 concentrations were significantly lower after fish oil exposure and inversely related to cord erythrocyte DHA content (114). Neonatal mono-

nuclear cells secreted less Th1 and Th2 cytokines in response to allergen stimulation after fish oil consumption and IL-10 secretion in response to house dust mite or cat hair extract was significantly reduced. At one year of age, infants exposed to fish oil during pregnancy were 60% less likely to be sensitized to egg allergen as determined by skin prick testing and the same tendency was shown for peanut and cow's milk protein. Although the incidence of atopic dermatitis was not different between the groups, fish oil treated children showed significantly fewer severe symptoms (115).

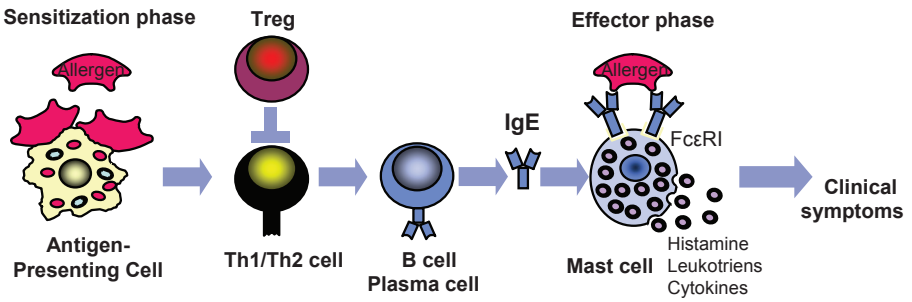
Instead of providing fish oil to increase n-3 LCPUFA status in pregnant woman, the Salmon in Pregnancy Study (SiPS) was the first study to increase the intake of oily fish (two portions salmon per week) from week 20 of pregnancy until delivery in pregnant women whose offspring were at high risk of developing atopic disease. The salmon, specifically farmed for the study, provided about 3.45 g EPA plus DHA per week. In the salmon group EPA and DHA percentages in plasma phosphatidylcholine were higher at weeks 34 and 38 of pregnancy as compared to 20 weeks, EPA showed the largest increase. In the control group these fatty acids decreased during pregnancy. Also in umbilical cord plasma phosphatidylcholine the EPA and DHA content was higher in the salmon group as compared to controls subjects. EPA was even 2-fold higher in the salmon group, while DHA was 1.2-fold increased. This indicates enhanced n-3 LCPUFA transfer to the fetus. Children will be followed for atopic disease status during the first years of life (116).

Lactation studies

In two small studies including healthy and allergic mothers the PUFA composition of breast milk was assessed in relation to the allergic outcome of their offspring (117, 118). In both studies it was shown that an increased n-6/n-3 PUFA ratio in breast milk was related to atopic disease in the infant. In the allergic infants the n-6/n-3 PUFA ratio was also higher in the serum cholesteryl ester fraction as a result of reduced n-3 PUFA content and in the serum lipid triglyceride fraction as a result of an increase in n-6 PUFA (118). Fish oil supplementation during pregnancy is shown to increase breast milk n-3 PUFA levels and enhances IgA, IL-6, IL-10 and sCD14 concentrations in human milk (119). Although the effect on the allergic outcome of n-3 LCPUFA supplementation during lactation only has not been studied yet this may be beneficial as well. Lauritzen *et al.* showed long-term immunologic effects in children at 2.5 year of age that were exposed to n-3 LCPUFA in the first 4 months of life via breastfeeding. Supplementation with 1.5 g n-3 LCPUFA per day has been associated with significantly higher production of Th1 type interferon-γ (IFN-γ) in a whole blood assay compared to children with low maternal n-3 LCPUFA intake during lactation. This may reflect a faster maturation of the immune system (120).

EFFECTS OF LCPUFA ON IMMUNE CELLS

Although some studies have a relatively small study population, supplementation studies provide evidence that n-3 LCPUFA can exert protective effects in the development of allergic disease when supplemented early in life (during pregnancy and lactation), at least in children at high risk. The mechanisms of effect remain to be elucidated but may comprise cells of the innate as well as the adaptive immune system «Figure 2».



n-3 LCPUFA	Ag presentation ↓ Co-stimulation ↓ IL-12 ↓ IL-10 ↑	Th1 ? ↑ Th2 = ↓ Th1/Th2 ? ↑ Treg ? ↑	IgE ? ↓	Eicosanoids 2/4 series ↓	AD score/symptoms ↓ SPT ↓ Incidence atopic disease ↓

Figure 2 Summary of the effects of moderate n-3 LCPUFA dosage on immune cell function. For details see text. AD: atopic dermatitis; SPT: skin prick test

LCPUFA suppress DC activation *in vitro*

Dendritic cells (DC) are the link between the innate and the adaptive immune system. Both n-3 as well as n-6 LCPUFA were able to inhibit the expression of LPS-induced CD40, CD80 and/or CD86 co-stimulatory signals and to suppress T cell proliferation and cytokine production when the DC were added to naive T cells, hence rendering a tolerogenic DC phenotype (121-123). For n-3 LCPUFA these effects were obtained via PPAR-γ activation and/or inhibition of MAPK p38 (122, 123). Zapata-Gonzalez *et al.* and other studies have shown a suppression of LPS-induced expression of major histocompatibility complex (MHC) class II receptors on DC by n-3 LCPUFA and also a reduced capacity to present antigen to T cells (122, 123). Also in rats fed fish oil a reduction in MHC II expression on DC and diminished antigen presentation was observed (124). In a recent study DHA and EPA were found to suppress Th1 polarizing IL-12 secretion by LPS-stimulated bone marrow derived DC while regulatory IL-10 production was enhanced. LA did not affect cytokine secretion of the DC (125). N-6 LCPUFA AA-derived eicosanoids such as PGE_2 and PGD_2 and other mediators such as histamine influence the activity of the DC. Both histamine and PGD_2 decreased IL-12 secretion by DC and promote a Th2 cell response (126-128). Further-

more, TNF-α is important in migration and functional maturation of DC. Production of these inflammatory mediators also may be reduced by n-3 LCPUFA.

LCPUFA may affect T cell proliferation and polarization

Th1/Th2 polarization can be affected by the fatty acid composition of the diet. An increased dietary n-6/n-3 PUFA ratio may induce a shift in the Th1/Th2 balance towards a Th2 prone response (129). In the study by Mizota *et al.* Balb/c mice and humans were provided with diets supplemented for four weeks with different n-6/n-3 PUFA ratios due to variations in ALA content. Secretion of IFN-γ by splenoyctes was significantly enhanced in mice fed a n-3 PUFA rich diet (n-6/n-3 PUFA ratio = 0.25) as compared to a n-6 PUFA rich diet (n-6/n-3 = 42.9). Secretion of IL-4 was reduced in both the n-6 rich and n-3 rich diet as compared to a diet with n-6/n-3 = 2.27. The ratio of IFN-γ to IL-4 was significantly higher in mice fed the n-3 PUFA rich diet. In humans the ratio of IFN-γ/IL-4 production by peripheral blood mononuclear cells was significantly higher during consumption of n-3 PUFA (n-6/n-3 = 3) as compared to a n-6 PUFA rich diet (n-6/n-3 = 44), as a result of reduced IL-4 secretion (129). However, Zhang *et al.* found opposite results by fish oil in C57Bl/6 mice. They reported suppression of Th1 development (reduced % IFN-γ positive cells) by fish oil but no enhanced Th2 polarization (% IL-4 positive cells) in splenic T lymphocytes cultured under Th2 polarizing conditions (130). Kleemann *et al.* also reported suppressed IFN-γ but increased regulatory IL-10 mRNA expression in the Peyer's patches of fish oil-fed BB rats (131). Unfortunately not all studies assessed regulatory cytokine secretion, which might benefit the T cell balance. IL-10 is often marked as a Th2 type cytokine. Wallace *et al.* have observed that feeding a 20% fish oil diet to C57Bl/6 mice had little effect on IL-4 secretion and gene expression by splenocytes but reduced IL-2 and IFN-γ production and T cell proliferation. Also *in vitro* IL-2 secretion by murine splenocytes was inhibited with high EPA and DHA doses however; low doses tended to enhance IL-2 secretion (132). In addition, in humans supplementation with high dose of fish oil (3-6 g/day) resulted in reduced IL-2 and IFN-γ production and decreased T cell proliferation (133, 134); whereas moderate fish oil supplementation (up to 2 g/day) increased T cell proliferation and IFN-γ production *ex vivo* although no effect on IL-4 secretion was seen which enhanced the Th1/Th2 ratio (135). Yaqoob *et al.* supplemented MF1 mice with fish oil and spleen lymphocytes were cultured for 24 hours. They have not shown an effect on IFN-γ concentrations; however IL-4 and IL-10 concentrations in supernatants tended to be lower in the fish oil group (136). Some studies do not report effects on these outcomes (90). Also AA-derived PGE_2 has *in vitro* been shown to suppress T cell activation and proliferation including inhibition of IL-2 and IFN-γ production, shifting the T cell response towards Th2 (137-139). N-3 LCPUFA decreased the production of PGE_2 which benefits the Th1/Th2 balance in allergic individuals. On the other hand, Trebble *et al.* reported an increase in PGE_2 secretion by fish oil supplemented humans (2 g/day) (135). Differences in results may be due to varying study parameters such as differences in species and cell types

studied, LCPUFA type and route of cell activation. Furthermore, dose seems to be rather important. Unfortunately, many effects have been studied in Th1 type of animal models. N-3 LCPUFA do not seem to suppress either Th1 or Th2 responses. Possibly the principal immune response is targeted, this is the Th2 response in neonates and atopic subjects. So far it is unknown how n-3 LCPUFA can selectively suppress a Th response; it has been suggested that n-3 LCPUFA support regulatory T cell function (1).

T cell responses can be modulated either directly by fatty acid incorporation in the T cell membrane or indirectly e.g. via interaction with antigen-presenting B cells or DC with different fatty acid composition (140-143). LCPUFA selectively alter protein composition of the cytoplasmic (inner) lipid leaflet by altering lipid composition since they can dislocate acetylated proteins that are anchored to the cytoplasmic lipid leaflet from the membrane. This has been shown to be a functional effect since it correlates with impaired calcium signaling (144, 145). Phosphorylation of linker for activation of T cells (LAT) is inhibited by n-3 LCPUFA in T cells and LAT displacement from rafts by n-3 LCPUFA leads to the suppression of T cell responses *in vitro* (17, 146). In animal studies alteration of T cell lipid rafts has been shown to be functional and the effects of n-3 LCPUFA on early signaling events have been confirmed (147, 148). By changing the number and activity (proliferation and cytokine secretion) of immune cells n-3 LCPUFA affect maturation and polarization of the immune system (1).

N-3 LCPUFA may reduce IgE production by B cells

As reported by dietary intervention studies during pregnancy n-3 LCPUFA show a strong tendency to reduce IgE antibody levels. Weise *et al.* found in B cells from human peripheral blood mononuclear cells that DHA inhibited IgE production (149). However, animal feeding studies are inconclusive possibly due to different animal species used, see (19). So far the underlying mechanism is unknown. Effects on DC, Th1/Th2 ratio as well as direct effects on the B cell may be involved. For example PGE_2 promotes IgE production by B cells, which will reduce after AA substitution by n-3 LCPUFA (138, 150, 151). However, Pène *et al.* reported contrasting results, namely that PGE_2 inhibited IgE production by peripheral blood lymphocytes (152). Furthermore cytokines including IL-4 and IL-13 induce IgE isotype switching in plasma cells. Possibly the production of these cytokines by T cells and mast cells is suppressed by n-3 LCPUFA (114). Recently it has been discovered that DHA can reduce IgE production by human B cells via the IL-4 pathway or via an effect on CD40 (149).

Are LCPUFA able to affect mast cell activation and signaling?

Although the anti-inflammatory effects of LCPUFA have been demonstrated in neutrophils and mononuclear cells, effects on mast cell function have not been fully addressed (153). Modulation of mast cell degranulation and secretion of cytokines can be beneficial in the prevention of mast cell-mediated diseases including allergy.

Mast cell activation often involves the Fcε receptor I (FcεRI). Allergen-specific IgE binds to the extracellular domain of the FcεRI. As soon as IgE binds to the FcεRI receptor, the receptor is stabilized. Binding of the allergen to surface bound IgE results in FcεRI receptor aggregation and activation, followed by phosphorylation of LAT «Figure 3». Lyn phosphorylates tyrosine residues in the immunoreceptor tyrosine–based activation motifs (ITAM) of the FcεRI which causes the recruitment of Syk (154). Activation of tyrosine kinase Syk may be affected by LCPUFA (155). Further downstream activation of PLC is induced causing PIP_2 to divide into the second messengers inositol-1,4,5 triphosphate (IP_3) and DAG. These mediators contain fatty acid tails and therefore might be regulated by LCPUFA. IP_3 binds to its receptor on the ER and causes calcium to be released with as a consequence mast cell degranulation and cytokine release (156, 157). An overview of currently reported effects of LCPUFA on mast cell degranulation and mediator secretion can be found later in this section. Together with DAG, calcium also activates PKC leading to generation of intracellular ROS such as superoxide (156, 157). It has been described that n-3 LCPUFA may suppress both PKC and ROS generation in different cell types. However, studies assessing the impact of LCPUFA on ROS production in mast cells are rare. Nakano *et al.* found that AA and EPA enhance ROS production in mast cells (155) and canine mastocytoma cell line C2 cells incubated with LA, AA, ALA and EPA increased ROS production. The latter might involve the susceptibility of PUFA to lipid peroxidation. Antioxidant supplementation resulted in a lower increase in ROS production (158).

The pathophysiological importance of ROS generated from mast cells has not been fully elucidated. At high levels ROS are involved in the innate immune response while at low levels they are involved in cell signaling (159). ROS are formed in many signaling pathways in mast cells and play an important role in mast cell signaling by mediating calcium influx and the secretion of histamine, eicosanoids and cytokines including IL-8 and TNF-α via NF-κB and/or MAPK signaling (159-162). Furthermore, tyrosine phosphorylation of PLC and LAT is ROS dependent (160).

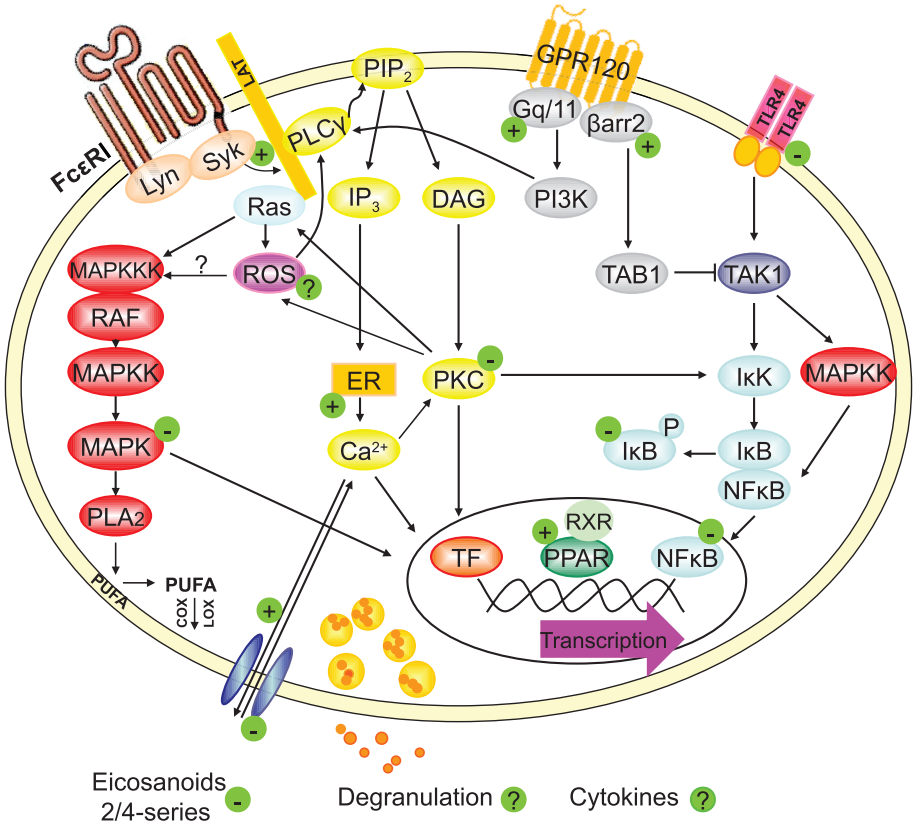


Figure 3 Proposed mechanisms by which n-3 LCPUFA can affect mast cell function. Currently known effects of n-3 LCPUFA on mast cells are indicated in the figure. Furthermore effects found in other cell types, but so far not studied in mast cells are extrapolated and included in the figure. An enhancing effect of n-3 LCPUFA is indicated by (+); a reduction by (–) and (?) means inconclusive results. N-3 LCPUFA incorporate in the cell membrane and increase membrane fluidity and change lipid raft formation leading to suppressed signal transduction. FcεRI receptor activation by an allergen results in phosphorylation of Syk, which can be enhanced by EPA supplementation. Further downstream activation of PLC is induced causing PIP_2 to divide into the second messengers IP_3 and DAG. Calcium (Ca^{2+}) is released from the ER which can be induced by n-3 LCPUFA while calcium influx across the membrane is reduced and calcium efflux stimulated by n-3 LCPUFA. PKC is activated by DAG, this activation can be reduced by n-3 LCPUFA either directly or via altering the fatty acids composition of phospholipids involved. Together calcium and PKC contribute to mast cell degranulation; the findings for n-3 LCPUFA on mast cell degranulation are contrasting. Furthermore calcium and PKC affect gene transcription involved in cytokine release. In addition IgE receptor activation leads to activation of Ras influencing the generation of intracellular ROS. While n-3 LCPUFA have shown to be able to suppress ROS production in different cell types, the results from the few studies in mast cells show the opposite. Ras also activates the MAPK signaling route leading to pro-inflammatory eicosanoid formation via PLA_2 together with increased gene transcription involved in cytokine release. Both MAPK activation and eicosanoid formation of the 2/4 series can be reduced by n-3 LCPUFA. For mast cells the effects of n-3 LCPUFA on cytokine secretion (continued)

◀ (continued) are largely unknown. Besides FcεRI the mast cell contains TLR on their surface that signal via the NF-κB pathway thereby inducing cytokine secretion. This can be reduced by n-3 LCPUFA involving reduced receptor clustering and reduced phosphorylation of IκB. Recently the GPR120 receptor for DHA has been discovered. So far it is unknown whether this receptor is present on mast cells, but its activation by DHA can reduce NF-κB signaling or activate a G protein. Finally, PPAR can be activated by n-3 LCPUFA inhibiting the activation of inflammatory genes. MAPKKK: MAPKK kinase; PI3K; phosphatidylinositide 3-kinases. For other abbreviations and details see text.

LCPUFA may alter mast cell degranulation

The effect of PUFA on mast cell degranulation has been studied in different cell types. Teshima *et al.* found n-6 PUFA LA, AA and γ-linolenic acid (18:2n-6) to dose-dependently increase the cytosolic free-calcium concentration and degranulation in rat basophilic leukemia (RBL)-2H3 cells independent of IP_3 formation (163). Activation of PKC by fatty acids including AA has been reported and might be involved here too (164). Incubation with n-3 PUFA ALA and EPA did not result in activation (163). Nakano and colleagues reported that AA significantly increased β-hexosaminidase release upon IgE-antigen stimulation and EPA showed the same tendency. This might be the result of effects early in FcεRI signaling. In AA-supplemented cells, the intracellular calcium concentration was increased and in both AA- and EPA-supplemented cells tyrosine phosphorylation of Syk and LAT was enhanced as well as intracellular ROS generation required for tyrosine phosphorylation of LAT and intracellular calcium mobilization. Fatty acid supplementation did not alter expression level of the FcεRI on the cell surface (155). Besides this mast cell degranulation perhaps occurs more easily when the membrane is more fluid as a result of PUFA incorporation. Also in C2 cells incubation with AA and EPA increased histamine release; AA-mediated increase was significantly higher from EPA-incubated cells (165).

Others did not see an effect of LCPUFA on mast cell degranulation. Peritoneal mast cell histamine release by compound 48/80 inducing non-receptor mediated degranulation was not different for mast cells isolated from rats fed a fish oil diet as compared to a diet rich in n-6 fatty acids or saturated fatty acids (166). Also murine mast cells (PT-18 cells) incubated with AA or EPA *in vitro* did not show an effect on histamine release (167). Incubation of cultured human mast cells from umbilical cord mononuclear cells with EPA did not affect histamine release too (27).

Furthermore, several (LC)PUFA have been shown to reduce mast cell degranulation. Fish oil contains various n-3 LCPUFA besides EPA and DHA; 6,9,12,15,18,21-Tetracosahexaenoic acid (THA 24:6n-3) is one of these. In mouse mast cell line MC/9 cells AA increased histamine content whereas n-3 PUFA including EPA, DHA and THA reduced histamine content. Only ionophore-stimulated histamine release was affected by PUFA, while spontaneous and antigen-mediated degranulation was not. LA, AA, EPA, DHA and THA but not ALA reduced histamine release (168). ALA incubation in RBL-2H3 cells increased both ALA and DHA content in different lipid fractions in the cell whereas LA and AA were decreased. Histamine release in this study was reduced by ALA (169). Currently reported effects of LCPUFA on mast cell degranulation vary widely and are inconclusive.

N-3 LCPUFA suppress PGD_2 and PGE_2 secretion by mast cells

PGD_2 is the main prostanoid secreted by activated mast cells and associated with allergic diseases because of its functions in vasodilatation and immune cell activation (27). In addition, PGD_2 is a random migration stimulating (chemokinetic) factor of neutrophils and eosinophils (170). AA-derived mediators such as PGD_2 are very important for the initiation of the allergic response. C2 cells supplemented with AA and EPA showed an increase in PGE_2 production, although the increase in EPA was significantly lower than AA incubated cells (165). However, in general n-3 LCPUFA have shown to be potent inhibitors of pro-inflammatory lipid mediators including PGD_2. Feeding rats a diet rich in fish oil increased n-3 LCPUFA and reduced AA in peritoneal mast cell phospholipids. As a result upon mast cell stimulation PGD_2 and PGE_2 secretion were reduced when compared to a diet mainly containing saturated fatty acids (171). THA has shown to be effective in reducing the antigen-stimulated production of eicosanoids, like other n-3 LCPUFA do in murine MC/9 cells (168). EPA can reduce PGD_2 secretion by cultured human mast cells by inhibiting COX-2 activity (27). Furthermore, synthetic phospholipids containing DHA inhibit 5-LOX activity of rat basophilic leukemia cell line RBL-1 (169). As a result of other lipid mediators formed in the presence of n-3 LCPUFA, the allergic response may benefit from dietary supplementation with these fatty acids.

LCPUFA may be able to affect the mast cell phenotype

The effect of LCPUFA on mast cell mediator secretion is relevant for both the initiation and persistence of the allergic response. Although LCPUFA can regulate several transcription factors involved in cytokine release by lymphocytes and macrophages (18, 19, 172), little is known about the effects of LCPUFA on cytokine secretion by mast cells. In rodents fish oil supplementation reduced the *ex vivo* production of TNF-α, IL-1β and IL-6 by macrophages (136, 173). Dietary fish oil consumption by healthy volunteers reduces production of TNF-α, IL-1 and IL-6 by mononuclear cells. Moreover, production of these cytokines is dose dependently reduced by n-3 LCPUFA intake (135, 174). Similar effects on cytokine secretion might occur in mast cells. Effects on cytokine production are most likely the effect of altered activity of transcription factors; reduced activation of NF-κB and activation of PPAR-γ may be involved. Furthermore, MAPK pathways are known to regulate pro-inflammatory mediator secretion. PPAR-γ is known to be expressed by mast cells and has been shown to be involved in the release of cytokines like IL-5 and TNF-α but not histamine in mast cells (175). PPAR-γ agonists have been shown to lower the amount of intracellular ROS (176), therefore PPAR-γ agonists including n-3 LCPUFA might have a positive effect on allergic mediator secretion. ROS can regulate tyrosine phosphorylation near the cell membrane, such as phosphorylation of LAT and PLC-γ, finally resulting in a reduction in cytokine secretion (155, 160). Nakano *et al.* reported that AA dose-dependently augmented TNF-α release in RBL-2H3 cells (155). Incubation of murine PT-18 cells with AA or EPA but not LA *in vitro* resulted in enhanced release of platelet-

activating factor independent of mast cell activation (histamine release). No antioxidants were used in these cell cultures which makes that oxidative stress may be involved in these effects (167).

There is still much unknown about the effects of LCPUFA on mast cells however several mechanisms by which n-3 LCPUFA can affect mast cell function, contributing to the prevention of allergic disease, have been discussed. Furthermore, mast cells and their products are involved in function, maturation and migration of B cells, T cells and DC and the interplay between all these cells is very important in allergic disease. T cell responses can be modulated via cytokines such as IL-4, TNF-α or PGD_2 released by mast cells (140, 141). In addition, interaction of mast cells with B cells via CD40–CD40L can result in Th1-skewing of naive T cells or, in the absence of T cells induce IgE production (141). Mast cell cytokines including IL-4 and IL-13 induce IgE isotype switching of B cells (126, 140, 141, 177-179). Histamine and PGD_2 can decrease IL-12 secretion by DC and promote a Th2 cell response. Further, TNF-α is important in migration and functional maturation of DC (128). As a result an effect of n-3 LCPUFA on mast cell function can also affect allergic responses via other cell types. Alteration of the mast cell phenotype by n-3 LCPUFA might result in a reduced susceptibility to develop allergy or allergic inflammation.

N-3 LCPUFA IN THE PREVENTION OF CVD

CVD have become the leading cause of morbidity and mortality in developed countries, and surprisingly in some developing countries too. Worldwide CVD result in death of approximately 16.7 million people each year. CVD include coronary heart disease (including angina pectoris, myocardial infarction and sudden death), stroke and peripheral vascular disease. Cardiovascular risk factors include hypertension and dyslipidemia (especially elevated plasma levels of low-density lipoprotein (LDL)) which are often found in metabolic disorders such as diabetic disease and obesity (180, 181).

The most important contributor to increasing CVD is atherosclerosis. Atherosclerosis is a complex, chronic inflammatory process accompanied by accumulation of lipids and fibrous elements in the large arteries (181). Atherosclerosis is an inflammatory disease because of the interaction between modified lipoproteins, monocyte-derived macrophages, T lymphocytes and the cell elements of the vessel wall (182, 183). Plaques formed consist of macrophage-derived foam cells, immune cells, vascular EC, smooth muscle cells, platelets, extracellular matrix, and a lipid-rich core with extensive necrosis and fibrosis of surrounding tissues (184). The adhesion of leukocytes to endothelium is involved in the early phases of atherosclerosis (185). Plaques may occlude the flow of oxygen-rich blood to the organs and other parts of the body and under certain conditions may rupture, leading to coronary events such as myocardial infarction.

A key event leading to the formation of plaques is the entry of LDL into the arterial intima. LDL is a major risk factor for atherosclerosis because it can be modified, mostly via oxidation, generating oxidized LDL, minimally modified LDL (mmLDL), and other biologically active forms that initiate inflammatory processes in the blood vessel wall. Modified LDL is taken up by macrophages and these cells become lipid-laden foam cells. Modified LDL also upregulates VCAM-1 and ICAM-1 expression and induces monocyte adhesion to activated endothelium, an early event in the development of atherosclerotic lesions (186, 187).

Vascular inflammation, which is closely related to endothelial dysfunction is often involved in CVD and occurs at an early stage of atherosclerotic cardiovascular disorders. Vascular inflammation is caused by activation of the endothelium. This can be caused by decreased or abnormal shear stress, hypoxia, inflammatory cytokines and leukocyte activation. EC activation is followed by an increase in leukocyte-endothelium adhesion via upregulation of endothelial cell adhesion molecules (CAM) which bind circulating leukocytes. In addition, secretion of pro-inflammatory factors like monocyte chemoattractant protein 1 (MCP-1), IL-6, IL-8, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) by EC themselves enhances leukocyte adherence, and transmigration to the endothelium, and provocation of endothelial dysfunction and tissue injury (187, 188).

N-3 LCPUFA contribute to the primary prevention of CVD

Populations consuming high amounts of fish or other seafood containing high levels of n-3 LCPUFA are protected against cardiovascular morbidity and mortality. Inuit populations consuming a marine food diet (500 g/day) rich in n-3 LCPUFA (5 – 15 g/day) have a low cardiovascular mortality, despite the high fat percentage of the diet (40% of calories) (76, 82, 189-191). The diet in the United States is equally high in fat, however is low in n-3 and high in n-6 fatty acids. Between 1974 and 1976 deaths due to ischemic heart disease counted for 5.3% of all deaths in Greenland compared to 40.4% of deaths in the US (76). The Japanese population also has a low cardiovascular mortality (192). Differences in genetic factors are unlikely to fully explain the difference in coronary heart disease (CHD) between Japanese and Western people. Studies of Japanese migrants to the US clearly illustrated an increase in CHD morbidity and mortality in Japanese men in the US (193). Furthermore, the protective role of fish is supported by the difference in cardiovascular mortality between fishing and farming villages in Japan. Japanese fishers (250 g fish/day) have lower blood pressures and reduced incidence of heart disease compared to Japanese farmers (90 g fish/day). In addition, the Japanese population has a six fold lower incidence of atherosclerosis and heart disease than the US population (<20 g fish/day) (82, 194). In both Inuit and Japanese, the EPA and DHA contents of plasma and platelet and tissue phospholipids are high whereas the relative concentration of AA is low (82).

N-3 LCPUFA may prevent CVD via epigenetic programming

Programming of atherosclerosis and its complications might occur via an effect on vascular physiology. Epidemiological and pathological studies in animals and humans have shown that exposure to an adverse maternal environment during development increases adult vascular disease in the offspring (195, 196). Nelson *et al.* examined that endothelial dysfunction marker ICAM-1 was elevated at birth in umbilical cord plasma in the offspring of type 1 diabetic mothers. This reflects is a possible mechanism for *in utero* programming of (vascular) disease which is probably involved in an increased atherosclerotic risk later in life (196, 197). Animal studies indicate that permanent changes either in DNA methylation and/or chromatin modification are responsible for the epigenetic programming of increased atherosclerosis susceptibility (196). Stenvinkel *et al.* has found that inflammation is indeed linked with DNA hypermethylation in peripheral blood cell DNA. This DNA hypermethylation was associated with (cardiovascular) mortality (198). Jiang *et al.* showed that DNA hypermethylation of specific genes is important in atherosclerosis. PPAR ligands, to which n-3 LCPUFA belong, can alter epigenetic modifications in genes participating in atherogenesis hence reducing hypermethylation (199-201). Several PPAR activators have been shown to reduce the expression of VCAM-1 on the surface of EC during inflammatory conditions (202). Dietary n-3 LCPUFA are associated with decreased plasma homocysteine levels, a risk factor for atherosclerosis inducing DNA methylation (203, 204). Homocysteine upregulates expression of adhesion molecules such as ICAM-1 on EC and this is reduced by PPAR agonists (205, 206). In addition, a number of chromatin modifying enzymes, which control CAM expression, have PPAR response elements in their promoters so can be regulated via n-3 LCPUFA (207-209). Early exposure to n-3 LCPUFA may contribute to a subtle but persistent effect on the development of CVD later in life.

N-3 LCPUFA contribute to the secondary prevention of CVD

Primary prevention avoids the development of CVD, whereas secondary prevention aims at reducing the incidence of new cardiovascular events and the increase of survival in patients with established disease. Several secondary prevention studies in post-myocardial infarction patients providing oily fish or fish oil have shown a protective effect of n-3 fatty acids on mortality, which is confirmed by meta-analysis (210-215). The GISSI Prevenzione study investigated the effect of modest supplementation of n-3 LCPUFA (850 mg EPA and DHA daily) in approximately 11,000 post myocardial infarction patients. The risk reduction of cardiovascular death was remarkable, with an overall reduction of 30%. Furthermore, the study reported a beneficial effect of subjects also taking aspirin. Aspirin treatment enhances EPA conversion to products including RvE1, which might have caused this cardiovascular benefit (34, 210). The DART trial (diet and reinfarction trial) was a randomized controlled trial in which it was observed that intake of two or three portions of fatty fish per week may reduce mortality in men who have recovered from

myocardial infarction. They have found a 29% reduction of mortality in those advised to eat fatty fish compared to people not advised to eat fatty fish (211). Further, the JELIS trail tested the effect of adding EPA to statin therapy in Japanese people among whom the baseline intake of n-3 LCPUFA is high compared to the Western population. Also here a 19% reduction in major coronary events was observed using EPA (215). Hence, n-3 LCPUFA have preventive importance in humans affected with CVD. In general, 1 g/day of n-3 LCPUFA is considered effective for secondary prevention of myocardial infarction (216-218).

N-3 LCPUFA IMPROVE ENDOTHELIAL FUNCTION

There are several mechanisms to explain cardiovascular protection of n-3 LCPUFA. Originally the beneficial effects were attributed to the decrease in production of inflammatory eicosanoids and other prothrombotic and vasoconstrictive mediators, reducing inflammatory processes within the vessel wall. In addition, n-3 LCPUFA may suppress leukocyte and smooth muscle migration into the vessel wall intima since they have been shown to dampen EC cytokine secretion and CAM expression *in vitro*, reducing the susceptibility to develop atherosclerosis (216, 219-222). Furthermore, DHA supplementation to HUVEC altered distribution of endothelial nitric oxide synthase (eNOS) – responsible for cardiovascular homeostasis – by displacement of eNOS from caveolae (223, 224). Hence DHA may reduce the susceptibility to develop atherosclerosis by supporting eNOS (224). In humans n-3 LCPUFA resulted in a 43% increase in nitric oxide metabolites in urine, implicating an increase in nitric oxide which implicates an improvement of the endothelium and vasodilatory responses (225). In animal models as well as dietary intervention trials in humans n-3 LCPUFA have been shown to reduce atherosclerosis via suppression of plaque build up. In humans n-3 LCPUFA prevented the processes that cause death such as plaque rupture, thrombosis, myocardial infarction and stroke e.g. by stabilizing atherosclerotic plaques (226, 227). In addition, a number of risk factors involved in the development of atherosclerosis are positively affected such as reduced blood pressure and serum triacylglycerol levels. In humans, n-3 LCPUFA decrease serum triglycerides and very low-density lipoprotein cholesterol, while LDL cholesterol tends to be either elevated or unchanged (228, 229). An impaired endothelial response may be secondary to atherosclerotic damage or a consequence of raised blood pressure. Systolic and diastolic blood pressure are directly related to risk of CHD and stroke (230). Only 4 mm Hg reduction in diastolic blood pressure reduces the risk of stroke by more than 34% and CHD by 14%, possibly due to the damaging effect of pressure on the vessel wall creating a spot for plaque formation (231, 232). Supplementation of n-3 LCPUFA can act vasodilatory by reduced TXA_2 and increased PGI_3 production. In addition, PGE_2 and PGF_2 stimulate renin production resulting in increased blood pressure, whereas EPA-derived prostaglandins do not (233, 234). A meta-analysis of 31 placebo controlled trials, with an average dose of

4.8 g EPA+DHA/day observed an overall reduction in systolic blood pressure of 3 mm Hg and a reduction of 1.5 mm Hg in diastolic blood pressure in hypertensive but not in normotensive individuals (235). More recently an overall reduction of 2.3 mm Hg in systolic blood pressure and a 1.5 mm Hg reduction in diastolic blood pressure were observed after combining 36 individual studies with fish oil doses ranged from 0.2 – 15 g/day (3.7 g/day on average) and a treatment period of 12 weeks on average (236). Hence, n-3 LCPUFA improve endothelial function which has an impact on the development of CVD since the endothelium is a key player in these kinds of pathologic conditions.

N-3 LCPUFA reduce endothelial cell adhesion molecule expression

Adhesion of leukocytes to the endothelium is a critical event in the pathogenesis of certain vascular diseases. Increased expression of adhesion molecules on EC contributes to the early phases of inflammation, vascular injury and atherogenesis (186, 187, 222). Conditions that trigger leukocytes to leave the circulation may occur in damaged veins in chronic venous diseases (237).

Endothelial CAM which play an important role in leukocyte adhesion and transendothelial migration include E-selectin (CD62E) mediating the initial tethering of leukocytes and ICAM-1 (CD54) and VCAM-1 (CD106) recognizing integrin ligands on the leukocyte surface and involved in the more firm adhesion of leukocytes to the endothelium (187).

In vitro studies

Incubation of primary human umbilical vein endothelial cells (HUVEC) with LCPUFA increases the content of the respective LCPUFA in cell phospholipids (221). In *in vitro* cultures in which EC were directly exposed to pure LCPUFA, endothelial activation was attenuated. EPA and DHA can decrease the expression of ICAM-1 and VCAM-1 on EC (221, 222, 238, 239) and leukocyte adhesion to EC (221, 222, 239). Not only n-3 LCPUFA are able to suppress CAM expression. AA and its hydroperoxy (HPETE) and hydroxy (HETE) derivatives have shown to reduce neutrophil and monocyte adhesion to HUVEC. The TNF-α induced E-selectin, ICAM-1 and VCAM-1 expression was suppressed by these mediators; 15-HPETE was most effective (186).

De Caterina *et al.* showed that DHA, but not EPA supplementation for 96 hours with up to 25µmol/L, decreased endothelial expression of E-selectin, ICAM-1 and VCAM-1 in a dose-dependent manner in human saphenous vein endothelial cells (HSVEC) after incubation with a pro-inflammatory stimulus (IL-1β) (222, 239, 240). Also the adhesion of monocytic cells to HSVEC was reduced by DHA incubation (239). The inhibition of endothelial activation by LCPUFA increased with the number of double bonds. Double bond position or configuration (i.e. *cis* or *trans*) do not make a difference. DHA appears to be the most potent inhibitor of endothelial activation amongst LCPUFA (240). Weber *et al.* demonstrated that DHA, but not EPA or AA dose-dependently decreased TNF-α induced surface expression of VCAM-1 on HUVEC and reduced subsequent monocytic cell adhe-

sion. E-selectin or ICAM-1 induction by TNF-α or IFN-γ and upregulation of ICAM-1 and VCAM-1 by IL-1β remained unaffected (221). It has been reported by Collie-Duguid *et al.* that 65μmol/L EPA and DHA for 24 hours reduced mRNA expression for E-selectin, ICAM-1 and VCAM-1 in IL-1β stimulated, but not in resting HUVEC (238). Furthermore, EPA reduced IFN-γ induced ICAM-1 expression on monocytes (241). Yates *et al.* 2011 showed that adhesion of flowing neutrophils to HUVEC after TNF-α stimulation is dose-dependently inhibited by supplementation with DHA but not EPA. Cells that did adhere were activated and able to migrate across the endothelium normally; this suggests that DHA interferes with selectin-mediated tethering of adhesion. DHA reduced the expression of E-selectin on the surface of HUVEC, while mRNA levels for E-selection were not changed by DHA supplementation (242). E-selectin is known to be concentrated in lipid rafts (243). DHA incorporates into these parts of the membrane, possibly affecting E-selectin expression. In addition PGD_2 is known to stabilize neutrophil adhesion and induction of neutrophil transmigration. In the presence of EPA PGD_3 is generated, which inhibited migration of neutrophils across EC (244).

Dietary supplementation studies

Increased dietary salmon intake in pregnancy dampened *ex vivo* offspring EC activation. ICAM-1 and VCAM-1 expression on resting HUVEC was not affected, but endothelial activation in response to LPS was reduced. After *ex vivo* LPS stimulation EC surface expression of ICAM-1 and VCAM-1 and EC IL-6 production were reduced. In contrast, production of G-CSF in response to LPS was higher by EC in the salmon group and secretion of some other mediators was not affected by the intervention. This implicates some selectivity or specificity to the anti-inflammatory effects of dietary n-3 LCPUFA. Reduced CAM expression on EC could result in less adherence and transmigration of leukocytes into vascular tissue. This suggests an anti-inflammatory effect of dietary n-3 LCPUFA on human EC and is a possible mechanism by which these fatty acids can lower cardiovascular risk (245). Studies of dietary fatty acids on EC adhesion molecule expression are few. Dietary fish oil in rodents decreased expression of several adhesion molecules on lymphocytes (246), macrophages (247) and dendritic cells (248). A fish oil supplementation study in humans decreased ICAM-1 expression on *ex vivo* IFN-γ stimulated monocytes (249).

N-3 LCPUFA reduce endothelial mediator secretion

Decreased generation of inflammatory cytokines after n-3 LCPUFA incubation has been demonstrated *in vitro*. EPA and DHA inhibit the production of IL-6 and IL-8 by venous endothelial cells, whereas n-6 LCPUFA AA was ineffective (222, 250). Khalfoun found EPA to be more potent in inhibiting IL-6 secretion than DHA (250). This suggests that n-3 LCPUFA may be involved in suppressing inflammation. Furthermore, incubation of EC with n-3 LCPUFA resulted in an almost completely inhibited production of platelet derived growth factor-like protein; as a result intimal smooth muscle cell proliferation may be suppressed (251).

N-3 LCPUFA reduce endothelial cell activation in association with suppression of ROS, NF-κB and MAPK signaling cascades

Endothelial CAM expression and secretion of pro-inflammatory factors are induced by activation of NF-κB and/or involve MAPK cascades in endothelium (220, 252). Intracellular signaling mechanisms for inhibiting endothelial activation by n-3 LCPUFA are not fully elucidated, but may involve inhibition of these pathways (220, 253). Xue *et al.* have demonstrated that both EPA and DHA diminish activation of c-Jun N-terminal kinase (JNK) and p38 MAPK of TNF-α stimulated HUVEC. This may contribute to the inhibiting effects of n-3 LCPUFA on endothelial activation by pro-inflammatory stimuli (220). Others have shown that VCAM-1 and partly E-selectin, but not ICAM-1 expression required mobilization of the transcription factor NF-κB in HUVEC and that DHA incorporation inhibits activation of this signal transduction pathway (221). Wang *et al.* reported recently a reduction in surface VCAM-1 expression on EC after >40 μmol/L DHA incubation, attributed to an inhibitory effect on the NF-κB pathway (254). Critical in NF-κB activation is the inhibition of the production of the ROS hydrogen peroxide. N-3 LCPUFA may decrease the production of ROS, partly through the formation of active metabolites like resolvins. ROS activate NF-κB, probably by promoting degradation of IκB. N-3 LCPUFA may modulate receptor responses to atherogenic stimuli (e.g. mmLDL) by inhibiting ROS generation, leading to a decrease in endothelial activation and atherogenesis (185, 216). Gene expression of COX-2 – involved in plaque angiogenesis and rupture – is reduced by similar mechanisms. Together this affects leukocyte adhesion to the endothelium, early atherogenesis and later stages of plaque development and plaque rupture (185).

PREVENTION OF CVD BY N-3 LCPUFA MAY INVOLVE MAST CELL MEDIATOR SECRETION

Recently the involvement of mast cells in atherosclerosis has been discovered. Mast cells are present in the vessel wall and in atherosclerotic lesions. They release pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, MCP-1 and IFN-γ that induce plaque formation, progression and destabilization by targeting various mechanisms (255). Also lipid mediators such as leukotriens are involved (256). Mast cell mediators induce adhesion molecule expression and leukocyte adhesion, leading to enhanced plaque inflammation (255, 257). Furthermore, other mechanisms including lipid uptake and vascular permeability are promoted by mast cell mediators (255, 258, 259). This makes the mast cell a promising novel therapeutic target in CVD. Possibly n-3 LCPUFA generate their protective effect in CVD partially by affecting the mast cell phenotype prevent CVD.

SUMMARY

PUFA are incorporated into the cell membranes of virtually all cells of the body. An increase in n-3 LCPUFA intake increases the n-3 LCPUFA content of the cell membranes. N-3 LCPUFA and their effects on human health are interesting in the view of the steadily increasing incidence of allergic and inflammatory diseases. N-3 PUFA, especially LCPUFA EPA and DHA, exert anti-inflammatory and immunoregulatory effects, whereas n-6 PUFA do not. However, exposure to n-3 LCPUFA is in general low in the Western world.

The beneficial effects of n-3 LCPUFA on the development of allergic disease are complex. It is the result of a range of effects on different types of cells. N-3 LCPUFA have been shown to inhibit DC activation and antigen presentation, to affect T cell polarization and reduce IgE production by B cells. Although there are several studies reporting about the effects of EPA and DHA on DC, T and B cells, the effect on mast cell degranulation and mediator release is still largely unknown. Besides the direct action on the generation of inflammatory eicosanoids, n-3 LCPUFA may act on signal transduction pathways. In general they are able to suppress ROS generation and NF-κB and MAPK signaling pathways while activating PPAR. However, the exact mechanism of action differs between cell type and route of stimulation. Interference with cell signaling may suppress or enhance nuclear transcription factors and the expression of genes encoding for proteins including cytokines. It is likely that these kinds of effects can alter the mast cell phenotype which may contribute to the prevention of allergic disease. All together the immune modulating effects of n-3 LCPUFA on cells of the innate and adaptive immune system may reduce the development of allergic disease.

N-3 LCPUFA not only have strong impact on the function of cells of the immune system, also structural cells benefit from these dietary fatty acids. There is an inverse relationship between the intake of n-3 LCPUFA and the incidence of CVD, at least for a part because of a reduction in atherosclerosis. The underlying mechanism for beneficial effects attributed to n-3 LCPUFA includes an endothelial mechanism of action, which lowers leukocyte recruitment to the vessel wall. A lot of information is available from *in vitro* studies in which primary HUVEC are exposed to single, pure fatty acids in high concentrations. Animal studies have also shown that dietary fish oil reduces leukocyte adhesion to EC. Cardioprotective effects have been confirmed by several studies in humans, investigating the association of fish or n-3 LCPUFA and CVD. In addition, mortality outcomes in post-myocardial infarction patients are reduced by fish and n-3 LCPUFA. Therefore, fish containing high levels of n-3 LCPUFA may be useful (in combination with other therapies) in treatment or prevention of the development of CVD. However, the precise anti-atherogenic effects of n-3 LCPUFA in humans are not well established. Recently the contribution of mast cells to atherosclerotic plaque formation has been discovered. Possibly n-3 LCPUFA reduce pro-inflammatory mast cell mediator secretion leading to reduced plaque progression and rupture. This may contribute to the anti-atherogenic effects.

In conclusion, n-3 LCPUFA can affect cell function and contribute to the primary prevention of allergic and cardiovascular disease. As a result they may be able to support the effectiveness of drugs currently used to treat this kind of inflammatory disorders.

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CHAPTER THREE

Increased intake of vegetable oil high in n-6 PUFA enhances allergic symptoms to cow's milk protein and prevents oral tolerance induction by partial whey hydrolysate in mice

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ABSTRACT

Background The increased intake of vegetable oils high in n-6 polyunsaturated fatty acids (PUFA), including soybean oil, has been associated with the increase of allergic disease. Aim was to determine the effect of 7% versus 10% dietary soybean oil on oral tolerance (OT) induction, allergic sensitization and symptoms.

Methods Mice received a 7% versus 10% soybean oil diet before and during oral sensitization with whey or whey-hyperimmune serum transfer. In another study, mice received partial whey hydrolysate (pWH) while being fed the 7% or 10% soybean oil diet prior to oral sensitization. The acute allergic skin response, serum immunoglobulins, mouse mast cell protease-1 (mmcp-1) and splenic T cell percentages were determined upon whey challenge.

Results When provided during sensitization the acute skin response was increased in mice fed 10% compared to 7% soybean oil. Whey-IgE and -IgG1 were unaltered while mmcp-1 increased in the 10% fat group. Also in naive mice fed the 10% soybean oil diet and injected with whey-hyperimmune serum allergic symptoms increased. Besides enhancing the mast cell response, the 10% soybean oil diet also increased the percentage of activated Th1 and Th2 cells while increasing the Th2/regulatory T cell and Th2/Th1 ratio compared to the 7% fat diet. OT induction by pWH was abrogated in the 10% fat group compared to mice fed the 7% soybean oil during pWH pretreatment.

Conclusion Increased dietary intake of vegetable oil rich in n-6 PUFA suppresses the tolerizing capacity of pWH and enhances the severity of the allergic effector response in cow's milk allergic mice.

