Anti-inflammatory effects of marine nutritional products in the treatment of ulcerative colitis

Based on clinical and animal studies

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To Ida, Magnhild and Jakob

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Scientific environment

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Abbreviations

AA	Arachidonic acid
AASA	Aminoadipic semialdehyde
AGE	Advanced glycation end products
AIFAI	Anti-inflammatory fatty acid index
ALA	Alpha linolenic acid
ADP	Adenosine diphosphate
cDNA	Complementary DNA
CD	Crohn´s disease
CEL	Carboxyethyl-lysine
CML	Carboxymethyl-lysine
COX	Cyclooxygenase
CXCL1	Chemokine (C-X-C motif) ligand 1
CRP	C-reactive protein
DAI	Disease activity index
DAMP	Danger-associated molecular pattern
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
DSS	Dextrane sulfate sodium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FFQ	Food frequency questionnaire
GC/MS	Gas chromatography/mass spectrometry
GSA	Glutamic semialdehyde
GWAS	Genome-wide association study
HCS	Histological combined score
H-E	Hematoxylin and eosin
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IFN-γ	Interferon-gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KC/GRO	Keratinocyte chemoattractant/Growth-regulated oncogene
LA	Linoleic acid
LC/MS/MS	Liquid chromatography/tandem mass spectrometry
LOX	Lipoxygenase
LT	Leukotriene
MDA	Malondialdehyde
MDAL	Malondialdehyde-lysine
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NFκB	Nuclear factor kappa B

NK	Natural killer
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern
PG	Prostaglandin
PPAR	Peroxisome proliferator-activated receptor
PPARGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PUFA	Polyunsaturated fatty acid
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCCAI	Simple Clinical Colitis Activity Index
SIRT	Sirtuin
TGF-β	Transforming growth factor beta
Th	T helper
TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TTA	Tetradecylthioacetic acid
TX	Thromboxane
UC	Ulcerative colitis

Abstract

Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) with a peak onset between 15-30 years of age. The ethiology is unknown, but essential in the pathogenesis is an interplay between environment (microbiota), a dysfunctional immune system and a genetic predisposition.

Treatment of IBD by corticosteroids, classical immunosuppressives and biological agents have potential side-effects and complications. Patients therefore often ask if alternative treatment can reduce or eliminate their disease or need for potent medication, and diet modifications has thus gained increasing interest as an alternative option.

Food components may change the microbial composition in the gut, and thereby influence the immune response and inflammation in IBD. Both innate and adaptive immune responses in the gut are involved in IBD, and can be affected by changes in microbiota and diet intervention.

Long chained polyunsaturated n-3 fatty acids (PUFAs) are essential components in marine oils and may exert anti-inflammatory effects in IBD through several mechanisms. They constitute substrates for production of anti-inflammatory lipid molecules – eicosanoids - change biochemical properties of cell membranes, or influence gene transcription; thus dampening inflammation. Atlantic salmon contains fish oil with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as peptides and/or proteins with potentially biological effects. We tested how a regular high intake of Atlantic salmon influenced disease activity and inflammatory markers in patients with active UC.

Further studies of marine nutritional products and a modified fatty acid was carried out in the dextrane sulfate sodium (DSS) chemically induced rat colitis model. This animal colitis model resembles human UC.

Fish is a source of bioactive peptides that have demonstrated antihypertensive-, anti-oxidant-, as well as anti-proliferative properties. Accordingly, diets with fish

peptides, fish oil and a combination, were administered in the DSS colitis model to investigate potential anti-inflammatory effects.

Krill oil is a novel source of n-3 marine fatty acids, with different chemical structure from fish oil, containing larger amounts of EPA and DHA bound to phospholipids. Krill oil also contains the natural anti-oxidant astaxanthin.

Tetradecylthioacetic acid (TTA) is a modified fatty acid containing sulphur in the 3-position, making it resistant against β-oxidation. It has demonstrated anti-oxidant, lipid lowering as well as immunomodulatory effects in previous studies, and was therefore tested in our experimental colitis model to assess whether it could reduce colon inflammation and affect oxidative stress markers.

Main objectives

- Investigate the anti-inflammatory effects of a high intake of Atlantic salmon in patients with active UC

- Assess the anti-inflammatory and anti-oxidative effects of fish oil, fish peptides and a combined diet in DSS induced colitis in rats

- Assess the anti-inflammatory and anti-oxidative effects of krill oil in DSS induced colitis in rats

- Investigate the anti-inflammatory and anti-oxidative effects of a modified fatty acid - TTA - in DSS induced colitis in rats

Subjects and methods

In the salmon diet pilot study (Paper I), all patients with active UC were recruited from the outpatient clinics, Stavanger University Hospital. Of the 23 patients included, six patients withdrew due to loss of motivation. Five more patients were excluded from further analyses because of fecal calprotectin < 50 mg/kg. Thus, twelve patients where available for analyses. After inclusion (visit 0), four weeks of wash out was followed by intake of 600 g Atlantic salmon weekly for eight weeks. At start (visit 1), and end of the diet intervention (visit 2), we rated the Simple Clinical Colitis Activity Index

(SCCAI), endoscopic score, histological score in rectum specimens, disease activity markers in plasma and feces, as well as fatty acid profile in plasma an rectum samples. We also calculated the anti-inflammatory fatty acid index (AIFAI). A food frequency questionnaire was filled out at inclusion (visit 0), and at the end of the study (visit 2).

In the experimental animal studies (Papers II-IV) male Wistar rats were given intervention diets for four weeks, with chemical colitis induction the last week. Seven groups of 10 animals received the following diets:

1) Standard diet (controls)

2) Standard diet + DSS

3) Fish oil (5%) + DSS

4) Fish peptides (3.5%) + DSS

5) Fish oil + fish peptides + DSS

6) Krill oil (5%) + DSS

7) TTA + DSS.

Due to this design, the two control groups (standard diet and standard diet + DSS) were identical in the three studies reported in Papers II-IV.

After four weeks, disease activity was rated according to the DAI. The animals were then anesthetized, sacrificed, and the major part of colon was removed. Colon length was measured, and tissue samples were isolated for rating of the histological combined score (HCS), measurements of selected cytokines, prostaglandins, mRNA expression, and markers of protein oxidative damage. The fatty acid profile was assessed in the krill oil, standard diet and standard diet + DSS groups.

Results

We found that:

- High intake of salmon in patients with active UC reduced the SCCAI, increased the EPA and DHA levels and n-3/n-6 fatty acid ratio in plasma and rectum biopsies, as well as increased the AIFAI, although these observations were not supported by other analogue changes in disease activity markers.

- The fish peptides supplemented diet attenuated DSS induced colitis in rats as compared with fish oil according to an improved HCS, lower levels of KC/GRO; while fish oil increased the anti-inflammatory prostaglandin (PG)E$_3$ levels, especially when combined with fish peptides diet

- Rats fed krill oil diet had retained their colon length versus rats treated with DSS alone, had lower levels of protein oxidative markers, increased EPA, -DHA levels, -n-3/n-6 fatty acid ratio and AIFAI (and a pattern with consistently reduced TNF-α, KC/GRO and IL-1β levels at protein and mRNA levels, although not reaching statistical significant differences).

- TTA fed rats had reduced protein levels of TNF-α, mRNA levels of IL-1β and IL-6 as well as lower levels of protein oxidative damage markers, and a reduced colon wall thickness compared with rats fed DSS, as assessed by transabdominal high frequency ultrasound.

Conclusions

We found that salmon fillets, as well as several marine nutritional products and a modified fatty acid may have beneficial effects in human UC and in an experimental animal colitis model resembling human UC. Although the biological effects are limited, fat fish fillets, fish peptides, krill oil and TTA should be further explored in controlled human IBD studies including patients in remission, and the effect in combination with medical therapy should be tested. Future human studies should take into account that genetic factors might explain some of the response variability in n-3 interventions. High frequency ultrasound imaging may become a valuable non-invasive tool in rat colitis models.

List of publications

Paper I:

Grimstad T, Berge RK, Bohov P, Skorve J, Gøransson L, Omdal R, Aasprong OG, Haugen M, Meltzer HM, Hausken T.
Salmon diet in patients with active ulcerative colitis reduced the simple clinical colitis activity index and increased the anti-inflammatory fatty acid index - a pilot study.
Scand J Clin Lab Invest. 2011 Feb;71(1):68-73

Paper II:

Grimstad T, Bjørndal B, Cacabelos D, Aasprong OG, Omdal R, Svardal A, Bohov P, Pamplona R, Portero-Otin M, Berge RK, Hausken T.
A salmon peptide diet alleviates experimental colitis as compared with fish oil.
Journal of Nutritional Science (accepted oct 2012)

Paper III:

Grimstad T, Bjørndal B, Cacabelos D, Aasprong OG, Janssen EA, Omdal R, Svardal A, Hausken T, Bohov P, Portero-Otin M, Pamplona R, Berge RK.
Dietary supplementation of krill oil attenuates inflammation and oxidative stress in experimental ulcerative colitis in rats.
Scand J Gastroenterol. 2012 Jan;47(1):49-58

Paper IV:

Bjørndal B, **Grimstad T**, Cacabelos D, Nylund K, Aasprong OG, Omdal R, Portero-Otin M, Pamplona R, Lied GA, Hausken T, Berge RK.
Tetradecylthioacetic acid attenuates inflammation and has antioxidative potential during experimental colitis in rats.
Dig Dis Sci 2012 Aug 2. [Epub ahead of print] DOI 10.1007/s10620-012-2321-2

1. Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises two main entities; ulcerative colitis (UC) and Crohn´s disease (CD). Both are chronic inflammatory diseases and show a remitting-relapsing course. UC is usually characterized by a diffuse and superficial inflammation of the colorectal mucosa, extending proximally to a variable degree (Figure 1). Bloody diarrhea frequently occurs during active flares of disease. Up to one quarter of the patients have a progressive disease course, do not respond to medical therapy, and will undergo a colectomy during their disease career (1).

CD presents with a patchy transmural inflammation and may affect any part of the GI tract, from the oral cavity to the anus, but most frequently involves the ileocoecal region. In a majority of patients the deep inflammatory processes result in a course complicated by strictures, fistulas and abscesses (2). Surgical treatment is required in up to 80% after 20 years of disease (3).

Figure 1. Ulcerative colitis
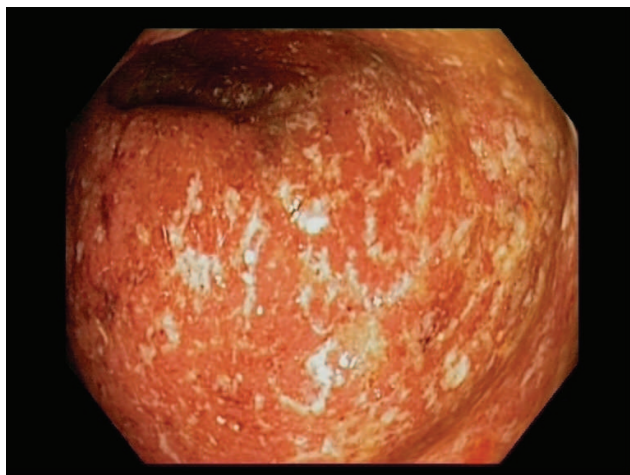
Photo: T. Grimstad

1.2 Epidemiology

The increasing incidence and prevalence of IBD in Northern Europe and North America during the last decades, most markedly for CD, appears to stabilize. In contrast, geographical areas in Europe with previously low incidences now reportedly show an increase of IBD. UC and CD presently affect up to 0.4% of the population in Europe, and are most frequently diagnosed in individuals between 15-30 years of age. Also, the incidence rates seem to follow a north-south gradient (4). Scandinavia, UK and North America are all regions with a high incidence of IBD, while Greece and Portugal represent countries with lower incidence rates. In Norway, the annual incidences for UC is about 16/100.000, and for CD 8/100.000 (5).

1.3 Pathogenesis

Growing evidence points to a dysregulated immune response against commensal gut microbiota in a genetically predisposed host as essential factors for the pathogenesis of IBD. In this setting, diet, surrounding microbiota and smoking have gained increased attention as important environmental elements in the pathogenesis (6).

1.3.1 Basic principles of the immune system

The main purpose of the immune system is to detect and destroy potentially invading pathogens and other danger molecules. The innate and adaptive immune system forms two different functional entities with close collaboration. Innate immunity serves as the first line of defence. It responds rapidly against evolutionary conserved specific molecular structures on pathogens, but has no memory for past events. Some epitopes of microorganisms have "pathogen-" or "danger-associated molecular patterns" (PAMPS or DAMPS). Cell-surface located toll-like receptors (TLRs) and intracellular receptors like nucleotide oligomerization domain (NOD) are examples of important sensors for PAMPS and DAMPS. Physical and chemical barriers, secretory- and serum molecules, phagocytic cells (monocytes, macrophages and granulocytes) and dendritic cells are also components of the innate immune system.

