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Inflammation Modulating Effects of Prostaglandins and Omega-3 Fatty Acids

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ABSTRACT

Omega-(ω6) and ω3 fatty acids (FA) and their metabolites (eikosanoids, such as prostaglandins, PG) are important modulators of immune and inflammatory responses in various ways. The aim of this thesis was to assess some in vitro and ex vivo effects of these lipids on blood leukocytes.

In the first part of this thesis (studies *I* and *II*), we examined the *in vitro* effects on peripheral blood lymphocytes and monocytes by various PGs. We found that the natural killer (NK) activity of lymphocytes was decreased by PGD_2 and PGE_2. PGD_2 also decreased surface expression of the CD8 antigen (on cytotoxic/suppressor T cells), as well as of Fc receptors for IgG on T cells. In peripheral blood mononuclear cells (PBMC), the proliferative response to phytohemagglutinin (PHA) was reduced in the presence of PGD_2, PGA_2 and PGE_2, in falling order of potency. In lipopolysaccharide (LPS) stimulated purified monocytes we found that, although PGD_2 did not influence TNF-α release, its metabolites PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy Δ^{12}, Δ^{14}-PGJ_2 (15d-PGJ_2) enhanced the TNF-α release. The monocyte NADPH oxidase activity was not affected by PGB_2, PGD_2 or PGE_2.

In the second part of the thesis (*III-V*), we assessed various *ex vivo* effects on PBMC of a dietary treatment for 6 months with a DHA enriched fish oil preparation (1.7 g of docosahexaenoic acid (DHA) and 0.6 g of eicosapentaenoic acid (EPA) per day; ω3 FA group) or an isocaloric placebo oil in patients with Alzheimer disease (AD; the *OmegAD* trial). The ω3 FA-treated group displayed significant increases of DHA and EPA plasma levels whereas the placebo group did not. The release of $PGF_{2\alpha}$ (a stable metabolite of PGE_2) was significantly diminished from LPS (but not from PHA) stimulated PBMC in the ω3 FAs group, while no change was noted for the placebo group (*III*). The ω3 group showed significant decreases of IL-1β, IL-6, and G-CSF release after LPS stimulation of PBMC (*IV*). $PGF_{2\alpha}$, IL-1β and IL-6 changes correlated inversely with changes in DHA and EPA plasma levels. Furthermore, reductions of IL-1β and IL-6 were significantly correlated with each other, and decreased IL-1β and IL-6 levels correlated with decreased $PGF_{2\alpha}$ levels.

In paper *V*, we studied gene expressions in the PBMC, using a genome wide technique with approx. 8000 genes. At 6 months, a significant up-regulation of nine, and a down- regulation of 10 genes were noted in the ω3 group. Many of these genes are involved in inflammation and neurodegeneration, e.g. *CD63, RHOB, CASP4, NAIP, VCP* and *SORL1*. The up-regulation of *CD63* and *SUPT4H1* genes and the down-regulation of *RHOB*, *LOC 399491*, *ZNF24* and *ANAPC5* genes were significant in the ω3 group compared to placebo group. The down-regulated *ANAPC5* and *RHOB* genes correlated to increased DHA and EPA levels.

In conclusion, cyclooxygenase products of arachidonic acid, i.e. prostaglandin of the E and D series, influence various immune and inflammatory responses of lymphocytes and monocytes in vitro in a complex way, depending on the specific eikosanoid, the dose and studied functions. Dietary intake of DHA rich fish oil reduces the release of PGE_2, pro- inflammatory cytokines and the myeloid growth factor G-CSF ex vivo in PBMCs. Moreover, the DHA rich fish oil affected several genes, which might be of significance for inflammation or Alzheimer's disease. Although these responses point to a down-regulation of inflammatory reactions, it remains to be seen whether the DHA rich fish oil also affects the inflammatory process in the AD brain.

Till Engla och Saga

LIST OF PUBLICATIONS

I. Wasserman J, Hammarström S, Petrini B, Blomgren H, von Stedingk L-V, Vedin I. Effects of some prostaglandins and leukotrienes on lymphocytes, monocytes and their activity in vitro. *Int Allergy Appl Immun 1987; 83:39-43.*

II Vedin I, Wasserman J, Hammarström S. Stimulation of tumor necrosis factor-α release from lipopolysaccharide activated human blood monocytes by prostaglandin J_2 and metabolites of prostaglandin J_2. *Prostaglandins, Leukotr. Essent Fatty Acids 1996;55:185-189.*

III Vedin I, Cederholm T, Freund-Levi Y, Basun H, Hjorth E, Faxén-Irving G, Eriksdotter Jönhagen M, Schultzberg M, Wahlund L-O, Palmblad J. Reduced prostaglandin $F_{2\alpha}$ release from blood mononuclear leukocytes after oral supplementation of ω-3 fatty acids: the OmegAD study. *Submitted 2009.*

IV Vedin I, Cederholm T, Freund Levi Y, Basun H, Garlind A, Faxén Irving G,. Eriksdotter Jönhagen M, Vessby B, Wahlund L-O, Palmblad J. Effects of docosahexaenoic acid- rich n-3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes: the OmegAD study. *Am J Clin Nutr 2008; 87:1616-1622.*

V Vedin I, Cederholm T, Freund Levi Y, Basun H, Garlind A, Faxén Irving G, Eriksdotter Jönhagen M, Wahlund L-O, Dahlman I, Palmblad J. Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on gene expression changes in blood mononuclear leukocytes: the OmegAD study. *Submitted 2009*.

Papers I and II were included in my Licentiate Thesis at Karolinska Institutet 1993. *Some effects of prostaglandins of E and D series on the in vitro activity of lymphocytes and monocytes.*

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Freund Levi Y, Eriksdotter-Jönhagen M, Cederholm T, Basun H, Faxén-Irving G, Garlind A, **Vedin I**, Vessby B, Wahlund LO, Palmblad J. Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial. Arch Neurol 2006; 63:1402-08.

Freund-Levi Y, Basun H, Cederholm T, Faxen-Irving G, Garlind A, Grut M, **Vedin I**, Palmblad J, Wahlund LO, Eriksdotter-Jonhagen M. Omega-3 supplementation in mild to moderate Alzheimer's disease: effects on neuropsychiatric symptoms. Int J Geriatr Psychiatry 2008; 23:161-9.

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Freund-Levi Y, Hjorth E, Lindberg C, Cederholm T, Faxen-Irving G, **Vedin I,** Palmblad J, Wahlund LO, Schultzberg M, Basun H, Eriksdotter Jönhagen M. Effects of omega-3 fatty acids on inflammatory markers in cerebrospinal fluid and plasma in Alzheimer's disease: the OmegAD study. Dement Geriatr Cogn Disord 2009; 27:481-490

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AD	Alzheimer's disease
ASA	Acetylsalicylic acid
CSF	Cerebrospinal fluid
COX	Cyclooxygenase
c-AMP	Cyclic adenosin 3´,5´-monophosphate
c-GMP	Cyclic guanosin 3´,5´-monophosphate
CL	Chemiluminiscence
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
EGF	Epidermal growth factor
FA	Fatty acid
bFGF	basic Fibroblast growth factor
FITC	Fluoroscein isothiocyanate
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage stimulating factor
HBSS	Hank´s balanced salt solution
IL	Interleukin
IFN-γ	Inteferon gamma
KIRs	Killer cell immunoglobulin-like receptors
KLRs	Killer lectin like receptors
LPS	Lipopolysaccharide, endotoxin
LT	Leukotriene
LOX	Lipooxygenase
M-CSF	Macrophage colony stimulating factor
NK	Natural killer cells
ω3 FA, ω6 FA	Omega-3 fatty acid, Omega-6 fatty acid
PPARs	Peroxisome proliferator activating factors
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PG	Prostaglandin
PHA	Phytohemmagglutinin
PUFA	Polyunsaturated fatty acids
RT-PCR	Reverse transcription polymerase chain reaction
NF-κB	Nuclear factor kappa B
SAM	Significance analysis of microarrays
TLR	Toll receptors
TXA	Tromboxane
TGFβ	Transforming growth factor beta
TNF-α	Tumor necrosis alpha
TPA	12-*O*- tetradecanoylphorbol-13-acetate, PMA
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

Here, I want to describe briefly some basic aspects of inflammation that pertains to the subject of my thesis. Basically, there are two types of inflammation, the neutrophilic (classic) and the allergic. Since this thesis primarily deals with the classic inflammation, the allergic will not be presented further.

1.1 Inflammation types and general features

Inflammation plays an important role in preservation of "self" and rejection of "non-self", thus, being part of host defense together with adapted immunity (which is represented by immune competent cells that react to individual antigens by production of antibodies or initiation of cytotoxic reactions).

Inflammation represents an early host response, e.g. an acute inflammation phase that can be prolonged into a chronic phase. Inflammation also has a resolution phase, i.e. a healing phase. These will be described in some details below.

Inflammation is defined clinically by the five cardinal signs, redness (rubor), heat (calor), swelling (tumor), pain (dolor) and impaired function (function laesa). On the microscopic level, inflammation is characterized by an increased vasodilatation, blood flow, vascular permeability, exudation of fluids and plasma constituents, and by emigration of leukocytes from the blood stream through the endothelial cells into tissues. A majority of these events occur in the postcapillary venules.

Acute inflammatory reactions are mediated and regulated in a complex interaction between many cell types. These include (but are not limited to) phagocytes (such as granulocytes, monocytes/ macrophages, natural killer (NK) and dendritic cells, as well as microglia), proteins (e.g. cyto- and chemokines, growth factors, enzymes, complement factors and antimicrobial peptides) and lipid mediators (e.g. fatty acids such as arachidonic /AA/, eikosapentaenoic /EPA/ and dokosahexaenoic /DHA/ acids, leukotrienes /LTs/, prostaglandins /PGs/ and other related metabolites collectively known as eikosanoids). All these cells and molecules act in a highly regulated manner, interacting with each other during the various phases of inflammation. They are responsible for the five cardinal signs of inflammation (Table 1).

Table 1. **Biochemical characteristics of acute inflammation include generation of:**
- vasoactive substances (histamine, neuropeptides, NO, leukotrienes, VEGF etc)
- cell activating substances (chemotactic agents, cytokines, chemokines etc)
- cytotoxic substances (antibodies, complement components, perforins etc)
- microbicidal substances (oxygen radicals**,** antimicrobial peptides etc)
- growth factors (PDGF, EGF, bFGF, VEGF, G-CSF and other angiopoietic mediators etc).
- pain-eliciting substances
After Palmblad, 2009

1.1.1 The acute inflammation

The initial response, *the innate immune response* of the body to harmful stimuli, involves many actors, which recognize invading injurious agents (e.g. microorganisms, endotoxins) or agents produced by the organism itself (e.g. necrotic cells or autoantigens) by pattern recognition systems. That might involve various receptors for classes of molecules, cytotoxic systems and cells (Table 2). These systems must be available within minutes in order to define whether a molecule belongs to the organism or is foreign; however, there is at this time no need to identify the individual molecules, suffice it to recognize molecular patterns of an intruder.

Table 2 **Innate immunity response systems** *After Palmblad, 2009*

- Pattern recognition molecules: Mannose binding protein, TLR (Toll receptors), NLR (NOD receptors), SNARE receptors etc.
- Complement system: Factors C'1-9.
- Antimicrobial proteins: Lysozyme, bactericidal permeability increasing factor, defensins, LL-37 etc.
- Cells*:* Mast cells, neutrophils, monocytes, endothelial, dendritic, NK cells.