INTRODUCTION

The last decades have seen an increase in total polyunsaturated fatty acid (PUFA) intake in the western society. This is caused by the increased intake of n-6 PUFA-rich vegetable oils such as soybean, corn and sunflower oil and products such as margarines which are especially high in linoleic acid (LA) (1-3). LA is an essential fatty acid, found in plants only, and precursor for arachidonic acid (AA) (4). Usually, n-6 PUFA are considered to be pro-inflammatory because of the range of inflammatory eicosanoids (e.g. prostaglandins) to which AA is metabolised by cyclooxygenases and lipoxygenases (5). For this reason the use of excessive amounts of n-6 PUFA may be linked to the increased incidence of allergic disease in Westernized countries, affecting 15-30% of the population (6). Indeed it has been shown that high intake of n-6 PUFA, especially LA, is associated with allergic sensitization and eczema (7, 8). One of the first outcomes of atopic disease in infancy is cow's milk allergy, which affects between 0.3 and 3.5% of young infants (9, 10). So far it is unknown whether n-6 PUFA-rich vegetable oils, like soybean oil, may influence the risk of developing cow's milk allergy. Aim of this study was to determine the effect of 7% versus 10% dietary soybean oil on oral tolerance (OT) induction by partial whey hydrolysate (pWH), and allergic sensitization and symptoms using a murine model for orally induced cow's milk allergy.

MATERIALS AND METHODS

Animals

Animal use was performed in accordance with guidelines of the Animal Ethics Committee of Utrecht University. Three-week-old specific pathogen free female C3H/HeOuJ mice (Charles River Laboratories, L'Arbresle Cedex, France; n=6-8/group) were fed cow's milk protein-free AIN-93G diet (containing 7% soybean oil) (11) or a 10% soybean oil diet (Research Diet Services, Wijk bij Duurstede, The Netherlands). The fat percentage of AIN-93G was adapted by exchange of soybean oil with cornstarch, as described before (12). Soybean oil contained 59.1% PUFA of which 53.1% was the n-6 PUFA LA and 5.6% the n-3 PUFA α-linolenic acid; 24.9% monounsaturated fatty acids (oleic acid) and 15.1% of the fatty acids were saturated (palmitic and stearic acid). The diets were stored at -20°C prior to use.

Oral sensitization and challenge of mice

Mice were fed the 7% or 10% fat diet, starting two weeks prior to the first sensitization and continued during the whole sensitization period until sacrifice «Figure 1A». Mice were sensitized intragastrically (i.g.) using a blunt needle with 20 mg whey (DMV International, Veghel, The Netherlands) in 0.5 mL PBS with 10 µg cholera toxin (CT; List Biological Laboratories Inc, Campbell, CA, USA) as an adjuvant while sham mice received CT (10

µg/0.5 mL PBS). Mice were orally exposed once a week for 5 consecutive weeks as previously described by Schouten *et al.* (13). One week after the last sensitization mice were challenged intradermally (i.d.) in the ear pinnae of both ears with 10 µg whey in 20 µl PBS to determine the acute allergic skin response, anaphylactic shock and body temperature. The same day the mice were challenged i.g. with 50 mg whey in 0.5 mL PBS and 18 h after the oral challenge blood samples were collected and centrifuged at 14,000*g* for 15 min. Sera were stored at -70°C until analysis. Mice were sacrificed by cervical dislocation and the spleen was obtained for flow cytometry.

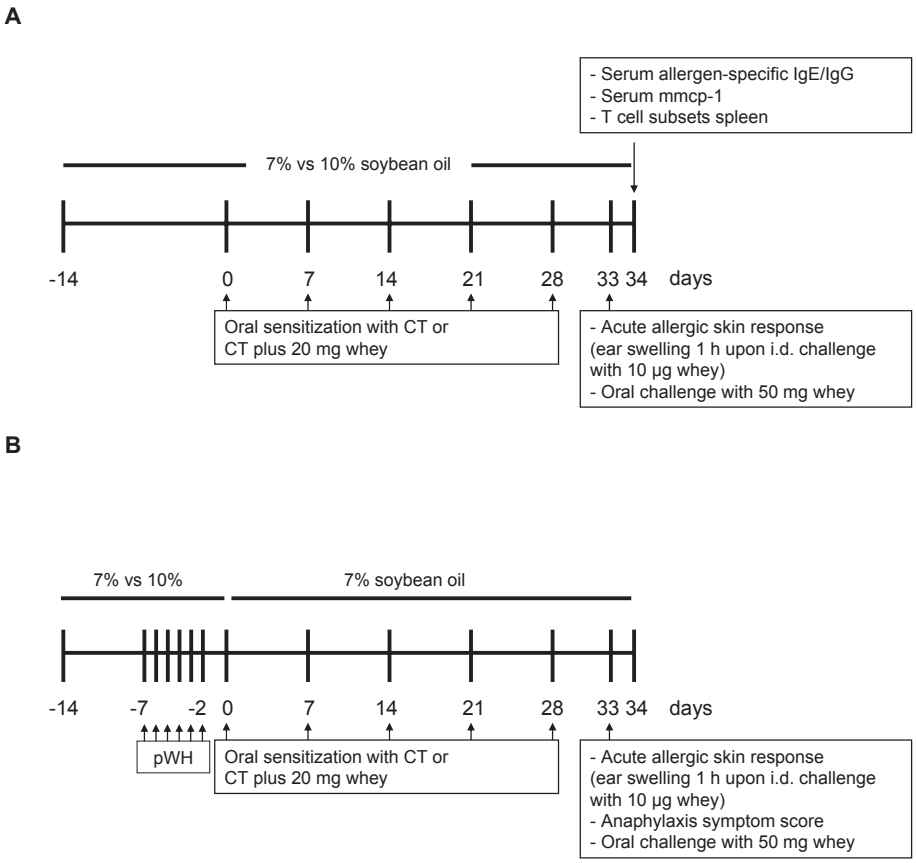

Figure 1 Schematic overview of the two different models used and parameters that were analyzed. Protocols used for (A) oral sensitization and (B) oral tolerance induction. CT: cholera toxin; mmcp-1: mouse mast cell protease-1; pWH: partial whey hydrolysate

Oral tolerance induction

Whey was hydrolysed with an established mixture of endopeptidases and exopeptidases according to a confidential enzyme composition used by Danone Research (Wageningen, The Netherlands) resulting in partially hydrolysed whey proteins (pWH). The enzymatic process was stopped by fast cooling. The partial whey hydrolysate (pWH) was characterized by analysis of the peptide size (85% < 1 kD, 8% < 2 kD, 4% < 5 kD, 1% < 10 kD, 0.6% < 20 kD and 1.4% > 20 kD) by means of high pressure liquid chromatography. pWH was previously shown to have limited sensitizing properties while restraining the capability of oral tolerance induction in mice (14, 15).

Prior to whey sensitization the mice were fed the 7% or 10% soybean oil diet from day -14 to 0. In addition, from day -7 until day -2 they were daily treated i.g. with 0.5 mL PBS as a control or 50 mg pWH/0.5 mL PBS using a blunt needle «Figure 1B» as previously described (15). Subsequently, mice were sensitized and challenged as described above while being fed the 7% soybean oil diet. One week after the last sensitization the acute allergic skin response was measured upon intradermal whey challenge and blood samples were collected the next day, after an oral challenge.

Acute allergic skin response and anaphylaxis symptom score

The acute allergic skin response was measured in duplicate for both ears 1 h after i.d. allergen challenge using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). Isoflurane was used for inhalational anesthesia during measurements. The ear swelling was expressed as delta µm by subtracting basal ear thickness before i.d. whey challenge from ear thickness 1 h after challenge. To assess the severity of shock symptoms 30 min after i.d. challenge a validated anaphylaxis symptom scoring table was used (16). The scoring was as follows: 0) no symptoms 1) scratching nose and mouth 2) swelling around eyes and mouth; piloerection; reduced activity; higher breathing rate 3) shortness of breath; blue rash around mouth and tail; higher breathing rate 4) no activity after stimulation; shivering; muscle contractions 5) death by shock.

Serum immunoglobulins and mmcp-1

Levels of whey-specific IgE and IgG1 were determined by ELISA in serum as previously described (12, 17). The concentration of mouse mast cell protease-1 (mmcp-1) in serum was determined using a commercially available ELISA kit (Moredun Scientific Ltd., Penicuik, UK) according to the manufacturer's protocol.

Passive sensitization: transfer of hyperimmune serum to recipients

To generate hyperimmune sera mice were immunized i.p. with 100 µl/mouse containing whey (100 µg) in PBS plus alum (2 mg) at day 0, 7 and 21 after which blood was collected at day 28. Pooled ('hyperimmune') sera from whey-alum i.p. immunized mice were i.v. transferred (100 µl) to isoflurane anesthesized (5% in air) naive mice. The naive mice

were fed the 7% or 10% soybean oil diet for two weeks prior to injection (n=6/group). The acute skin response was measured 30 minutes after serum transfer as described before, starting with measuring ear thickness at basal conditions prior to i.d. allergen challenge followed by assessing ear swelling 1 h after i.d. challenge.

T cell subsets assessed by flow cytometry

Single cell splenocyte suspensions were obtained by passing the organs through a 70 µm filter. Cells were blocked for 20 min in PBS containing 1% BSA and 5% FCS. $5x10^5$ Cells were plated per well and incubated for 30 min at 4°C with different antibodies (eBioscience, Breda, The Netherlands or BD, Alphen aan den Rijn, The Netherland, unless otherwise stated) against CD4, CD69, CXCR3, T1/ST2 (MD Biosciences, St. Paul, Minnesota, USA), CD25 and FoxP3 and isotype controls were used. Cells were fixed using 0.5% paraformaldehyde and permeabilized for intracellular staining with anti-FoxP3 using the FoxP3 staining buffer set (eBioscience) according to the manufacturer's protocol. Flow cytometry was performed using FACS Canto II (BD, Alphen aan den Rijn, The Netherlands) and analyzed using FACSDiva software (BD).

Statistics

Data are presented as mean ± SEM and were analyzed using one or two-way ANOVA and post hoc Bonferroni test using GraphPad Prism software (version 5.0). For anaphylaxis symptom score data Kruskall-Wallis followed by Dunn's multiple comparison test was used. If required LOG transformation was used to normalize data distribution for analysis and then Tukey box-and-whisker plots were used. $P<0.05$ was considered statistically significant.

RESULTS

The acute allergic skin response is enhanced by increasing dietary soybean oil intake

First the effect of the 7% versus 10% soybean oil diet on allergic outcome when provided before and during oral sensitization was studied. Sensitization with whey resulted in an enhanced acute allergic skin response upon i.d. challenge with whey compared to sham-sensitized mice fed the 7% or 10% fat diet «Figure 2A». Furthermore, the acute allergic skin response was significantly increased in whey-mice when fed 10% compared to 7% soybean oil diet. To study whether this difference in acute allergic skin response was related to alterations in the Th2 type humoral response, serum allergen-specific antibodies were measured. Both whey-specific IgE and IgG1 levels were enhanced in sensitized mice compared to sham mice but not affected by increasing dietary soybean oil content «Figure 2B-C». Food intake and body weight were equal in all groups during the experiment (data not shown).

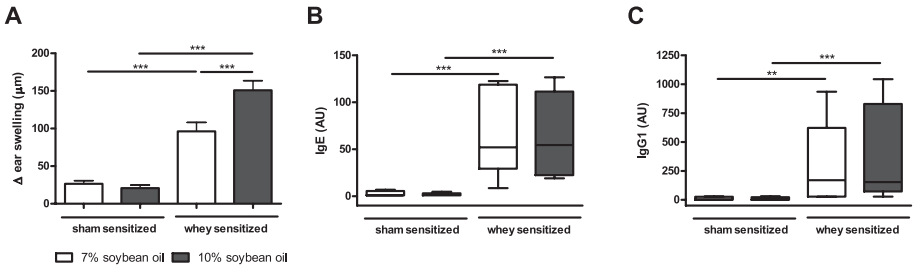

Figure 2 The effect of 7% vs 10% soybean oil diet on the (A) acute allergic skin response assessed upon intradermal challenge (delta ear swelling in µm) and whey-specific (B) IgE and (C) IgG1 levels in serum were determined 18 hours after oral challenge. Data are presented as mean ± SEM or Tukey box-and-whisker plots, n=6. ***P*<0.01, ****P*<0.001 two-way ANOVA followed by Bonferroni's multiple comparison test (after LOG transformation for IgE and IgG1).

High soybean oil fat enhances the allergic effector response

To determine the effect of increased intake of soybean oil rich in n-6 PUFA on mucosal mast cell degranulation, mmcp-1 serum concentrations after oral challenge were measured in whey-sensitized mice. Serum mmcp-1 increased in the 10% soybean oil group «Figure 3A». To determine whether the local effector response was altered by increased soybean oil intake naive mice fed the 7% or 10% soybean oil diet were passively immunized with whey-hyperimmune serum and i.d. challenged in the ear with whey. Recipient mice in the 10% fat group receiving the hyperimmune serum, displayed a higher acute allergic skin response compared to 7% soybean oil diet fed recipients «Figure 3B». This confirms that in accordance with the mucosal mast cell response also the local effector response was enhanced upon feeding the 10% soybean oil diet.

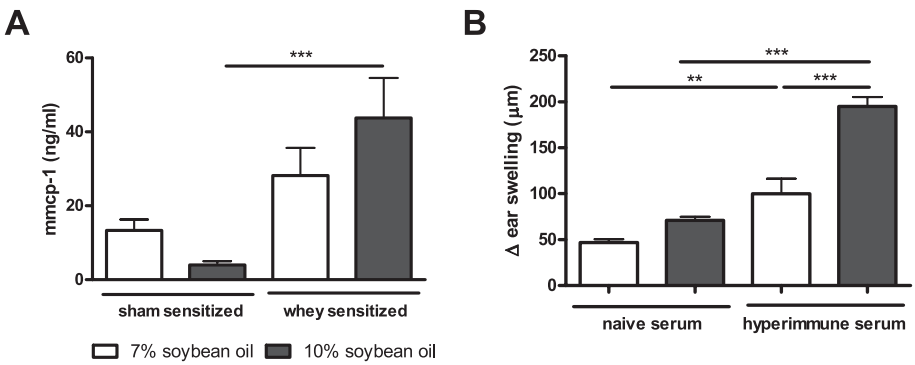

Figure 3 The effect of n-6 PUFA on the allergic effector response. The concentration of serum (A) mucosal mast cell protease-1 (mmcp-1) as a measure of mucosal mast cell degranulation. (B) The acute allergic skin response (delta ear swelling in µm) was measured in naive recipient mice fed the 7% or 10% fat diet before injection with naive or whey-hyperimmune serum and an intradermal challenge. Data are presented as mean ± SEM, n=6. ***P*<0.01, ****P*<0.001 two-way ANOVA followed by Bonferroni's multiple comparison test.

The high fat diet enhances the Th2/Th1 and Th2/Treg ratio in sham mice

To further determine the effect of a diet high in n-6 PUFA during sensitization on the adaptive immune system, T cell subsets in the spleen were studied. In sham-sensitized mice fed the 10% fat diet the percentages of activated Th2 and Th1 cells were enhanced compared to 7% soybean oil fed mice «Figure 4A-C». As a result of a more pronounced increase in the percentage of Th2 compared to Th1 cells in sham mice fed 10% soybean oil, the Th2/Th1 ratio increased compared to the 7% fat sham group and remained high upon whey sensitization «Figure 4E». Whey-sensitized mice in the 7% soybean oil group displayed an increase in the percentage of activated Th1 and Th2 cells and an enhanced Th2/Th1 ratio compared to sham-mice fed 7% soybean oil. Although the percentage $CD25^{+}FoxP3^{+}$ T cells within the $CD4^{+}$ population was enhanced by the 10% fat diet in the sham group «Figure 4D», the Th2/$CD25^{+}FoxP3^{+}$ Treg ratio was still increased in sham-sensitized mice fed the 10% soybean oil diet compared to mice fed 7% soybean oil «Figure 4F».

Increased intake of soybean oil abrogates oral tolerance induction by pWH

To study the effect of 7% vs 10% soybean oil on oral tolerance induction by pWH, the different diets were fed for two weeks prior to sensitization, pWH was given by daily gavage during the last 5 days «Figure 1B». Subsequently mice were sensitized while being fed the 7% soybean oil diet. pWH induced OT to whey in mice fed the 7% soybean oil diet as the acute allergic skin response was reduced compared to non-tolerized mice «Figure 5A». The tolerizing capacity of pWH was lost when mice were fed 10% soybean oil during pWH treatment prior to whey sensitization. Also the anaphylactic symptoms were significantly enhanced in the 10% soybean oil fed mice compared to mice fed the 7% soybean oil diet when treated with pWH «Figure 5B».

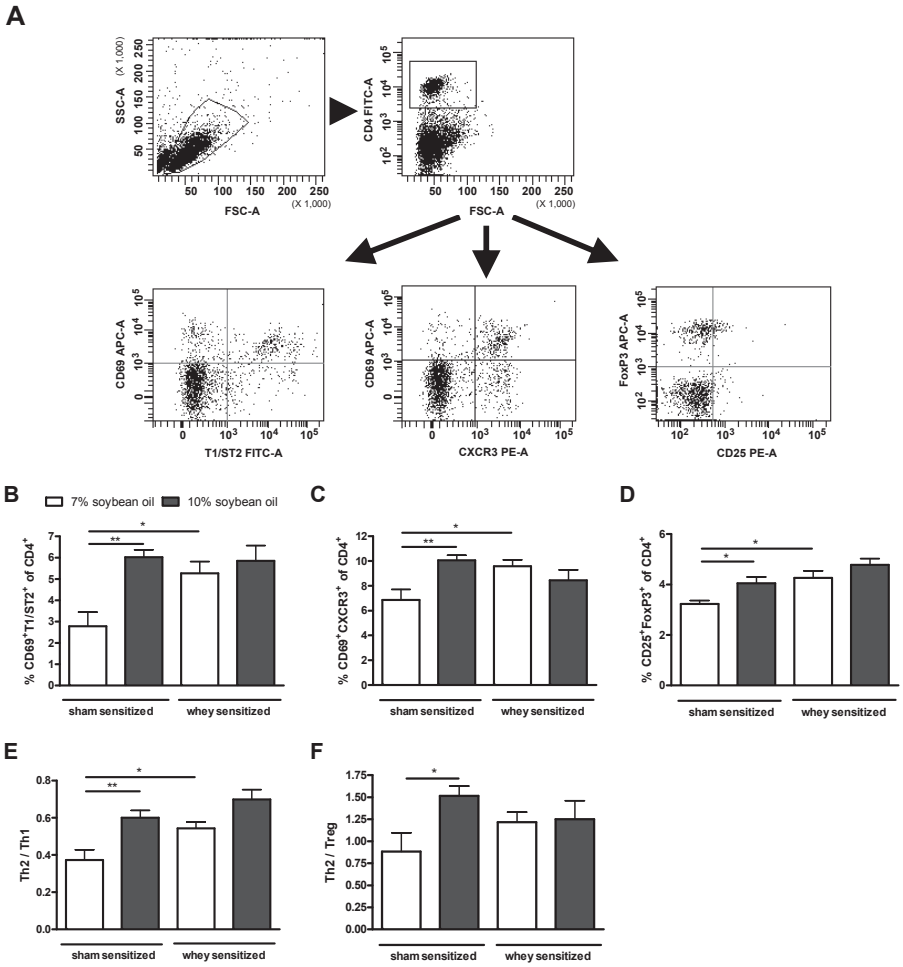

Figure 4 Splenic T cell subsets determined after oral allergen challenge. (A) Representative dot plots of activated Th2 cell ($CD4^+CD69^+T1/ST2^+$) analysis in spleen. Lymphocytes were gated based on FSC-SSC pattern, followed by analysis for expression of CD4. Then co-expression of CD69 and T1/ST2 (activated Th2 cells); CD69 and CXCR3 (activated Th1 cells) or CD25 and FoxP3 (regulatory T cells; Treg) was analyzed. Percentage of activated (B) Th2 and (C) Th1 cells and (D) $CD25^+FoxP3^+$ Treg was determined. Furthermore (E) Th2/Th1 ratio and (F) Th2/Treg ratio were calculated. Data are presented as mean ± SEM, n=6. *$P<0.05$, **$P<0.01$ two-way ANOVA followed by Bonferroni's multiple comparison test.

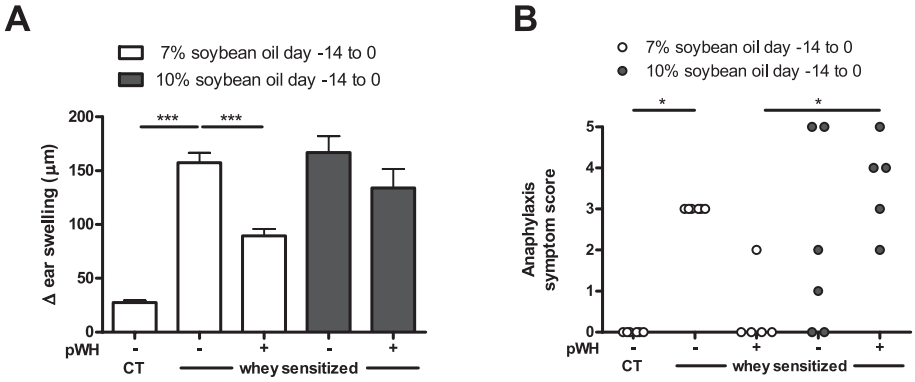

Figure 5 Effect of increasing soy bean oil intake on oral tolerance induction. In the oral tolerance model mice were pretreated with the 7% or 10% fat diet in presence or absence of partial whey hydrolysate (pWH) followed by whey sensitization while being fed the 7% fat diet. (A) The acute allergic skin response assessed upon intradermal challenge (delta ear swelling in µm) and (B) anaphylaxis symptom score was assessed. Data are presented as mean ± SEM or scatter plot, n=6. *$P<0.05$, ***$P<0.001$ one-way ANOVA followed by Bonferroni's multiple comparison test. Anaphylaxis symptom score: Kruskall-Wallis followed by Dunn's multiple comparison test

DISCUSSION

Increasing the intake of vegetable soybean oil rich in n-6 PUFA enhances the severity of the allergic effector response in a murine model for cow's milk allergy and prevents oral tolerance induction using partial whey hydrolysate.

High dietary n-6 PUFA intake may be a risk factor for the development of atopic disease. High margarine consumption (rich in LA) has been associated with an increased risk of asthma (18-20), allergic rhinitis (8, 21-23), eczema and allergic sensitization (7, 8). In children margarine and vegetable oil intake is linked to enhanced risk of wheezing symptoms (24-26) and children with atopic disease consumed more margarine and less butter and fish than non-atopic children (27).

In this study the effect of a 7% versus 10% soybean oil diet in the development of cow's milk allergy was assessed. There are only a few studies addressing the effect of dietary fat percentage on allergic inflammation in the absence of obesity. Compared to other studies 7% vs 10% soybean oil is a very mild increase in fat percentage of the diet, not inducing an increase in body weight. When these diets were provided before and during oral sensitization with whey, the acute allergic skin response was enhanced by the 10% fat diet. This effect was accompanied by enhanced serum mmcp-1 reflecting enhanced mucosal mast cell degranulation (28). Although the effector response was increased in whey-sensitized mice, no increase in the whey-specific Th2 type immunoglobulins could be detected. The acute allergic skin response was also enhanced in naive mice fed the 10% soybean oil diet prior to passive immunization.

It indicates that increasing the amount of n-6 PUFA rich soybean oil in the diet enhances the allergic effector response independent of the humoral response. This may be the consequence of an increased sensitivity of mast cells to degranulate or altered mediator release due to the increased consumption of dietary n-6 PUFA. Indeed, n-6 PUFA AA -the main LA metabolite- dose-dependently increased IgE-mediated mast cell degranulation as measured as β-hexosaminidase release *in vitro*. Furthermore PGD_2 secretion upon mast cell activation was enhanced by AA incubation of mast cells (29). This implicates that the mast cell response may be altered by a diet high in n-6 PUFA and as a result the severity of allergic symptoms may increase. In diet-induced obese mice fed a diet containing 50% soybean oil and sensitized to ovalbumin, mast cell numbers were enhanced compared to non obese control mice fed 10% fat (30). Furthermore reactivity to histamine in the skin may be enhanced by increasing dietary soybean oil. Indeed it has been shown that vascular permeability increased in mice fed a 24% compared to 6% soybean oil diet (31), suggesting that a high fat diet may enhance allergic reactions.

In the current study the 10% soybean oil diet did not affect the Th2 type humoral response, which was in accordance with the study of Yamaki *et al.* that also did not find an increase in serum IgE and IgG1 (31). Although the diet rich in n-6 PUFA enhanced the effector response while the allergen-specific antibody response remained unaltered, fish oil derived long chain n-3 PUFA were shown to largely prevent the induction of both parameters in the murine model for whey sensitization (12). Historically the dietary ratio of n-6:n-3 PUFA approached 1:1 however nowadays in the Western diet this ratio is 10:1 up to 25:1 because of a gradual decrease in n-3 PUFA consumption and excessive amounts of n-6 PUFA (32-34). Intake of a higher ratio of n-6:n-3 PUFA enhanced the risk of asthma (35). Furthermore, the risk of atopic disease and sensitization were associated with reduced n-3 PUFA levels and increased n-6:n-3 PUFA ratio in serum (36). This indicates that the source of dietary fat is important in immune regulation. Indeed partial substitution of soybean oil, rich in n-6 PUFA, by fish oil rich in n-3 LCPUFA largely prevented allergic sensitization in association with an enhanced frequency of $FoxP3^+$ regulatory T-cells (Treg) and reduced percentage of activated Th1 and Th2 cells (12). This suggests that not the amount of fat per se but the fat source is important in the effects exerted *in vivo*. As allergic sensitization occurs early in life or even *in utero*, also the maternal diet composition may have consequences for the risk of developing allergic disease in newborns. Several studies supplementing pregnant women with fish oil have demonstrated a reduced risk of atopy in children at risk (37-41). On the other hand, studies demonstrated that high levels of total n-6 PUFA in breast milk were associated with an increased risk for atopic disease in children (42-44). Furthermore atopic children had higher LA and total PUFA levels resulting in a higher n-6:n-3 PUFA ratio in serum lipid fractions than healthy children (42).

Like in our study, Yamaki *et al* showed that increased dietary soybean oil intake enhanced the Th2/Th1 ratio, which was demonstrated by an increased Th2 type cytokine production and trend towards decreased IFN-γ in serum (31). Furthermore, Mizota *et al.* also demonstrated that the ratio of IFN-γ/IL-4 was reduced in culture supernatants of both mice and humans after being fed a n-6 PUFA-rich diet compared to a n-3 PUFA-rich diet, resulting in a shift towards allergy prone Th2 (45). In the current study Th2 polarization by a diet high in n-6 PUFA was readily present in the sham-sensitized mice hence, independent of allergic sensitization. AA-derived eicosanoids like PGD_2 and other mediators such as histamine can induce DC activation and Th2 development (46, 47). Indeed the enhanced Th2/Treg and Th2/Th1 ratio suggests Th2 skewing of the adaptive immune response by the 10% soybean oil diet rich in n-6 PUFA. As a result the susceptibility to develop allergic sensitization instead of tolerance upon allergen exposure may increase. To determine whether increasing the dose of soybean oil affects oral tolerance (OT) induction, mice were fed the different diets and treated with pWH before sensitization. Hypoallergenic formulae containing partially hydrolyzed peptides are often used to prevent allergies in children at risk. Previously pWH has been demonstrated to be effective in inducing OT to whey in mice when fed a 7% fat diet (15). However, combining pWH with the 10% soybean oil pretreatment was shown to abrogate OT induction by pWH. Van Esch *et al.* demonstrated pWH to restore Treg frequency in the MLN of whey-sensitized mice to the same percentage found in sham mice (15). In the current study the percentage of Treg in the MLN was not reduced by sensitization nor altered by the 10% fat diet (data not shown). In addition, neither the Th2/Treg nor Th2/Th1 ratio increased in the MLN (data not shown). Hence, future studies are needed to further unravel the suppression of the tolerizing capacity of pWH using a 10% soybean oil diet.

Increasing dietary intake of vegetable oil, rich in n-6 PUFA, enhances the severity of the allergic effector response and breaks oral tolerance induction in a murine model for cow's milk allergy. This suggests that dietary fat dose and source may have impact on the susceptibility to develop allergic disease and severity of allergic symptoms.

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CHAPTER FOUR

N-3 LCPUFA reduce allergy-related mediator release by human mast cells *in vitro* via inhibition of reactive oxygen species

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ABSTRACT

Background The increased n-6 and reduced long chain n-3 polyunsaturated fatty acids ((LC)PUFA) intake in Western diets may contribute to the increased prevalence of allergic diseases. Key effector cells in allergy are mast cells (MC). Aim was to investigate the effects of n-6 versus n-3 LCPUFA on MC phenotype.

Methods Human MC lines (LAD2, HMC-1) were incubated for 24 hours with either arachidonic acid (AA, n-6 LCPUFA) or n-3 LCPUFA eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Effects of these three LCPUFA on degranulation, mediator secretion and reactive oxygen species (ROS) generation were assessed. ROS, mitogen-activated protein kinase (MAPK) or nuclear factor (NF)-κB inhibitors were used to unravel signaling pathways involved in cytokine secretion.

Results AA, EPA or DHA did not reduce IgE-mediated degranulation by LAD2 cells. However, AA increased PGD_2 and TNF-α secretion by ionomycin/PMA stimulated HMC-1 whereas EPA and DHA more prominently inhibited IL-13 and IL-4 secretion. Suppression of IL-4 and IL-13 release by LCPUFA correlated with reduced ROS generation. IL-4 and IL-13 release by activated HMC-1 was abrogated using ROS inhibitors. Inhibition of MAPK signaling, but not NF-κB, downstream of ROS reduced IL-13 secretion by activated HMC-1. Combined incubation of EPA or DHA with MAPK inhibitors further suppressed IL-13 secretion.

Conclusion N-6 LCPUFA AA enhanced pro-inflammatory mediator production by MC, while n-3 LCPUFA EPA as well as DHA more effectively suppressed ROS generation and IL-4 and IL-13 release. This suggests that dietary supplementation with EPA and/or DHA may alter the MC phenotype, contributing to a reduced susceptibility to develop and sustain allergic disease.

INTRODUCTION

During the last decades the prevalence of allergic diseases has dramatically increased (1). Mast cells (MC) are key effector cells in allergy and play a pivotal role in initiating and maintaining allergic reactions and inflammation by release of numerous inflammatory mediators (2-4). Upon activation MC immediately release a plethora of preformed mediators such as histamine and proteases that are stored in secretory cytoplasmic granules. Arachidonic acid (AA)-derived eicosanoids and multiple pro-inflammatory chemokines and cytokines, such as allergy-driving interleukin (IL)-4, and IL-13, are generated *de novo* (2, 5). These mediators are important in the pathogenesis of allergic responses and can increase the susceptibility to develop allergic disease and enhance allergic symptoms (6-10).

Polyunsaturated fatty acids (PUFA) can modulate immune responses. In general, long chain (LC) n-6 PUFA such as AA (20:4n-6) are considered to be pro-inflammatory and n-3 LCPUFA eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3) protect against inflammation (11-14). Decreased dietary intake of n-3 LCPUFA from fatty fish together with the high intake of n-6 PUFA from mainly vegetable oils in the Western diet is likely to contribute to the increased incidence of allergic and inflammatory disease in humans over the last decades (14-17). Dietary supplementation with n-3 LCPUFA has been studied to some extent in allergic disease, focussing on prevention. Human pregnancy studies revealed a reduction in infant atopy when women were supplemented with fish oil during pregnancy and lactation (18-23).

N-3 LCPUFA compete with n-6 LCPUFA for incorporation into the cell membrane. After release from the membrane AA can be metabolized by cyclooxygenases (COX) and lipoxygenases (LOX), resulting in the formation of 2/4-series eicosanoids which support inflammatory responses. By contrast, exchange of AA by n-3 LCPUFA results in production of 3/5 series eicosanoids which are less potent (11, 13, 15). Besides modification of the generation of eicosanoids, LCPUFA may change intracellular signaling pathways and may affect activation of nuclear transcription factors and consequently gene transcription (11, 24). Dietary EPA and DHA have been shown to inhibit the release of pro-inflammatory cytokines (IL-1, IL-6, IL-8, tumor necrosis factor (TNF)-α by macrophages and mononuclear cells (11, 25, 26) but little is known about the effects of LCPUFA in MC.

In MC cytokine secretion is under regulation of intracellular reactive oxygen species (ROS), mitogen-activated protein kinases (MAPK) and nuclear factor-κB (NF-κB) signaling pathways (27, 28). The pathophysiological importance of ROS such as superoxide generated from MC has not been fully elucidated. At high levels ROS are involved in the innate immune response while at low levels they are involved in cell signaling (28). It has been described that n-3 LCPUFA may suppress ROS generation by targeting eicosanoids, cytokines and protein kinase C (PKC) (11, 13). These molecules control enzymes involved in ROS formation such as NADPH oxidase (11, 28). Wong *et al.* reported that DHA inhibited NADPH oxidase and ROS generation in macrophages (29).

Downstream of ROS MAPK extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) and transcription factors including NF-κB become activated, resulting in cytokine production (27, 30). Precise signaling pathways are still unclear but activation of these routes induces transcriptional activity of cytokine genes in MC.

Although the anti-inflammatory effects of EPA and DHA have been studied in neutrophils and mononuclear cells, effects on MC function have not been fully addressed. Modulation of MC mediator release e.g. by modification of membrane lipid composition, may have impact on the allergic outcome. In view of the steadily increasing prevalence of allergies and inflammatory diseases, there is a need for (novel) treatment or prevention strategies. Therefore we studied the effects of AA, EPA and DHA on MC degranulation and cytokine production. In particular, the involvement of ROS, MAPK and NF-κB signaling in cytokine secretion and the modulation of the MC phenotype by LCPUFA was addressed.

MATERIALS AND METHODS

Cell culture

All chemicals and inhibitors were derived from Sigma-Aldrich, Steinheim, Germany unless otherwise stated. Human leukemic mast cell line HMC-1 was kindly donated by the Mayo Clinic and used with permission (31). These non-adherent cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco, Invitrogen, Paisley, Scotland UK) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10% (v/v) fetal bovine serum (FBS, HyClone, Perbio, see «Supplementary Table 1 on page 101» for fatty acid composition) at 37°C with 5% CO_2 in a humidified atmosphere. HMC-1 were passaged once a week.

LAD2 cells (human mast cell line) were grown in StemPro-34 serum-free complete medium (serum-free medium plus nutrient supplement; Gibco) plus L-Glutamine, recombinant human stem cell factor (100 ng/ml; ProSpec, Ness Ziona, Israel) and penicillin (100 IU/ml) and streptomycin (100 µg/ml).

LCPUFA incubation

For modification of cell lipids MC were pre-incubated with either AA, EPA or DHA for 24 hours. Cells were collected and resuspended at a concentration of 1×10^6 cells/ml in fresh medium containing L(+) ascorbic acid (75 µM, Merck, Darmstadt, Germany) in order to prevent LCPUFA oxidation. Stock solutions in ethanol of AA, EPA or DHA were further diluted in FBS or StemPro-34 nutrient supplement containing anti-oxidant α-tocopherol. 5% FBS or 2.5% nutrient supplement was added to the cells (final concentration ethanol 0.1% [v/v]). The final concentration of α-tocopherol was 20 µM. Control wells also included ethanol (0.1% [v/v]). Membrane fatty acid composition in HMC-1 (0, 10, 25, 50 and 100 µM) and LAD2 cells (0, 50 and 100 µM based on HMC-1 results) was assessed by gas chromatography as previously described by Bligh and Dyer (32, 33).

IgE-mediated β-hexosaminidase release assay

LAD2 cells were incubated for 24 hours with solely AA, EPA or DHA (0, 25, 50 and 100 μM chosen from membrane fatty acid analysis) in 96-well culture plate (Costar, Corning Incorporated, NY, USA) at 1 x 10^5 cells/100μl/well. Stempro-34 medium without nutrient supplement but supplemented with L(+) ascorbic acid was used. Nutrient supplement enriched with α-tocopherol ± LCPUFA was added to determine the degranulation of LCPUFA supplemented cells. After four hours human purified IgE (Chemicon, Millipore, Temecula, CA, USA) was added at concentration 0.5 μg/ml and incubated an additional 20 hours at 37°C, to prime the cells with IgE. The cells were washed two times by tyrode's buffer (10 mM HEPES (Acros organics, Geel, Belgium) buffer pH 7.2, tyrode salts (Gibco) 9.5 g per litre, 0.1% sodium bicarbonate (Merck), 0.1% [w/v] BSA (fraction V, Roche, Mannheim, Germany)). Next, α-human IgE–FITC (KPL, Gaithersburg, MD, USA) was added to a final concentration of 10 μg/ml and incubated for 1 hour at 37°C to induce degranulation. Degranulation was determined by the amount of β-hexosaminidase released in the cell-free supernatant. To determine the total amount of β-hexosaminidase release present in untreated cells, Triton X-100 1% was used to lyse the cells. Supernatants were diluted 1:1 with 160 μM 4-methyl umbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG) in 0.1 M citrate buffer (pH 4.5) and incubated for 1h at 37°C. This reaction was terminated by the addition of glycine buffer (15 g glycine (MP Biochemicals, Illkirch, France), 11.7 g sodium chloride (Merck) per litre, pH 10.7). Fluorescence was measured within one hour at ex: 360 nm and em: 460 nm by Fluorescence Measurement System (Millipore, CytoFluor 2350, B&L Systems, Zoetermeer, the Netherlands). Average fluorescence of unstimulated cells (background) is subtracted from all values. Background release did not differ between groups. Amount of β-hexosaminidase release was calculated as percentage of total β-hexosaminidase in Triton X-100 cell lysates (maximal release).

MC mediator release and cell viability

After pre-incubation with AA, EPA or DHA in some experiments inhibitors were used before MC stimulation. HMC-1 were incubated for 30 min with 1-10 μM of COX-inhibitors (indomethacin or NS398) or for 10 min with a ROS inhibitor (superoxide dismutase (SOD); 20 or 100 U/ml or 1,3-dimethyl-2-thiourea (DMTU); 30, 40, 50, 60 mM), a MAPK inhibitor (ERK inhibitor PD98059; 50, 100 μM, p38 inhibitor SB203580; 20, 50 μM, JNK inhibitor SP600125; 10, 20 μM) or NF-κB inhibitor (Bay117082; 10, 30 μM). Inhibitors were diluted in medium (final concentration dimethyl sulphoxide (DMSO) , 0.2% [v/v], except for PD98059 with 0.5 % DMSO [v/v]). After pre-incubation with LCPUFA and/or inhibitors, the cells were stimulated with 1 μM of ionomycin plus 16 nM phorbol 12-myristate 13-acetate (PMA) (both diluted in medium, final concentration DMSO 0.1% [v/v]) and incubated at 37°C. Optimal doses of ionomycin and PMA were chosen after pilot experiments. To assess inflammatory mediator production in the culture supernatants of HMC-1, supernatants were collected 30 min (PGD_2), 4h (TNF-α, IL-8) and 24h (IL-4, IL-13) after stimulation. Se-

creted cytokine concentrations were determined by ELISA cytoSet kits according to the manufacturer's instruction (Bioscource, Camarillo, CA, USA). PGD_2 was measured by Enzyme Immunoassay Prostaglandin D_2 – MOX kit (Cayman Chemical, Ann Arbor, MI, USA).

After 24h stimulation cell proliferation/viability reagent WST-1 (Roche) was added to the cells to assess cell viability (mitochondrial activity). The absorbance at 450 nm was determined using a Bio-Rad Benchmark Microplate Reader (Bio-Rad Laboratories). In separate experiments cell death (necrosis) was examined by light microscopy with trypan blue exclusion.

Intracellular ROS production

Following 24h incubation with 0, 25 or 100 µM AA, EPA or DHA, the ROS production by HMC-1 was measured by means of flow cytometry. Cells were washed and resuspended in 200 µl fluorescence-activated cell sorter (FACS) buffer (5% FBS in PBS (BioWhittaker, Lonza, Verviers, Belgium) in a V-shaped 96-well culture plate (Cellstar, Greiner bio-one, TC-plate). Subsequently, the cells were incubated with 5 µM 2', 7' dichlorofluorescin diacetate (DCFH-DA) for 10 min at 37°C followed by ionomycin and PMA, as described before. After 30 min ROS production was determined as the potency to oxidize 2', 7'-dichlorofluorescin to fluorescent dichlorofluorescein on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), as described (27, 34). Generation of intracellular ROS was determined by counting of 10.000 cells in channel FL1 (excitation 488 nm, emission 530 nm) of the FACS.

Statistical Analysis

Graphs were made by Statistical software GraphPad Prism (GraphPad Prism for Windows, Version 4, GraphPad software Inc., La Jolla, CA, USA). Each figure represents the mean ± SEM of the different experiments under the same conditions. Differences between groups were assessed by one-way ANOVA and post hoc Dunnett's test for multiple comparisons. To compare effects between different LCPUFA one-way ANOVA and post hoc Bonferroni test for multiple comparisons or paired Student's *t* test has been used. SPSS version 15 software (SPSS inc., Chicago, Ill, USA) was used for these analyses. Pearson correlation coefficients were calculated by GraphPad Prism. $P<0.05$ was considered statistically significant.

RESULTS

Fatty acid composition human MC

Membrane fatty acid composition of HMC-1 and LAD2 cells was significantly altered after AA, EPA or DHA incubation. These LCPUFA did incorporate dose-dependently in the cellular membranes of HMC-1 and LAD2 cells «Table 1». Furthermore, incubation with EPA or

DHA significantly reduced AA in the cell membrane for HMC-1 and LAD2 cells while EPA and DHA were substituted significantly by addition of AA in HMC-1 only due to lack of EPA and low DHA in LAD2 cells under basal conditions. In addition to exchanges in LCPUFA membrane composition, AA, EPA or DHA supplementations competed with 18:1n-9 and 16:0 for incorporation (data not shown).

Table 1 Membrane fatty acid composition of HMC-1 and LAD2 cells after 24 hour LCPUFA incubation (mean values for n=4 independent experiments and SEM).

		HMC-1						LAD2					
PUFA	Conc (µM)	AA (%)	SEM	EPA (%)	SEM	DHA (%)	SEM	AA (%)	SEM	EPA (%)	SEM	DHA (%)	SEM
	0	3.4	0.4	0.3	0.1	2.7	0.3	7.0	0.4	0.0	0.0	1.1	0.1
AA													
	10	13.6	2.2	0.0**	0.0	2.0	0.2						
	25	26.4***	7.0	0.0**	0.0	1.4*	0.4						
	50	20.9*	1.9	0.0**	0.0	1.6	0.0	16.4	3.1	0.0	0.0	0.9	0.1
	100	33.7***	0.7	0.0**	0.0	1.2*	0.1	26.2*	5.0	0.0	0.0	0.7	0.1
EPA													
	10	3.0	0.3	4.0	0.3	4.4	0.9						
	25	2.7	0.1	7.9	0.9	3.5	0.7						
	50	2.2	0.1	11.3	2.7	2.5	0.8	6.1	0.3	5.7	1.5	1.0	0.1
	100	1.7*	0.2	21.5***	2.0	1.5	0.2	5.6*	0.2	13.4*	3.7	0.8	0.1
DHA													
	10	3.0	0.3	0.8	0.3	12.6	1.0						
	25	2.6	0.2	0.8	0.2	25.0***	2.4						
	50	2.3	0.0	0.8	0.2	34.8***	4.0	6.2	0.3	0.0	0.0	16.5*	2.6
	100	1.7*	0.2	0.7	0.1	52.1***	5.1	5.5*	0.2	0.1	0.0	28.0***	3.7

Mean membrane fatty acid % were significantly different from the control group (0 µM) after incubation with arachidonic acid (AA), EPA or DHA as tested by one-way ANOVA with post-hoc Dunnett's Multipe Comparison Test: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Effect of AA, EPA or DHA on IgE-stimulated degranulation of LAD2 cells

β-Hexosaminidase release was used as a marker for MC degranulation. LAD2 cells were stimulated by sensitization with IgE followed by cross-linking with α-IgE. AA at 100 µM increased FcεRI-mediated β-hexosaminidase release of LAD2 cells while EPA and DHA did not affect MC degranulation (AA 100 µM: 36.4 ± 6.7% vs 49.9 ± 5.6%, n=3, *$P<0.05$ «Figure 1A»). Degranulation after incubation with AA did not differ significantly from EPA or DHA. The solvent of LCPUFA (ethanol, 0.1% [v/v]) did not affect β-hexosaminidase release (data not shown).

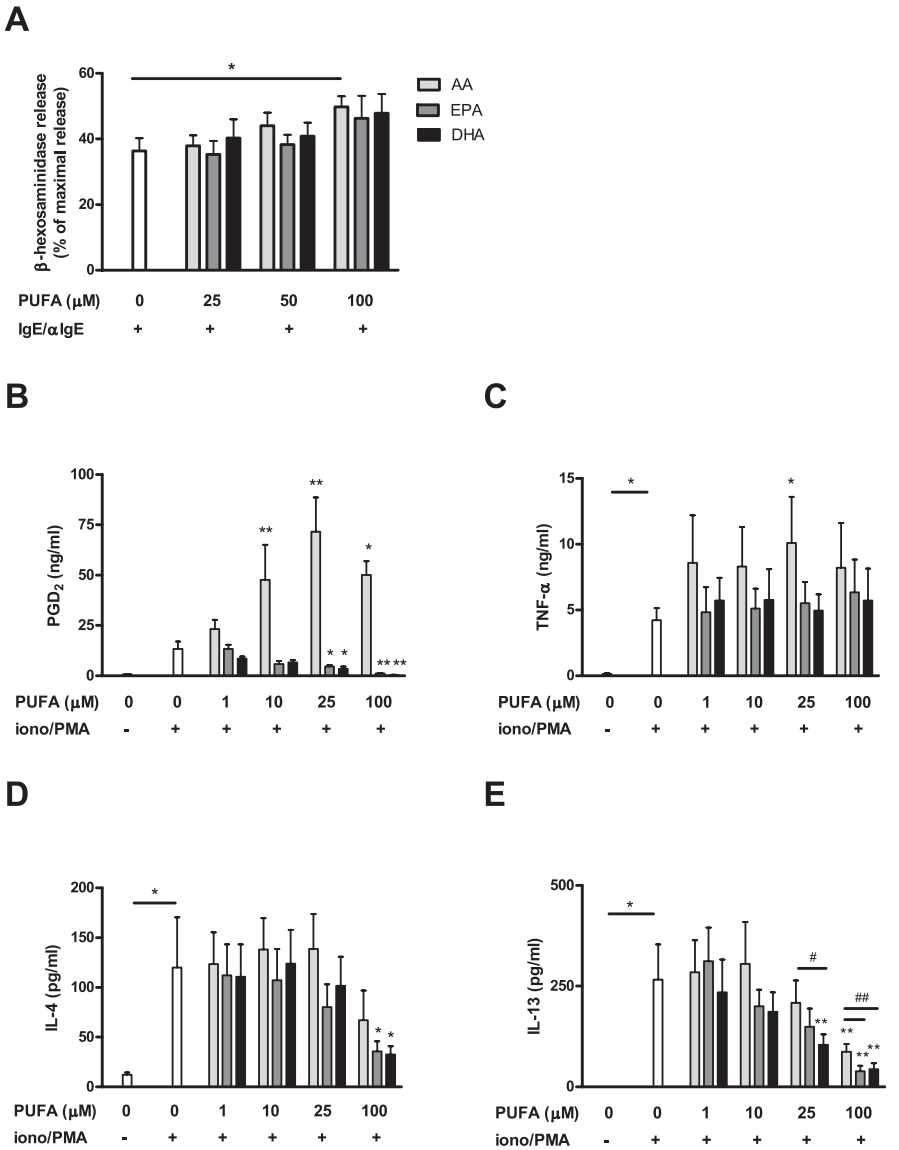

Figure 1 (A) IgE-mediated degranulation of LAD2 cells 1 hour after α-IgE stimulation incubated with different concentrations of arachidonic acid (AA), EPA and DHA. Degranulation was determined by the amount of β-hexosaminidase release as % of Triton X-100 (TX100) treated cells. Figure represents three independent experiments, average background release is subtracted. Effect of LCPUFA on ionomycin/phorbol 12-myristate 13-acetate (iono/PMA)-induced release of (B) prostaglandin (PG)D_2 after 30 minutes (n=3) (C) TNF-α after 4 hours (n=5) (D) IL-4 after 24 hours (n=6) and (E) IL-13 after 24 hours (n=6) by HMC-1. Light grey bars represent AA; dark grey is EPA and black is DHA. *$P<0.05$, **$P<0.01$, ***$P<0.001$ one-way ANOVA followed by Dunnett's test for multiple comparison to 0 (control); #$P<0.05$, ##$P<0.01$ one-way ANOVA followed by Bonferroni's multiple comparison test.