The adaptive immune system is much more specific although slower in its response. Lymphocytes, constituting T, B and natural killer (NK) cells originate from stem cells in the bone marrow and are distributed to their maturing organ: Thymus - T cells, and bone marrow – B cells. In the thymus, the T cells become immunologically educated and aquire ability to respond to specific antigens. The B cells develop antigen-specific receptors on their surface – B cell receptors.

Dendritic cells and macrophages present antigen to T cells which become activated and polarize into T helper subsets (Th1, Th 2, Th17) and T cytotoxic cells.

Th 1, Th 2 and Th 17 cells are essential upregulators of adaptive immunity. Their activation lead to secretion of local acting substances like cytokines and chemokines resulting in a cascade involving T cell differentiation and proliferation, and further cytokine release. T cytotoxic cells eliminate other cells infected by viruses or bacteria. In the early phases of the immune response, the specific nature of the antigen triggers sensing receptors and subsequent effector systems, resulting in an optimized immune reponse against the particular antigen.

Some B and T cells furthermore mature into memory cells, being able to mount a rapid response when exposed to the same antigen a second time. This increases the immunological effect against a specific pathogen (aquired immunity) (7).

Tolerance is the ability of the immune system to not respond to certain epitopes, such as "self", thereby avoiding inappropriate activity under normal conditions. In the healthy subject there is a balance in the immune system between factors that dampen activity, such as regulatory T and B cells, interleukin (IL)-10, and stimulating factors, like the Th 17 cells and IL-17A, IL-17F and IL-22.

Autoimmune diseases are classically characterized by a loss of tolerance against self antigens, resulting in autoreactive T cells, and B cell production of antibodies against autoantigens (8). Such autoreactive T and B cells may trigger chronic inflammatory processes.

1.3.2 Cytokines and chemokines

Cytokines are small signal molecules, mostly produced by immune cells, allowing communication between cells. Cytokines stimulate or downregulate different cell types in the vicinity in an autocrine or paracrine way. The local cytokine profile created by innate immune cells like macrophages and dendritic cells determine how activated T-cells differentiate to specific subsets, and how these cell populations later proliferate. Cytokines act by activating designated receptors on the target cells, followed by intracellular signalling cascades controlling gene transcription and thus the cellular function (7).

Cytokines may functionally be divided into pro-inflammatory, such as IL-1β, tumor necrosis factor (TNF)-α and IL-6, and anti-inflammatory, like IL-10 and transforming growth factor (TGF)-β. Different Th subsets tend to release different "cytokine profiles", such as Th 1: IFN-γ, IL-2, IL-12; Th 2: IL-4, IL-13 and Th 17: IL-17A, IL-17F and IL-22 (9).

Chemokines are small signal molecules with effect on homing and migration of leukocytes. Chemokines guide leukocytes from the circulation to sites of inflammation. There are four groups, named C-, CC- CXC- and CX_3C chemokines, according to the positions of their cysteine residues. Like cytokines, chemokines also act through designated receptors on target cells (10).

1.3.3 The gut immune system in inflammatory bowel disease

Under normal conditions, the immune response is tightly regulated, and inflammation downregulated once the invading pathogen is defeated. However, in chronic inflammatory conditions like IBD, the immune response is continuously upregulated. Organ-specific antibodies or autoreactive T cells do not seem to play a major role in IBD, but other dysregulations of mucosal and systemic immunity are clearly evident in both UC and CD. The integrity between the enteric flora and mucosal immune system appears disturbed, and a loss of tolerance towards the normal gut flora in IBD results in detrimental inflammatory activity (8). Dysfunctions in the innate immune system play pivotal roles in the pathogenesis of IBD.

Several important components of innate immunity might be affected in IBD, such as secretory Ig A, defensins secreted from epithelial cells, the mucus layer, the epithelial layer with tight junctions between cells, and dysfunctions of lamina propria located macrophages, neutrophilic granulocytes, dendritic cells and NK cells. This leads to an aberrant influx of microorganisms or their degraded products into the lamina propria, and triggers an immunological response. B cells also play a role in the lamina propria in IBD, producing antimicrobial antibodies like IgA.

Specific PAMPs or DAMPs on pathogens and other "danger-molecules" activate macrophages, dendritic cells and even epithelial cells through binding to innate sensors like TLR or NOD receptors. Dysfunctions in sensing and processing of signals may result in inappropriate activation of innate immune cells.

When a foreign antigen is discovered by antigen-presenting cells, it is brought to an "induction area", a mesenteric lymph node or Peyer plaque by dendritic cells for presentation to naive antigen-specific T cells which then become activated and differentiate to specific Th cell subsets modulated by the surrounding cytokine profile. T cells then proliferate, and express chemokine surface receptors like CCR9 and enterotropic adhesion molecules like integrin $\alpha_4\beta_7$ before being transported back to the "effector area" of the intestinal mucosa by efferent lymph vessels, the thoracic duct and blood. Effector T cells then secrete cytokines and chemokines that accelerate the inflammatory process. In addition, high levels of nitric oxide, oxygen radicals, eicosanoids and other effector molecules produced by macrophages contribute to the tissue damage in IBD (1, 2, 6).

CD has been regarded a Th 1 mediated disease with elevated interferon (IFN)-γ, IL-12 and IL-18, while UC has been attributed to a modified Th 2 cytokine profile, with high levels of IL-4, IL-5 and IL-13 (6). TNF-α and IL-1β are essential pro-inflammatory cytokines that are increased in the intestinal mucosa in IBD (2). In addition, Th-17 produced IL-17, and IL-23 generated by macrophages and dendritic cells influence pathways important for inflammation in both UC and CD (2, 11).

1.3.4 Inflammatory markers: CRP and fecal calprotectin

C-reactive protein (CRP) produced in hepatocytes is an essential marker of the acute phase reaction in humans. To some degree serum levels of CRP reflect the degree of IBD disease activity (12), but to a lesser extent in UC than in CD (13). Calprotectin is a cytosolic calcium- and zinc binding protein produced and released from macrophages and neutrophils. Fecal calprotectin levels appear to reflect the amount of neutrophil infiltration in the intestinal wall (13). Thus, measurements of fecal calprotectin permit differentiation between IBD and irritable bowel syndrome (IBS) with high accuracy. Fecal calprotectin can also separate active from inactive IBD, and is superior to CRP in assessing intestinal inflammation (14).

1.4 Genetics in IBD

Genes are made up of densely packed double stranded DNA in 23 pairs of chromosomes located in the cell nuclei. The DNA sequences code for and determines the structure and function of proteins and peptides. Through activation by transcription factors, genes are transcribed to messenger RNA (mRNA). The sequence of bases in the mRNA molecule is then read in order to create a sequence of aminoacids, ultimately forming a protein of specific length, structure and folding. Gene expression is regulated by RNA-DNA interactions, by microRNA molecules or other epigenetic changes, such as methylation or acetylation of the DNA-histone complexes. Also, the synthesized peptide or protein can be modified by post-translational changes, such as adding a functional group or a protein, or making a structural or chemical change (15). Transcription factors are key regulators of gene transcription. They contain DNA-binding domains which allow binding to a defined DNA sequence, frequently in the regulatory region of a specific gene. These factors also contain regions that interact with RNA polymerase, necessary for transcription (16).

Genome-wide assossiation studies (GWAS) permit the whole human DNA sequence to be searched for susceptibility genes for different diseases, including genes for IBD. In a recent study 163 IBD loci were discovered. Of them, 110 were shared by both UC and CD, and 23 were associated with UC only (17). Although the disease

heredity explained by known IBD loci was reported to only 23% in CD (18) and 16% in UC (19), valuable information about key genes involved and pathogenetical pathways has been revealed from such studies. It is, however, important to know that only a minor fraction of IBD susceptibility can be explained by these candidate genes, and that they represent variants of normal genes, and not monogenetic disease.

1.5 Oxidative stress - reactive oxygen species - antioxidants

Reactive oxygen species (ROS) have important physiolocical functions in cell signalling and defence against foreign microorganisms. They are highly reactive substances formed during both normal and abnormal conditions. ROS are produced and released into phagocytic blebs of macrophages and neutrophils when they recognize, phagocytize and destroy pathogens (20, 21).

An example of ROS is the superoxide anion radical, $O_2^-\bullet$. It has an extra electron, and is continuously produced as a by-product of the respiratory chain energy producing process. Hydrogen peroxide, H_2O_2, hypochlorous acid, $HOCl$, the hydroxyl radical, $OH\bullet$, nitric oxide, NO, and peroxynitrite, $ONOO^-$ are other examples of reactive molecules (20). ROS are extremely reactive molecules and may damage normal cellular components and cellular life if not opposed by strong protective anti-oxidant mechanisms of both enzymatic and non-enzymatic nature (22).

In chronic inflammatory conditions like IBD there is a relative excess of ROS. This "oxidative stress" can lead to tissue damage through oxidative chain reactions with production of toxic lipid peroxides, protein carbonyls or DNA base hydroxylations (22).

1.6 Treatment

The standard medical treatment of IBD the last decades has been 5-aminosalisylates, corticosteroids and immunosuppressive drugs (azathioprine and methotrexate), inducing clinical response or remission in a substantial proportion of patients. The introduction of biological treatment such as TNF-α-inhibitors and anti-integrins provides a more specific immune therapy with additional increase in the mucosal healing rate. However, these regimens also increase the risk of infections and rare

complications and side effects (23). Alternative and non-medical therapy, such as dietary intervention, is therefore often asked for by IBD patients.

1.7 Diet and IBD

Dietary habits in the Western world have changed considerably the last century, whereas our genes are unchanged. At the same time, human physical activity level has decreased. New life style related diseases have emerged, such as essential hypertension and diabetes. This has partly been attributed to a change in the fatty acid intake (24).

Food has gained increased attention as a pathogenetic factor in IBD. Recent research has highlighted the influence of dietary factors on immune functions (25), as well as the interplay between diet, microbiota and the immune system (26, 27). Dietary components can alter the composition of the intestinal microbial flora, which may lead to beneficial changes in both innate and adaptive immune responses (26).

1.7.1 Polyunsaturated fatty acids (PUFAs)

Fatty acids serve important biological functions in the human body, and are crucial components in the phospholipid bi-layer of cell membranes. Fatty acids consist of a carbon chain with a methyl group at one end and a carboxyl group at the other one. A saturated fatty acid does not have double bonds in the carbon chain, while unsaturated fatty acids have one (monounsaturated) or more (polyunsaturated) C=C bonds. The nomenclature of fatty acids, such as "C20:5n-3" describes the number of carbons in the chain followed by the number of double bonds, and finally the "omega-" or "n- ", indicating the number of carbons from the methyl end to the first double bond. Of polyunsaturated fatty acids (PUFAs), the n-3 and n-6 PUFAs are the most important groups (28).

Fatty acids are esterified to glycerol molecules to form mono-, di-, or triglycerides, containing one, two or three fatty acids, respectively. Fatty acids may also be divided into essential and non-essential fatty acids. The basic essential fatty acids are linoleic acid (LA), "C18:2n-6" and alpha-linolenic acid (ALA), "C18:3n-3" (Figure 2). These must be supplied in the diet, and serve as substrates for the

production of other long chain PUFAs. Through metabolism by elongase and desaturase enzymes, the human body can synthesize important fatty acids like "C20:4n-6" arachidonic acid (AA) from LA and "C20:5n-3" eicosapentaenoic acid (EPA) from ALA. However, the n-3 conversion to EPA is limited to 8-20% of the existing ALA (29). Therefore, PUFAs like EPA, and its elonged products, "C22:5n-3", docosapentaenoic acid (DPA) and "C22:6n-3" docosahexaenoic acid (DHA) are better supplied by diet than by endogenous production.

The n-3 PUFAs have several beneficial biological effects in humans: EPA and DHA are antithrombotic, lower triglycerides, reduce cardiac mortality, reduce blood pressure, reduce joint pain in rheumatoid arthritis, and may beneficially influence inflammatory diseases (30). In contrast, an increased n-6/n-3 ratio in the diet has been associated with higher rates of inflammatory and autoimmune diseases (24).

Figure 2. Essential fatty acids.