Since my thesis mainly concerns lymphocytes, monocytes and NK cells, they will be briefly presented here.

Natural killer (NK) cells. When first described by Kiessling and Klein [1] their ability to spontaneously kill certain tumor cells was appreciated. They were described as FcγR+ large granular (LGL) lymphocyte [2]. Eventually, it became difficult to separate the NK cell population by size and morphology, since other cells could share the LGL phenotype. Together with the ability to kill certain cells without prior sensitization, NK cells are producers of cytokines such as IFNγ, TNF-α, GM-CSF and IL-3 [3]. NK cells bear a wide variety of invariant activating and inhibitory receptors, including KIRs and KLRs. These and natural cytotoxicity receptors are of significance for killing of tumor cells as well as of cells infected by viruses and intracellular pathogens [4].

Monocytes/macrophages. Monocytes are derived from a myeloid precursor cell, which gives rise also to neutrophils [5]. GM-CSF and M-CSF are important growth factors for the development of monocytes. In the blood, monocytes act as innate immune surveillance and antigen presentation cells and they give rise to tissue macrophages in diverse organs e.g. as Kupffer cells in the liver, peritoneal and lung macrophages, and microglia cells in the brain [6]. Mononuclear phagocytes, as isolated blood monocytes often are referred to, have diverse activities, e.g. they confer recognition and phagocytosis of invaded microbes [7], and they clear the inflammatory area, repair damaged tissues [8] and take part in the wound healing process [9]. They have microbicidal mechanism and express a variety of surface receptors. Macrophages also are a major source of eikosanoids, including prostaglandins PGE_2 and $PGF_{2\alpha}$. Activated monocytes/ macrophages produce a great variety of cytokines e.g. IL-1, TNF-α, IL-6 and growth factors. Recently, the non-phlogistic uptake of dead neutrophils has focused on a role for monocytes/macrophages in the resolution of inflammation, as well as that some monocytes subpopulations may be anti-inflammatory [9].

Endothelial cells constitute a cellular barrier that separates the blood flow from the tissues. In non-inflamed tissues, these cells are anti-inflammatory, anti-coagulative and maintain the vessel wall permeability. Upon activation they become pro-inflammatory, pro-coagulative, attracts blood leukocyte, and release a variety of mediators that act on neighboring cells. They are unique producers of prostacyclin [10]. Angiogenesis, the generation of new blood vessels from existing old ones, is a major phenomenon in wound healing and termination of inflammation as well as in remodeling of inflamed tissues.

1.1.2 The chronic inflammation

The unresolved acute inflammation may lead to progression into a chronic inflammation due to poor antigen-elimination in the acute phase of the inflammation or insufficient initiation of the resolution phase (Table 3). At the site of the chronic inflammation, there are greater numbers of macrophages and fewer neutrophils compared to the acute lesions [11]. Further, it is maintained by T- (and B-) lymphocytes and other actors of the adaptive immune system. Several disorders are characterized by chronic inflammation, e.g. autoimmune and cardiovascular disorders, while neoplastic and degenerative disorders are often accompanied by chronic inflammation, e.g. around plaques in the brain of patients with Alzheimer's disease.

Lymphocytes, being the principal actors in the adaptive immune response, are divided into T-cells, e.g. CD4 positive (T_H1, T_H2 and T_H17) and CD8 positive (cytotoxic) cells, and into B-cells (antibody producing cells). Briefly, T_H1 cells are involved in the pro-inflammatory immune response. Whereas, T_H2 cells take part in the allergic immune response and in helping B cells to antibody production of immunoglobulins of the IgM, IgG, IgA, IgE and IgD class [4]. T_H17 cells have recently attracted much attention as a new subset of lymphocytes with distinct properties [4, 12]. CD8 positive cells mediate cytotoxic effects on, for instance, virus infected cells. T-regulatory cells (T_{REG}) are cells involved in limiting the immune response (suppressing T-cell responses) and preventing autoimmune disease [13]. The adaptive immune system differs from innate system by means of their unique receptors for all possible antigens and ability to create memory cells after antigen recognition. Apart from various naturally occurring antigens, some (often plant) products can stimulate to lymphocyte proliferation and cytokine production, so called mitogens.

Lymphocyte studies, included in this thesis, were on peripheral venous blood and contained a mixture of several lymphocyte subpopulations. The lymphocyte preparations was particular rich in CD5 (T cells), CD4 (T helper cells) and CD8 (cytotoxic/ suppressor T cells), and CD 57 (NK and T cells).

Table 3. **The Fine Tuning of Inflammation.** *After Palmblad 2009*

If inflammation *is not initiated properly*:
- infection susceptibility increases.

If inflammation *is not turned off*:
- systemic injury (MODS, SIRS) with (multi)organ injury, tissue necrosis
- persisting inflammation with granuloma formation, tissue remodeling, fibrosis etc
- metabolic adjustments, including cachexia, ensue.

1.1.3 Resolution of inflammation

Recently, understanding of the termination of inflammation has advanced considerably. Previously, this phase was often considered as an exhaustion of pro-inflammatory mechanisms, but recent research has appreciated that the switch from pro- to anti-inflammatory events is orchestrated in a tightly regulated manner (Table 4). A pivotal finding was the discovery of new lipid-derived mediators, the resolvins and protectins, originally isolated in murine models and biosynthesized from the omega (ω)-3 fatty acids EPA and DHA. These novel mediators are very promising candidates for drug development in order to induce potent anti-inflammatory and pro-resolving actions in vivo [9].

Table 4. The resolution of inflammation. *After Palmblad 2009*

Degradation of pro-inflammatory molecules:
- e.g. formyl peptides, oxidation of leukotrienes
Switch from pro- to anti-inflammatory mediators:
-leukotrienes to lipoxins, resolvins/protectins etc
-IL-1, TNF to IL-10, TGFβ
-Switch from destruction to healing, including angiogenesis.

1.2 Inflammation mediators

This review will concentrate on molecules with relevance for this thesis. Fatty acid metabolites will be presented separately below.

1.2.1 Lipopolysaccharide (LPS or endotoxins) and mitogens

These exogenous molecules have in common that they trigger activation of the innate immunity as well as adaptive immunity.

LPS ligates to the CD14 receptor on the surface of monocytes/macrophages, activating the Toll-like receptor 4 (TLR4) and, via MyD88, leading to either a phosphorylation of the IKK complex pathway and an activation of the transcription factor NF-κB, or an activation of the transcription factor Ap-1, through the MapKinase (MAPK) pathway and expression of the TNF-α, IL-1β and IL-6 cytokine genes. Recently, it was reported that LPS, through a direct TLR4 signaling, also can affect T-cell responses [14].

Mitogens are molecules that cause proliferation of cells. One example is phytohemagglutinin (PHA), a lectin obtained from the red bean *Phaseolis vulgaris* [15, 16]. PHA has been used extensively in studies of the immune response where it causes proliferation of exclusively T-cells. PHA binds to N-acetylgalactosamine [17] and stimulates various mononuclear cells to production of IL-2 (and other cytokines) [18], as well as certain eikosanoids like PGE_2, $PGF_{2\alpha}$ [19]. To induce a good PHA response in human blood lymphocytes, monocytes have to be present in the cell culture [20], which is accomplished by using blood mononuclear cell populations, as done in this thesis.

1.2.2 Cytokines and chemokines

Cyto- and chemokines are small proteins, produced by the innate and adaptive immune systems, where they are regulators of host responses to infection and trauma. They are secreted from different cells, and they are also found intracellularly or bound to cell membranes. These molecules act either in a para-, juxta- or autocrine mode of signaling, as response to immune and inflammatory stimuli.

There are today numerous of different cyto/chemokines, and the regulatory effect of them is due to the cell origin, concentration or mixture. Cyto/chemokines are often divided in pro- or anti-inflammatory molecules and growth factors, but due to their pleiotropic effects, the same molecule can be found in more than one class.

Pro- and anti-inflammatory cytokines. IL-1β, IL-6 and TNF-α, often regarded as inflammatory promoting cytokines, are produced in inflammatory cells such as monocytes/macrophages after LPS activation. There are many similarities in the mode of action by TNF-α and IL-1β, although the receptors for TNF and IL-1β is clearly different. But, in the context of activation of the adaptive immune response, there are differences. TNF-α, but not IL-1β, activates an increased macrophage-mediated killing effect of invaded pathogens and TNF-α also can induce apoptosis in certain tumor cells. IL-1β, but not TNF-α, can activate naïve T-cells [21].

IL-1β is synthesized as pro-IL-1β and then undergoes an enzymatic cleavage by caspase-1 to the mature IL-1β. Part of IL-1β may be retained intracellularly.

IL-6 is, together with IL-I β and TNF-α, a mediator of fever and the acute phase response in the liver. IL-6 is considered to exhibit anti-inflammatory effects since IL-6 stimulates the synthesis of anti-inflammatory corticosteroids which dampens TNF-α and IL-1β production.

IL-4, IL-6, IL-10 and IL-13 are cytokines that can both activate B-lymphocytes to antibody production [22], and also function as anti-inflammatory cytokines by means of mediating suppression of the genes for the pro-inflammatory TNF-α, IL-1β and IL-8 (CXCL8). IL-2 activates T- lymphocyte proliferation.

Chemokines. The chemoattractant IL-8 is released from activated monocytes/macrophages together with TNF-α, IL-1β. The latter also trigger endothelial and epithelial cells to release IL-8, which activates neutrophils to migrate into the tissue and to degranulate, which leads to damage of the invaded tissue.

Anti-viral cytokines. IFN-γ and IL-2 stimulates the maturation of cytotoxic T-cells and macrophages. IFN-γ augment TNF activity in macrophages and induces nitric oxide (NO) synthesis in a variety of cells [21]

1.2.3 Growth factors, e.g. G-CSF, GM-CSF, IL-3, IL-5.

TNF-α, IL-1β, IL-17 and other mediators stimulate bone marrow fibroblast to release G-CSF, and GM-CSF. G-CSF is the major growth factor for neutrophil production and suppresses the apoptosis of these cells. Pharmacologically, it is used in the setting of congenital and acquired neutropenias.

Vascular endothelial growth factor (VEGF) can induce angiogenesis in vivo and is a potent inducer of vascular permeability [23, 24]. Hence, it plays important roles in inflammation during normal angiogenesis and wound healing. Epidermal growth factor (EGF) is involved in repairing of the epithelial cells, and basic fibroblast growth factors (bFGF) involved in healing of damaged tissues as well as being an angiogenic factor.

1.3 Inflammation, fatty acids and eikosanoids

Fatty acids (FA) form a very large group of compounds, classified as to carbon chain length, number of double bonds and their locations. Here, I present those FAs that relates to my thesis, i.e. primarily the essential fatty acids, necessary for life. They are the polyunsaturated (PUFA) of the omega-(ω) 6 (also called n-6 FA) and ω(n-)3 series. Prototype FAs belonging to these families are AA, EPA and DHA.

1.3.1 Essential fatty acids, omega-3 fatty acids

The essential FAs linoleic acid (18:2, ω6; LA) is the precursor for AA and its metabolites, whereas α-linolenic acid (18:3, ω3; ALA) is precursor for EPA and its metabolites. None of these can be synthesized de novo in humans [25] due to location of double bonds. They must therefore be supplied to the body by food intake.