Modulation of mediator release of HMC-1 by AA, EPA or DHA

To determine the effects of modification of cell membrane fatty acid composition on cytokine production HMC-1 were used. To study dose-dependency of MC mediator release HMC-1 were incubated with 0, 1, 10, 25 and 100 μM AA, EPA or DHA because this was expected to be more sensitive to changes in membrane fatty acid composition than degranulation. As shown in Figure 1B, the production of PGD_2 increased dramatically in a dose-dependent manner after incubation with AA (13.4 ± 6.3 vs 71.6 ± 24.1 ng/ml, 25 μM AA, n=3, $^{**}P<0.01$), in contrast EPA and DHA inhibited PGD_2 production (13.4 ± 6.3 vs 3.5 ± 1.9 ng/ml, 25 μM DHA, n=3, $^{*}P<0.05$). AA (25 μM) was also found to enhance TNF-α secretion by HMC-1 (4.4 ± 1.0 vs 10.1 ± 3.5 ng/ml, n=5, $^{*}P<0.05$); no effects were observed for EPA and DHA «Figure 1C». IL-8 secretion was not affected by AA, EPA or DHA (data not shown). However, 24h pre-incubation with AA, EPA or DHA resulted in a decrease in ionomycin/PMA induced IL-4 and IL-13 release «Figure 1D, E». The effects of DHA on IL-13 production were most pronounced since DHA already reduced IL-13 secretion at 25 μM (300.1 ± 94.5 vs 104.6 ± 25.8 pg/ml, n=6, $^{**}P<0.01$). In addition, this was significantly lower than AA treated cells ($^{\#}P<0.05$). AA as well as EPA and DHA reduced IL-13 secretion at a concentration of 100 μM (AA, EPA, DHA 86.8 ± 19.7; 38.5 ± 13.7; 44.0 ± 14.6 pg/ml, n=6, $^{**}P<0.01$), however EPA and DHA treatment resulted in significantly lower IL-13 secretion than AA ($^{\#\#}P<0.01$). At the highest concentration used EPA and DHA were able to reduce IL-4 secretion (119.8 ± 50.6 vs 35.7 ± 10.2; 32.8 ± 8.1 pg/ml respectively, n=6, $^{*}P<0.05$). The solvent of LCPUFA (ethanol, 0.1% [v/v]) and ionomycin/PMA (0.1% v/v DMSO) did not affect mediator release (data not shown).

Preincubation for 30 min with indomethacin (general COX inhibitor; 10 μM) as well as NS398 (COX-2 inhibitor; 1 and 10 μM) effectively inhibited COX as PGD_2 release was blocked by more than 90% (data not shown). However, COX inhibitors did not affect TNF-α or IL-13 secretion, hence these cytokines are not regulated by a COX dependent mechanism (data not shown).

AA, EPA or DHA do not affect cell viability

AA, EPA or DHA (0, 1, 10, 25 and 100 μM) incubation up to 48h did not affect cell viability of stimulated HMC-1 as determined by WST-1 assay. Ionomycin (1 μM) and PMA (16 nM) stimulation for 24 hours slightly tended to reduce cell viability as compared to non-stimulated HMC-1 (data not shown). EPA tended to induce a slight increase in mitochondrial activity and the WST-1 signal was significantly higher with 100 μM AA (n=6, $P<0.05$). Cell viability was also studied by trypan blue exclusion test. Addition of AA, EPA or DHA did not induce cell death at the concentrations 25 and 100 μM (data not shown).

Generation of intracellular ROS and modulation by AA, EPA or DHA

ROS is known as second messenger and related to different inflammatory diseases (27). HMC-1 showed a slight increase in ROS generation upon ionomycin/PMA stimulation

«Figure 2A». HMC-1 cells have high basal ROS levels (MFI) and due to limitations in the sensitivity of the method used, the additional ROS production upon stimulation was not higher than ROS production in unstimulated cells «394.8 ± 95.3 vs 424.9 ± 53.7 MFI, not significant; Figure 2B». To study the effect of AA, EPA or DHA on ROS production in ionomycin/PMA stimulated HMC-1, cells were incubated with 25 µM and 100 µM based on differences between 25 and 100 µM in IL-4 and IL-13 secretion.

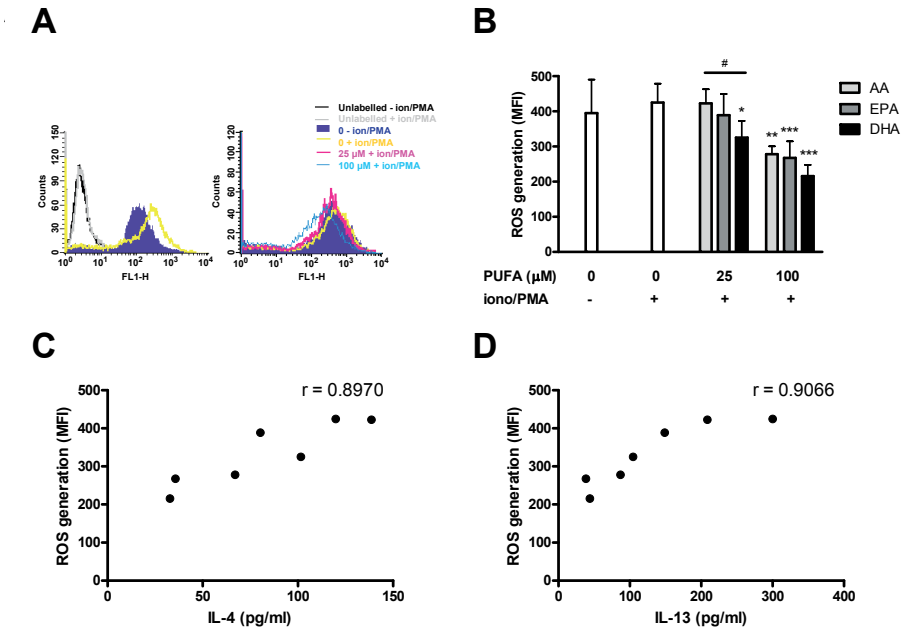

Figure 2 (A) The generation of reactive oxygen species (ROS) in HMC-1 increased by stimulation with ionomycin/phorbol 12-myristate 13-acetate (ion/PMA) and LCPUFA reduced ROS generation in stimulated HMC-1 in a dose-dependent manner (shift to left) as shown in this example for DHA (25, 100 µM) (B) ROS generation (n=3) by ionomycin/PMA stimulated HMC-1 after LCPUFA incubation in mean fluorescence intensity (MFI). Light grey bar represents arachidonic acid (AA); dark grey is EPA and black is DHA (C) ROS (mean of n=3 per data point) was found to correlate positively with IL-4 (mean of n=6; P=0.006, r=0.897 Pearson correlation coefficient) and (D) IL-13 (mean of n=6; P=0.005, r=0.907 Pearson correlation coefficient) secretion in HMC-1. *P<0.05, **P<0.01, ***P<0.001 one-way ANOVA followed by Dunnett's test for multiple comparison to 0 (control); $^{\#}P$<0.05 one-way ANOVA followed by Bonferroni's multiple comparison test.

Intracellular ROS generation is dose-dependently reduced by AA, EPA or DHA in stimulated HMC-1. This effect was most pronounced for DHA, which showed a significant reduction in ROS generation at 25 µM (325.1 ± 47.9 MFI, *P<0.05) when compared to basal ROS generation after stimulation. This also was significantly lower than AA treated cells ($^{\#}P$<0.05). At a concentration of 100 µM AA, EPA and DHA were all able to reduce ROS gen-

eration (AA: 278.2 ± 22.6 MFI **P<0.01, EPA: 267.6 ± 47.1 MFI ***P<0.001, DHA: 215.6 ± 32.1 MFI ***P<0.001). ROS generation after stimulation «mean of n=3 for each condition, used from Figure 2B» was found to correlate positively with IL-4 and IL-13 secretion «mean of n=5/6 for each condition, used from Figure 1D or 1E respectively» in HMC-1 (P=0.006, r=0.897 and P=0.005, r=0.907 respectively) «Figure 2C, D».

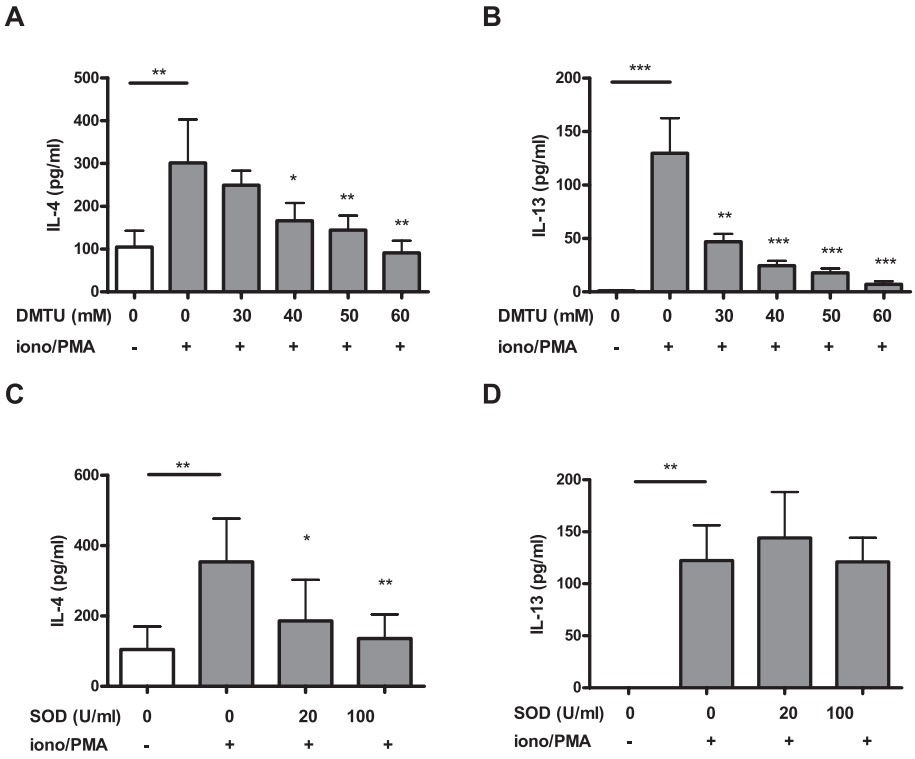

Figure 3 Effect of incubation with general reactive oxygen species (ROS) inhibitor 1,3-dimethyl-2-thiourea (DMTU) on (A) IL-4 secretion and (B) IL-13 secretion upon ionomycin/phorbol 12-myristate 13-acetate (iono/PMA) stimulation. Contribution of superoxide on (C) IL-4 and (D) IL-13 release was assessed by determining the effect of specific ROS inhibitor superoxide dismutase (SOD). *P<0.05, **P<0.01, ***P<0.001 one-way ANOVA followed by Dunnett's test for multiple comparison to 0 (control).

Effect of ROS inhibition on IL-4 and IL-13 secretion

Since AA, EPA or DHA were able to suppress ROS and IL-4 and IL-13 secretion it was assessed whether these allergy-related cytokines are under regulation of ROS in HMC-1. Preincubation with DMTU at the highest concentrations (40-60 mM) resulted in a decrease in IL-4 secretion of ionomycin plus PMA stimulated HMC-1 (354.1 ± 122.7 vs 100.4 ± 55.2 pg/ml, 60 mM, **P<0.01) «Figure 3A».

IL-13 release was dramatically and dose-dependently decreased by DMTU at all dosage (129.7 ± 32.8 vs 7.1 ± 2.9 pg/ml, 60 mM, ***P<0.001) «Figure 3B». Addition of SOD only resulted in reduction of secretion of IL-4 (136.0 ± 68.8 pg/ml, 100 U/ml, **P<0.01), but not of IL-13 «Figure 3C,D». Incubation with ROS inhibitors did not reduce mitochondrial activity or increase cell death as shown by WST-1 assay and trypan blue exclusion respectively (data not shown).

Effects of MAP kinases and NF-κB inhibitors on IL-13 release

ROS has been described to operate upstream in the signaling cascade. MAPK and NF-κB are known to contribute to cytokine secretion by MC. To determine the involvement of MAPK and NF-κB signaling in the secretion of IL-13 and the possible effects of LCPUFA on these pathways, we examined the effects of ERK inhibitor PD98059, p38 inhibitor SB203580, JNK inhibitor SP600125 and NF-κB inhibitor Bay117082 on IL-13 release by HMC-1 in absence or presence of pre-incubation with 25 μM of LCPUFA since at this concentration differences between AA vs EPA or DHA on suppression of IL-13 secretion were most pronounced. Solvents did not have an effect on mediator release (data not shown).

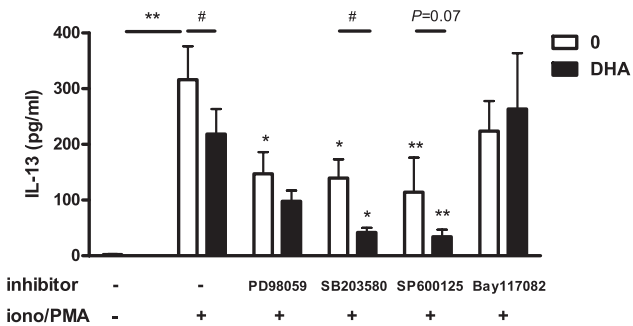

Figure 4 Effect of ERK inhibitor PD98059 50 μM; p38 inhibitor SB203580 20 μM; JNK inhibitor SP600125 10 μM and NF-κB inhibitor Bay117082 10 μM in presence or absence of DHA (black bars) on ionomycin/phorbol 12-myristate 13-acetate (iono/PMA) induced IL-13 release by HMC-1. *P<0.05, **P<0.01 one-way ANOVA followed by Dunnett's test for multiple comparison to 0 (control); #P<0.05 paired Student's t test.

25 μM DHA reduced ionomycin/PMA-induced IL-13 secretion by HMC-1 by 30% (316.1 ± 60.2 vs 218.6 ± 44.8 pg/ml, #P<0.05) «Figure 4». SB203580 20 μM, SP600125 10 μM and PD98059 50 μM inhibited IL-13 secretion by more than 50% (139.6 ± 33.6, 114.2 ± 61.9 and 147.3 ± 38.9 pg/ml respectively). Higher concentrations p38 or JNK inhibitor were even more effective in suppression of IL-13 secretion, (SB203580 50 μM: 60.9 ± 15.1 pg/ml and SP600125 20 μM: 27.0 ± 23.9 pg/ml), while the ERK inhibitor was not (PD98059 100 μM: 200.0 ± 56.3 pg/ml). In contrast Bay117082 10 μM did not inhibit IL-13 release whereas 30 μM strongly reduced cell viability. Simultaneous treatment of MAPK or NF-κB inhibitors with DHA induced a further inhibition in IL-13 release using suboptimal SB203580 and SP600125

incubations (41.7 ± 8.3 and 34.2 ±12.7 pg/ml, $^{\#}P<0.05$ and $P=0.07$ respectively). 25 μM EPA was able to further reduce IL-13 release in combination with SB203580 as compared to the inhibitor alone (67.7 ± 10.8 pg/ml, $^{\#}P<0.05$) while AA did not add to the effect of MAPK nor NF-κB inhibitors used (data not shown). MAPK and NF-κB inhibitors did not enhance cell death in HMC-1. Trypan blue exclusion showed that at the end of the experiment cell viability was >80% (data not shown).

DISCUSSION

Upon activation MC initiate and maintain allergic inflammation due to the release of various inflammatory mediators. MC-derived mediators like PGD_2, TNF-α, IL-4 and IL-13 increase the susceptibility to develop allergic disease and enhance allergic symptoms (6-10). The current study shows differential effects of n-6 LCPUFA AA versus n-3 LCPUFA EPA or DHA on mast cell phenotype.

24 Hours of incubation with AA prior to mast cell activation enhances IgE-mediated MC degranulation in LAD2 cells, the only human analogue that can degranulate in an IgE-dependent manner. Teshima *et al.* found AA and other n-6 PUFA to increase degranulation in rat basophilic leukemia (RBL-2H3) cells, while n-3 PUFA including EPA had no effect (35). Nakano and colleagues reported that AA significantly increased β-hexosaminidase release upon IgE-antigen stimulation and EPA showed the same tendency (34). An increase in the content of PUFA in membrane phospholipids is accompanied by an increase in membrane fluidity (decrease in microviscosity) (36, 37). MC degranulation may occur more easily when the membrane is more fluid which may explain the significant increase in degranulation after AA incubation. The same trend was shown for EPA and DHA. In addition to membrane fluidity LCPUFA may affect events in signal transduction and MC mediator release. IgE-mediated MC activation involves recruitment of tyrosine kinase Linker for activation of T cells (LAT) and Syk as well as calcium mobilization (38). Nakano *et al.* have shown that supplementation of RBL-2H3 cells with AA or EPA augmented activation of LAT and Syk as compared to control cells (34). In addition, AA supplemented cells increased the intracellular calcium concentration (34, 35).

Besides LAD2 cells, HMC-1 is often used as a human MC line to circumvent costly isolation procedures for human tissue MC. They lack a functional IgE receptor but can be cultured in large quantities and produce sufficient amounts of mediators for analysis. To simulate IgE receptor signaling, the cells are stimulated by ionomycin (calcium ionophore) and PMA (activating PKC). FcεRI signaling in MC also leads to simultaneous activation of calcium and PKC, by IP3 and DAG respectively (39). Hence, similar downstream signaling pathways are activated and these pathways act synergistically to provide exocytose.

Supplementation of LCPUFA to HMC-1 or LAD2 cells readily resulted in effective AA, EPA or DHA membrane incorporation in a dose-dependent manner. The LCPUFA incorporation

seems to be slightly less efficient in LAD2 cells as compared to HMC-1, which may be the result of serum-free culturing of these cells. In both MC lines EPA and DHA incorporate at the cost of AA and vice versa which results in changes in membrane n-6/n-3 LCPUFA and EPA/DHA ratio's due to alterations in membrane composition. In addition 18:1n-9, followed by 18:0 and 16:0 were exchanged for the supplemented AA, EPA or DHA (data not shown) enabling efficient incorporation of high amounts of LCPUFA having implications for the biological function of MC.

Differential effects of n-3 versus n-6 LCPUFA on cytokine secretion by HMC-1 were demonstrated in this study. The n-6 LCPUFA AA increased TNF-α and PGD_2 secretion by HMC-1, while n-3 LCPUFA EPA and DHA dose-dependently reduced PGD_2 release and were most effective in suppressing allergy-driving IL-4 and IL-13 secretion. These MC mediators are important in the initiation and persistence of the allergic response (6-10). Besides their role in allergic disease, MC and their products can regulate the adaptive (acquired) immune response via effects on maturation, function and migration of B cells, T cells and dendritic cells (DC) (3, 40).

The pro-inflammatory effect of AA is demonstrated clearly by the dramatic induction of PGD_2 release. In contrast to AA, EPA and DHA reduced PGD_2 secretion by activated HMC-1. EPA has been shown to reduce IgE-mediated PGD_2 generation by cultured human MC as well (41). PGD_2 is the main prostanoid secreted by activated MC and associated with allergic diseases. It can decrease IL-12 secretion by DC and promote Th2 polarization (42, 43). Furthermore, PGD_2 is important in MC dependent activation of Th2 lymphocytes, eosinophils and basophils via CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) (7). Recently, CRTH2 antagonists have shown to be promising in the treatment of asthma and related disorders. A randomized, double-blind comparison of a CRTH2 antagonist and placebo in a population with moderate persistent asthma showed beneficial effects of the CRTH2 antagonist, including an improvement in lung function and asthma symptoms (44). These results provide evidence for an important role for PGD_2 in asthma and other allergic disorders, which implicate a beneficial effect of providing EPA and DHA reducing PGD_2 generation in these patients.

Nakano *et al.* (2005) reported that AA dose-dependently augmented TNF-α release using RBL-2H3 cells, similar to what was shown in these experiments in human MC. MC-derived TNF-α has recently been incriminated to worsen allergic symptoms via the induction of adhesion molecules, enabling influx of inflammatory cells resulting in e.g. airway inflammation and development of airway hyperresponsiveness (6). However, studies using anti-TNF-α treatment were not consistent, showing marked heterogeneity in responses (45, 46). This makes the involvement of TNF-α dependent pathways in LCPUFA effects less likely.

Our study showed that EPA and DHA, in particular DHA, most effectively inhibit IL-4 and IL-13 secretion from human MC. This has not been reported previously. AA was also able

to reduce IL-13 secretion but less effective than EPA or DHA, while enhancing TNF-α and PGD_2 secretion. IL-13 is produced by Th2 cells, MC, eosinophils and basophils and is critical in induction and persistence of allergic disease. In allergic asthma IL-13 is required for the induction of clinical symptoms (8-10). Furthermore, cytokines including IL-4 and IL-13 affect B cell development and induce IgE isotype switching (47). Recent studies reporting about fish oil supplementation during pregnancy showed inhibition of IL-13 release by neonatal mononuclear cells in response to allergens as well as reduced levels of IL-13 in cord blood plasma (19, 48).

LCPUFA are known to affect signal transduction cascades leading to the transcription and production of cytokines. A variety of tissues and cells, including MC, produce ROS such as superoxide and hydrogen peroxide upon stimulation which are upstream regulators of signal transduction pathways (27, 30). Although the effect of ionomycin/PMA stimulation on ROS production by HMC-1 was small in our experiments, the reduction in ROS generation after LCPUFA supplementation appeared to be specific for activated MC. Furthermore, within these experiments IL-13 and IL-4 secretion by HMC-1 after stimulation was found to correlate positively with amount of ROS. This is in agreement with a study in bone marrow-derived MC in which IL-4 and IL-13 secretion after IgE-mediated activation was associated with increased ROS generation (49). Unfortunately no sensitive method for measuring intracellular ROS generation upon cell activation is currently available, while only minor changes in ROS upon activation are required to activate intracellular signaling cascades (28). Similar to our *in vitro* results fish oil has been shown to decrease ROS production in several animal studies and in healthy human volunteers (50-54). By contrast, other animal studies have reported contradictory results for ROS production in macrophages after fish oil supplementation (55, 56). Studies assessing the impact of LCPUFA on ROS production in MC are rare. Nakano *et al.* found that AA and EPA enhance ROS production in stimulated RBL-2H3 cells (34). Canine mastocytoma cell line C2 cells were incubated with 18:2n-6 (linoleic acid, LA), AA, 18:3n-3 (α-linolenic acid) and EPA which all increased ROS production. However, this was possibly due to lipid peroxidation since antioxidant supplementation resulted in a lower increase in ROS production (57). LCPUFA in general are oxidized easily because of their high degree of unsaturation and thereby form oxygen radicals in many cell types (27, 58). In our experiments in HMC-1 the antioxidants L(+) ascorbic acid and α-tocopherol were used, which act as free radical scavengers and protect LCPUFA from harmful lipid peroxidation and neutralize the free radicals formed. This may explain some of the discrepancies observed in the effects of LCPUFA on ROS generation in *in vitro* studies. As suggested by others the number of double bonds present may be important in the anti-inflammatory effects generated by LCPUFA. It may explain the most potent inhibition of ROS generation by the fatty acid with the highest degree of unsaturation, namely DHA. This possibly results in inhibition of IL-4 and IL-13 secretion by AA < EPA < DHA (59). Our results implicate that EPA and DHA

act via similar mechanisms in suppression of IL-4 and IL-13 secretion from MC, DHA just showed to be slightly more effective than EPA. Probably the anti-allergic effects of AA are overruled since AA also enhances pro-inflammatory PGD_2 and TNF-α secretion. Indeed, it has been shown that high maternal intake of margarine and vegetable oils rich in n-6 PUFA during the last four weeks of pregnancy is associated with enhanced occurrence of atopic eczema in offspring (60), by contrast fish oil supplementation during pregnancy and lactation reduces the susceptibility to develop allergic disease in the neonates (18-23).

Use of ROS inhibitors confirmed the involvement of ROS in the IL-4 and IL-13 secretion pathway in MC. ROS consists of a number of different mediators and although both IL-4 and IL-13 secretion could be blocked using general ROS inhibitor DMTU, use of SOD showed that IL-4 but not IL-13 secretion is under regulation of superoxide. LCPUFA may be less able to affect this superoxide cascade in relation to other ROS mediators since IL-13 was suppressed more effective by n-3 LCPUFA than IL-4. ROS generation by MC can contribute to secretion of inflammatory cytokines via NF-κB and/or MAP kinase signaling (61). Previous studies have shown that IL-13 secretion by RBL-2H3 cells is regulated by JNK and p38 (62). However, as revealed using inhibitors of these pathways MAPK ERK, p38 and JNK were all involved in IL-13 secretion by activated HMC-1, while NF-κB inhibitor Bay117082 did not reduce IL-13 release. This suggests ROS generation to be upstream of the MAPK signaling cascade.

Recently a study with anti-IL-4/IL-13 demonstrated an improvement in asthma endpoints in patients with severe, uncontrolled asthma (63), suggesting a possible role for dietary supplementation with n-3 LCPUFA EPA and DHA in allergic disease. However, even though some studies report modest improvement of atopic dermatitis, there is no convincing evidence yet for dietary n-3 LCPUFA supplementation alone for the treatment of those with established atopic disease (64, 65). Thus, although AA, EPA and DHA suppress allergy-related mediator release by MC, the effects are moderate and may not be strong enough for treatment purposes. However, when MC were treated with suboptimal doses of DHA and MAPK inhibitors, the suppression of IL-13 secretion by p38 inhibitor SB203580 (20 μM) and JNK inhibitor SP600125 (10 μM), but not ERK inhibitor PD98059 nor NF-κB inhibitor Bay117082, was further supported by DHA. Addition of DHA to p38 and JNK inhibitors was as effective as the higher inhibitor doses tested (50 μM and 20 μM respectively). In addition to DHA, EPA was able to support SB203580 in suppression of IL-13 secretion, while AA did not enhance the efficacy of any of the inhibitors. Hence, combination of n-3 LCPUFA with other drugs seems promising in reducing allergic type mediator release of MC. EPA and more prominently DHA, but not AA, added to the inhibitory effect of MAPK inhibitors on IL-13 secretion. Dietary n-3 LCPUFA may therefore be able to optimize efficacy and/or safety of novel strategies to treat allergies using drugs aiming to suppress IL-4, IL-13 and/or PGD_2.

In conclusion, the n-6 LCPUFA AA promotes the allergic cascade by enhancing degranu-

lation and TNF-α and PGD_2 secretion by activated MC. In contrast n-3 LCPUFA EPA and DHA suppress PGD_2, IL-13 and IL-4 secretion as well as ROS generation most effectively. Hence LCPUFA differentially modulate the MC phenotype. MC are involved in the initiation and perpetuation of allergic disease and suppression of allergy-related mediators by dietary n-3 LCPUFA may contribute to the reduced susceptibility to develop or sustain allergic disease.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1 Fatty acid composition of fetal bovine serum (relative %)

Fatty acid	relative %	Fatty acid	relative %
6:0	0.00	18:2n-6	4.69
8:0	0.00	18:3n-6	0.07
10:0	0.00	20:2n-6	0.59
12:0	0.00	20:3n-6	1.95
14:0	1.29	20:4n-6 (AA)	6.24
15:0	0.88	22:4n-6	0.85
16:0	23.96	22:5n-6	0.03
17:0	1.08	24:2n-6	0.08
18:0	12.55	18:3n-3	0.47
20:0	0.43	18:4n-3	0.32
22:0	1.41	20:3n-3	0.19
23:0	0.10	20:5n-3	0.86
24:0	1.12	22:3n-3	0.12
16:1n-7	2.97	22:5n-3 (EPA)	2.81
18:1n-9	20.63	22:6n-3 (DHA)	4.09
18:1n-7	6.41	16:1n-7tr	0.29
20:1n-9	0.38	18:1tr	0.41
20:3n-9	0.09	14:1n-x	0.06
22:1n-9	0.07	16:0dma	0.50
24:1n-9	1.84	22:2n-x	0.16

CHAPTER FIVE

Dietary long chain n-3 polyunsaturated fatty acids prevent allergic sensitization to cow's milk protein in mice

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The editorial 'Fishing for allergy prevention' by Prof. P.C. Calder in the same issue pp 700-702 discusses the findings of this paper

ABSTRACT

Background Cow's milk allergy is one of the most common food allergies in children and no treatment is available. Dietary lipid composition may affect the susceptibility to develop allergic disease. Aim of this study was to assess whether dietary supplementation with long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) prevents the establishment of food allergy.

Methods Mice were fed a control or fish oil diet before and during oral sensitization with whey. Acute allergic skin response, serum immunoglobulins as well as dendritic cell (DC) and T cell subsets in mesenteric lymph nodes (MLN), spleen and/or small intestine were assessed.

Results The acute allergic skin response was reduced by more than 50% in sensitized mice fed the fish oil diet compared to the control diet. In addition, anti-whey-IgE and -IgG1 levels were decreased in the fish oil group. Serum transfer confirmed that the Th2 type humoral response was suppressed since sera of fish oil fed sensitized mice had a diminished capacity to induce an allergic effector response in naive recipient mice compared to control sera. Furthermore, the acute skin response was diminished upon passive sensitization in fish oil fed naive recipient mice. In addition, the percentage of activated Th1 cells was reduced by fish oil in spleen and MLN of sham mice. The percentage of activated Th2 cells was reduced in both sham- and whey-sensitized mice. By contrast, whey-sensitized mice showed an increased percentage $CD11b^{+}CD103^{+}CD8\alpha^{-}$ DC in MLN in association with enhanced $FoxP3^{+}$ regulatory T cells (Treg) in spleen and intestine of fish oil fed whey-sensitized mice compared to sham mice.

Conclusion Dietary n-3 LCPUFA largely prevented allergic sensitization in a murine model for cow's milk allergy by suppressing the humoral response, enhancing local intestinal and systemic Treg and reducing acute allergic symptoms, suggesting future applications for the primary prevention of food allergy.

INTRODUCTION

Epidemiological research has demonstrated a rapid increase in allergic diseases. In developed countries these affect between 15% and 30% of the population (1). Allergic sensitization to food proteins occurs in about 6% of children and 3-4% of the adult population in westernized countries (2). Cow's milk is introduced in a child's diet early in life, making cow's milk allergy one of the most common food allergies in early childhood with a prevalence of 0.3-3.5% amongst children under five years of age (3, 4). Major allergens characterized in cow's milk are distributed among the casein and whey fractions and include α_{s1}-casein and β-lactoglobulin respectively (5). IgE mediated allergy accounts for about 60% of the hypersensitivity reactions against these proteins in milk (6). Symptoms involve the skin, gastrointestinal and respiratory tract and even systemic anaphylaxis may occur (7). There is no cure for food allergies so far, the only possibility is to avoid the culprit food allergen, however this might affect children's growth and health (7). Although approximately 90% of children outgrow the disease, most of them are predisposed to the development of other food or inhalant allergies (4, 8). Therefore there is a major interest in interventions to prevent or treat this condition.

The increase in prevalence of atopic disease is associated with a reduction in the consumption of long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) from fatty fish – generally considered to be anti-inflammatory - paralleled by an increase in the intake of margarine and vegetable oils containing high levels of n-6 PUFA (9-11). Current developments in Japan provide additional evidence since the prevalence of atopic diseases is rising in parallel with a reduced ratio of n-3 to n-6 PUFA in the diet (12, 13). Furthermore, it was observed that reduced n-3 LCPUFA serum levels were associated with allergic disease in Swedish adolescents (14). In Finland atopic disease prevalence is low in Eastern provinces where high percentages of n-3 LCPUFA, especially of eicosapentanoic acid (EPA), and low percentages of n-6 PUFA are found in serum (9, 15, 16). In addition, it has been demonstrated that increased fish oil consumption by pregnant and lactating woman can reduce the incidence of food allergy and IgE-associated eczema by more than 60% in children at risk (17).

Therefore fish oil, rich in n-3 LCPUFA EPA and docosahexaenoic acid (DHA), may have a role in the prevention of allergic diseases. In this study it is assessed whether dietary supplementation with fish oil starting early in life, can reduce allergic sensitization in a murine model for orally induced cow's milk allergy.

MATERIALS AND METHODS

Diets

Semi purified cow's milk protein-free AIN-93G-based diets were composed with either 10% soybean oil (control diet) or 4% soybean oil plus 6% tuna oil (fish oil diet) at Research Diet Services (Wijk bij Duurstede, The Netherlands) «Table 1 and 2» (18). The ratio n-3:n-6 PUFA was 1:9.5 for the control diet while this ratio was reduced to 1:1 for the fish oil diet. Tuna oil was a kind gift from Bioriginal, Den Bommel, The Netherlands. The fat percentage of AIN-93G was adapted and extra fat was exchanged for cornstarch. The diets were stored at -20°C prior to use to prevent fatty acid oxidation.

Table 1 Dietary composition mouse chow (based on AIN-93G)

	Control diet (g/kg)	Fish oil diet (g/kg)
Carbohydrates		
Cornstarch	367.5	367.5
Dextrinized cornstarch	132.0	132.0
Sucrose	100.0	100.0
Cellulose	50.0	50.0
Protein		
Soya	200.0	200.0
Methionine	3.0	3.0
Fat		
Soybean oil	100.0	40.0
Tuna oil	0.0	60.0
Others		
Mineral mix AIN 93G	35.0	35.0
Vitamin mix AIN 93VX	10.0	10.0
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.014	0.014

Table 2 Fatty acid composition lipid source

Fatty acid	Soybean oil (%)	Tuna oil (%)
SFA	15.1	28.9
MUFA	24.9	22.8
PUFA	59.1	44.5
n-6 PUFA	53.1	5.5
C18:2 n-6 LA	53.1	1.3
C20:4 n-6 AA		1.8
C22:5 n-6		1.6
n-3 PUFA	5.6	38.5
C18:3 n-3 ALA	5.6	0.5
C20:5 n-3 EPA		7.0
C22:5 n-3 DPA		1.4
C22:6 n-3 DHA		27.8
Minor components	0.9	3.8

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LA: linoleic acid; AA: arachidonic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DPA; docosapentaenoic acid; DHA: docosahexaenoic acid.

Oral sensitization and challenge of mice

Three-week-old specific pathogen free female C3H/HeOuJ mice (n=6/group) were purchased from Charles River Laboratories (L'Arbresle Cedex, France) and fed either the control or fish oil diet starting two weeks prior to the first sensitization and continued during the whole sensitization period until sacrifice «Figure 1». The mice were housed in the animal facility at Utrecht University. Animal care and use were approved by and performed in accordance with the guidelines of the Animal Ethics Committee of Utrecht University.

Mice were sensitized intragastrically (i.g.) using a blunt needle with 20 mg whey (DMV International, Veghel, The Netherlands) in 0.5 mL PBS with 10 µg cholera toxin (CT, List Biological Laboratories Inc, Campbell, CA, USA) as an adjuvant. Sham mice received CT (10 µg/0.5 mL PBS). Mice were sensitized once a week for 5 consecutive weeks as previously described by Schouten *et al.* (19). One week after the last sensitization sham and whey-sensitized mice were challenged intradermally (i.d.) in the ear pinnae of both ears with 10 µg whey in 20 µl PBS to determine the acute allergic skin response (see below). The same day the mice were challenged i.g. with 50 mg whey/0.5 mL PBS and 18 hours (h) after the oral challenge blood samples were collected and centrifuged at 14,000*g* for 15 min. Sera were stored at -70°C. Mice were sacrificed by cervical dislocation and mesenteric lymph nodes (MLN), spleen and small intestine were obtained for flow cytometry or histological examination.

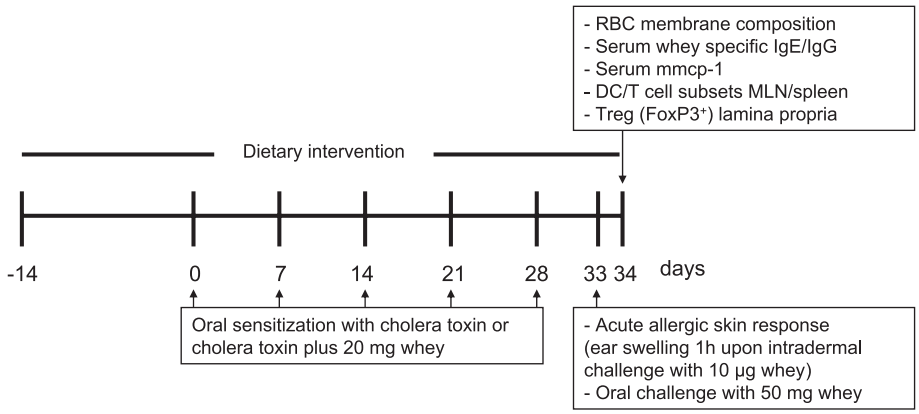

Figure 1 A schematic overview of the sensitization and challenge protocol and the parameters that are analyzed. RBC: red blood cell; mmcp-1: mouse mast cell protease-1; DC: dendritic cell; MLN: mesenteric lymph nodes; Treg: regulatory T cell.

Fatty acid composition erythrocytes

Blood was collected in heparin tubes and after centrifugation plasma was removed. Erythrocytes were stored at -70°C until analysis. Erythrocyte lipids were extracted as described by Bligh and Dyer (20) and the membrane fatty acid composition was assessed using gas chromatography as previously described (21).

Acute allergic skin response

Ear thickness was measured in duplicate for each ear using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands) at basal conditions prior to whey challenge and 1 h after i.d. challenge in the ear. Isoflurane was used for inhalational anesthesia during measurements. The mean basal thickness per ear was subtracted from the mean thickness measured at 1 h after injection to express ear swelling as delta µm. Mean of left and right delta ear swelling were calculated for each mouse.

Plasma PGE_2 and serum immunoglobulins and mmcp-1

Concentrations of prostaglandin E_2 (PGE_2) in plasma and mouse mast cell protease-1 (mmcp-1) in serum were determined using commercially available ELISA kits (PGE_2: Oxford Biomedial Research, Oxford, USA; mmcp1: Moredun Scientific Ltd., Penicuik, UK) according to the manufacturer's protocol.

Levels of whey-specific IgE, IgG1 and IgG2a were determined in serum by ELISA. Microlon plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 100 µl whey (20 µg/mL) in carbonate-bicarbonate buffer (0.05M, pH = 9.6) for 18 h at 4°C. Plates were washed and blocked for 1 h with 0.5% BSA in ELISA buffer (50mM TRIS, 137 mM NaCl, 2 mM EDTA and 0.05% Tween20). Serum samples were incubated for 2 h at

room temperature after which plates were washed and incubated with biotin-labeled rat anti-mouse IgE, IgG1 or IgG2a (1 µg/mL, BD Biosciences, Alphen aan den Rijn, The Netherlands) for 90 min at room temperature. After washing the plates were incubated with streptavidin poly horseradish peroxidase (Sanquin, Amsterdam, The Netherlands) for 1 h, washed and developed with o-phenylendiamine (Sigma-Aldrich-Chemicals, Zwijndrecht, The Netherlands). The reaction was stopped with 4 M H_2SO_4 and absorbance was measured at 490 nm on a Benchmark microplate reader (Bio-rad, Hercules, Calif, USA). Results were expressed as arbitrary units (AU) composed using a titration curve of pooled sera from whey alum-i.p. immunized mice as positive reference serum serving as an internal standard.

Passive sensitization: transfer of dietary intervention serum

Sera obtained from sham and whey-sensitized mice fed the control or fish oil diet were transferred intravenously (i.v. 100 µl) to naive recipient mice fed control diet using isoflurane anesthesia (n=6/group). Half an hour after serum transfer the acute skin response was measured as described before, starting with measuring ear thickness at basal conditions prior to whey challenge followed by assessing ear swelling 1 h after i.d. challenge.

Passive sensitization: transfer of hyperimmune serum to fish oil fed recipients

Pooled ('hyperimmune') sera from whey alum-i.p. immunized mice were i.v. transferred (100 µl) to naive mice fed the control diet or fish oil for two weeks prior to injection (n=6/group). After half an hour the acute skin response was measured as described before.

Dendritic cell (DC) and T cell subsets by flow cytometry

After sacrifice MLN were removed and after homogenization incubated with 0.2% collagenase IV and 2 kU/mL DNAse for 30 min at 37°C, resuspended and incubated for another 30 min. The enzymatic reaction was stopped by adding 0.5 mL FCS after which the cell suspension was filtered. In addition, single cell suspensions were prepared from mouse spleens. All cells were blocked for 20 min in PBS containing 1% BSA and 5% FCS. $5x10^5$ Cells were plated per well and incubated for 30 min at 4°C with different antibodies (eBioscience, Breda, The Netherlands, unless otherwise stated) anti-CD11c-PerCpCy5.5 (1:50), anti-CD11b-PE (1:50), anti-CD8α-APC or FITC (1:100), anti-CD103-APC (1:100), anti-CD4-FITC or PerCpCy5.5 (1:100), anti-CD25-PE (1:100), anti-CD69-APC (1:50), anti-CXCR3-PE (1:50), anti-T1/ST2-FITC (1:50, MD Biosciences, St. Paul, Minnesota, USA) and isotype controls were used. Cells were fixed using 0.5% paraformaldehyde, or permeabilized for intracellular staining for FoxP3-APC (1:50) using the FoxP3 staining buffer set (eBioscience) according to the manufacturer's protocol. Flow cytometry was performed using FACS Canto (BD, Alphen aan den Rijn, The Netherlands) and analyzed using FACSDiva software (BD).

Immunohistochemistry FoxP3$^+$ cells

The small intestine (jejunum and proximal ileum) was carefully dissected, opened longitudinally and luminal contents were removed by gently washing in PBS. The intestine was placed with the mucosal side down and rolled from the distal to the proximal end. The Swiss rolls were fixed in formalin (10% v/v solution) and embedded in paraffin (Leica IG1150C; Leica Microsystems, Rijswijk, The Netherlands). Sections (5 µm) were cut using a microtome (Leica Microsystems) and deparaffinized Swiss roll sections were stained for intracellular FoxP3 expression. In short, after dewaxing the sections were boiled in sodium citrate buffer (0.01 M) for 10 min. Then sections were blocked with 5% rabbit serum (Dako, Heverlee, Belgium) in PBS with 1% BSA, followed by overnight incubation at 4°C with rat anti-mouse FoxP3 purified antibody (final concentration 2.5 µg/mL, eBioscience) or rat IgG2a isotype (2.5 µg/mL) as control in 1% BSA/PBS. Then slides were incubated for 30 min with 3% H_2O_2 in PBS. The primary antibody was detected with a biotinylated rabbit anti-rat antibody (2.5 µg/mL in 1% BSA/PBS, Dako) for 1 h at room temperature, followed by incubation with avidin biotin complex (ABC, Vector) in 1% BSA/PBS for 1 h. Color was developed with 3,3'-diaminobenzidine (Sigma) and sections were counterstained with hematoxylin, dehydrated and covered with Pertex mounting medium (Histolab, Göteborg, Sweden) and a cover glass. FoxP3$^+$ cells were counted per villus-crypt unit. Per mouse 100 intact villus-crypt units were counted on a slide.

Statistics

All data were analyzed using two-way ANOVA (factors diet and sensitization) or one-way ANOVA and post hoc Bonferroni test using GraphPad Prism software (version 5.0) and SPSS version 15 software. The hypothesis that the effect of sensitization would differ between the control and fish oil groups was tested by the interaction term between sensitization and diet in the two-way ANOVA. Therefore in the legends the *P*-value for diet; sensitization and diet x sensitization interaction are reported. For serological data square root transformation was used before analysis to normalize data distribution. Data are presented as mean ± SEM. For serological data Tukey box-and-whisker plots were used, represented as median [25% - 75% percentiles] in text.

RESULTS

Increased DHA and EPA membrane content upon fish oil supplementation

Fish oil supplementation more than doubled DHA content of red blood cell membranes as compared to control diet fed mice in both sham and whey-sensitized mice ($P<0.001$, «Figure 2A»). Also the percentage of EPA in the membranes did increase after fish oil treatment ($P<0.001$, «Figure 2B»). The incorporation of n-3 LCPUFA in the cell membrane

«occurred mainly at the expense of n-6 PUFA arachidonic acid (20:4n-6) ($P<0.001$, «Figure 2C») and linoleic acid (18:2n-6) (whey mice 16.3 ± 1.1 vs 9.6 ± 0.4%, $P<0.001$) which were reduced in the erythrocyte membranes. In addition, the concentration of PGE_2 was significantly reduced in whey-sensitized mice fed the fish oil diet as compared to control diet fed mice ($P<0.05$, «Figure 2D»).

Fish oil diet reduces acute allergic skin response in whey-sensitized mice

One hour after the dermal challenge the acute allergic skin response was measured to compare the effect of the control and fish oil diet on ear swelling. The delta ear swelling in the whey-sensitized mice fed control diet was significantly enhanced as compared to sham-sensitized mice ($P<0.001$, «Figure 2E»). Whey-sensitized mice fed fish oil diet showed a more than 50% reduced acute allergic skin response ($P<0.001$) compared to control diet fed whey-sensitized mice.

Reduced whey-specific IgE and IgG1 levels in whey-sensitized mice fed fish oil diet

Serum levels of whey-specific IgE were increased in mice orally sensitized with whey compared to sham mice ($P<0.001$, «Figure 3A»). The fish oil diet reduced whey-IgE levels in whey-sensitized mice compared to the control fed mice ($P<0.05$). Whey-specific IgG1 levels of whey-sensitized mice were enhanced compared to sham-sensitized mice ($P<0.001$, «Figure 3B»). Fish oil prevented the generation of Th2 type whey-IgG1 and levels were significantly reduced compared to control diet fed whey-sensitized mice ($P<0.01$). In addition, Th1 type immunoglobulin IgG2a showed the same pattern. Sham sensitized mice showed lower IgG2a levels than whey sensitized mice fed control diet ($P<0.05$, «Figure 3C»), the fish oil diet reduced IgG2a in whey sensitized mice compared to whey sensitized mice fed control diet ($P<0.05$).

To assess mucosal mast cell degranulation, mmcp-1 was determined in serum 18 h after oral challenge with whey. Serum mmcp-1 concentrations of whey-sensitized mice fed control diet were enhanced when compared to sham treated mice ($P<0.05$, «Figure 3D»). mmcp-1 was not enhanced in whey-sensitized mice fed fish oil diet when compared to sham mice. However, fish oil diet did not significantly reduce mmcp-1 in serum compared to control diet.

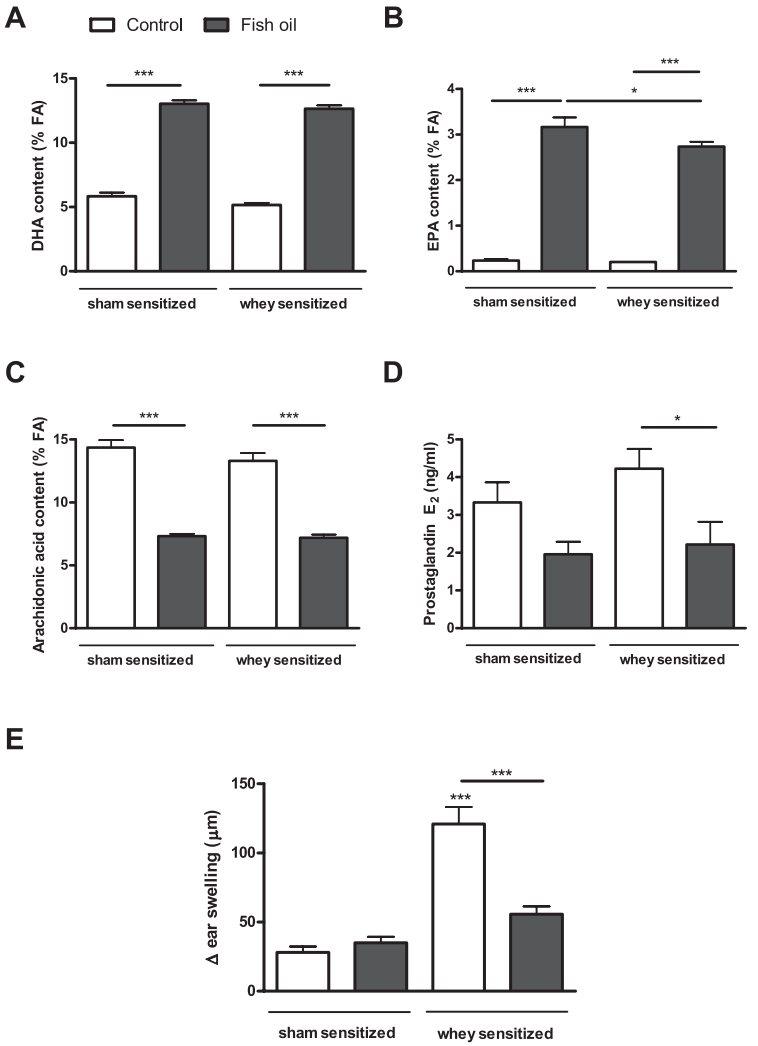

Figure 2 N-3 LCPUFA content in erythrocyte membranes was enhanced in mice fed the fish oil diet as shown for (A) DHA (diet $P<0.001$, sensitization $P<0.05$, interaction NS) and (B) EPA (diet $P<0.001$, sensitization $P=0.06$, interaction $P=0.11$). Incorporation occurred at the expense of n-6 PUFA such as (C) arachidonic acid (diet $P<0.001$, sensitization NS, interaction NS). In addition, the arachidonic acid metabolite (D) prostaglandin E_2 was reduced in plasma of whey-mice fed fish oil (diet $P<0.01$, sensitization NS, interaction NS). (E) The fish oil diet effectively reduced the whey-induced acute allergic skin response in whey-sensitized mice (diet $P<0.01$, sensitization $P<0.001$, interaction $P<0.001$). Ear swelling (µm) was calculated as the increase in ear thickness induced by whey one hour after challenge. Data are presented as mean ± SEM, n=6/group. *$P<0.05$, ***$P<0.001$ two-way ANOVA followed by Bonferroni's multiple comparison test. NS: not significant.

