Subjective food hypersensitivity:

Studies on enterochromaffin cell secretion products and effects of seal oil therapy

Kine Gregersen

Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

2013

Acknowledgements

The scientific work presented in this thesis was carried out at the National Institute of Nutrition and Seafood Research (NIFES), the Institute of Medicine, University of Bergen, and at the Department of Medicine, Haukeland University Hospital (HUH). The work was principally funded by a research grant through the Top Research Program from the Western Norway Regional Health Authority.

First of all, I would like to express my gratitude to my supervisors Dr. Med. Gülen Arslan Lied, Dr. Med. Arnold Berstad, Ph.D. Jørgen Valeur, Dr. Philos. Livar Frøyland and Ph.D. Pedro Araujo for excellent supervision and constructive advice. Gülen, thank you for introducing me to the challenging field of ultrasound, for helpful discussions, and for always being positive and available. Arnold, thank you for introducing me to the field of clinical research, for your brilliant expertise in gastroenterology, your never ending encouragement and enthusiasm; you have taught me to think "outside the box". I thank you for all your support, patience and help throughout these years. A special thank to you, Jørgen, for valuable discussions and collaboration, excellent writing skills, great support and guidance, especially in the final stage of this thesis. Thank you for your always positive encouragement and good friendship. Livar, thank you for giving me the opportunity to work in an interdisciplinary scientific milieu, your fantastic enthusiasm, and for always being available for discussions concerning both science and many aspects of life. Thank you for always believing in me. Pedro, thank you for introducing me to the field of LCMS and analytical thinking. Thank you for constructive advice, valuable discussions (not always scientific) and your kindly patience when explaining me an issue for the thousandth time! You are brilliant in your field and working with you has been a privilege and pleasure. You always make me smile, and I greatly appreciate our friendship.

Thank you to all former and present colleagues and friends at NIFES, especially to Anita, Tormod, Maria, Haldis and Christel for creating a pleasant working atmosphere, for nice lunches and coffee breaks. I would like to thank the Human group, and especially Dr. Scient Ingvild Eide Graff for support and encouragement.

I would like to thank Ph.D. Ragna Anne Lind for great collaboration and for being an excellent coordinator of the projects at HUH. Thank you for sharing your knowledge and

experience with patients in the clinic, I learned a lot by working by your side. I would also like to thank Ph.D. Kristine Lillestøl and Ph.D. Mette Helvik Morken for interesting discussions, and I greatly appreciate all our talks and happy social gatherings.

To my family and friends, thank you for all the love and great support and for always being present whenever needed.

To my dear Harald, thank you for your encouragement and support. Thank you for your effort trying to understand my work, and for your always positive attitude. Last, but not least, to our daughter Ingrid for endless happiness and joy and unconditional love. Thank you for reminding me of the things that really means something in life.

Don't cry because it's over. Smile because it happened – Dr. Seuss

Bergen, July 2012, Kine Gregersen

Abstract

Background: Perceived food hypersensitivity is a prevalent condition which often remains medically unexplained. The etiology of this subjective condition is not fully understood, mainly because of a lack of specific diagnostic methods, together with limited knowledge about underlying pathogenetic mechanisms. Thus, the management of such patients is challenging.

Aim: The objective of the present thesis was to assess the potential beneficial effects of seal oil on symptoms, and to develop a novel sample protocol to investigate the putative role of enteroendocrine secretorial compounds in patients with subjective food hypersensitivity.

Results: In study I, a single dose of duodenal administered seal oil significantly influenced neither gastric accommodation, as examined by two dimensional (2D) ultrasonographic scanning, nor meal-induced gastrointestinal symptoms, as assessed by visual analogue scales (VAS).
After 10 days' treatment, duodenal administered seal oil, as compared to soy oil, significantly reduced meal-induced gastrointestinal symptoms. However, the symptomatic improvement was not associated with improvements in gastric accommodation.

In study II, patients with subjective food hypersensitivity received a 10-day open treatment with duodenal administered seal oil. Before and after treatment, and 1 month post-treatment, the patients filled in the Subjective Health Complaints (SHC) Inventory for non-gastrointestinal symptoms, the short form of the Nepean Dyspepsia Index (SF-NDI) for evaluation of quality of life (QoL), and the Ulcer Esophagitis Subjective Symptoms Scale (UESS) score and the Gastrointestinal Symptom Rating Scale (GSRS) for assessment of gastrointestinal symptoms. Both intestinal and extra-intestinal complaints were significantly improved from baseline, both at 10 and 30 days post-treatment.

In study III, a novel method for direct determination of serotonin (5-HT) in gut lavage fluid using liquid chromatography mass spectrometry was achieved. This method was applied in study IV.

In study IV, analyses of chromogranin A (CgA) in serum and 5-HT in gut lavage fluid were performed. Serum levels of CgA were significantly lower in patients with subjective food hypersensitivity as compared to healthy controls. No significant differences in gut lavage 5-HT levels were detected between patients with subjective food hypersensitivity, patients with inflammatory bowel disease (IBD) and healthy controls.

Conclusion: The present thesis suggests a beneficial effect of duodenal administered seal oil on both intestinal and extra-intestinal complaints in patients with subjective food hypersensitivity. Impaired gastric accommodation seems to play a limited role in the pathophysiology, as the positive effect of seal oil was not associated with improvements in gastric accommodation. The mechanism of action may involve the anti-inflammatory and immune-modulating properties of the long-chain omega-3 polyunsaturated fatty acids (PUFAs), and further studies addressing this aspect are needed.

Decreased systemic CgA levels suggest a role for enteroendocrine alterations in the pathophysiology of subjective food hypersensitivity, such as impaired enterochromaffin (EC) cell function, and the potential of using granins as biomarkers for functional gastrointestinal disorders should be explored in future studies.

Taken together, the hypothesis-generating studies of the present thesis encourage further investigations to elucidate causal relationships between the patients' unexplained symptoms and possible pathophysiological mechanisms.

List of publications

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Kine Gregersen, Ragna Lind, Tormod Bjørkkjær, Livar Frøyland, Arnold Berstad and Gülen Arslan Lied (2008).
"Effects of seal oil on meal-induced symptoms and gastric accommodation in patients with subjective food hypersensitivity: a pilot study."
Clinical medicine: Gastroenterology 1 (33-41).

II Kine Gregersen, Ragna A. Lind, Jørgen Valeur, Arnold Berstad and Gülen Arslan Lied (2010).
"Duodenal administered seal oil for patients with subjective food hypersensitivity: an explorative open pilot study."
International Journal of General Medicine 3 (383-92).

III Kine Gregersen, Livar Frøyland, Arnold Berstad and Pedro Araujo (2008).
"Direct determination of serotonin in gut lavage by liquid chromatographic ion trap tandem mass spectrometry."
Talanta 75 (466-72).

IV Kine Gregersen, Jørgen Valeur, Kristine Lillestøl, Gülen Arslan Lied, Pedro Araujo and Arnold Berstad (2011).
"Subjective food hypersensitivity: assessment of enterochromaffin cell markers in blood and gut lavage fluid."
International Journal of General Medicine 4 (555-60)

Contents

Abbreviations

2D:	Two-dimensional
5-CH_3O-HT:	5-methoxytryptamine
5-HT:	5-hydroxytryptamine (serotonin)
AA:	Arachidonic acid (20:4n-6)
ALA:	Alpha-linolenic acid (18:3n-3)
CgA:	Chromogranin A
COX:	Cyclooxygenase
DBPCFC:	Double-blind placebo-controlled food challenge
DHA:	Docosahexaneoic acid (22:6n-3)
DPA:	Docosapentaenoic acid (22:5n-3)
EC:	Enterochromaffin
EPA:	Eicosapentaenoic acid (20:5n-3)
FGID:	Functional gastrointestinal disorder
IBS:	Irritable bowel syndrome
IBD:	Inflammatory bowel disease
IS:	Internal standard
LA:	Linoleic acid (18:2n-6)
LCMS:	Liquid chromatography mass spectrometry
LCPUFA:	Long-chain polyunsaturated fatty acid
LOX:	Lipooxygenase
PEG:	Isotonic polyethylene glycol
PGE_2:	Prostaglandin E_2
PUFA:	Polyunsaturated fatty acid
RF:	Response factor
SERT:	Serotonin-selective reuptake transporter
SHC:	Subjective health complaints
TAG:	Triacylglycerol
VAS:	Visual analogue scale
QoL:	Quality of life

1. Introduction

1.1 Background

The incidence of food hypersensitivity is increasing worldwide. For instance, in Western countries, as many as approximately 35 % of the general population report adverse reactions to food, but only 1-3 % are medically diagnosed with food allergy [1,2,3]. The observed high discrepancy between subjective and medically confirmed food hypersensitivity, is mainly due to limited knowledge of the underlying mechanisms of the pathogenesis, and, hence, a lack of specific diagnostic methods.

The present thesis examines different aspects of subjective food hypersensitivity, such as potential beneficial effects of seal oil on the symptomatology, with emphasis on gastrointestinal and systemic symptoms, the potential of assessing enteroendocrine secretorial compounds for evaluating gastrointestinal pathologies and exploration of a novel sample protocol.

1.2 Definition of food hypersensitivity

In 2003 the Nomenclature Review Committee of the World Allergy Organization proposed an updated report [4] on the revised nomenclature for allergy published in 2001 by the European Academy of Allergological and Clinical Immunology (EAACI) [5]. The EAACI stated that the nomenclature can be used independently of target organ and patient age group, and the nomenclature proposed is based on the mechanism initiating the reaction and causing the symptoms and signs of an allergic disease. Considering the EAACI's definition, the word "allergy" has previously been faulty used to describe all kinds of unexpected reactions in the skin and mucosal surfaces, including reactions to food additives, side-effects of drugs, psychological reactions, pharmacological factors, behavioural disorders and others. As proposed by the EAACI, hypersensitivity should be used as a collective term to cover all kind of adverse reactions, including food allergy and non-allergic food hypersensitivity. The definition of the term hypersensitivity should be as follows: *Hypersensitivity causes objectively reproducible symptoms or signs, initiated by exposure to a defined stimulus at a dose tolerated by normal subjects* [5]. Classical responses to infection, autoimmunity and toxic reactions do not accommodate this definition. An important condition for the hypersensitivity reaction is that the reproducibility of the symptoms or signs can be confirmed

by history, clinical examinations or investigation of causality between symptoms and environmental factors to which the patients attribute their symptoms.

Food hypersensitivity can be divided into two main groups; food allergy and non-allergic food hypersensitivity. Based on the immunological mechanism involved, food allergy can be further classified into three groups, namely IgE-mediated food allergy, mixed IgE- and non-IgE-mediated and non-IgE-mediated food allergy (Figure 1) [1,6].

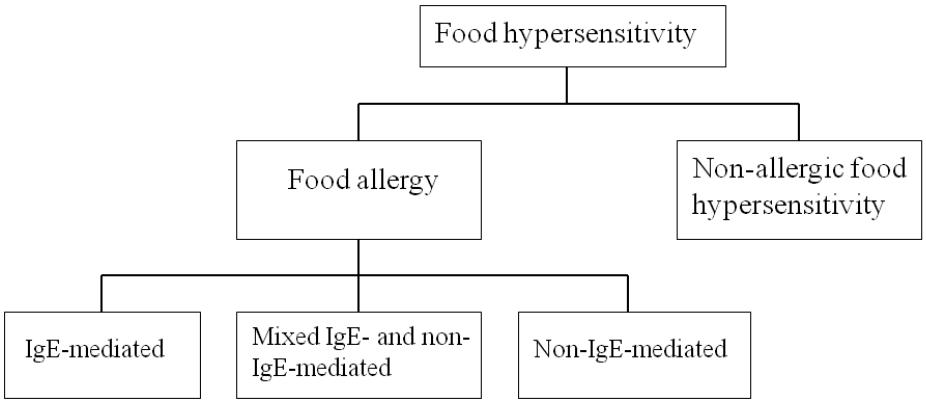

Figure 1. Nomenclature of food hypersensitivity. Illustration by Gülen Arslan Lied.

The diagnostic work-up of suspected food hypersensitivity is based on detailed medical history, including familial atopic background and comprehensive physical examination. It is important to obtain an accurate history to elucidate the relationship between ingestion of specific foods and onset of symptoms, and also to assess the time interval between ingestion and appearance of symptoms, the amount of ingested food and to inspect whether same foodstuff reproduces symptoms. The standard clinical or laboratory tests include skin prick testing (SPT) and *in vitro* testing for food specific IgE-antibodies [7,8], the atopy patch test (APT) [9], and double-blind placebo-controlled food challenge (DBPCFC) [10,11,12,13]; the latter representing the gold standard in the diagnostic work-up. The diagnostic accuracy of the APT is somewhat controversial, but it is considered a useful tool in diagnosing delayed food allergy in children with atopic dermatitis [9,14].

IgE-mediated food allergy

IgE-mediated food allergy is a typical well-known immediate reaction (type I) resulting in classical clinical presentation, such as anaphylactic reactions. The most common food allergic

disorders put on by IgE-mediated mechanisms are immediate gastrointestinal hypersensitivity, oral allergy syndrome, acute urticaria, angiodema, acute bronchospasm and allergic rhinitis. Positive SPT and detection of serum food specific IgE are strong indicators for IgE-mediated food allergy. The immune system may respond to a foreign protein by producing IgE antibodies, thus sensitizing the individual, resulting in positive IgE tests. But this is by no means identical with a clinical relevant IgE-mediated allergy. Therefore, in cases of positive SPT and *in vitro* specific IgE, oral food challenges are mandatory before establishing a diagnosis of IgE-mediated food allergy. Skin irritations after oral food challenge may be IgE mediated, but may also be caused by acids contained in the foods (e.g. citrus fruits). In such cases, avoidance is only necessary for the patients' convenience.

Non-IgE-mediated food allergy

Non-IgE mediated food allergy initiate immune responses to foods in the absence of specific IgE to the causal food protein, at least as verified by routine tests. Non-IgE-mediated food allergic reactions has a slower onset (type III-IV reactions) compared to IgE-mediated reactions, ranging from a few hours to 48 hours after ingestion of the causative agent [15]. The delayed onset of symptoms often makes it difficult to detect the clinical association between offending food and clinical symptoms. Elimination diets and DBPCFC are the primary mode of diagnosis, and strict avoidance of the culprit food is often sufficient for the patient to manage such food allergies. Eosinophils and T cells seems to play a major role in the immune response [16,17]. Gastrointestinal disorders, including allergic eosinophilic gastroenteritis, dietary protein-induced enterocolitis and celiac disease, have been associated with non-IgE mediated food allergy [15].

Mixed IgE- and non-IgE-mediated food allergy

Allergic eosinophilic esophagitis, gastritis and gastroenterocolitis are characterised by various infiltrations with eosinophils in the mucosal, muscular or serosal layer. Eosinophilic infiltration and degranulation mediate inflammatory effects by releasing various cytotoxic proteins and other lipid mediators together with the release of cytokines. It has been shown that peripheral blood T cells in these patients secrete excessive interleukin (IL)-4 and IL-5 compared to controls [18], but the immunopathogenesis of these disorders is poorly understood. Elevated serum total IgE or food specific IgE levels are common especially in eosinophilic gastritis and gastroenterocolitis [15]. The increased IgE production in these

conditions is most likely regulated by cytokines secreted by T cells; especially IL-4 and IL-13 is known to induce IgE production in humans [19].