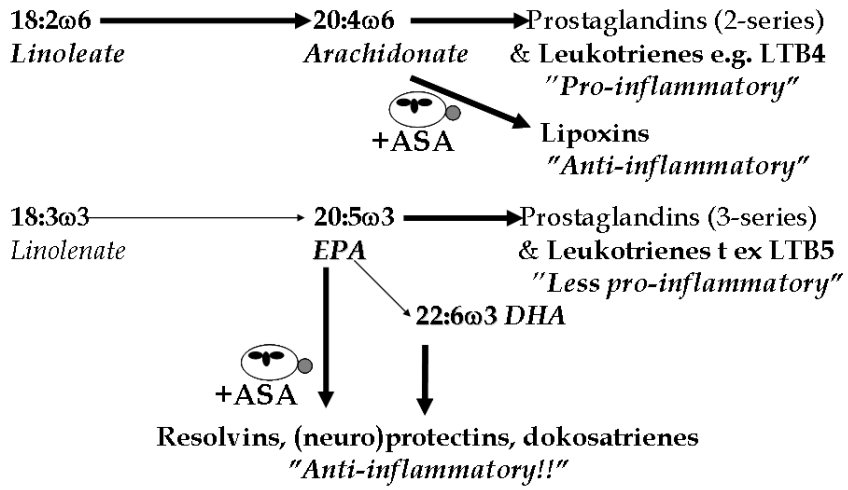
The ω6 and ω3 FAs give raise to a great variety of inflammation and immunity regulating mediators, primarily eikosanoids such as leukotrienes (LTs) and prostaglandins (PGs). The most potent inflammation-promoting eikosanoids belong to the ω6 family (i.e. LTs and PGs derived from AA). Leukotrienes and PGs derived from ω3 FAs, mainly from EPA, are less pro-inflammatory than those from the ω6 FAs, since they have aberrant 3D structure because of one additional double bond. AA is also the

parent substance of lipoxins [26-29], and EPA and DHA of resolvins and (neuro)-protectins; they possess anti-inflammatory activity in contrast to LTs and PGs [30, 31].

AA, EPA and DHA are found in adipose tissue, in the brain (where AA and DHA, but not EPA, make up a majority of the FAs), and in the phospholipid bilayers in cell membranes in all tissues. Depending on the amount and the type of PUFAs in the bilayer, they will affect the membrane fluidity and the signaling pathways differently. In general terms, the longer and more unsaturated the FA is, the more is membrane fluidity enhanced. This, in turn, may lead to surface receptor modulation, effects on ion pumps, G-protein coupling, binding to transcription factors (e.g. NF-kB) and nuclear receptors (PPARs, RXR and HNF4a) and further into gene interactions and protein synthesis [32, 33].

EPA and DHA. EPA and DHA are special since they are mainly supplied by intake of oily fish or marine fish oils, e.g. from herring, tuna, mackerel, salmon and sardines [34]. In fact, it has recently been doubted that EPA and DHA are generated from ALA in any appreciable amounts [35]. Furthermore, the conversion of EPA to DHA has also been questioned [36], whereas it (still) appears to be sufficient evidence for retroconversion of DHA to EPA.

Figure 1 The pathway of biosynthesis ad metabolism of polyunsaturated fatty acids. Acetylsalicylic acid **(**ASA) enhances the generation of lipoxins and resolvins, protectins by transcellular metabolism of the parent compounds by neutrophils and platelets. After Palmblad 2005



1.3.2. Eikosanoids, prostaglandins and leukotrienes (Figure 1)

Upon cell activation by certain cell stimuli, phospholipase A_2 releases AA from the cell membrane. This AA is then further metabolized, either by the enzyme cyclooxygenase (COX) into prostaglandins (PGs) of the 2-series (PGA_2, PGB_2, PGE_2, $PGF_{2\alpha}$, and PGD_2 etc), or alternatively by the enzyme 5-lipoxygenase (LOX) into leukotrienes (LTs) of the 4-series (LTB_4, LTC_4, LTD_4 and LTE_4). EPA, released from the cell membrane by the same mechanisms, give rise to LTs and PGs with one additional double bond. These PGs and LTs belong to the 3 and 5 series (e.g. $PGF_{3\alpha}$, PGE_3 and LTB_5, LTC_5,

respectively). The anti-inflammatory lipoxins, resolvins and protectins are generated by transcellular metabolism of parent compounds by neutrophils and platelets.

Acetylsalicylic acid (ASA) enhances these processes and this has generated interest that the anti-inflammatory effect of ASA is due to this effect.

Leukotrienes are powerful pro-inflammatory stimuli in the classic inflammatory process (particularly LTB_4) as well for the allergic inflammatory system (typically LTC_4, LTD_4, and LTE_4) [37]. However, since they are not parts of this thesis, they are not further presented here.

1.3.3 Eikosanoids, immune and inflammatory processes

Various PGs and LTs are involved in the modulation of the immune response [19, 38-41] PGs are considered to be immunoregulatory due to their ability to change the cellular cAMP/cGMP ratio.

PGE_2 and PGD2. In the immune system, PGE_2 acts on T-lymphocyte helper and suppressor/ cytotoxic cells and on IL-1 and IL-2 production, which modulates immune and inflammatory responses [42]. It has been demonstrated that PGE_2 and D_2, and also LTB_4, have effect on lymphocyte and monocyte/macrophage functions either by inhibiting cell reactivity or by stimulating target cells, which leads to suppression of activity [19, 38-41]. Studies have shown that PGE_2 can decrease TNF-α production from LPS stimulated monocytes/macrophages [43-47]. PGD_2 has been suggested to be a potential antineoplastic agent as it exerts growth inhibition on L-1210 mouse and some human leukemia cell lines [48]. These effects of PGD_2 have been attributed to its metabolites PGJ_2 and Δ^{12}-PGJ_2 [49, 50]. These two metabolites enhanced TPA induced monocyte NADPH activity [51]. PGE_2 can arrest the function of T_H1, but not of T_H2 cells [52], inhibit T_H1 lymphokines [53, 54] and inhibit lymphocyte proliferation [55] and NK cell activity [56].

PGE_2 also has a stimulatory effect on cytokine generation from T_H2 cells (IL-4, IL-5) [54] and on hematopoietic stem cell homing [57]. Furthermore, PGE_2 increase the IL-23 induced IL-17 production [58] and promotes inflammation through T_H1 differentiation phenomena involved in the enhancing of neutrophil recruitment and migration [59]. PGE_2 can enhance T-cells proliferation by inducing of co-stimulatory molecules of the TNF/TNF receptor superfamily [60].

Recent studies have highlighted PGE_2 to be both a pro- and anti-inflammatory modulator. Thus, PGE_2, derived from AA, might be advantageous for the LOX system [61, 62], synthesis of LTB_4 and recruitment of neutrophils [62]. PGE_2 exert anti-inflammatory response through inhibition of 5-LOX and lower levels of LTs of the 4-series [61],[9]] and induce 15-LOX, which leads to the formation of the anti-inflammatory lipoxins [61, 62]

$PGF_{2\alpha}$. PGH_2 (a common precursor of PGs derived from AA) is converted to PGE_2 and $PGF_{2\alpha}$ by specific terminal PGE and PGF synthases, respectively. By means of an enzyme, 9KPGR, PGE_2 can be converted to $PGF_{2\alpha}$ [63]. Hence, $PGF_{2\alpha}$ is considered to be a major and stable metabolite of PGE_2 [63].

$PGF_{2\alpha}$ is a major PG for modulation of functions of the reproductive tract, e.g. in ovarian function, endometrial cyclic changes and in luteal maintenance of pregnancy [63-65] where $PGF_{2\alpha}$-PGE_2 works like opposing dyads [63]. $PGF_{2\alpha}$ can induce changes in cGMP, whereas PGE_2 induces changes of cAMP [66]. $PGF_{2\alpha}$ has also effects in the inflammatory/immune system, as described in an earlier section of this thesis (1.3.2)

[63, 67]. Recently, it was observed that $PGF_{2\alpha}$ elevates blood pressure and promotes arteriosclerosis [68], being involved in leukocyte migration [69].

The biological activities have been mapped by means of additions in various in vitro systems and by observing the phenotype of animals where the production has been hampered or where specific receptors have been knocked out. Examples of the latter are given in Table 5. PGE_2 ligates to EP receptors (divided into four subgroups), and $PGF_{2\alpha}$ to FP receptors [65].

Table 5. Some effects of knocking out prostanoid receptors in mice. From [65]

Genotype	*Phenotype*
EP2 (-/-)	Loss of bronchodilaton with PGE_2
EP3 (-/-)	Impaired febrile response to pyrogens,
EP4 (-/-)	Decreased inflammation–dependent bone resorption
FP (-/-)	Loss of parturition
IP (-/-)	Decreased inflammatory swelling, thrombotic tendency
DP (-/-)	Decreased allergic responses in ovalalbumin- induced bronchial asthma

While my studies on the role of PGs and lymphocyte reactivity did not associate eikosanoids with inflammatory reactions of the kinds explored in my late studies, recent findings by others have highlighted an association between the two research fields [70].

Resolvins and protectins

These novel metabolites of EPA and DHA were recently reported as important mediators of the resolution phase of inflammation. Resolvin E1 (from EPA) has been described to reduce inflammation in vivo, by blocking human neutrophil transendothelial migration. Resolvin E2 reduces zymosan-initiated neutrophil infiltration and thus displaying anti-inflammatory actions. It also takes part in the regulation of macrophage function. Resolvin D1 (from DHA) controls inflammation resolution and in neural tissues. Protectin D1 (from DHA) is produced by human peripheral blood mononuclear cells, blocks T cell migration, reduce TNF and IFN-γ production and promotes T-cell apoptosis [9, 28].

1.4 Omega-3 fatty acids in health and disease

1.4.1 Dietary intake of ω3 fatty acids.

As presented in detail below, eikosanoids generated from ω3 FA are believed to be less pro-inflammatory than those derived from ω6 FAs. Decreased intake of ω3 FA may thus promote inflammation, and increased intake the opposite. This has lead to an extensive debate over the last decades as to the balance of dietary ω6 and ω3 FAs. It is often claimed that a Western diet is poor in ω3 FA and that ω6 FAs predominate. This result in a corresponding composition of cell membrane lipids, thus mimicking the consumed food and bearing witness of the old saying: We become what we eat.

This notion has raised concerns that a relative deficiency of ω3 FAs may have health consequences and that supplementation with ω3 FA may improve health in certain aspects. A recent study showed that Mediterranean-inspired diet lowered the numbers

of platelets, leukocytes and VEGF concentrations and the changes could be linked to higher concentrations of ω3 FAs in serum [71].

1.4.2 Omega-3 fatty acids and inflammation diseases.

Systematic reviews and metaanalyses support the notion that dietary supplementation with EPA and DHA cause reductions in mortality, morbidity and symptoms of atherosclerotic cardiovascular diseases and rheumatoid arthritis [72-76]. Moreover, evidence based medicine (EBM) data makes it likely that EPA and DHA are of benefit for bipolar and depressive mood disorders [76, 77]. EBM analyses have, as of yet, not found enough data to support EPA and DHA supplementation for a variety of other diseases, but with emerging studies, more data will be available for statements as to, for instance, if cognitive decline in Alzheimer's disease (AD) can be reduced. Since the first published randomized controlled study of ω3 supplementation in AD [78] forms an essential part of this thesis, I will here review what is known about these FAs and AD.