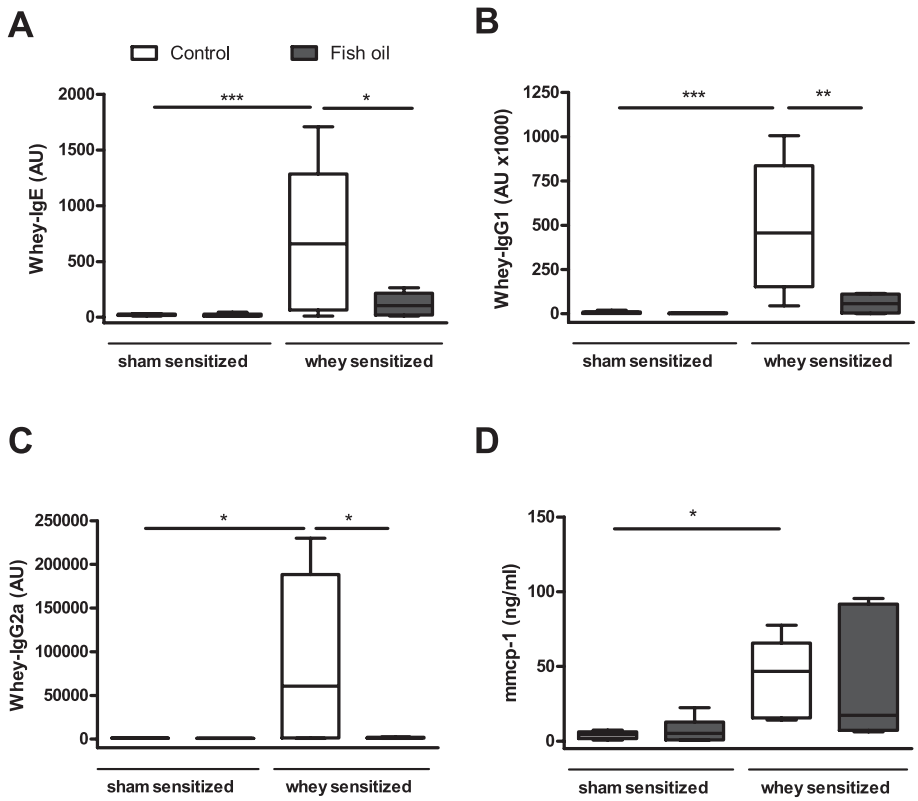

Figure 3 The effect of fish oil on the humoral response and mouse mast cell protease-1 (mmcp-1). Fish oil reduced whey-specific (A) IgE (diet $P<0.05$, sensitization $P<0.01$, interaction $P<0.05$) (B) IgG1 (diet $P<0.01$, sensitization $P<0.01$, interaction $P<0.01$) and (C) IgG2a (diet $P<0.05$, sensitization $P<0.05$, interaction $P<0.05$) as compared to control diet fed whey-sensitized mice. (D) Serum mmcp-1 concentrations were increased in whey-sensitized mice fed control diet as compared to sham mice (diet NS, sensitization $P<0.01$, interaction NS). Data are presented as box-and-whisker Tukey plots, n=6/group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ two-way ANOVA followed by Bonferroni's multiple comparison test. Data were square root transformed for analysis. NS: not significant.

Passive transfer of sera from fish oil fed mice to naive recipients confirms the humoral response to be suppressed

In recipient mice receiving sera from whey-sensitized donor mice fed the control diet a significant ear swelling was induced as compared to recipients receiving sera from sham mice ($P<0.001$, «Figure 4A»). Sera of whey-sensitized donor mice fed the fish oil diet elicited a significantly lower whey-induced ear swelling response in recipient mice compared to serum of control diet fed whey-sensitized donor mice ($P<0.001$).

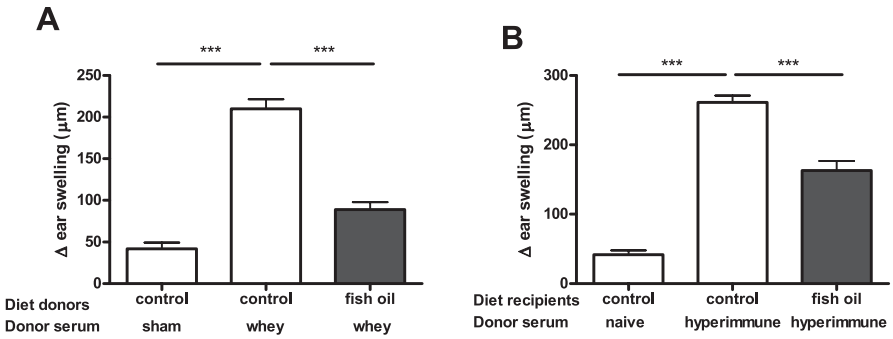

Figure 4 Effect of fish oil on humoral and effector response (A) Passive transfer of sera of whey-sensitized mice fed the fish oil diet resulted in a reduced ear swelling upon i.d. whey challenge in comparison with recipient mice transferred with sera of sensitized mice fed control diet. (B) The acute allergic skin response was diminished in fish oil fed naive recipient mice injected with whey hyperimmune serum as compared to control diet fed recipients. Data are presented as mean ± SEM, n=6/group. ***$P<0.001$ one-way ANOVA followed by Bonferroni's multiple comparison test.

Fish oil diet reduces acute allergic symptoms induced by passive sensitization using whey hyperimmune sera

N-3 LCPUFA from fish oil diet possibly target the mast cell directly, which may affect mast cell phenotype or degranulation (22, 23). To assess whether the fish oil diet itself is capable of suppressing acute allergic symptoms, hyperimmune serum – high in whey-specific immunoglobulins after whey alum-i.p. sensitization – was transferred to naive recipients fed either the control or fish oil diet, after which the mice were challenged with whey in both ears. Transfer of whey-hyperimmune serum resulted in a significantly enhanced acute allergic skin response in recipient mice fed the control diet as compared to injection of naive serum from non sensitized mice ($P<0.001$, «Figure 4B»). Recipient mice fed the fish oil diet showed a reduced allergic skin response as compared to control diet fed recipients transferred with hyperimmune serum ($P<0.001$).

Fish oil diet alters DC population and reduces percentages of Th1 and Th2 cells in MLN and spleen

MLN and splenocytes from whey-sensitized mice fed the control or fish oil diet were analyzed for DC and T cell subsets. Th1 inducing $CD11c^{+}CD11b^{-}CD8\alpha^{+}$ lymphoid DC (24, 25) were reduced in MLN and spleen by the fish oil diet in sham-sensitized mice when compared to sham mice fed control diet ($P<0.05$ for MLN; $P<0.01$ for spleen, «Figure 5A»). By contrast the percentage of $CD11c^{+}CD11b^{+}CD8\alpha^{-}$ myeloid DC (24, 25) were enhanced by the fish oil diet in MLN of both sham and whey-sensitized mice ($P<0.001$ sham mice; $P<0.001$ whey mice, «Figure 5A»).

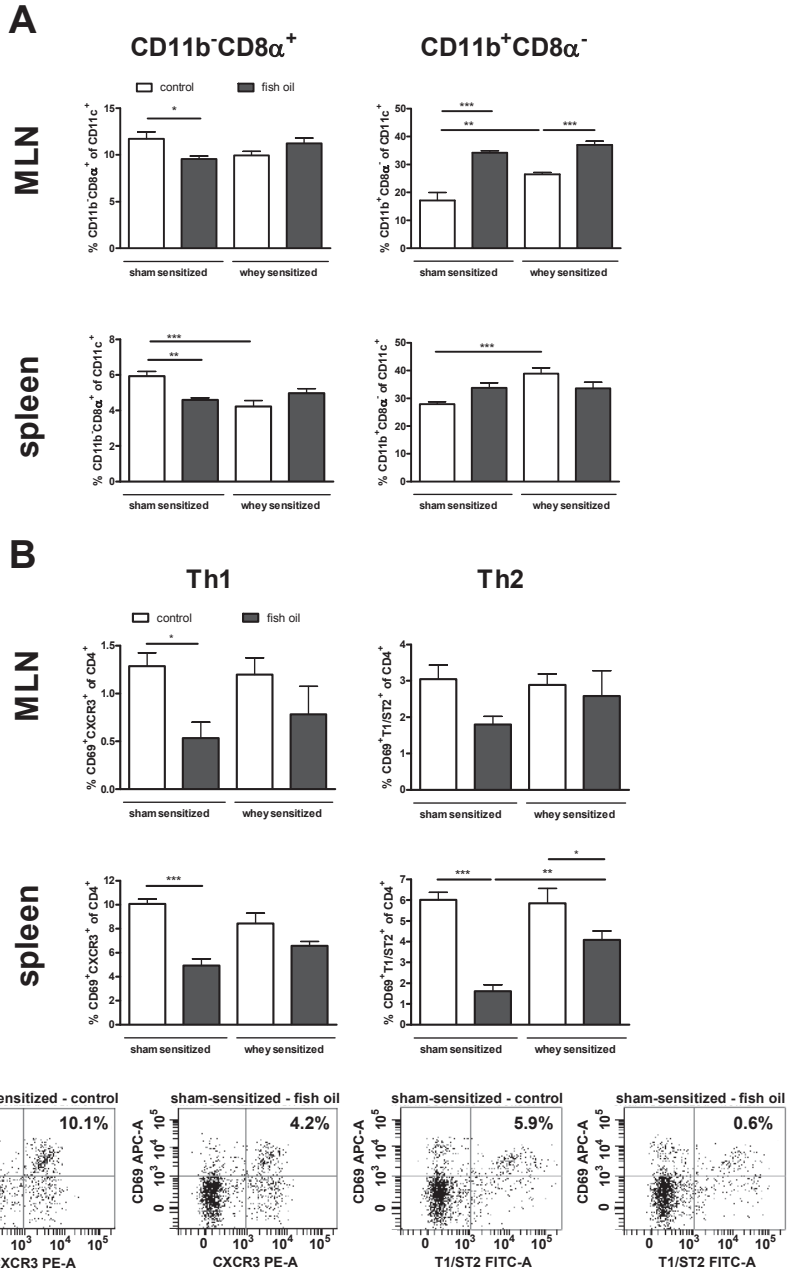

Figure 5 Effect of the fish oil diet on dendritic cell (DC) and T cell subsets in mesenteric lymph nodes (MLN) and spleen by flow cytometry. (A) Percentage of CD11b$^-$CD8α$^+$ lymphoid DC was reduced in MLN (diet NS, sensitization NS, interaction $P<0.01$) and spleen (diet NS, (continued)

◀ (continued) sensitization $P<0.05$, interaction $P<0.001$) of fish oil fed sham mice while % $CD11b^+CD8\alpha^-$ myeloid DC was enhanced by the fish oil diet in MLN (diet $P<0.001$, sensitization $P<0.01$, interaction $P=0.06$) but not spleen (diet NS, sensitization $P<0.01$, interaction $P<0.01$) (B) Percentage of activated Th1 cells ($CD4^+CD69^+CXCR3^+$) were reduced by the fish oil diet in MLN (diet $P<0.05$, sensitization NS, interaction NS) and spleens (diet $P<0.001$, sensitization NS, interaction $P<0.05$) of sham mice. Percentage of splenic activated Th2 cells ($CD4^+CD69^+T1/ST2^+$) were reduced in both sham- and whey-sensitized mice ($P<0.001$, sensitization $P<0.05$, interaction $P<0.05$). Th2 cells in MLN were not significantly altered (diet $P=0.10$, sensitization NS, interaction NS). Representative FACS plot are shown for splenic Th1 and Th2 cells in sham sensitized mice. Data are presented as mean ± SEM, n=6/group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ two-way ANOVA followed by Bonferroni's multiple comparison test. NS: not significant.

Total $CD4^+$ cells are not enhanced by sensitization with whey and not reduced by the tuna oil diet «Supplementary Figure 1 [page 124]». The fish oil diet reduced the percentage of activated Th1 cells ($CD4^+CD69^+CXCR3^+$) (26-28) in spleens of sham-sensitized mice by 50% ($P<0.001$, «Figure 5B»). Dietary fish oil supplementation also significantly reduced the percentage of splenic activated Th2 cells ($CD4^+CD69^+T1/ST2^+$) (29, 30) in sensitized mice ($P<0.001$ for sham; $P<0.05$ for whey). Since the effect on the percentage of activated Th2 was more pronounced this resulted in an increased Th1/Th2 ratio in fish oil fed sham-sensitized mice as compared to sham mice fed control diet (3.3 ± 0.5 vs 1.7 ± 0.1, $P<0.001$). Also in MLN the percentage of activated Th1 but not activated Th2 cells was reduced by the fish oil diet in sham-sensitized mice ($P<0.05$, «Figure 5B»). This did not affect the Th1/Th2 ratio in MLN (data not shown). The fish oil diet exerted a similar tendency for percentages of total Th1 ($CXCR3^+$ of $CD4^+$) and total Th2 ($T1/ST2^+$ of $CD4^+$) cells in MLN and spleen but effects were less prominent «Supplementary Figure 1».

Increase in FoxP3 positive cells in spleen and lamina propria of whey-sensitized mice fed fish oil diet

$FoxP3^+$ regulatory T cell (Treg) inducing $CD11c^+CD11b^+CD103^+CD8\alpha^-$ DC (31, 32) were increased in MLN of whey-sensitized mice fed fish oil as compared to fish oil fed sham mice ($P<0.05$, «Figure 6A»). The percentage of $FoxP3^+$ Treg in MLN was low (data not shown). However, in spleen the percentage of $FoxP3^+$ Treg in whey-sensitized mice fed the fish oil diet was enhanced as compared to sham mice fed fish oil ($P<0.05$, «Figure 6B»).

In addition to this systemic compartment an increased number FoxP3 positive cells per villus-crypt unit was counted locally in the small intestine of whey-sensitized mice fed the fish oil diet as compared to sham-sensitized mice fed the fish oil diet ($P<0.05$, «Figure 6C»). Isotype control slides were all negative for FoxP3.

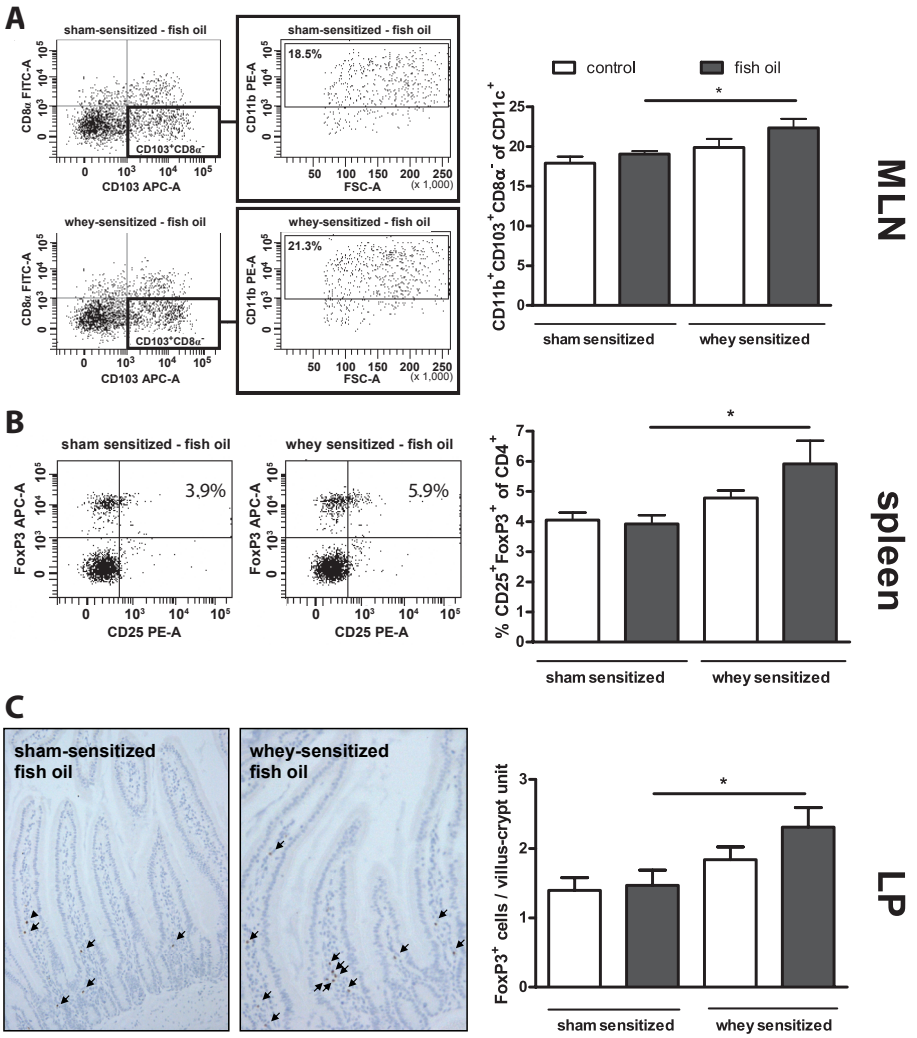

Figure 6 Effect of the fish oil diet on CD103$^+$ dendritic cells (DC) and FoxP3$^+$ regulatory T cells (Treg) (A) Percentage of CD11b$^+$CD103$^+$CD8α$^-$ DC was enhanced in mesenteric lymph nodes (MLN) of whey-sensitized mice fed the fish oil diet when compared to sham mice in both control and fish oil fed mice (diet P=0.07, sensitization P<0.01, interaction NS). Representative FACS plots are shown. Furthermore, the percentage of FoxP3$^+$ Treg was enhanced in whey-sensitized mice fed the fish oil diet in (B) spleen (flow cytometry, diet NS, sensitization P<0.01, interaction NS) and (C) lamina propria (LP) of small intestine (diet NS, sensitization P<0.01, interaction NS; immunohistochemical staining for FoxP3 (arrows); magnification 200x; typical example shown). Data are presented as mean ± SEM, n=6/group. *P<0.05 two-way ANOVA followed by Bonferroni's multiple comparison test. NS: not significant.

DISCUSSION

Fish oil supplementation reduces allergic sensitization in a mouse model for cow's milk allergy since the acute allergic skin response in the fish oil fed mice was reduced by over 50% combined with low serum whey-IgE and whey-IgG1 levels. The current study implies that n-3 LCPUFA can suppress the susceptibility to develop allergic disease even when provided after birth.

In recent human trials fish oil supplementation was able to lower the risk of allergic disease. Since allergic diseases are determined early in life, or even *in utero,* these trials were performed during pregnancy. Daily supplementation with n-3 LCPUFA during late pregnancy has been shown to reduce the risk of food allergy, including milk allergy and IgE-associated eczema in children at risk of atopic disease (17, 33-36). Furthermore, fewer severe allergic symptoms have been reported in fish oil treated infants (33). Results from our study suggest the possibility for primary prevention of allergic sensitization when providing fish oil starting early in life after birth. The Childhood Asthma Prevention Study (CAPS) reported that fish oil supplementation starting after birth did not prevent the onset of atopy at age of 5 in high risk children (37, 38). However, the study population with a family history of asthma may be less susceptible to interventions due to genetic setting. In addition, the dosage aimed at may have been insufficient to prevent the onset of atopy in this critical period. Furthermore, the suboptimal adherence to oil capsules (51% for fish oil group) suggests that the average ingested n-3:n-6 fatty acid ratio was lower than the 1:5 aimed at for the fish oil group. Therefore the fatty acid ratio of 1:7 achieved in plasma by fish oil is considered suboptimal in this study. Another postnatal intervention study supplemented newborns from birth to 6 months of age with 650 mg fish oil and reported lowered allergen-specific Th2 responses (39).

The n-3:n-6 PUFA ratio of the diets in our study should be seen in the view of the current human Western diet with a ratio of 1:10-20 versus the diet of Inuit's, a fish consuming population with low prevalence of allergic disease consuming a 2.5:1 ratio (40-42). Inuit plasma phospholipids have a 1:1 n-3:n-6 PUFA ratio (43). Here, fatty acid ratio of erythrocytes was reduced from about 1:5 in the control group to 1:1 in the fish oil fed mice. In human pregnancy studies, supplementing with 4-4.5 gram of fish oil daily to prevent allergic outcomes in offspring, ratios about 1:1.5 were shown to be effective (33, 34).

IgE and IgG are known to sensitize mast cells and basophils and immunoglobulin levels correlate positively with the intensity of the acute allergic skin response (44). Whey-specific IgE and IgG1 levels were reduced by the fish oil diet in our study, in association with a reduced acute allergic skin response. The suppressed Th2 type humoral response was further confirmed using serum transfer suggesting that n-3 LCPUFA have suppressed the sensitization to whey.

Studies report that arachidonic acid metabolite PGE_2 promotes IgE production by B cells (45). Substitution of arachidonic acid by n-3 LCPUFA did reduce plasma PGE_2 concentrations and may indirectly have suppressed immunoglobulin generation. Furthermore IL-4 and IL-13 induce IgE isotype switching and production of these cytokines by Th2 cells as well as mast cells may be reduced by n-3 LCPUFA (23, 46, 47). In addition, DHA was found to reduce IgE production by human B cells via the IL-4 pathway or via an effect on CD40 (48). Animal studies up to now are inconclusive about the effect of n-3 LCPUFA on IgE production (49). Mice fed 1% soybean oil plus 10% fish oil tended to have enhanced OVA-specific IgE levels in serum as compared to mice fed 1% soybean oil plus 10% sunflower oil (50). Since sunflower oil is very low in ALA, the requirements for an adequate amount of ALA as recommended by Reeves *et al.* are not met in the diet, which may explain the discrepancy with our results (18). Like in our study Matos *et al.* reported in addition to reduced OVA-specific IgE , also reduced OVA-IgG1 levels using a 7% fish oil diet (51). Furthermore, dietary intervention with n-3 LCPUFA during pregnancy showed a tendency to reduce IgE antibody levels in infants at risk which is in line with our observations (17).

Our data indicate that n-3 LCPUFA not only target the humoral response but also may directly target the effector response. To further investigate this, naive mice were fed the fish oil diet prior to passive sensitization with hyperimmune serum high in whey-IgE. This indicates that dietary fish oil modulates the mast cell response resulting in less acute allergic symptoms upon mast cell degranulation. The effects of LCPUFA on mast cell function have not been fully addressed in literature and many mechanisms may be involved. However, it has been proven that n-3 LCPUFA alter the mast cell phenotype and reduce the formation of eicosanoids of the 2/4-series which are causatively involved in inducing edema and allergic symptoms (23).

Besides dampening the humoral and effector response, the present study shows n-3 LCPUFA to affect DC and T cells. The fish oil diet was found to reduce the percentage of $CD11c^+CD11b^-CD8\alpha^+$ conventional lymphoid DC which are IL-12 producing and therefore Th1 polarizing (24, 25). This corresponds well with our observation that the percentage of Th1 cells is diminished in spleen and MLN of sham-sensitized mice. On the other hand conventional myeloid DC ($CD11c^+CD11b^+CD8\alpha^-$), which may induce tolerogenic or Th2 responses (24, 25), were enhanced by the fish oil diet in MLN. Since the diet reduced the development of Th2 cells, especially in the spleen, this may imply that this subset represents a tolerogenic phenotype in the current setting. Also in a recent study EPA and DHA were found to suppress IL-12 secretion by LPS-stimulated DC while regulatory IL-10 production was enhanced (52). As the percentage of Th2 cells was reduced most prominent in our study the Th1/Th2 ratio was increased in the sham group fed the fish oil diet which implicates skewing away from the Th2 phenotype. Suppression of both Th1 and Th2 cell subsets relate to the findings of Dunstan *et al.* reporting that neonatal mononuclear cells secreted less Th1 and Th2 cytokines in response to allergen stimula-

tion after fish oil consumption (33). Another study showed a reduced IL-4 production by human peripheral blood mononuclear cells after n-3 LCPUFA consumption, enhancing the Th1/Th2 cytokine ratio (48). Furthermore, supplementation with high doses of fish oil (3-6 g/day) reduced IL-2 and IFN-γ production (53, 54). Although 90% of Th1 cells express CXCR3 we cannot exclude that other $CD4^+$ cells also express this marker and are included in the analysis (27). However, serological data confirm these findings. In our study the effect of fish oil on Th1 and Th2 cells was shown most pronounced for sham sensitized mice. We hypothesize that this effect was dampened in whey-sensitized mice due to the continuous boost of the immune response by the repeated sensitizations while for sham mice the challenge is the first contact with whey. Typically the suppression of Th1 and Th2 cell subsets in both sham and whey sensitized mice was most pronounced when the early activation marker CD69 was taken into account (55). This suggests that apart from a minor population of whey specific T cells also aspecific or bystander T cell activation was caused by whey. Intrinsic capacities of the whey protein fraction may contribute to this (56, 57). Since percentages of both activated Th1 and Th2 cells were reduced by the fish oil diet in sham mice this suggests a general suppression of immune activation by the fish oil diet.

Not only Th1 and Th2 subsets were affected by the fish oil diet. Strikingly, dietary n-3 LCPUFA were observed to enhance Treg in whey-sensitized mice fed the fish oil diet both systemically in the spleen as well as locally in the small intestine. $FoxP3^+$ Treg play an important role in modulating the immune response and oral tolerance induction (58, 59). $CD103^+$ cells migrate from lamina propria to the MLN and induce $CD4^+FoxP3^+$ Treg via a retinoic acid dependent mechanism (24, 32, 60). In particular the $CD11b^+CD103^+CD8\alpha^-$ DC subset is known to express retinal dehydrogenase isoform 2 which catalyzes the conversion of retinal to retinoic acid (31, 32). This subset was enhanced in the MLN of fish oil fed whey-sensitized mice. $FoxP3^+$ Treg generated in the MLN, transfer tolerance systemically and Treg home to the gut lamina propria to maintain homeostasis (59). The fish oil diet was found to enhance $FoxP3^+$ Treg in spleen and lamina propria of whey-sensitized mice. There is not much known about the effects of PUFA on Treg but it has been suggested that n-3 LCPUFA support Treg function (61). Kleemann et al. reported increased regulatory IL-10 mRNA expression in Peyer's patches of fish oil-fed BB rats (62) whereas Yaqoob *et al.* supplemented mice with fish oil and found IL-10 production by cultured spleen cells to be reduced (63). Treg may reduce B cell and mast cell activation whereas they enhance oral tolerance induction, this may contribute to the reduced sensitization to whey observed (58).

In this study we demonstrated the effect of dietary fish oil supplementation starting early in life, in a mouse model for cow's milk allergy. Fish oil reduced the generation of a whey-specific humoral response by B cells, leading to attenuated production of Th2 type immunoglobulins and reduced acute allergic symptoms. Besides the suppression of

allergic sensitization, the fish oil diet also directly reduced the allergic effector response. Furthermore, the fish oil diet increased Treg in both spleen and small intestine thus enhancing tolerizing capacities of the adaptive immune system. Together this implicates beneficial effects of fish oil supplementation in early life for the primary prevention of atopic diseases such as food allergy.

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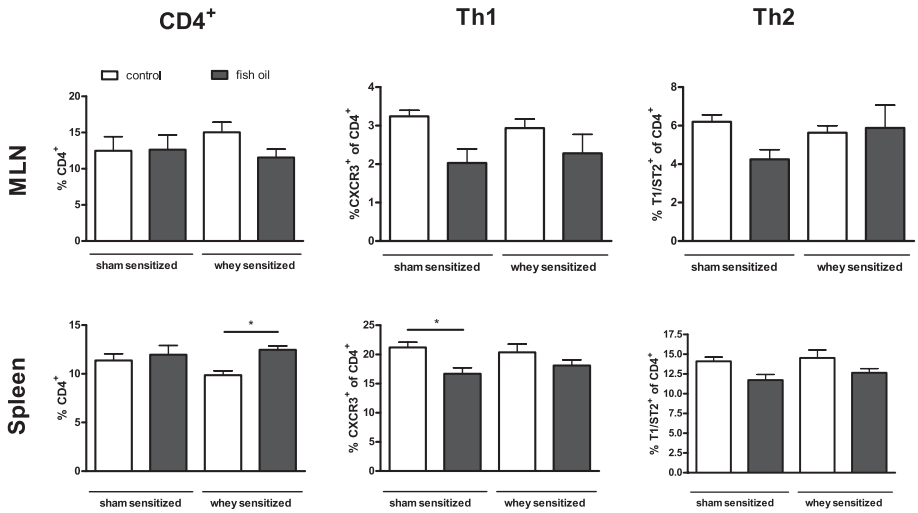
SUPPLEMENTARY MATERIAL



Supplementary Figure 1 Effect of the fish oil diet on total Th1 and Th2 cell subsets in mesenteric lymph nodes (MLN) and spleen by flow cytometry. Percentage of $CD4^+$ cells in MLN (diet NS, sensitization NS, interaction NS) and spleen (diet $P<0.05$, sensitization NS, interaction $P=0.09$ for spleen) were not reduced by the fish oil diet. The percentage of total Th1 cells ($CD4^+CXCR3^+$) was reduced by the fish oil diet in spleen of sham mice (diet $P<0.01$, sensitization NS, interaction NS). Percentage of Th1 cells in MLN showed a similar tendency (diet $P<0.05$, sensitization NS, interaction NS) as well as the percentage of total Th2 cells ($CD4^+T1/ST2^+$) in spleen ($P<0.01$, sensitization NS, interaction NS) and MLN (diet NS, sensitization NS, interaction NS). Data are presented as mean ± SEM, n=6/group. *$P<0.05$ two-way ANOVA followed by Bonferroni's multiple comparison test. NS = not significant

CHAPTER SIX

CD25+ Regulatory T-cells transfer n-3 LCPUFA induced tolerance in cow's milk allergic mice

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ABSTRACT

Background Recently, we have shown that dietary long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) largely prevent allergic sensitization in a murine model for cow's milk allergy. Aim of this study was to assess the contribution of regulatory T-cells (Treg) in the prevention of food allergy by n-3 LCPUFA.

Methods C3H/He0uJ female donor mice were fed a control or fish oil diet before and during oral sensitization with cow's milk protein whey. Acute allergic skin response (ASR), anaphylaxis, body temperature, serum immunoglobulins and mouse mast cell protease 1 (mmcp-1) were assessed. Splenocytes of sham- or whey-sensitized donor mice fed either the control or fish oil diet were adoptively transferred to naive recipient mice. Recipient mice received a whole splenocyte suspension, splenocytes *ex vivo* depleted of $CD25^+$ cells, or MACS-isolated $CD4^+CD25^+$ Treg. Recipient mice were sham- or whey-sensitized and fed control diet.

Results The ASR as well as whey-IgE and -IgG1 levels were reduced in sensitized donor mice fed the fish oil diet as compared to the control diet. Splenocytes of control diet fed whey-donors transferred immunological memory. By contrast, splenocytes of fish oil fed whey- but not sham-donors transferred tolerance to recipients as shown by a reduction in ASR and serum mmcp-1 and depletion of $CD25^+$ Treg abrogated this. Transfer of $CD25^+$ Treg confirmed the involvement of Treg in the suppression of allergic sensitization.

Conclusion $CD25^+$ Treg are crucial in whey allergy prevention by n-3 LCPUFA.

INTRODUCTION

The current prevalence of allergic disease in developed countries is estimated to be 15-30% (1). Allergic sensitization to food proteins may occur early in life and affects 6% of children and 3-4% of adults (2). In food allergic patients harmless foods including egg, milk, nuts and wheat can result in eczema, diarrhea, respiratory problems or even systemic anaphylaxis. This is the result of an impaired development of tolerance or the breakdown of existing tolerance to food proteins (3, 4). Intact food derived epitopes that escaped gastrointestinal degradation may be taken up in the Peyer's patches or lamina propria (LP) by dendritic cells (DC). Presentation of the antigen to naive $CD4^+$ T-cells in the mesenteric lymph nodes (MLN) will instruct regulatory T-cells (Treg) which support oral tolerance induction (5). From the MLN tolerance can be transferred to the systemic compartment since Treg released in the blood home back to the LP but also remain present systemically (6). Treg comprise several subsets including the naturally occurring $CD4^+CD25^+$ Treg (nTreg) derived from the thymus and expressing the transcription factor forkhead box protein 3 (FoxP3) as well as peripheral antigen-induced $CD4^+CD25^+FoxP3^+$ Treg (iTreg), interleukin (IL)-10- (Tr1) and TGF-β-producing (Th3) Treg (7-9).

Epidemiological data suggest that dietary components may affect oral tolerance induction. Recently it has been shown that the long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), found in oily fish, may reduce the susceptibility to develop allergic disease. Daily dietary supplementation with n-3 LCPUFA during pregnancy and lactation reduced the incidence of food allergy and IgE-associated eczema in children at risk for atopy (10). Furthermore, dietary supplementation with fish oil largely reduced ovalbumin-allergy in mice (11) and prevented oral sensitization to the cow's milk protein whey (12). Currently, the involvement of Treg in tolerance induction by n-3 LCPUFA is speculative and remains to be elucidated. In this study we assessed whether Treg from whey-sensitized donor mice fed n-3 LCPUFA could adoptively transfer tolerance to whey-sensitized recipients.

MATERIALS AND METHODS

Oral sensitization donor mice

Animal use was performed in accordance with guidelines of the Animal Ethics Committee of Utrecht University. Three-week-old specific pathogen free female C3H/HeOuJ mice (Charles River Laboratories, L'Arbresle Cedex, France; n=6-12/group) were fed AIN-93G-based diets composed of either 10% soybean oil (control diet) or 4% soybean oil plus 6% tuna oil (fish oil diet) as described before (12). Tuna oil (38.5% n-3 PUFA) was a kind gift from Bioriginal (Den Bommel, The Netherlands) and contained 27.8% DHA and 7% EPA. The ratio n-3:n-6 PUFA was 1:9.5 for the control diet while this ratio was reduced to 1:1

for the fish oil diet. Mice were sensitized intragastrically (i.g.) using whey (DMV international, Veghel, The Netherlands) as described before (13, 14) «Figure 1A». Mice received an intradermal (i.d) challenge in the ear pinnae with 10 µg whey and the day thereafter mice were sacrificed.

Adoptive transfer of splenocytes

For depletion of $CD25^+$ Treg, splenocytes were *ex vivo* incubated with rat anti-CD25 monoclonal antibody PC61 as previously described (13). The mouse $CD4^+CD25^+$ Regulatory T-cell Isolation kit (Miltenyi Biotec, Leiden, The Netherlands) was used for $CD4^+CD25^+$ enrichment according to the manufacturer's instructions. The whole splenocyte suspension, $CD25^+$ Treg-depleted splenocytes (both 5 x 10^6 cells) or isolated $CD4^+CD25^+$ Treg cells (1.2 x 10^5 cells; 85% was $FoxP3^+$ as assessed by flow cytometry as previously described (12)) were intravenously injected in naive recipient mice (n=6/group; «Figure 1A») Recipient mice were fed AIN-93G diet (15) and sensitized as described above. At day 33 they received an i.d. challenge with 10 µg whey and i.g. challenge with 50 mg whey and at day 34 mice were sacrificed.

Acute allergic skin response, anaphylactic shock score and body temperature

The acute allergic skin response (ASR) was measured in duplicate for both ears 1 h after i.d. challenge with whey using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). Ear swelling was calculated by subtracting basal ear thickness from ear thickness after 1 h. To assess the severity of shock symptoms 30 min after i.d. whey challenge a validated anaphylactic shock scoring table was used (16) [see page 69]. All mice were given an implantable electronic ID transponder (Plexx, Elst, The Netherlands) to measure individual changes in body temperature 1 h after i.d. challenge.

Serum immunoglobulins and mmcp-1

Levels of whey-specific IgE, IgG1 and IgG2a were determined by ELISA in serum collected 18 h after oral challenge, as previously described (14). Serum mouse mast cell protease-1 (mmcp-1), reflecting mucosal mast cell degranulation, was determined using a commercially available ELISA kit (eBioscience, Vienna, Austria) according to the manufacturer's protocol.

Proliferation and cytokine release

Splenocytes (4x10^5 per well) were incubated in RPMI1640 supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS. Cells were stimulated for 48 h with 1 µg/ml anti-CD3 plus 1 µg/ml anti-CD28. Supernatants were harvested for cytokine measurements by ELISA according to the manufacturer's recommendations (eBioscience). T-cell proliferation was determined by measuring [^{3}H]-thymidine incorporation.

Statistics

Data are presented as mean ± SEM and were analyzed using two-way ANOVA and post hoc Bonferroni test using GraphPad Prism software. For shock score medians are depicted and Kruskall-Wallis followed by Dunn's multiple comparison test was used. For serological data Tukey box-and-whisker plots are used and square root transformation was performed before analysis to normalize the data distribution. Spearman correlation coefficients were calculated.

RESULTS

Fish oil diet reduces whey-induced ASR and drop in body temperature in whey-sensitized donor mice

One hour after i.d. challenge the ASR in whey-sensitized mice fed control diet was significantly enhanced as compared to sham-sensitized mice «Figure 1B» and this was effectively reduced by the fish oil diet. Basal body temperature was approximately 37.5°C in all groups (data not shown). A decline to 33.5°C after challenge was observed in whey-mice fed the control diet «Figure 1C» while the fish oil diet reduced the drop in body temperature. Eleven out of twelve whey-sensitized control diet fed donors showed mild to severe symptoms, which was fatal for three mice «Figure 1D». The fish oil diet tended to reduce shock scores and in this group all mice survived. Interestingly, in this group four of eleven mice showed no signs at all. Body temperature showed a negative correlation with shock symptoms «Figure 1E».

Reduced whey-specific immunogobulin levels and mmcp-1 in whey-sensitized donor mice fed fish oil diet

Serum levels of whey-specific IgE, IgG1 and IgG2a were increased in control diet fed mice sensitized with whey compared to sham-mice «Figure 2A-C». The fish oil diet reduced whey-IgE, -IgG1 and IgG2a levels in whey-sensitized mice. In addition, fish oil diet fed whey-sensitized mice showed lower serum mmcp-1 concentrations compared to control diet fed mice «Figure 2D».

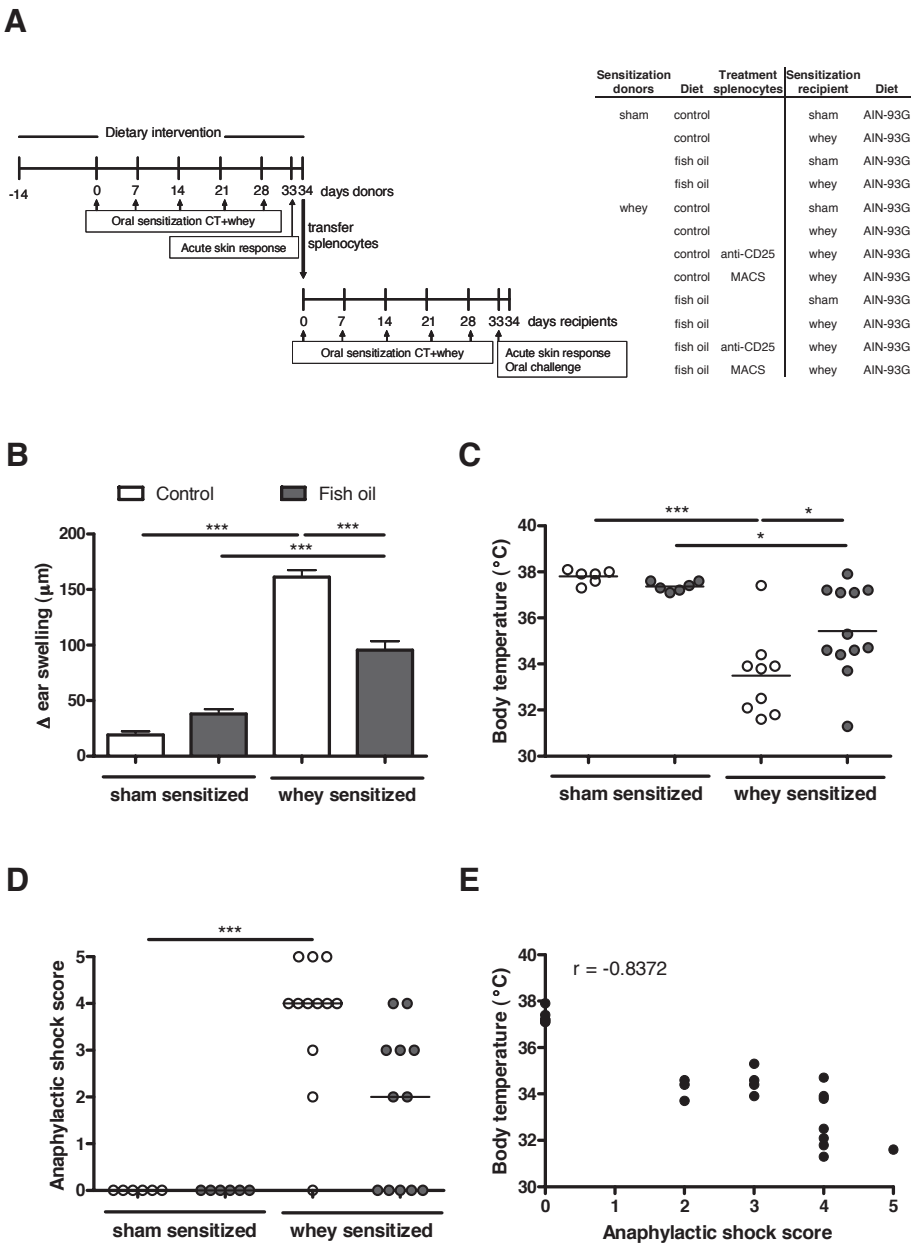

Figure 1 The allergic response in donor mice after an intradermal (i.d.) challenge with whey. (A) Schematic overview of the adoptive transfer protocol and experimental set-up. Donor mice (n=6-12/group) were fed the control or fish oil diet, starting two weeks prior to the first sensitization and continued during the whole sensitization period until sacrifice. Mice were sensitized (continued)

◀ (continued) intragastrically with 20 mg whey with 10 µg cholera toxin (CT). Sham mice received CT alone. Mice were sensitized once a week for 5 consecutive weeks. At day 33 they received an i.d. challenge with whey (10 µg) in the ear and at day 34 spleens were collected. Single cell suspensions either treated or not treated with anti-CD25 antibody or MACS separation were transferred into naive recipients. Recipient mice were fed AIN-93G diet and sham- or whey-sensitized. After the ear challenge they received an oral challenge (50 mg whey). At day 34 they were sacrificed. Whey allergy was assessed by changes in (B) acute allergic skin response (C) body temperature and (D) anaphylactic shock score. (E) Body temperature and shock symptoms were negatively correlated ($P<0.001$). Data are presented as mean ± SEM, median is shown for shock, n=6-12/group. *$P<0.05$, ***$P<0.001$.

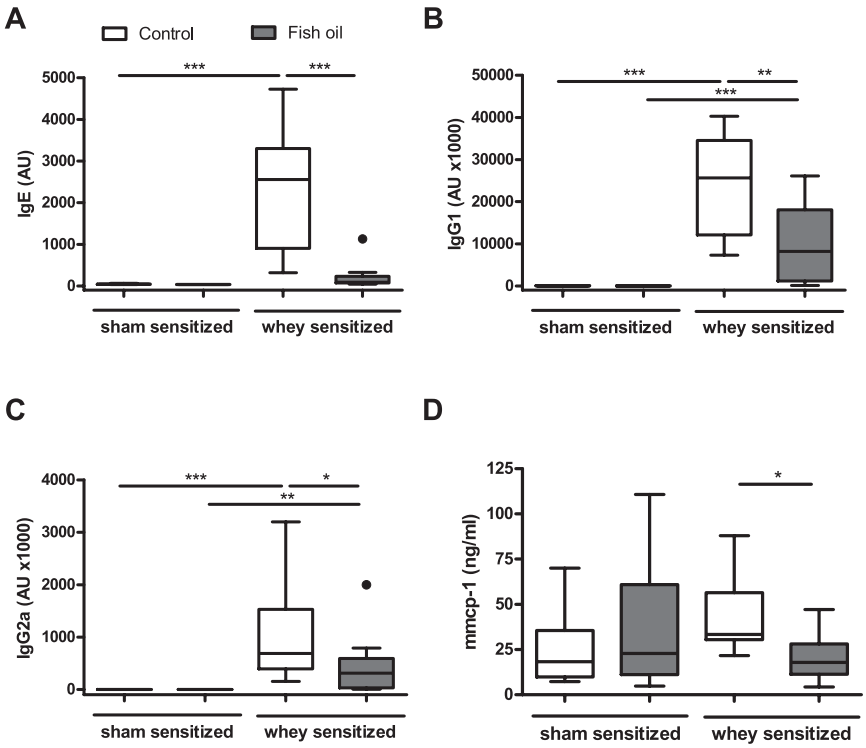

Figure 2 The humoral response in donor mice fed control of fish oil diet after the oral challenge with whey. Fish oil reduced whey-specific (A) IgE (B) IgG1 (C) IgG2a and (D) mouse mast cell protease-1 (mmcp-1) in serum as compared to control diet fed whey-sensitized mice. Data are presented as box-and-whisker Tukey plots (outliers indicated as dots), n=6-12/group. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Cow's milk allergy is suppressed by adoptive transfer of splenocytes from whey-sensitized fish oil fed mice

To test whether splenocytes of fish oil fed donor mice were able to transfer n-3 LCPUFA induced tolerance an adoptive transfer experiment was performed. The ASR was enhanced in whey-sensitized as compared to sham-sensitized recipients transferred with

splenocytes of sham-donors «Figure 3A». Splenocyte transfer of control diet fed whey-donor mice as compared to sham-donor mice increased the ASR in whey-sensitized recipients. By contrast, splenocytes of fish oil fed whey-donors reduced the ASR in whey-sensitized recipients compared to control diet fed whey-donors and also compared to fish oil fed sham-mice, indicating an antigen-specific protective effect of fish oil. The body temperature of whey-sensitized recipients transferred with splenocytes from control diet fed whey-donors, was significantly reduced as compared to their sham-sensitized counterparts «Figure 3B». This effect was lost upon transfer of splenocytes of fish oil fed whey-donor mice. The serum mmcp-1 concentrations were in accordance with this «Figure 3C» and there was a positive correlation between mmcp-1 and the ASR «Figure 3D».

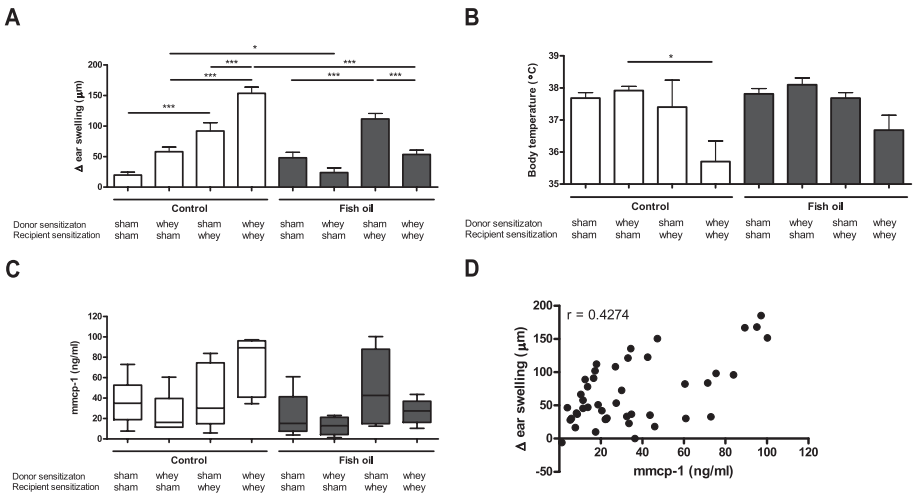

Figure 3 Adoptive transfer of splenocytes from control or fish oil diet fed donors to sham- or whey-sensitized recipient mice fed AIN-93G diet. (A) Acute allergic skin response (B) body temperature and (C) mmcp-1 in serum were assessed. (D) mmcp-1 showed a positive correlation with the acute allergic skin response ($P<0.01$). Data are presented as mean ± SEM or box-and-whisker Tukey plots, n=6/group. *$P<0.05$, ***$P<0.001$.