Non-allergic food hypersensitivity

Non-allergic food hypersensitivity, previously called food intolerance, refers to non-immune mediated food hypersensitivity reactions, at least, immune mechanisms not verified by current available techniques. Typical symptoms are flushing, headaches, wheezing, altered bowel habits, abdominal pains, bloating, among others. Triggering factors can be divided into intrinsic or extrinsic causes; intrinsic causes being enzyme deficiency (e.g. lactase deficiency and phenylketonuria), malignancies and psychological factors. Examples of extrinsic causes are infections (bacteria, virus, parasites), food additives (glutamate, aspartame, sulphites, nitrates, dyes) and pharmacological factors (alcohol, caffeine, histamine, tyramine, serotonin and metal contaminants) [5,20]. Patients with non-allergic food hypersensitivity showed reproducible symptoms in an oral food challenge, despite negative food specific IgE and SPT [21].

The above mentioned classification of food hypersensitivity is widely applied in clinical practice, but the diagnostic work-up can be challenging and time-consuming. In fact, perceived food hypersensitivity most often remains unexplained, despite comprehensive medical examination. In the present thesis, the studied patients is referred to as having 'subjective food hypersensitivity', considering the difficulty in allocating them to any of the specific diagnosis outlined in Figure 1.

1.3 Symptomatology

The fact that subjective food hypersensitivity often remains unexplained constitutes great concern and challenge for both patients and doctors. The patients with subjective food hypersensitivity may appear heterogeneous, but today no meaningful diagnostic approach exists to detect possible subgroups. The frequency and severity of diverse complaints can vary considerably between patients, who typically present diffuse and unspecific symptoms, and multiple organ systems are often affected.

A major part of the symptomatology in subjective food hypersensitivity is related to the gastrointestinal tract, with abdominal pain and discomfort, bloating, nausea, and defecation disturbances being predominant. The clinical features of the gastrointestinal complaints typify

other gastrointestinal disorders, such as irritable bowel syndrome (IBS). In fact, the gastrointestinal complaints in unexplained subjective food hypersensitivity usually comply with the Rome II criteria for IBS. Also, many IBS patients associate the generation of symptoms to food ingestion [22]. Whether the similarity of symptoms in subjective food hypersensitivity and IBS is purely coincidental or it is part of a causal relationship has not yet been fully established. In our studies, at least 90% of the patients with subjective food hypersensitivity have been classified as IBS. Commonly, perceived food hypersensitivity is found in around 70 % of patients with IBS [23]. Hence, work up and treatment of IBS is central in the caring of our patients. In spite of high prevalence of atopic diseases among patients with IBS, classical IgE-mediated food allergy appears of minor importance in these patients. On the other hand, the concept "atopic bowel" has been introduced to designate a subgroup of patients characterized by atopic diseases and possibly also a high level of serum total IgE [24] and a high number of mast cells in the intestinal mucosa that may be related to visceral hypersensitivity. Another important subgroup of IBS patients is those with post-infectious IBS, which comprises around 30% of the total IBS population. Patients with persistent complaints following the Giardia lamblia epidemic in Bergen in 2004 are a typical example [25].

Our patients with subjective food hypersensitivity have considerably reduced QoL [26] and they reported more SHC compared with the general population [27,28]. SHC are defined as symptoms or complaints that, despite thorough examination, cannot be fully explained. The most frequently reported SHC are musculoskeletal, gastrointestinal and systemic complaints [27]. Lillestøl and colleagues found high prevalence of anxiety and depression in patients with subjective food hypersensitivity, as compared to the general population [29]. Whether mental illness is a consequence or the cause of the gastrointestinal and somatic complaints of the patients is not clear. Continuous worry and chronic bodily complaints, may over time lead to worsen mental illness [30]. On the other hand, psychological stress may modulate visceral responses like gut motility and mucosal function through the release of hormones and peptides [31,32]. Coexistence of unexplained gastrointestinal and non-gastrointestinal complaints suggests multifactorial pathogenic mechanisms, and both gut sensorimotor dysfunction and psychological factors may be involved. However, a recent study found that psychological factors were not major predictors of symptom severity in patients with subjective food hypersensitivity [33].

1.4 Pathophysiological aspects

1.4.1 Role of nutrition

Nutrition is a critical environmental factor for maintaining a good health, thus the diet play an important role in prevention and treatment of different diseases. The evolutionary aspects of diet indicate enormous nutritional changes, and the modern diet is characterised by more processed and synthetic food, and less fish, fruits and vegetables. One change that has been identified in particular is the type and amount of fat consumed. Intake of omega-6 fatty (n-6) acids have increased, with major contribution from linoleic acid (LA; 18:2n-6) due to extensive use of soybean oil in food production, while the intake of omega-3 (n-3) fatty acids, especially the marine derived long chain polyunsaturated fatty acids (LCPUFA) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), have declined. The diet of most westernized societies has been estimated to be deficient in n-3 fatty acids with ratios of n-6 to n-3 of 15 to 20:1, whereas optimal ratios have been postulated to be 1 to 2:1 [34]. Alpha-linolenic acid (ALA; 18:3n-3) and LA are essential fatty acids for humans and precursors for the endogenously synthesized EPA and arachidonic acid (AA; 20:4n-6), respectively. However, the capacity in humans to convert ALA to EPA is somewhat disputed [35]. It is therefore important to have a sufficient intake of preformed EPA and DHA to maintain or increase the concentration of these fatty acids in tissues.

LCPUFAs are involved in inflammation, and the link is the metabolites of AA (also known as eicosanoids), which are mediators and regulators of inflammatory processes. Eicosanoids are synthesized from 20-carbon PUFAs and AA is usually the major substrate due to its high content in cell membrane phospholipids. EPA is a metabolic antagonist that inhibits the metabolism of AA and serves as an alternate substrate for cyclooxygenase (COX) and lipooxygenase (LOX). Whereas AA is converted by COX to the pro-inflammatory prostaglandin E_2 (PGE_2), EPA is converted to the homologue PGE_3, which is considered far less immunologically active. The n-6 derived compounds synthesized in the LOX pathway are also more potent triggers of immunological reactions compared to the n-3 derived compounds [36]. Selective COX-2 inhibitors are effective drugs in inflammation and pain management, but their use has been linked to increased risk of cardiovascular events, like heart-attack, thrombosis and stroke [37]. The selective COX-2 inhibitors have been proposed for IBD patients due to sparing of the mucosal protective properties of COX-1, while retaining the anti-inflammatory effect. However, conflicting clinical observations have been reported, and the use of selective COX-2 inhibitors in gastrointestinal disorders should be used with same

caution as for conventional non-steroidal anti-inflammatory drugs (NSAIDs) [38,39,40]. EPA and DHA also inhibit COX-2 activity induced during inflammation [41].

The typical aspects of inflammation or mucosal distortion associated with inflammatory disorders of the gut, like IBD, are not present in IBS [42]. However, increased attention has been directed towards the role of low-grade inflammation in the intestinal mucosa in patients with IBS [43]. Intestinal inflammation may affect the visceral sensory system, leading to intestinal motor abnormalities, pain and discomfort, which are typical complaints of patients with IBS.

The role of food in the pathophysiology of IBS is unclear, but it is obvious that food by virtue of its components is a potent stimulus for gastrointestinal functions like motility and secretion. A general dietary advice for IBS patients has been to increase the intake of fiber, which are incompletely or indigestible in the small intestine, but partly or totally fermentable in the large bowel [44]. Gastrointestinal fermentation may play a role in IBS, thus avoidance of fermentable, poorly absorbed carbohydrates is more and more advocated. A recent study by Valeur and colleagues indicate that indigestible but fermentable carbohydrates are poorly tolerated by patients with IBS and subjective food hypersensitivity [45]. Gibson's group in Australia has introduced the FODMAP (Fermentable Oligo-, Di-, and Monosaccharides and Polyols) concept, which, when avoided, has proved to be an effective approach to the management of patients with adverse gastrointestinal complaints [46]. FODMAPs are widespread in the diet. They are characterized by being poorly absorbed, rapidly fermented by colonic flora, osmotically active, and they induce distention and discomfort of the gut. A low FODMAP diet can be challenging to maintain, due to the range of FODMAPs in the diet, but global restriction is recommended [46]. A dietician with good knowledge about the low FODMAP diet is therefore required, and should be involved in the treatment and follow-up of the patients. Some of the most frequently reported foods to cause gastrointestinal complaints in patients with subjective food hypersensitivity are fruits, vegetables and cereal products, which all have a high content of FODMAPs. To improve the compliance of the FODMAP strategy, further studies are needed to systematically examine the content of FODMAPs in foods, and to define cut-off values.

Probiotics have also been tried extensively as treatment in IBS. Probiotics are live organisms that, when ingested in adequate amounts, exert a health benefit on the host [47]. Probiotics are believed to modify the gut microbiota by changing its composition and metabolic activity, and to protect against pathogens and stimulate the immune system [48]. A possible role of the gut

microbiota in IBS has been suggested, particularly in post-infectious IBS (PI-IBS). Studies have demonstrated reduction in abdominal pain and bloating, and an overall better gut function in IBS after treatment with specific strains of *Lactobacillus* and *Bifidobacteria* [49,50]. O'Mahony et al. also investigated the immune-modulating effect of probiotics, and detected an improvement in the IL-10/IL-12 ratio [49]. However, because the impact of probiotics might be species specific, the functioning of different species needs to be further investigated, both clinically and mechanistically, in placebo-controlled studies and in well-defined sub-groups of IBS.

The complex pathophysiology of IBS and subjective food hypersensitivity requires a careful approach when it comes to treatment, one of which may be through the diet. Although diet alone is most likely not sufficient, there are indications that dietary modifications can reduce the patients' complaints. However, due to heterogeneity, a general dietary advice may not work for all. Other triggering factors from the diet that potentially can create gastrointestinal complaints are food additives, like glutamate, aspartame, and sulphite, and there are also pharmacological factors, like alcohol, caffeine, histamine, and serotonin that can affect the gut [5,20].

1.4.2 Role of gastric accommodation

The stomach can be divided into two parts when it comes to motor function; the proximal (fundus and corpus) and the distal (antrum) region. During the interdigestive phase, the proximal stomach muscle tone is high, whereas the distal stomach has a recurrent contraction pattern, known as the migrating motor complex. Upon food intake, the muscle tone decreases and the proximal stomach relaxes, providing a temporarily reservoir of the ingested food. This relaxation, called the accommodation reflex, enables the stomach to accommodate the extra volume of ingested food, without accompanying rise in the intragastric pressure. Postprandially, a tonic contraction of the proximal stomach pushes the food distally, where it is mixed and grinded by peristaltic, 3 per minute, contractions ("fed state"), before controlled release into the duodenum. The accommodation reflex, which consists of a vagovagal reflex pathway, is an important mechanism of normal gastric physiology, and under physiological conditions, this adaptive relaxation is not perceived.

Impaired gastric accommodation of the proximal stomach has been reported in functional dyspepsia (FD) [51,52]. The symptoms in FD are related to ingestion of food (especially fat-

rich food), and they are mainly of gastrointestinal character such as epigastric pain/discomfort, nausea, bloating and early satiety. The relationship between impaired gastric function and symptoms in FD is not clear, but impaired gastric accommodation has been found to be significantly associated to early satiety [53]. The barostat procedure is considered as the gold standard in assessing gastric accommodation, but the invasive nature of the barostat procedure has limited its application to research facilities. Ultrasound, which is non-invasive, patient-friendly and a largely available method, is found to be very useful for examination of the gastric accommodation response [54,55]. The cause to impaired gastric accommodation has not been fully established, but disorders in the sensory apparatus, the vagovagal pathway or intrinsic inhibitory innervations are probably involved [56]. Psychological factors, especially anxiety and neuroticism, have also been showed to affect the accommodation response [57].

Visceral hypersensitivity is believed to play an important role in the pathogenesis of functional gastrointestinal disorders (FGID), and, indeed, increased sensitivity to visceral distension has been reported in patients with IBS and FD [58,59,60] . However, a potential role of impaired gastric accommodation in IBS needs to be further investigated.

1.4.3 Role of enterochromaffin cells

The name *enterochromaffin* (EC) cells was introduced by Ciaccio in 1907 to designate the cell's location in the intestinal epithelium and their affinity for chromium salts [61]. The endocrine nature of the EC cells was first suggested by Masson in 1914. EC cells constitute the largest population of neuroendocrine cells in the gastrointestinal tract. They are distributed from the stomach through the colon, and play an important role in several aspects of gut function including secretion, motility and sensation. [62,63,64]. The EC cell population includes several different sub-populations with morphological differences in shape, cytoplasmatic extensions and secretory granules. The main secretory product of EC cells is serotonin (5-HT). Over 95 % of the body's total 5-HT is located within the gut, and 90 % of that is present in the EC cell, which express the enzymatic machinery, including the rate limiting enzyme, tryptophan hydroxylase (TpH), to synthesize 5-HT. The EC cells extend from the basal lamina of the crypt epithelium to the lumen of the crypt. Apical cytoplasmic extensions with microvilli border protrude into the lumen, and respond to chemical and physical alteration of the gut. Hence, the EC cells have been referred to as "taste buds of the gut". Secretory granules are concentrated at the base of the EC cell cytoplasm, and secretion

occur mainly basally into the lamina propria, but the cells are bipolar and able to secrete also from the apical end, into the gut lumen [62,63,64]. Cytoplasmic extensions with serotonin containing granules have also been found basally, often with terminal buttons with accumulation of 5-HT [65]. Due to constant turnover of the enteric epithelium including its EC cells, nerves cannot form morphological recognizable junctions with EC cells. The secretion rate is therefore constitutively high, and even higher upon stimulation.

Altered 5-HT signalling between EC cells and sensory afferent fibres has been suggested as possible mechanisms for alterations in gastrointestinal functions as motility, secretion and visceral sensation, characteristic of IBS. Changes in key elements in 5-HT signalling, such as EC cell count, 5-HT content, TpH message levels, serum 5-HT levels and expression of serotonin-selective reuptake transporter (SERT), have been reported in IBS. Despite an improved understanding of the enteric nerve system and its involvement in gastrointestinal function, further knowledge is essential to understand the pathophysiology of gastrointestinal disorders like IBS.

Serotonin (5-hydroxytryptamine; 5-HT)

Serotonin (5-HT) is a monoamine primarily found in blood platelets, the central nervous system and in the digestive tract. As mentioned above, 95 % is located in the digestive tract, of which 90 % in the EC cells, where it is synthesized and stored in secretory granules at the basal border [66]. The remaining 5 % is located within neurons of the enteric nerve system. The pathway for 5-HT synthesis is initiated by conversion of dietary $_{L}$-tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase, which is the rate limiting enzyme. Subsequently, 5-hydroxytryptophan is decarboxylated into 5-HT by the action of 5-hydroxytryptophan decarboxylase. Newly synthesised 5-HT is packed into secretory granules by the vesicular monoamine transporter 1 (VMAT1), which is specific for 5-HT- producing EC cells [67]. Released 5-HT can interact with neurons, both intrinsic and extrinsic sensory afferent terminals, immune cells, intestinal enterocytes, mucus cells, and smooth muscle receptors to regulate secretion, motility and visceral pain perception [64,68]. Because of the constant migration and turnover of the EC cells, and thus no close contact with nerve endings or immune cells, a high secretion rate of 5-HT is necessary, and the specificity of responses to this paracrine signal is dependent on the receptors reached by the transmitter [69]. The 5-HT receptor family comprises fourteen different receptor sub-types, which are grouped in seven families. Except for the 5-HT_3 receptor, which belongs to the ligand-gated ion channel family, all the receptors are G-protein coupled neurotransmitter receptors [70]. The 5-HT_3 and 5-HT_4

receptors are both therapeutic targets; 5-HT_3 antagonists alleviate nausea and emesis associated with cancer chemotherapy [71,72], and 5-HT_4 agonists have shown to be effective in treatment of constipation-predominant IBS [73,74].