ALA – alpha -linoleic acid
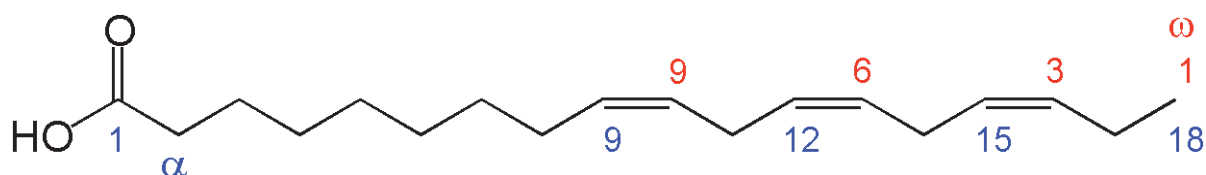
LA – linoleic acid
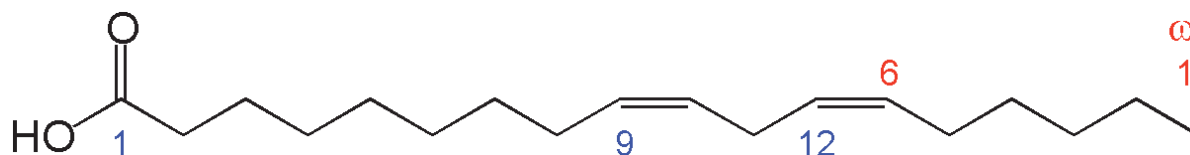
Reprinted from "ALA" and "LA" in *Wikipedia.* Retrieved on November 30, 2012 from http://en.wikipedia.org/wiki/File:ALAnumbering.png and http://en.wikipedia.org/wiki/File:LAnumbering.png

Eicosanoids

Eicosanoids are oxidised, short-lived mediators of inflammation derived from 20 carbon polyunsaturated fatty acids (31). The n-6 PUFA AA is abundant in cell membrane phospholipids of immune cells like macrophages and neutrophils. It is a major substrate for production of the generally pro-inflammatory eicosanoids. AA may be metabolized through 5-lipoxygenase (LOX), resulting in leukotriene (LT) 4-series of A, B, C, D and E, or through cyclooxygenase (COX) 1 and -2 pathways, producing prostaglandin (PG) D, E, F and I, as well as thromboxanes (TX), of the 2-series (32) (Figure 3).

A competitive substrate is the n-3 PUFA EPA which gives rise to anti-inflammatory substances. When dietary EPA replaces AA, the same enzyme systems produce leukotrienes (LOX) of the 5 series and prostaglandins (and thromboxanes) (COX) of the 3 series believed to have less inflammatory potential (32).

Other eicosanoids have emerged in recent years with anti-inflammatory properties, like resolvin E1 derived from EPA and resolvin D1-D6 from DHA. In addition, AA may give rise to anti-inflammatory lipid mediators in form of lipoxins, like lipoxin A4 (33).

1.7.2 Fish oil

Fish oil contains the important marine PUFAs EPA, DHA and DPA. The most important sources of marine n-3 fatty acids are fat fish such as salmon, trout and mackerel. EPA and DHA typically constitute about 18 % of total fat (34), which provides about 2g fatty acids in 100g fat fish fillet (30). Dietary fish oil may be supplied as fish oil concentrates in capsulas or as fish fillets. A more novel contributor to dietary marine PUFAs is krill, a crustacean living in the arctic seas. Krill provides n-3 with a large fraction bound to phospholipids instead of triglycerides, as well as a natural antioxidant, astaxanthin (34).

The anti-inflammatory effect by marine PUFAs may be attributed to several mechanisms, including transcription factor modulation, altered substrate for

production of eicosanoids (EPA for AA) and modifications of plasma membrane organization ("lipid rafts") (35).

Figure 3. Outline of eicosanoid synthesis from arachidonic acid. Selected products from eicosapentaenoic acid are shown.

Arachidonic acid (AA)

Eicosapentaenoic acid (EPA)

COX-1
COX-2

5-LOX

PGG_2

LTA_4

PGH_2

LTB_4 LTC_4

LTB_5

LTD_4

PGD_2 PGE_2 PGI_2 TXA_2

PGE_3 TXA_3

LTE_4

LTE_5
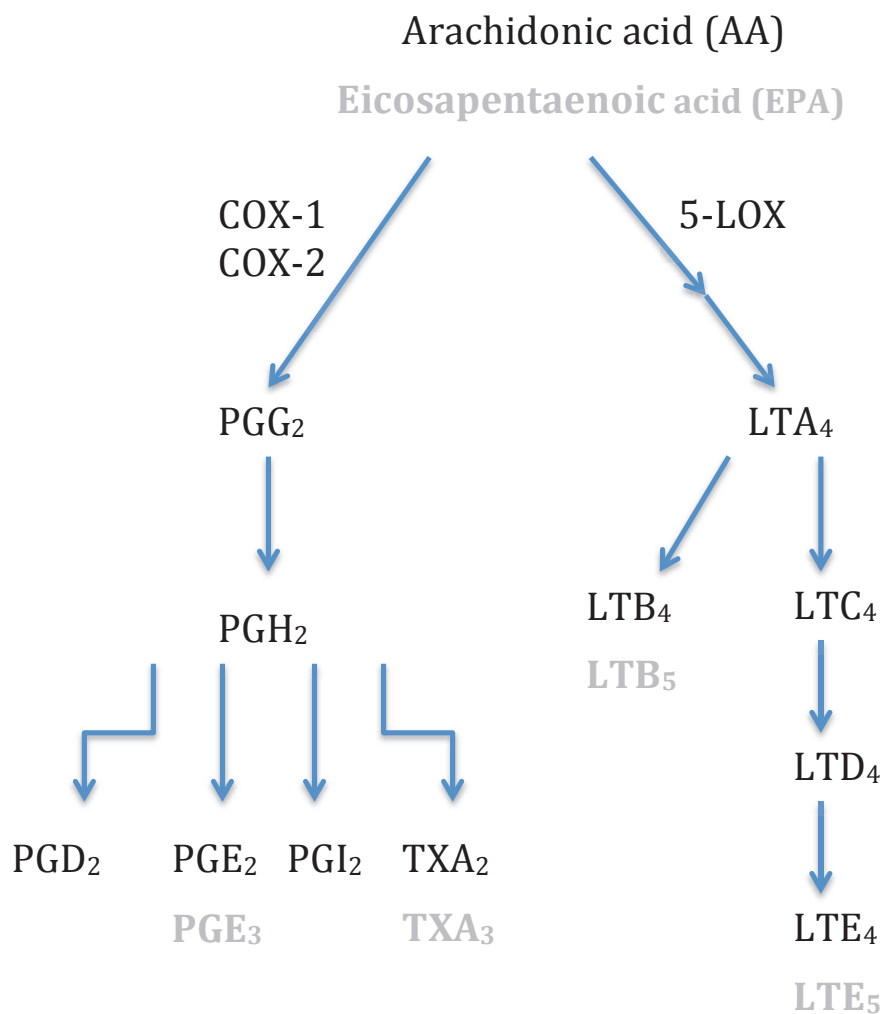
Abbreviations: COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandin; TX, thromboxane; LT, leukotriene.

The anti-inflammatory fatty acid index is the sum of EPA, DHA, dihomo-gamma-linolenic acid (DGLA) "C20:3n-6" divided by AA x 100. An increased AIFAI has been associated with a decrease in PGE_2 (36).

1.7.3 A modified fatty acid

Tetradecylthioacetic acid (TTA) is a modified, syntetic fatty acid with a sulphur atom in the 3-position from the carboxyl end, making the molecule resistant to beta-oxidation (37). It is a pan-peroxisome proliferator-activated receptor (PPAR) agonist and has demonstrated immunomodulatory properties in reducing IL-2 levels and increasing IL-10 (38), as well as having an anti-oxidant potential. The effects from TTA resembles a potent n-3 fatty acid in reducing lipid peroxidation and oxidative stress (39, 40) and in lowering serum triglycerides (41).

1.7.4 Bioactive peptides

Peptides are protein fragments with less than 50 aminoacids. Bioactive peptides have potentially beneficial biological effects when supplied in the diet. They are often encrypted in larger proteins and are released after cleavage by proteolytic enzymes following intestinal digestion (42). These substances may have a variety of biological functions, dependent on their size and structure. Characteristic for the biologically active peptides, is that they contain mostly 2-9, potentially up to 20 amino acids, with a molecular weight of < 10,000 Dalton, preferably < 1,000 Dalton (43). Such peptides may be absorbed unchanged or with only small modifications of structure. They can also act directly in the gastrointestinal tract or via receptor mediated signalling (42). They may enrich the diet, providing "nutraceuticals", with potentially increasing effect with higher amount in the diet.

      Bioactive proteins and peptides in milk, such as lactoferrin, has been the focus for considerable research. Other sources for bioactive peptides are soy beans, meat and fish (42, 43).

1.7.5 Fish peptides

Hydrolysates containing fish peptides from fish carcasses are produced by adding water after mincing and then conducting proteolytic cleavage by adding proteases (44). Fish peptides exist as commercially available products. One specific dietary fish-derived hydrolysate contained large amounts of protein constituents, 75-80%, in forms of peptides or amino acids. Smaller studies using this fish peptide product have reported reparative properties in cell cultures from NSAID induced damage (44), and small intestine protective potential in rodents and humans when administered together with NSAID (45, 46). Fish peptides may have beneficial effects in IBD patients (47).

1.8 Animal models of IBD

There is a number of animal models of IBD used to investigate disease mechanisms, and to evaluate novel treatment interventions in IBD. The main types are *spontaneous colitis models*, *inducible colitis models*, *genetically modified models* and *adoptive transfer models* (48). The ideal IBD animal model should present disease features identical to human IBD: It should have the same ethiology (be induced by the same pathogenetic factors), involve identical pathophysiological patterns, and present the same clinical, histological and immunological characteristics. In addition, it should respond to the same treatments as the human disease (49).

Main advantages of animal models are that trials can be conducted in very controllable environmental conditions, on genetically identical animals, and the trial logistics are simpler than in human experiments. Although animal models do not fully reflect the human disease, they have provided important insight regarding complex pathogenetic mechanisms in IBD, including Th function (Th 1, Th 2, Th 17), regulatory cell functions, TLRs and bacteria/epithelium interactions (50).

1.9 Specific theoretical considerations regarding some aspects of the projects

In this project, several advanced mechanisms and concepts are involved. In the following section a closer description of the theoretical background is given.

1.9.1 Transcription factors as regulators of inflammation

Nuclear factor kappa B (NFκB)

NFκB transcription factors comprise a family of dimeric proteins present in cytoplasma of mammalian cells. Most NFκB molecules include the p65 (Rel A) subunit. Under unstimulated conditions, they are bound to inhibitory proteins called inhibitory (I)κB and are inactive. In classic activation of NFκB, activation of TLRs triggers a signalling cascade which results in proteolytic degradation of the IκB. This allows NFκB to translocate to the nucleus where it binds to DNA promoter regions, and regulate transcription of a variety of "inflammatory" genes such as genes encoding IL-1, TNF-α, IL-6, and IL-23, chemokines, cell adhesion molecules, inducible NO synthase (iNOS), COX-2, as well as anti-apoptotic genes and cell cycle genes (20, 51) (Figure 4). TNF-α may potentiate NFκB activation through positive feedback (51). Levels of NFκB p65 are elevated in nuclear extracts from mucosa in both CD and UC patients as compared with healthy subjects (52).