$CD4^+CD25^+$ Treg are involved in transfer of tolerance

To study the contribution of $CD25^+$ Treg in the allergy preventive effects of fish oil, *ex vivo* $CD25^+$ Treg-depleted splenocytes and MACS-isolated $CD4^+CD25^+$ Treg were adoptively transferred. Recipients transferred with splenocytes of control diet fed donors treated with anti-CD25, showed a slight reduction in ASR compared to transfer of the total splenocytes «Figure 4A». By contrast, recipients transferred with splenocytes of fish oil fed whey-donor mice depleted of $CD25^+$ cells showed an increased ASR compared to recipients transferred with the whole splenocyte suspension. Transfer of isolated $CD25^+$ Treg

from control diet and fish oil diet fed whey-donor mice reduced the ASR. Injection of CD25⁺ Treg-depleted cells from control diet fed whey-donors normalized body temperature in whey-recipients «Figure 4B». Transfer of CD25⁺ Treg compared to whole splenocyte suspension from control diet fed whey-donor mice reduced mmcp-1; whereas mmcp-1 was already low in fish oil group «Figure 4C».

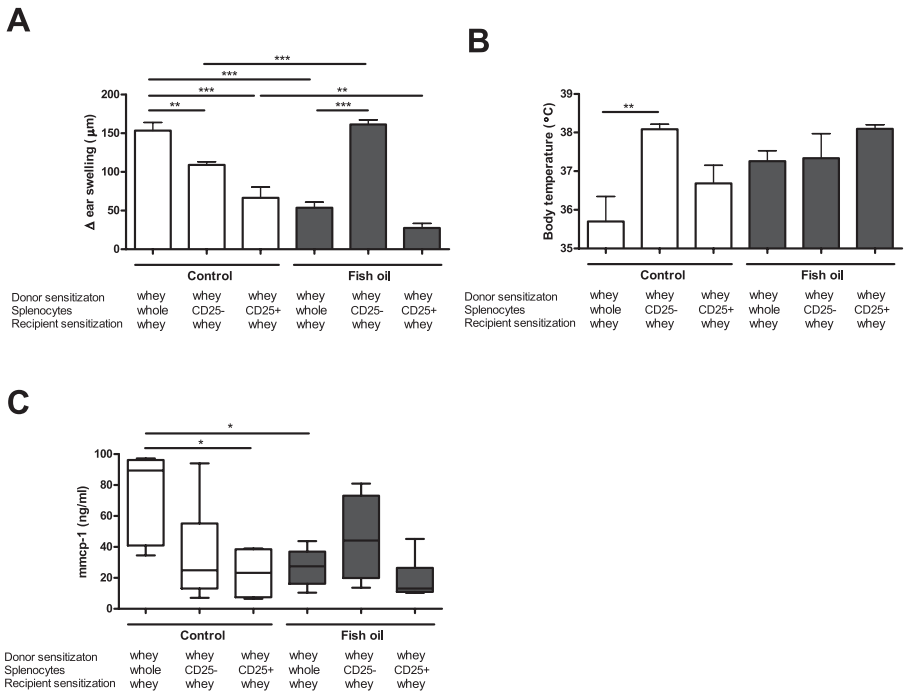

Figure 4 The effect of CD25⁺ Treg-depleted and CD25⁺ Treg populations from control or fish oil diet fed donors on allergic parameters in whey-sensitized recipient mice fed AIN-93G diet. (A) Acute allergic skin response (B) body temperature and (C) mmcp-1 in serum. CD25⁻ splenocytes represent the *ex vivo* CD25⁺ Treg-depleted splenocyte population; CD25⁺ splenocytes represent the MACS isolated CD25⁺ Treg population. Data are presented as mean ± SEM or box-and-whisker Tukey plots, n=6/group. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Whey-IgE levels were significantly reduced in recipients transferred with CD25⁺ Treg from control fed whey-mice, when compared to the whole splenocyte suspension «Figure 5A». Transfer of CD25⁺ Treg from both control and fish oil fed whey-donors completely abrogated whey-specific IgG1 production in recipient mice «Figure 5B». The CD25⁺ cell-depleted population of control diet fed whey-donor mice induced whey-IgG2a levels in recipient mice compared to recipients transferred with the whole cell suspension, which was significantly reduced by the fish oil diet «Figure 5C».

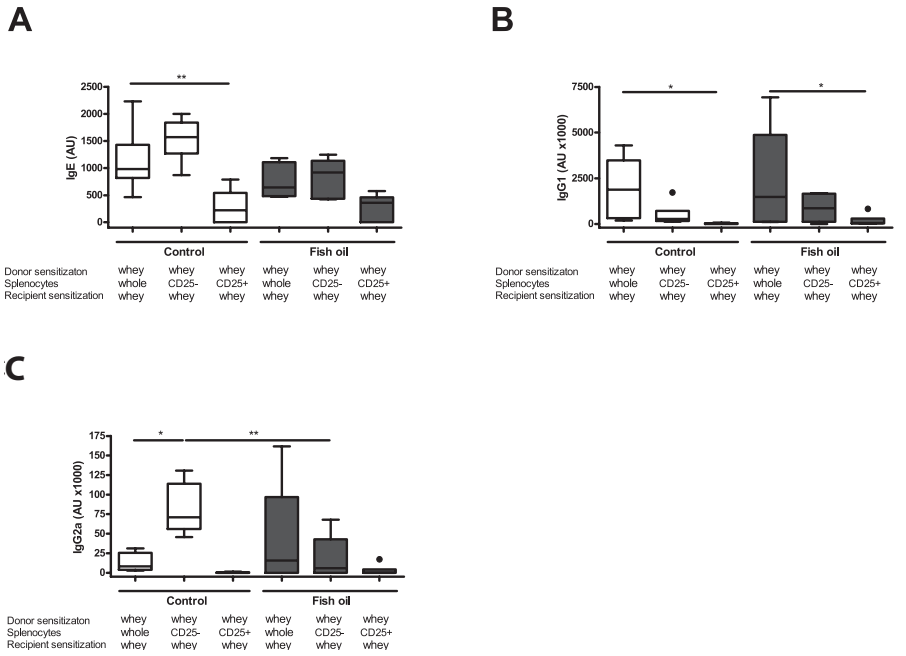

Figure 5 The effect of CD25⁺ Treg-depleted and CD25⁺ Treg populations from control or fish oil diet fed donors on immunoglobulin levels in whey-sensitized recipient mice fed AIN-93G diet. (A) IgE (B) IgG1 and (C) IgG2a in serum. CD25⁻ splenocytes represent the *ex vivo* CD25⁺ Treg-depleted splenocyte population; CD25⁺ splenocytes represent the MACS isolated CD25⁺ Treg population. Data are presented as box-and-whisker Tukey plots, n=6/group. *$P<0.05$, **$P<0.01$.

Enhanced IL-10 secretion in restimulated splenocytes isolated from recipients transferred with cells from fish oil diet fed whey-donors

The proliferation upon anti-CD3/CD28 stimulation was enhanced in splenocytes isolated from recipients transferred with cells from fish oil fed whey-donors as compared to donor cells from the control group «Figure 6A». Accordingly, the concentration of IL-10 was significantly increased in supernatants of this group «Figure 6B».

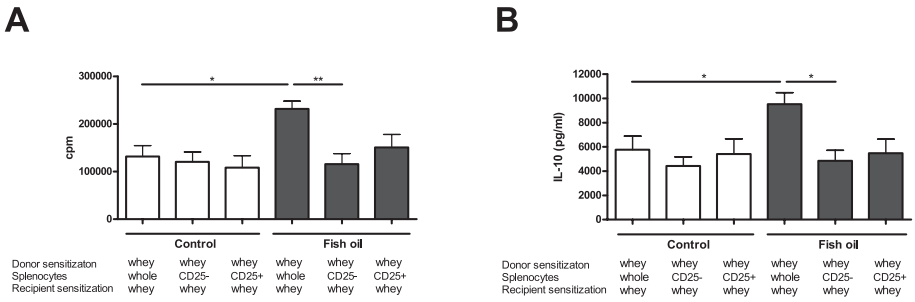

Figure 6 Proliferation and IL-10 secretion by *ex vivo* anti-CD3/CD28 stimulated (48 h) splenocytes from whey-sensitized recipient mice fed AIN-93G diet. (A) Tritiated-thymidine incorporation and concentration of (B) IL-10 in culture supernatants. $CD25^-$ splenocytes represent the *ex vivo* $CD25^+$ Treg-depleted splenocyte population; $CD25^+$ splenocytes represent the MACS isolated $CD25^+$ Treg population. Data are presented as mean ± SEM, n=6/group. $^*P<0.05$, $^{**}P<0.01$. cpm; counts per minute.

DISCUSSION

In the current study a mouse model for orally induced cow's milk allergy was used to investigate the contribution of $CD25^+$ Treg in fish oil induced tolerance to whey. Adoptive transfer of whole splenocyte suspensions compared to *ex vivo* $CD25^+$ Treg-depleted splenocytes or isolated $CD4^+CD25^+$ Treg populations, confirmed that $CD25^+$ Treg are required for the protective effects of fish oil on the induction of cow's milk allergy in mice.

In this study fish oil supplementation after birth effectively prevented allergic sensitization to whey in mice, as demonstrated before (12). Furthermore, it has been shown that postnatal n-3 LCPUFA supplementation during the first 6 months of life reduced allergen-specific Th2 responses in humans (17). However, it is unknown how n-3 LCPUFA exactly may exert their effects. It has been suggested that n-3 LCPUFA may support Treg function, however results are complex and sometimes contradictory. Iwami *et al.* suggested EPA to induce Treg via peroxisome proliferator-activated receptor (PPAR)-γ (18). Furthermore, EPA fed mice demonstrated increased $FoxP3^+$ cells as well as PPAR-γ expression in transplanted hearts. *In vitro* EPA enhanced proliferation of Treg whereas a PPAR-γ antagonist abrogated this (19). On the other hand, DHA was reported to induce the expression of Treg markers FoxP3, cytotoxic T lymphocyte-associated antigen 4 and/or transforming growth factor (TGF)-β while Treg suppressive function was diminished (20, 21). We have recently shown that fish oil was able to enhance the number of Treg both locally in the LP of the jejunum and systemically in the spleen in a mouse model for whey-allergy (12).

In the current study the induction of functional Treg by the fish oil diet was confirmed using an adoptive transfer approach. Splenocytes from whey-sensitized donor

mice fed the control diet transferred allergy to recipient mice. In contrast, splenocytes of fish oil fed whey-donor mice, did not transfer an immunological memory response but rather tolerance to recipients. Transfer of cells from sham-donors fed fish oil did not protect recipient mice indicating the involvement of an antigen-specific Treg population contributing to whey-allergy prevention by fish oil. Whey-specific immunoglobulin levels were not reduced by transfer of splenocytes isolated from whey-donors fed fish oil compared to control diet. Yamashita *et al.* reported high ovalbumin-specific serum IgE in recipient mice after transfer of MLN cells from ovalbumin-tolerant mice (22). However, the ASR and serum mmcp-1 were reduced by transfer of cells of fish oil fed whey-donors, indicating suppression of mast cell degranulation in recipient mice. $CD25^+$ Treg were shown to inhibit IgE-mediated mast cell degranulation via cell-cell contact (23-25) Furthermore, IL-10 was shown to reduce mast cell degranulation and cytokine release (26, 27). Hence, $CD25^+$ donor Treg may have suppressed the capacity of mast cells to degranulate. Indeed, only *ex vivo* stimulated splenocytes from recipients transferred with cells from fish oil fed whey-donors showed increased IL-10 secretion which was in association with proliferation.

We hypothesized the $CD25^+$ Treg to be a potential candidate mediating the fish oil induced oral tolerance to whey. The importance of $CD25^+$ Treg in oral tolerance induction to cow's milk was demonstrated in several studies. The percentage of circulating $CD25^+$ Treg was enhanced and *in vitro* proliferation of PBMC reduced in tolerant compared to cow's milk allergic patients (28). Shreffler *et al.* reported enhanced Treg proliferation in heated milk tolerant versus allergic individuals, while depletion of $CD25^+$ cells increased effector T-cell proliferation (29). Similar results confirming Treg involvement were found by Sletten *et al.* (30). In mice iTreg also contribute to oral tolerance induction and transfer of tolerance (13, 31). Indeed, *ex vivo* Treg depletion with anti-CD25 antibody prior to transfer completely abrogated the transfer of tolerance in this study. Humoral factors were not enhanced; therefore loss of $CD25^+$ Treg or suppressive mediator secretion by these cells (e.g. IL-10) may have resulted in development of the ASR. The reduced ASR and abrogation of temperature drop in recipients transferred with $CD25^+$ Treg-depleted cells from control diet fed whey-donors probably was the result of a partial deletion of effector T-cells while the increase of IgG2a possibly was caused by a disbalanced Th response as a result of depletion of both Treg and effector cells.

To confirm the contribution of Treg in the tolerizing effect of the fish oil diet, $CD4^+CD25^+$ Treg of whey-sensitized donors were isolated and transferred. The $CD25^+$ enriched cell population of both control and fish oil fed donors showed suppressive effects on the ASR as well as serologic parameters. Hence, transfer with Treg alone was capable of complete prevention of sensitization in recipient mice. Yamashita *et al.* also reported reduced ovalbumin-specific IgE in recipients when transferring isolated $CD25^+$ Treg independent from tolerance induction in donors (22). Transfer of whole splenocyte suspension from control diet fed whey-donors containing this functional $CD25^+$ Treg population did not

suppress whey-allergy; hence this population of Treg probably was unable to overrule the immunological memory present in the whole cell suspension.

In the present study it was shown that the protective effect of fish oil on allergic sensitization could be adoptively transferred using splenocytes. The whey-specific ASR of recipients transferred with donor cells of whey-mice fed the fish oil diet was reduced, which correlated with a reduction in mmcp-1. *Ex vivo* $CD25^+$ Treg depletion prevented the transfer of tolerance from fish oil fed donor to control diet fed recipient mice. These results imply functional Treg to be involved in the prevention of cow's milk allergy induced by fish oil. Therefore fish oil supplementation early in life may support tolerance induction and herewith prevent the development op food allergy.

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CHAPTER SEVEN

Dietary DHA is more effective than EPA in suppressing allergic symptoms in whey or peanut allergic mice

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ABSTRACT

Background Recently, supplementation with long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) has been demonstrated to reduce the development of allergic disease. This study assessed whether dietary intervention with fish oil rich in either eicosapentaenoic acid (EPA) or docosahexaenoic (DHA) differentially affects allergic symptoms and whether these effects differ between food allergens.

Methods Mice were fed a control, EPA or DHA diet before and during oral sensitization with peanut extract (PE) or whey. Acute allergic skin responses, serum immunoglobulins and mucosal mast cell protease-1 (mmcp-1) as well as $FoxP3^+$ cells and T cell proliferation were assessed. Furthermore, hyperimmune serum was transferred to naive recipient mice fed the different diets to study whether the n-3 LCPUFA rich diets affected the acute allergic skin response independent of the effect on sensitization.

Results Dietary DHA but not EPA effectively reduced the acute allergic skin response in PE-allergic mice. In contrast, both DHA and EPA reduced the allergic skin response in whey allergic mice, however only the DHA diet reduced whey specific Th2-type immunoglobulins and serum mmcp-1. In PE-allergic mice PE-induced T cell proliferation tended to be suppressed while the intestinal $FoxP3^+$ cell number was increased by the DHA- compared to the EPA-rich diet. The DHA and EPA diet reduced allergic symptoms beyond suppression of sensitization, since the acute skin response was also reduced by the diets in passively immunized mice.

Conclusion DHA-rich fish oil was more effective than fish oil rich in EPA in reducing allergic sensitization to whey and symptoms in PE- and whey-allergic mice. The latter was partly due to a direct effect on the mast cell response.

INTRODUCTION

The prevalence of food allergies is increasing throughout the world and affecting up to 6% of children and about 4% of adults (1). Allergic reactions to milk, egg, peanuts, tree nuts and wheat are common nowadays and can result in symptoms such as eczema, diarrhea, and hypothermia, which sometimes even is life threatening (2). Peanut allergy represents the majority of cases of food-induced anaphylaxis (3). No definitive therapy for food allergy is available yet and therefore avoidance of the food allergen is the only possibility to prevent clinical manifestations (2).

Peanut allergy generally develops in childhood and is - in contrast to cow's milk allergy - in 80% of cases persistent for life (3). About 90% of children suffering from cow's milk allergy outgrow this condition. However, they are still predisposed to other allergic diseases (4, 5). Therefore, it is of great interest to test novel approaches for the management of allergic disease. Dietary components, including long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA), may be useful in the prevention or treatment of allergic diseases.

In the same period in which the prevalence of allergic disease was increasing dietary habits have largely changed. This includes an altered consumption of PUFA (6). The intake of n-6 PUFA (margarine, vegetable oils) has increased, while the consumption of n-3 LCPUFA found in oily fish is traditionally low in Westernized countries and has decreased further over the last decades (6, 7). These dietary changes are suggested to be involved in the increased prevalence of atopy (6, 8, 9). As a consequence increasing the dietary n-3 LCPUFA content was opted as a strategy to prevent allergic disease.

Recently, the beneficial effects of fish oil supplementation in the prevention of food allergy have been demonstrated. Dietary supplementation with fish oil rich in n-3 LCPUFA during pregnancy and lactation reduced the incidence of food allergy and/or eczema in children at risk for atopy (10-14). Moreover, postnatal n-3 LCPUFA supplementation during the first 6 months of life reduced allergen-specific Th2 responses (15). Beneficial effects of fish oil supplementation are supported by experimental studies, showing reduced food allergy in mice fed a diet rich in fish oil (16). We have previously shown that a diet rich in DHA suppresses the Th2-type humoral response and acute allergic symptoms to the cow's milk whey protein while regulatory T cells (Treg) were enhanced (17).

N-3 LCPUFA docosahexaenoic acid (DHA 22:6n-3) and eicosapentaenoic acid (EPA 20:5n-3) are thought to function via several mechanisms (18). Dietary lipids incorporate in the phospholipids of the cell membrane and thus can alter functionality of a large variety of cell types (19). EPA serves as a substrate for cyclooxygenases and lipoxygenases converting EPA into eicosanoids of the 3- and 5-series. These lipidic mediators are considered less pro-inflammatory than metabolites of the n-6 LCPUFA arachidonic acid (20, 21). Besides altering lipid mediator formation, LCPUFA may act on intra- and extracellular receptors and transcription factors involved in immune responses (22-25).

In addition to our previous study on the effects of a DHA-rich diet on whey allergy (17), in this study we compared the efficacy of dietary intervention with a fish oil high in EPA versus a fish oil rich in DHA in their ability to prevent allergic symptoms, using mice orally sensitized with peanut extract (PE) or whey.

MATERIALS AND METHODS

Diets

Semi purified cow's milk protein-free AIN-93G-based diets comprising either 10% soybean oil (control diet), 4% soybean oil plus 6% EPA oil (EPA diet) or 4% soybean oil plus 6% DHA oil (DHA diet) were prepared by Research Diet Services (Wijk bij Duurstede, The Netherlands). The extra fat compared to AIN-93G (7%) (26) was exchanged for cornstarch, as described before (17). The ratio n-6:n-3 PUFA was 9.5 for the control diet while this ratio was reduced to 0.7 for the EPA diet and 1 for the DHA diet. EPA oil was obtained from Equatec (Scotland). Tuna oil was used as a DHA oil and was a kind gift from Bioriginal, Den Bommel, The Netherlands. Table 1 shows the fatty acid composition of these lipid sources. The diets were stored at -20°C prior to use and refreshed weekly to prevent fatty acid oxidation.

Table 1 Fatty acid composition lipid source

Fatty acid	Soybean oil	EPA oil	DHA oil
	(% FA)	(% FA)	(% FA)
FA	15.1	14.7	28.9
MUFA	24.9	20.9	22.8
PUFA	59.1	54.9	44.5
n-6 PUFA	53.1	2.8	5.5
C18:2 n-6 LA	53.1	1.0	1.3
C20:4 n-6 AA		1.6	1.8
n-3 PUFA	5.6	52.1	38.5
C18:3 n-3 ALA	5.6	0.8	0.5
C20:5 n-3 EPA		28.8	7.0
C22:5 n-3 DPA		3.2	1.4
C22:6 n-3 DHA		13.7	27.8
Other FA	0.9	9.5	3.8
EPA:DHA	N/A	2.1	0.25

Percentages of fatty acids in the oils used as lipid source for the control diet (10% soybean oil), EPA diet (4% soybean oil plus 6% EPA oil) and DHA diet (4% soybean oil plus 6% DHA oil). AA: arachidonic acid; ALA: α-linolenic acid; DPA; docosapentaenoic acid; FA: fatty acid; LA: linoleic acid; MUFA: monounsaturated fatty acid.

Oral sensitization and challenge of mice

Animal use was performed in accordance with guidelines of the Animal Ethics Committee of Utrecht University. Three-week-old specific pathogen free female C3H/HeOuJ mice (Charles River Laboratories, L'Arbresle Cedex, France; n=4-8/group) were fed the control, EPA or DHA diet, starting two weeks prior to the first sensitization and continued during the whole sensitization period until sacrifice «Figure 1». Peanuts were kindly provided by Intersnack Nederland BV (Doetinchem, The Netherlands) and peanut extract (PE) was prepared as previously described (27). Whey protein concentrate 80 (indicated as whey) was obtained from DMV International (Veghel, The Netherlands).

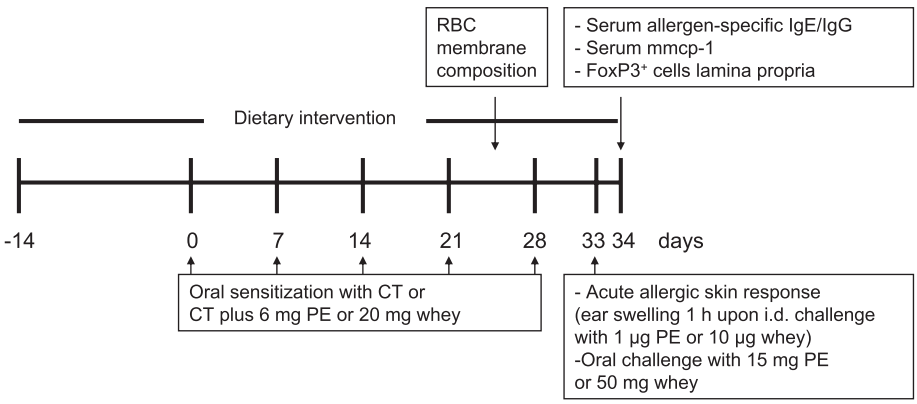

Figure 1 A schematic overview of the sensitization and challenge protocol and the parameters that are analyzed for the peanut and cow's milk allergy mouse model. CT: cholera toxin; mmcp-1: mucosal mast cell protease 1; PE: peanut extract; RBC: red blood cell.

Mice were sensitized intragastrically (i.g.) using a blunt needle with 6 mg PE or 20 mg whey in 0.5 mL PBS with 10 µg cholera toxin (CT; List Biological Laboratories Inc, Campbell, CA, USA) as an adjuvant while sham mice received CT only (10 µg/0.5 mL PBS). Mice were orally exposed once a week for 5 consecutive weeks as previously described by Schouten *et al.* (28). On day 28 mice were equipped with an implantable electronic ID transponder (Plexx, Elst, The Netherlands). One week after the last sensitization (day 33) mice were challenged intradermally (i.d.) in the ear pinnae of both ears with 1 µg PE or 10 µg whey in 20 µl PBS to assess their acute allergic skin response. The ear swelling was measured in duplicate for both ears using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands) and expressed as delta µm by subtracting basal ear thickness from ear thickness after 1 h. Isoflurane (5% in air) was used for inhalational anesthesia during measurements. Half an hour after the i.d. challenge the severity of the anaphylactic shock was scored using a validated anaphylactic shock scoring table from Li *et al.* (29) [see page 69] and body temperature was monitored. The same day the mice were

challenged i.g. with 15 mg PE or 50 mg whey in 0.5 mL PBS and 1 h and 18 h after the oral challenge blood samples were collected and centrifuged at 14,000*g* for 15 min. Sera were stored at -70°C until analysis. Mice were sacrificed at day 34 by cervical dislocation and spleen and small intestine were obtained for flow cytometry or histological examination.

Fatty acid composition erythrocytes

At day 24, blood was collected in heparin tubes and after centrifugation plasma was removed. Erythrocytes were stored at -70°C until analysis. Erythrocyte lipids were extracted as described by Bligh and Dyer (30) using C19:0 as an internal standard. The membrane fatty acid composition was assessed by gas chromatography as previously described (31).

Serum immunoglobulins and mmcp-1

Concentrations of mouse mast cell protease-1 (mmcp-1) were determined in serum collected 1 h after i.g. challenge using a commercially available ELISA kit (Moredun Scientific Ltd., Penicuik, UK) according to the manufacturer's protocol.

Levels of PE- and whey-specific IgE and IgG1 were determined by ELISA in serum (18 h after i.g. challenge) as previously described (17, 27). For peanut-specific immunoglobulins IgG1 highbond plates (Costar) were coated with 100 µl PE (10 µg/mL) in PBS for 18 h at 4°C, followed by 1 h blocking with 0.5% bovine serum albumin (BSA) in ELISA buffer (50mM TRIS, 137 mM NaCl, 2 mM EDTA and 0.05% Tween20; pH 7.2). Serum samples and serial dilutions of a reference serum (1000 arbitrary units of PE-specific antibodies of both immunoglobulin subclasses) were incubated for 2 h at room temperature. To detect IgG1 alkaline phosphatase-conjugated antibodies (Southern Biotechnology Associates) were incubated for 1 h at room temperature. Subsequently, p-nitrophenylphosphate (1 mg/mL) in diethanolamine buffer was used as a substrate and color reaction was stopped with 10% EDTA. Absorbance was measured at 405 nm.

PE-specific IgE levels were determined using a sandwich ELISA. Highbond plates (Costar) were coated overnight at 4°C with 1 µg/mL anti-IgE antibody (BD-Pharmingen, Erembodegem, Belgium) in PBS, followed by 1 h blocking with ELISA buffer. Serum samples and serial dilutions of a reference serum (containing 1000 arbitrary units of PE-specific IgE) were incubated for 2 h at room temperature. Subsequently, PE coupled to digoxigenin (DIG), diluted in HPE-buffer (Sanquin, Amsterdam, The Netherlands) was added for 1 h at room temperature. Coupling of DIG to PE was performed according to the instructions of the manufacturer (Boehringer, Mannheim). Streptavidin-coupled anti-DIG (Roche diagnostics) was added for 1 h at room temperature, followed by TMB (3,3',5,5'-tetramethylbenzidine) substrate (0.1 mg/mL). The color reaction was stopped after 15 min with 2 M H_2SO_4 and absorbance was measured at 450 nm.

For whey-specific immunoglobulins microlon plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 100 µl whey (20 µg/mL) in carbonate-bicarbonate buffer

(0.05M, pH = 9.6) for 18 h at 4°C, followed by 1 h blocking with ELISA buffer. Serum samples and serial dilutions of a reference serum were incubated for 2 h at room temperature. Subsequently, biotin-labeled rat anti-mouse IgE or IgG1 (1 µg/mL, BD Biosciences, Alphen aan den Rijn, The Netherlands) was incubated for 90 min at room temperature. Streptavidin poly horseradish peroxidase (Sanquin, Amsterdam, The Netherlands) was added for 1 h and developed with o-phenylendiamine (Sigma-Aldrich-Chemicals, Zwijndrecht, The Netherlands). The reaction was stopped with 4 M H_2SO_4 and absorbance was measured at 490 nm. Results were expressed as arbitrary units (AU) using a titration curve of pooled sera from whey-alum i.p. immunized mice as an internal standard.

Immunohistochemistry FoxP3$^+$ cells small intestine

The small intestine (jejunum and proximal ileum) was dissected, opened longitudinally and washed in PBS. The intestine was placed with the mucosal side down and rolled from the distal to the proximal end. The Swiss rolls were fixed in formalin (10% v/v solution) and embedded in paraffin (Leica IG1150c; Leica Microsystems, Rijswijk, The Netherlands). Sections (5 µm) were cut using a microtome (Leica Microsystems) and deparaffinized. Swiss roll sections were stained for intracellular FoxP3 expression as previously described (17). In short, after dewaxing the sections were boiled in sodium citrate buffer (0.01 M) for 10 min, blocked with 5% rabbit serum (Dako, Heverlee, Belgium) in PBS with 1% BSA, followed by overnight incubation at 4°C with rat anti-mouse FoxP3 purified antibody (final concentration 2.5 µg/mL, eBioscience) or rat IgG2a isotype (2.5 µg/mL) as a control in 1% BSA/PBS. Then slides were incubated for 30 min with 3% H_2O_2 in PBS. The primary antibody was detected with a biotinylated rabbit anti-rat antibody (2.5 µg/mL in 1% BSA/PBS, Dako) for 1 h at room temperature, followed by incubation with avidin biotin complex (ABC, Vector) in 1% BSA/PBS for 1 h. Color was developed with 3,3'-diaminobenzidine (Sigma) and sections were counterstained with hematoxylin, dehydrated and covered with Pertex mounting medium (Histolab, Göteborg, Sweden) and a cover glass. FoxP3$^+$ cells were counted per villus-crypt unit. Per mouse 180 intact villus-crypt units were counted, 60 per slide.

Proliferation of splenocytes

Spleen single cell suspensions ($4x10^5$ cells per well in triplicate) were incubated in RPMI1640 supplemented with penicillin (100U/ml), streptomycin (100 µg/ml) and 10% fetal calf serum (FCS) in the presence or absence of 100 µg/mL PE + 1.1 ng/mL IL-4 for 96 h at 37°C, 5% CO_2. Cultures were pulsed with tritiated thymidine ([^{3}H]-TdR, 0.25 µCi/well, Amersham, Aylesburym, UK) for 8 h. Cells were harvested using a Packard filtermate harvester and thymidine incorporation was determined by beta-plate liquid scintillation counter (Packard Topcount NXT) and expressed as count per minute (cpm).

Passive sensitization: transfer of hyperimmune serum to fish oil fed recipients

To generate hyperimmune sera, mice were immunized i.p. with 100 µg PE or whey in alum for 2-3 times after which blood was collected at day 28. Pooled ('hyperimmune') sera from peanut- or whey-alum i.p. immunized mice were i.v. transferred (100 µl) to isoflurane anesthesized (5% in air) naive mice. These naive mice were fed the control or fish oil diet for two weeks prior to injection (n=6/group). The acute skin response was measured 30 minutes after serum transfer as described before, starting with measuring ear thickness at basal conditions prior to allergen challenge followed by assessing ear swelling 1 h after i.d. challenge.

Histamine-induced acute skin response

Naive mice fed the control diet or fish oil diets for two weeks (n=6/group) received an i.d. injection in the pinnae of both ears with 20 µl saline as a control or 500 µg histamine bisphophate (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 20 µl saline. Ear thickness was measured at basal conditions prior to injection and 30 minutes after i.d. treatment.

Statistics

Data are presented as mean ± SEM and were analyzed using one-way ANOVA and post hoc Bonferroni test using GraphPad Prism software (version 5.0) or SPSS version 20. For anaphylactic shock score data Kruskall-Wallis followed by Dunn's multiple comparison test was used and medians are depicted in the graph. If required LOG transformation was used to normalize data distribution for analysis and then Tukey box-and-whisker plots were used.

RESULTS

Effect of n-3 LCPUFA rich diets on allergic sensitization depends on the allergen used

The efficacy of the dietary intervention was demonstrated by the increased n-3 LCPUFA content in erythrocyte membranes in mice fed the EPA or DHA diet, independent of the allergen used for sensitization «Table 2». Incorporation occurred at the expense of n-6 PUFA such as arachidonic acid.

Table 2 Erythrocyte membrane fatty acid composition

Diet	PE-sensitized						Whey-sensitized					
	AA (% FA)	SEM	EPA (% FA)	SEM	DHA (% FA)	SEM	AA (% FA)	SEM	EPA (% FA)	SEM	DHA (% FA)	SEM
CNTR	14.0[a]	0.23	0.10[a]	0.00	5.7[a]	0.08	15.1[a]	0.09	0.13[a]	0.02	6.2[a]	0.07
EPA	5.4[b]	0.05	6.8[b]	0.09	10.1[b]	0.08	5.4[b]	0.08	6.6[b]	0.10	10.1[b]	0.06
DHA	8.3[c]	0.11	2.5[c]	0.05	12.8[c]	0.10	8.6[c]	0.12	2.5[c]	0.06	13.4[c]	0.16

Membrane fatty acid content of PE- or whey-sensitized mice fed the control, EPA or DHA diet. One-way ANOVA followed by Bonferroni's multiple comparison test. Values are mean ± SEM, n=6-12. Means in a column without a common letter differ, $P<0.001$ for the percentage of erythrocyte membrane AA, EPA and DHA. AA: arachidonic acid; CNTR: control; FA: fatty acid.

To investigate allergic sensitization, allergen-specific Th2-type antibodies in serum were measured. Levels of whey-specific IgE and IgG1, enhanced due to the oral sensitization procedure, were reduced in whey-mice fed the DHA diet compared to control diet. In mice fed the EPA diet whey-IgE and -IgG1 levels were not significantly decreased «Figure 2A-B». Sensitization to PE increased PE-specific IgG1 and IgE compared to sham sensitization «Figure 2C-D». PE-specific IgE and IgG1 levels did not differ between control diet fed or n-3 LCPUFA fed PE-sensitized mice. The frequency of $FoxP3^+$ cells in the small intestine was not significantly affected upon PE-sensitization, however PE-sensitized mice fed the DHA-rich diet had significantly higher $FoxP3^+$ counts than mice fed the EPA diet «Figure 2E». Furthermore, PE-sensitized mice displayed an enhanced T cell proliferation in spleen 96h after *ex vivo* stimulation with PE plus IL-4 compared to sham-sensitized mice. This increase tended to be less pronounced in DHA fed PE-sensitized mice «Figure 2F».

Acute allergic skin response is most effectively reduced in DHA diet fed mice

Intradermal injection with allergen induced an acute allergic skin response, as shown by a significant increase in ear thickness in both PE- and whey-sensitized mice compared to sham-sensitized mice fed the control diet. In whey-sensitized mice, both the EPA and DHA diet were able to reduce the acute allergic skin response compared to control diet fed mice «Figure 3A». In addition, the drop in body temperature and anaphylactic shock score in whey-sensitized mice fed the DHA and the EPA diet showed a pattern in association with the reduced acute allergic skin response «Figure 3C-D». The DHA diet effectively reduced the PE-induced acute allergic skin response in PE-sensitized mice as compared to control and EPA diet fed mice «Figure 3E».

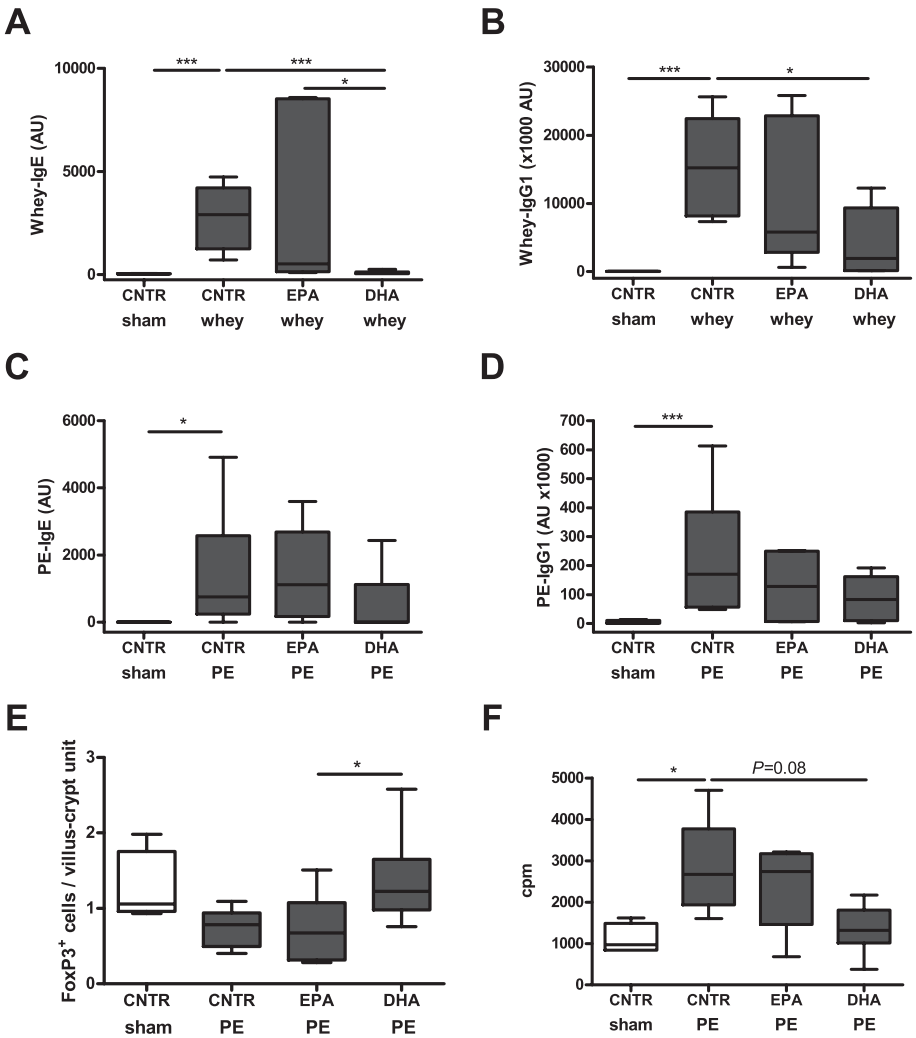

Figure 2 The effect of the EPA and DHA diet on allergic sensitization in whey- and peanut extract (PE)-sensitized mice. Mice were fed control, EPA or DHA diet and orally sensitized with cholera toxin (CT), CT+whey or CT+PE during 5 weeks. After an intradermal allergen challenge in the ear followed by an oral challenge, serum samples were collected and spleen isolated. Levels of (A) whey-IgE (B) whey-IgG1 (C) PE-IgE and (D) PE-IgG1 were determined by ELISA in serum. (E) $FoxP3^+$ cells in the lamina propria of the small intestine were assessed by immunohistochemical staining. Furthermore, 96 h after stimulation of splenocytes with PE plus IL-4 (F) T cell proliferation was measured. Data are presented as Tukey box-and-whisker plots, n=4-8. *$P<0.05$, **$P<0.01$, ***$P<0.001$ one-way ANOVA followed by Bonferroni's multiple comparison test after LOG transformation. AU: arbitrary units; CNTR: control; cpm: counts per minute.

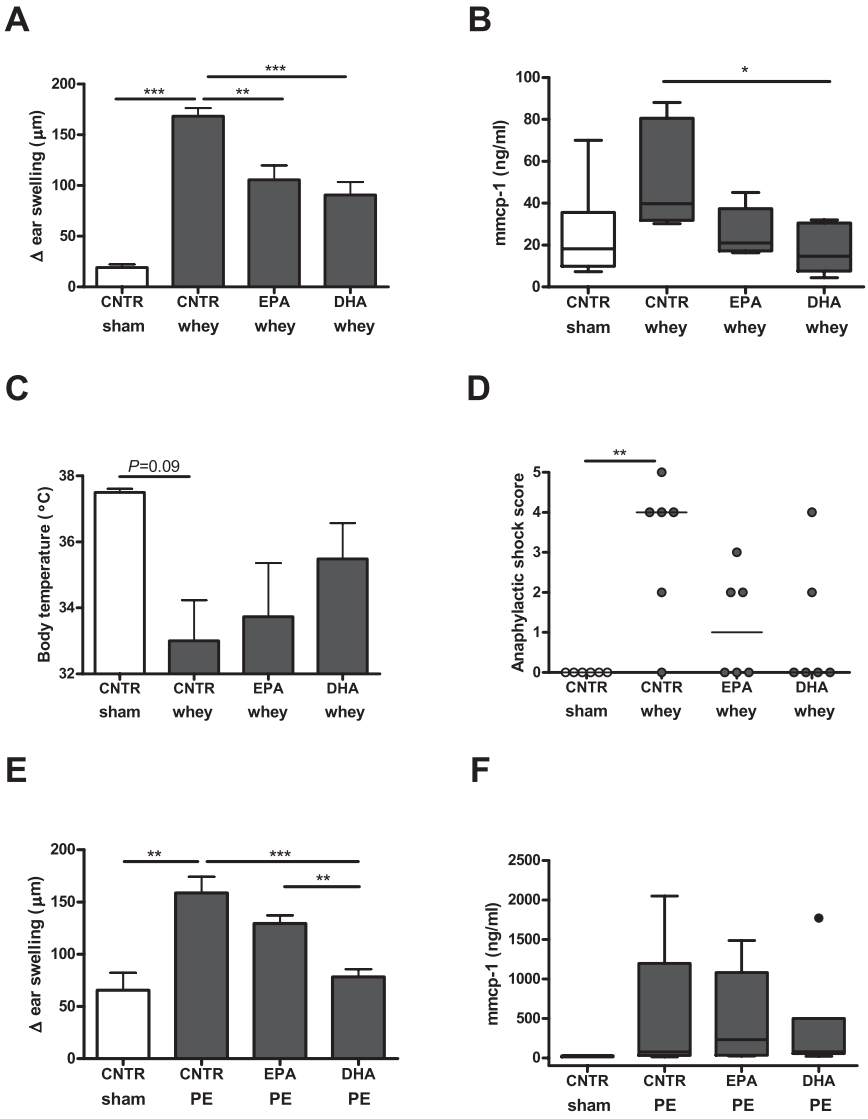

Figure 3 The allergic response evaluated in whey- and peanut extract (PE)-sensitized mice. Mice were fed control, EPA or DHA diet and orally sensitized with cholera toxin (CT), CT+whey or CT+PE during 5 weeks. The acute allergic skin response was assessed after intradermal challenge with (A) whey or (E) PE. Furthermore, (C) body temperature and (D) anaphylactic shock symptoms were assessed in whey-sensitized mice. One hour after an oral allergen challenge serum was collected. As a measure of mucosal, intestinal mast cell degranulation the concentration of mucosal mast cell protease 1 (mmcp-1) in serum of (B) PE- and (F) whey-challenged mice was measured. Data are presented as mean ± SEM, Tukey box-and-whisker plots (outlier indicated as dot) or medians, n=4-8. *$P<0.05$, **$P<0.01$, ***$P<0.001$ one-way ANOVA followed by Bonferroni's multiple comparison test (after LOG transformation for mmcp-1) or Kruskall-Wallis followed by Dunn's multiple comparison test for shock data. CNTR: control.

DHA oil reduces serum mmcp-1 in whey-sensitized mice

To determine the effect of n-3 LCPUFA on mucosal mast cell degranulation, mmcp-1 serum concentrations after oral challenge were measured. Although mmcp-1 did not significantly increase in both PE- and whey-sensitized mice fed the control diet compared to sham-sensitized mice «Figure 3B,F», in whey-sensitized mice fed the DHA diet mmcp-1 was significantly reduced compared to mice fed the control diet.

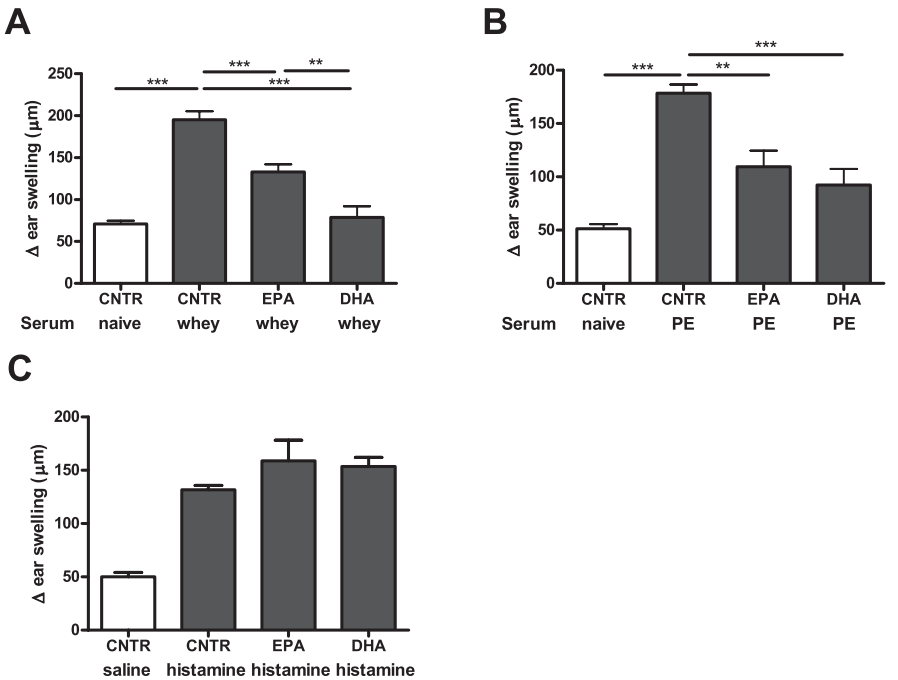

Figure 4 The effect of dietary EPA or DHA on the local effector response. Naive mice were fed control (CNTR), EPA or DHA diet for 2 weeks before passive transfer with peanut extract (PE)- or whey-hyperimmune sera high in immunoglobulins for the respective allergen. Naive serum was transferred as a control. After an intradermal allergen challenge in the ear the acute allergic skin response was measured in mice that received (A) whey- and (B) PE-hyperimmune sera. In addition, naive mice fed the control EPA or DHA diet for 2 weeks were injected in the ear with histamine or saline as a control after which (C) the acute allergic skin response was assessed. Data are presented as mean ± SEM, n=4-8. *$P<0.05$, **$P<0.01$, ***$P<0.001$ one-way ANOVA followed by Bonferroni's multiple comparison test.

N-3 LCPUFA reduce the local effector response in naive mice transferred with hyperimmune serum

Naive mice fed the different diets received an i.v. injection with PE- or whey-hyperimmune serum to investigate whether the n-3 LCPUFA rich diets affected the acute allergic skin response independent of the effect on sensitization. Ears were injected with PE or whey 30 minutes after serum transfer, and ear swelling as a consequence of the local effector response was assessed. Recipient mice injected with the hyperimmune serum and challenged with the corresponding allergen displayed a significant higher acute allergic skin response compared to mice injected with naive serum «Figure 4A-B». The acute allergic skin response was diminished in n-3 LCPUFA diet fed recipient mice as compared to control diet fed recipients for both PE and whey. The DHA diet was more effective than the EPA diet when whey was used as an allergen.

To reveal whether the n-3 LCPUFA directly affected the mast cell response or indirectly suppressed edema formation via an effect on surrounding tissues the histamine-induced ear swelling response in naive mice fed the control, EPA or DHA diet was assessed. The acute skin response induced by histamine injection was not altered by the different diets «Figure 4C».

DISCUSSION

In this study, we demonstrate that fish oil rich in DHA more effectively than fish oil rich in EPA suppresses both the sensitization to whey and the allergic symptoms in whey- or peanut-allergic mice.

Like in our previous study tuna oil rich in DHA largely prevented allergic sensitization to whey in mice (17). Tuna oil contains more DHA than EPA and *in vitro* DHA was more effective compared to EPA in the suppression of Th2 type cytokines (32).

The EPA and DHA diet used in this study approximate the n-3:n-6 PUFA ratio consumed by the Inuit population (2.5:1) (33). Inuit have a low prevalence of allergic disease, presumed to be associated to their high n-3 LCPUFA consumption (34). In our murine study, the n-3:n-6 intake ratio of 1.4:1 (EPA-diet) and 1:1 (DHA-diet) is relatively low compared to the ratio in the Inuit diet but we interpret this as the highest ratio that realistically could be achieved in humans by dietary intervention using fish oil soft gels. The EPA-diet, which contained more n-3 (LC)PUFA than the DHA-diet, lowered the AA content most pronounced, whereas the DHA diet reduced allergic sensitization and/or symptoms most effectively. This suggests that the amount of dietary DHA is more important for its effectiveness in reducing allergy than the membrane content of total n-3 (LC)PUFA or n-6 LCPUFA.

In whey-allergic mice, the Th2-type immunoglobulins are effectively suppressed by the DHA but not by the EPA diet. Previously we also demonstrated a diet high in DHA to

suppress the induction of whey-IgE and –IgG1 (17). Effects of dietary fish oil supplementation on the Th2 type humoral response differ between studies. In mice fed a 10% fish oil diet serum OVA-specific IgE levels tended to increase as compared to mice fed a diet rich in sunflower oil (35). However, in another study with different experimental design reduced OVA-specific IgE and -IgG1 levels were reported using a 7% fish oil diet (36). Although the DHA diet effectively suppressed allergic sensitization for whey, it lacked efficacy in the suppression of PE-IgE and -IgG1, even though in these PE-sensitized mice the reduction in ear swelling correlated positively with Th2-type immunoglobulins (data not shown). Possibly the intrinsic capacities of PE - a very strong allergen (37) - are the reason that EPA and DHA cannot effectively prevent induction of the Th2 type humoral response. However, the DHA diet enhanced intestinal FoxP3 counts in PE-sensitized mice compared to the EPA diet. These effects were less pronounced compared to the whey model in which we have previously shown that DHA oil increases Treg frequency in the intestine and spleen (17). Although in the PE-model the Treg frequency in the spleen did not increase by the diets (data not shown), the DHA and not the EPA diet showed a strong tendency to reduce splenic PE specific T-cell proliferation. This suggests that DHA did affect the adaptive response in PE allergic mice. However, these effects may have been too limited to lower the humoral immune response that is generated upon repeated PE exposure. Hence, the effectiveness of n-3 LCPUFA on the allergic sensitization probably depends on the nature of food allergens.