An important aspect of efficient intercellular signalling is the termination of the signal. The action of 5-HT in the gastrointestinal tract is terminated by uptake mediated by SERT, which is expressed by most epithelial cells [75]. Inflammation of the intestinal mucosa is associated with decrease in the expression of SERT [76,77], which impairs the inactivation of 5-HT. The accumulation of synaptic 5-HT will excessively potentiate the actions of 5-HT, affecting secretory, motility and sensory functions, which can cause abdominal discomfort, pain, bloating, nausea, diarrhoea or constipation; effects that are similar to the abnormalities of gastrointestinal function and sensation observed in IBS.

Chromogranin A (CgA)

Chromogranin A (CgA) is a member of the granin family of secretory glycoproteins that are found within secretory cells of the enteric endocrine and immune systems. The granins are proposed to play several roles in the secretory process, from targeting peptide hormones and neurotransmittors in granules intracellularly, to regulate hormone secretion extracellularly. CgA is a precursor to a range of peptides with possible regulatory functions [78]. Clinically, measurement of serum and tissue content of CgA has become a valuable tool in the diagnosis of a variety of neuroendocrine tumors (NETs). Carcinoid tumors, which arise from EC cells, represent the most common gastrointestinal NETs, and are characterised by increased release of CgA and 5-HT into systemic circulation [79].

Circulating CgA levels have recently been studied in patients with IBS, and both elevated and reduced levels of CgA have been reported [80,81]. The inconsistency in the results may suggest varied CgA levels in subgroups of IBS, but possible pathophysiological role or clinical implication is yet to be elucidated.

1.5 Methodology for the analysis of 5-HT

Traditionally, determination of 5-HT is carried out by enzyme immunoassays, which are considered inexpensive and simple to perform. However, their main disadvantages are the lack of specificity for complex biological samples, potential cross-reactivity and consequently the liability to overestimate the levels of a determined analyte, especially in cases where multiple metabolically related products are present. High performance liquid chromatography

and LCMS (single or tandem) have been successfully used in the determination of 5-HT in a wide variety of biological samples such as diverse tissues [82], brain [83], whole blood [84], serum [85], plasma [86], urine [87], and intestine [88]. It has been suggested that the use of LCMS/MS in the analysis of 5-HT offers advantages in terms of specificity and linear range, while permitting simultaneous determination of 5-HT metabolic related products [84]. The internal standard (IS) technique is the preferred approach to achieve rapid sample analysis efficiency at a minimum cost, as opposed to external standard calibration (a technique also used in immunological assays). The ideal IS is a compound that resembles the analyte of interest. Unfortunately in the majority of prior studies there is no description regarding the strategies behind the selection of an optimal amount of IS, especially in cases where the analyte may have a wide span of concentrations. The potential applicability, in the analysis of biomedical samples, of a multivariate strategy to estimate an optimal concentration of IS and a robust response factor (RF), which does not vary with changes in the analyte and internal standard concentrations, has previously been suggested [89].

2. Aims of the Study

2.1 Overall aim

The present study was part of a project called the MAI-project ("Matallergi og Intoleranse" Norwegian translation), which started in 2001 at Haukeland University Hospital (HUH), Bergen, Norway. The MAI-project embraced patients who self-attributed their gastrointestinal complaints to food hypersensitivity in the absence of organic diseases. The patients went through an extensive examination program which involved allergologists, gastroenterologists, dieticians and psychiatrists/psychologists. The National Institute of Nutrition and Seafood Research (NIFES) collaborated with HUH in this interdisciplinary project.
The overall aim of the present thesis was to study the potential beneficial effect of seal oil in gastrointestinal and non-gastrointestinal complaints and to investigate the possible role of EC cell markers in subjective food hypersensitivity.

2.2 Aims of the papers

Paper I: To compare the effects of duodenal administration of seal oil with that of soy oil on gastric accommodation (single dose experiment) and gastrointestinal complaints (single dose and short-term experiment) in patients with subjective food hypersensitivity.

Paper II: To investigate the impact of short-term duodenal administered seal oil on SHC, QoL and gastrointestinal complaints in patients with subjective food hypersensitivity.

Paper III: To develop a method for analysing 5-HT in gut lavage fluid by LCMS/MS, by using a multivariable approach in modelling the relationship 5-HT/IS to select an optimal amount of IS. The selected approach was a multivariable model, to investigate how simultaneous changes of 5-HT and IS concentrations could affect the response factor (RF), and how the mathematical modelling can contribute to select an optimal amount of IS.

Paper IV: To investigate the possible role of the EC cell markers 5-HT and CgA in gut lavage fluid and blood, respectively, in patients with subjective food hypersensitivity.

3. Material and Methods

3.1 Patients

The patients were referred from general practitioners and specialists to HUH because of gastrointestinal complaints suspected to be due to food allergy. The patients underwent a standardized clinical examination program by an allergologist, a dietician and a gastroenterologist. The extensive examination included detailed medical history, physical examination, routine laboratory tests in addition to SPT, measurements of serum total- and food-specific IgE levels, and DBPCFC performed by a dietician when indicated. The gastroenterological investigations included a gastroscopy with gastric and duodenal biopsy taking to exclude *Helicobacter pylori* infection and celiac disease, respectively, and analysis of intestinal permeability and levels of calprotectin in gut lavage fluid to exclude IBD. In addition, stool samples were investigated to rule out parasitic infections. Patients with organic gastrointestinal disease, which could explain their symptoms, and pregnant or lactating women, were not included. Also patients who reported anaphylactic reactions or very strong atopic reactions combined with high IgE levels were also excluded from the study. All the patients that attended this study were part of the MAI-project.

In paper I, in the single dose experiment, ten female patients with subjective food hypersensitivity were included (range 28-82 years, mean age 49 years). Twenty-four patients (22 females and 2 males, range 24-80 years, mean age 43 years) were included in the short-term experiment, and they were randomly allocated to treatment with either seal oil (n = 12; 11 females and 1 male, mean age 45 years) or soy oil (n = 12; 11 females and 1 male, mean age 41 years).

In paper II, 34 patients with subjective food hypersensitivity were included in the study, but only 26 patients completed the seal oil treatment and filled in all four questionnaires (Subjective Health Complaints Inventory, short form of the Nepean Dyspepsia Index, Gastrointestinal Symptom Rating Scale and Ulcer Esophagitis Subjective Symptoms Scale). Of the remaining 26 patients, 23 were female and 3 were male (range 26-88 years, mean age 47 years).

In paper III, six patient samples were randomly chosen from the MAI-material for the purpose of method development for determination of 5-HT in gut lavage fluid using liquid chromatography ion-trap tandem mass spectrometry technique.

In paper IV, 69 patients with subjective food hypersensitivity were included (55 females and 14 males, mean age 39 years, range 21-83 years). Gut lavage fluid was collected in all 69 patients for determination of 5-HT, and blood samples for analysis of serum CgA were obtained from 32 out of 69 patients.

3.2 Control subjects

The healthy subjects were mainly employees at NIFES and students at the University of Bergen. Inquiry and study information were sent by e-mail. Participants that reported having regularly gastrointestinal complaints or suspected adverse reactions to foods were not included. However, no medical examinations of the recruited healthy subjects were performed. In paper I in the single dose experiment, the patients with self-reported food hypersensitivity were compared with ten healthy subjects (4 females and 6 males, range 23-56 years, mean age 31 years).

In paper IV, 34 healthy volunteers (24 females and 10 males, range 23-61 years, mean age 33 years) were included in the study. Twenty-seven patients admitted to the Section of Gastroenterology at HUH because of suspected IBD were also included as a control group (11 females and 16 males, range 21-65 years, mean age 35 years). 23 out of 27 patients were diagnosed with IBD (19 had Crohn's disease and 4 had ulcerative colitis).
Overview of study design, material and methods are given in table 1.

Table 1. Overview of study designs, material and methods in paper I-IV.

	PAPER I	PAPER II	PAPER III	PAPER IV
Experimental groups	*Singel dose:* 10 S-FH patients *Short-term:* 12 S-FH-patients	26 S-FH patients	6 S-FH patients (method development)	69 S-FH patients
Comparison groups	*Singel dose:* 10 healthy adults *Short-term:* 12 S-FH patients			34 healthy adults 23 IBD patients
Experimental oils	Seal oil and soy oil	Seal oil		
Daily dosage/duration of oil administration	*Single dose*: 10 mL *Short-term*: 10 mL × 3/10 days	10 mL × 3/10 days		
Ultrasonography	2D ultrasound			
Symptom registration	Visual Analogue Scale			
Questionnaires		SF-NDI, SHC, GSRS, UESS		
Intestinal lavage			5-HT (method development)	5-HT (sample quantification)
Blood samples				CgA

S-FH=subjective food hypersensitivity; IBD=inflammatory bowel disease; 2D=two dimensional; SF-NDI=Short Form of the Nepean Dyspepsia Index; SHC=Subjective Health Complaints; GSRS=Gastrointestinal Symptom Rating Scale; UESS=Ulcer Esophagitis Subjective Symptoms Scale

3.3 Methods

3.3.1 Marine and vegetable oils

In paper I and II, refined seal oil (Rieber Skinn A/S, Bergen, Norway) was used. The seal oil was from adult harp seal (*Phagophilus groenlandicus*), and a combination of natural and synthetic tocopherols, the latter being dl-α tocopheryl acetate were added, acting as antioxidants by scavenging lipid radicals from potentially oxidised PUFAs. To further protect the seal oil against oxidation, a top layer of nitrogen was added to the bottles, and stored in a refrigerator (kept around 4 °C) during study, otherwise in −20 °C freezer. The level of dioxins in the seal oil was below the current European Union upper limits in marine oils for human consumption (2 pg WHO-TEQ/g fat).

In paper I, the effect of seal oil was compared to commercially available soybean oil (Mills DA, Oslo, Norway). The soybean oil was naturally high in linoleic acid (18:2n-6, LA). See Table 2 for an overview of fatty acids, fat soluble vitamins A and E, and lipid peroxidation (thiobarbituric acid reactive substances, TBARS) in the seal oil (paper I-II) and soy oil (paper I).

Table 2. fatty acid profile (g/100 g), vitamins A, D and E and TBARS in experimental oils

Analyte	Seal oil	Soy oil
14:0	4.5	n.d
16:0	8	9.7
18.0	1.2	3
∑ saturated	14.2	13.4
16:1n-7	14	n.d
18:1n-11	3.2	n.d
18:1n-9	14.9	17.5
18:1n-7	3.8	1.3
20:1n-11	1.6	n.d
20:1n-9	7.7	.2
22:1n-11	1.8	n.d
∑ monoenes	48.9	19
18:2n-6	1.5	49.7
20:4n-6	.6	n.d
∑ n-6	2.2	49.7
18:3n-3	.6	5.5
18:4n-3	1.6	n.d
20:4n-3	.5	n.d
20:5n-3	7.9	n.d
22:5n-3	3.7	n.d
22:6n-3	8.6	n.d
∑ n-3	23.9	5.5
n-6/n-3	.1	9
Sum vitamin A	.3 mg/100 g	n.d
Vitamin D_3	n.a	15 µg/100 g
α-tocopherol	4.5 mg/100 g	17.1 mg/100 g
TBARS	3.6 nmol/g w/w	n.d

Monoenes = monounsaturated fatty acids. Sum vitamin A = sum retinol (13-, 11-, 9-cis and all-trans retinol ie, A_1) and 3.4 didehydroall-trans retinol (A_2). TBARS = thiobarbituric acid reactive substances. w/w = wet weight.

3.3.2 Mode of oil administration and oil dosage

In paper I and II, the experimental oils were administered via a nasoduodenal tube (Freka® Feeding Tube, Fresenius Kabi, GmbH, Germany). By aid of fluoroscopy and a stylet, the nasoduodenal tube was positioned with its tip to the middle part of the duodenum. The tube was secured with tape to the cheek. This mode of administration was chosen to ensure compliance considering the relatively high amount of oil self-administrated by the participants during the 10 days' trial period. On the first trial day, health personnel at the hospital demonstrated how to administrate the oils, via the tube, using a syringe. The participants kept the nasoduodenal tube inserted during the entire trial period. Below is a fluoroscopy image of a nasojejunal feeding tube, located with its tip at the ligament of Treitz (duodenal- and jejuna junction) (Figure 2), and not in the middle or distal part of the duodenum, as in the present thesis.

The patients with subjective food hypersensitivity in paper I and II received approximately 6.1 g of EPA+DHA from the 30 mL daily dosage of seal oil. The seal oil also provided a daily dosage of approximately 1.1 g of DPA. The dose of seal oil was chosen based on previous

studies where the same mode of administration and amount of seal oil was used with promising results and no reported harmful effects [90,91].

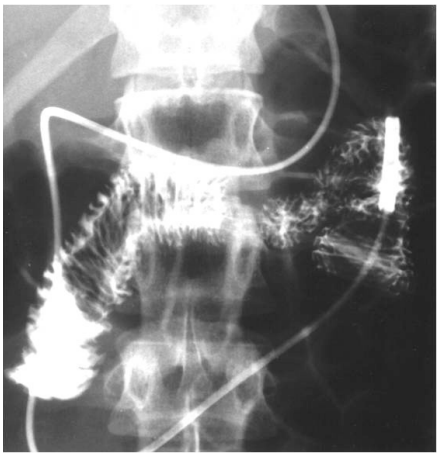

Figure 2. Fluorscopy image of an inserted nasojejunal feeding tube. With permission from Arnold Berstad.

3.3.3 Two-dimensional ultrasonography

Ultrasonography is established as a clinical method with high applicability in diagnostic and prognostic evaluations. Ultrasound is a non-invasive, cheap, radiation-free, user- and patient-friendly method allowing repeated and prolonged examinations [92]. Transabdominal ultrasound is useful in assessing gastric emptying, size, accommodation and contractile activity, and thickness of wall and diameter of small and large bowel [54,93,94]. The method has limitations when it comes to depth penetration, especially in obesity, and the presence of intestinal gas may impair image quality [95].

Scanning procedure

The patients were scanned while sitting in a chair, leaning slightly backward an angle of 120º between the thighs and the spine. 2D ultrasound images were obtained using a sector scanner (System Five, GE Vingmed Ultrasound, Horten, Norway) with a built in magnetometer-based position and orientation measurement (POM) device (Flock of Birds® Model 6D FOB., Ascension Technology Corp., Burlington, VT, USA). Each standardized image was frozen before being recorded on to videotape and stored on System Five ultrasound scanner. A proximal gastric area in a sagittal section was outlined by tracing from the top margin of the fundus and 7 cm downward along the axis of the stomach. Within 7 cm along the axis of the

outlined sagittal section of the proximal stomach, the maximal diameter was chosen as sagittal diameter. The sagittal section of antral area was measured by tracing the outer profile of the muscularis propria of the gastric wall. In those cases in which air pockets were present in the selected sonographic section, the outer border of the air pocket was traced. The measures were traced twice and the average result recorded. Three ultrasonographic measures were calculated at each recording time: proximal gastric area, frontal diameter and antral area. To assure compliance, all sonographic examinations and measurements were performed by Dr. Med. Gülen A. Lied.