Figure 4. *NFκB mechanism of action.*
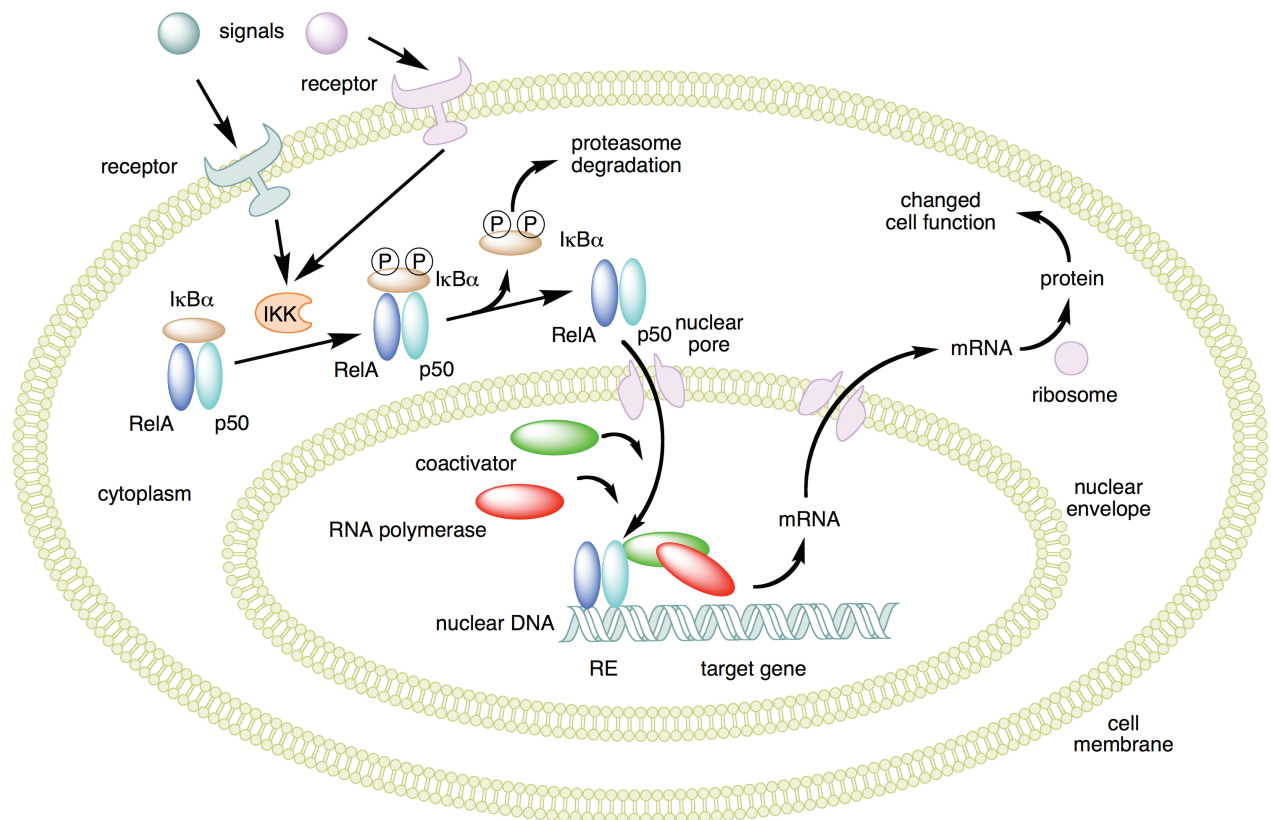
Reprinted from "NFκB", in *Wikipedia*, retrieved November 30, 2012 from
http://en.wikipedia.org/wiki/File:NFKB_mechanism_of_action.png

Peroxisome proliferator-activated receptor γ (PPAR-γ)

PPARs are proteins that are part of the nuclear receptor family, and consist of three
subtypes: PPAR-α, PPAR-β/δ and PPAR-γ. PPAR-γ is expressed in epithelial cells, in
macrophages and lymphocytes in the colon (53), and reportedly plays a role in
intestinal inflammation (54). PPAR-γ is located in the cell nucleus, and is activated by
natural or synthetic ligands. It then forms a heterodimer with the retinoid X receptor

before binding to the peroxisome proliferator response elements gene promoter, and regulates the expression of pro-inflammatory mediators (55). The PPAR-γ coactivator 1 (PPARGC-1α) aquires transcriptional activity after binding ("docking") to PPAR-γ, and may then regulate genes related to energy metabolism (56).

When activated, PPAR-γ downregulate the inflammatory response, interferes with inflammatory signal pathways such as the transcription factor NFκB system, and reduces the production of proinflammatory mediators like cytokines, chemokines and adhesion molecules, as well as proliferation factors of inflammatory cells (53, 57).

Likewise, PPAR-α, with a high expression in sites like the vascular walls and the liver, also seems to play a role in the inflammatory response through antagonizing NFκB (58).

In IBD both nuclear factor kappa B (NFκB) and peroxisome proliferator-activated receptor γ (PPAR-γ) play important roles in the regulation of the pathophysiological processes (6, 53).

1.9.2 Sirtuins

Sirtuins (SIRT), are proteins that modulate pathways related to stress response and metabolism, and are also considered to be cellular redox sensors. In mammals there are seven sirtuins, SIRT 1-7. SIRT1 is a 100 kDa protein located in the cell nucleus and provides prolonged lifespan in model organisms. SIRT1 may act as an inhibitor of NFκB activity, and decreased levels of SIRT1 gene expression were associated with increased NFκB activation and colitis in mice (59).

SIRT4 is a smaller, mitochondrial protein involved in energy production during stress and nutrient adaptation. The sirtuins have two characteristic enzymatic features: 1) Protein deacetylase activity, which is Nicotinamide adenine dinucleotide NAD^+ dependent, and 2) ADP-ribosyltransferase activity. They target certain acetylated proteins substrates (59, 60).

1.9.3 Protein markers of oxidative stress - Advanced glycation end products

Excessive ROS activity leads to oxidation of proteins. Protein carbonyl derivates are generated by oxidative cleavage, through introduction of a double carbon bonding to

oxygen, C=O, into proteins, by lipidperoxidation and formation of malondialdehyde (MDA). They are advantageous in the laboratory as oxidative biomarkers due to their relative stability. Glutamic and aminoadipic semialdehydes (GSA and AASA) are generated after direct oxidation of amino acid side chains in arginyl or prolyl residues (GSA) or lysyl residue (AASA) (61).

A particular branch of protein carbonyls are advanced glycation end (AGE) products. Sugars may react with protein amino groups non-enzymatically to form so called Sciff bases, thereafter early glycation products (Amadori products). Through several modifying reactions they generate AGE products (62). Carboxymethyl-lysine (CML) is a marker of both glycoxidation and lipoxidation while carboxyethyl-lysine (CEL) is a glycoxidation product (62).

In UC, increasing urinary levels of pentosidine, an AGE product, correlates with disease activity (63).

AGE products have been linked to inflammatory mechanisms as the interaction with their receptor, RAGE, may induce activation of NFκB, and consequently increase expression of pro-inflammatory mediators (64).

1.9.4 KC/GRO – CXCL1

The chemokine CXCL1, also called Growth-regulated oncogene (GRO)-α in humans, is expressed by macrophages, neutrophils and epithelial cells. It binds to the receptor CXCR2 and attracts neutrophilic granulocytes (65). Both CXCL1 and CXCR2 were upregulated in active IBD patients (66). In rodents, Keratinocyte chemoattractant (KC) is regarded homologous to human GRO-α/CXCL1 (67).

2. Aims of the study

- Investigate the influence of a regular high intake of Atlantic salmon fillets on disease activity, inflammatory markers and fatty acid profile in blood and colon samples in patients with active UC.

- Investigate the anti-inflammatory and anti-oxidant effects of dietary fish peptides as compared with (a) fish oil and (b) a combined fish oil − fish peptides diet in a DSS induced rat colitis model.

- Evaluate the anti-inflammatory and anti-oxidant effects of dietary krill oil in DSS induced colitis in rats as compared with control rats that were both exposed and not exposed to DSS.

- Evaluate the anti-inflammatory and anti-oxidant effects of tetradecylthioacetic acid in DSS induced colitis in Wistar rats as compared with control rats that were both exposed and not exposed to DSS.

3. Subjects/animals and methods

3.1 Ulcerative colitis study (Paper I)

Subjects

Twenty-three patients were included in the study, of which six patients withdrew due to loss of motivation to follow the prescribed diet. Five more patients were excluded due to fecal calprotectin levels < 50 mg/kg.

After four weeks wash out with dietary restrictions to minimize biological n-3 effects from the background diet, eight weeks of salmon intake of 200 grams three times weekly followed.

Study diet

The Atlantic salmon used in the study was produced at EWOS innovation AS, Dirdal, Norway. The salmon feed contained vegetabile oils and marine oils in a 1:1 ratio. Salmon fillet dinner portions were cooked and vacuum packed at the canteen at Haukeland University Hospital, ready for distribution to study participants.

Fatty acid analysis of total lipids in plasma and colon

We used a direct methylation method, and the same method for both plasma and colon FA analyses, modified from Shirai (68).

First, 20 µl of plasma or tissue from four rectal biopsies (weighing 2-9 mg) were added into a vial. Then 0.4 ml 2% H_2SO_4 in methanol (v/v) (derivatization solution) and 0.4 ml toluene containing 5 µg C21:0 (internal standard solution) were added. The samples were heated for 1 hr at $90^{\circ}C$ (69), cooled to room temperature and 0.3 ml water was added into the samples. Also 120 µl 1.5 M K_2CO_3 in water (neutralization solution) was added. After centrifuging, about 200 µl of the toluene upper layer containing fatty acid methyl esters was transferred into autosampler vials and stored at $-20^{0}C$ before analyzed by gas-liquid chromatography.

Gas chromography of fatty acids

Gas chromatography was performed with GC 8000 TOP (Finnigan, USA), equipped with a programmable temperature vaporization injector (PTV), flame-ionization detector (FID), AS 800 autosampler and with a fused silica capillary column coated with dimethylpolysiloxane stationary phase, DB1-ms (60m x 0.25mm x 0.25um) (J & Scientific, USA).

Identification of fatty acids

The GC signal was acquired and evaluated with Chromeleon software (Dionex, ver. 6.80, USA). The baselines of chromatograms of each sample were corrected and adjusted in order to identify and quantify the fatty acids. Peaks were identified by means of known fatty acid standards. Detailed identification by means of mass spectra, obtained by GC/MS analysis (GCQ, Finnigan, USA) on the same column was carried out before the study.

Diet questionnaire

A validated FFQ semi-quantitative questionnaire designed to capture dietary habits and intake of dietary supplements during the last year was filled in at the start and at the end of the study (70). It includes questions about intake of 255 food items, including 10 questions regarding cold cuts and spreads made of fish/shellfish, 16 questions about fish/shellfish eaten for dinner, and four questions regarding cod liver oil/cod liver oil capsules/fish oil capsules.

Disease activity

Simple Clinical Disease Activity Index

The SCCAI is developed to evaluate disease activity in patients with UC (71). It is based on symptoms and signs and contains six items: stool frequency at day and night-time, urgency before defecation, the amount of blood in the stools, general well-being, and presence of extra-intestinal manifestations such as: arthritis, pyoderma gangrenosum, erythema nodosum and uveitis. Each item is given a score. Maximum total score sum is 19, and a score of ≥ 4 indicates active colitis (Figure 5).

Figure 5. The SCCAI.

Walmsley ENKEL AKTIVITETSINDEKS ULCERØS COLITT

Pasient   Dato

Antall avføringer dag
- 0; 1-3
- 1; 4-6
- 2; 7-9
- 3; >9

Antall avføringer natt
- 0: 0
- 1: 1-3
- 2: 4-6

Urge
- 0: ingen
- 1: haster
- 2: med én gang
- 3: inkontinens

Makroskopisk blod i avføringen
- 0: ingen
- 1: spor
- 2: av og til mye
- 3: ofte mye

Allmenntilstand
- 0: god
- 1: litt mindre enn god
- 2: uvel
- 3: dårlig
- 4: forferdelig

Ekstraintestinale manifestasjoner (hver teller 1)
- ingen
- artritt
- pyoderma gangrenosum
- erythema nodosum
- uveitt

Sum

Endoscopical examination

Following bowel cleansing with a rectal enema (Klyx®) a limited simoidoscopy was performed and the grade of macroscopical inflammation rated according to a scale from 0-3: Grade 0 - normal mucosa (no inflammation); Grade 1 – mucosal oedema and altered vascular pattern (mild inflammation); Grade 2 – mucosal oedema, no

visible vascular pattern, presence of erosions (moderate inflammation); Grade 3 – ulcerations and spontaneous mucosal bleeding (severe inflammation) (72).

Histological investigations

During the sigmoidoscopy procedure, six biopsies were taken approximately 8 cm from the anus, at the point of most visible inflammation. Four biopsies were cooled on ice before swiftly transferred to -70° C for later FA analyses. The remaining two biopsies were placed in formaline, embedded in paraffin and Hematoxylin-Eosine (H-E) stained before a section from both biopsies were evaluated by a senior pathologist unaware of the protocol. The degree of inflammation was then rated as follows: Grade 0 - normal mucosa; Grade 1A – chronic lamina propria inflammatory infiltration with no effect on the mucosal lining or crypts, and no or mild architectural disorder; Grade 1B - chronic inflammatory infiltration of the lamina propria not excluding the mucosal lining with no or mild architectural disorder; Grade 2 - mild crypt injury with acute inflammatory cell infiltration; Grade 3 - extensive crypt injury with crypt abscesses and ulcerations.

Grade 0 reflects clinically inactive disease, grade 1 mild disease, grade 2 moderate disease and grade 3 indicates severe disease (72).

Inflammatory markers

An immunoassay based 21-plex kit from LINCO® (Millipore Corporation, Billerica, MA 01821, USA) was used on a Bio-plex 200 suspension array system from Bio-Rad® (Bio-Rad Life Science Research Group, Hercules, CA 94547, USA) to measure plasma cytokines. Further processing was performed by a Bio-plex manager 4.1 software supplied from Bio-Rad®. The 21-plex kit included IL-1β, IL-2, IL-6, IL-10 and TNF-α.

High-sensitivity CRP was analysed at the Stavanger University Hospital´s routine laboratory. Plasma levels of homocysteine and malondialdehyde (MDA) were analysed as markers of oxidative damage. Levels of fecal calprotectin were analysed by the PhiCal Test® (NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany), an ELISA based method.