In whey-sensitized mice the local effector response and mucosal mast cell degranulation as determined by serum mmcp-1 (38) were reduced by the DHA diet. To determine the direct effects of n-3 LCPUFA on the local effector response naive recipient mice were fed the EPA and DHA diet and passively transferred with hyperimmune serum after which the acute skin response was determined. The ear swelling response (edema formation) depends on mast cell degranulation (e.g. release of preformed mediators including histamine), eicosanoids formation (e.g. prostaglandin D_2 production) (39) and the micro milieu of the mast cell (e.g. vascular permeability (40), and interaction with nerve endings (41)). Most studies suggest that n-3 LCPUFA do not reduce mast cell histamine release (42, 43). We demonstrated this as well however found that n-3 LCPUFA may alter the type of mediators secreted (32). Transfer of hyperimmune serum in naive mice fed the fish oil diets confirmed that n-3 LCPUFA suppress the local effector response for both food allergens independent of any possible effects on the adaptive immune response. This may be the result of the reduced formation of eicosanoids of the 2/4 series involved in vasodilation (39). The acute skin response after histamine injection remained unaffected strengthening the finding that n-3 LCPUFA affect mast cell function.

Although the EPA-rich diet contained more n-3 LCPUFA and the AA membrane content in EPA-fed mice was lower, the DHA-diet reduced IgE and allergic symptoms most effective. Currently, it is unclear why the DHA oil is more effective than EPA oil. Since both oils contain a mixture of EPA and DHA, perhaps the ratio of n-3 LCPUFA present may be

important. Furthermore, the molecular structure of the fatty acids may underlie this functional difference. DHA, which is more unsaturated than EPA, may enhance membrane fluidity most and thus affect lipid raft clustering more pronounced (44-47). *In vitro* we have shown that DHA is the most effective n-3 LCPUFA in reducing reactive oxygen species and IL-13 secretion by mast cells (32). A higher degree of unsaturation but not chain length, double bond position or configuration increased the potency of LCPUFA to inhibit endothelial expression of cell adhesion molecules, possibly via interference with reactive oxygen species (48). Furthermore, the n-3 LCPUFA receptor GPR120 binds DHA more potently than EPA (25). Together these phenomena may have contributed to a more efficient suppression of the allergic response by the DHA diet over the EPA diet.

In conclusion, in this study we demonstrated that dietary DHA but not EPA was effective in reducing the acute allergic skin response while tending to reduce systemic T cell proliferation in PE-allergic mice. In addition, DHA reduced whey-IgE and the local effector response in the ear more pronounced than EPA and reduced mucosal mast cell degranulation in the whey allergy model. Together this suggests that the DHA diet is more effective in reducing allergic symptoms than the EPA-rich diet, at least partially due to a direct effect on mast cells. This knowledge could be useful for future interventions intended to prevent or treat food allergic symptoms.

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CHAPTER EIGHT

Dietary fish oil improves endothelial function and lowers blood pressure via suppression of sphingolipid-mediated contractions in spontaneously hypertensive rats

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ABSTRACT

Background Long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) from oily fish reduce blood pressure (BP) in hypertension. Previously we demonstrated that hypertension is associated with marked alterations in sphingolipid biology and elevated ceramide-induced vasoconstriction. Here we investigated in spontaneously hypertensive rats (SHR) whether fish oil improves endothelial function including reduced vascular contraction induced via the sphingolipid cascade, resulting in reduced BP.

Methods Twelve-week-old SHR were fed a control or fish oil-enriched diet during 12 weeks, and BP was recorded. Plasma sphingolipid levels were quantified by mass spectrometry and the response of isolated carotid arteries towards different stimuli was measured. Furthermore, erythrocyte membrane fatty acid composition, thromboxane (TX)A_2 formation and cytokine secretion in *ex vivo* lipopolysaccharide (LPS)-stimulated thoracic aorta segments were determined.

Results The fish oil diet reduced the mean arterial BP ($P<0.001$) and improved endothelial function, as indicated by a substantially increased relaxation potential towards *ex vivo* methacholine exposure of the carotid arteries ($P<0.001$). The n-3 LCPUFA diet resulted in altered levels of specific (glucosyl)ceramide subspecies ($P<0.05$), reduced membrane arachidonic acid content ($P<0.001$) and decreased thromboxane concentrations in blood plasma ($P<0.01$). Concomitantly, the fish oil diet largely reduced ceramide-induced contractions ($P<0.01$) which are predominantly mediated by TX. Furthermore, TXA_2 and interleukin-10 were reduced in supernatants of LPS-stimulated thoracic aorta of SHR fed the fish oil diet while RANTES was enhanced. This may contribute to reduced vasoconstriction *in vivo*.

Conclusion Dietary fish oil lowers BP in SHR rats and improves endothelial function in association with suppression of sphingolipid-dependent vascular contraction.

INTRODUCTION

Hypertension is one of the major risk factors for cardiovascular disease and it is the strongest risk factor for mortality worldwide (1, 2). The prevalence of hypertension is rising and has reached pandemic proportions, affecting around 30% of the population (3). Hypertension is associated with endothelial dysfunction, which is mainly defined by decreased vasodilatory potential, increased contractile factor release (e.g. thromboxane A_2), and endothelial activation (e.g. cytokine release) (4-6).

Next to classical pharmaceutical interventions to reduce blood pressure (BP) in hypertensive patients, there is an increasing interest for nutraceuticals, or functional foods, as part of an advocated lifestyle intervention. Prehypertensive patients, predisposed to develop hypertension as a consequence of their genetic background, may benefit from this. Meta-analysis showed that the beneficial effects of fish consumption, rich in long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA), are particularly prevalent in high-risk populations (7). Supplementation with n-3 LCPUFA at a relatively high dose (3-9 g per day) markedly lowered BP in moderately hypertensive subjects (8, 9). A hypotensive effect on systolic but not diastolic BP was demonstrated even at low n-3 LCPUFA doses (150 mg docosahexaenoic acid (DHA) + 30 mg eicosapentaenoic acid (EPA)) (10). However, not all studies demonstrate a hypotensive effect of n-3 LCPUFA on blood pressure (11). Still, many of these studies did not assess the effect of fish oil on endothelial dysfunction, which contributes to elevated BP and a persistent risk of developing end-stage organ damage (12, 13).

We have previously shown that hypertension is associated with altered sphingolipid levels in both hypertensive humans and spontaneously hypertensive rats (SHR). Vascular and blood plasma levels of the bioactive sphingolipid ceramide were higher in SHR than normotensive rats. Furthermore, in hypertensive subjects plasma ceramide levels correlated positively with their BP. In addition, pharmacological elevation of ceramide using sphingomyelinase in isolated vessels of SHR but not normotensive rats, results in vasoconstriction due to the endothelium-dependent production of the contractile factor thromboxane A_2 (TXA_2) and increases BP *in vivo* (14). High ceramide levels stimulate the activation of calcium-independent phospholipase A_2 ($iPLA_2$) which releases arachidonic acid (AA) from the cell membrane. This n-6 LCPUFA serves as a substrate for cyclooxygenase-1 (COX-1) and thromboxane synthase (TXAS) involved in TXA_2 synthesis (15, 16). Expression of these enzymes is elevated in the SHR vasculature (14).

In this study we assessed whether n-3 LCPUFA from fish oil lower BP in SHR by improving endothelial function in association with modulation of sphingolipid-initiated vascular contraction. Fish oil intake indeed reduced BP in SHR and substantially improved endothelial function as determined *ex vivo* by methacholine-induced relaxation of carotid arteries. Furthermore, endothelial function restoration was associated with altered plasma ceramide and glucosylceramide subspecies, decreased erythrocyte cell membrane

AA content and lowered plasma TXA_2 concentrations. In accordance, ceramide-induced contractions of carotid arteries were largely reduced in fish oil compared to control diet fed SHR. *Ex vivo* mediator secretion by lipopolysaccharide (LPS)-stimulated vessels was altered by the fish oil diet which may relate to the reduction in arterial vascular tone *in vivo*. Therefore, a high n-3 LCPUFA diet may protect against the development or progression of essential hypertension by improving endothelial function including suppression of sphingolipid-induced vascular contraction.

MATERIALS AND METHODS

Chemicals

Acetyl-β-methylcholine, phenylephrine, bovine serum albumin (BSA, fatty acid free, low endotoxin) and EDTA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Neutral sphingomyelinase C (SMase; from *Staphylococcus aureus*) was purchased from Enzo Life Sciences (Antwerp, Belgium), U46,619 from Cayman Chemical Co. (Ann Arbor, MI, USA) and lipopolysaccharide (purified *E. coli* 0111:B4 LPS) from Invivogen (San Diego, CA, USA). Furthermore ketamine (Eurovet, Putten, The Netherlands), dexmedetomedine (Orion Pharma, Amsterdam, The Netherlands), atropine sulphate (PCH, Teva Pharmachemie, Haarlem, The Netherlands) and NaCl (Calbiochem, Merck KGaA, Darmstadt, Germany) were used. Other chemicals were from Merck Chemicals (Merck KGaA).

Animals

Animal use was performed in accordance with guidelines of the Animal Ethical Committee of the University of Amsterdam, The Netherlands. Twelve-week-old male spontaneously hypertensive rats (SHR; Charles River, Maastricht, The Netherlands) were fed soy-protein-based AIN-93G (17) containing 7% soybean oil (control diet) or a fish oil diet containing 3% soybean oil plus 4% tuna oil (Research Diet Services, Wijk bij Duurstede, The Netherlands). Tuna oil (38.5% n-3 PUFA) was a kind gift from Bioriginal (Den Bommel, The Netherlands) and contained 27.8% DHA and 7% EPA. The ratio n-3:n-6 PUFA was 1:9.5 for the control diet while this ratio was reduced to approximately 1:1 for the fish oil diet. Rats were fed the diets during twelve weeks after which they were sacrificed.

Blood pressure (BP) measurements

Intra-arterial BP measurements were performed after rats were anesthetized by intraperitoneal (i.p.) injection with a mixture of ketamine (90 mg/kg), dexmedetomedine (0.125 mg/kg) and atropine sulphate (0.05 mg/kg). Furthermore, heparin (750 IU, Leo Pharma B.V., Weesp, The Netherlands) was injected i.p. to prevent blood coagulation. For this purpose a PE-50 canula with PE-10 fused tip was inserted into the left femoral artery. Arterial pressure was recorded using LabChart data acquisition software (ADInstruments

Ltd, Oxford, UK). When BP tracking stabilized, baseline values of BP were recorded and averaged over 10-15 min. Hereafter, blood plasma, organs and blood vessels were collected and processed. After tissue isolation the rats were euthanized by exsanguination.

Arterial preparation and isometric force recording

Carotid arteries were isolated and placed in carbogen aerated (95% O_2; 5% CO_2) Krebs-Henseleit buffer (pH 7.4; in mmol/L: 118.5 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 1.1 KH_2PO_4, 0.025 EDTA and 5.6 glucose) at room temperature (RT). After removing connective and adipose tissue, vessel segments were mounted in a multi-channel wire myograph organ bath (M610, Danish Myo Technology A/S, Aarhus, Denmark) containing 37°C Krebs-Henseleit buffer under continuous carbogen aeration for isometric force measurement. Arterial lumen diameter was normalized according to Mulvany & Halpern (1977) and the experimental protocol was followed as previously described (18). Briefly, during normalization all segments were individually stretched until the internal circumference was 90% of which the segments would have at transmural pressure (100 mmHg). Then, vessel segments equilibrated during 30 min before starting a training protocol. When applicable Krebs buffer was replaced every 15 min. Two high K^+-containing Krebs buffer contractions were performed (pH 7.4; in mmol/L: 23.2 NaCl, 100.0 KCl, 25.0 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 1.1 KH_2PO_4, 0.025 EDTA and 5.6 glucose) with 30 min washout in between. Then, vessels were contracted with the α_1-adrenoceptor agonist phenylephrine (0.6 µmol/L) inducing a stable contraction >60% of the K^+-induced contraction. Relaxation was induced by methacholine (10 µmol/L) to assess endothelial integrity. Subsequently, after 30 min wash-out, a third high K^+-Krebs buffer contraction was performed. After 30 min wash out, the enzyme sphingomyelinase (SMase; 0.1 U/mL) was applied to the organ baths to measure alterations in vasomotor tone during 1 hour. In other arteries, concentration-response curves of methacholine during phenylephrine-induced contractions or the thromboxane analogue U46,619 were generated.

Liquid chromatography – mass spectrometry of plasma

Blood was collected by cardiac puncture using a 21G needle (BD Microlance 3) in a lithium heparin tube (4 ml Vacutainer Plasma Tube, BD). Blood samples were centrifuged for 20 min at 1600 g at 4°C and plasma samples were stored at -70°C. Lipids were extracted from plasma according to Wijesinghe *et al.* (19) and Merrill *et al.* (20) with slight modifications as described in Spijkers *et al.* (14). Briefly, to 33 µl of plasma 167 µl water, 1 mL methanol and 0.5 mL chloroform were added. An internal standard was added containing 500 pmol of the following; d17:1 sphingosine, sphinganine, spingosine-1-phosphate and sphinganine-1-phosphate, and d18:1/12:0 ceramide, ceramide-1-phosphate, sphingomyelin and glucosylceramide. The mixture was sonicated and incubated at 48°C overnight. The following day, extracts were subjected to base hydrolysis for 2 hours at 37°C using 150 µl of 1 mol/L methanolic KOH. Following base hydrolysis the extract was completely neutralized by the

addition of glacial acetic acid which was confirmed by pH measurement. Half of the extract was dried down and brought up in reversed phase sample buffer (60%A:40%B) (A= methanol:water 60:40 with 5 mmol/L ammonium formate and 1% formic acid, B= methanol with 5 mmol/L ammonium formate and 1% formic acid). To the remainder of the extract 1 mL chloroform and 2 mL water were added, and the lower phase was transferred to another tube, dried down and brought up in normal phase sample buffer (98%A:2%B). Sphingosine, sphinganine, sphingosine-1-phosphate, sphinganine-1-phosphate and ceramide-1-phosphate were quantified via reversed phase HPLC ESI-MS/MS using a Discovery C18 column attached to a Shimadzu HPLC (20AD series) and subjected to mass spectrometric analysis using a 4000 Q-Trap (Applied Biosystems) as described by Wijeshinge *et al.* (19). Ceramides, sphingomyelins and monohyexosyl ceramides were quantified via normal phase HPLC. ESI-MS/MS using an amino column (Sigma) as described by Merrill *et al.* (20). Finally, the quantified lipids were normalized to the volume of the material injected into the column.

Fatty acid composition erythrocytes

After removal of plasma, erythrocytes were stored at -70°C until analysis. Erythrocyte lipids were extracted as described by Bligh and Dyer (21) and the membrane fatty acid composition was assessed using gas chromatography as previously described (22).

Mediator production

Thoracic aorta segments were *ex vivo* stimulated with 10 µg/mL LPS for 24 h in MEM199 (Gibo, Invitrogen, Bleiswijk The Netherlands) with penicillin (100 U/ml) and streptomycin (100 mg/ml). Supernatants were stored at -70°C. Cytokine release was assessed in supernatants by multiplex assay (Bio-Plex Pro, Rat 23-plex, Bio-Rad, Veenendaal, The Netherlands). Concentration of TXB_2, the stable metabolite of TXA_2, was measured in plasma samples and supernatants according to the manufacturer's instructions (Cayman Chemical, Thromboxane B_2 EIA kit).

Statistics

Data are presented as mean ± SEM and analyzed using Student's *t*-test or two-way ANOVA followed by Bonferroni's multiple comparison test using Graphpad Prism Software version 5.0 (San Diego, CA, USA). Values of $P<0.05$ were considered statistically significant.

RESULTS

The fish oil diet lowers BP and improves endothelial relaxation potential to methacholine

After 12-weeks of dietary intervention the intra-arterially measured mean arterial BP in anesthetized SHR was lower in fish oil diet fed than control diet fed rats «Figure 1A». Supplementation with fish oil rich in n-3 LCPUFA did not significantly affect rat body weight or heart and lung weight «Table 1».

Arteries of fish oil fed SHR displayed a significantly enhanced endothelium-dependent relaxation in response to methacholine compared to control diet fed rats «Figure 1B». Contractile responses of isolated carotid artery rings to phenylephrine (0.6 µmol/L) were unaltered, while the smooth muscle contractile response to K^+ (100 mmol/L) was slightly enhanced in vessels of SHR fed the fish oil compared to the control diet (data not shown).

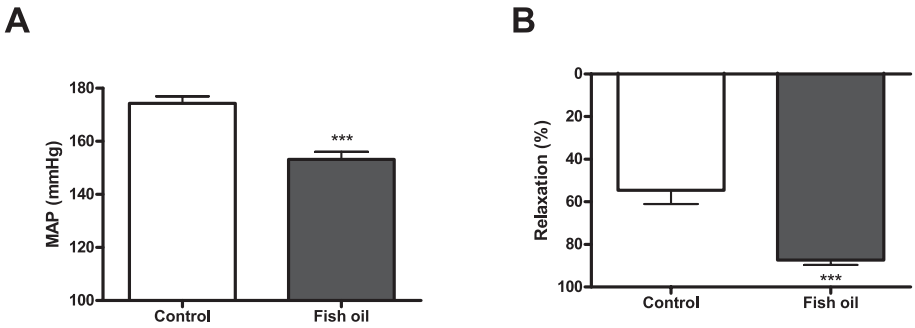

Figure 1 Blood pressure and endothelium-dependent arterial relaxing response in SHR. (A) mean arterial pressure (MAP) recorded in anaesthetized rats after 12 weeks of dietary intervention. (B) Percentage relaxation of carotid artery after phenylephrine-induced contraction followed by a cumulative dose of metacholine of 30 µmol/L. Data presented as mean ± SEM, n=7-8, ***P<0.001 Student's t-test.

Table 1 General characteristics of control and fish oil fed spontaneously hypertensive rats and *ex vivo* carotid artery segments.

	Control diet		Fish oil diet	
	Mean	SEM	Mean	SEM
Rat body weight start (g)	315	6	310	9
Rat body weight 12 weeks (g)	401	7	404	10
Normalized heart weight (mg/g)	3.9	0.1	3.8	0.1
Normalized lung weight (mg/g)	3.5	0.1	3.5	0.1

Heart and lung weight were normalized against rat body weight, n=7-8

Dietary fish oil alters fatty acid composition of plasma ceramide and glucosylceramide

Lipidomic analysis of plasma samples demonstrated that the ceramide subspecies C16:0, C22:0, C24:0 and C26:0 were significantly decreased after fish oil supplementation, with only C24:1 ceramide levels elevated «Figure 2A». The total ceramide content (cumulative levels of all acyl chain lengths) was not altered by these changes. In addition, elevated levels of C20:0 and C24:1 glucosylceramide were detected in plasma of fish oil fed SHR «Figure 2B», resulting in a 30% increase in total glucosylceramide levels.

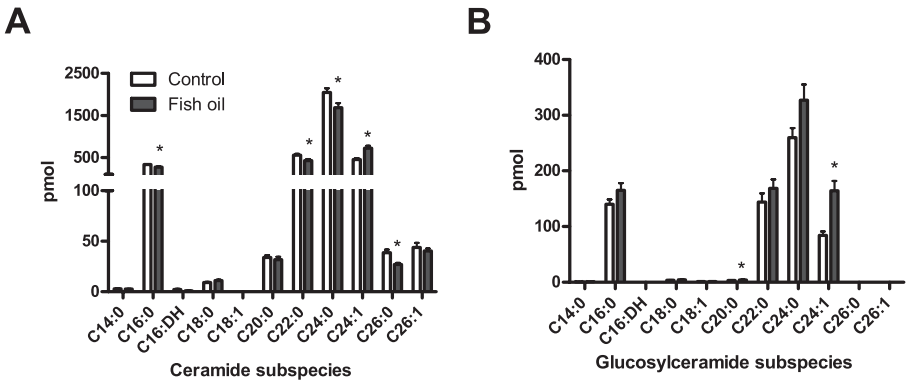

Figure 2 Liquid-chromatography-mass spectrometry measurements of plasma sphingolipid content in control and fish oil fed SHR. Quantification of the spectrum of single subspecies of (A) ceramide and (B) glucosylceramide. Data presented as mean ± SEM, n=7-8, *P<0.05 Student's t-test. Dihydroceramide: DH.

Exchange of AA with DHA and EPA in SHR erythrocyte membranes after fish oil diet

Ceramide stimulates the release of AA from the cell membrane by activation of $iPLA_2$. AA serves as a substrate for the synthesis of TXA_2. Substitution of AA by n-3 LCPUFA DHA and EPA in the cell membrane reduces TXA_2 formation and therefore vasoconstriction. Fish oil supplementation enhanced the DHA content of erythrocyte cell membranes by more than three-fold as compared with control diet fed rat erythrocytes «Figure 3A». In addition, the percentage of EPA in the membranes increased in the fish oil group (0.1±0.02% control versus 1.3±0.04% fish oil, n=7-8, P<0.001). The incorporation of n-3 LCPUFA in the erythrocyte membrane occurred at the expense of n-6 PUFA. Membrane AA content was reduced by 20% «Figure 3B».

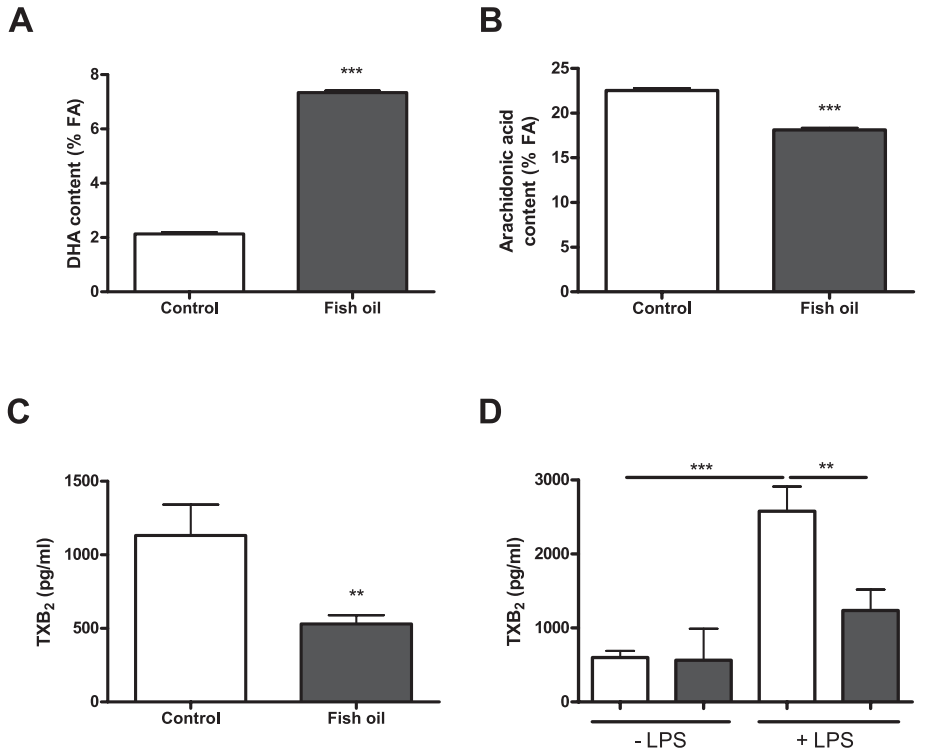

Figure 3 Erythrocyte membrane fatty acid (FA) content and thromboxane B_2 (TXB_2) generation. Content of n-3 LCPUFA (A) DHA and n-6 LCPUFA (B) arachidonic acid in erythrocyte cell membranes of control and fish oil fed SHR. TXB_2 content in (C) plasma and (D) supernatants of thoracic aorta *ex vivo* stimulated with lipopolysaccharide (LPS) for 24 hours. Data presented as mean ± SEM, n=7-8, $^{**}P<0.01$, $^{***}P<0.001$ Student's *t*-test. Two-way ANOVA followed by Bonferroni's multiple comparison test to analyze TXB_2 in supernatants.

Fish oil supplementation reduced TXB_2 concentrations in SHR plasma and supernatants of thoracic aorta upon *ex vivo* LPS stimulation

The concentration of circulating TXB_2 (the stable metabolite of TXA_2) in plasma of fish oil fed SHR was effectively reduced compared with the control group «Figure 3C». Plasma TXB_2 concentrations correlated positively with systolic BP ($r=0.62$; $P<0.05$) and mean arterial pressure ($r=0.76$; $P<0.01$). Furthermore, *ex vivo* LPS stimulation of thoracic aorta segments of control diet fed rats resulted in an increase in TXB_2 in supernatants «Figure 3D». This concentration was significantly reduced in the fish oil diet group.

Previously, enhanced expression of $iPLA_2$, COX-1 and TXAS was demonstrated in SHR compared with Wistar Kyoto (WKY) rat vessels (14). These enzymes are responsible for TXA_2 synthesis. Fish oil supplementation did not alter expression of these enzymes in endothelium or smooth muscle cells as determined by immunohistochemistry (See Supplementary material on page 178).

Fish oil diet reduces SMase-induced contractions

Addition of exogeneous SMase to increase ceramide levels in isolated carotid arteries resulted in a marked endothelium-dependent contraction «Figure 4A» in vessel segments of control diet fed SHR, which was reduced >70% in the fish oil diet group segments «Figure 4B». We have previously shown that these constrictions were mediated by the release of TXA_2. The thromboxane receptor sensitivity remained unaltered by fish oil, as demonstrated by an equal dose-response curve of the thromboxane analogue U46,619 in control SHR carotid artery segments (pEC_{50}: 7.2±0.2, E_{max}: 4.8±0.1 mN/mm) compared with fish oil fed rat artery segments (pEC_{50}: 7.1±0.2, E_{max}: 5.2±0.2 mN/mm) (n=7-8, $P>0.05$).

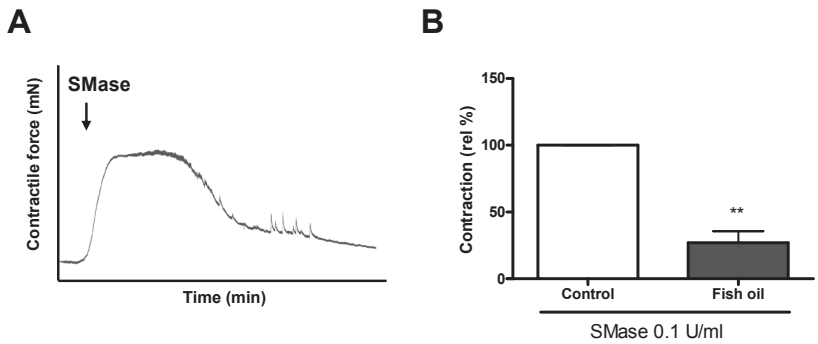

Figure 4 Sphingomyelinase-induced contractions in SHR carotid artery. (A) Typical vasomotor tracing of rat carotid artery segments exposed to sphingomyelinase (SMase, 0.1 U/ml). (B) Maximal contractile responses to SMase as a relative percentage of the control group. Data presented as mean ± SEM, n=7-8, $^{**}P<0.01$, Student's *t*-test.

Dietary fish oil alters mediator secretion by *ex vivo* LPS-stimulated thoracic aorta

Ex vivo stimulation with LPS for 24 h induced the secretion of a plethora of mediators from thoracic aorta segments «Figure 5». Aorta tissue from fish oil fed SHR secreted significantly less interleukin (IL)-10 after LPS stimulation when compared to control diet fed rats. For the other T cell cytokines IL-2, interferon (IFN)-γ and IL-4 there was no significant effect of the fish oil diet. Tumor necrosis factor (TNF)-α secretion was not affected by the fish oil diet, while the production of the chemokine RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) was significantly increased in LPS-stimulated vessels. On the other hand, granulocyte-macrophage colony-stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF) concentrations were reduced in supernatants of isolated aortas of fish oil fed rats.

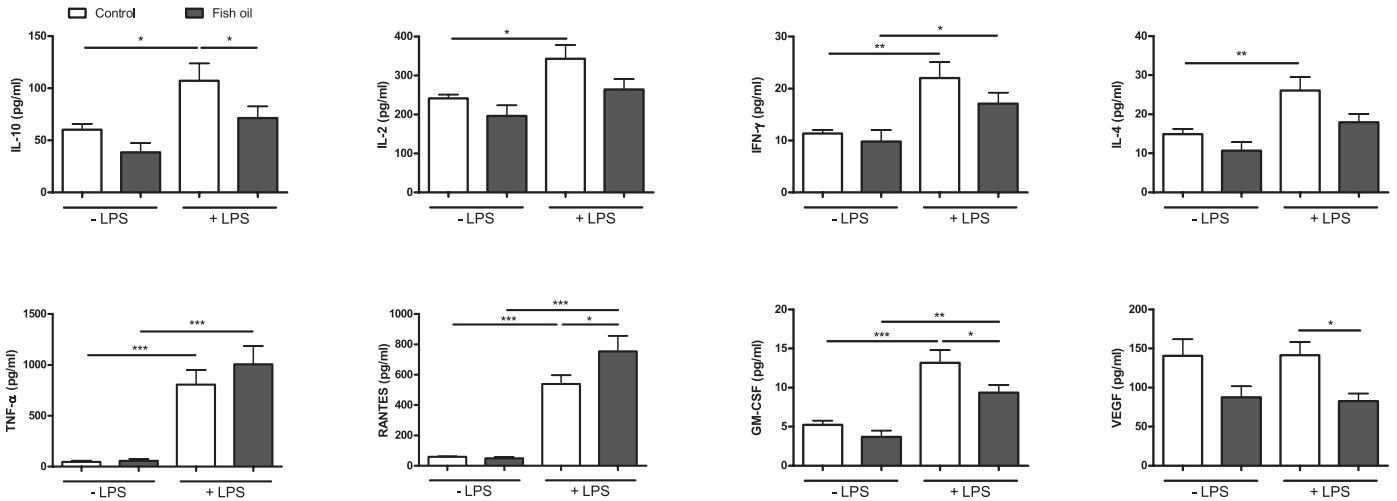

Figure 5 Cytokine concentrations in supernatants of *ex vivo* LPS-stimulated thoracic aorta segments. Data presented as mean ± SEM, n=7-8, *$P<0.05$, **$P<0.01$, ***$P<0.001$ two-way ANOVA followed by Bonferroni's multiple comparison test. GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin; IFN: interferon; RANTES: Regulated on Activation, Normal T cell Expressed and Secreted; TNF: tumor necrosis factor; VEGF: vascular endothelial growth factor.

DISCUSSION

In this study we assessed the mechanism by which n-3 LCPUFA from fish oil contributes to blood pressure lowering in essential hypertensive rats. Here we show that fish oil indeed has substantial beneficial effects on blood pressure and endothelial function. This may partially be achieved by a reduction in blood plasma ceramide subspecies which stimulate the formation of TXA_2 through activation of $iPLA_2$ and subsequent AA liberation. In addition, the substitution of AA by DHA and EPA in the cell membranes resulted in reduced TXA_2 formation and attenuated ceramide-induced contraction of carotid arteries. Furthermore, reduced TXA_2, IL-10 and enhanced RANTES secretion in supernatants of LPS-stimulated thoracic aorta of SHR fed the fish oil diet may relate to reduced vasoconstriction *in vivo*.

Many studies have demonstrated a negative association between fish consumption and cardiovascular and/or overall morbidity and mortality (23-28). In accordance, several studies have reported a blood pressure lowering effect by supplementation with EPA and/or DHA in both SHR (29-32) and hypertensive patients (33-36) and this effect has been confirmed by meta-analyses (9, 37). Importantly, the study by Tribolout *et al.* (2001) indicated that fish oil did not reduce BP in WKY rats (29), and also in normotensive humans no effect of fish oil supplementation on BP was reported (38). This suggests that fish oil reduces BP by targeting a mechanism primarily present in hypertensive subjects.

Here we demonstrate that a diet rich in fish oil was able to improve endothelium-dependent vasodilation thus improving endothelial function. SHR receiving a control diet demonstrated a lower maximal relaxation potential to methacholine than the fish oil group. This was likely due to increased endothelium-derived contractile mediators, of which thromboxane and prostacyclin are major contractile effectors (39). Similar results were shown by the use of acetylcholine in thoracic aortic segments in SHR (32). Mori *et al.* demonstrated enhanced vasodilator and reduced vasoconstrictor responses in the forearm microcirculation of overweight hyperlipidemic men using DHA but not EPA via endothelium-independent mechanisms (40). However, in the present study endothelium-independent mechanisms most likely only play a minor role since the contractile responses upon exposure to K^+ and the thromboxane analogue U46,619, both endothelium-independent constrictors, were not reduced by the fish oil diet.

A possible mechanism which is responsible for BP elevation and endothelium-dependent vasoconstriction in hypertension involves the modulation of the sphingolipid system. We have previously shown that hypertension in both humans and SHR rats is associated with elevated ceramide levels and marked ceramide-induced vascular contractions (8). This study provides evidence on n-3 LCPUFA being capable of provoking subtle alterations in sphingolipid biology in the SHR. Although total ceramide levels were not altered by the diet, we observed some divergent changes in distinct ceramide subspecies.

Furthermore, plasma glucosylceramide is reduced in SHR compared with WKY (manuscript submitted), and this imbalance was partly restored by the fish oil diet. These interesting results warrant further investigation. In addition, the endothelium-dependent contraction of isolated carotid arteries to exogenous SMase, which generates ceramide from ubiquitously present membrane sphingomyelin, was largely reduced in the fish oil group. Most likely this is the result of decreased membrane AA levels, resulting in reduced TXA_2 generation after activation of $iPLA_2$ by ceramide «see Figure 6». AA is the predominant PUFA present in human lipids. A diet rich in n-3 LCPUFA lowers the membrane AA content and consequently the production of AA-derived eicosanoids (41, 42). Indeed, in SHR erythrocyte membranes we demonstrated AA as one of the predominant fatty acids, which was reduced by the fish oil diet, as demonstrated by others (31). This substitution likely affects vascular substrate availability for COX to generate thromboxane in our experiments.

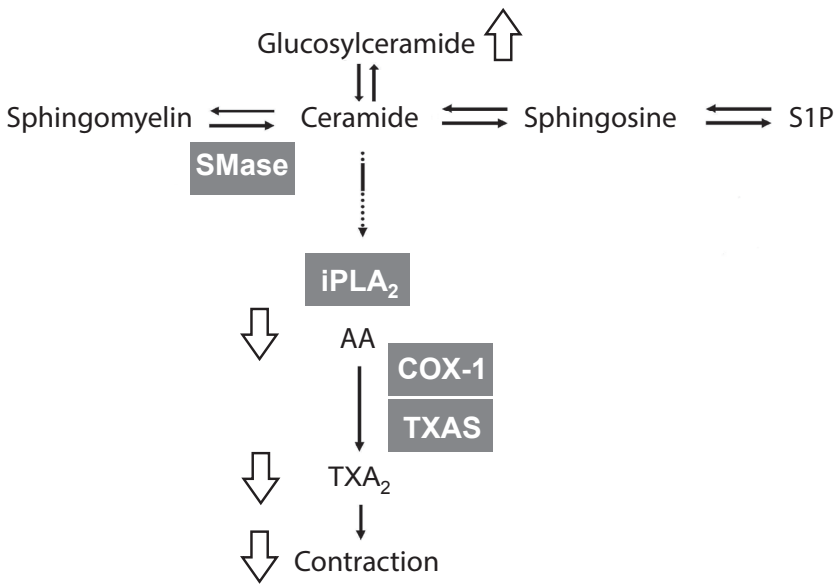

Figure 6 Suggested mechanism by which n-3 LCPUFA improve endothelial function in SHR and contribute to decreased blood pressure. Functional effects of fish oil diet are indicated by hollow arrows. AA: arachidonic acid; COX-1: cyclooxygenase 1; $iPLA_2$: calcium-independent phospholipase 2; SMase: sphingomyelinase; S1P: sphingosine-1-phosphate; TXA_2: thromboxane A_2; TXAS: thromboxane synthase.

We previously reported that the antihypertensive drug losartan decreased SMase-induced vasoconstriction by attenuating endothelial $iPLA_2$ expression. However, $iPLA_2$, COX-1 and TXAS expression, responsible for ceramide-induced thromboxane production, appeared unaffected by the intervention with fish oil. Thus, reduced substrate availability

is probably responsible for inhibition of the ceramide-thromboxane pathway. Indeed, the lower AA membrane content was reflected by lower plasma thromboxane levels in SHR fed with fish oil, like was shown in (32). Several studies reported the ability of inflammatory stimuli to activate endogenous SMases to generate ceramide (43) possibly leading to enhanced thromboxane release (14). In agreement with the latter, LPS treatment resulted in increased thromboxane release *ex vivo*, which was attenuated in the fish oil fed group.

However, not only LPS-induced TXA_2 release was affected by the fish oil diet, also secretion of other inflammatory mediators by the thoracic aorta was altered. In hypertension inflammatory cells are attracted to the activated endothelium and reside in the vascular wall. Inflammatory mediators released by endothelial and immune cells are characteristic for endothelial dysfunction. In patients with essential hypertension pro-inflammatory cytokines were increased in LPS-stimulated whole blood (44). Cytokines can directly modulate arterial vascular tone in isolated human vessels e.g. via the induction of SMases or endothelin-1 secretion, leading to vascular contraction (43, 45). Vascular endothelial cells express receptors for various cytokines including IL-10 and TNF-α but not GM-CSF. TNF-α and IL-10 have been shown to promote endothelium-dependent vasoconstriction in arterial but not venous rings, while GM-CSF did not show an effect on vascular tone (45). In this study, IL-10 was reduced in supernatants of LPS-stimulated thoracic aorta of SHR fed the fish oil diet which may relate to the *in vivo* reduced vasoconstriction. Furthermore, other T cell cytokines IL-2, IL-4 and IFN-γ showed a similar pattern, while TNF-α remained unaltered. High levels of circulating cytokines including IL-2 were previously associated with the development of hypertension and reduced secretion of IL-2 was shown after fish oil intervention in SHR but not WKY (29). In addition, the growth factors GM-CSF and VEGF were reduced in this study, while the chemokine RANTES was enhanced. Whereas RANTES is involved in the chemotaxis of monocytes, VEGF and GM-CSF have been described to activate monocytes which may contribute to the process of atherosclerosis (46-48). Reduced levels of the latter two may therefore counter balance the possible pro-inflammatory effect of RANTES. Furthermore, previously it has been shown that RANTES was downregulated in SHR, and this was suggested to induce hypertension (49, 50).

In conclusion, these results present altered sphingolipid signaling by fish oil as a mechanism that can prevent the rise in BP in SHR, as depicted in Figure 6. The intervention group presented less endothelial dysfunction as demonstrated by reduced ceramide-mediated contraction and enhanced relaxation to methacholine of carotid arteries, in association with reduced TXA_2 and IL-10 and enhanced RANTES secretion by *ex vivo* stimulated aortas. These findings provide novel mechanistic insight in how n-3 LCPUFA improve cardiovascular health.

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SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Antibodies

Antibodies against cyclooxygenase and thromboxane synthase were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA), calcium-independent phospholipase A_2 antibody from Abcam (Cambridge, UK), Von Willebrand factor antibody from GeneTex (Irvin, CA, USA) and Alexa Fluor 488-labeled and Alexa Fluor 546-labeled secondary antibody from Invitrogen (Carlsbad, CA, USA).

Fluorescent immunohistochemistry and quantification

Carotid artery rings were collected in ice-cold Krebs buffer directly after dissection. After cleaning the segments were rapidly submerged in TissueTek OCT compound (Sakura, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen with subsequent storage at -70°C. Frozen sections of 5 µm thick were cut on a Leica CM3050S cryostat and dried by cold air before storage at -70°C. Upon defrosting the slides were fixed in 100% ice-cold acetone during 1 min, and processed as described previously (1). The slides were washed shortly in PBS and incubated with blocking buffer (2% PBS/BSA) during 30 min at RT. After a short wash in 0.1% PBS/BSA the slides were incubated overnight at 4°C with the primary antibody dissolved in 0.1% PBS/BSA. Following a triple wash in 0.1% PBS/BSA during 5 min, the appropriate Alexa Fluor 546-labeled secondary antibody was applied during 1 hour at RT. After triple wash, the antibody against von Willebrand Factor (vWF) was applied during 1 hour at RT as an endothelium marker and finally Alexa Fluor 488-labeled secondary antibody was applied. Vessel slides were embedded in DAPI-containing mounting medium (UltraCruz, sc-24941) and vessels were imaged at RT using a Nikon Eclipse TE2000-U fluorescence microscope (Plan Fluor ELWD 20x objective, Nicon DXM1200F digital camera) with NIS Elements AR 2.30 software. Quantification of fluorescence (fluorescent light units; FLU) was performed using NIS Elements. Using vWF endothelial marker region, mean fluorescence intensity of the protein of interest was quantified of the endothelium by normalizing total fluorescence against untreated endothelial cell area. Then, the tunica media was selected and mean fluorescence intensity (MFI) was determined for smooth muscle cells. For both determinations, an intensity threshold as low as possible was selected to exclude background fluorescence and restricting the area of interest to mere tissue. All settings and exposure times were equally applied to all tissues.

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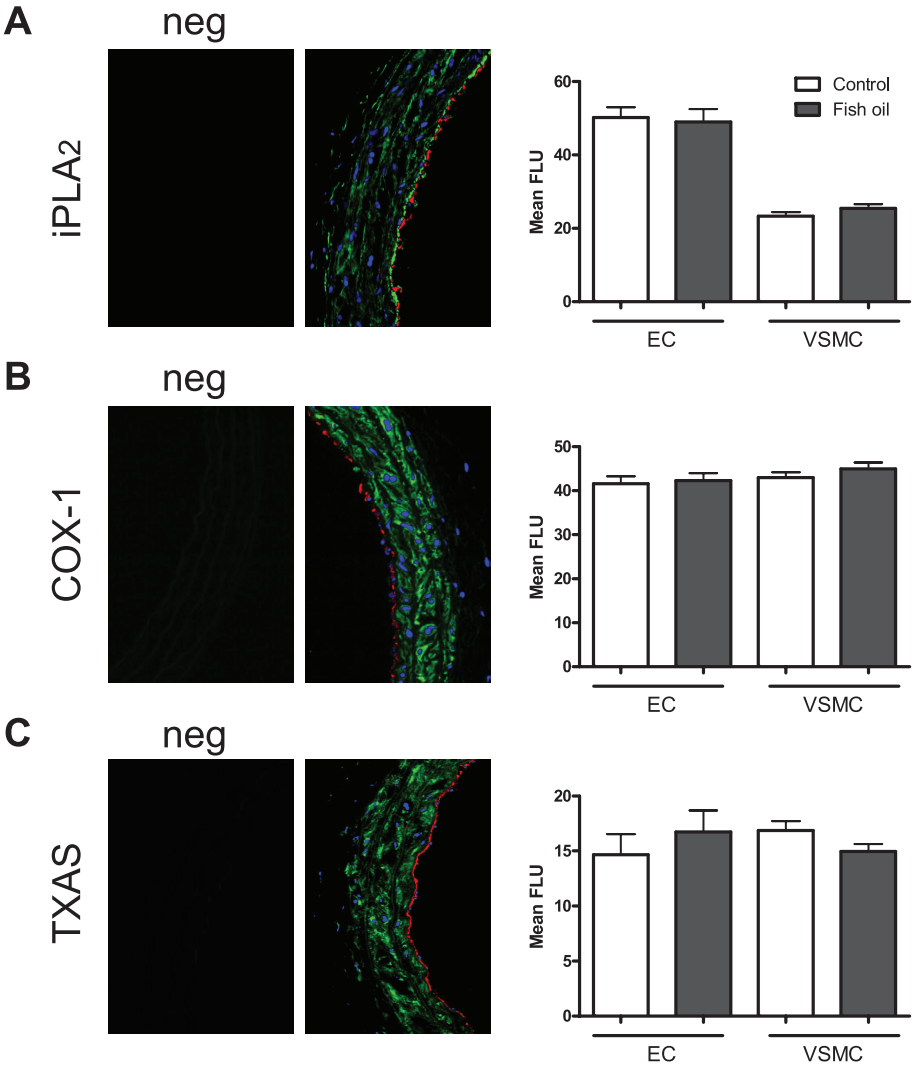

Supplementary Figure 1 Quantitative immunohistochemical protein expression in SHR carotid artery. Immunohistochemical staining (left, typical staining images; 200x magnification) and quantification (right) of SHR carotid artery segments depicting cell nuclei staining (blue), endothelial cell marker von Willebrand Factor (vWF) (green) and (A) calcium-independent phospholipase A_2 ($iPLA_2$; red) (B) cyclooxygenase-1 (COX-1; red) or (C) thromboxane synthase (TXAS; red) for both endothelial cells (EC) and vascular smooth muscle cells (VSMC). Data presented as mean ± SEM, n=7-8, Student's *t*-test.

CHAPTER NINE

Salmon consumption by pregnant women reduces *ex vivo* umbilical cord endothelial cell activation

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ABSTRACT

Background *In vitro* exposure of endothelial cells (EC) to long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) reduces cell adhesion molecule (CAM) expression. However, to our knowledge, no previous human studies have examined the influence of an altered diet on CAM expression. We assessed whether salmon (rich in n-3 LCPUFA) consumption twice a week during pregnancy affects offspring umbilical vein EC CAM expression.

Methods Women were randomly assigned to maintain their habitual diet or to consume two portions of salmon per week during pregnancy months 4-9. EC were isolated from umbilical cord veins collected at birth and cultured. The cell surface expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) was assessed by flow cytometry after the culture of EC in the presence and absence of bacterial lipopolysaccharide (LPS) for 24 hours. Cytokine and growth factor concentrations in culture supernatant fluid were measured by multiplex assay.

Results LPS increased expression of VCAM-1 and production of several cytokines and growth factors. The level of ICAM-1 expression per cell (i.e. median fluorescence intensity (MFI)) was increased by LPS stimulation in the control group (16.9 ± 2.4 vs 135.3 ± 20.2; $P<0.001$) and to a lesser extent in the salmon group (14.1 ± 3.8 vs 65.8 ± 22.4; $P=0.037$). The ICAM-1 MFI in the salmon group following LPS stimulation was lower than in the control group ($P=0.006$).

Conclusion Increased dietary salmon intake in pregnancy dampens offspring EC activation, which implicates a role for n-3 LCPUFA in suppression of inflammatory processes in humans.

INTRODUCTION

Cardiovascular disease (CVD) has become the leading cause of morbidity and mortality in developed countries. The most important contributor to CVD is atherosclerosis, a complex, chronic inflammatory process accompanied by accumulation of lipids and fibrous material within the walls of the large arteries (1). Vascular inflammation is often involved in the early stages of atherosclerosis and CVD. It can lead to upregulation of endothelial cell adhesion molecule (CAM) expression and pro-inflammatory cytokine production (1, 2). The activated endothelium expresses selectins, required for adhesion of leukocytes to the vessel wall. Leukocyte integrins become activated by chemokines and bind to endothelial intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) permitting firmer adhesion and diapedesis (3-5). Adhesion molecule expression on endothelial cells (EC), together with the secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, growth factors including granulocyte colony-stimulating factor (G-CSF) and transforming growth factor-β, and chemokines such as IL-8, forms an important event in inflammatory responses, vascular injury and atherogenesis (6-11).

Epidemiological and intervention studies have indicated that long chain n-3 polyunsaturated fatty acids (n-3 LCPUFA) such as eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3) reduce the risk of cardiovascular morbidity and mortality (12-18). Part of this protective effect may involve reduced inflammation (4). However, whether dietary n-3 LCPUFA target EC responses is not yet established. *In vitro* studies have shown that EPA and DHA can decrease expression of CAM on cultured EC and can decrease production of inflammatory cytokines by these cells (8, 19-21). There are no human studies investigating whether dietary n-3 LCPUFA affect the inflammatory response of EC, including CAM expression. The Salmon in Pregnancy Study (SiPS) (22) provided an opportunity to study human EC following a dietary intervention that increased both intake of n-3 LCPUFA and n-3 LCPUFA status in the mother and newborn infant. The aim of this work was to assess whether salmon consumption, providing enhanced intake of n-3 LCPUFA, twice a week during the second half of pregnancy affects the response of human umbilical vein EC (HUVEC) to an inflammatory stimulus *ex vivo*; CAM expression and inflammatory mediator release in response to lipopolysaccharide (LPS) were measured as outcomes. It was hypothesised that CAM expression and inflammatory mediator release would be lower in the salmon group than in the control group.