3.3.4 Meal-induced symptoms

In paper I, a commercial meat soup (Toro® clear meat soup, Rieber & Søn A/S, Bergen, Norway) was applied for symptom provocation and evaluation of gastric accommodation. At each experiment, the subjects drank during 4 min 500 mL of the soup containing 1.8 g protein, 0.9 g bovine fat, 1.1 g carbohydrate and 0.2 g non-soluble seasoning (20 kcal totally). The soup was boiled and then cooled to 37 ºC before ingestion. In previous studies this soup meal induced fed state motility with approximately 3 antral contractions per min in over 85 % of patients and controls [93].

The participants were asked to score the meal-induced symptoms (epigastric pain, nausea, fullness, satiety and discomfort) on a 100 millimetre visual analogue scale (VAS), where zero denotes absence of symptoms and 100 denotes excruciating symptoms. This Norwegian version of VAS has previously been validated in terms of reliability, validity and sensitivity [96,97].

3.3.5 Questionnaires

Short Form Nepean Dyspepsia Index (SF-NDI)

The SF-NDI is a 10-item questionnaire with five subscales measuring the influence of dyspepsia on domains of health related QoL, namely tension, interference with daily activities, altered eating/drinking habits, knowledge/control over disease symptoms and interference with work/study, and each subscale contains two items. The scores were determined by measuring each item by a five-point graded Likert scale ranging from 1 (not at all or not applicable), 2 (a little), 3 (moderately), 4 (quite a lot) to 5 (extremely). The total sum score for QoL ranges from 10 to 50, and the sum score of each of the five subscales ranges

from 2 to 10. Higher scores indicate poorer QoL. The 10-item SF-NDI has been validated in patients with functional dyspepsia [98], as well as in patients with subjective food hypersensitivity [26].

Subjective Health Complaint (SHC) Inventory

The SHC Inventory includes 29 items concerning subjective, somatic and psychological complaints. The questionnaire contains five subscales: musculoskeletal pains (migraine, headache, arm pain, shoulder pain, neck pain, upper back pain, lower back pain and leg pain), gastrointestinal problems (gas discomfort, stomach discomfort, gastric ulcer, heartburn, diarrhoea, constipation and stomach pain), allergy (allergies, breathing difficulties, eczema and asthma), "pseudoneurology" (tiredness, sleep problems, dizziness, heath flushes, extra heartbeats, sadness/depression and anxiety) and flu (cold/flu and coughing). The scores were determined by rating each item by a four-point graded Likert scale ranging from 0 (not at all), 1 (a little), 2 (quite a lot) to 3 (severely).

Gastrointestinal Symptom Rating Scale (GSRS)

The questionnaire includes 15 items which are grouped into five subscales: abdominal pain syndrome (abdominal pain/discomfort, sucking sensation in the epigastrium, nausea and vomiting), reflux syndrome (heartburn and acidic regurgitation), indigestion (borborygmi, abdominal distension, eructation and increased flatus), diarrhoea (increased passage of stools, loose stools and urgent need for defecation) and constipation (decreased passage of stools, hard stools and feeling of incomplete evacuation). The scores were determined by rating each item by a 7-point graded Likert scale, and higher scores indicate more pronounced symptoms.

Ulcer Esophagitis Subjective Symptoms Scale (UESS)

The UESS was developed to examine the symptoms frequently experienced by patients with peptic ulcer and esophagitis. The questionnaire includes 9 items which are grouped into four subscales: abdominal discomfort (abdominal pain, sucking sensation), reflux discomfort (acid regurgitation, heartburn), intestinal discomfort (abdominal distension, borborygmi) and sleep dysfunction (difficulty falling asleep, insomnia and rested waking up). The scores were determined by rating each item by a 100 mm VAS, and higher scores indicate more pronounced symptoms.

3.3.6 Intestinal lavage

Intestinal lavage was performed to collect gut lavage fluid (paper III and IV) (Figure 3). The method is briefly described as following: by aid of a gastroscope, a nasoduodenal tube (Freka® Feeding Tube, Fresenius Kabi, GmbH, Germany) was positioned with its tip to the distal part of the duodenum. Two litres of isotonic polyethylene glycol solution (PEG, MW 3350, Laxabon®, Tika, Sweden) was administered through the nasoduodenal tube during 40 minutes using a peristaltic pump (Watson Marlow 505S/RL, Falmouth, England). The first clear fluid passed per rectum was collected and filtered through gauze, and a 4 mL aliquot was collected on tubes containing 0.5 mL of a solution with antiseptic and antiproteolytic activity prepared by adding 1 ml of 10% sodium azide (NaN_3) to 50 ml of soybean trypsin inhibitor (Sigma, Taufkirchen, Germany). The samples were stored at -80 °C prior to analysis.

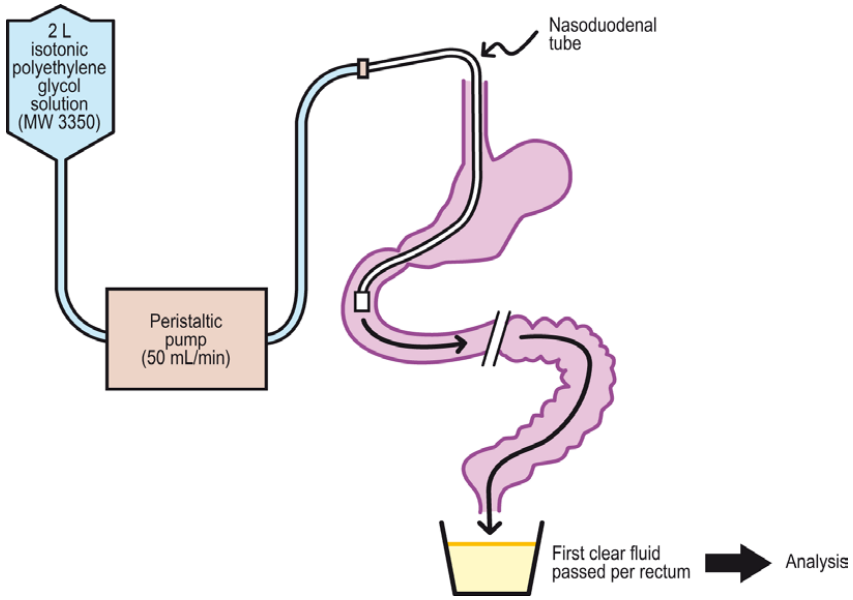

Figure 3. Illustration of the intestinal lavage procedure. Illustration by Jørgen Valeur.

3.3.7 Laboratory analysis

Gut lavage fluid was collected from patients with subjective food hypersensitivity (paper III-IV), patients with IBD and healthy controls (paper IV), according to procedure previously described [99], for analysis of 5-HT. The gut lavage fluid specimens were

thawed and centrifugated at 3000 rpm for 10 minutes at 4 ºC. The supernatant was collected and filtered by using a hydrophilic nylon membrane syringe filter, 4 mm diameter and 0.45 µm pore size (Chromacol Ltd, Trumbull, USA). Aliquots of 50 µl of the filtered supernatants were transferred into tubes containing internal standard (5-methoxytryptamine; 5-CH_3O-HT, Cat. No. 286583, Sigma-Aldrich Co), evaporated to dryness in advance. The tubes were vortex-mixed for 1 min, transferred to an autosampler vial and submitted to LCMS/MS for analysis of 5-HT. The sample preparation is schematically outlined in Figure 4.

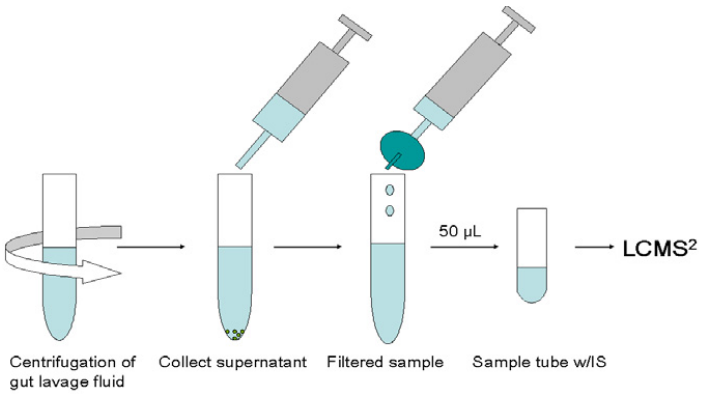

Figure 4. Schematic demonstration of sample preparation of gut lavage fluid.The samples were thawed and centrifugated, and the supernatant was collected and filtered by using a syringe filter, 0.45 µm pore size. Aliquots of 50 µl were added to test tubes containing IS, and after being vortexed, submitted to $LCMS^2$ analysis. Illustration by the author.

Fasting venous blood samples were collected from patients with subjective food hypersensitivity and healthy controls (paper IV) using vials with no added anticoagulants (gel vials), for the analysis of serum CgA. The blood samples were centrifugated and stored at -80 °C prior to analysis. Serum CgA was measured by enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (ALPCO Diagnostics, Salem, N.H., USA; cat. #43-CHRHU-EO 1.2).

The methyl ester fatty acid (FA) composition of total lipids in seal oil (paper I-II) and soy oil (paper I) were determined by gas liquid chromatography (GLC) at NIFES as described previously [100], with some modifications. In brief, total lipid content was extracted, filtered and evaporated, and thereafter the samples were saponified and the FAs were esterified in 20 % boron fluoride in methanol, and biological sample parallels were analysed. The methyl esters were separated using a gas chromatograph (Trace GC 2000),

equipped with a 50 m CP sil 88 (Chrompack) fused silicia capillary column (id: 0,32 mm), using "cold on column" injection, with a temperature programme of $60^{25°C/min}$ $160^{25°C/min}$ $190^{25°C/min}$ $220°C^{5min}$ and flame ionization detector. The FA profiles were calculated using an integrator (Turbochrome Navigator, Version 6.1), connected to the GLC instrument and identification ascertained by standard mixtures of methyl esters (Nu-Chek, Elyian, USA). The nonadecanoic acid (19:0) methyl ester was used as IS for quantification of FA methyl esters. Limit of quantification (LOQ) was 10 μg FA/g sample (wet weight, w/w).

TBARS were analysed in both seal oil (paper I-II) and soy oil (paper I) at NIFES by a modified in vitro method, measuring mainly malondialdehyde and other aldehydes, and secondary lipid peroxidation parameters [101,102]. Briefly, fat and water-soluble components are separated, while the analytes are being extracted in a methanol:water phase. An aliquot of the extract is added thiobarbituric acid (TBA) in excess and heated to form a coloured complex between aldehydes in the sample and TBA. The absorption at 532 nm was registered and TBARS were quantified by reference to an external standard curve in a spectrophotometer. LOQ was 3,9 nmol TBARS/g sample (w/w).

Vitamin A, i.e. sum retinol (13-, 11-, 9-cis and all-trans retinol, i.e. A_1) and 3,4 didehydro-all-trans retinol (A_2), were analysed in seal oil (paper I-II) and soy oil (I) at NIFES by a modified high performance liquid chromatography (HPLC) method [103,104]. In brief, the sample is saponified, while the unsaponified material is extracted, and analysed by a HPLC column (HICHROM 4,6 × 150 mm, LC-SI, 3 μm, Teknolab A/S) using an ultra violet (UV)-detector (Thermo Separations products, UV1000, Intrument-teknikk AS), with reference to an external standard curve. LOQ in oils was 280 ng vitamin A_1/g sample and 460 ng vitamin A_2/g sample, both w/w.

Vitamin E was analysed in seal oil used in paper I and II. The analysis was performed at NIFES by HPLC, based on the principles reported by the European Committee for Standardization (CEN) [105]. Briefly, the sample is saponified, while the unsaponified material is extracted. Alpha-, beta-, gamma- and delta-tocopherol isomers were determined using a HPLC column (LiChroART, 4,6 × 125 mm, Purospher STAR Si, 3 μm, Merck) equipped with a fluorescence detector (TSP, FL3000, Spectra system) and quantified by an external standard curve. In the present study, only α-tocopherol was reported. LOQ in oils was 500 ng α-tocopherol/g sample (w/w).

Vitamin D was analysed in soy oil at NIFES by HPLC as described earlier. Briefly, sample material is saponified and the unsaponified material is extracted before clean-up on a preparative column (HICHROM, Kromasil silicia, 5 µm, 4.6 x 250 mm). The fractions with vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) are collected, evaporated and dissolved in methanol, before injected on an analytical reverse phase column (Ace 5 C18, 5 µm, 4.6 × 250 mm). Vitamin D_3 was determined by UV-detector (LaChrom, Merck HITACHI L-7420) and quantified using vitamin D_2 as IS. LOQ was 1 µg vitamin D_3/100 g sample (w/w).

3.3.8 Liquid chromatography tandem mass spectrometry

A Liquid chromatography tandem mass spectrometry (LCMS/MS) system was used for method development (paper III) and analysis and quantification of 5-HT in gut lavage fluid (paper IV). The LCMS/MS used in the present study was an Agilent 1100 series LC/MSD trap, SL model with an electrospray interface (ESI), a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector and 25 µl injection volume. The column, a Zorbax Eclipse-C_8 RP 150 × 4.6 mm, 5 µm (Agilent Technologies. Palo Alto, CA, USA) is a double endcapped column 100 % water compatible which can be operated at maximum pH range of 2-9 and provides improved basic peaks of amines compounds (such as 5-HT) due to the polar groups embedded in the stationary phase. The column was kept in the column compartment at 20 °C and the solvent system in gradient mode consisted of water with formic acid 0.1 % v/v (A) and acetonitrile (B) and UV detection at 254 nm and 0.2 mL/min flow rate. The initial condition 100 % of A was ramped to 35 % of A in 20 min, returned immediately to 100 % of A in 5 min and held there for 5 min. Nitrogen was used as nebulizing and drying gas at 300 °C. The ESI source was operated in positive ion mode and the ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option with a resolution of 13000 *m/z*/sec (FWHM/*m/z* = 0.6-0.7). Complete system control, data acquisition and processing were done using the ChemStation for LC/MSD version 4.2 from Agilent. The transitions monitored were 177 → 160 *m/z* for 5-HT and 191 → 174 *m/z* for IS, 5-methoxytryptamine (IS). The magnitude of the signals was recorded in ion counts per second (icps). A representative ion chromatogram of a gut lavage fluid sample is shown in Figure 5.