Routine laboratory tests

After an overnight fast, haemoglobin, leukocytes, platelets, creatinine, alanine aminotransferase (ALAT), alkaline phosphatase (ALP), albumin, and electrolytes were analysed at the Stavanger University Hospital´s routine laboratory.

3.2 Animal studies (Papers II-IV)

Animals

The animal experiments were carried out at Vivariet, Haukeland University Hospital, Bergen. Male Wistar rats, 12 weeks old (Mollegaard and Blomholtgaard, Denmark) with a mean weight of approximately 360 g at arrival, were housed in Makrolon III cages in an open system, with five animals in each cage. They were kept under standard laboratory conditions with temperature 22 ± 1 °C, dark/light cycles of 12/12 h, relative humidity 55 ± 5 % and 20 air changes per hour and had free access to food and water.

Dietary intervention

The animals were divided into groups of 10, and after seven days of acclimatization, they started a dietary intervention which lasted for four weeks. To develop colitis, the animals were exposed to DSS the last week.

Three different studies were scheduled, in which animals received diets containing fish oil (5%), fish peptides (3.5%) or combined fish oil + fish peptides (Paper II), krill oil (5%) (Paper III), or TTA (0.4%) (Paper IV) respectively. All animals had free access to the feed on day 1-29. To minimize the total number of animals, the three experiments with identical methodology and conditions were performed simultaneously, and the control groups 1 (standard diet) and 2 (standard diet + DSS) were identical (Figure 6).

Figur 6. Design of dietary rat experiment.
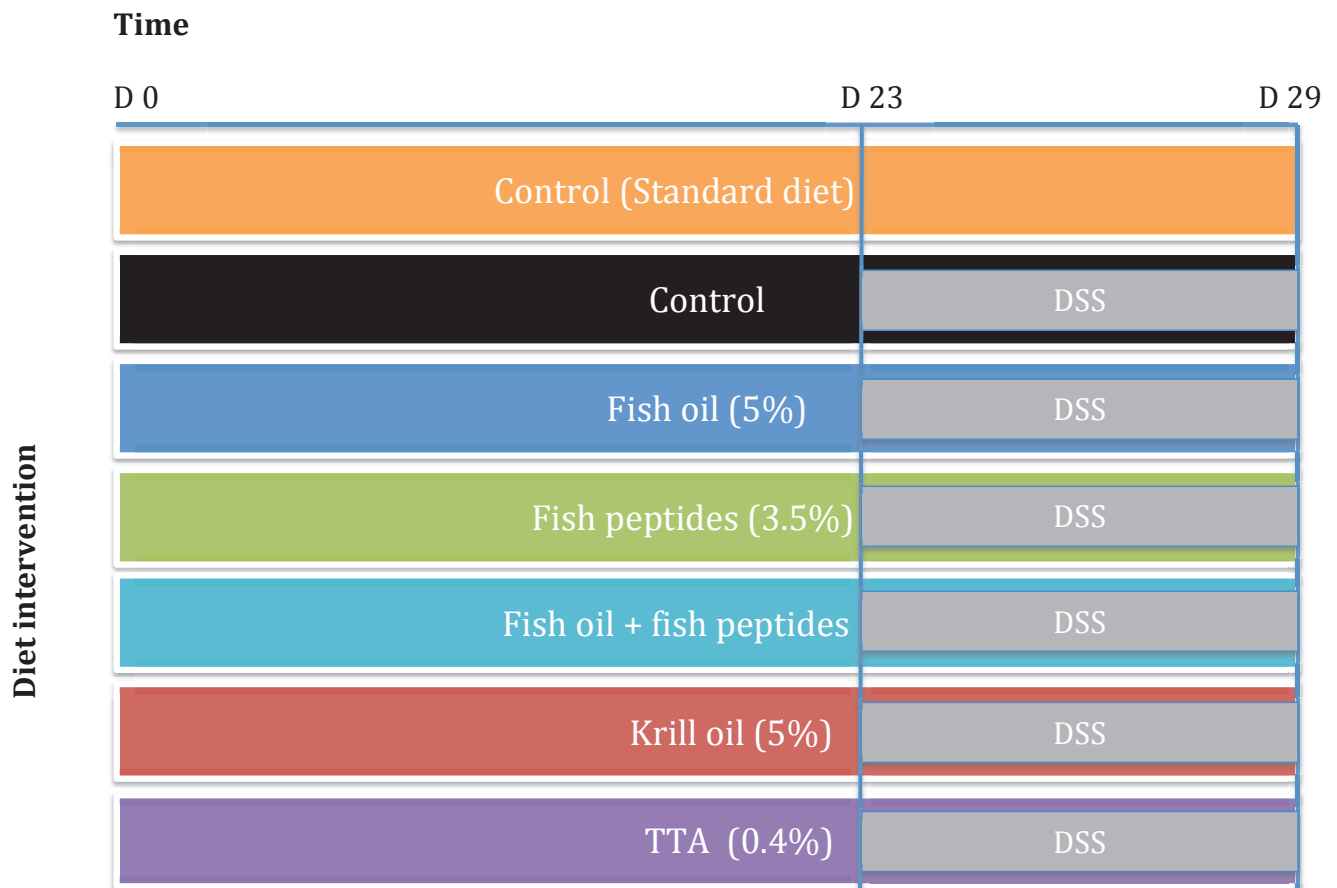
The diets contained (w/w) 5% lard (except the fish oil diets) (generous gift of Ten Kate Vetten BV, Musselkanaal, Netherlands), soy oil (2%) (Dyets Inc., Bethlehem, PA, USA), and 20% casein (except the fish peptides diets) (Sigma-Aldrich, St. Louis, MO, USA). The other constituents of the diets were cornstarch, dyetrose, sucrose, fiber, AIN-93G mineral mix, AIN-93 vitamin mix, L-Cysteine, Choline bitartrate (Dyets Inc.), and tert-butyl-hydroquinone (Sigma- Aldrich). The prescribed diets are shown in Table 1.

Table 1. Intervention diets in animal studies (Papers II-IV), (g/kg).

Ingredients	Control	FO	FP	FO + FP	KO	TTA
KO					50	
FO		50		50		
FP			35	35		
TTA						4
Lard	50		50		50	50
Soy oil	20	20	20	20	19	20
Casein	200	200	165	165	190	200
Cornstarch	397	397	397	397	378	397
Dyetrose	132	133	132	132	126	132
Sucrose	100	100	100	100	95	100
Fiber	50	50	50	50	48	50
AIN-93G mineral mix	35	35	35	35	33	35
AIN-93 vitamin mix	10	10	10	10	10	10
L-Cysteine	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.4	2.5
tert-Butyl-hydroquinone	0.014	0.014	0.014	0.014	0.013	0.014

Colitis induction

The DSS colitis model

Dextran sulfate sodium is a synthetic polysaccharide which provides a chemically induced colitis in rodents when administered in drinking water. The DSS colitis model used in rodents was originally described in hamsters in 1985 (73), later in mice (74) and in rats (75) as an experimental colitis reminiscent of UC. Studies regarding both acute colitis (4-9 days of DSS exposure 2-5%) and chronic colitis (continuous or several cycles of 5-7 days of DSS exposure) have been conducted (48). DSS induced inflammation only affects the colon and has clinical as well as morphological

resemblance to UC (75). The colitis results from a toxic effect from DSS on epithelial cells, leading to barrier defects and increased mucosal permeability. This allows the passage of DSS molecules and, most importantly, the entrance of luminal microorganisms and antigens into the mucosa provoking a vigorous inflammatory response (48). Acute rodent DSS colitis is located to the mucosa and lamina propria and is characterized by mucosal erosions, epithelial regeneration and partly cryptitis, typically mostly defined to the left colon (74, 75).

In our experiment, all animals except for controls had 50 g/L of DSS (MW 44 000, TdB Consultancy AB, Uppsala, Sweden) added to the drinking water for 7 days (days 23-29) in order to induce an acute colitis resembling human UC (Figure 6).

Descriptives

The animal weight, feed intake and the DSS consumption were recorded before and during the DSS exposure, from days 23-29. Two animals died during the DSS week, both had severe colitis.

Animal sacrifice

All the rats were killed on day 30 of the experiment. In an anesthesia chamber, the animals were anesthetized with 2 % Isoflurane (Forane, from Abbot Laboratories Ltd, Illinois, USA). Then thoracotomy, cardiac puncture, and exsanguination were performed.

Disease activity evaluation

Disease Activity Index

DAI is an instrument for assessment of "clinical" disease activity (76) and is based on weightloss, stool concistency and rectal bleeding. Each item is rated from 0-4, and the sum is divided by 3 (77). All rats were placed in individual cages the last day of DSS intake, and DAI (Table 2) assessed after 24 hours.

Table 2. Disease Activity Index (DAI) scoring

DAI score*	Weight loss (%)	Stool concistency	Rectal bleeding
0	0	Normal	Normal
1	1-5		
2	5-10	Loose stools	
3	10-20		
4	>20	Diarrhea	Gross bleeding

*From (77)

Colon length (Papers II-III)

Shortly after animal sacrifice, the colon segment was removed from the colocecal junction to the anal verge, rinsed with phosphate-buffered saline and the length of the unstreched colon was measured.

Histopathological examination

Following length measurement, colon samples from the proximal and distal ends and the middle part were separated, divided longitudinally and placed in 10% paraformaldehyde. After being embedded in paraffin, sections were stained with H–E. One section from each location of the colon was evaluated using an Olympus BX 51 microscope at magnification x 400 by a senior pathologist unaware of the study protocol.

A crypt and inflammatory score were determined for each section. The crypt injury was graded as follows: grade 0, intact crypts; grade 1, loss of the bottom third of crypts; grade 2, loss of the bottom two-thirds of crypts; grade 3, loss of the entire crypt with the surface epithelium remaining intact; grade 4, loss of the entire crypt and surface epithelium. Likewise, the severity of inflammation was also graded: grade 0,

normal; grade 1, focal inflammatory cell infiltration; grade 2, inflammatory cell infiltration, gland dropout, and crypt abscess; and grade 3, mucosal ulcers. Both scores include a measure of involvement: grade 1, 1% to 25%; grade 2, 26% to 50%; grade 3, 51% to 75%; grade 4, 76% to 100%. Finally, the histological combined score (HCS) was calculated as the sum of the crypt and inflammatory scores, and was assessed in all three selected locations of the rat colons (78).

Cytokine analyses in rat colon

Preparations

A 50-mg full wall colon sample cut into minute pieces was placed in cooled lysis buffer consisting of 10 ml premade Tris Lysis Buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton-X-100) (Meso Scale Discovery, Gaithersburg, MD, USA) with 1% bovine serum albumin (Meso Scale Discovery), one Complete Mini, EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany), 100-ml phosphatase inhibitors I and II (Sigma, Saint Louis, Missouri, USA), 100 ml 1 M NaF, and 100 ml Non-Idet P40 (Roche Diagnostics) protected from daylight, and air was finally added to complete the lysis buffer. After placing the sample in the cooled lysis buffer, a VDI 12 homogenizator disruption probe with dispersion tool S12N-5 S (VWR international, West Chester, Pennsylvania, USA) was applied for 15–90 s and repeated once if the sample was considered not fully homogenized. The homogenisate was then centrifuged for 10 min at 14,000 rpm (4°C) using a Kubota 2800 with an RS-240 rotor (Kubota, Tokyo, Japan). This step was repeated with the supernatant only. The final supernatant was collected and stored at -70°C until further analysis.

Tissue cytokine analyses

Custom-made rat cytokine 4-plex kits including IL-1β, IL-5, interferon (IFN)-γ, and chemokine (C-X-C motif) ligand 1 (CXCL1 also known as KC/GRO), and single-plex kits with TNF-α (Meso Scale Discovery, Gaithersburg, MD, USA), were used to measure cytokines in the supernatant from the homogenisate. The analyses were

performed according to the manufacturer's recommendations. The assays from MSD (Meso Scale Discovery) are immunoassays based on electrochemiluminiscence for detection. This provides a large detection range, high sensitivity and a low rate of interference from other compounds.

Cytokine levels were reported as µg/kg (wet weight) in Paper II, as concentration pg/ml in Paper III and as µg/g (wet weight) in Paper IV.

Ultrasound examination

In the TTA study (Paper IV), rats from the following groups of the study population were randomly selected for ultrasound examination: control group 1 (n = 7), DSS group 2 (n = 7), TTA + DSS group 3 (n = 7). The rats were anesthetized by inhalation of 2 % Isoflurane. The distal colon wall thickness was then measured using a Vivid 7 ultrasound machine (GE Vingmed Ultrasound A/S, Horten, Norway) with 14 MHz linear ultrasound probe (12L, GE Health- care, Milwaukee, USA). The urinary bladder was used as an acoustic window, and also provided a standardized level of distal colon measurements. The mean of three values per animal was reported. The investigator was unaware of which group the rats belonged to. The examination cannot be considered blinded however, as the health condition for some of the colitic rats were evidently poor. In the DSS group 2, the measurements from one animal were not adequate and results were thus omitted.