MATERIALS AND METHODS

Study design and population

Samples used in this study were from a small subgroup of participants involved in the Salmon in Pregnancy Study (SiPS), a dietary intervention study in pregnant women investigating the impact of maternal oily fish intake on health outcomes in pregnancy and early infancy (22). At 12 weeks of pregnancy women at risk of giving birth to an atopic baby were identified at antenatal clinics in Princess Anne Hospital, Southampton, UK and recruited into the study. This study was approved by the Southampton and South West Hampshire Research Ethics Committee (07/Q1704/43) and was registered at www.clinicaltrials.gov (Clinical trials identifier NCT00801502).

Recruited women were randomly assigned to one of two groups. Women in the control group (n=61) were asked to continue their habitual diet and women in the salmon group (n=62) were asked to incorporate two portions of farmed-salmon (150 g/portion) into their diet per week from study entry (week 20) until they gave birth. Farmed salmon for use in the SiPS was raised using dietary ingredients selected to contain low levels of contaminants. Each 150 g salmon portion contained (on average) 30.5 g protein, 16.4 g fat, 0.57 g EPA, 0.35 g docosapentaenoic acid, 1.16 g DHA, 3.56 g total n-3 polyunsaturated fatty acids, 4.1 mg α-tocopherol, 1.6 mg γ-tocopherol, 6 µg vitamin A, 14 µg vitamin D_3, and 43 µg selenium. Thus, two portions of salmon per week would typically provide 3.45 g EPA + DHA, 28 µg vitamin D_3 and 86 µg selenium. Contaminants contributed < 12.5% of the Food and Agricultural Organization/World Health Organization provisional tolerable weekly intake for dioxin and dioxin-like polychlorinated biphenyls, < 11.5% for arsenic, < 0.00000008% for cadmium, 0.0000025% for mercury and < 0.00000002% for lead.

At birth umbilical cords were collected from all pregnancies. Some of these were used for immunohistochemical staining (n=14 in the control group and n=22 in the salmon group), while others (n=5 in the control group and n=4 in the salmon group) were used for isolation of primary HUVEC.

Immunohistochemical staining of umbilical cord vasculature

After birth, a segment of the umbilical cord was stored at 4°C in sterile Hanks' salt solution. The vein and artery were isolated, placed in ice-cooled acetone containing protease inhibitors, processed in glycolmethacrylate resin as previously outlined (23) and stored at -20°C until staining. Sequential sections (2 µm) were cut and stained with toluidine blue to assess the morphology. The specimens were stained using monoclonal antibodies against human CD31 (Monosan, Newmarket, UK), human VCAM-1 (Abcam, Cambridge, UK) and human ICAM-1 (Invitrogen, Paisley, UK) and then visualised using 3-amino-9-ethylcarbazole according to a previously described protocol (23). Staining was scored using the Quality and Intensity method as previously described (24). A rating from 0-5 was given for quantity of staining, where 0 is none, 1 is patchy and 5 is continuous staining.

The same was done for intensity of staining where 0 is no staining, 1 is very light and 5 is very dark staining. Quantity and intensity values were added to yield a total score. All scoring was done blind by one investigator (MAvdM).

Endothelial cell isolation and culture

Chemicals were purchased from Sigma-Aldrich Co. Ltd (Poole, UK) unless otherwise stated. HUVEC were isolated and cultured by adapting the method of Jaffe *et al.* (23). After birth, a segment of the umbilical cord was stored at 4°C in sterile Hanks' salt solution. Cords were processed within 24-48 hours after delivery. HUVEC were obtained by collagenase perfusion of the large vein. Briefly, the umbilical vein was cannulated at both ends, rinsed with phosphate-buffered saline (PBS) and infused with 5 ml of filter sterilized 1% [w/v] collagenase type II (284 kU/mL in PBS) (prepared from *Clostridium histolyticum;* Gibco, Invitrogen, Paisley, UK) to digest the interior. The cord was placed in a container with sterile PBS and incubated in a water bath at 37°C for 15-20 minutes and the cord vein was rinsed with approximately 30 ml of sterile PBS to obtain the collagenase solution containing EC. The solution was centrifuged at 220 x *g* for 10 minutes at room temperature, and washed with cell culture medium consisting of Medium 199 supplemented with 30 U/ml sodium heparin (from porcine intestinal mucosa), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml), 50 µg/ml endothelial cell growth factor (ECGF) and 20% heat-inactivated fetal calf serum (PAA Laboratories, Pasching, Austria). The cell pellet was resuspended in fresh medium and cultured in a gelatin-coated (0.1% gelatin (from bovine skin, type B)) tissue culture flask (Greiner bio-one, Stonehouse, UK). Cells were grown to confluency in about 10 days under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37°C) before experimental use. Cultured cells were identified as vascular EC by their characteristic cobblestone morphology at confluence. In separate experiments, cells isolated and cultured under the same conditions were double stained with FITC conjugated mouse anti-human CD31 (platelet endothelial cell adhesion molecule) and phycoerythrin-cojugated mouse anti-human CD144 (VE-cadherin) in order to confirm that they were HUVEC. Both antibodies were from R&D Systems, Minneapolis, MN, USA. After antibody incubation the cells were washed with BD cell wash (140 x *g*, 7 min), fixed with BD Cell Fix™, and 10,000 gated events/sample were acquired using FACS-Calibur flow cytometer (Becton Dickinson, Oxford, UK). Typically over 95% of cells stained positively for both these markers confirming the cells as HUVEC.

Cytokine secretion and CAM expression

For experimental use, confluent HUVEC were harvested, plated and grown to semi-confluence in 4 days before stimulation with 1 µg/ml LPS (from *Escherichia coli*) for 24 hours. HUVEC used for these cultures were between passages one and three (passages 1, 1, 2, 2 and 3 in the control group and passages 1, 2, 3 and 3 in the salmon group; not significantly different). 24 hours after stimulation with LPS, supernatants were collected for

assessment of inflammatory mediator concentrations by multiplex assay (Bio-Plex Pro, 27-plex M50-0KCAF0Y, Bio-Rad Laboratories, Veenendaal, The Netherlands). Mediators measured with their limits of detection (all pg/ml) were: monocyte chemotactic protein 1 (1.81), basic fibroblast growth factor (1.21), eotaxin (2.00), G-CSF (1.31), granulocyte macrophage colony stimulating factor (0.74), interferon-γ (1.61), IL-1β (2.67), IL-1 receptor antagonist (4.97), IL-2 (1.42), IL-4 (0.32), IL-5 (2.51), IL-6 (2.01), IL-7 (2.43), IL-8 (1.77), IL-9 (1.43), IL-10 (1.78), IL12(p70) (2.55), IL-13 (2.68), IL-15 (1.82), IL-17 (1.87), interferon-γ induced protein 10 (2.38), macrophage inflammatory protein 1α (1.42), macrophage inflammatory protein 1β (1.41), platelet-derived growth factor (PDGF)-BB (2.04), regulated upon activation, normal T cell expressed and secreted (1.59), TNF-α (6.42) and vascular endothelial growth factor (2.24). Then, EC were detached and stained for the EC marker CD31 to confirm their phenotype. Cell surface expression of ICAM-1 (CD54) and VCAM-1 (CD106) was assessed by flow cytometry. After washing with 10% FCS in PBS, cells ($1 \times 10^5 - 5 \times 10^5$) were treated at 4°C for 30 minutes with fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD31, FITC conjugated mouse anti-human CD54 or FITC-conjugated mouse anti-human CD106. FITC conjugated isotype-matched negative controls (IgG_1 and IgG_{2a}) were used. All antibodies were from R&D Systems, Minneapolis, MN, USA. After antibody incubation the cells were washed with BD cell wash (140 x *g*, 7 min), fixed with BD Cell Fix™, and 10,000 gated events/sample were acquired using FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK). Data on CAM expression were analyzed using CellQuest Pro software. HUVEC with a forward scatter > 200 were included for ICAM-1 and VCAM-1 analysis. Both the percentage (%) of positive cells and the mean fluorescence intensity (MFI) were obtained in order to analyze adhesion molecule expression. All positive staining was corrected for isotype background levels.

Statistical Analysis

Graphs were made using GraphPad Prism (GraphPad Prism for Windows, Version 4, GraphPad software Inc., La Jolla, CA, USA). Adhesion molecule staining on umbilical cord vasculature determined by immunohistochemistry was compared between groups using unpaired Student's t-test. A two-way mixed model ANOVA with subject as random factor was used to assess the effects of diet (control, salmon) and LPS (-LPS, +LPS) on CAM expression, inflammatory mediator production and growth factor production by cultured HUVEC. The hypothesis that the effect of LPS differs between the salmon and control group was tested by including an interaction term between LPS and diet to the model. Data before and after LPS stimulation (-LPS vs +LPS) within a dietary group were tested by paired comparisons, whereas comparisons between dietary groups (control vs salmon) were tested unpaired. Statistical Analysis Systems statistical software package version 9.1.3 (SAS Institute, Cary, NC, USA) was used for these analyses. Pearson correlation coefficients were calculated by GraphPad Prism. In all cases $P<0.05$ was considered statistically significant.

RESULTS

Adhesion molecule expression on umbilical cord vasculature

There were no differences between groups in the Quality and Intensity score for either VCAM-1 (mean ± SEM: 0.9 + 0.5 in the control group vs. 1.7 ± 0.5 in the salmon group) or ICAM-1 (mean ± SEM: 3.5 + 0.8 in the control group vs. 3.2 ± 0.5 in the salmon group).

HUVEC culture and phenotype

HUVEC were isolated using collagenase perfusion of the umbilical vein according to Jaffe *et al.* and cultured in Medium 199 (23). For experimental use cells were grown to semi-confluence and stained for endothelial cell marker CD31 using flow cytometry. Figure 1A shows the morphology of cultured HUVEC and Figure 1B the cell population as a forward vs. side scatter plot. The phenotype of every culture was confirmed by staining for CD31, corrected for isotype background «Figure 1C and 1D». CD31 expression for HUVEC cultures varied between 84.1% and 96.7% of cells (mean ± SEM: 89.6 ± 1.9%).

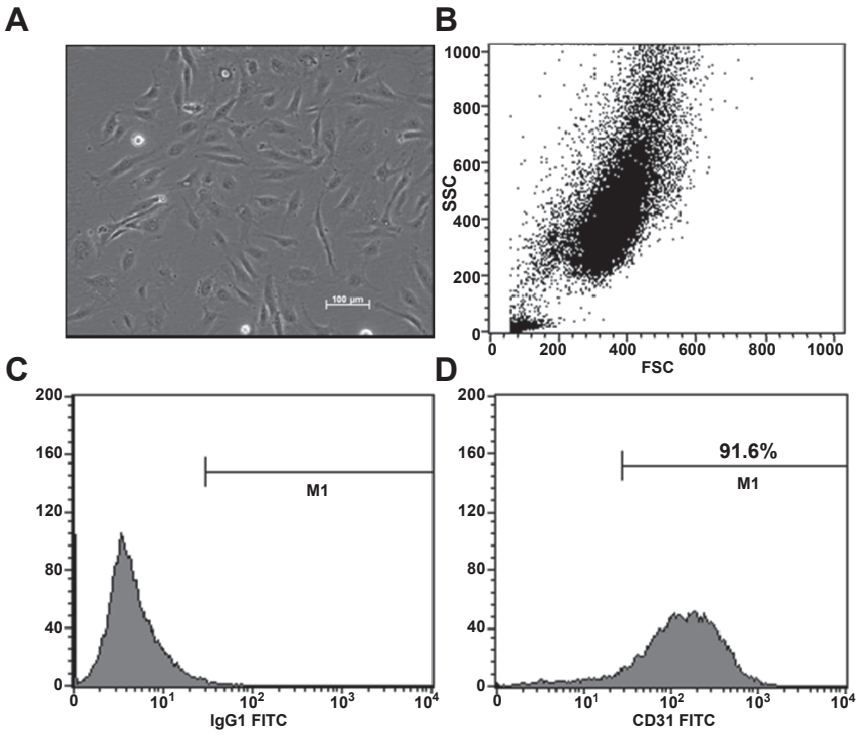

Figure 1 (A) Morphology of semi-confluent human umbilical vein endothelial cells (HUVEC). (B) Forward scatter (FSC) vs. side scatter (SSC) plot showing a distinct population of HUVEC present. (C) Isotype control (marker M1 <1% of events) staining of HUVEC. (D) Cultured cells were positive for CD31 (in this case 91.6% of cells were $CD31^{+}$) confirming their phenotype.

Effect of salmon during pregnancy on ICAM-1 and VCAM-1 expression on cultured HUVEC

After growing to semi-confluence, HUVEC were stimulated for 24 h with the inflammatory stimulus LPS. Adhesion molecule expression on the cell surface in response to LPS stimulation was analysed by flow cytometry. Figure 2A shows representative histograms for ICAM-1 and VCAM-1 expression on HUVEC from the control and the salmon groups in the presence or absence of LPS stimulation.

The expression of ICAM-1 and VCAM-1 in cultured HUVEC is represented in Figure 2B-E. In figure 2B and C the percentages (%) of cells positively stained for the respective adhesion molecules are depicted while the level of expression (MFI) is shown in figure 2D and E. There was a significant effect of LPS ($P<0.001$), but not of diet ($P=0.147$), on the percentage of HUVEC expressing ICAM-1, and there was no diet x LPS interaction ($P=0.768$). About 10 to 20% of HUVEC expressed ICAM-1 in the basal state, with no differences between groups (17.8 ± 2.1% in the control group and 11.6 ± 2.0% in the salmon group). The % of ICAM-1 positive cells was significantly increased by LPS stimulation (73.6 ± 2.6% in the control group and 65.4 ± 7.4% in the salmon group). There was a significant effect of LPS ($P<0.001$) and a trend towards a significant effect of diet ($P=0.069$) on the level of expression of ICAM-1 on HUVEC, and there was a significant diet x LPS interaction ($P=0.043$). The level of ICAM-1 expression per cell (i.e. MFI) was significantly increased by LPS stimulation in the control group (16.9 ± 2.4 vs 135.3 ± 20.2; $P<0.001$) and to a lesser extent in the salmon group (14.1 ± 3.8 vs 65.8 ± 22.4; $P=0.037$). The MFI in the salmon group in the LPS stimulated state was significantly lower than in the control group ($P=0.006$).

There was a significant effect of LPS ($P=0.005$), but not of diet ($P=0.138$), on the percentage of HUVEC expressing VCAM-1, and there was no diet x LPS interaction ($P=0.139$). The % of HUVEC expressing VCAM-1 was low under basal conditions (1.2 ± 0.2% in the control group and 0.9 ± 0.3% in the salmon group) and this was increased by LPS stimulation (21.4 ± 4.6% in the control group and 9.3 ± 5.7% in the salmon group). There was a significant effect of LPS ($P=0.006$), but not of diet ($P=0.188$), on the level of expression of VCAM-1 on HUVEC and there was a trend towards a significant diet x LPS interaction ($P=0.088$). The level of VCAM-1 expression (i.e. MFI) was increased by LPS stimulation «Figure 2E».

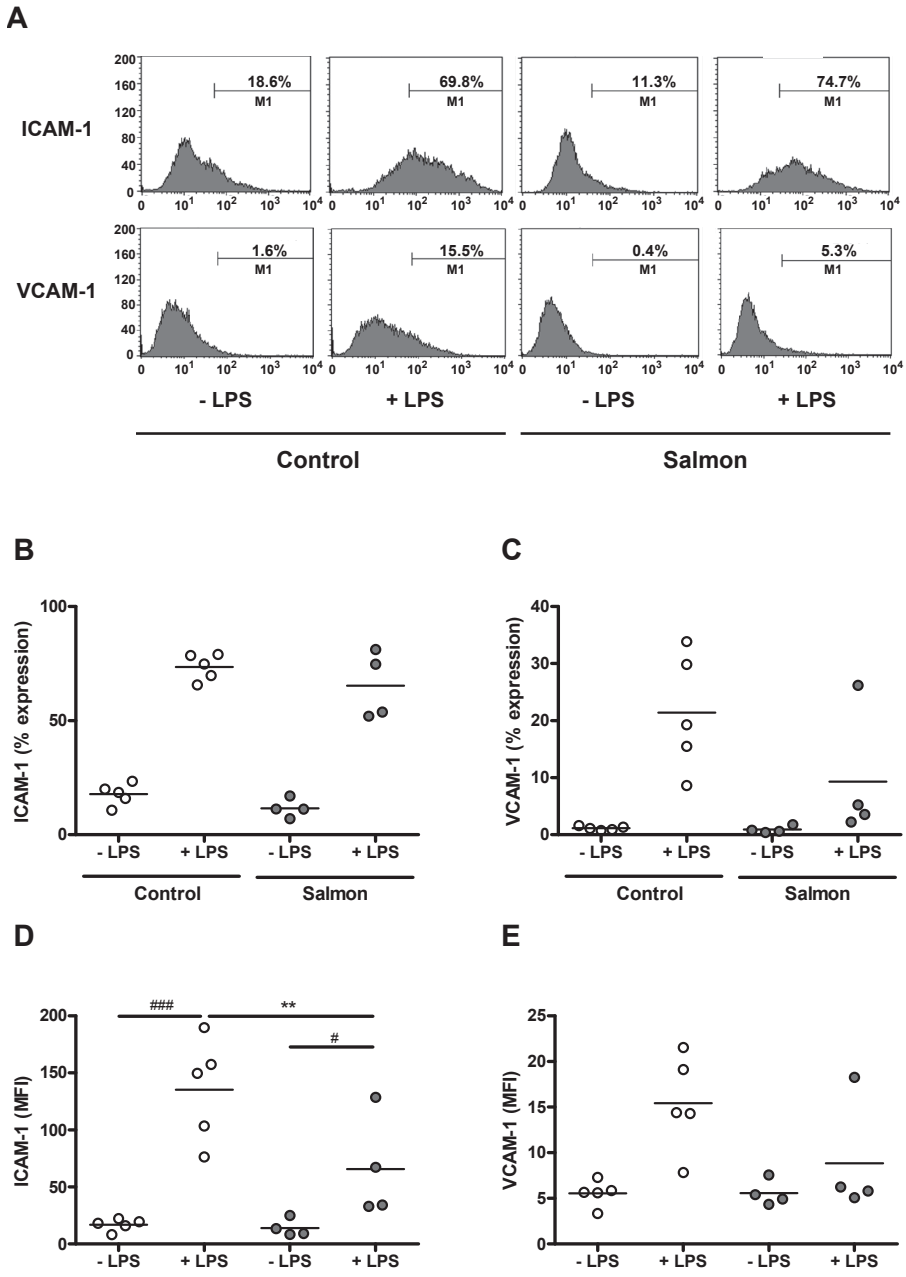

Figure 2 (continued)

◀ (continued) Intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 expression on human umbilical vein endothelial cells (HUVEC) isolated from women consuming the control (n=5) or salmon (n=4) diet, in presence or absence of 24 hours of lipopolysaccharide (LPS) stimulation. (A) Representative histogram plots showing the marker set using the relevant isotype control. (B, C) Adhesion molecule expression as percentage (%) of positive cells and (D, E) mean fluorescence intensity (MFI). Data for individual volunteers are shown in each graph. A two-way mixed model ANOVA with subject as random factor was used to assess the effects of diet (control, salmon) and LPS (-LPS, +LPS). For % of ICAM-1 positive cells there was a significant effect of LPS ($P<0.001$) but not diet ($P=0.147$) and no diet x LPS interaction ($P=0.768$). For % of VCAM-1 positive cells there was a significant effect of LPS ($P=0.005$) but not diet ($P=0.138$) and no diet x LPS interaction ($P=0.139$). For ICAM-1 MFI there was a significant effect of LPS ($P<0.001$), a trend towards a significant effect of diet ($P=0.069$) and a significant diet x LPS interaction ($P=0.043$). For VCAM-1 MFI there was a significant effect of LPS ($P=0.006$) but not diet ($P=0.188$) and a trend towards a significant diet x LPS interaction ($P=0.088$). Statistical significance of post-hoc pairwise comparisons: $^{*}P<0.01$ (control vs salmon); $^{\#}P<0.05$, $^{\#\#}P<0.001$ (-LPS vs +LPS).

Effect of salmon during pregnancy on inflammatory mediator secretion by HUVEC

In addition to CAM surface expression, the effect of dietary salmon intake on inflammatory mediator secretion by HUVEC was assessed. Data for the pro-inflammatory cytokines IL-6 and TNF-α are shown in Figure 3A and 3B, respectively.

There was a significant effect of LPS ($P=0.004$), but not of diet ($P=0.229$), on IL-6 secretion, and there was no diet x LPS interaction ($P=0.300$). IL-6 secretion by HUVEC increased upon stimulation with LPS (724.5 ± 125.05 vs 3969 ± 1044 in the control group; 436.8 ± 170.2 vs 2299 ± 741.8 in the salmon group). There was a significant effect of LPS ($P<0.001$), but not of diet ($P=0.229$), on TNF-α secretion, and there was no diet x LPS interaction ($P=0.716$). TNF-α secretion by HUVEC increased upon stimulation with LPS (166.4 ± 23.4 vs 303.3 ± 25.8 in control group; 108.1 ± 46.7 vs 261.6 ± 44.4 in salmon group). The concentrations of the other inflammatory mediators measured were not different between control and salmon groups although several were induced by LPS stimulation (data not shown).

Growth factors are known to exert protective effects on endothelial cells. There was a significant effect of LPS ($P=0.008$) and trends towards an effect of diet ($P=0.066$) and towards a diet x LPS interaction ($P=0.066$) on G-CSF secretion «Figure 3C». G-CSF secretion by HUVEC increased upon stimulation with LPS (4.2 ± 1.1 vs 658.6 ± 121.7 in the control group; 87.8 ± 46.8 vs 2680 ± 1044 in the salmon group). There was a significant effect of LPS ($P<0.001$), but not of diet ($P=0.200$) on PDGF-BB secretion «Figure 3D». There was no diet x LPS interaction ($P=0.309$). PDGF-BB secretion by HUVEC increased upon stimulation with LPS (297.5 ± 49.3 vs 489.9 ± 61.2 in control group; 485.3 ± 165.4 vs 772.2 ± 197.0 in salmon group).

ICAM-1 and VCAM-1 expression (MFI) after LPS stimulation were positively correlated ($r=0.9315$, $P<0.001$ «Figure 4A»). Both ICAM-1 and VCAM-1 expression were positively correlated with IL-6 secretion from HUVEC («Figure 4B and 4C»; $P<0.001$ and $P=0.013$ respectively), while TNF-α was not correlated with CAM expression (data not shown). PDGF-

BB showed a negative correlation with VCAM-1 after stimulation («Figure 4D»; P=0.044 whereas G-CSF showed a trend towards negative correlation with VCAM-1 (P=0.061, data not shown). In addition, PDGF-BB tended to correlate negatively with ICAM-1 expression (P=0.065, data not shown).

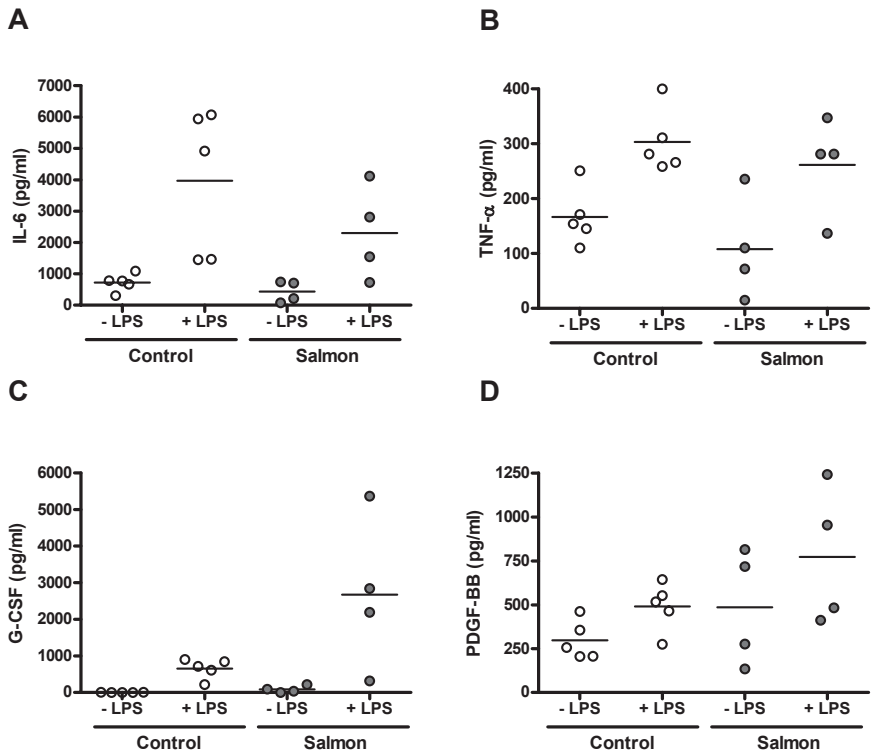

Figure 3 Secretion of cytokines (A) interleukin (IL)-6 and (B) tumor necrosis factor (TNF)-α and growth factors (C) granulocyte colony-stimulating factor (G-CSF) and (D) platelet-derived growth factor (PDGF)-BB by human umbilical vein endothelial cells (HUVEC) isolated from women consuming the control (n=5) or salmon (n=4) diet, in presence or absence of 24 hours of lipopolysaccharide (LPS) stimulation. Data for individual volunteers are shown. A two-way mixed model ANOVA with subject as random factor was used to assess the effects of diet (control, salmon) and LPS (-LPS, +LPS). For IL-6 there was a significant effect of LPS (P=0.004) but not diet (P=0.229) and no diet x LPS interaction (P=0.300). For TNF-α there was a significant effect of LPS (P<0.001) but not diet (P=0.289) and no diet x LPS interaction (P=0.715). For G-CSF there was a significant effect of LPS (P=0.008), a trend towards a significant effect of diet (P=0.066) and a trend towards a significant diet x LPS interaction (P=0.066). For PDGF-BB there was a significant effect of LPS (P<0.001) but not diet (P=0.200) and no diet x LPS interaction (P=0.309).

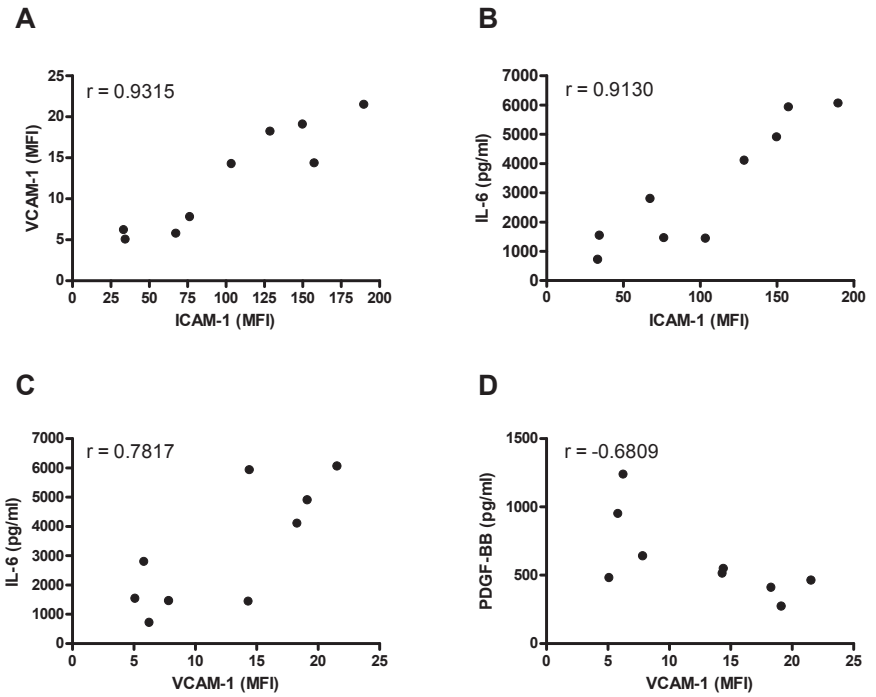

Figure 4 Correlations between cell adhesion molecule expression as mean fluorescence intensity (MFI) and mediator secretion after lipopolysaccharide (LPS) stimulation. There were positive correlations between (A) intercellular adhesion molecule (ICAM)-1 expression and vascular cell adhesion molecule (VCAM)-1 expression (r=0.932; P=0.0003), (B) ICAM-1 expression and interleukin (IL)-6 secretion (r=0.913; P=0.0006) and (C) VCAM-1 expression and IL-6 secretion (r=0.782; P=0.013). There was a negative correlation between (D) VCAM-1 expression and PDGF-BB secretion (r=-0.681; P=0.044).

DISCUSSION

Studying the effects of n-3 LCPUFA on EC responsiveness is important since such responses are involved in CVD and other inflammatory conditions (1). Cell culture studies have identified that EPA and DHA can decrease expression of ICAM-1 and VCAM-1 on EC (8, 19, 21), adhesive interactions between EC and leukocytes (8, 21) and production of IL-6 by EC (8, 20). Similarly EPA and DHA decreased ICAM-1 expression by cultured monocytes (24). However few studies have shown an effect of dietary n-3 LCPUFA on adhesion molecule expression. Feeding fish oil to rodents decreased expression of several adhesion molecules on lymphocytes (25), macrophages (26) and dendritic cells (27), while a fish oil supplementation study in humans showed decreased ICAM-1 expression on interferon-γ stimulated monocytes (28). To our knowledge no human studies have investigated the

effect of dietary intervention on adhesion molecule expression or other inflammatory responses of EC. The current highly novel study has used unique samples obtained from the SiPS in order to identify the effects of dietary n-3 LCPUFA on adhesion molecule expression and mediator secretion by human EC. It was hypothesised that EC responses would be lower in the group whose mothers received an increased dietary intake of n-3 LCPUFA (i.e. the salmon group). In accordance with this, an increased dietary salmon intake was found to result in lower EC surface expression of ICAM-1 after *ex vivo* LPS stimulation; surface expression of VCAM-1 also tended to be lower in the salmon group following LPS exposure. In contrast, production of G-CSF in response to LPS tended to be higher by EC in the salmon group. Production of a number of other mediators was not significantly different between the salmon and control groups. Thus, there is some selectivity or specificity to the anti-inflammatory effects of dietary n-3 LCPUFA, suggesting perhaps an effect on a single signaling pathway.

Increased intake of oily fish from week 20 of pregnancy did not affect expression of ICAM-1 or VCAM-1 on cord tissue or on resting (i.e. unstimulated) HUVEC, but decreased endothelial activation in response to LPS as indicated by lower ICAM-1 expression. The lack of a group difference in CAM expression on cord tissue and on unstimulated HUVEC suggests that n-3 LCPUFA do not affect basal expression levels of these molecules. This finding is in accordance with *in vitro* studies of n-3 LCPUFA with EC. For example, incubation of unstimulated HUVEC with EPA or DHA had no effect on mRNA for ICAM-1 or VCAM-1 (19), while DHA had no effect on basal ICAM-1 expression on HUVEC (21).

Stimulation with LPS for 24 hours resulted in an increase in both the percentage of cells expressing ICAM-1 and the level of expression (MFI); however in the salmon group the level of expression was about 50% lower than in the control group. The salmon diet was also associated with a tendency towards a lower VCAM-1 MFI compared to the control group. Both ICAM-1 and VCAM-1 are involved in the firm adhesion of leukocytes to the endothelium (9). Thus lower CAM expression on EC could result in decreased adherence and transmigration of leukocytes into vascular tissue, a key event in atherosclerosis. These data are supported by studies in which HUVEC were directly exposed to LCPUFA during *in vitro* cultures and then stimulated with LPS or an inflammatory cytokine. De Caterina *et al.* showed that DHA, but not EPA, decreased endothelial expression of ICAM-1 and VCAM-1 in a dose-dependent manner in human saphenous vein EC after incubation with a pro-inflammatory stimulus (8). Weber *et al.* showed that DHA, but not EPA, dose-dependently decreased TNF-α induced VCAM-1 expression on HUVEC and also lowered subsequent monocytic cell adhesion (21). Collie-Duguid *et al.* demonstrated that mRNA levels for both ICAM-1 and VCAM-1 in IL-1 activated HUVEC were lower in the presence of either EPA or DHA (19). These earlier studies were all conducted *in vitro* involving direct exposure of EC to pure n-3 LCPUFA in a cell culture setting. As far as we are aware, the current study is the first dietary intervention study that investigated the possible effects of salmon rich in n-3 LCPUFA on EC activation in humans.

Dietary n-3 LCPUFA have been shown in the current study to exert similar effects to those seen for the individual n-3 LCPUFA in *in vitro* experiments (8, 19-21). Since the HUVEC were cultured for 2 weeks in a standard culture medium prior to activation, this suggests a possible programming effect of the salmon diet with regard to endothelial responsiveness to an inflammatory stimulus. *In utero* exposure to n-3 LCPUFA could modulate gene expression via epigenetic changes (e.g. DNA methylation or histone modifications), leading to altered susceptibility to atherosclerosis (29). DNA hypermethylation of specific genes is important in atherosclerosis. n-3 LCPUFA are peroxisome proliferator-activated receptor (PPAR) activators and PPAR can alter epigenetic processes (30, 31). Several PPAR activators have been shown to reduce the induced expression of CAM (32). Dietary n-3 LCPUFA are associated with decreased plasma homocysteine levels, a risk factor for atherosclerosis inducing DNA methylation (33, 34). Homocysteine upregulates expression of adhesion molecules such as ICAM-1 on EC and this is reduced by PPAR agonists (35, 36). In addition, a number of chromatin modifying enzymes have PPAR response elements in their promoters so can be regulated via n-3 LCPUFA, these enzymes contribute to regulation of CAM expression (37-39). Furthermore, n-3 LCPUFA could affect CAM expression via reduced activation of nuclear factor-κB (NF-κB) (2, 21). Early exposure to salmon derived n-3 LCPUFA may contribute to a subtle but persistent effect on the development of CVD later in life.

The effect of salmon on production of soluble mediators by EC was also studied. Secretion of pro-inflammatory mediators by EC is important in the inflammatory response and contributes, together with adhesion molecules, to the early phases of atherogenesis. N-3 LCPUFA, in particular DHA, have been shown to decrease IL-6 expression in human EC after stimulation (8, 20). The salmon diet had little effect on cytokine secretion by HUVEC in response to LPS stimulation.

Some growth factors are known to play an anti-inflammatory role and exert protective effects on EC (40, 41). The salmon diet tended to increase production of G-CSF by LPS stimulated EC. Endogenous G-CSF is known to down-regulate IL-1 induced ICAM-1 expression and granulocyte adhesion on human EC (40). The concentration of G-CSF in HUVEC supernatants of the salmon group upon LPS stimulation was four times higher than in the corresponding control group, which may be related to the anti-inflammatory effects of the salmon diet. PDGF-BB has been shown to promote the structural integrity of the vessel wall and can stimulate the secretion of other growth factors. It has been shown to improve survival in severe sepsis via an effect on vessel wall homeostasis and tissue healing capacity (41). LPS stimulation led to an increase in PDGF-BB production but the salmon diet did not significantly affect this response. However PDGF-BB was negatively associated with VCAM-1 expression and tended to be negatively correlated with ICAM-1 expression after stimulation, suggesting a possible contribution of PDGF-BB in controlling LPS induced VCAM-1 and ICAM-1 expression.

In summary, increased intake of salmon providing n-3 LCPUFA from week 20 of pregnancy was associated with lower responsiveness of offspring umbilical vein EC to LPS with regard to upregulation of ICAM-1 and there was a trend towards greater responsiveness with regard to upregulation of G-CSF compared with controls. This suggests an anti-inflammatory effect on human EC through dietary n-3 LCPUFA, an effect not demonstrated previously. If such an effect was a generalisable action of n-3 LCPUFA on EC at all sites of the vasculature, then this would be suggestive of an important mechanism by which these fatty acids could lower cardiovascular risk.

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CHAPTER TEN

Summarizing discussion

Fat is an essential part of the diet in humans, with a daily recommended intake of 30% of total calories consumed. It is recommended to keep saturated fat intake ≤ 10% of calories because of its relation with cardiovascular risk (1). Humans evolved on a diet that was much lower in saturated fat than today. Furthermore, the early diet contained small amounts of n-6 and n-3 polyunsaturated fatty acids (PUFA) in almost equal amounts. An absolute and relative change of n-6 and n-3 PUFA has occurred. The present diet is very high in n-6 PUFA because it is recommended to lower saturated fatty acids. Furthermore, consumption of n-3 PUFA is low because of the industrial production of animal feeds rich in n-6 PUFA and reduced fish consumption (2). Dietary n-6 PUFA have been linked to allergy in some but not all studies (3-6). Some studies are confounded by the association between high-fat intake and obesity (7). In Japan the dietary habits are changing, leading to an enhanced n-6:n-3 PUFA ratio, which is accompanied by an increase in atopic disease (8). Besides many studies reporting about the beneficial effects of long chain (LC) n-3 PUFA consumption in allergic disease, there are studies that did not find this (9-11). Perhaps study results are confounded since the choice of fat source as a placebo (corn, soybean, olive oil) differs between the different human trials. The amount of n-6 PUFA provided in control groups being rather low for olive oil and high in corn oil, may partly add to the differences found (see Table 2 General Introduction) (12). Therefore the effect of n-6 and n-3 PUFA intake in allergic disease is still under debate.

The effects of n-3 LCPUFA on cardiovascular disease (CVD) have been investigated for a much longer period and are more clear. However, mechanisms involved in its protective effect in CVD are not fully elucidated.

This thesis provides *in vitro* and *in vivo* data concerning the effectiveness of n-3 LCPUFA in food allergy and cardiovascular disease and, in part, describes the underlying mechanism of action. Here we discuss the impact of PUFA on the allergic cascade and cardiovascular function.

FOOD ALLERGY

Oral tolerance and dendritic cells

Dietary interventions including n-3 LCPUFA may be beneficial in the prevention and/or treatment of (food) allergies as demonstrated in this thesis. Possibly a diet rich in n-3 LCPUFA, of which fish oil is a prominent source, provides a strategy to reduce or prevent initial sensitization to food allergens early in life by supporting oral tolerance induction. On the other hand increasing n-6 PUFA in the diet may worsen allergic symptoms or counteract oral tolerance induction.

In this thesis it was indeed demonstrated that increasing the fat percentage of n-6 rich soybean oil in the diet from 7% to 10% during the sensitization phase enhanced allergic symptoms; which was suggested to be a direct effect on the mast cell response

(**Chapter Three**). In another study the 10% fat diet was provided during two weeks of oral tolerance (OT) induction by partial whey hydrolysate (pWH), followed by sensitization with whey while being fed the control diet. The 10% soybean oil diet reversed the tolerizing capacity of pWH completely. However, serum whey-IgE and –IgG1 were not increased (data not shown). Therefore serum immunoglobulin free light chain (IgLC) levels were studied. IgLC are known to contribute to allergic reactions to milk proteins and are suggested to be involved in oral tolerance induction to food proteins (13). However, IgLC were not altered by increasing linoleic acid (LA) in the diet (data not shown). Currently it remains unclear why the acute skin response to whey increased in mice fed the 10% soybean oil diet during pretreatment with pWH, while the immunoglobulin levels did not change. However, this break of tolerance for whey suggests that n-6 PUFA have altered the DC phenotype thereby diminishing the efficacy of tolerance induction by pWH. It has been reported that AA-derived eicosanoids and other mediators such as histamine enhance co-stimulatory molecule expression (i.e. CD86) and reduce IL-12 secretion by DC promoting a Th2 cell response (14-16). However, it has been demonstrated also that AA inhibits the expression of LPS-induced co-stimulatory molecules (17-19). In our bone marrow derived DC (BMDC) study using cells derived from sham- or whey-sensitized C3H/HeOuJ mice AA did enhance CD80 and CD40 expression (data not shown). Future studies are needed to study the effect of increased soybean oil intake on DC phenotype during the period of OT induction to prove the involvement of DC in the abrogation of oral tolerance induction by n-6 PUFA.

Partial substitution of the soybean oil diet with tuna oil reduced the percentage of $CD11c^{+}CD11b^{-}CD8\alpha^{+}$ DC in the spleen and MLN of sham-sensitized mice compared to control diet fed sham-mice. This conventional DC subset is IL-12 producing and therefore Th1 polarizing (20, 21). This was accompanied by a reduction in the percentage of activated Th1 cells in both organs (**Chapter Five**). The same was found for peanut extract (PE)-sensitized mice (data now shown). This effect on DC and T cells was only demonstrated for sham-mice, possibly in allergic mice this effect is lost due to the continuous boost of the immune system by repeated sensitization. On the other hand the percentage of $CD11c^{+}CD11b^{+}CD8\alpha^{-}$ DC was increased in the spleen of both sham- and whey-sensitized mice. A subset of these DC, which is $CD103^{+}$, was enhanced in MLN. Whereas $CD103^{-}$ DC in the MLN may mark resident DC that have developed from precursors from the blood, $CD103^{+}$ DC likely mark migratory DC arriving from the intestinal lamina propria (20). The $CD103^{+}CD11b^{-}CD8\alpha^{+}$ has previously been described as an inflammatory DC (22) while the $CD103^{+}CD11b^{+}CD8\alpha^{-}$ DC expresses retinal dehydrogenase isoform 2 which results in the conversion of retinal to retinoic acid and may function as a tolerogenic DC (31, 32). Retinoic acid is involved in the induction of $FoxP3^{+}$ Treg (24, 32, 60). $FoxP3^{+}$ Treg generated in the MLN transfer local intestinal tolerance systemically and home to the lamina propria (59). Indeed, the increase in $CD11c^{+}CD11b^{+}CD103^{+}CD8\alpha^{-}$ DC was associated with enhanced

FoxP3$^+$ Treg in the spleen and intestine of fish oil fed whey-sensitized mice. The crucial involvement of Treg in the prevention of allergic sensitization induced by n-3 LCPUFA has been demonstrated in **Chapter Six**. Besides programming the DC subsets as mentioned above, fish oil may reduce co-stimulatory molecule expression on DC thereby decreasing DC-T cell interactions. This may result in OT induction as well. Indeed also in previous studies n-3 LCPUFA have been shown to affect the DC phenotype. It has been demonstrated that EPA and DHA inhibit the expression of LPS-induced co-stimulatory molecules, including CD40, CD80 and CD86, and are able to suppress antigen presentation, T cell proliferation and cytokine secretion (17-19). In LPS stimulated BMDC EPA and DHA reduced IL-12 production while IL-10 secretion was enhanced (23). In our hands DHA but not EPA was able to reduce CD40, CD80 and CD86 expression in LPS stimulated BMDC (data not shown). However this phenomenon was less clear in the MLN of whey-sensitized mice fed the fish oil diet either or not *ex vivo* stimulated with LPS (data not shown). Besides the MLN, n-3 LCPUFA may have affected DC in the Peyer's patches and lamina propria. It remains to be studied whether in these sites DC co-stimulatory markers are suppressed like has been shown *in vitro*. If this is the case, this may add to the effect of n-3 LCPUFA on the DC phenotype such as shown in **Chapter Five**. Furthermore it would be interesting to take along a PBS control group during sensitization to exclude a possible effect of n-3 LCPUFA on CT-induced DC alterations. However, this seems unlikely since CT was shown to reduce the differentiation of CD8α^+ conventional DC and n-3 LCPUFA also reduce this subset (24). Furthermore the plasmacytoid DC was reduced by CT exposure (24), this DC subset was not affected by the fish oil diet in the whey model (data not shown).

T helper cell response

Besides breaking tolerance for pWH the 10% soybean oil diet increased the percentage of activated Th1 and Th2 cells as well as the Th2/Th1 and Th2/regulatory T cell ratio in sham-mice compared to the 7% fat diet during the sensitization protocol. Similar effects were shown in whey-sensitized mice but whey sensitization as such also increased these parameters in the 7% fat group. This indicates that n-6 PUFA skew towards a more allergy-prone state in these mice, independent of the allergen. Mizota *et al.* demonstrated that increased dietary n-6:n-3 PUFA intake due to variations in ALA induces a shift in the balance between Th1 and Th2 towards Th2 (25). Since the 10% soybean oil diet also was able to reverse pWH induced oral tolerance, it would be interesting to investigate whether a high n-6 PUFA diet could provoke sensitization to a food allergen in mice, without the use of cholera toxin (CT) as an adjuvant. However, it seems more likely that only increasing the LA content of the diet would not activate or modulate DC sufficiently to prevent oral tolerance induction. It is more likely that the diet may augment the effect of an adjuvant (e.g. CT) on DC function and consequent immune polarization.

By contrast, the percentage of activated Th1 cells in the spleen and MLN of sham mice was reduced by partial replacement of soybean oil by fish oil. The percentage of

activated Th2 cells was reduced in the spleen of both sham- and whey-sensitized mice (**Chapter Five**). The modulation of T cell polarization by n-3 LCPUFA may be induced indirectly via interaction with DC (see oral tolerance and DC). Furthermore the Th1/Th2 ratio in spleens of fish oil fed sham-sensitized mice was increased compared to sham mice fed the control diet. While the n-6 PUFA enrichment enhanced the Th2 cell percentage more pronounced than Th1 (**Chapter Three**), the DHA-diet reduced this subset stronger than Th1 (**Chapter Five**). Suppression of both Th1 and Th2 cells indicates general immune suppression by fish oil as previously described (see **Chapter Two**). It also suggests some kind of specificity for the Th2 response in the Th2 skewed C3H/He0uJ mice used. Perhaps the model used or the immune status of the diseased subject decides whether the Th1 or Th2 response will be affected most. The varying effects of n-3 LCPUFA on T cell subsets in literature appear to confirm this. In Th1 prone C57Bl/6 mice fish oil suppressed Th1 development while Th2 polarization remained unaltered (26, 27). This implicates a considerably safe way of immune suppression, in particular since n-3 LCPUFA also instruct Treg known to contribute to immune homeostasis.

Humoral response

Dietary fat consumption may modulate IgE production. The formation of n-6 PUFA-derived eicosanoids results in enhanced IgE production and therefore an increase in allergic sensitization. High margarine intake (rich in LA) was positively associated with allergic sensitization (4, 28). Interestingly, we demonstrate in **Chapter Three** that whey-IgE and -IgG1 were unaltered by increasing the fat percentage from 7% to 10%. Also others could not detect a relation between margarine consumption and atopic sensitization although it was associated with hay fever in children (29). The high fat diet used in this thesis contains only 10% of fat, which is low compared to many other studies which often aim to induce obesity in mice. These studies often use up to 50% of fat (30, 31). The increase in fat percentage from 7% to 10% in this study is exchanged for cornstarch (w/w). As a result the caloric values of the diets only slightly differ and body weight of the mice was not affected. Soybean oil is especially rich in the n-6 PUFA LA; also other vegetable oils such as corn and sunflower oil are rich in LA and therefore possibly could exert similar results (12).

As demonstrated in **Chapter Five** the partial substitution of n-6 PUFA by n-3 LCPUFA from tuna oil effectively prevented the production of whey-specific IgE and IgG1 indicating prevention of allergic sensitization. Serum transfer confirmed that the sera of whey-sensitized mice fed a diet rich in DHA, which were low in Th2 type immunoglobulins, reduced the capacity to induce an allergic effector response in recipient mice upon allergen challenge in the ear. Besides the Th2 type immunoglobulins also the IgG2a was reduced by the DHA diet (**Chapter Five**) which was in accordance with the reduction in both Th1 and Th2 subsets as discussed above. In **Chapter Seven** we demonstrate that the DHA-rich fish

oil was more effective than EPA-rich fish oil in reducing allergic sensitization to whey. Possible differences between EPA and DHA effects are discussed below (see Dosage). In addition, DHA was only effective in reducing Th2 immunoglobulins in whey- but not PE-sensitized mice. Besides the two food allergy models we have studied, OVA-induced food allergy has been studied by Matos *et al.* reporting lower allergen-specific IgE and IgG1 levels upon dietary intervention with fish oil (32). Although PE-IgE and -IgG1 were not reduced, the PE-specific T cell proliferation was reduced by the DHA diet, suggesting that also in the PE-model there is some suppression of the adaptive immune response in the sensitization phase. This may be the result of an effect of n-3 LCPUFA on the DC-T cell interaction or a direct effect on T cells present, resulting in reduced T cell proliferation (Th1 and/or Th2). Indeed also other studies have shown that n-3 LCPUFA incubated LPS-stimulated DC are able to suppress T cell proliferation (17-19). However, in the case of PE these effects were not strong enough to suppress the generation of Th2 type immunoglobulins upon repeated sensitization with the allergen. Previously it was demonstrated that T cell responses and immunoglobulin levels not necessarily need to be modulated in parallel (33). Also in children that outgrow food allergy IgE levels often remain high, while allergic symptoms are reduced (desensitization or oral tolerance induction) (34, 35). Transfer of serum from mice fed EPA or DHA diet to naive recipient mice fed control diet would be useful to study the effect of the humoral factors more in depth. Furthermore it is possible that IgE is blocked, and therefore not able to opsonize mast cells. However, it is more likely that the dietary intervention was not effective enough and perhaps an increase in n-3 LCPUFA dose or shortening of the sensitization protocol may reveal an effect on IgE and/or IgG1. This also suggests that the allergen used may affect the effectiveness of n-3 LCPUFA. Perhaps the allergenicity of the allergen may be involved, e.g. counteracting the effect of EPA or DHA on the DC. However, since the dosage of PE and whey during sensitization differs it is hard to draw firm conclusions and therefore this needs to be further investigated.