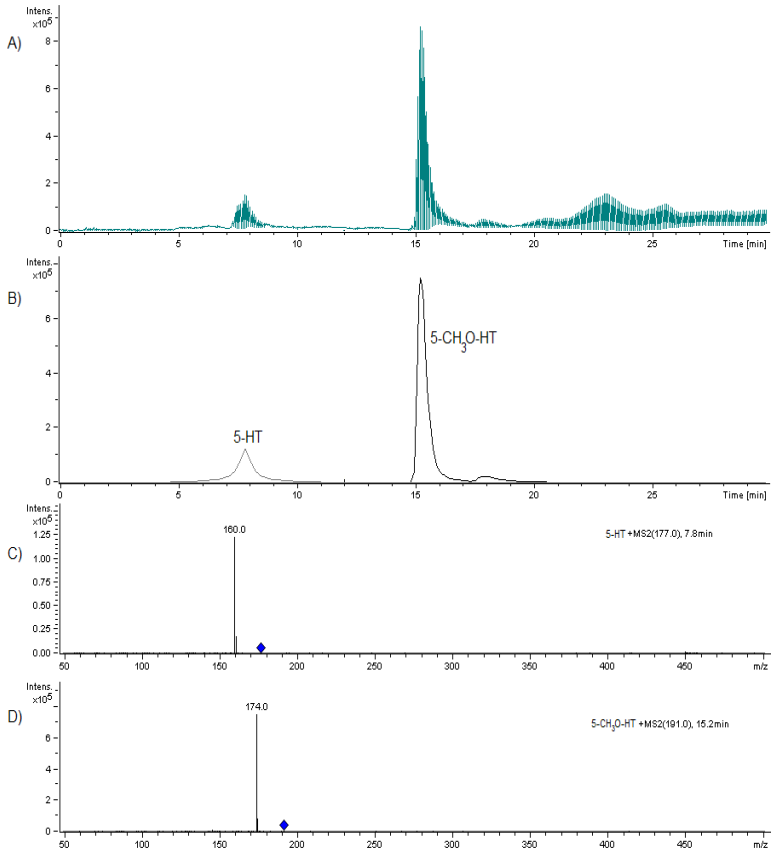

Figure 5. A) Representative ion chromatogram of one of the GLF sample. B) Extracted ion chromatograms at 160 and 174 *m/z* obtained by fragmenting $[5\text{-HT+H}]^+$ and $[5\text{-CH}_3\text{O-HT+H}]^+$ signals 177 and 191 *m/z* respectively. C and D) MS/MS product ion spectra for 177 and 191 *m/z*, respectively. Figure processed by ChemStation for LC/MSD version 4.2, Agilent.

3.3.9 Selection of optimal amount of 5-CH_3O-HT

A uniform shell design proposed by Doehlert [106] was applied to model the relationship between 5-HT and 5-CH_3O-HT, in terms of linearity of the detector response (paper III). The main characteristics of this design are:

- The number of experiments is calculated by the general expression $X^2 + X + 1$ where X represents the number of variables studied. For instance, to investigate the effect of two variables ($X = 2$) seven experiments will be required.

- The experiments are allocated at the centre and at the vertexes of a hexagonal shape, circumscribed in a circle of radius 1, in this way the variances of the estimated responses are the same at all points on the circle centred at the origin.
- The variables are codified and equally spaced between -1 and 1 and by using basic geometric it is possible to determine the coded combination of the variables at each vertex of the hexagon. Figure 1 in paper III described how the coded levels of the variable x_1 (represented by question marks) are calculated by using Pythagoras' theorem.
- The variables are simultaneously investigated at different numbers of levels. Figure 1 in paper III shows how the coded variables x_1 and x_2 are studied at 3 and 5 levels (-0.866, 0, 0.866 and -1, -0.5, 0, 0.5, 1), respectively. The coded levels are then converted into µg/mL values.

The application of this design in the determination of the optimal amount of IS to be used in the quantitative determination of 5-HT was as follows:
Test tubes containing seven different concentration ratios of 5-HT:IS in 50 µl of ethanol were prepared in triplicates (7 × 3 = 21 tubes) and evaporated to dryness under a stream of nitrogen at room temperature. A gut lavage sample with no detectable levels of 5-HT was thawed at room temperature, and prepared as described above. Aliquots of 50 µl of the filtered supernatant were added into the aforementioned 21 test tubes. The tubes were vortex-mixed for 1 min, transferred to an autosampler vial and submitted to LCMS/MS analysis. A GLF blank sample spiked with 3 µg/mL of 5-HT and 16 µg/mL of IS was prepared in triplicate to check the prediction capability of the model to be proposed. The analytical signals recorded with this additional solution along with those obtained by applying the uniform shell design, were used for modelling purposes.

3.4 Statistics

All statistical calculations and graphic designs were performed using the GraphPad Prism statistical software package (GraphPad Prism version 4.00 and 5.00 for Windows, GraphPad Software, San Diego, USA). In general, results are given as means with standard deviations or standard errors, as indicated. Differences between means were evaluated by t-tests or ANOVA, and between proportions by chi square tests. Regression analysis in paper III was

done by Statgraphics Plus 5.1 software package (Statistical Graphics Corp., Herndon, USA).

3.5 Ethics

The study trials were performed according to the Declaration of Helsinki, after approval from the Regional Committee for Medical Research Ethics and written informed consent from patients and controls. The collection of the gut lavage fluid from the patients was part of the routine examination, and the samples were stored in an approved biobank at HUH prior to analysis. When analysed, the gut lavage fluid samples were stored in an approved biobank at NIFES (paper III). The gut lavage fluid samples obtained from patients and used during method development in paper IV were approved for research. The blood samples obtained from patients and controls in paper IV were stored in an approved biobank at HUH.

4. Summary of results

4.1 Paper I

Effects of seal oil on meal-induced symptoms and gastric accommodation in patients with subjective food hypersensitivity: a pilot study

In the single dose experiment, 10 patients and 10 healthy volunteers received 10 mL duodenal administered seal oil or soy oil. The gastric accommodation of the proximal and distal stomach, examined by 2D ultrasonographic scanning, and meal-induced abdominal symptoms, assessed by VAS, were not significantly influenced by single doses of seal or soy oil.

In the short-term treatment study, 24 patients were randomly allocated to 10 days' treatment with either 10 mL of seal or soy oil, self-administrated through a nasoduodenal feeding tube, 3 times daily. Seal oil, but not soy oil, reduced total symptom score significantly ($p = 0.03$). However, the symptomatic improvement was not associated with improvements in gastric accommodation.

4.2 Paper II

Seal oil in patients with subjective food hypersensitivity: An open explorative pilot study

Twenty-six patients received 10-day open treatment with seal oil, 10 mL self-administrated three times daily. Before and after treatment, and 1 month post-treatment, patients filled in the SHC Inventory for non-gastrointestinal symptoms in addition to the SF-NDI for evaluation of QoL, the UESS score and the GSRS for gastrointestinal symptoms. Total sum score of the SHC Inventory was significantly reduced between before and after 10 days' seal oil treatment ($p < 0.0001$), and between before and 30 days after ended seal oil treatment ($p = 0.0008$). Total sum score of the SF-NDI was significantly reduced from baseline both at 10 days ($p < 0.0001$) and at 30 days post-treatment ($p = 0.0008$). The decrease from baseline for the total sum score of the GSRS and the UESS, were significant at 10 days, $p = 0.007$ and $p < 0.0001$, respectively, and at 30 days post-treatment, $p = 0.02$ and $p = 0.0006$, respectively. Thus, both non-gastrointestinal and gastrointestinal complaints were significantly improved from baseline both at 10 and 30 days post-treatment.

4.3 Paper III

Direct determination of serotonin in gut lavage fluid by liquid chromatographic ion trap tandem mass spectrometry

A new method for direct determination of serotonin in gut lavage fluid was established. The method involves addition of 5-methoxytryptamine (5-CH3O-HT) IS, centrifugation, filtration and injection of the sample supernatant in a liquid chromatographic system coupled to an ion-trap tandem mass detector. Electrospray in positive mode was used to isolate and fragment the protonated ions $[5\text{-HT+H}]^+$ and $[5\text{-CH3O-HT+H}]^+$ signals 177 and 191 *m/z* respectively. Different response factor values were estimated by using a multivariate model, suggesting that the interaction 5-HT/IS is an important factor that affects the validity of the RF and consequently the accuracy of 5-HT determination. The systematic and simultaneous experimental design applied on the entire 5-HT concentration range (2 – 12 µg/mL), resulted in a constant RF of 0.040 and a IS concentration of 2 µg/mL to perform a reliable 5-HT quantification.

4.4 Paper IV

Subjective food hypersensitivity: assessment of enterochromaffin cell markers in gut and blood

Analysis of 5-HT was performed in gut lavage fluid from patients with subjective food hypersensitivity (n = 67), patients with IBD (n = 23) and healthy controls (n = 17). Blood samples for CgA analysis were obtained from 30 patients with subjective food hypersensitivity and 34 healthy volunteers. Serum levels of CgA were significantly lower in patients with subjective food hypersensitivity compared to healthy controls (p = 0.04). No significant difference in gut lavage 5-HT was detected between patients with subjective food hypersensitivity, patients with IBD and healthy controls.

5. General discussion

5.1 Patients

The patients in the present study attributed their gastrointestinal complaints to the ingestion of food, and many believed to suffer from food allergy or non-allergic food hypersensitivity. However, immune-mediated food allergy was not detected, and only occasional cases of non-allergic food hypersensitivity could be verified. The high discrepancy between perceived food hypersensitivity and confirmed food allergy, by current available diagnostic methods, is in accordance with findings in previous studies [26,33]. Despite extensive medical work-up, the patients' gastrointestinal complaints remained unexplained, and we therefore denoted the patients' condition as 'subjective food hypersensitivity' in the present thesis. The patients' gastrointestinal complaints complied with a FGID; in fact, as many as 90 % of the patients who participated in our studies have IBS, and some few FD, according to Rome II criteria.

In addition to gastrointestinal complaints, the patients with subjective food hypersensitivity also reported multiple non-gastrointestinal complaints, like musculoskeletal complaints, fatigue and sleep disorders, and also psychological problems like anxiety and depression were frequently reported. This co-morbidity of gastrointestinal and non-gastrointestinal complaints emphasizes the complex nature of this patient group. The existence of subgroups seems very likely, but to designate the patients to defined groups will be challenging due to a comprehensive and multifactorial symptomatology.

There was a clear predominance of women in the present study (over 90 %). This gender difference is consistent with other studies on patients with subjective food hypersensitivity [26,27]. In general, the prevalence of FGID, mainly IBS, seems to be higher in women, at least of those who seek health care services [107,108]. There is evidence to support a gender-related difference in pathophysiology and management of FGID [109], however, it is unknown whether the differences are disease-specific, or more a reflection of general gender differences.

5.2 Oil administration and dosage

In the present thesis, the seal oil was administered via a nasoduodenal tube (paper I and II). Although installation is an invasive and cumbersome procedure, the treatment was remarkably well tolerated and largely without side-effects in patients with subjective food

hypersensitivity, and the mode of administration secured nearly perfect compliance (according to the patients' verbal reports). The targeting delivery into the small intestine may also reduce potential gastroesophageal reflux, nausea and vomiting [110].

The therapeutic benefit observed following this short-term strategy suggests that a rapid booster effect may be achieved by duodenal administration of seal oil [90,91,111,112,113]. No direct comparative study of oral versus duodenal administration of marine oils for pain relief exists, but it is worth noticing that 14 days' orally administrated seal oil showed no significant effect in patients with IBD or psoriatic arthritis [114,115]. The fact that EPA and DHA most likely represent the main bioactive component of marine oils [116], has lead to an increased use of highly purified LC n-3 PUFA capsules, e.g. free fatty acids or ethyl esters. Capsules may be a good alternative in long-term trials, especially when it comes to blinding. Interestingly, capsules containing free LC n-3 PUFA were not effective in patients with Crohn's disease [117].

In the present study (paper I + II) natural triacylglycerol (TAG) oil was used, and the daily dosage of 30 mL provided a relative high dose of EPA + DHA (6.1 g/day), in addition to 1.1 g/day DPA. A dosage of 2.7 g/day EPA + DHA gave a moderately anti-inflammatory effect in long-term treatment of rheumatoid arthritis [118]. Thus, 6.1 g/day of n-3 LCPUFAs was considered to be a proper dose in the present study. Duodenal lipid infusion has been reported to exacerbate symptoms in patients with FGID, like FD [119,120], but the duodenal infused seal oil in the present study did not reproduce or worsen the patients' symptoms. On the contrary, the patients experienced attenuation of their symptoms.

5.3 Effect of seal oil on gastrointestinal complaints

Gastrointestinal complaints constitute a large part of the symptomatology in subjective food hypersensitivity. The patients' gastrointestinal complaints usually manifest after ingestion of a meal, with emphasis on abdominal pain/discomfort, nausea, diarrhea and sensation of being bloated and full. Following a 10-day intraduodenal administration of seal oil in the present study, gastrointestinal complaints were significantly attenuated (paper I and paper II). The mechanisms of action may be due to the anti-inflammatory and immune-modulating properties of the long chain n-3 PUFAs. The anti-inflammatory effect is supposed to be brought about by modulation of the amount and types of eicosanoids produced via the COX and LOX enzyme pathways. Consistently, plasma levels of pro-inflammatory PGE_2 was reduced in response to 10 days' treatment with duodenal administered seal oil in patients with

IBD-related joint pain [112]. Unfortunately, the present study did not include analysis of PGE_2, due to logistical problems on the lab. However, we conducted a small trial where four patients with subjective food hypersensitivity ingested 20 mL seal oil per day for four weeks, and PGE_2 analysis by LCMS/MS was performed (results not published, Figure 6). The protocol applied for the analysis of PGE_2 was developed at NIFES [121]. Interestingly, we found that PGE_2 level in plasma decreased following four week per-oral seal oil treatment (Figure 6). Although this result is based on only 4 patient samples, it indicates that an effect is measurable after short-term seal oil treatment. Placebo effects are considered to be strong in patients with subjective food hypersensitivity [122,123], but taken together with the findings noted above [112], it is reasonable to say that the positive effects of short-term seal oil treatment reported in the present thesis cannot solely be ascribed a placebo effect. The fact that 10 days' duodenal soy oil treatment did not have any effect on meal-induced gastrointestinal symptoms (paper I), supports the possibility that the positive effect is conserved for seal oil due to its contribution of n-3 LC-PUFAs which interrupt specific receptor-mediated signaling pathways involved in pain perception. By virtue of its high content of n-6 LA, soy oil will induce PGE_2 production, while the n-3 LCPUFAs, especially EPA, from seal oil will act inhibitory.

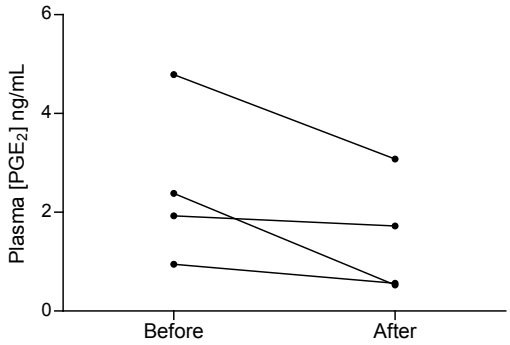

Figure 6: Plasma PGE_2 level (ng/mL) measured in patients with subjective food hypersensitivity before and after ingestion of seal oil (20 mL/day for 4 weeks). Data are given as individual values. *P*-value = 0.09.

In general, increased levels of PGE_2 mediate typical aspects of inflammation, including pain, edema and fever. PGE_2 is found to play a direct role in pain perception through interactions with the EP1 receptor [124]. Low-grade inflammation of the intestinal mucosa has been proposed to play an important role of the etiology in IBS [43]. Spiller et al., found increased number of inflammatory cells in the colonic and ileal mucosa of IBS patients [125], and

recently, significant elevated levels of PGE_2 and LTB_4 in patients with IBS were reported [126]. These findings support a mild activation of the immune system, in at least a subset of IBS patients. It is also interesting the findings of Lillestøl and colleagues, who reported increased intestinal permeability and increased duodenal IgE-bearing mast cells, which significantly correlated with serum total IgE levels, in patients with subjective food hypersensitivity [127]. In addition, Arslan Lied and colleagues, recently reported increased concentrations of B-cell activating factor (BAFF) in blood and in gut lavage fluid, and increased serum CD38 levels, which are released from monocyte-derived dendritic cells [128]. Hence, all findings regarding increased number of "IgE-armed" mast cells, BAFF and dendritic cells support the notion of immune activation in patients with subjective food hypersensitivity [129].