Prostaglandin analysis

Preparations

All eicosanoids were purchased from CalBiochem, La Jolla, CA, USA. Stock solutions of deuterated standards (CalBiochem) were kept in 50/50 EtOH/H_2O (Kemetyl Norge AS, Fredrikstad, Norway) at -80°C and diluted in ultrapure distilled water immediately before sample preparation to avoid any oxidation. Frozen colon tissue samples (12–35 mg) were added to a solution of ice-cold 0.1% butylated hydroxytoluene (BHT) (Sigma-Aldrich, St. Louis, MO, USA) in ultrapure distilled water (1:100 (w/v)) and homogenized by a Virtishear homogenizer at 25,000 rpm for 2 x 30 s. Then 50 µL

internal standard solution (PGE2d4 150 pg/µL; PGD2d4 250 pg/µL) was added to 1200 µL homogenisate. The samples were sonicated for 60 s and centrifuged at 12,000 rpm for 5 min at 5°C. Later, 1,125 µL supernatant were evaporated to dryness under a stream of nitrogen at room temperature. Then the samples were reconstituted in 50 µL of 50/50 EtOH/H_2O (v/v) before LC/MS/MS analysis.

LC/MS/MS

The LC-system was an Agilent (Waldbronn, Germany) 1200 Series with binary pump, variable volume injector, and a thermostated autosampler. HPLC separation was performed using a gradient solvent mixture at 20°C. Mobile phase A was made of 10 mM ammonium acetate (Merck, Darmstadt, Germany) in water with pH adjusted to 8.5 with ammonia solution 25% (Merck) Mobile phase B was methanol (Fisher Scientific, Leicestershire, UK). The gradients were B 0.1 min 0-40%, B 18 min 40–60%, B 1 min 60–100%, B 6 min 100%, B 0.1 min 100-0% and B 5 min 0%. Flow was 0.25 mL/min and a Phenomenex Luna Phenyl-Hexyl column (3 µm, 150 x 2 mm) was used. A 10 mL sample was injected. The mass spectrometer used was an Agilent 6410 Triple Quad LC/MS (USA) equipped with an electrospray source. Source parameters: gas temp 350°C, gas flow 12 L/min, nebulizer 40 psi, capillary 4000 V. Multiple reaction monitoring (MRM) for data acquisition and negative ion detection was used. MassHunter software (Agilent) was used for HPLC system control, data acquisition, and data processing. The prostaglandins analyzed were expressed as µg/kg (wet tissue) in Paper II and as pg/mg (wet tissue) in Paper III.

Fatty acid analysis of colon total phospholipids

In order to avoid contamination from surrounding neutral lipids, extraction of total colon lipids was followed by TLC separation of lipid fractions. We then used the PL fraction only for colon FA analyses.

Extraction of colon lipids

Colon tissue samples of 10-25 mg were homogenized in 2.5 ml of methanol for 30 sec (Ultra-Turrax T25 basic homogenizer, IKA-Werke, at speed 11000 rpm/min) in a glass

test-tube with a teflon-lined seal cap. Then 1.25 ml of chloroform was added and extracted overnight at 4°C with an occasional shaking. After centrifugation, the supernatant was transferred into a new glass test-tube and the sediment was re-extracted for 1 hour in solvents as above. To the collected supernatants, chloroform and water was added to the ratio methanol:chloroform:water (1:1:0.9) (79). After phase separation, lower chloroform phase was used for lipid analysis.

TLC separation of lipid fractions

Aliquots of chloroform extracts were applied on TLC plates 10 x 20 cm Silica gel 60 (layer thickness 250 μm, particle size of 10 - 12 μm) (Merck, Germany) in a line 0.5 x 2 cm. The plates were developed in a tightly closed depeloping chamber in hexane/diethyl ether (50:50, v/v). The spots were visualized by ultraviolet-light after spraying with Rhodamine 6G (0.01% in methanol, w/v) and identified by means of co-eluting lipid standars (Sigma-Aldrich, USA). The silica gel with the spots of total phospholipids were scraped off into glass test-tubes with teflon-lined seal caps (80).

Transesterification of total phospholipids

To the total phospoholipids scraped off with silica gel 1.0 ml of 2% H_2SO_4 in methanol (v/v) and 1.0 ml toluene containing appropriate amount of C21:0 was added and heated for 2 hr at $90^{\circ}C$ (69). At the end of the heating, the samples were cooled off to room temperature, 0.3 ml water and 300 μl 1.5 M K_2CO_3 in water was added. After centrifuging, about 200 μl of the toluene upper layer containing fatty acid methyl esters was transferred into autosampler vials and stored at $-20^{0}C$ before analyzed by GC. GC conditions and method of fatty acids identification were as described earlier in fatty acid analysis of total lipids.

Oxidative stress markers

Glutamic semialdehyde (GSA), carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL), aminoadipic semialdehyde (AASA) and malondialdehyde-lysine (MDAL) concentrations in samples from the proximal and distal colon were homogenated in

0,5 mg protein and measured by gas chromatography/mass spectrometry (GC/MS) as previously described (81).

Samples were delipidated using chloroform/methanol (2:1, v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM NaBH$_4$ (final concentration) in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. Proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and subsequent centrifugation. The following isotopically labeled internal standards were then added: [^{2}H$_8$]lysine (d$_8$-Lys, CDN Isotopes); [^{2}H$_4$]CML (d$_4$-CML), [^{2}H$_4$]CEL (d$_4$-CEL), and [^{2}H$_8$]MDAL (d$_8$-MDAL) prepared as described (82, 83) and [^{2}H$_5$]5- hydroxy-2-aminovaleric acid (for GSA) and [^{2}H$_4$]6-hydroxy- 2-aminocaproic acid (for AASA) prepared as described (40, 84).

The samples were hydrolyzed at 155 °C for 30 min in 1 ml of 6 HCl, and then dried in vacuo. The N,O-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described (40).

GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30-m HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Agilent, Barcelona, Spain). The injection port was maintained at 275 °C; the temperature program was 5 min at 110 °C, then 2 °C/min to 150 °C, then 5 °C/min to 240 °C, then 25 °C/min to 300 °C, and finally hold at 300 °C for 5 min. Quantitation was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analytes were detected by selected ion-monitoring GC/MS (SIM-GC/MS). The ions used were: lysine and d$_8$-lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and d$_5$–5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; CML and d$_4$-CML, *m/z* 392 and 396, respectively; CEL and d$_4$-CEL, *m/z* 379 and 383, respectively; 6-hydroxy-2-aminocaproic acid and d$_4$-6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m/z 294 and 298, respectively; and MDAL and d$_8$-MDAL, m/z 474 and 482, respectively. The amounts of products were expressed as the ratio of micromole per mole Lys of GSA, CML, CEL, AASA and MDAL.

Gene expression

Distal colon samples were frozen in liquid nitrogen immediately after dissection and stored at −80°C. Total cellular RNA was purified from 20 mg frozen tissue using a TissueLyser II with 5-mm stainless steel beads, and the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA). RNA was quantitated spectrophotometrically (NanoDrop 1000; NanoDrop Technologies, Boston, MA), and the quality was evaluated by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA). For each sample, 400 ng total RNA was reversely transcribed in 20-μl reactions using Applied Biosystem's High Capacity cDNA Reverse Transcription Kit with RNase inhibitor according to the manufacturer's description. Real-time polymerase chain reaction was performed with custom-made 384-well microfluidic plates (Taq-Man Low Density Arrays (TLDA), Applied Biosystems, Foster City, CA) on the following genes: *Ppargc-1a*, Rn00580241; *Pparg*, Rn00440945; *Nos2*, Rn00561646_m1; *Il-6,* Rn 00561420; *Il-1b*, Rn00580432_m1; *KC-GRO* (*Cxcl1*), Rn00578225_m1; *Ptgs2* (*Cox2*), Rn01483828_m1; *Ptges*, Rn00572047_m1; *Ptgds*, Rn00564605_m1; *Tlr4*, Rn00569848_m1; *sirtuin 1* (*Sirt1*), Rn 01428093_m1; *Sirt4*, Rn01481485_m1. All probes and primers were obtained from Applied Biosystems. Two different control genes were included: *18s* (Kit-FAM-TAMRA (Reference RT-CKFT-18s) from Eurogentec, Belgium, and *Rplp0* (Rn00821065_g1) from Applied Biosystems.

To normalize the absolute quantification according to the reference genes, a second set of PCR reactions was performed for all experimental samples, and the relative abundance values were calculated for the reference genes as well as for the target genes using standard curves derived from Universal Rat Reference RNA, (Agilent Technologies inc., Santa Clara, CA, USA). The software NormFinder was used to evaluate the reference genes, and the relative abundance value of each gene in each sample was normalized against the value of the two reference genes (*18s* and *Rplp0*) (in Paper II) or the most stable reference gene, *Rplp0* (in Papers III and IV) (85).

3.3 Statistical methods

Distribution of data was examined by Shapiro–Wilks test. For normally distributed data parametric statistics were applied and mean with 95% confidence intervals reported. For differences between two or more groups, Student´s t-test for paired (Paper I) and unpaired (Papers III –IV) samples, or One-way analysis of variance (ANOVA) were used, respectively.

For non-normally distributed data, non-parametric statistics were used and median values with ranges reported. For differences between two or more groups, Wilcoxon matched pairs signed-rank test (Paper I), Mann-Whitney rank test (Papers III and IV) or Kruskal-Wallis one-way analysis of variance test (Paper II) were performed, respectively.

Correlation coeffecients were calculated using Spearman rank correlation test (Paper I). Post-hoc tests were performed by Mann–Whitney rank test (Paper II). A p-value < 0.05 was considered statistically significant. Corrections for multiple testing were performed in Papers II and III except for the fatty acid and gene expression analyses.

4. Summary of results

Paper I

The twelve patients that completed the study had a significant decrease of SCCAI after salmon intake (p=0.007), a close to significant drop in CRP (p=0.07) and the concentrations of AA, "C20:4n-6" in rectum tissue specimens, correlated with endoscopic and histopathological score at visit 2. EPA, DHA, total n-3 levels as well as AIFAI were all elevated in both plasma and rectum. However, fecal calprotectin levels and histological score were not altered after salmon diet.

Paper II

There was a significant reduction of HCS after fish peptides diet as compared with fish oil (p=0.03). DAI also decreased, not reaching statistical significance (p=0.07). KC/GRO protein levels in colon was lower after fish peptide intake (p<0.05). Fish oil increased the DAI as compared with DSS alone, and elevated PGE$_3$ levels. In addition, the combined fish oil + fish peptides diet increased the PGE$_3$ levels even more than fish oil alone. A number of beneficial changes occurred after fish peptides diet vs. DSS alone, not reaching statistical significance.

Paper III

We found a retained colon length, and reduced levels of selected protein oxidant variables in rats following krill oil diet, as compared with rats fed standard diet + DSS. The EPA, DHA, n-3/n-6 ratio and the AIFAI were beneficially changed/increased. Moreover, a consistent tendency of improved HCS, cytokine and PGE$_3$ levels, as well as mRNA levels of PPAR-γ and Ppargc-1α occurred, although not significantly.

We also detected significant differences between the control and the control + DSS groups in variables like weight gain, DAI, HCS, colon length, cytokines at both protein and mRNA levels, in accordance with a colitic state after DSS exposure.

Paper IV

Following the TTA diet, cytokines were reduced at protein level for TNF-α, and at mRNA levels for IL-1β and IL-6. The levels of all oxidative markers except AASA decreased versus DSS alone. DAI and HCS were not significantly altered. PPAR-γ expression was significantly upregulated after TTA intake. Transabdominal ultrasound showed a significant increased distal colonic wall thickness in DSS exposed rats versus control rats. In addition, TTA + DSS diet reduced colonic wall thickness as compared with DSS alone.

5. Discussion

The main hypothesis that nutritional products from mostly marine sources have anti-inflammatory effects in UC patients or in an experimental animal IBD model was tested in one human and three animal studies.

We found that a regular salmon intake for eight weeks reduced the SCCAI in patients with UC. We then tested dietary salmon components in experimental rat colitis, and found that a fish peptide diet attenuated DSS colitis as compared with fish oil diet. Furthermore, a combined fish oil and fish peptides diet could beneficially influence prostaglandin metabolism. We revealed that dietary krill oil may diminish DSS induced rodent colitis and reduce protein oxidative damage. We then demonstrated that a modified fatty acid (TTA) seems to have have anti-inflammatory and anti-oxidative effects in DSS induced rat colitis. Finally, we found that high frequency transabdominal ultrasound is able to distinguish between normal and inflamed colon in the DSS model, as seen by increased wall thickness.