DHA (as well as AA) is an important FA for the development of the human brain and vision. DHA and AA are found mostly in neuronal membranes and for DHA in the synapses. In human studies, consumption of fish or dietary supplementation of EPA and DHA was associated with reduced risk of developing Alzheimer's disease [79-82]. Furthermore, a study reported that the amyloid burden was reduced in an aged mouse model by a DHA enriched diet [83]. Thus, epidemiological and experimental evidence suggest that ω3 FA supplementation might attenuate cognitive decline in relation to AD.

1.4.3 Alzheimer´s disease (AD).

AD is the major neurodegenerative disease that causes dementia, with a prevalence of 14 million patients worldwide [84]. The DHA content in AD brains are lower compared to normal brains [85]. There is an inflammatory reaction around the amyloid plaque in the AD brain. Cytokine and serum complement levels in the cerebrospinal fluid (CSF) are often elevated [86]. Consequently, there has been a great interest in trying to reduce cognitive decline by means of anti-inflammatory drug treatment. However, until we performed the OmegAD study there were no randomized controlled trials (RCT) on the effect of oral supplementation with EPA and DHA to AD patients.

1.4.4 PGs and ω3 FAs.

Diets rich in EPA and DHA lower the production of PGE_2 and metabolites and TXA_2 and increase the production of the less potent inflammatory TXA_3 from EPA [87]. Fish oil is able to decrease the PGE_2 production and increase the IFNγ production as well as lymphocyte proliferation [88, 89], an effect, also noticed for DHA [90].

1.4.5 ω3 FA and cytokines.

Previously studies on dietary supplementation with ω3 FAs (mainly EPA) to laboratory animals found a reduced release of various cytokines (e.g. IL-1 and TNF) [91] and prevented the development of atherosclerotic lesions in mice [92]. In vitro additions of EPA and DHA have been shown to have similar effects [93]. Moreover, in vitro studies have observed that DHA depressed TNF-α, IL-1β and IL-6 production in THP-1 cells more than EPA [94]. Recently, a study showed that EPA suppressed TNF-α and IL-1β mRNA expression and cytokine production in human asthmatic alveolar macrophage cells more effective than DHA [95].

1.4.6 ω3 fatty acids and cytokine generation ex vivo in humans.

Several studies have investigated effects of dietary supplementation with EPA and DHA on inflammatory reactions in healthy humans. A majority of studies have found decreased production of pro-inflammatory cytokines by the ω3 FA, for instance of IL-1 and TNF by mononuclear cells [96-100] and IL-6 production [101, 102]. Fish oil supplementation reduces fever and cytokine production [103] and LTB_4 production in rheumatoid arthritis patients [104].

Most studies have used EPA-rich fish oil, and there are fewer studies performed with DHA-rich preparations. Although results often agree, some discrepancies have been observed and they may be due to type of ω3 FA, dosages, duration of treatment concerned healthy subjects or patients with inflammatory diseases [104-106]. DHA and EPA regulate inflammatory reactions by various mechanisms, inter alia, gene activation and TNF-α production by fish oil have been shown to be influenced and associated with polymorphisms in the promoter region of TNF-α genes [107].

1.4.7. ω3 FA, eikosanoids and gene expression.

Genes are differently expressed by AA, EPA and DHA [108, 109]. PUFAs affect gene transcription by binding to nuclear receptors [110] and are involved in gene regulation in many other ways [111]. Modulatory mechanisms differ not only between the ω6 and ω3 FAs, but also between EPA and DHA. For instance, EPA binds to the PPARγ receptor and DHA binds to the nuclear retinoid X receptor [33]. ω3 FAs are considered to exert anti-inflammatory effects on several cellular levels, e.g. binding to transcriptions factors and gene activation [32, 112]. Reductions of the ω6/ω3 ratio in healthy humans was associated with reduced expression of IL-1β, IL-10 and IL-23 cytokines in mononuclear cells and ex vivo production of LTB_4 by stimulated neutrophils [113].

Previous investigations on effects of DHA and/or EPA on gene expressions in animal studies and *in vitro* models have shown changes in a variety of genes, some of which are supposed to be involved in inflammation and chronic neurodegenerative disorders. These gene expression studies have mostly been on short time exposure and on small sets of genes. In a few animal studies, array techniques with a restricted set of gene probes for studies on ω3 FAs have been used. DHA enriched diet altered the gene expression in brains from 2-months and 2- years old rats. Different sets of genes were changed depending of the animal's age. In old rat hippocampi, the transthyretin gene in response to ω3 FA diet was up-regulated [114, 115] and in brains from rats fed fish or perilla oil (from conception to adulthood), almost the same expression profiles were displayed [116]. DHA-rich fish oil supplementation for two months modified 77 out of 588 studied genes in human lymphocytes [117].

Microarray studies, on wide gene expression from baboons fed DHA-enriched mother´s milk for 10-12 weeks, showed changes in approximately 1000 genes [118], and dietary fish oil supplementation to rats conferred changes in 5 genes [119]. When mice were fed with a DHA enriched fish oil, changes in 329 and 356 dietary transcripts from liver and hippocampi were identified, respectively [120]. Studies on effects of long term treatment with an EPA rich fish oil in humans, using genome wide techniques were reported during the final stages of writing this thesis [121]. Thus, results vary considerably as to number of affected genes as well as to which genes are influenced.

In this thesis we have conducted a genome wide gene expression study, *ex vivo*, from a number of AD patients in the OmegAD study that received either a marine DHA rich fish oil supplementation or placebo oil for six months

1.5 The OmegAD study

The OmegAD study is described in detail in [78]. The aim of this double blind randomized controlled trial was to see if dietary supplementation with a DHA-rich fish oil preparation would reduce the cognitive decline in patients with mild to moderate AD. It included altogether 204 patients (73±9 y, 52 % women). Patients were randomized to 6 months of nutritional supplementation with a ω3 fish oil rich in DHA (EPAX 1050TG™, Pronova, Norway) or to a placebo preparation. Patients received daily 1.7 g DHA plus 0.6 g EPA, or an isocaloric placebo preparation containing 1 g corn oil and 0.6 g linoleic acid. After 6 months all patients received the ω3 FA formulation for the next 6 months. At 12 months 174 patients remained in the study.

At 0, 6 and 12 months patients underwent cognition, neuropsychiatric and nutritional evaluations. From the first randomized patients, a subgroup of 40 patients was also assessed by means of blood and cerebrospinal fluid (CSF) for inflammatory markers, e.g., cytokines and growth factors. Moreover, blood was obtained for separation of PBMC for *ex vivo* evaluations of cytokine, PG release after stimulation with LPS and PHA, and for a global gene expression measurement.

The results of the cognitive, neuropsychiatric and some nutritional tests have been published [78, 122-124]. In short, only patients with very mild AD appeared to benefit from the treatment, in that the cognitive decline was diminished during ω3 FA supplementation compared to placebo treatment.

2 AIMS OF THE STUDY

The aim of present study was to investigate effects on/of

- lymphocyte functions, such as lymphocyte proliferation, NK cell activity and Leu-1(CD5), Leu-2 (CD8), Leu-3 (CD4), Leu-7 (CD57) markers, *in vitro,* following additions of PGA_2, PGB_2, PGE_2, $PGF_{2\alpha}$ and PGD_2.

- on monocyte TNF-α production *in vitro* following additions of PGD_2 and some of its metabolites, *viz.* PGJ_2, $\Delta^{12}PGJ_2$ and 15-deoxy, Δ^{12}, Δ^{14} PGJ_2.

- dietary supplementation to Alzheimer patients for 6 months with ω3 fatty acids on peripheral blood mononuclear leukocyte $PGF_{2\alpha}$, cytokine and growth factor release *ex vivo*.
- dietary supplementation to Alzheimer patients for 6 months with ω3 fatty acids on global gene expression in peripheral blood mononuclear leukocytes.

3 SUBJECTS AND METHODS

3.1 Subjects and ethical considerations (papers I, II, III, IV and V).

Blood sampling, for preparation of peripheral mononuclear cells (PBMCs) and isolation of blood monocytes in papers *I* and *II*, were collected from healthy laboratory staff members during the years of 1984-1993. At that time, blood sampling from laboratory staff did not require ethical permission. Papers I and II were included in my thesis for examination for the Licentiate degree of Medical Science at Karolinska Institutet 1993.

PBMCs, used in papers *III, IV* and *V,* were from a subset of AD patients included in the OmegAD study. The study was approved by the local ethics committee of Karolinska Institutet. A pre-trial power calculation in the whole study estimated that 200 patients had to be included to reach a statistical significance level of 0.05 and 80% power to find differences in the measurement of the cognitive function. 174 patients concluded the OmegAD study. A pre-trial power calculation indicated that 20 patients would be required to detect significant differences between the ω3 FA and placebo groups, when using a cytokine assay.

3.2 Material and Methods for all papers

3.2.1. Materials

PGA_2, PGB_2, PGE_2, $PGF_{2\alpha}$, PGD_2, PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy Δ^{12}, Δ^{14}-PGJ_2, were provided as stated in papers *I* and *II*, and used for lymphocyte proliferation tests, NK activity tests, and for phenotype markers in paper *I*, and for TNF-α production by monocytes in paper *II*. Sodium diclofenac was used as a COX inhibitor in paper *II*. FITC-conjugated monoclonal antibodies for lymphocyte markers; Leu-1, Leu-2a, Leu-3a+b and Leu-7 were used in paper *I*. 12-*O*-teradecanoylphorbol-13 acetate (TPA) was used for stimulation of monocyte NADPH oxidase activity, as assessed by 0.17m*M*, luminol-enhanced chemiluminescence (CL) in paper *I*. PHA (HA 16) was used as stimulus for PBMC lymphocyte proliferation tests in paper *I*, and for production of $PGF_{2\alpha}$ from PBMCs in paper *III*. LPS was used as a stimulus for TNF-α production from monocytes (paper *II*), cytokine, growth factor and $PGF_{2\alpha}$ production from PBMCs, respectively, in papers *III* and *IV*.

3.2.2. Cell separations.

In paper *I*, for studies on the effects of PGs on lymphocyte proliferation, lymphocyte subpopulations and natural killer (NK) activity, lymphocytes were isolated from heparinized venous blood by a Ficoll-Isopaque™ gradient centrifugation [125]. PBMCs, used in assays for the release of cytokines, growth factors and $PGF_{2\alpha}$ (papers *III* and *IV*), and for preparation of RNA used in gene expression analyses (paper *V*), were isolated from EDTA anti-coagulated venous blood by means of a Lymphoprep™ gradient centrifugation [125].

Separation of monocytes (papers I and II) was according to a protocol by Bøyum [126]. In the study of effects of PGs on monocyte activities, monocytes were isolated by a Nycodenz gradient (paper *I*) and by a Nycoprep gradient (paper *II*). The preparations contained >95% viable monocytes as determined by morphology and OKM1 (paper *I*) and the monocyte preparation (paper *II*) contained approximately 91% pure monocytes as determined by CD14 antibodies.

3.2.3 Experimental design in papers I and II.

Determination of lymphocyte DNA synthesis.

In paper *I,* we used a micro-culture technique described by Lilliehöök and Blomgren [127]**.** Lymphocytes were incubated with PGs A_2, B_2, D_2, E_2, $F_{2\alpha}$, at various concentrations and stimulated with 0,25, 2,5 and 25 µg of PHA/mL for 72 hrs. Cell proliferation was measured by the incorporated ^{14}C thymidine radioactivity.

Cytotoxic assay of ^{51}Cr release (paper I). Briefly, after magnetically removal of phagocytes from the PBMCs, lymphocytes were incubated overnight with PGs A_2, B_2, D_2, E_2, $F_{2\alpha}$. Then, NK activity was measured for 4 h as release of ^{51}Cr from labeled K562 target cells in different lymphocyte/target cells ratios [128].