The visceral sensory system may be sensitized by inflammation, leading to lowered threshold to stimuli, which may lead to intestinal motor abnormalities and abdominal pain and discomfort, which are reported by a large proportion of patients with IBS. Especially, there are indications that patients with PI-IBS are good candidates for developing low grade mucosal inflammation [130]. Even though the clinical relevance needs to be further explored, these interesting findings indicate an up-regulated COX and LOX activity, and also that the fatty acid profile may be critical to the symptom generation in IBS**.** NSAIDs are known effective drugs in inflammation and pain management, but the analgesic agents are associated with troublesome side-effects. The n-3 LCPUFA found in seal oil, and also fish oil and fatty fish, act as natural inhibitors and modulators of both the COX and LOX pathways, thus they have the potential to modify pathology in diverse inflammatory conditions, free of adverse side-effects [118].

Visceral hypersensitivity is considered a hallmark of the pathogenesis of FGID, and the term refers to an increased sensation of stimuli originating from the abdomen, and both central and peripheral sensitization may be important factors. Central sensitization is believed to be due to an increase in the excitability of central neurons, so that normal sensory input from the bowel is manifested as abnormal peripheral responses. Such change in central processing has been suggested to be involved in IBS [108]. The mechanisms of visceral hypersensitivity are not fully understood, but both stress and inflammation are believed to be implicated in the triggering of pain and discomfort observed in FGID, like IBS, possibly through vagal afferent nerve activity. PGE_2, a potent mediator during inflammatory response, is believed to act as a sensitizer especially in pain sensation. The vagus nerve is an important communicator of the

brain-gut interaction, and is increasingly recognised to be involved in control of immune responses. Dietary fat can affect the vagus nerve by inducing release of cholecystokinin (CCK) that binds to CCK-receptors located centrally or on peripheral vagal afferents. Activation of CCK-receptors triggers vagal efferents which lead to an increase of acetylcholine, which by binding to appropriate receptors, inhibit release of pro-inflammatory cytokines like TNF-α and IL-6 from immune activated macrophages. Based on these findings, high fat enteral nutrition has been suggested as potentially therapeutic in various inflammatory disorders [131].

In the present thesis, the patients reported discomfort in response to ingestion of 500 mL of dilute meat soup, suggesting visceral hypersensitivity to be part of the pathophysiology in patients with subjective food hypersensitivity (paper I). It may also indicate that the patients' perception of being food hypersensitive might be less specific for particular food items than often self-reported. Impaired gastric accommodation has been suspected to cause sensation of gastric pain, fullness and early satiety in patients with subjective food hypersensitivity. Considering the bolus dosage of 10 mL duodenal administered seal oil in the present study, we anticipated that the symptom improvement reported by the patients would be associated with improvements in gastric accommodation, due to the effect of LC-PUFAs, through increased CCK secretion, on gastric tone. On the contrary, no such improvements in either proximal or distal (antral) gastric accommodation, assessed by 2D ultrasonographic scanning, were observed. Thus, impaired gastric accommodation most likely plays a limited role of the aetiology in subjective food hypersensitivity. The gastrointestinal discomfort reported by the patients following the test meal, and the consecutive symptom attenuation after short-term treatment with seal oil (paper I) may suggest visceral chemosensitivity rather than visceral hypersensitivity to mechanical distension, which is characteristic in FD. In a study using a post-inflammatory mouse model, altered chemosensitive responses was found, suggesting vagal chemosensitive afferents to play a central role in post-inflammatory visceral hypersensitivity, like PI-IBS [132]. Whether visceral chemosensitivity mechanisms are present in subjective food hypersensitivity, at least in a subset of the patients, warrants further investigation.

In paper II, we found that baseline, or customary, gastrointestinal complaints were significantly improved following 10 days' seal oil administration, as assessed by the GSRS and the UESS questionnaires. Interestingly, the patients consistently reported improvement of gastrointestinal complaints at 30 days post-treatment compared to baseline as well. A major

limitation of this pilot study is the lack of control, and the possibility of placebo effect is highly present. However, as seal oil previously improved gastrointestinal complaints in this patient group compared to soy oil (paper I), we wanted to first perform an open pilot study to examine if positive effects in non-gastrointestinal complaints could be anticipated (discussed below). The apparent beneficial effect of short-term duodenal seal oil administration on gastrointestinal complaints reported by the patients in paper I and paper II, may be brought about by the n-3 LC-PUFAs inhibitory effects of the COX and LOX systems. Especially considering the findings reported by Lillestøl *et al*. [127] and Arslan Lied *et al*. [133] which indicate immune activation in patients with subjective food hypersensitivity, it is likely that inhibition of COX activity and subsequently reduction in PGE_2 can attenuate pain sensation.

Another effect of the n-3 fatty acids may be via increased release of CCK, which may reduce immune response via actions on peripheral vagal afferents. Indeed, LC fatty acids have been found to be a more potent stimulus to CCK secretion, compared to medium-chain fatty acids [119]. The fact that the attenuation of the patients' complaints was maintained 30 days post-treatment, could indicate that the effect cannot be assigned exclusively to a placebo effect. But further controlled studies are highly warranted for confirmation of results.

Functional alterations of the gut flora, so-called dysbiosis, has recently been suggested as a key pathogenetic mechanism of several conditions, both extra-intestinal, such as metabolic syndrome, allergy and autism, and intestinal, like IBD and IBS [134,135]. Early life stress results in an altered brain-gut axis, as shown in a mouse model, suggesting this as a valuable model for investigating stress-related disorders including depression and IBS [136]. In man, enteric infections, antibiotic usage, and stress may disturb the indigenous gut flora and predispose to IBS [137]. Bloating and perception of increased gas production are indeed common in subjective food hypersensitivity and IBS, and the complaints most often arises after eating. Small intestinal bacterial overgrowth (SIBO) may explain the pronounced bloating, due to increased hydrogen excretion. In addition to bloating and distension, visceral hypersensitivity, motility disturbances, autonomic dysfunctions and immune activation are frequently observed in IBS, and may be understood as a consequence of the host response to SIBO [138]. Indeed, Pimentel et al., showed that eradication of bacterial overgrowth considerably reduced IBS symptoms [139]. Elimination of the hydrogen produced during bacterial fermentation depends primarily on methanogenic and sulphate-reducing bacteria that convert hydrogen to methane or hydrogen sulphide [140]. Intriguingly, EPA capsules for seven days have been found to reduce total breath hydrogen excretion after challenge with

lactitol, another unabsorbable but fermentable carbohydrate [141]. EPA contains five double bonds which potentially can be saturated during bacteria metabolism, and thus offer a salvage route for excess hydrogen [141]. Whether tube-administered seal oil, rich in EPA, could influence microbial metabolism is yet not known. The n-3 PUFA has been found to promote adhesion of gut bacteria to mucosal surface, which is pivotal for the beneficial effects of probiotics. Indeed, administration of PUFAs increased the number of *Lactobacillus paracasei* adhering to jejunal mucosa [142]. Also, a recently study found lower content of lactobacillus in n-3 depleted mice compared to mice given an n-3 rich diet [143]. Thus, PUFAs have been suggested to enhance the effectiveness of probiotics.

In SIBO, colonic bacteria will arise in the small intestine and exert its actions there. In contrast to methanogenesis, which produce non-toxic CH_4, dissimilatory sulphate reduction produces highly toxic H_2S. SRB utilize several fermentation products, butyrate being one of the main substrates. Butyrate is also a major factor to maintain a healthy and functioning epithelium barrier, protecting the intestinal mucosa against inflammation and cancer [144]. Indeed, the mucosa itself contains highly sulphated mucins that serve as substrate for SRB. These actions of SRB may compromise intestinal barrier function, and increase intestinal permeability which may cause hypersensitive responses to foods, and also to bacterial endotoxins, like lipopolysaccharide (LPS). Interestingly, increased intestinal permeability has been reported in patients with subjective food hypersensitivity [127], but this needs to be further investigated to elucidate possible pathogenic mechanisms.
In the present study, the bolus dosage of 10 mL duodenal administered seal oil, may act as a protective layer floating on-top of the mucosa, in the same way as hydrophobic phospholipids. This shielding effect is considered an important mechanism in mucosal defence [145].

The role of dietary constituents in FGID is unclear, and a causal relationship between onset of symptoms and ingestion of particular food items is often not unambiguously confirmed. But considering the high proportion of patients who attribute their complaints to foods, dietary factor is most likely implicated in the symptomatology. Milk, bread, cereals and fruits are most often recognized as the culprits. Indeed, many patients experience relief of symptoms when excluding the suspected food item from their diet. In a recent study, Biesiekierski et al. showed that gluten can provoke IBS symptoms in subjects without celiac disease [146] . Previously, we have shown that ingestion of lactulose, an unabsorbable, but fermentable carbohydrate, may replicate the gastrointestinal symptoms in patients with subjective food hypersensitivity [45], and Gibson et al., found a number of poorly absorbed short-chain

carbohydrates to trigger IBS-like symptoms [46]. Thus, a low FODMAP diet may be effective in patients with IBS. FODMAPs do not represent the underlying cause to a functional disorder, but it constitutes an opportunity for reducing symptoms. It is important to remember that ingestion of FODMAPs is normal and tolerated by most people, and that symptoms will be generated only when underlying bowel response is exaggerated or abnormal [46].

Several recent observations suggest a component of malabsorption in patients with IBS. Thus Morken et al. and Berstad et al., found increased faecal fat excretion in around one forth of patients with IBS and food hypersensitivity [147,148]. Consistently, Solakivi et al., found decreased levels of PUFA in blood in patients with IBS and suggested that intestinal malabsorption of fatty acids could be the reason [149]. An unbalance of n-3 PUFAs have been shown to modify the gut microbiota, were low level of n-3 PUFA most likely lower its metabolic activity [143]. An experimental study by Pachikian and colleagues showed that malabsorption could be related to altered microbial fermentation [143]. They even suggested that such fermentation is related to endotoxemia, a potentially very important cause of systemic symptoms in these patients. Further studies of the potential pathological role of intestinal dysfunction in IBS are highly warranted.

5.4 Effect of seal oil on non-gastrointestinal complaints

Non-gastrointestinal, systemic complaints are prevalent in patients with subjective food hypersensitivity (paper II). In the present thesis, besides high scores on gastrointestinal problems, the patients presented high scores of SHC from different organ systems, as assessed by the SHC Inventory. Musculoskeletal complaints, tiredness/fatigue, sleep problems and anxiety were the most prominent non-gastrointestinal complaints (paper II). Unexplained, gastrointestinal complaints like abdominal pain, bloating and diarrhea may be considered as more defined and ‘genuine’ complaints than the more diffuse non-gastrointestinal complaints. But based on our findings, non-gastrointestinal complaints represent a problem of high relevance, and the high proportion of patients with subjective food hypersensitivity that suffers from SHC, especially musculoskeletal complaints and tiredness/fatigue, suggests that SHC and subjective food hypersensitivity may share pathophysiological mechanisms. Thus it might be valuable to assess SHC in future studies. Patients with subjective food hypersensitivity and IBS have previously been found to have impaired QoL [150,151]. The patients in the present thesis also reported considerably impaired QoL. Unfortunately, we did

not have a control group in this explorative, open study (paper II), but the baseline total sum score of the QoL (28.0) reported in the present thesis [152] is comparable with that reported in the study of Arslan and colleagues (27.4) [26], were the QoL of patients with SHC was significantly impaired compared to controls (13.5). Considering the patients' complex symptomatology, including both gastrointestinal and systemic symptoms, and the fact that their complaints often remain unexplained, impaired QoL was highly expected in the present thesis.

It is generally assumed that psychological factors, including anxiety, depression and stress, aggravate gastrointestinal disorders and play important roles in the pathogenesis of unexplained medical symptoms. However, Lind and colleagues have recently shown that psychological factors such as symptom-specific and general anxiety, and depression could explain only approximately 10 % of the variance in the patients' symptom severity; hence, 90 % of the variance remained unexplained [33]. This also suggests psychological factors to be secondary as a consequence of long-lasting somatic complaints. Consistently, many patients experience that a psychological explanation model is put forward in absence of somatic pathology. A diagnosis of psychosomatic disorder is therefore often not well accepted, and many patients tend to seek alternative medicine when not receiving adequately medical care.

Sensitization theories, like cognitive sensitization or cognitive bias, have been suggested to account for the development of, at least in part, medically unexplained conditions, and such psychobiological mechanisms could be involved in subjective food hypersensitivity as well. The patients are often constantly worried with heightened vigilance for specific symptoms, which may unreasonably activate their cognitive sensitization networks, leading to over-reporting of somatic sensations and misattribution of bodily symptoms. Cognitive bias, which is considered as a higher form of cognitive-emotional sensitization, may be the underlying neurobiological substrate for SHC reaching the level of somatisation [153]. Concerns and modern health worries (MHW) (e.g. air pollution, additives in food, X-rays, chemicals in household products, etc.) have been associated with SHC and rise in health care use. MHW may lead to misinterpretation of everyday complaints as physiological consequences of environmental factors, and this is often exacerbated by media's overemphasis of high-risk and disease-related stories [154]. Although, patients with food hypersensitivity report considerably more SHC than healthy controls, Lind and colleagues found that sum score on the MHW scale did not significantly differ between the patients and

controls [27]. Thus, concerns for modern living do not explain the high prevalence of SHC in patients with subjective food hypersensitivity.

In the present thesis, the short-term treatment with seal oil significantly improved the patients' non-gastrointestinal complaints from baseline, and the improvement maintained significant 30 days post-treatment. It is reasonable to believe that the beneficial effect of the seal oil treatment can be explained by the involvement of the n-3 LC-PUFAs in pain attenuation by inhibiting PGE_2 production. Indeed, PGE_2 is known to be involved in pain sensitization [155]. A possible placebo response cannot be ignored when interpreting the results from the seal oil studies (paper I+II). Still, the prolonged effect of the seal oil treatment, on both SHC and gastrointestinal complaints, is intriguing, and may indicate that the positive effect of seal oil is not alone an outcome due to placebo response. To confirm and further elucidate the findings objective measures must be included in further controlled studies.

Medical unexplained symptoms, arising from different organs, often occur together, and may have common underlying pathology. Central sensitization is believed to be responsible for generation of symptoms in fibromyalgia [129]; a condition characterised by widespread chronic pain mainly of musculoskeletal origin, and also gastrointestinal complaints. In fact, of those patients with fibromyalgia, 30 % to 70 % have concurrent IBS [156]. Interestingly, in a recently published study, Berstad and colleagues found that 71 % and 85 % of patients with IBS and subjective food hypersensitivity reported symptoms suggestive of fibromyalgia/and or chronic fatigue, respectively. Thus, it is likely to believe that these multi-symptom conditions may have a common underlying cause. Multiple unexplained symptoms are often associated with psychosomatic models. But, more likely, psychological disturbances are a consequence rather than a cause. The story of the peptic ulcer disease comes to mind here; when eliminating the microbial factor (*Helicobacter pylori*), both psychological and somatic complaints disappeared [157].

5.5 The role of enterochromaffin cells

The secretory EC cells play an important role of the enteric neuroendocrine system in maintaining normal gut function. CgA and 5-HT are major secretory products which may reflect activation of the neuroendocrine system. Due to inconsistent and limited data, the association between CgA and gastrointestinal pathophysiology, and the relevance of CgA as a biomarker for disease activity in IBS is still unclear. Elevated serum levels of CgA have been

reported in patients with PI-IBS [80]. However, the density of CgA+ cells in duodenal and colonic mucosa has been decreased in IBS patients [158].