5.1 Evaluation of main findings

Paper I. The hypothesis tested in this study was that salmon fillets might have an anti-inflammatory effect in patients with UC. As fish fillets contains substances like fish proteins and peptides in addition to fish oil, we considered this intervention as different from a pure fish oil supplemented diet only. A number of studies in human IBD have been conducted regarding n-3 PUFA supplementation, with inconsistent results, for selected studies, see Table 3. Systematic reviews and metaanalyses (92-95) have not found clear evidence for a beneficial effect. This may be attributed to differences in study designs, different n-3 formulas and doses, as well as different types of placebo substances used (95).

Fish /salmon consumption provide other substances than n-3 PUFAs, such as bioactive peptides (43). We are not aware of other IBD studies involving salmon diet, except for a study in coronary heart disease where intake of salmon diet resulted in lower vascular inflammatory activity (96).

Table 3.

Selected dietary n-3 intervention studies in human IBD

Author/year	Disease	Design	Sample size	Source and dose n-3 fatty acids (EPA+DHA)	Duration	Placebo	Findings	Reference
Lorentz/1989	UC/CD	RXT	39 (10/29)	Max EPA. 11ml/d capsules 3.2 g/d	7 mo (3-1-3)	Olive oil 11ml/d capsules	UC: Clinical disease activity ↓ Endoskopy score↓ (pooled UC+CD)	86
Aslan/1992	UC	RXT	11	Max EPA 15 capsules/d 4.2 g/d	8 mo (3-2-3)	Oleic acid, palmitic acid, linoleic acid 15 capsules/d	Disease acitivity index ↓	87
Stenson/1992	UC	RXT	24	Max EPA 18 capsules/d 5.4 g/d	9 mo (4-1-4)	Vegetable oil 18 capsules/d	LTB_4 ↓ Histology index ↓ Weight gain ↑	88
Hawthorne/1992	UC	RCT	96	HiEPA. 20 ml/d fluid 6.0 g/d	12 mo	Olive oil 20 ml/d	LTB_4 ↓ LTB_5 ↑ Corticosteroid use↓ (Incl. in relapse)	89
Belluzzi/1996	CD	RCT	78	Fish oil 9 enteric coated capsulas/d 2.7 g/d	12 mo	Miglyol 9 capsulas/d	Relapse rate ↓	90
Feagan/2008 EPIC-1, EPIC-2	CD	RCT	383/379	Epanova 4 gelatin capsulas/d 2.6-3.4 g/d	12 mo	MCT oil 4 capsulas/d	S-triglycerides ↓	91

Abbreviations: UC, ulcerative colitis; CD, Crohn's disease; RCT, randomized controlled trial; RXT, randomized controlled crossover trial

Our study diet probably contained sufficient daily amounts (at least 3.4 grams) of n-3 PUFAs to expect anti-inflammatory effects, as amounts between 2.7-5.6 grams have proved effective in previous trials (97).

At inclusion, we regarded a SCCAI of ≥ 4 as indicative of active colitis. However, during the wash-out period we observed a reduction in SCCAI from a median of 5 (inclusion) to 3 (start of intervention). Following salmon intake, the SCCAI was further reduced to 1.5. The drop in SCCAI from 5 to 3 during the wash-out period could possibly be attributed to a "regression towards the mean" effect, and/or a placebo effect.

SCCAI levels to determine remission or disease activity improvement have been defined differently in previous studies (98, 99). One report, based on patient defined cut-off levels, suggested a SCCAI of < 2,5 as remission and a drop of > 1,5 as improvement (99). It could thus be discussed whether the SCCAI reduction observed in our study of 1.5 is clinically significant.

The AIFAI is not a validated index, but the increase that occurred after salmon intake may reflect a beneficial change of the fatty acid profile, and has been associated with decreased levels of pro-inflammatory eicosanoids like PGE_2 (36). The tendency to lower CRP following salmon diet supports such anti-inflammatory effect.

High levels of polyunsaturated marine fatty acids predisposes for lipid peroxidation reactions (100), but MDA, a marker of lipid oxidation, rather tended to decrease in our study.

The background daily intake of n-3 PUFAs of 2.5 grams as indicated by the FFQ analyses, could have influenced the results. Despite a four-week wash out period, a carry over effect could possibly have "saturated" the tissues to some extent. Even ten weeks have been claimed to be a too short interval to allow complete wash out (101).

A limited anti-inflammatory effect could be due to different genetic variants of G-protein coupled receptor 120 (GPR 120) that mediate n-3 fatty acid anti-inflammatory effects. In GPR 120 knock-out mice, n-3 PUFAs were not effective

(102). Thus, the expression of GPCR may determine responders and non-responders towards n-3 mediated effects (102).

The small study population and lack of controls complicate firm conclusions to be drawn from this pilot study.

Paper II. In this study we found that a diet containing 3.5 % salmon derived fish peptides reduced DSS induced inflammation compared with fish oil diet, as assessed by HCS. Also, KC/GRO protein levels were significantly lowered. DAI was reduced although not reaching statistical significance. Several other variables had a "beneficial shift" without being statistical significant.

Fish is an important dietary source of bioactive peptides that may have diverse biological effects (43). Small peptides may be absorbed unaltered into the circulation to exert physiological effects. Most bioactive peptides are not absorbed and are believed to act directly in the gut via receptors (42). Other bioactive peptides are encrypted in proteins and must be released by digestive or microbial proteolytic enzymes to gain biological activity (42).

Some evidence point to reparative or anti-oxidant properties from fish derived hydrosylates (44-46). The effect of fish peptides in IBD models has not been much investigated. One brief report suggests symptom reduction and lowered intestinal permeability following intake of fish hydrolysate (47). We used fish peptides of optimal small sizes and weights regarding bioactivity and lipid lowering (103).

The results suggest a beneficial effect of fish peptides, most evident when compared with fish oil, and not with DSS alone. Several previous studies in rodent IBD models have shown attenuated intestinal inflammation following fish oil supplementation or combinations with other substances, for selected studies see Table 4.

These results contrast our study, where fish oil diet did not have beneficial effect on histology or cytokine measures. In fact, DAI increase suggested an unfavourable effect, possibly caused by increased lipid peroxidation, as no vitamin E had been added to the fish oil diets. However, we did not disclose increased protein oxidative damage.

Table 4.

Selected studies of fish oil supplemented diets in rat experimental colitis

Author/Year	No animals	Experimental model	Dietary intervention	Duration	Main findings	Reference
Vilaseca /1990	70, groups of 6-10	TNBS	Cod liver oil (8%) vs Sunflower oil (8 %) (w/w)	4w, up to 50 days follow-up	Cod liver oil: Colon damage score ↓ Tromboxane B$_2$ ↓ (chronic phase)	104
Yuceyar/1999	60, groups of 10	TNBS	FO (8%) vs sunflower/cotton oil (total 8%) [w/w]	6w, 2w follow-up	FO: Colon damage ↓ LTB$_4$ ↓ MPO ↓	105
Nieto/2002	120, groups of 10	TNBS	FO (1.1%) vs Olive oil (63%) vs pig brain phospholipids (1.1%)	1 or 2w	FO: 1w: Colon damage ↓ MPO↓ PGE$_2$ ↓ Mucosal repair ↑	106
Camuesco/2005	40, groups of 10	DSS 5% (5d)- 2% (10d)	Soybean oil (4%) vs olive oil (4%)vs FO (0.02% + olive oil 3.98%) w/w	4 w, DSS after 2 w,	Olive oil and FO: Colon damage ↓ FO: TNF-α ↓ LTB$_4$ ↓	77
Barros/2012	18, groups of 6	DSS 3% (7d)	Soybean (8%-5%) vs FO (8% - 5%) vs Soybean/FO (4%-2.5% of each)	47 d	Soybean/FO: DAI ↓ MPO ↓Histology ↓ Soybean/FO and FO: IL-10 ↑ DNA damage ↓	107

Abbreviations: TNBS, Trinitrobenzene sulfonic acid; DSS, Dextran sulfate sodium; FO, Fish oil

Increased inflammation in IBD models after fish oil supplementation has been previously reported (108) and it has been speculated whether reduced T reg function could be responsible (109). Also, there could be an "upper tolerable limit" for n-3 PUFAs /DHA in IBD. A combination of soybean oil and fish oil appeared more beneficial than pure fish oil supplementation in rat experimental colitis (107). However, no current data supports that EPA and DHA exert unfavourable immunosuppressive effects leading to higher risk of infections (110).

The salmon diet used in Paper I reduced SCCAI. Salmon contains both fish oil and fish peptides. In the DSS model, fish oil alone increased DAI, but the combined fish oil and fish peptides diet appeared to dampen the negative effect from fish oil alone. The reason for this is unclear, but could reflect an anti-oxidant effect by fish peptides (43). Salmon also contains the natural anti-oxidant astaxanthin (111). In addition, a synergy effect on prostaglandin synthesis, favouring production of less inflammatory eicosanoids such as PGE_3 may contribute.

Paper III. In this study we investigated the anti-inflammatory and anti-oxidant effects of krill oil in the DSS model. This marine oil has large amounts of its EPA and DHA bound to phospholipids in contrast to fish oil, where these PUFAs are largely bound to triglycerides (34). Krill oil contains the carotenoid anti-oxidant astaxanthin (112). In contrast, the fish oil diets were without vitamin E, an anti-oxidant often used in fish oil products to protect against lipid peroxidation.

We found that rats fed krill oil diet had preserved colon length. Colon shortening is typically experienced after DSS induced colitis, and may result from mucosal oedema, increased vagal tone, or muscular contraction (75).

There were no other significant changes in disease activity variables. A number of consistent changes, although each of them not being statistically significant, was evident for DAI, HCS and cytokines at both protein- and mRNA levels. Together with the increased PGE_3 levels, these results suggest a protective effect of krill oil in the DSS-induced colitis. This is in accordance with previous animal IBD studies using fish oil derived n-3 PUFA supplementation (see Table 4), whereas our study is the first trial of krill oil in an IBD model.

The fat content of krill - 1,5% of total weight - is lower than in fat fish such as salmon (5.9%), but the PUFA content and EPA + DHA amount in krill is higher, 29.8% vs 18.3% (34). Krill oil therefore seems to be an adequate source of marine fatty acids. This was also confirmed by fatty acid analyses, showing increased levels of EPA, DHA, DPA, n-3/n-6 ratio as well as AIFAI in rat colon tissue after krill oil intake.

EPA and DHA may work through several mechanisms to dampen inflammation. A classical hypothesis is that EPA competes with AA as substrate for COX and LOX enzymes resulting in production of less inflammatory eicosanoids, such as PGE_3, found to be increased in our study (32). Other possible mechanisms are changes in lipid rafts that constitute important parts of receptor complexes and signalling molecules in membranes of T cells and dendritic cells. Increased amounts of EPA and DHA may alter the lipid organization of such rafts, and downregulate inflammatory responses (35). Also, resolvins and neuroprotectins are molecules with anti-inflammatory properties, originating from EPA and DHA (33, 35). Future studies of anti-inflammatory effects from n-3 fatty acids should investigate the effects of these substances. Finally, activation of nuclear receptors and modulation of gene transcription may be important effects from EPA and DHA: PPAR-γ is higly expressed in the colon, and may be activated upon ligand binding by EPA or DHA. PPAR-γ activation inhibits NFκB, an important pro-inflammatory transcription factor. Consequently, the production of pro-inflammatory substances such as IL-1β, IL-6, TNF-α, COX-2, iNOS and chemokines is downregulated. (57, 113). In another study, PPAR-γ expression was decreased in rectal mucosa in UC patients with active disease (114). In accordance with this, we found that the expression of PPAR-γ, as well as PPARGC-1α, a coactivator related to PPAR-γ, were reduced in the DSS group as compared with controls.

It is not clarified if krill oil´s chemical structure improves its bioavailability. Similar plasma levels of EPA and DHA were observed after a smaller krill oil dose (62.8%) as compared with fish oil dose (115).

Oxidative stress was assessed by markers that have scarcely been tested in IBD models earlier. The protein carbonyl compunds CEL, CML and GSA were reduced

following krill oil diet, indicating reduced protein oxidative damage and less inflammation. Increased oxidative stress in form of elevated levels of ROS occurs in IBD, and may also be a pathophysiological factor (116). Protein carbonyl levels in mucosal biopsies from CD and UC patients were increased in a previous study (117). In IBD patients, pentosidine, another AGE product was increased in urine and intestinal tissue and reflected disease activity (63). This suggests that AGE products may influence inflammation in IBD, and CEL and CML measurements were thus relevant in our rat model of IBD.