Phenotype determination of T lymphocytes (paper I). Purified lymphocytes were incubated overnight with PGs A_2, B_2, D_2, E_2, $F_{2\alpha}$. We used two different methods to determine the phenotype. One identified lymphocytes possessing membrane receptors for the Fc-part of IgG and their capacity to form rosettes with ox RBC IgG (T_G-Cells, suppresssor/cytotoxic cells) [129]. The second method was based on an immune-fluorescence microscope technique with FITC-conjugated monoclonal antibodies to Leu-1 (CD5), Leu-3a+3b (CD4), Leu-2 (CD8) and Leu-7 (CD57).

Assay of luminol-enhanced chemiluminescence (CL) of monocytes. Cells were incubated overnight with PGs; then, after stimulation with TPA, generation of light during 1-5 minutes was assayed.

TNF-α release from monocytes (paper II). Cells were treated for 1 h with PGD_2 or its metabolites, with or without diclofenac. Then, TNF-α in supernatants from cells stimulated with 10 µg LPS/mL for 22 hrs were determined with an immunoradiometric assay (IRMA).

3.2.4 Experimental design, cytokine and $PGF_{2\alpha}$ production (papers III, IV).

In the OmegAD study, AD patients were randomized for oral ω3 FA supplementation as detailed in 1.5 [78].

Plasma FA analyses (by gas chromatography) (papers *III, IV* and *V*) were performed on all 174 patients. Results are given as the relative abundance of individual FAs [130].

Cytokine, growth factor and $PGF_{2\alpha}$ analyses (papers III and IV). Here, we included 25 AD patients. They were the first to be randomized in the OmegAD study. Because of various set-backs (detailed in the papers), 9 of the remaining patients received ω3 FAs and 12 the placebo oil, for 6 months. PBMCs were stimulated with 1 or 10 ng of LPS/mL overnight, where 10 ng/mL represents the optimal stimulating concentration, and 1 ng/mL was aimed to obtain an ED_{50} concentration (paper IV). Cells in additional tubes were stimulated with PHA. In paper *III*, $PGF2_{\alpha}$ release was measured in the supernatants using an enzyme immunoassay kit. In paper *IV*, cytokines and growth factors were measured in the cell supernatants using kits for Luminex in a Bio-Plex 100 System reader.

3.2.5 Experimental design, gene expression (paper V).

Blood samples, for preparation of PBMCs or for the gene expression study, included finally 16 patients. They were among the first to be randomized in the OmegAD study. We present data on 11 patients, who received the ω3 FAs preparation, and 5 patients who received the placebo oil, for 6 months.

RNA extraction from PBMCs and microarray hybridization. Total RNA was isolated from PBMCs and treated with RNase-free DNase. A prepared biotinylated RNA complementary RNA served as template for hybridization to Human Genome Focus Arrays containing 8,747 probesets, corresponding to approximately 8000 genes. We used standard protocols from Affymetrix. After hybridization and scanning, each sample was analyzed using Affymetrix statistical algorithms.

Microarray data analysis Signal intensities for each microarray were calculated in Microarray Analysis Suite (MAS) 5.0. Quality control was performed and Scaling factor and number of present were well within approved intervals. We used software Significance Analysis of Microarrays (SAM) [131] to identify changes in gene expression induced by ω3 FA treatment.

Verification of microarray data with real time PCR. RNA was reversed transcribed into cDNA using. The cDNA was mixed with TaqMan R universal mix and predesigned TaqMan gene expression assays from Applied Biosystems. For more detailed information see the supplementary materials in the submitted manuscript (paper *V*).

3.3. Statistical analyses

Basically, statistical tests were chosen according to the distribution and skewness of the data. Thus, in paper *I*, Students´ t-test was used. In paper *II*, a Wilcoxon's signed rank test was used for the TNF–α release from PG treated monocytes. For plasma FAs, cytokine, growth factor and $PGF_{2\alpha}$ data in papers *III* and *IV*, all analyses were performed on an intention-to-treat basis with Wilcoxon's signed rank test. For comparisons of differences in responses between groups over time, we used Mann Whitney *U* test. For correlation analyses, a Spearman's rank correlation (rho=r) test was applied. - In paper *V*, a paired two sided Students´ t-test was used for analyses of plasma FAs and microarray data. In order to confirm if specific genes regulated by ω3 FA substitution were also regulated by placebo treatment we used a t-test for independent microarray data. - A paired one-sided Students´ t-test was used in confirmatory analysis of individual mRNA measurements by Real Time (RT)-PCR. Values are presented as means and SD. For correlation analyses a Pearson correlation test was applied.

4 RESULTS AND DISCUSSION

The thesis is divided into two parts, the first is about *in vitro* effects of certain prostaglandins on lymphocyte markers and functions as well as on monocyte functions (papers *I* and *II*). These papers were part of my licentiate exam in 1993 (entitled *Some effects of Prostaglandins of E and D series on the in vitro activity of lymphocytes and monocytes*). The second part concerns the OmegAD study.

4.1 Part One: The Early Prostaglandin Studies

4.1.1. (Paper I)

Wasserman J, Hammarström S, Petrini B, Blomgren H, von Stedingk L-V, Vedin I. **Effects of some prostaglandins and leukotrienes on lymphocytes, monocytes and their activity in vitro.** *Int Archs Allergy Appl Immunol 1987 83:39-43*

The aim was to study the effects of the PGA_2 PGB_2, PGD_2, PGE_2 and $PGF_{2\alpha}$, *in vitro,* on blood lymphocyte functions, such as mitogen induced proliferation, tumor cell cytotoxicity, expression of surface markers and NADPH oxidase activation, with a phorbolester (TPA) as the stimulus.

Lymphocyte proliferation. PGD_2 strongly reduced the proliferation induced by PHA. Reductions were also observed for PGA_2 and PGE_2. The order of potency for inhibition was PGD_2>PGA_2>PGE_2. PGA_2 (a converted form of PGE_2) has together with Δ^{12} PGJ_2 been reported to be taken into cytosol in target cells without interacting with membrane receptors [132-134]. Thus, the mechanism underlying the suppressor activity of PGD_2 may differ from that mediated by PGA_2 and PGE_2.

NK activity of peripheral lymphocytes. Here, we studied effects of PGs on NK activity by using the tumor cell line K562 as the target. The NK cell activity of lymphocytes, pre-incubated with PGD_2 and PGE_2 (but not with PGA_2, PGB_2 or $PGF_{2\alpha}$) was reduced.

At the time, when these studies were conducted, the knowledge about NK cells and their phenotype as well as their full capacity, except of the recognition and killing of tumor cells, was sparse. Therefore, with today's knowledge of these cells, we are unable to state, that our obtained results in paper *I* are from cells with NK cell morphology/ phenotype. Our NK cell activity results might rather be considered as natural cytotoxicity effects by lymphocytes.

Novel findings have suggested PGE_2 as a key mediator of mesenchymal stem cell induced inhibition of NK cell activity [56]. Studies have also showed that PGD_2 has a suppressive role on NK function, via signaling through the prostanoid D receptor [135], and that PGD_2 also inhibits the production of IFN-γ by invariant NK T cells which has consequences in the control of melanoma cells [136]. Lymphocytes in our NK assay were positive for the CD8 cytotoxic T-cell marker and also for CD57, a marker found on both NK cells and T-cells. Therefore, it may be speculated if lymphocytes in our NK assay could be so called $CD8^+$ NKT–like T cells [137].

Expression of lymphocyte phenotype. PGD_2 significantly reduced the expression of CD8, i.e. on cells with suppressor/cytotoxic phenotype, but had no effects on CD5, total T cells, CD4, i.e. cells with helper inducer phenotype, or CD57 antigen subsets of lymphocytes, reported to be rich in cells mediating NK activity. The frequency of lymphocytes with Fc receptors for IgG (FcγR) was significantly reduced.

The findings of the reduced expression of Leu-2 (CD8) and FcγR by PGD_2 and the decreased PHA proliferative response by PGD_2, PGA_2, PGE_2 as well as the NK activity by PGD_2 and PGE_2, can be considered to be somewhat contradictory, as the above mentioned markers define cells with suppressor/cytotoxic functions, and the decrease of these cells would not be expected to result in a decrease of different functions. However, a change in the expression of phenotype does not necessarily mean a corresponding effect on the functional level. Moreover, the existence of suppressor cells has been questioned [138]. Lymphocytes that mediates NK activity in human cells seem to possess FcγR [139] and Leu-2+, Leu-15+ subsets of lymphocytes mediate suppressor effects whereas Leu-2+, Leu-15- subsets exert cytotoxicity. Thus, it is possible that only cytotoxic cells were affected by PGs. Recently, it has been reported that PGD_2 inhibits IFN-γ production by iNKT T cells, which might confirm the immunoregulatory roles of PGD_2 in innate responses [136]. Our results on DNA synthesis are in agreement with that of Narumiya et al. [140] who also reported a decreased *in vitro* DNA synthesis in cells treated with PGD_2. Kikuchi et al. [141], also reported effects of anti-neoplastic PGs on human lymphocyte responses to PHA. In low doses, PGA_2, PGE_2, PGD_2, PGJ_2 and Δ^{12}-PGJ_2 stimulated the PHA response, but at higher doses, these PGs inhibited the PHA response. These findings agree well with our results (and results in a work by Petrini & Vedin) [51], except for the stimulation of PHA response with low PG doses, which was rather variable in our experiments. This latter discrepancy could be explained by different experimental conditions. The relevance of our in vitro findings on the reduced lymphocyte reactivity for the in vivo situation is uncertain. However, it may be speculated that the immuno-suppressive effect of PGs is compensated by their anti-neoplastic activity.

Inhibition of T-cell proliferation by PGE_2 is well established [142]. The inhibited proliferation of PBMCs by PGE_2 was also noted in that review.

*Monocyte NADPH oxidase activity***.** With this paper we started our studies on PGs and monocytes. Activated monocyte/macrophages had, at that time, been defined as producers of PGE_2. Here, we tested TPA induced NADPH oxidase activity following PG treatment by using a luminol dependent CL system, which is considered to mirror the NADPH oxidase associated respiratory burst [143] and the generation of several species of oxygen radicals [144].

None of PGB_2, PGD_2 and PGE_2 had any effect on the NADPH oxidase activity. It could be speculated that it might be due to the stimulus used for activation of the monocytes. TPA has intracellular activation mechanism of monocytes, whereas the formyl peptide fMLP activates the monocyte O_2 generation through membrane receptors, mimicking a bacterial activation. We can only speculate that results might have not been similar if we used fMLP.

4.1.2. Paper II.

Vedin I, Wasserman J, Hammarström S. **Stimulation of tumor necrosis factor-α release from lipopolysaccharide activated human blood monocytes by prostaglandin J_2 and metabolites of prostaglandin J_2** *Prostaglandins Leukotr Essent Fatty Acids 1996; 55:185-9*

The aim of this paper, was to study the influence of PGD_2 and its metabolites PGJ_2, Δ^{12} PGJ_2 and 15-deoxy Δ^{12}, Δ^{14} PGJ_2, *in vitro,* on TNF-α production from LPS stimulated blood monocytes. Here, we stimulated monocytes [43] for further analysis of TNF-α in supernatants. TNF-α has an immune regulatory capacity and its mode of action is either directly on effector cells or indirectly through intermediated cytokines [145].