In the present study (paper IV), serum CgA was significantly lower in the patients with subjective food hypersensitivity compared to the healthy controls, which corroborates with our previous results [81]. Sidhu et al., have reported abnormally elevated levels of serum CgA in a small proportion of their included patients with diarrhea predominant IBS [80]. However, they did not include healthy controls and in many of the patients with elevated CgA, the levels declined with time, which the authors explained with short-lived EC cell hyperplasia, as previously reported by Dunlop et al. [159]. Thus, it would be interesting to perform repeated measurements over time of both CgA and 5-HT in future studies.

CgA serves as a pro-hormone for shorter peptide fragments with regulatory properties [160]. Fragments of CgA exert anti-microbial effects and may modulate gastrointestinal motility, sensitivity and barrier function [161,162]. The low levels of CgA demonstrated in the present study could be a consequence of alterations in the gut microbial flora in patients with subjective food hypersensitivity [163,164]. Intriguingly, Dlugoz et al., have recently demonstrated *Chlamydia trachomatis* antigens in enteroendocrine cells and macrophages of the small bowel in patients with IBS [165]. Although the clinical significance is yet unknown, this is an exciting finding that together with the present finding of low serum levels of CgA could imply an impairment of EC cell function in patients with subjective food hypersensitivity and IBS. El-Salhy and colleagues demonstrated decreased CgA-positive cells in biopsies from patients with IBS [158]. Thus, a decrease in number of CgA secretory cells could be a reasonable explanation for the low serum CgA found in the present study. This should be looked into in future studies.

Interestingly, increased levels of plasma somatostatin have been reported in IBS [166]. Also, infusion of somatostatin analogues has been found to decrease plasma CgA [167]. Somatostatin suppresses the release of several gastrointestinal hormones which affects motility and intestinal secretions, among others. Hypothetically, abnormal high secretion of somatostatin could account for the decreased level of serum CgA observed in our patients. Potential role of somatostatin in subjective food hypersensitivity should be further investigated.

A recently published study by Öhman et al demonstrated increased fecal levels of CgA and secretogranins II and III (Sg II and Sg III) in patients with IBS [168]. The authors reported a negative correlation between the fecal levels of CgA, SgII and SgIII with colonic transit time, but the association between fecal granins and symptoms were weak. Also, SgII

was found to have good discriminative validity to positively identify IBS. This study adds to the body of evidence that there is a gastrointestinal component in the aetiology of IBS, but whether the granins represent a cause of the pathopysiology or merely reflect the phenotype of IBS is still unanswered. The fact that elevated levels of Cgs also have been reported in other diseases than IBS, complicate the use of Cgs alone as a biomarker of IBS. Cgs may be more eligible to reflect the activity of the enteroendocrine system, and serve as a biomarker for other bioactive secretion products, like 5-HT, that can affect the bowel function. CgA is an established biomarker for neuroendocrine tumors, and circulating CgA is assumed to reflect the neuroendocrine secretion in the body. When it comes to gastrointestinal disorders, it might be beneficial to include analysis of faeces and/or luminal fluids because this could probably better mirror the condition in the gastrointestinal tract, and thus enhance the understanding of gastrointestinal disturbances. With this respect, it should be clarified whether circulating CgA corresponds to luminal secretion of CgA.

One of the signaling molecules with an unambiguous physiological role in the enteric nervous system (ENS) is 5-HT, involved in several aspects of gut function, including secretion, motility and sensation. Release or leakage of 5-HT into the gut lumen has been demonstrated previously [169,170], but whether luminal 5-HT has any physiological function or is just an overflow phenomena is not clear. High systemic levels of 5-HT may cause symptoms resembling hypersensitivity reactions to food, such as diarrhea [171,172], nausea [173], or flushing and heart palpitations [174]. 5-HT has been implicated in several gastrointestinal disorders, including IBS [169], IBD [175] and food hypersensitivity [176]. 5-HT is commonly measured in blood platelets or in plasma, the latter being a poor matrix due to rapid degradation of 5-HT into 5-hydroxyindolacetic acid (5-HIAA). More than 95 % of plasma 5-HT is present in platelets, and studies assessing platelet 5-HT content in IBS patients are somewhat conflicting [177,178,179]. Alterations of 5-HT secretion within the gut are not necessarily reflected in the systemic circulation, and assessing 5-HT in gut tissue samples is therefore often considered as more accurate. However, considering the large surface of the gastrointestinal tract, biopsies can only give information from a small fragment of this huge area. Indeed, EC cell counts seem to vary a lot in different studies [180,181]. In the present thesis we propose a simple and less invasive sampling procedure in conjunction with a rapid technique for determination of 5-HT in gut lavage fluid (paper III). EC cells release 5-HT mainly from granules at the basal border, but studies have shown that EC cells are bipolar and that they are able to secrete 5-HT both basally, into the systemic circulation,

and apically, into to the gut lumen [62,63,64]. Our method enabled quantification of luminal EC cell secretion of 5-HT along the entire length of the small and large bowel. In the present study (paper IV), gut lavage 5-HT were found to be unaltered in patients with subjective food hypersensitivity, as compared to healthy controls. Patients with IBD had slightly higher mean level of 5-HT, but the difference was not significant.

The high degree of variance in the 5-HT data may be due to high individual differences as well as a consequence of the pre-analytical sampling procedure. The use of gut lavage fluid for analytical purpose is quite unique. Based on our previous experience with the procedure [99,182], the first clear fluid passed per rectum was chosen because it was desirable with as little particles as possible to avoid analytical problems, but we acknowledge that the time point of sampling is not necessarily the same for each participant, as transit time may vary a lot. Also, the 5-HT release with time may differ between subjects, thus, one measurement in time may not be the optimal method. These aspects should be considered in further studies using the gut lavage procedure. The PEG solution used in the lavage procedure is a potent laxative that may act as a stimulant and induce secretion of 5-HT from the EC cells. But because the PEG-load was equal in all participants, it is unlikely that PEG-stimulated secretion of 5-HT has affected the outcome in a way that has masked possible differences in detected 5-HT content.

To check if the high degree of variance in the 5-HT measurements could be due to analytical errors, we performed multiple runs of gut lavage fluid samples spiked with known 5-HT concentration, and multiple runs of both patient and control samples. The results showed good reproducibility, and we concluded that the variance observed in the analyzed material could not be ascribed to the preparation of the samples nor the analytical technique.

The high variability observed in the present study, may be a factor of the number of participants, at least to a certain degree. An increase in number of patients and controls would most likely lower the variability of the data.

ELISA is a widely used technique, due to numerous available commercial kits applicable for a variety of biological samples and its ability to detect low levels of the targeted analyte.

We wanted to compare our reported LCMS method to the ELISA technique. The 5-HT level in gut lavage fluid was measured using an enzyme immunoassay, following the manufacture's intructions (Labor Diagnostica Nord GmbH & Co. KG, Nordhorn, Germany. Cat # BA 10-0900). This particular assay was designed for analyzing serum, urine, platelets, plasma and cerebrospinal fluid. To our knowledge, no immunoassay exists for analyzing gut lavage fluid.

The high degree of variability in the 5-HT data obtained from LCMS analysis, were also observed after performing the ELISA technique on the same set of samples. The graph in Figure 7 shows the dispersion of the 5-HT measurements obtained from patient with subjective food hypersensitivity (unpublished data).

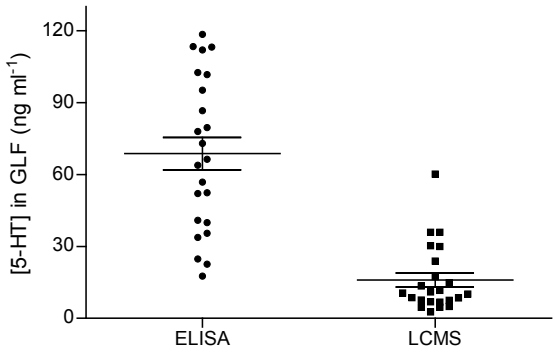

Figure 7. [5-HT] in gut lavage fluid from patients with subjective food hypersensitivity. The same set of samples was analyzed by ELISA and LCMS. Data are given as individual values with mean±SEM. Unpublished data.

The level of 5-HT in gut lavage fluid, from both patients and controls, were found to be considerable elevated when performing ELISA compared to the 5-HT data obtained by using our reported LCMS method (Figure 8). This preliminary study showed that ELISA overestimated the levels of 5-HT by a factor of 4 in both the patients and the controls data when compared to LCMS. Indeed, overestimation is a well-known problem with enzyme immunoassay techniques, due to cross-reactivity. The analytical specificity is decreased when compounds similar to the target molecule are present, and in the case of 5-HT analysis compounds like tryptamine, melatonin, 5-HIAA and histidine may affect the quantitative outcome.

Although the ELISA and LCMS results were not significant at a confidence level of 95 %, it was observed an increase of 42 % and 33 % in the 5-HT level of the patients compared to the controls. The interesting findings of this unpublished preliminary study should be further investigated.

The potential applicability of our reported LCMS/MS method to analyze other biomedical samples, like plasma, serum and urine, should be explored, together with simultaneous determination of 5-HT and its metabolic related products.

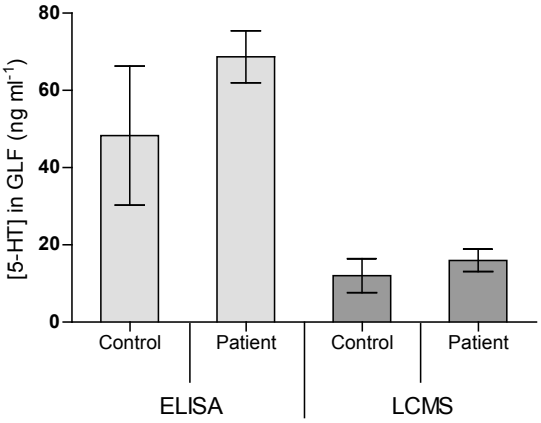

Figure 8. [5-HT] in gut lavage fluid from patients with subjective food hypersensitivity and healthy controls. Data are given as mean±SEM. Unpublished data.

5.6 Determination of IS amount in quantification of 5-HT

Chromatographic techniques using the IS method have been applied in the determination of 5-HT in a wide variety of biological samples. However, in the majority of studies there is no description regarding the strategies behind the selection of an optimal amount of IS, especially in cases where the analyte may have a wide span of concentrations. The most common strategy is trial and error methods or rule of thumb technique such as targeting the IS to the lower 1/3 of the calibration curve. The validity of the IS method relies on the assumptions of linearity of the detector towards both the analyte and the IS, but this is often not acknowledged. The potential applicability of a multivariate strategy to estimate an optimal concentration and a robust RF, which do not change with changes in the analyte and IS concentrations, has been proposed by Araujo et al. [89].

Figure 9, shows that between 2 and 12 µg/mL of 5-HT, a RF that vary between 0.035 and 0.045, is obtained when the concentration of 5-CH_3O-HT is varied from 2 and 20 µg/mL. Concentrations exceeding 12 µg/mL cause a considerable variation of the RF with the concentration of IS and a remarkable lessening of the dynamic analytical range (Figure 9). The curvatures observed in Figure 9 are the result of interaction between 5-HT and 5-CH_3O-HT, which is generally ignored in analytical studies, and could affect the accuracy of the determination. The estimation of concentration regions where the RF remains constant is vital in order to conform to the criteria of linearity of the detector towards the analyte and IS.

Paper IV demonstrates that application of the Doehlert design enables estimation of the interaction 5-HT/5-CH_3O-HT and selection of an optimal amount of IS and a constant RF for analysing 5-HT, where the unknown levels of 5-HT are spanned over a wide range of concentrations.

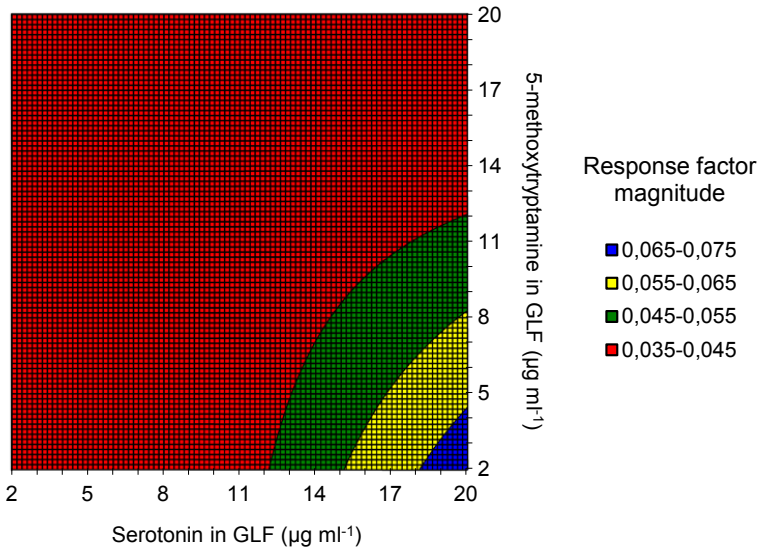

Figure 9. Response factor contour plot. The contours of constant responses were generated by simultaneously varying the concentrations of 5-HT and 5-CH_3O-HT between 2 and 20 µg/mL. GLF=gut lavage fluid.

6. Conclusions

The present thesis suggests that short-term duodenal seal oil administration alleviate both intestinal and extra-intestinal complaints in patients with subjective food hypersensitivity. Impaired gastric accommodation seems to play a limited role in the pathogenesis of the condition. A possible role of n-3 LCPUFA on intestinal inflammation, vago-vagal reflexes, visceral sensitivity, mucosal leakage and intestinal microbiota should be further investigated.

Patients with subjective food hypersensitivity have lower circulating CgA levels as compared to healthy controls, which may indicate the involvement of the endocrine system in subjective food hypersensitivity, but the pathophysiologic role needs to be further elucidated.

The novel method for measuring 5-HT in gut lavage fluid by LCMS/MS demonstrated to be a useful approach for a rapid, simple and efficient quantification in a wide range of concentrations. The small amount of sample, the low solvent consumption and the high sample throughput (30 samples/day) are important features that make the present approach highly attractive to clinical trials involving the analysis of 5-HT in a large number of samples. The relationship 5-HT/5-CH3O-HT is highlighted as an important factor that affects the validity of the RF and consequently the accuracy of the analysis. However, in the present study, levels of 5-HT in gut lavage fluid were unaltered in patients with subjective food hypersensitivity.

Taken together, the hypothesis-generating studies of the present thesis encourage further investigations on the respective roles of 5-HT, CgA and seal oil in the pathophysiology and therapy of subjective food hypersensitivity.