Regulatory T cell

The prevention of allergic sensitization by the n-3 LCPUFA-rich diet suggests the induction of tolerance during the dietary intervention. The development of tolerance to foods including milk is associated with circulating, antigen-specific $CD25^+$ Treg capable of suppressing e.g. effector T cells, secretion of allergen-specific IgE by B/plasma cells and mast cells via direct cell-cell contact or suppressive cytokines (IL-10, TGF-β) (36, 37). Whey-sensitized mice showed an increased percentage $CD11c^+CD11b^+CD103^+CD8\alpha^-$ DC in the MLN in association with enhanced $FoxP3^+$ Treg in the spleen and intestine of fish oil fed whey-sensitized mice compared to sham mice. In **Chapter Six** we report that splenocytes from fish oil fed whey-sensitized mice transferred tolerance to naive recipients if injected prior to sensitization, as demonstrated by a reduced acute allergic skin response. Depletion of $CD25^+$ Treg confirmed the involvement of Treg in this suppression. Hence

the fish oil induced (oral) tolerance to whey. This effect cannot completely be explained by a reduction in whey-specific IgE or IgG1 levels in the recipient mice. TGF-β has been demonstrated to reduce IgE-receptor expression on mast cells and switches B cells to IgA production (38, 39). Since this may reflect improved Treg function it would be interesting to measure TGF-β in serum and whey-IgA in fecal samples as an indication of Treg functionality in the recipient mice.

In PE-allergic mice the intestinal FoxP3$^+$ cell number increased by the DHA- compared to the EPA-rich diet, suggesting that DHA is more efficient than EPA in the installation of Treg. DHA was not effective in inducing Treg in the spleen, which was in contrast with the whey model. Perhaps the lower allergen dose during sensitization (6 mg PE vs 20 mg whey) may result in less efficient instruction of Treg development. The allergen doses used were in agreement with previous studies and considered optimal (40, 41), however this discrepancy hinders comparison between models. In the model for peanut allergy the fish oil diets were unable to lower the humoral immune response that is generated upon repeated PE exposure. However the Treg generated by the DHA-rich diet may have contributed to reduced mast cell sensitivity in the PE sensitization model. In this model the DHA diet and not the EPA was able to suppress the acute allergic skin response.

Effector response

In this thesis (**Chapter Three**) an enhanced allergic response by increasing the dosage of dietary soybean oil rich in n-6 PUFA was demonstrated in whey-allergic mice. Although whey-IgE and -IgG1 were not affected the mucosal mast cell response -measured as mmcp-1 (42)- indicated an effect of LA on the effector response. Indeed, transfer of hyperimmune serum high in whey-IgE and -IgG1 showed an enhanced capacity to induce an acute allergic skin response in naive recipients fed 10% soybean oil as compared to 7% fat, while fish oil effectively reduced this. So far it is unknown how LA exerts its effects. It was found that the erythrocyte membrane fatty acid composition at the end of the study was equal for both the 7% and 10% soybean oil groups (mean LA content: 14.5% of fatty acids). This indicates that the cell membranes are saturated with LA when consuming a 7% soybean oil diet containing mainly LA. Possibly free LA signals via receptor signaling (43) on the mast cell thereby directly altering signaling pathways or transcription factors. Increased sensitivity of mast cells to degranulate (e.g. by FcεRI expression) or altered mast cell mediator secretion may cause the increase in the allergic response. AA, the most important LA metabolite, did increase IgE-mediated LAD2 degranulation (**Chapter Four**). LA and AA were shown to induce RBL-2H3 degranulation dose-dependently (44). Nakano *et al.* reported that AA supplementation of RBL-2H3 cells resulted in enhanced IgE-mediated β-hexosaminidase release representing mast cell degranulation. This was accompanied by the induced intracellular production of ROS, calcium influx, and tyrosine phosphorylation of Syk and linker for activation of T cells (LAT) (45). Also the activation of PKC by AA may be involved (46). In contrast, in HMC-1 cells ROS generation was

reduced by AA incubation. However, AA dramatically increased PGD_2 -which is quickly released from the cell membrane after mast cell activation- and enhanced TNF-α secretion by ionomycin/PMA stimulated HMC-1 cells. Previously, AA-supplemented cells were demonstrated to increase TNF-α release (45). AA-derived metabolites such as PGD_2 are important in the initiation of the allergic effector response (47, 48). On the other hand PGD_2 was reduced in HMC-1 incubated with EPA and DHA. This is the result of reduced AA in mast cell membranes, as demonstrated previously (49). Also in serum of DHA-fed rats PGE_2 was reduced as a reflection of decreased AA erythrocyte membrane content (**Chapter Five**).

The findings for n-3 LCPUFA on mast cell degranulation reported in literature are contrasting as reviewed in **Chapter Two**. EPA supplementation of RBL-2H3 cells resulted in enhanced IgE-mediated ROS production and β-hexosaminidase release (45). In other studies EPA did not show an effect (44) or reduced (50) mast cell degranulation. Our experiments *in vitro* using mast cell lines (**Chapter Three**) indicate that EPA and DHA were unable to reduce mast cell degranulation, since β-hexosaminidase release remained unaltered. On the other hand, EPA and DHA reduced mast cell mediator release (IL-4, IL-13, PGD_2) by HMC-1 cells.

In the whey allergy model only the DHA diet reduced mmcp-1, although the EPA diet showed the same pattern (**Chapter Seven**). It is unclear whether the reduction in mmcp-1 concentration by n-3 LCPUFA is the result of a direct effect on the mucosal mast cell and/or the suppressed immunoglobulins present *in vivo*. The latter were also more effectively suppressed by the diet rich in DHA than EPA. To study this it could be useful to measure mmcp-1 upon oral challenge after transfer of hyperimmune serum to naive mice fed control versus fish oil diet. Improved efficacy of the DHA diet compared to the EPA diet in suppressing the whey-induced allergic effector response was demonstrated using passive immunization, while for PE the diets were equally effective (**Chapter Seven**). This reduction in acute skin response was probably the result of a direct effect on the local mast cell response in the ear. We suggest that this was independent of an effect of n-3 LCPUFA on surrounding tissues, since the fish oil diets did not reduce the histamine induced effector response. However, since histamine is not the only mast cell derived mediator involved in effector reactions it remains a topic for future studies. To exclude the contribution of humoral factors other than immunoglobulins (e.g. allergic cytokines) in reducing the acute allergic skin response by n-3 LCPUFA it would be interesting to remove immunoglobulins from serum of control or fish oil fed sensitized mice and transfer this to naive recipients followed by an allergen challenge.

CARDIOVASCULAR DISEASE

Although studies focusing on the beneficial effects of n-3 LCPUFA in allergic disease only started recently, its use in CVD has readily been proven in large clinical trials. In particular in high-risk patients dietary intake of n-3 LCPUFA can reduce the risk of developing coronary heart disease (51).

Blood pressure

The spontaneously hypertensive rat (SHR) model is widely used as a model for human essential hypertension. Previously the involvement of the sphingolipid system in the regulation of BP in SHR and hypertensive patients was described on a genetic basis (52). Ceramide levels were demonstrated to be enhanced in SHR compared to normotensive rats (53) activating a signaling cascade contributing to vasoconstriction via TXA_2 release by vascular endothelial cells (**Chapter Eight**). Sphingomyelin is a membrane phospholipid composed of a sphingoid base, linked to a fatty acid chain and phosphocholine head group (see **Chapter One**) and the precursor for ceramide formation (54). Only a subset of fatty acids has been described to be linked to sphingolipids, mostly saturated of mono-unsaturated fatty acids. PUFA are membrane lipids present as part of glycerol-based phospholipids (55). Both sphingolipids and PUFA are involved in signaling.
In **Chapter Eight** it is demonstrated that the mean arterial blood pressure (BP) is stabilized by the fish oil diet; no further increase over the 12-weeks intervention period is observed (data not shown) resulting in reduced BP at the 12 week endpoint compared to control diet fed rats. Previously a beneficial effect of n-3 LCPUFA on BP reduction has been demonstrated (56-59). In human subjects a reduction in diastolic BP as small as 4 mmHg was shown to be clinically relevant by significantly reducing the risk of stroke and coronary heart disease (60, 61). However the mechanism of action remains not completely clear. Therefore we set out to study the effects of n-3 LCPUFA on the sphingolipid cascade, endothelial function and vascular inflammation.

Endothelial dysfunction

Relaxation response

We have demonstrated that metacholine induced vasodilation was improved after feeding the DHA-rich diet for 12-weeks, like demonstrated for the antihypertensive drug losartan (62). Others also report EPA to augment vasodilatation in animals (63) and diseased human (64, 65). Furthermore, in healthy men forearm vasoconstrictive responses were reduced by n-3 LCPUFA (66). To our knowledge this has not been studied in hypertensive patients. It would be interesting to study the condition of SHR vessels at start of the study to determine whether fish oil prevents or reduces endothelial dysfunction. Spijkers *et al.* demonstrated before that young SHR, aged 9-weeks, do not suffer endothelial dysfunction yet (67), so it is most likely that fish oil supplementation prevented

the development of endothelial dysfunction. In aged SHR endothelium-dependent relaxation of the thoracic aorta was not reduced by the fish oil diet, despite reduced BP (68). In our study, using younger SHR, endothelial function was improved by the DHA rich diet. Possibly supplementation with fish oil would be able to prevent a rise in BP and protect against endothelial dysfunction in genetically predisposed prehypertensive subjects.

Contractile response

Endothelial (dys)function was investigated by studying the *ex vivo* ceramide-induced vessel contraction. This was reduced in the fish oil fed SHR due to substitution of AA from the cell membrane by DHA and EPA from the diet. This exchange of AA with n-3 LCPUFA has been described extensively (see **Chapter Two**). As a result less contractile TXA_2 is formed. In addition, although total ceramide levels in the plasma remained unaltered, a subtle change in several ceramide subsets were observed in SHR. C16:0, C22:0, C24:0 and C26:0 subsets were reduced in fish oil fed SHR, possibly adding to this effect since ceramide activates $iPLA_2$ to release AA from the cell membrane. By contrast, C24:1 levels were increased. Spijkers *et al.* have previously demonstrated that ceramide levels are enhanced in both hypertensive subjects and SHR. Furthemore, the total glucosylceramide levels were increased by over 30%, for which the C24:1 subtype was largely responsible. Previously glucosylceramide was shown to be reduced in plasma of SHR compared to normotensive WKY rats and antihypertensive treatment with losartan and hydralazine tended to increase this (unpublished results (67)). This was the first study indicating an effect of n-3 LCPUFA on sphingolipid biology. Currently the meaning of these alterations in CVD is largely unclear which deserves further investigation.

Endothelial activation

Cell adhesion molecule expression

In the Salmon in Pregnancy Study (SiPS) the intake of oily fish (two portions salmon per week) was increased in women whose offspring was at risk of developing atopic disease from week 20 of pregnancy until delivery. Neonatal immune responses were altered by salmon consumption. However, total IgE levels at birth and total IgE and incidence and severity of atopic dermatitis at 6 months of age were not different between the groups. As compared to the previously mentioned pregnancy studies, consumption of salmon twice a week provides less EPA and DHA (3.45 g EPA+DHA per week) perhaps explaining the lack of effect on atopic outcomes (69, 70). Other maternal intervention studies, using fish oil for supplementation do report beneficial effects of n-3 LCPUFA in the prevention of atopic disease in offspring (71-75). The umbilical vessel wall obtains essential fatty acids from the blood flow (76, 77). This makes human umbilical vein endothelial cells (HUVEC) a useful tool for studying the effects of dietary n-3 LCPUFA intake on changes in adhesion molecule expression on endothelial cells (EC) upon stimulation. Although the effects in offspring on atopic disease were disappointing, it was shown that *ex vivo*

LPS stimulated HUVEC reduced intercellular adhesion molecule (ICAM)-1 expression compared to HUVEC from pregnant women that continued their habitual diet. This may result in reduced leukocyte adhesion and therefore implicating a role for dietary n-3 LCPUFA in suppression of vascular inflammation in humans.

While this is the first study reporting about the effect of dietary intervention with n-3 LCPUFA on *ex vivo* cell adhesion molecule (CAM) expression, the results were in line with studies incubating cell cultures with EPA and DHA *in vitro*. These studies, using primary human saphenous vein endothelial cells (HSVEC) and HUVEC, assessed the effects of n-3 LCPUFA on bacterial lipopolysaccharide (LPS) induced endothelial activation. LPS is known to enhance ICAM-1 and VCAM-1 expression by EC and n-3 LCPUFA were found to decrease cell surface and mRNA expression of these adhesion molecules. Furthermore, the secretion of pro-inflammatory cytokines was reduced in EC stimulated with LPS or pro-inflammatory mediators (78-85). This implies a reduced activation of EC, which as a consequence may reflect reduced vascular inflammation upon *in vivo* challenge and may contribute to the protective effect of n-3 LCPUFA in CVD. In addition, animal studies have confirmed that dietary fish oil reduces leukocyte adhesion to endothelial cells (86, 87). It is currently not known whether increased dietary intake of n-3 LCPUFA alters EC inflammatory responses in humans; these results using HUVEC isolated from pregnant women that were supplemented with salmon provide the first evidence. Since HUVEC were cultured for several days *ex vivo*, these results suggest a programming effect by the maternal diet in neonatal tissue. Fetal and early postnatal factors may influence or program the development of disease (6, 88). It would be very interesting to confirm an effect on DNA methylation in HUVEC after salmon intervention.

A characteristic of endothelial dysfunction is the overexpression of CAM, followed by its release in the circulation. In subjects with hypertension serum levels of the soluble form of ICAM-1 (sICAM-1) and other markers are elevated compared to normotensive subjects (89, 90). Blood pressure reduction reduced sICAM-1 and sVCAM-1 (91). However, in SHR feeding a diet enriched in DHA from tuna oil did not alter CAM expression on EC and smooth muscle cells present in thoracic aorta segments that were cultured in presence or absence of *ex vivo* LPS stimulation (Immunohistochemistry, data not shown). This was in contrast with the effect of fish oil on CAM expression on HUVEC as measured by flow cytometry.

Cytokine secretion

Endothelial activation also comprises cytokine secretion by the endothelium. EPA and DHA can reduce the secretion of IL-6 by EC (80, 85). The production of IL-6 by LPS-stimulated HUVEC from pregnant women supplemented with salmon was not significantly reduced, however the IL-6 concentration was positively correlated with the suppression in ICAM-1 expression. Furthermore, the production of anti-inflammatory G-CSF tended to be higher in the salmon group. This indicates a reduced inflammatory state. Also

hypertension is associated with marked alterations in mediator secretion. In the hypertensive rat model, pro-inflammatory signaling was shown to be upregulated (e.g. platelet-derived growth factor) (52, 92). The diet rich in n-3 LCPUFA, was able to reduce IL-10 in supernatants of *ex vivo* LPS-stimulated thoracic aorta of SHR. This may contribute to reduced vasoconstriction (93). Like in HUVEC of the SiPS, TNF-α secretion remained unaltered by dietary fish oil treatment in SHR. On the other hand growth factors GM-CSF and VEGF were reduced in this study, suggesting selectivity or specificity in the effect of n-3 LCPUFA. The chemokine RANTES was increased in the fish oil group, suggesting an inflammatory effect. However, this may be neutralized by the reduction in other growth factor production. Furthermore, it has been suggested that low levels of RANTES are pro-hypertensive (94). Previously RANTES was shown to be reduced in SHR (95). Fish oil reduced (vascular) inflammation upon LPS challenge in both HUVEC and SHR vessels. This could result in decreased adherence and transmigration of leukocytes into vascular tissue, a key event in atherosclerosis. Therefore, fish oil could impact disease morbidity and mortality by improving endothelial dysfunction.

These results together suggest that a diet rich in fish oil could help to delay the onset of hypertension and hence the age at which drug therapy is needed by preserving a healthy BP and endothelial function. Prehypertensive patients, predisposed to develop hypertension as a consequence of their genetic background, may benefit from this. Furthermore, combining fish oil with antihypertensive drugs possibly can reduce the dose of antihypertensive drugs needed. Indeed, the combination of the beta-blocker propranolol and n-3 LCPUFA induced a synergistic hypotensive effect (96). Only a few antihypertensive drugs restore endothelial function and therefore end-organ damage persists.

EPA VERSUS DHA

In this thesis we show that besides the amount also the quality of fat is important. Next to the n-3:n-6 ratio, the ratio EPA:DHA was demonstrated to be important in **Chapter Seven**. For a long time it has been assumed that EPA and DHA act through similar mechanisms and in clinical trials often a mixture of EPA and DHA with varying ratios has been applied. In **Chapter Four** was shown that DHA was effective in reducing ROS generation and IL-13 secretion by mast cells *in vitro* at a lower dose than EPA. Therefore, the first *in vivo* experiments were performed using a diet rich in DHA from tuna oil. Our results suggest an increased efficiency of a DHA- over EPA-rich diet in the prevention and treatment of food allergy.

The possible mechanistic differences between EPA and DHA are still highly unclear and further investigation is needed. Both fish oil diets used in this thesis contain a mixture of EPA and DHA. Perhaps the interplay between PUFA or the ratio between EPA and DHA may

contribute to the effects observed. The molecular structure of the fatty acids may underlie functional difference, DHA has a higher degree of unsaturation which possibly alters membrane fluidity and therefore lipid raft clustering most effectively than EPA (97-99). Also differential effects of EPA and DHA on the regulation and transcription of genes were demonstrated (100). Others have suggested that EPA and DHA from fish oil accumulate in different compartments in the body. DHA accumulates usually in phosphatidylethanolamine and PLA_2 preferentially releases DHA from these phospholipids (80, 101). On the other hand, EPA is more potent than DHA in reducing platelet aggregation (102, 103). An intervention study demonstrated that serum triacylglycerol levels were suppressed by both DHA and EPA whereas HDL cholesterol increased in the DHA group only (104). Postprandial triglyceridemia was reduced by 19% by EPA and 49% by DHA supplementation (105). In cultured human endothelial cells, DHA selectively attenuates expression of pro-inflammatory proteins such as CAM (80). In SHR BP was reduced by DHA but not EPA (106) and DHA was more effective in improving vasodilation than EPA (107). Hence, also for CVD it may be preferable to use fish oil rich in DHA like is demonstrated for the prevention of food allergy in this thesis.

It would be interesting to study the effect of individual n-3 LCPUFA rather than a mix provided via fish oil in the food allergy model or SHR. N-3 LCPUFA present in fish are almost exclusively triglycerides, combining three fatty acids esterified to a glycerol backbone. Fish and therefore fish oil contain a combination of EPA and DHA in different ratios (see **Chapter One**). Ethyl esters contain two fatty acid tails per moiety since ethanol is used in the distillation and concentration process to obtain a higher concentration of n-3 LCPUFA. Although useful to study the effect of a single PUFA, the digestion of ethyl esters is slightly different due to the lack of a glycerol backbone, their absorption differs and they are less stable (108). An alternative way to control for possible beneficial effects of the oil matrix would be to remove EPA and DHA from the fish oil.

DOSAGE

While the average fish intake in the Japanese population with a low incidence of CVD was reported to be approximately 100 g fish/day (109), guidelines recommend that adults eat at least 2 servings/week of (preferably oily) fish providing at least 250 mg/day EPA+DHA (110, 111). Furthermore, the American Heart Association advises CHD patients to consume approximately 1 g of EPA and DHA (combined) per day for the secondary prevention of CHD (112, 113). In most countries the recommended intake is not met. In the Netherlands man consume 100 mg/day and woman 80 mg/day on average (114). Guidelines are based on combined consumption of EPA and DHA. The importance of EPA versus DHA is not taken into account. Furthermore, the current dietary guidelines are mainly based on cardiovascular health, not the possible prevention of allergic disease. Therefore dietary

guidelines may need to be adapted for specific endpoints. However, it is hard to translate dosages used in the murine food allergy model directly to the human situation. The rodent diet consists of a much lower fat percentage than the human diet. Over the last decades both the absolute amount of n-3 LCPUFA intake as well as the n-3:n-6 ratio has decreased. We calculated the ratio of n-3:n-6 PUFA in our diets to relate this to the n-3 LCPUFA intake in human fish consuming populations with proven protection against allergic disease. However, we assume that the absolute/total amount of n-3 LCPUFA intake is more important than the ratio consumed or the background diet. In human pregnancy studies a dose of 3 g/day was shown to be effective in the prevention of atopic outcome (72, 73, 115). This is a higher amount that has been advised for CHD patients. However, more clinical trials in human subjects need to be conducted aiming on allergy prevention to identify optimal dosing.

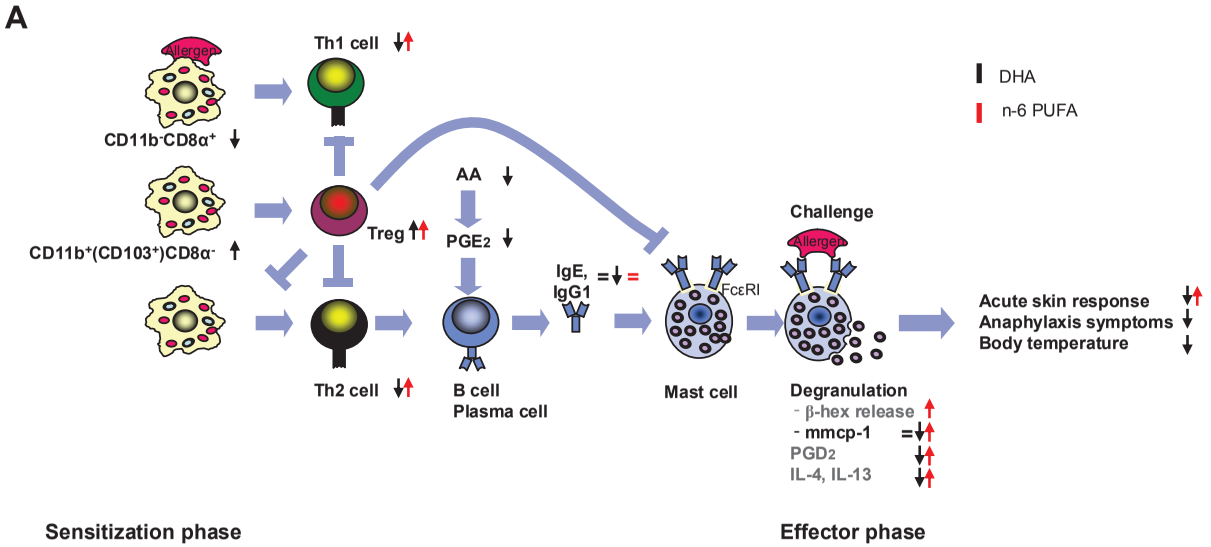
FUTURE DIRECTIONS

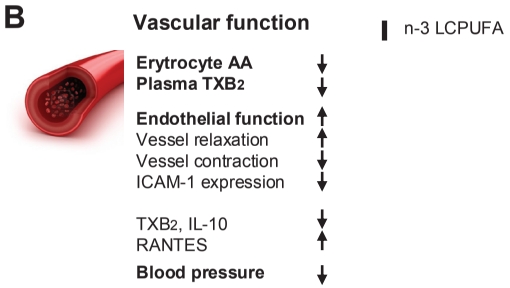
Currently, no cure for food allergy is available and therefore avoidance of the culprit food allergen causing allergic symptoms is recommended to food allergic patients. A reduction in mast cell activation or signaling may provide a strategy for the treatment of allergic symptoms. Dietary components may be able to alter the mast cell response either directly or via Treg. Treg can alter mast cell degranulation via cell-cell contact or IL-10 and TGF-β production (37, 116, 117). Reduced FcεRI receptor expression may contribute to this (118). Unfortunately the first studies in a therapeutic setting in humans using fish oil supplementation (asthma and atopic dermatitis) are not promising (119, 120). In patients with allergic rhinitis, asthma and wasp and bee venom hypersensitivity, oral tolerance can by re-established via subcutaneous, sublingual or oral immunotherapy. Immunotherapy aims at specific long-lasting immune suppression by administering small increasing doses of the allergen. Immunotherapy uses tolerance induction by Treg ($CD4^+CD25^+FoxP3^+$ and Tr1) and the desensitizing capacity of the allergen to redirect allergen-specific T cell responses from a Th2 to a Th1 profile (121, 122). However, in food allergy immunotherapy has not reached the clinic yet. This is due to the high risk for side effects, including anaphylaxis (123). We have demonstrated in **Chapter Five-Seven** that n-3 LCPUFA were able to reduce the effector response in both food allergic mouse models studied. The induction of Treg shown for DHA may contribute to this effect. Hence, dietary EPA and/or DHA may lower side effects and improve safety and efficacy of immunotherapy. Nevertheless, our preliminary results using allergen specific immunotherapy in the whey model indicated that DHA did not improve allergic symptoms and that n-6 PUFA did not worsen clinical symptoms, as measured by the acute allergic skin response (data not shown). In this treatment protocol the dietary intervention was started after the sensitization period while giving oral immunotherapy with PBS as a control or pWH for 4 weeks (at least 3

times a week). Immunotherapy with pWH successfully reduced the acute skin response. However, parallel intake of fish oil rich in DHA did not further improve this and a 10% soybean oil diet did not reverse the effects of pWH compared to standard 7% soybean oil diet. Hence in contrast to data presented in this thesis, no effect on the allergic response could be detected in readily allergic mice. Perhaps due to the repeated sensitization the mast cell phenotype has altered, e.g. increased FcεRI expression or signaling, rendering these cells insensitive to the effects of PUFA. However, the use of dietary fatty acids in the treatment of food allergy warrants further investigation e.g. by using an adapted and/or prolonged therapeutic strategy, since it may aid to instruct Treg.

TO CONCLUDE

This thesis provides insight in the mechanisms underlying the preventive effects of PUFA in allergic disease and CVD. While n-6 PUFA worsen food allergic symptoms, a diet rich in n-3 LCPUFA from fish oil induces tolerance. This involves alterations in DC subsets, skewing away from the Th2 phenotype, the increase in Treg capable of transferring tolerance and a reduced mast cell response «Summarizing Figure 1A». Besides beneficial effects in the allergic cascade also endothelial dysfunction is reduced by n-3 LCPUFA. This is demonstrated by the improved vessel relaxation, reduced contractility, less endothelial CAM expression and altered mediator secretion and results in beneficial effects on BP and vessel inflammation involved in atherosclerosis «Summarizing Figure 1B». Together the results in this thesis create more insight into the efficacy and mechanism of action of n-3 LCPUFA in the prevention of food allergy and cardiovascular disease.





Summarizing Figure 1

Summarizing Figure 1 Proposed mechanism of action of (dietary) polyunsaturated fatty acids (PUFA) in food allergy and cardiovascular disease (A) Schematic overview of the effects that are described in this thesis for a diet rich in n-6 PUFA linoleic acid from soybean oil and a diet rich in n-3 LCPUFA DHA from tuna oil on the allergic cascade. Results are shown for the DHA-rich diet as this diet was studied most extensively. DC and T cell changes by the DHA-rich diet were most pronounced in sham-sensitized mice. A diet rich in n-6 PUFA enhanced the Th2/Th1 and Th2/Treg ratio suggesting Th2 skewing, whereas the DHA diet enhanced the Th1/Th2 ratio in the spleen. The Treg generated by the DHA-rich diet contribute to tolerance induction and may reduce mast cell sensitivity. Substitution of membrane AA with DHA decreased PGE_2 generation. This may reduce allergic sensitization since PGE_2 stimulate B cells to produce IgE. DHA reduced splenic T cell proliferation but not PE-IgE and -IgG1 and serum mmcp-1 in the PE-model; whereas it effectively reduced the humoral response in the whey-model. The EPA-rich diet was not effective for the parameters shown, except in reducing the acute skin response in whey-allergic mice. *In vitro* results for AA vs DHA incubation of mast cells are shown in grey font. (B) Summary of the effects that are described in this thesis for n-3 LCPUFA in spontaneously hypertensive rats and human umbilical vein endothelial cells. This includes improved endothelial function and reduced blood pressure. Mediators from *ex vivo* stimulated aorta are not exclusively secreted by endothelial cells. The reduction in pro-inflammatory mediator secretion by the vascular tissue relates to improved endothelial function. AA: arachidonic acid; β-hex: β-hexosaminidase; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; IL: interleukin; LCPUFA: long chain polyunsaturated fatty acids; mmcp-1: mucosal mast cell protease 1; PE: peanut extract; PG: prostaglandin; RANTES: Regulated on Activation, Normal T cell Expressed and Secreted; Th: T helper; Treg: regulatory T cell; TX: thromboxane.

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Nederlandse samenvatting

Verscheidene voedingscomponenten worden geassocieerd met gezondheidsbevorderende effecten, terwijl andere voedingsgewoonten worden geassocieerd met allergische en cardiovasculaire aandoeningen. Meervoudig onverzadigde vetzuren (ofwel PUFA, de afkorting van de Engelse term polyunsaturated fatty acids) staan volop in de aandacht vanwege deze gezondheidsbevorderende effecten.

In dit proefschrift is onderzocht of de toevoeging van PUFA aan het dieet voedselallergie kan voorkomen of de progressie van cardiovasculaire aandoeningen kan remmen. Hiertoe is gebruik gemaakt van celkweekexperimenten, waarin onder andere humane cellen zijn onderzocht. Ook zijn verschillende proefdiermodellen toegepast. Dit is noodzakelijk omdat het onderzoeken van de werking van PUFA ter preventie en afremming van deze aandoeningen in de mens beperkt mogelijk is. Met alleen celkweekexperimenten kan de onderzoeksvraag niet volledig worden beantwoord, omdat bij de ontwikkeling van allergische en cardiovasculaire aandoeningen veel verschillende celtypen zijn betrokken en samenwerken.

Meervoudig onverzadigde vetzuren

Ons dieet bestaat uit een breed scala aan vetzuren die verschillen in ketenlengte en de hoeveelheid dubbele koolstofbindingen. Als de koolstofketen van een vetzuur een dubbele binding bevat, spreekt men van een onverzadigd vetzuur. Wanneer twee of meer dubbele bindingen in het molecuul aanwezig zijn, spreekt men van een meervoudig onverzadigd vetzuur ofwel PUFA. PUFA worden onderverdeeld in n-6 en n-3 PUFA afhankelijk van de positie van de eerste dubbele koolstofbinding.

Gedurende de laatste jaren is de consumptie van PUFA toegenomen. Dit is voornamelijk het gevolg van een toename in het gebruik van plantaardige oliën en producten, zoals margarine, die rijk zijn aan n-6 PUFA, voornamelijk linolzuur. Daarentegen is de consumptie van n-3 PUFA met een lange keten (long chain) (n-3 LCPUFA) uit vette vis geleidelijk afgenomen. N-3 LCPUFA omvatten eicosapentaeenzuur (EPA) en docosahexaeenzuur (DHA) die bestaan uit 20 of meer koolstofatomen. De mens is geëvolueerd op een dieet met een n-3:n-6-ratio van 1:1, echter deze ratio benadert in de Westerse wereld thans 1:10 tot 25.

PUFA kunnen op verschillende manieren werken. Zo incorporeren PUFA in celmembranen van alle lichaamscellen, waardoor deze veranderen. Ook kunnen PUFA op receptoren (eiwit dat specifieke moleculen kan binden) op en in de cel binden en de signalen in de cel beïnvloeden. Verder kunnen PUFA worden omgezet in metabolieten (product na omzetting door enzym) met verschillende functies. Metabolieten van de n-6 LCPUFA arachidonzuur kunnen een ontstekingsreactie verergeren, terwijl metabolieten van n-3 LCPUFA EPA dit in mindere mate kunnen doen. In **Hoofdstuk Twee** is uitgebreid beschreven hoe n-3 LCPUFA werken.

Voedselallergie

Ofschoon normaliter orale tolerantie (specifieke afweer onderdrukking) tegen onschadelijke voedseleiwitten wordt ontwikkeld, is dit in ongeveer 6% van de jonge kinderen niet het geval. Zij ontwikkelen een voedselallergie.

Bij mensen met een voedselallergie functioneert het immuunsysteem niet naar behoren. In de darmwand nemen dendritische cellen eiwitten uit de voeding op. Deze eiwitten worden aangeboden aan T-helpercellen (Th). Ongevaarlijke eiwitten (allergenen) uit ons voedsel worden in patiënten met een voedselallergie door het immuunsysteem als gevaarlijk gezien. Daardoor ontstaat een Th2-immuunrespons tegen het ongevaarlijke allergeen. Th2-cellen zetten B-cellen aan om antistoffen (antilichamen) tegen dit allergeen aan te maken. De antistoffen binden op mestcellen (gespecialiseerde immuuncellen) en bij een volgend contact met het allergeen zal dit worden herkend door deze antistoffen. Dit leidt ertoe dat de opgeslagen stoffen in de blaasjes (granulen) in mestcellen worden afgegeven (degranuleren) (Figuur 3, Hoofdstuk Eén). Degranulatie leidt tot klachten, zoals van de huid (jeuk en roodheid), het maag- en darmstelsel (diarree) en de luchtwegen (benauwdheid). In ernstiger gevallen kan een anafylactische shock ontstaan, soms met de dood tot gevolg. Aan de andere kant zijn regulatoire T-cellen (Treg) belangrijk voor het (gedeeltelijk) onderdrukken van een immunologische reactie zoals een allergische reactie. Daarmee zou dus de ernst van een voedselallergische reactie kunnen worden verminderd of volledig kunnen worden voorkomen.

In dit proefschrift is een muismodel voor koemelkallergie en pinda-allergie gebruikt. De muizen worden gevoelig gemaakt voor deze allergenen via orale toediening. Omdat gelijktijdig het choleratoxine wordt gegeven, worden de muizen niet tolerant maar allergisch. Koemelkallergie is de eerste voedselallergie die in het leven ontstaat. Hoewel de meeste kinderen koemelkallergie ontgroeien, zijn zij vatbaarder voor andere allergieën en astma. De belangrijkste koemelkeiwitten zijn caseïne- en wei-eiwitten. In het model beschreven in dit proefschrift worden muizen allergisch gemaakt voor wei-eiwit. Pinda-allergie is een andere veelvoorkomende voedselallergie. In tegenstelling tot koemelkallergie, ontgroeien kinderen een pinda-allergie meestal niet. Ook zijn de klachten veelal ernstiger. In de muismodellen voor voedselallergie zijn diëten onderzocht die visolie bevatten. Visolie is een belangrijke bron van n-3 LCPUFA.

Cardiovasculaire aandoeningen

Een aanzienlijk deel van de Westerse bevolking heeft een verhoogd risico om cardiovasculaire aandoeningen te ontwikkelen. Cardiovasculaire aandoeningen zijn een van de belangrijkste doodsoorzaken in Westerse landen. Eskimo's consumeren grote hoeveelheden vette vis die veel n-3 LCPUFA bevat. In deze groep komen weinig cardiovasculaire aandoeningen voor. De resultaten van andere onderzoeken tonen een verlaagd risico op overlijden door cardiovasculaire aandoeningen door de consumptie van vette vis of visolie. Hoe dit beschermende effect tot stand komt, is thans niet duidelijk.

Atherosclerose is de belangrijkste risicofactor voor het ontstaan van cardiovasculaire aandoeningen. Dit is een complex chronisch proces waarbij onder andere vetten en cellen zich in de arteriën ophopen. Een belangrijke risicofactor voor de ontwikkeling van atherosclerose is hypertensie ofwel een hoge bloeddruk. Tegenwoordig heeft ongeveer 30% van de wereldbevolking een te hoge bloeddruk. Samen met andere factoren kan dit een ontstekingsreactie in het bloedvat veroorzaken. Dientengevolge kunnen de endotheelcellen, die als één enkele laag van cellen de binnenzijde van een bloedvat bekleden, meer celadhesiemoleculen op hun oppervlak aanbieden en meer ontstekingsbevorderende eiwitten (cytokinen) gaan produceren. Hierdoor kunnen witte bloedcellen aan de bloedvatwand binden waarna deze cellen zich kunnen vestigen in de bloedvatwand. Dit kan resulteren in atherosclerose, omdat een vicieuze cirkel van schade en ontsteking ontstaat, wat leidt tot de ophoping van een verscheidenheid aan cellen die een plaque vormen. Deze plaque kan een bloedvat afsluiten en een zuurstoftekort in delen van het lichaam veroorzaken. Ook kan de plaque beschadigd raken waardoor door verstopping van de bloedvaten een hartinfarct of beroerte kan ontstaan.

Hypertensie wordt geassocieerd met endotheeldysfunctie. Dit omvat onder andere een verminderde relaxatie (ontspanning), verhoogde contractie (samentrekking) en ontsteking van de bloedvaten. De bloeddruk wordt onder andere gereguleerd door de afgifte van factoren door het endotheel. Die factoren sturen de onderliggende spierlaag aan. In het geval van endotheeldysfunctie zullen voornamelijk contraherende factoren zoals tromboxane A_2 (TXA_2) worden afgegeven. Sfingolipiden zoals ceramide spelen hierin een belangrijke rol (Figuur 1 en 2, Hoofdstuk Eén). Zo worden verhoogde ceramideconcentraties in verband gebracht met hypertensie.

Om de effecten van visolie op hypertensie te onderzoeken, is gebruik gemaakt van ratten die spontaan hypertensie ontwikkelen ten gevolge van een genetisch defect. Dit is ook vaak bij mensen de oorzaak. Het is daarom een geschikt model om de effecten van visolie te bestuderen. Verhoogde ceramideconcentraties kunnen n-6 LCPUFA arachidonzuur uit het celmembraan vrijzetten (Figuur 2, Hoofdstuk Eén). Arachidonzuur kan vervolgens worden omgezet in TXA_2 leidend tot contractie van het bloedvat. Van visolie met EPA en DHA, is bewezen dat het de bloeddruk kan verlagen. Tot dusver is niet bekend hoe dit effect tot stand komt.

Dit proefschrift

Dit proefschrift heeft als doel meer inzicht te geven in de effectiviteit en het werkingsmechanisme van n-3 LCPUFA. In **Hoofdstuk Drie** wordt beschreven dat de toename in consumptie van sojaolie, rijk aan n-6 PUFA, orale tolerantie doorbreekt in het muismodel voor koemelkallergie. In dit experiment is orale tolerantie geïnduceerd door toediening van partieel weihydrolysaat (wei-eiwitten geknipt in kleinere fragmenten) voorafgaand aan behandeling met volledige wei-eiwitten en choleratoxine om allergie

te induceren. De kleine weifragmenten kunnen door hun formaat niet meer tot een allergische reactie leiden, maar nog wel orale tolerantie bewerkstelligen. Bij inname van een hoger percentage sojaolie in het dieet zijn deze fragmenten echter niet meer in staat om de muizen tolerant te maken. Hierdoor maakt de vervolgbehandeling met wei de muizen allergisch, terwijl dit bij een lagere hoeveelheid sojaolie niet het geval is. Ook verergert het dieet met meer n-6 PUFA de allergische symptomen zoals de acute allergische huidreactie (oorzwelling) en mestceldegranulatie in koemelkallergische muizen. Tevens nam het percentage geactiveerde Th2-cellen toe.

Vervolgens is in celkweekexperimenten onderzocht wat het effect is van n-6 LCPUFA arachidonzuur versus n-3 LCPUFA EPA en DHA op de mestceldegranulatie, de cytokinenproductie en signaaldoorgifte in de humane mestcel (**Hoofdstuk Vier**). Arachidonzuur verhoogde de afgifte van ontstekingsbevorderende stoffen (mediatoren), zoals prostaglandine D_2. Daarentegen bleken EPA en DHA het meest effectief in het verminderen van de afgifte van cytokinen die de allergische reactie bevorderen. Dit effect ontstaat door de vermindering van reactieve zuurstofverbindingen in de cel.

Het effect van de gedeeltelijke vervanging van n-6 PUFA (sojaolie) met n-3 LCPUFA uit visolie (tonijnolie, voornamelijk rijk aan DHA) op het ontstaan van voedselallergie is onderzocht in de muis in **Hoofdstuk Vijf**. Visolie was in staat om de ontwikkeling van koemelkallergie in muizen voor een groot deel tegen te gaan. Deze muizen hadden lagere hoeveelheden weispecifieke antilichamen in hun bloed, een minder sterke acute allergische huidreactie en een verminderde mestceldegranulatie. Ook was het percentage Th2-cellen in de milt verlaagd. Tegelijkertijd was er een toename in het percentage van een specifieke populatie van dendritische cellen in de lymfeknopen van de darm. Deze populatie brengt onder andere het eiwit CD103 aan de buitenkant van de cel tot expressie. Dit type dendritische cel kan tot de vorming van Treg aanzetten. Er werd dan ook een verhoogd percentage Treg in zowel de darm als in de milt gevonden.

De betrokkenheid van deze $CD25^+$ (specifiek oppervlakte molecuul) Treg bij het beschermende effect van visolie in weiallergie is verder onderzocht in **Hoofdstuk Zes**. Miltcellen van muizen die een visolierijk dieet of een controledieet zonder visolie hadden gehad terwijl ze allergisch werden gemaakt tegen wei (donormuis), werden ingespoten in gezonde muizen (ontvangermuis). Vervolgens werden de gezonde ontvangermuizen ook allergisch gemaakt voor wei, terwijl ze gevoerd werden met een controledieet. De muizen die de miltcellen van donormuizen uit de visoliegroep hadden ontvangen, werden niet allergisch. Echter de miltcellen van de donormuizen die het controledieet hadden gekregen, konden de ontvangermuizen niet beschermen. Als de $CD25^+$ Treg uit de miltcellen van de met visolie behandelde donormuizen werd weggehaald voordat ze aan de ontvangermuizen werden gegeven, viel het beschermende effect weg. Dit benadrukt de belangrijke rol van Treg in tolerantie-inductie door n-3 LCPUFA.

Vervolgens is het verschil tussen visolie met een hoge concentratie EPA en visolie met een hoge concentratie DHA onderzocht wat betreft het tegengaan van voedselallergie

(**Hoofdstuk Zeven**). DHA bleek effectiever dan EPA in het onderdrukken van de allergische symptomen in zowel wei- als pinda-allergische muizen. Dit was ten minste deels het resultaat van een directe vermindering van de mestceldegranulatie. Bovendien was DHA effectiever dan EPA ter voorkoming van koemelkallergie. Tot dusver is het niet bekend waardoor DHA effectiever is dan EPA. De effecten van visolie met een hoge concentratie DHA zijn daarna ook onderzocht in spontaan hypertensieve ratten.

De resultaten van **Hoofdstuk Acht** tonen dat tonijnolie de bloeddruk van spontaan hypertensieve ratten kan verlagen. Voorts bleek dat de bloedvaten geïsoleerd uit met visolie behandelde ratten een verbeterde endotheelfunctie hadden. Deze vaten waren beter in staat te relaxeren wanneer methacholine (endotheelafhankelijke farmacologische vaatverwijder) werd toegevoegd. Het DHA-rijke dieet verlaagde de hoeveelheid arachidonzuur in het celmembraan. Dit draagt waarschijnlijk bij aan de verminderde ceramide-gemedieerde contracties van de geïsoleerde bloedvaten. De hoeveelheid TXA_2 in het bloedserum was ook verlaagd. Tevens was de productie van TXA_2 en verschillende cytokinen betrokken bij vasoconstrictie (samentrekking van het bloedvat) door gestimuleerde vaten (aorta) verlaagd, terwijl de productie van andere beschermende mediatoren was verhoogd.

In **Hoofdstuk Negen** wordt aangetoond dat verrijking van het dieet met n-3 LCPUFA een belangrijke rol kan spelen in het verbeteren van de endotheelfunctie in bloedvaten van mensen. Hiervoor is gebruik gemaakt van materiaal uit het eerste dieetinterventieonderzoek waarbij vis werd gegeven aan zwangere vrouwen. Na isolatie van endotheelcellen uit de navelstreng werden deze cellen gekweekt en gestimuleerd. In dit hoofdstuk wordt beschreven dat visconsumptie door zwangere vrouwen de endotheelcelactivatie in een reactievaatje kan verminderen. Dit wordt het duidelijkst aangetoond door de verminderde expressie van celadhesiemoleculen. Deze resultaten maken aannemelijk dat n-3 LCPUFA uit vis(olie) ook in de mens cardiovasculaire ontsteking kan voorkomen. Het impliceert bovendien dat het dieet van de moeder tijdens de zwangerschap een belangrijke rol kan spelen in de ontwikkeling van cardiovasculaire aandoeningen in het latere leven.

Dit proefschrift geeft inzicht in de mechanismen die ten grondslag liggen aan de preventieve effecten van n-3 LCPUFA in allergische en cardiovasculaire aandoeningen. Waar n-6 PUFA de allergische symptomen in voedselallergie kunnen verergeren, kan een dieet rijk aan n-3 LCPUFA uit visolie het ontstaan van tolerantie hiertegen bevorderen. Hierbij zijn veranderingen in dendritische celtypen, vermindering van de Th2- en B-celrespons en een toename in Treg betrokken. Daarnaast kunnen de symptomen die ontstaan door mestceldegranulatie verminderen. Naast deze positieve effecten op de allergische cascade wordt ook endotheeldysfunctie verminderd door n-3 LCPUFA. Dit is aangetoond doordat een dieet van vis(olie) in spontaan hypertensieve ratten of in zwangere vrouwen leidde tot een verbeterde bloedvatrelaxatie, verminderde

bloedvatcontractie, verminderde celadhesiemolecuulexpressie op endotheelcellen en/of veranderde mediatorafgifte. Dit tezamen kan resulteren in een positief effect op de bloeddruk en een vermindering van bloedvatontsteking en uiteindelijk een verminderd risico op atherosclerose. Kortom, deze resultaten geven een beter inzicht in de effectiviteit en het werkingsmechanisme van n-3 LCPUFA bij de preventie van voedselallergie en cardiovasculaire aandoeningen.

Curriculum Vitae

List of publications

CURRICULUM VITAE

Lieke van den Elsen was born on January 6, 1985 in Best. In 2003 she graduated cum laude from the Heerbeeck College in Best, after which she started to study Pharmacy at Utrecht University. In 2006 she received her Bachelor's degree (cum laude) and she continued with the research Master Drug Innovation. During both her Bachelor and Master she participated in the Honours Programme of the department of Pharmaceutical Sciences. During the Master she did an internship at the Utrecht Institute for Pharmaceutical Sciences (UIPS) were she investigated the *in vitro* modulation of mast cell degranulation and mediator release by polyunsaturated fatty acids (PUFA) and the effects of PUFA on intestinal epithelial barrier function. She did this under supervision of Prof. dr. Johan Garssen en dr. Linette Willemsen. At the Institute of Human Nutrition, University of Southampton, United Kingdom she did her second internship. Here she studied the effect of salmon consumption by pregnant women on umbilical cord endothelial cell activation under supervision of Prof. dr. Philip Calder and dr. Paul Noakes. After receiving her Master's degree in 2008 (cum laude), she continued with her PhD project at UIPS with Prof. dr. Johan Garssen en dr. Linette Willemsen. She investigated whether and how PUFA suppress allergic sensitization and improve cardiovascular disease, as described in this thesis. During her PhD she collaborated with the Institute for Risk Assessment Sciences in Utrecht and the Academic Medical Center in Amsterdam. She also participated in the Utrecht Center for Food allergy and was trained in pharmacology and immunology in the UIPS and the Infection & Immunity PhD program. In both programs she was a member of the PhD student committee and she organized the UIPS PhD retreat in 2011. She was also involved in the PhD council of the Graduate School of Life Sciences and PhD council of the Faculty of Science. During her PhD project she received the New Investigator Award at ISSFAL 2010, the Young Investigator Award at ESPGHAN 2011, the oral abstract prize at EAACI 2011 & 2013 as well as the poster prize at the EAACI 2013. In October 2013 she will start as a post doctoral fellow in the lab of dr. Elizabeth Forbes-Blom at the Malaghan Institute of Medical Research, Victoria University, Wellington, New Zealand, to continue her work in food allergy research.

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