Effects of PGE_2 and PGD_2 and its metabolites. First, we tested if PGE_2 can reduce TNF-α secretion from monocytes, as described by Kunkel and Spengler. Indeed, that was the case: PGE_2 decreased TNF-α release in quiescent as well as LPS stimulated cells (unpublished data). Thus, these data were in accordance with others [43, 45, 47].

When we tested PGs of the D series for TNF-α production we found that PGD_2 had no effect. However, the PGD_2 metabolite PGJ_2, and the PGJ_2 metabolite Δ^{12} PGJ_2 increased TNF-α production significantly. The 15-deoxy-Δ^{12}, Δ^{14} PGJ_2, (a Δ^{12} PGJ_2 metabolite; 15d- PGJ_2) increased the TNF-α release.

Effects of the cyclooxygenase inhibitor sodium diclofenac on the TNF-α release.
Diclofenac increased the TNF-α release, induced by PGJ_2 and its metabolites. This effect suggested suppression of endogenous PGE_2 by inhibition of COX activity.

These results, suggesting that the production of TNF-α may be regulated at different levels by the PGs, are in agreement with later reports demonstrating an up- and down-regulation of TNF-α synthesis in macrophages by cGMP and cAMP [146, 147]. It can be speculated that the metabolites of PGD_2 up-regulate the TNF-α gene expression by affecting cGMP levels. However, it is also possible that the same PGs can have opposite effects on TNF-α release, depending on PG dosages and stimulus used.

PGE_2 acts through membrane receptors whereas Δ^{12}-PGJ_2 is believed to be taken up into cytosol and nuclei by target cells without interacting with membrane receptors [132, 133]. PGJ_2 and Δ^{12}-PGJ_2 are considered to possess biological activities different from their parental molecule [48, 49, 148]. Furthermore, it has been demonstrated that PGD_2 by dehydration in aqueous solution and in plasma is converted into PGJ_2 and Δ^{12}-PGJ_2, respectively [49, 148]. For this reason the above mentioned metabolites seem to be of considerable interest. It also appears that PGJ_2 and Δ^{12}-PGJ_2 are more potent in inhibiting tumor cell growth *in vitro* than their parental molecule [49, 50, 148].

Hence, it is possible, that the opposite effects on TNF-α secretion between various PGs depend on their interaction with different signaling pathways. However, their immuneregulatory effects seem to be more complex than assumed earlier [146, 147]. In inflammation, PGs of the D series act as stop signals in progressed inflammation by facilitating monocyte migration and differentiation into macrophages, and removal of dead cells from inflamed area [9, 28]. Thus, these PGs are also involved in the resolution phase of inflammation.

Our findings that 15d-PGJ_2 increased the release of TNF-α from LPS stimulated monocytes is in some contrast to other reports, showing an inhibition of release of TNF-α in THP-1 cells [149] and in macrophages [150]. However, in the study of Thieringer et al [150], a different experimental design was used, with activated monocytes and and a lower LPS concentration.

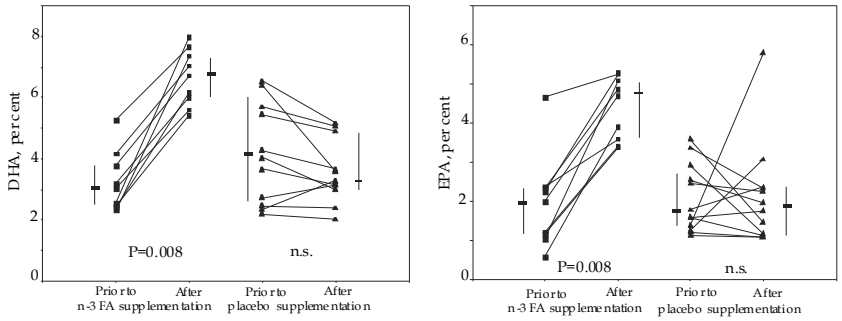
4.2 Part Two: The OmegAD study

4.2.1. Plasma fatty acids data in papers III, IV and V. Here we present data for DHA and EPA plasma levels for patients included in these studies, receiving either the ω3 preparation or placebo oil for 6 months.

After six months of supplementation the rise of DHA was larger than for EPA in the ω3 FA group. In paper *III* and *IV* the rise of DHA was + 3.7 percentage units and for EPA +2.7 percentage units (Figure 1). In paper *V*, the rise of DHA was +3.6 and of EPA +3.14 percentage units. The placebo group displayed no significant alterations (papers *III*, *IV* and *V*).

EPA and DHA can be metabolized to each other, which are to some extent demonstrated here, since EPA plasma levels rose nearly as much as to those of DHA despite administration of three times more DHA.

Figure 1. Plasma concentrations (in percentage units) of DHA and EPA for subjects in paper III and IV.



4.2.2. Paper III.

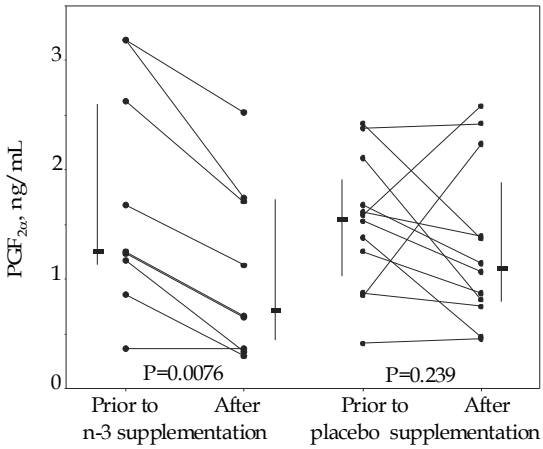
Vedin I, Cederholm T, Freund-Levi Y, Basun H, Hjorth E, Faxén Irving G, Eriksdotter Jönhagen M, Schultzberg M, Wahlund LO, Palmblad J **Reduced prostaglandin $F_{2\alpha}$ release from blood mononuclear leukocytes after oral supplementation of ω3 fatty acids: the OmegAD study.** *Submitted.*

We evaluated the effects of the per oral ω3 FA supplementation for 6 months on PGE_2 release from PBMC by measuring its stable metabolite $PGF_{2\alpha}$ [27, 63]. We used two different stimuli, one activating mainly monocytes (LPS), and the second (PHA) T-lymphocytes.

Minute amounts of $PGF_{2\alpha}$ were released by quiescent PBMCs. 10 ng LPS/mL conferred a 100-fold rise of the $PGF_{2\alpha}$ release from the stimulated cell supernatants.

After 6 months, the ω3 FA group showed significantly lower $PGF_{2\alpha}$ releases from LPS stimulated PBMCs compared to baseline values (Figure 2), whereas no changes were observed for the placebo group. The change in the ω3 FA group over time was trendwise significantly different from the change of the placebo group (P=0.06).

Figure 2. $PGF_{2\alpha}$ concentrations in supernatants of LPS stimulated PBMC.



In the used assay for $PGF_{2\alpha}$, we cannot determine how much of the assayed $PGF_{2\alpha}$ actually originated from AA or from EPA (hence being $PGF_{3\alpha}$). However, according to the manufacturer, the cross-reactivity for $PGF_{3\alpha}$ is 21% in their $PGF_{2\alpha}$ assays. Nonetheless, even if one assumes that the proportion of $PGF_{3\alpha}$ increased after 6 months of ω3 supplementation, the total outcome of all isoforms of PGF was a reduction. It is also reasonable to assume that the biological activity of the PGF mixture might be lower than if all PGF originated from AA. The same reasoning is valid for PGE_2 and PGE_3.

PHA conferred a 30-fold rise of the $PGF_{2\alpha}$ release. No changes from baseline were noted in the ω3 FA or the placebo oil groups. We believe this suggests that the structures and cells targeted by PHA were not affected in this capacity by the increase of ω3 FA. The result in this study (III) is in accordance with results in paper I, where no inhibitory effect on PHA induced lymphocyte proliferation by $PGF_{2\alpha}$ was obtained.

Although previous studies usually have focused on inhibitory effects of PGs on generation of cytokines such as TNF-α or IL-1β [43, 151, 152], recent data have emphasized that PGE_2 might also enhance immune and inflammatory reactions, e.g. T-cell proliferation [57, 60] and cytokines involved in neutrophil recruitment and migration [58]. Consequently, one might ask about the mechanisms for the simultaneous reductions of $PGF_{2\alpha}$, and several cytokines, as well as the statistically significant correlation between changes in $PGF_{2\alpha}$, and IL-6 and IL-1, observed in study (*III*). Is it so that all reactions depend on effects of ω3 FAs on common mechanisms for generation of these molecules? Or is it so that reduced release of PGs directly influenced generation and release of IL-6, IL-1, G-CSF (but not TNF)? At this time, we do not know.

These results points to interactions between the eikosanoid, FA and cytokine systems and may be part of the anti-inflammatory reactions associated with ω3 FA treatment.

4.2.3. Paper IV.

Vedin I, Cederholm T, Freund Levi Y, Basun H, Garlind A, Faxén Irving G, Eriksdotter Jönhagen M, Vessby B, Wahlund LO, Palmblad J. **Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes:** the OmegAD study. *Am J Clin Nutr* 2008; 87:1616-22.

In paper IV, we evaluated long-term effects of the DHA-rich ω3 supplementation on ex vivo cytokine and growth factor release by PBMC from AD patients.

A significantly lower release of IL-1β, IL-6 and the myeloid growth factor G-CSF (but not for GM-CSF) was observed from LPS-stimulated PBMC from the ω3 FA group after six months of treatment compared to pre-trial values. The data on G- and GM-CSF represents novel information in relation to dietary manipulations in humans. As to G-CSF, blood neutrophil counts did not change over time in neither the ω3 FA nor in the placebo group, so we assume that DHA supplementation did not directly interfere with host defense mechanisms involving the G-CSF-neutrophil axis.

We have no data on production of these factors in the PBMCs but most data suggests a close relation between the two reactions. The exception might be IL-1β where a substantial part of the produced cytokine is found intracellularly in monocytes after stimulation with LPS [153].

The drop of IL-1β and IL-6 (but not of G-CSF) between pre-trial and 6 months values was significantly larger in the ω3 FA group compared with the placebo group. Our observation that no changes of release of TNF-α, IL-8 or IL-10 in either the ω3 FA or the placebo groups is in agreement with results of others [154].

LPS did not confer any induced release of IL-2, IL-4, IL-5 or IFN-γ. Likewise, no measurable levels of VEGF, EGF and basic FGF were found.

Many studies have concerned effects of EPA on inflammatory reactions, but few have investigated effects of DHA on ex vivo cytokine release. The choice of fatty acid in clinical trials might be of significance since EPA and DHA display partly different modes of action on a variety of inflammatory and other reactions. The choice of ω3 FA in clinical trials depends also of the EPA/DHA ratio in the targeted organ, e.g. the brain which is rich in DHA but contains virtually no EPA.

Our here obtained data for IL-1β and IL-6 are in accordance with other studies examining effects of fish oils rich in EPA in healthy volunteers [96-99, 101, 103]. Some studies [96, 97, 99, 101] reported a decreased TNF-α production after fish oil supplementation, results that we were unable to repeat in this study. Nevertheless, others found that EPA rich fish oil supplementation (from 1-6 months up to 1 year) did not reduce TNF-α secretion [103, 105, 154-156] and some not even IL-1β or IL-6 releases [106, 154]. Even more confusing are findings that low doses of DHA and EPA given to healthy individuals or to children caused a raise in the TNF-α, IL-1β [98, 156] and IL-6, IL-10 secretions [156]. Thus, results of EPA supplementation on cytokine release are not unanimous although a majority indicates attenuations.