Paper IV. In our fourth study, we explored the anti-inflammatory and anti-oxidant effects of the modified fatty acid TTA in the DSS colitis rat model.

Non-invasive, transabdominal ultrasound examination with a relatively high frequency (14 MHz) was able to differentiate between inflamed and normal rat colon based on measurement of wall thickness. In this exploratory study, we also observed a difference between rats fed TTA versus rats fed DSS alone. Although this method is not validated, another pilot study also showed that ultrasound may differentiate between colitic and non-colitic rats following DSS as well as TNBS exposure (118). Biomicroscope ultrasound imaging in rat colitis on ex vivo samples using a 50 MHz scanner has shown promising results, but used a more cumbersome set up (119). Future ultra-high frequency transabdominal studies may improve resolution and accuracy of this method.

We found that TTA reduced TNF-α protein levels, and IL-1β and IL-6 mRNA levels, indicating an anti-inflammatory effect.

We also observed a reduction in protein oxidative damage markers as seen by reduced GSA, CEL, CML and MDAL, supporting the anti-oxidative potential from TTA. These markers were not significantly elevated in the DSS vs control group, but showed a consistent tendency to increased levels. Their role in human IBD and IBD animal models should be further investigated in future studies.

As in the krill study, *Sirt1* gene expression was elevated following intervention, although not significantly. In addition, *Sirt4* mRNA levels were higher following TTA intake. Both *Sirt1* and *Sirt4* expression levels decreased significantly in DSS vs

controls, suggesting that elevated levels mediate beneficial effects. In fact, SIRT1 may act as an inhibitor of NFκB activity, and decreased levels of *Sirt1* gene expression were associated with increased NFκB activation and colitis in mice (59).

We observed an increase in *Pparg,* but not of *Ppargc1a* mRNA levels, following TTA diet. PPAR-γ inhibits NFκB activation, which in turn may downregulate the production of pro-inflammatory cytokines and chemokines (53). In accordance with this, a consistent reduction of all measured cytokines occurred, at protein and/or at mRNA level, although not all significantly. The role of PPARGC1A is less understood in inflammatory conditions. Increased expression of both *Pparg* and *Ppargc1a* were associated with attenuated colitis and later onset of DSS colitis in pigs that were fed conjugated linoleic acid (120).

5.2 Evaluation of methods

Ulcerative colitis study

The original design of this study included 25 patients with active UC that received salmon diet and 25 patients with active UC, serving as controls. This number was not achievable for the following reasons:

1) - Recruitment of patients with active colitis that could manage 12 weeks study period without changing medication was more difficult than expected. Moderate to severly affected patients were not considered suitable as they needed treatment with corticosteroids.

2) - The salmon fillets were of limited durability, allowing an inclusion period of 6 months only. It was decided not to produce another batch of salmon for this study to prolong inclusion. As a result, the patient population could not be increased.

3) - Verification of active colitis was based on an objective marker – f-calprotectin, thus ruling out 5 patients with too low levels.

4) - Drop out; Patients had problems keeping to the treatment protocol for the eight weeks with high salmon intake.

The most important factors that could have improved the study would have been a prolonged inclusion period, which would probably have increased the sample size and provided a control group. This would have strenghtened the statistical power of the analyses.

Endoscopical evaluation: A limited sigmoidoscopy is better tolerated by the patients than coloscopy, and was considered adequate because rectal inflammation was expected in all cases of colitis. A complete colon examination by coloskopy could have revealed inflammation in other colon segments as well, and the complete disease distribution was thus not assessed.

Animal studies

The DSS colitis model

This chemically induced colitis model is a widely used UC model, with a predictable onset and consistent location of colitis. Acute DSS colitis is shown to increase the gene expression of *IL-6*, *CXCL1* (also called KC in rodents or GRO-α in humans) and Prostaglandin endoperoxide synthase 2 (*Ptgs2*)/*cox2*, as well as giving rise to increased levels of IL-1β, TNF-α and KC in colon specimens. This is analogue to human IBD, strengthening the relevance of these variables used in our experiments (48, 121).

We used 5% DSS with a MW of 44 kDa, known to induce a more severe colitis in the middle and distal colon, as opposed to induction with 5 kDa DSS or 2-3% DSS, which result in a more patchy and mild inflammation (48). If the colitis was less pronounced, weaker effects from the diets could have been easier to reveal.

This acute injury model has limitations. Although with several similarities, it is clearly different from the human IBD, being an exogenously triggered inflammation, opposed to the more complex human disease. A genetically modified UC model, such as the IL-2 knockout or T cell receptor mutant model, would possibly have been a more optimal model to investigate (122).

Study design

To minimize the total number of animals, 70 rats were included in a single large experiment, providing seven groups of 10 rats in each: (a) Control, (b) control + DSS , and five dietary intervention groups : (c) fish oil, (d) fish peptides, (e) combined fish oil and fish peptides, (f) krill oil and (g) TTA that were exposed to DSS the last week (Figure 6). If three separate experiments had been performed, 40 more animals would have been needed for separate control and control+DSS groups in each trial.

On the other hand, for each dietary group with DSS colitis, an additional control group without DSS could have been added. In the krill oil study, relevant groups would then be: 1) control, 2) control + DSS, 3) krill oil + DSS, and 4) krill oil (without DSS). Although the design was not optimal, we still think the current design have provided interesting findings.

Diets were administered during the four weeks in order to give the dietary components an opportunity to reach a steady state of maximal concentration in tissues. The DSS exposure was therefore withheld until the last week of the study. Our study design can therefore not differentiate between prophylactic and therapeutic effects.

In Paper III we report findings in the DSS versus the control group. The purpose of these controls was to have a reference point of normality, without colitis. DAI, HCS, colon length, as well as cytokines at protein an mRNA levels were increased in the DSS exposed controls versus control animals. This supports that colitis was induced, and strenghtens the relevance of the selected variables in this inflammatory model.

The protein oxidative markers have not been widely used in IBD models earlier, and we were unable to reveal significant differences between control rats and the DSS group, even though the levels in the DSS group were consistently higher for all three oxidative stress markers. We suspect that a higher number of animals analysed may have been clarifying. Unfortunately, technical problems during GC/MS analysis prohibited this, only permitting four rats to be analysed in each group.

DAI was only measured the last day of the experiment, because the animals had to be single caged and our laboratory resources were limited.

Cytokine analyses

We used full wall samples from rat colon in the cytokine analyses. In previous studies, small intestinal and also colon mucosal specimens have been mechanically scraped off in order to analyse cytokine levels in mice and rat IBD models (123, 124). As it proved difficult to perform this in rat colon during preliminary tests, it was decided to use full wall sections of colon. An excisting method for colon tissue homogenization in mice provided from Meso Scale Discovery (MSD, Gaithersburg, MD), was modified by us for use on rat colons. The method was optimized regarding colon sample weights, homogenisate preparations and phosphatase inhibitor concentrations.

Ultrasound

This method is non-invasive and still provides adequate measurements in skilled hands. One limitation is that the quality of the examination is largely investigator dependent. The small dimensions of the rat colon wall requires a very high ultrasound frequency. We suspect that the frequency of 14 MHz is probably not optimal in rats and should have been higher. This is considered another weakness of the examination.

LC/MS/MS

This method of combining HPLC with tandem MS for analysis of prostanoids allows highly sensitive, selective and simultaneous measurements, without the need of derivatization. A general limitation of this method is "ion suppression", considered as a matrix effect that may reduce detection, precision and accuracy of the LC/MS/MS method. Another limitation was the use of PGE_2 as internal standard for PGE_3 measurements, as an PGE_3 internal standard is not available.

GC/MS

This method used for analyzing markers of protein oxidation is very selective, with high ability to separate relevant substances, and sensitive, being able to detect small amounts of the relevant substance. The sample work-up is demanding with the need for several steps, which may be regarded as a limitation of the method. Another

limitation was, during the DSS study, technical problems with the GC limited the number of animals anlysed to four in each group.

Fatty acid analyses

Fatty acids from the tissues were analyzed by gas chromatography (GC) after preparation of fatty acid methyl esters, which are more volatile and less polar than the original lipids or potentially free fatty acids. That makes the GC analysis possible at lower temperatures which protect especially unsaturated fatty acids against degradation, peaks are symmetric and quantitation is more exact.

When total fatty acids were measured, a direct trans-esterification of lipids with whole tissue permitted a rapid analysis, with less loss of material.

In the DSS rat model, we isolated the phospholipid fraction for fatty acid analyses. This should avoid influences from surrounding fat tissue on the results.

Fatty acid analyses from colon specimens were only performed in the krill oil study due to limited resources. It is considered a limitation of Paper II not to report the colon tissue fatty acid profile, especially in the groups receiving fish oil diets.

Another weakness of the DSS experiment using this method is that direct structural analysis of lipids and to measure a position of individual fatty acids in the lipid molecules is not possible. Finally, some of the phospholipids may be lost quantitatively when scraping off the phospholipids from the thin layer chromatography plate.

Genetic analyses

The method has been validated and all components are commercially available. We used only one distal colon tissue specimen from each rat for gene expression analysis. As the inflammation grade may vary in different colon sections, the use of several specimens could have improved the method. In addition, the low-grade expression of some cytokines resulted in large variance of the data, also regarded as a limitation of this method.

5.3 Statistics

Ulcerative colitis study

Power estimation: 25 x 2 patients in the intervention and control groups would detect a difference of 20% or more with a power of 80% at a significance level of 0.05. However, this number of subjects was not possible to achieve.

Animal studies

Power estimation: Based on previous findings where fish oil supplemented diet clearly reduced pro-inflammatory markers we estimated that 10 animals per group would be sufficient to detect a 50% difference with a power of 80% at a significance level of 0.05.

However, many variables in our studies did not reach these differences between groups, resulting in a number of non-significant findings. In addition, the DSS control group displayed large data variation in several variables.

In Papers II and IV the data were pairwise analysed in order to focus on the rats with colitis.

Paper II: (a) Control vs. DSS and (b) DSS vs. Krill oil

Paper IV: (a) Control vs. DSS and (b) DSS vs. TTA

In Paper III, we preselected a limited number of paired analyses due to multiple groups, a limited number of animals per group and the number of analyses:

1) DSS vs. Fish oil
2) DSS vs. Fish peptides
3) Fish oil vs. Fish peptides
4) Fish oil vs. Fish oil + fish peptides

In this explorative setting, we wished to avoid a massive correction for multiple testing, discarding all significant differences between the DSS exposed groups.

Descriptives

Limited data regarding feed and DSS intake were due to keeping five animals per cage and difficulties in obtaining individual rat data.

5.4 Summary

In this study we found that regular high intake of Atlantic salmon reduced the SCCAI in patients with active colitis, improved the fatty acid profile in plasma and rectal biopsies, but this could not be supported by other disease variables. We thereafter explored the anti-inflammatory and anti-oxidant effects of different salmon components, (fish oil, fish peptides or a combined diet), in a colitis model in rats. In these studies we demonstrated that fish peptides may attenuate DSS colitis as compared with fish oil, but had no significant effect when compared with rats fed DSS. The mechanisms behind a beneficial effect from pure fish peptides diet are unclear, but the combined fish oil+fish peptides diet enhanced production of the anti-inflammatory PGE_3 more than expected from the fish oil component alone. We speculate that the latter effect may be caused by an increased activity from enzymes involved in eicosanoid synthesis.

We conducted the first dietary krill oil intervention study on experimental UC and demonstrated that it may dampen DSS colitis assessed as persistence of colon length and reduced protein oxidation in rats. Although not all measured variables improved significantly, there was a consistent change of most variables towards an anti-inflammatory pattern. However, further studies are needed to confirm this finding.

Results from dietary supplements of TTA supported earlier findings of anti-oxidant effects and anti-inflammatory potential by reducing cytokine levels at mRNA or protein levels. In addition, analysis of a subgroup of rats by a novel ultrasound examination method detected a clear difference in abdominal wall thickness between normal rats and DSS exposed rats. Furthermore, we were able to demonstrate a decreased wall thickness in TTA fed rats, in accordance with the cytokine and protein oxidant findings.

6. Future perspectives

In future animal studies, the effect of marine nutritional products should be tested in chronic, mild intestinal inflammation. Genetic IBD models may be preferable in this respect. Fish peptides may enhance the effect of n-3 PUFAs and should be further explored together with krill oil. Novel anti-inflammatory products from EPA and DHA metabolism such as resolvins and neuroprotectins should be further assessed in future studies, and supplemented in therapeutic trials in IBD models.

Krill oil should be further tested in IBD patients, possibly in combination with medical treatment, and in both patients with active and inactive IBD. However, as the anti-inflammatory potential from these marine products appears limited, future research should aim to select responders on dietary therapy. Determining GPR 120 receptor status before administering dietary n-3 PUFA may be an example of this.

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