Supplementation for three months with DHA-rich fish oil with lower doses than we used decreased IL-6 (but nor of IL-1β or TNF-α releases) [102]. It seems that the dose

and time dependence is of significance since 4.9 g DHA per day had no effects on TNF-α, IL-1 β, or IL-6 secretion but when given 6 g for 3 months, reductions were observed [154, 157]. Thus, our data on IL-1β, IL-6 and TNF-α are in good agreement with what has been found previously of several months of supplementation, even when rather low DHA doses were given.

Correlation of plasma fatty acid, $PGF_{2\alpha}$ and cytokine data (III and IV). Based on the assumption that the plasma FA levels reflect the PBMC FA composition we compared plasma FA profiles with the release of IL-6, IL-1β and $PGF_{2\alpha}$ .The main finding was that the more DHA (or EPA) increased, the lower was the IL-1β, and IL-6 and $PGF_{2\alpha}$ releases. Thus, we suggest that it was mainly the administered DHA that affected cytokine (and $PGF_{2\alpha}$) releases. When we asked if changes of $PGF_{2\alpha}$ release could be related to changes in released cytokines, we found that the more $PGF_{2\alpha}$ decreased, the lower was the release of IL-6 and IL-1β, possibly implying a biological relationship. However, only studies with pure EPA or DHA preparations can give the answer to the question which acid is doing what. Also, the balance between EPA and DHA in a preparation might also be of significance.

Correlation of cytokine data (IV). Additionally, we compared whether cytokines and growth factors related to each other. A reduced IL-1β level correlated significantly to a decreased level of IL-6 for all 21 subjects. Furthermore, reductions of IL-6 levels were significantly related to lower levels of G-CSF and IL-10 for all subjects. IL-8 changes did not relate to those of G-CSF. Thus, the coordinated, paralleled changes of these cytokines strongly suggest that DHA affected mechanisms common for generation of these cytokines. No significant correlations were found for any of the other variables.

Our study emphasizes the close relationship between ω3 FA levels and the cytokine and $PGF_{2\alpha}$ release from PBMC as well as the concerted reactions of certain cytokines. However, the question whether ω3 FA supplementation is associated with similar attenuation of release of cytokines from AD brain cells remains to be established.

The clinical significance of the here analyzed cytokines and growth factors is further emphasized by the recent report by Tsai et al [158], that G-CSF are involved in AD pathogenesis and also by Ray et al [159], who showed that plasma levels of IL-1, TNF-α and G-CSF are strong predictors of development of AD.

This study shows that $PGF_{2\alpha}$, generated directly from AA or via PGE_2, decreased in the ω3 FA group compared to the placebo group in a stimulus specific way. This novel finding agrees with and adds to previous data on effects of EPA supplementation, suggesting that EPA and DHA effects are similar in this particular respect, though differences are noted for other effector variables.

In this context, it may be speculated that DHA (and EPA) gave rise to anti-inflammatory and neuroprotective lipid mediators, which appears to be part of the resolution phase of inflammation [9, 28].

4.2.4. Paper V.

Vedin I, Cederholm T, Freund Levi Y, Basun H, Garlind A, Faxén Irving G, Eriksdotter Jönhagen M, Wahlund LO, Dahlman I, Palmblad J. **Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on gene expression changes in blood mononuclear leukocytes:** the OmegAD study. *Submitted 2009*

When we started our studies on global gene expressions after ω3 FA supplementation, there were no such results published. Indeed, it was only during the finalization of this resumé that one such study was published, but that concerned supplementation of humans with an EPA based fish oil [121]. Here, we determined the effects of a DHA enriched ω3 FA supplementation for 6 months on gene expressions in PBMC from 11 AD patients, and from 5 patients given placebo oil for six months. Since these assays allow broad mapping effects, results provide a comprehensive and integrated view of effects.

Gene expression data by microarray. After the 6 months we observed signals in 4,075 probesets (out of setting of 8000 genes) according to the criteria in MAS 5.0, showing that the corresponding mRNA was expressed in PBMCs on >15 of 22 arrays. According to SAM analysis the expression of 9 genes was up- and 10 genes were down-regulated with a false discovery rate of 10 %. These genes are given in Table 7.

Verification of microarray data by RT-PCR was made on five genes (*CD63, CASP4, RHOB, VCP and SORL*). Out of these five genes, three *viz. CD63, CASP4* and *SORL1* were significantly modulated in accordance with changes in the microarray assays.

Genes were categorized by the Gene Ontology database www.geneontology.org. Although paper *IV* reported on changes of LPS-induced pro-inflammatory cytokine and growth factor release after a long-term treatment by a DHA enriched ω3 FA supplementation, we were unable to find any changes in the corresponding genes. This might be due to the fact that our gene expression data is based on quiescent PBMCs, without LPS activation. However, no corresponding genes were found with those obtained in the baboon and murine and human global gene expression studies or in vitro gene expression studies [117-121].

Nonetheless, there are number of genes in our study that are associated with inflammatory reactions and/or associated with AD. Here, data are given with comments on possible functions (provided by Ref Seq at:http://www.ncbi.nlm.nih.gov/RefSeq/).

Up-regulated genes by ω3FA, associated with inflammatory reactions.

The *CD63* gene, coding for a tetraspanin protein found in, for example, macrophages. It is involved in cell adhesion and cell migration and neutrophil granule mobilization [160, 161]. Thus, CD63 gene is involved in inflammation and has not previously been described in gene expression studies.

The enzyme encoded by the *HSD17B11*gene modulates the intracellular glucocortocoid levels, taking part in steroid biosynthetic processes of relevance for inflammation.

RAB27A codes membrane bound protein belonging to Rab small GTPase superfamily. It may be involved in protein transport and signal transduction. Mutations in this gene are associated with Griscelli syndrome type 2, an X-linked lymphoprolifera-

tive syndrome associated with hemophagocytic syndromes [162] linking this protein to regulation of inflammation.

The *CASP4* gene encodes for an apoptosis-related inflammatory caspase. It participates in the LPS-induced TLR4-signaling pathway, acting in the NF-κB activation of cyto- and chemokine production. Hence, it is an important part of the innate immune system [163]. This gene was also observed to be up-regulated in an in vitro study with DHA treatment [164]. Caspase-4 is considered to inhibit activation of caspase-3 (a key mediator in cell apoptosis), which is involved in progressive synaptic degeneration and neuron loss in familial AD [165].

Table 7 **Up-regulated genes by a DHA rich ω3 fatty acids or placebo formula**.

Locus link	Genes	ω3 FA- [1] group Fold change	Placebo group Fold change	ω3 [2] placebo P-value
MS4A3	Membrane-spanning-4 domains subfamily A	1.72±0.83	1.65±1.12	0.88
NAIP	NLR family, apoptosis inhibitory protein	1.37±0.35	1.16±0.11	0.21
DRG1	Developmentally regulated GTP binding protein	1.20±0.19	1.14±0.11 [3]	0.51
CD63	CD63 molecule	1.20±0.18	0.99±0.11	*0.03*
HSD17B11	Hydroxysteroid (17beta) dehydrogenase II	1.19±0.15	1.27±0.55	0.64
RAB27A	Member RAS oncogen family	1.18±0.16	1.08±0.18	0.29
CASP4	Apoptosis-related cysteine peptidase	1.17±0.14	0.99±0.20	0.06
SUPT4H1	Suppressor of Ty 4 homolog 1	1.15±0.11	1.02±0.12	*0.050*
UBE2V1	Ubiquitin-conjugating enzyme E2 variant 1	1.13±0.10	1.06±0.22	0.42
	Down-regulated genes by a DHA rich ω3 fatty acid or placebo formula.			
RHOB	Ras homolog gene family, member B	0.70±0.26	1.30±0.79	*0.03*
VCP	Valosin-containing protein	0.72±0.24	0.94±0.16	0.08
LOC399491	LOC399491protein	0.73±0.19	1.09±0.32	*0.01*
ZNF24	Zinc finger protein 24	0.76±0.18	0.99±0.12	*0.02*
SORL1	Sortilin-related receptor L(DLR class)	0.77±0.21	0.95±0.18	0.13
MAN2A1	Mannosidase alpha, class 2A member 1	0.78±0.21	1.01±0.19	0.057
PARP1	Poly(ADP-ribose) polymerase family, member1	0.80±0.16	1.01±0.29	0.08
SSRP1	Structure specific recognition protein1	0.82±0.12	0.98±0.23	0.09
ARIH1	Ariadne homolog, ubiqutin-conjugting enzym E2 binding protein	0.83±0.16	0.88±0.16	0.58
ANAPC5	Anaphase promoting complex subunit 5	0.87±0.09	1.04±0.17	*0.02*

Values are means±SD. [1]All genes are significantly up- or down regulated by ω3 FAs treatment according to SAM based on 2000 permutations and a false discovery rate of 10%. [2] Values represent the statistical significance for fold-change values between the ω3 FAs (n=11) and placebo (n=5). [3] The Change for DRG1 was significant (p=0.04)

Up-regulated genes associated with neuro-inflammatory disorders
The *DRG1*gene encodes for a protein involved in stress and hormone responses, cell growth and differentiation. It is necessary for p53-mediated caspase activation and apoptosis. Mutation in this gene causes a hereditary variant of Chariot-Marie-Tooth disease (type Lom) and is thus linked to a degenerative nerve disorder.

The *NAIP* (*BIRC1*) gene encodes for NLR family apoptosis-inhibitory proteins. Low levels of NAIP protein is found in AD brains. Expression of one of the NAIP proteins may protect AD patients against the development of tangle pathology and cognitive decline [166], thus, being of significance for the AD patients studied here.

Down-regulated genes associated with inflammatory reactions
RHOB encodes for a Rho-related GTP-binding protein, with GTPase activity. RhoB can activate NF-κB signaling by modification of the RelA/p65 [167] and has a role in vascular development [168].

It is likely that the *MAN2A1* gene has a relation to regulation of inflammation, as a mutation in a mouse homolog of this gene is linked to a systemic autoimmune disease.

Down-regulated genes associated with neuro-inflammatory disorders.
VCP encodes for a valosin containing ATP binding protein involved in vesicle transport and fusion, and ubiquitin-dependent protein degradation. Polymorphism in this gene is linked to late onset AD [169]. Previously, this gene was found to be up-regulated in an in vitro study with DHA treatment [164].

The *PARP1* gene encodes for the poly (ADP-ribosyl) transferase-1, which modifies nuclear proteins. It is involved in the regulation of e.g. differentiation, proliferation, tumor transformation and the recovery of cells from DNA damage. It may also be involved in NF-κB driven expression of inflammatory mediators, like IL-1 [170]. An overactivity of this protein has been shown in AD brains [171]. This gene is hence of interest for AD pathology as well as for inflammation.

SORL1, also known as *SORLA* and *LR11*, encodes for a protein of the vacuolar protein sorting 10 (VPS10) receptor family. It is strongly expressed in the central nervous system, regulating processing of the amyloid precursor protein APP in AD [172]. Expression of this protein is reduced in the brain of AD patients [173]. In a murine study, DHA increased *SORL1* mRNA and protein in cortical neurons [174]. The *SORL1* gene regulates the amyloid precursor protein which is also elevated in aged DHA depleted mice [174]. Here, we found that ω3 FA instead down-regulated the expression of the *SORL1* gene. The reason for this discrepancy is not known, but may, be related to cell types and species.

Differences between groups and relation to ω3 FA.
Statistical analyses showed significant differences between the ω3 FAs and the placebo groups for *CD 63* and *SUPT4H1, RHOB*, *ANAPC5*, *LOC399491* and *ZNF24*, emphasizing the specificity of the reactions to the ω3 FAs.

When relating values for expressed genes to plasma concentrations of DHA or EPA, we observed that the more plasma levels of DHA (or EPA) increased, the lower was the expression of the *ANAPC5* and *RHOB* genes. Moreover, we found that the expression of the *CASP4* gene rose with an enhanced EPA (but not DHA) plasma level. These

results on the *RHOB* and *ANAPC5* genes showing a statistically significant correlation with changes of both plasma DHA and EPA levels make them interesting candidates for further studies on mechanisms for effects of ω3 FAs.

General comments
The changes observed might appear rather small, since they ranged between +72 and -30 percent. However, this is what could be expected in dietary supplementation studies in humans. Similar magnitudes of changes were observed in a study on obese woman and energy-restricted formulas effects on gene expression [175].

One can anticipate that acute and large effects will vane with prolonged exposure because of an adaptation over time. Surprisingly, the number and differently expressed genes, reported in the literature to be changed by ω3 FA treatment varies considerably, which points at factors need of further evaluations.

The present study gives novel information on mechanisms for marine lipids, suggesting that dietary ω3 FA supplementation affected expression of genes that might influence inflammatory processes and could be of significance for AD.

5 GENERAL DISCUSSION AND CONCLUDING REMARKS

The aim of this study has been to investigate effects of prostaglandins and omega-3 fatty acids on immune and inflammatory response and on gene expression

The prostaglandin studies.
The 1st part of this thesis was inspired by earlier studies on mononuclear blood leukocytes from irradiated breast cancer patients [176, 177]. In vitro studies showed a restored lymphocyte response to mitogens when the COX inhibitor indomethacin was added to cell cultures. The interpretation was that arachidonate-based eikosanoids from monocytes from the patients exerted a dampening effect on the mitogen induced lymphocyte response, which may have impact on the immune response.

So, in this part of the study, *in vitro* models were used to study exogenously added PGs of the E and D series and their influence on lymphocytes and monocytes from healthy persons. The observed inhibitory effects on PHA induced proliferative response in PBMC by PGE_2, PGA_2 and PGD_2 agreed well with earlier assumptions that a disease or treatment associated enhanced biosynthesis of PGs in monocytes may suppress lymphocyte proliferation [178]. The effects of PGE_2 and PGD_2 on lymphocyte NK activity is difficult to dissect as to mechanisms since the identity of the effector cells is unclear. However, ligation of the prostanoid DP receptor by PGD_2 generally may lead to suppression of cellular immune functions, including NK cells [135].

The reduction of the TNF-α release from monocytes by PGE_2 is well known. The findings of an increased TNF-α release from monocytes after adding PGJ_2, Δ^{12}, PGJ_2 and 15 deoxy-$\Delta^{12,14}$ PGJ_2 was not corroborated by a later study, which found the opposite [150]. This inconsistency of results might be explained by experimental settings and/or used PG concentrations. PGE_2, PGA_2 and PGD_2 in lower micromolar concentrations enhanced the PHA lymphocyte response [141]. Thus, it seems that not only different PGs and its metabolites but also the same PG may exert different effects in the

immune and the inflammatory response. Recently, PGE_2 has been regarded as an anti-inflammatory mediator [179].

An intriguing possibility has been revealed after our publications. The discovery of the anti-inflammatory arachidonate based lipoxins, and enhancement of their synthesis by means of aspirin (or other non-steroidal anti-inflammatory drugs) opens up for alternative explanations of our findings. It may be speculated that generation of such substances in our in vitro systems might be of significance for the observed effects.

The OmegAD study.

In the 2nd part of the thesis, we explored the *ex vivo* effects of EPA and DHA on some of the functions of PBMC studied in the 1st part.

The formula used in our *ex vivo* assays for measurement of $PGF_{2\alpha}$, cytokine/growth factor release and gene expression experiments was a preparation enriched in DHA, containing 3 times more DHA than EPA. Many studies of the inflammatory response by ω3 FAs have been conducted by using a formula with more EPA than of DHA. Our results show that this DHA administration leads to approximately the same effects as EPA supplementation, with some differences [96, 97, 99, 103]. The used formula did not affect gene expression of specific cytokine genes. These may be related to different doses of EPA and/or DHA, age and health status of studied subjects, as well as initiation of EPA and DHA adaptation systems after long-term treatment.

The gene expression data on PBMCs revealed that marine fish oil enriched with DHA up-regulated nine and down regulated ten genes, respectively. These are genes that might be of importance for inflammation and Alzheimer´s disease. Of further interest is the effects on the nutritional status of the studied AD patients, as they gained some weight during the supplementation, whereas placebo treated patients did not [124].

6 FUTURE PERSPECTIVES

The OmegAD study still provides intriguing possibilities to better understand effects of a ω3 treatment. In particular, we would like to focus the attention to the following themes.

- It would be of great interest to improve our understanding of ω3 effects on epigenetic mechanisms, as a continuation of our research on gene expression results. For future work in the OmegAD study, we want to investigate the DNA methylation in blood samples from our included AD patients before and after treatment.
- The novel finding of a reduction of G-CSF release after ω3 supplementation might be followed up with studies on regulation of G-CSF production, for instance the IL-17 and IL-23 initiation loops.
- Investigations of concentrations of resolvins and protectins and relation to aspirin intake are also warranted, as well as
- Analysis of how much of the given DHA and EPA ended up in the central nervous system.

7 SAMMANFATTNING PÅ SVENSKA

Omega (ω) 6- och ω3-fettsyror utgör två familjer av biologiskt aktiva fleromättade fettsyror. Linolsyra (18:2ω6) är substrat för arakidonsyra (AA), medan α-linolensyra (18:3ω3) är modersubstans för eikosapentaen- (EPA) och dokosahexaensyror (DHA). EPA och DHA finns i fiskolja och fet fisk. AA, EPA och DHA är viktiga byggstenar i cellers membraner. Dessa essentiella fettsyror måste tillföras vår kropp via föda, eftersom kroppen inte förmår att själv producera dem. DHA och AA är också viktiga för hjärnans och synens utveckling under fosterstadiet. Alla tre är även modersubstanser för eikosanoider (t ex prostaglandiner och leukotriener), vilka är mediatorer vid inflammation och vid aktivering av kroppens immunsystem.

Vid angrepp av främmande organismer (t ex mikroorganismer och andra produkter som känns främmande för vår kropp) aktiveras vita blodkroppar (t ex mördarceller, monocyter och neutrofila granulocyter) och därmed har en inflammation börjat. Inflammationen hålls igång och regleras med hjälp av bl a cytokiner, andra proteiner, eikosanoider och andra aktörer. I en andra fas av inflammationen aktiveras ett mer specifikt immunförsvar, som involverar lymfocyter, som aktiveras med delvis samma men även andra signalsubstanser. Så småningom initieras specifika processer, som stoppar inflammation och inleder läkningsfasen.

Prostaglandiner, PG och leukotriener, LT, som bildas från ω6-fettsyror anses förmedla kraftigare inflammatoriska effekter än de som bildas från ω3-fettsyror. Vissa eikosanoider (t ex resolviner och protektiner), som bildas ur EPA och DHA, anses ha en rent dämpande effekt på inflammation.

Under senare år har det framkommit att AA, EPA och DHA påverkar celler på gennivå, genom sin påverkan av transkriptionsfaktorer och genaktivering. Det resulterar i syntes av kroppens proteiner, inklusive pro- och anti-inflammatoriska cytokiner. Så, mängden och typen av ω6- och ω3-fettsyror som vi tillför vår kropp och celler, påverkar inflammationsprocessen och immunsystemet.

I den första delen av denna avhandling, studierna **I** och **II**, har vi undersökt effekter av ω6 PGs från E- (PGE_2, PGA_2) och D-serien (PGD_2 och PGJ_2, Δ^{12}PGJ och $\Delta^{12,\ 14}$ PGJ2) och deras påverkan på immun- och inflammationssvaret hos mononukleära blodceller från friska blodgivare. De medförde en minskad lymfocytproliferation efter cellaktivering och att avdödandet av för kroppen främmande antigen (s.k. NK aktivitet) också minskade. Det visade sig att PG av en typ kan ha olika effekter. T.ex, påverkar samma koncentrationer av PGE_2 och PGD_2, och metaboliter som bildats från dessa PG, blodmonocyters förmåga att bilda den proinflammatoriska cytokinen TNF-α på olika sätt. Efter aktivering av monocyter sänktes nivåerna av frisatt TNF-α med PGE_2, medan PGD_2 inte hade någon effekt, och metaboliterna PGJ_2, Δ^{12}PGJ och 15 deoxy-$\Delta^{12,14}$ PGJ_2 ökade nivån. Detta talar för att prostaglandiner kan ha en modulerande effekt i inflammation processen.

I den andra delen av avhandlingen (studierna **III-V**) har vi undersökt ω3 fettsyrors effekter på inflammationssvaret och aktivering av gener. Patienter med mild till måttlig Alzheimers sjukdom randomiserades i en dubbel-blind placebo-kontrollerad studie, **OmegAD-**studien, till ett dagligt intag av fiskolja med 1.7 g DHA och 0.6 g EPA, eller till behandling med placebo, i 6 månader. Därefter fick alla behandling med ω3-preparationen i ytterligare 6 månader.

Blodprover togs vid studiens start och efter 6 månaders behandling. Mononuklära blodleukocyter (lymfocyter och monocyter) isolerades och effekterna på frisättning av en lång rad cytokiner, på en prostaglandin ($PGF_{2\alpha}$) samt på genaktivering studerades.

Sex månaders behandling med den DHA-rika fiskoljan dämpade frisättningen av cytokinerna IL-1 β och IL-6, men hade ingen effekt på frisättning av TNF-α, när cellerna stimulerats med bakterieprodukten LPS. Sänkta nivåer av IL-1β och IL-6 var statistiskt relaterade till höjda nivåer av DHA och EPA i plasma. Ju mer EPA och DHA ökade, desto mer minskade frisättningen av cytokinerna.

Fiskoljan minskade även nivåerna av den myeloida tillväxtfaktorn G-CSF, som bl.a är viktig för bildandet och överlevnaden av neutrofila granulocyter.

Vidare minskade den DHA-rika fiskoljan också frisättningen av prostaglandin $PGF_{2\alpha}$, en stabil metabolit av PGE_2. Ju mer DHA och EPA ökade i plasma, desto mer minskade nivån av $PGF_{2\alpha}$. Dessutom minskade frisättningen av $PGF_{2\alpha}$, IL-1β och IL-6 parallellt från de vita blodkropparna.

Slutligen förändrade fiskoljebehandlingen uttrycket av ett flertal gener som är viktiga vid inflammation eller Alzheimers sjukdom.

Denna avhandling har således inriktat sig på att se om ω3-fettsyror och ω6-baserade metaboliter av en fettsyra (AA) påverkar inflammations- och immunitetssvar hos vita blodkroppar. ω3-behandling leder till förändringar som kan tolkas som att inflammationen dämpas något. Det kan vara av värde för personer som lider av inflammatoriska sjukdomar. Betydelsen av effekterna av prostaglandiner på blodceller är svårare att sätta in i ett kliniskt sammanhang, men många andra har, liksom vi, funnit att de medför komplexa men distinkta reaktioner.

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