References

1. O'Leary PF, Shanahan F (2002) Food allergies. Curr Gastroenterol Rep 4: 373-382.
2. Young E, Stoneham MD, Petruckevitch A, Barton J, Rona R (1994) A population study of food intolerance. Lancet 343: 1127-1130.
3. Jansen JJ, Kardinaal AFM, Huijbers G, Vlieg-Boerstra BJ, Martens BP, Ockhuizen T (1994) Prevalence of food allergy and intolerance in the adult Dutch population. J Allergy Clin Immunol 93: 446-456.
4. Johansson O, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, Motala C, Ortega Martell JA, Platts-Mills TA, Ring J, Thien F, Van Cauwenberge P, Williams HC (2004) Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol 113: 832-836.
5. Johansson SGO, Hourihane JOB, Bousquet J, Bruijnzeel-Koomen C, Dreborg S, Haahtela T, Kowalski ML, Mygind N, Ring J, van Cauwenberge P, van Hage-Hamsten M, Wüthrich B (2001) A revised nomenclature for allergy: An EAACI position statement from the EAACI nomenclature task force. Allergy 56: 813-824.
6. Cianferoni A, Spergel JM (2009) Food allergy: review, classification and diagnosis. Allergol Int 58: 457-466.
7. Sampson HA, Ho DG (1997) Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. J Allergy Clin Immunol 100: 444-451.
8. Sampson HA (2001) Utility of food-specific IgE concentrations in predicting symptomatic food allergy. J Allergy Clin Immunol 107: 891-896.
9. Niggemann B, Reibel S, Wahn U (2000) The atopy patch test (APT)-- a useful tool for the diagnosis of food allergy in children with atopic dermatitis. Allergy 55: 281-285.
10. Niggemann B, Sielaff B, Beyer K, Binder C, Wahn U (1999) Outcome of double-blind, placebo-controlled food challenge tests in 107 children with atopic dermatitis. Clin Exp Allergy 29: 91-96.
11. Niggemann B, Wahn U, Sampson HA (1994) Proposals for standardization of oral food challenge tests in infants and children. Pediatr Allergy Immunol 5: 11-13.
12. Bock SA, Sampson HA, Atkins FM, Zeiger RS, Lehrer S, Sachs M, Bush RK, Metcalfe DD (1988) Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. J Allergy Clin Immunol 82: 986-997.
13. Bock SA AF (1990) Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges. J Pediatr 117: 561-567.
14. Cudowska B, Kaczmarski M (2005) Atopy patch test in the diagnosis of food allergy in children with atopic eczema dermatitis syndrome. Rocz Akad Med Bialymst 50: 261-267.
15. Sampson HA, Anderson JA (2000) Summary and recommendations: Classification of gastrointestinal manifestations due to immunologic reactions to foods in infants and young children. J Pediatr Gastroenterol Nutr 30: Suppl:S87-94.
16. Beyer K, Castro R, Birnbaum A, Benkov K, Pittman N, Sampson HA (2002) Human milk-specific mucosal lymphocytes of the gastrointestinal tract display a TH2 cytokine profile. J Allergy Clin Immunol 109: 707-713.
17. Yan BM, Shaffer EA (2009) Primary eosinophilic disorders of the gastrointestinal tract. Gut 58: 721-732.

18. Jaffe JS, James SP, Mullins GE, Braun-Elwert L, Lubensky I, Metcalfe DD (1994) Evidence for an abnormal profile of interleukin-4 (IL-4), IL-5, and gamma-interferon (gamma-IFN) in peripheral blood T cells from patients with allergic eosinophilic gastroenteritis. J Clin Immunol 14: 299-309.
19. Pate MB, Smith JK, Chi DS, Krishnaswamy G (2010) Regulation and dysregulation of immunoglobulin E: a molecular and clinical perspective. Clin Mol Allergy 8: 3-15.
20. Bruijnzeel-Koomen C, Ortolani C, Aas K, Bindslev-Jensen C, Björkstén B, Moneret-Vautrin D, Wüthrich B (1995) Adverse reactions to food. European Academy of Allergology and Clinical Immunology Subcommittee. Allergy 50: 623-635.
21. Roehr CC, Edenharter G, Reimann S, Ehlers I, Worm M, Zuberbier T, Niggemann B (2004) Food allergy and non-allergic food hypersensitivity in children and adolescents. Clin Exp Allergy 34: 1534-1541.
22. Park MI, Camilleri M (2006) Is there a role of food allergy in irritable bowel syndrome and functional dyspepsia? A systematic review. Neurogastroenterol Motil 18: 595-607.
23. Simren M, Mansson A, Langkilde AM, Svedlund J, Abrahamsson H, Bengtsson U, Bjornsson ES (2001) Food-related gastrointestinal symptoms in the irritable bowel syndrome. Digestion 63: 108-115.
24. Lillestøl K, Helgeland LI, Florvaag E, Lied AG, Lind R, Valeur J, Berstad A (2009) Indications of "atopic bowel" in patients with irritable bowel syndrome. Gut 58: A20-A20.
25. Morken MH, Nysaeter G, Strand EA, Hausken T, Berstad A (2008) Lactulose breath test results in patients with persistent abdominal symptoms following Giardia lamblia infection. Scand J Gastroenterol 43: 141-145.
26. Arslan G, Lind R, Olafsson S, Florvaag E, Berstad A (2004) Quality of life in patients with subjective foodhypersensitivity: Applicability of the 10-item short form of the nepean dyspepsia index. Dig Dis Sci 49: 680-687.
27. Lind R, Arslan G, Eriksen HR, Kahrs GE, Haug T T, Florvaag E, Berstad A (2005) Subjective health complaints and modern health worries in patients with subjective food hypersensitivity. Dig Dis Sci 50: 1245-1251.
28. Lind R, Lillestøl K, Valeur J, Eriksen HR, Tangen T, Berstad A, L.G. A (2010) Job stress and coping strategies in patients with subjective food hypersensitivity. Scan J Psychol 51: 179-184.
29. Lillestøl K, Berstad A, Lind R, Florvaag E, Lied GA, Tangen T (2010) Anxiety and depression in patients with self-reported food hypersensitivity. Gen Hosp Psychiatry 32: 42-48.
30. Katon WJ (2003) Clinical and health services relationships between major depression, depressive symptoms, and general medical illness. Biol Psychiatry 54: 216-226.
31. Tache Y, Perdue MH (2004) Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. Neurogastroenterol Motil 16: 137-142.
32. Yu LC, Perdue MH (2001) Role of mast cells in intestinal mucosal function: studies in models of hypersensitivity and stress. Immunol Rev 179: 61-73.
33. Lind R, Lied GA, Lillestøl K, Valeur J, Berstad A (2010) Do psychological factors predict symptom severity in patients with subjective food hypersensitivity? Scand J Gastroenterol 45: 835-843.
34. Simopoulos AP (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed Pharmacother 56: 365-379.
35. Burdge GC, Calder PC (2006) Dietary a-linolenic acid and health-related outcomes: a metabolic perspective. Nutr Res Rev 19: 26-52.

36. Calder PC (2006) N-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 83: 1505S–1519S.
37. Mukherjee D, Nissen SE, Topol EJ (2001) Risk of Cardiovascular Events Associated With Selective COX-2 Inhibitors. JAMA 286: 954-959. .
38. Reinisch W, Miehsler W, Dejaco C, Harrer M, Waldhoer T, Lichtenberger C, Vogelsang H (2003) An open-label trial of the selective cyclo-oxygenase-2 inhibitor, rofecoxib, in inflammatory bowel disease-associated peripheral arthritis and arthralgia. Aliment Pharmacol Ther 17: 1371-1380.
39. Bonner GF (2001) Exacerbation of inflammatory bowel disease associated with use of celecoxib. Am J Gastroenterol 91: 1306-1308.
40. Mahadevan U, Loftus EV, Tremaine WJ, Sandborn WJ (2002) Safety of selective cyclooxygenase-2 inhibitors in inflammatory bowel disease. Am J Gastroenterol 97: 910-914.
41. Obata T, Nagakura T, Masaki T, Maekawa K, Yamashita K (1999) Eicosapentaenoic acid inhibits prostaglandin D2 generation by inhibiting cyclo-oxygenase-2 in cultured human mast cells. Clin Exp Allergy 29: 1129-1135.
42. Barbara G, Cremon C, Carini G, Bellacosa L, Zecchi L, De Giorgio R, Corinaldesi R, Stanghellini V (2011) The immune system in irritable bowel syndrome. J Neurogastroenterol Motil 17: 349-359.
43. Barbara G, Giorgio R, Stanghellini V, Cremon C, Corinaldesi R (2002) A role for inflammation in irritable bowel syndrome? Gut 51: i41-i44.
44. Morcos A, Dinan TG, Quigley EM (2009) Irritable bowel syndrome: role of food in pathogenesis and management. J Dig Dis 10: 237-246.
45. Valeur J, Morken MH, Norin E, Midtvedt T, Berstad A (2009) Carbohydrate intolerance in patients with self-reported food hypersensitivity: Comparison of lactulose and glucose. Scand J Gastroenterol 44: 1416-1423.
46. Gibson PR, Shepherd SJ (2010) Evidence-based dietary management of functional gastrointestinal symptoms: The FODMAP approach. J Gastroenterol Hepatol 25: 252-258.
47. Quigley EM, Flourie B (2007) Probiotics and irritable bowel syndrome: a rationale for their use and an assessment of the evidence to date. Neurogastroenetrol Motil 19: 166-172.
48. Ouwehand AC, Salminen S, Isolauri E (2002) Probiotics: an overview of beneficial effects. Antonie van Leeuwenhoek 82: 279-289.
49. O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM (2005) Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterol 128: 541-551.
50. Nobaek S, Johansson ML, Molin G, Ahrné S, Jeppsson B (2000) Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. Am J Gastroenterol 95: 1231-1238.
51. Piessevaux H, Tack J, Walrand S, Pauwels S, Geubel A (2003) Intragastric distribution of a standardized meal in health and functional dyspepsia: correlation with specific symptoms. Neurogastroenetrol Motil 15: 447-455.
52. Troncon LE, Bennett RJ, Ahluwalia NK, Thompson DG (1994) Abnormal intragastric distribution of food during gastric emptying in functional dyspepsia patients. Gut 35: 327-332.
53. Tack J, Piessevaux H, Coulie B, Caenepeel P, Janssens J (1998) Role of impaired gastric accommodation to a meal in functional dyspepsia. Gastroenterol 115: 1346-1352.

54. Gilja OH, Hausken T, Ødegaard S, Berstad A (1995) Monitoring postprandial size of the proximal stomach by ultrasonography. J Ultrasound Med 14: 81-89.
55. Gilja OH, Hausken T, Wilhelmsen I, Berstad A (1996) Impaired accommodation of proximal stomach to a meal in functional dyspepsia. Dig Dis Sci 41: 689-696.
56. Kindt S, Tack J (2006) Impaired gastric accommodation and its role in functional dyspepsia. Gut 55: 1685-1691.
57. Geeraerts B, Vandenberghe J, Van Oudenhove L, Gregory LJ, Aziz Q, Dupont P, Demyttenaere K, Janssens J, Tack J (2005) Influence of experimentally induced anxiety on gastric sensorimotor function in humans. Gastroenterol 129: 1437-1444.
58. Mertz H, Naliboff B, Munakata J, Niazi N, Mayer EA (1995) Altered rectal perception is a biological marker of patients with irritable bowel syndrome. Gastroenterol 109: 40-52.
59. Mertz H, Fullerton S, Naliboff B, Mayer EA (1998) Symptoms and visceral perception in severe functional and organic dyspepsia. Gut 42: 814-822.
60. Bouin M, Lupien F, Riberdy M, Boivin M, Plourde V, Poitras P (2004) Intolerance to visceral distension in functional dyspepsia or irritable bowel syndrome: an organ specific defect or a pan intestinal dysregulation? Neurogastroenetrol Motil 16: 311-314.
61. Ciaccio C (1907) Sopra speciali cellule granulose della mucosa intestinale. Arch Ital Anat Embriol 6: 482-498.
62. Forsberg EJ, Miller RJ (1982) Cholinergic agonists induce vectorial release of serotonin from duodenal enterochromaffin cells. Science 217: 355-356.
63. Ahlman H, Bhargava HN, Dahlström A, Larsson I, Newson B, Pettersson G (1981) On the presence of serotonin in the gut lumen and possible release mechanisms. Acta Physiol Scand 112: 263-269.
64. Nilsson O, Ahlman H, Geffard M, Dahlström A, Ericson LE (1987) Bipolarity of duodenal enterochromaffin cells in the rat. Cell and tissue research 248: 49-54.
65. Gustafsson BI, Bakke I, Tømmerås K, Waldum HL (2006) A new method for visualization of gut mucosal cells, describing the enterochromaffin cell in the rat gastrointestinal tract. Scand J Gastroenterol 41: 390-395.
66. Gershon MD, Tack J (2007) The Serotonin Signaling System: From Basic Understanding To Drug Development for Functional GI Disorders. Gastroenterology 132: 397-414.
67. Rindi G, Leiter AB, Kopin AS, Bordi C, Solcia E (2004) The "normal" endocrine cell of the gut: changing concepts and new evidences. Ann N Y Acad Sci 1014: 1-12.
68. Gershon MD (1999) Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. Aliment Pharmacol Ther 13 (Suppl. 2): 15-30.
69. Gershon MD (2004) Review article: serotonin receptors and transporters — roles in normal and abnormal gastrointestinal motility. Alimentary Pharmacology and Therapeutics 20: 3-14.
70. Nichols DE, Nichols CD (2008) Serotonin receptors. Chem Rev 108: 1614-1641.
71. Pater JL, Lofters WS, Zee B, Dempsey E, Walde D, Moquin JP, Wilson K, Hoskins P, Guevin RM, Verma S, Navari R, Krook JE, Hainsworth J, Palmer M, Chin C (1997) The role of the 5-HT3 antagonists ondansetron and dolasetron in the control of delayed onset nausea and vomiting in patients receiving moderately emetogenic chemotherapy. Annals of Oncology 8: 181-185.
72. Cubeddu LX, Hoffmann IS, Fuenmayor NT, Finn AL (1990) Efficacy of Ondansetron (Gr 38032F) and the Role of Serotonin in Cisplatin-Induced Nausea and Vomiting. New England J Med 322: 810-816.
73. Nyhlin H, Bang C, Elsborg L, Silvennoinen J, Holme I, Rüegg P, Jones J, Wagner A (2004) A double-blind, placebo-controlled, randomized study to evaluate the efficacy,

safety and tolerability of tegaserod in patients with irritable bowel syndrome. Scan J Gastroenterol 39: 119-126.
74. Müller-Lissner SA, Fumagalli I, Bardhan KD, Pace F, Pecher E, Nault B, Rüegg P (2001) Tegaserod, a 5-HT(4) receptor partial agonist, relieves symptoms in irritable bowel syndrome patients with abdominal pain, bloating and constipation. Aliment Pharmacol Ther 15: 1655-1666.
75. Gill RK, Pant N, Saksena S, Singla A, Nazir TM, Vohwinkel L, Turner JR, Goldstein J, Alrefai WA, Dudeja PK (2008) Function, expression, and characterization of the serotonin transporter in the native human intestine. Am J Physiol 294: G254-G262.
76. Liu MT, Rayport S, Jiang Y, Murphy DL, Gershon MD (2002) Expression and function of 5-HT3 receptors in the enteric neurons of mice lacking the serotonin transporter. Am J Physiol Gastrointest Liver Physiol 283: 1398-1411.
77. Linden DR, Chen JX, Gershon MD, Sharkey KA, Mawe GM (2003) Serotonin availability is increased in mucosa of guinea pigs with TNBS-induced colitis Am J Physiol Gastrointest Liver Physiol 285: G207-G216.
78. Hendy GN, Bevan S, Mattei MG, Mouland AJ (1995) Chromogranin A. Clin Invest Med 18: 47-65.
79. Pinchot SN, Holen K, Sippel RS, Chen H (2008) Carcinoid Tumors. The Oncologist 13: 1255-1269.
80. Sidhu R, McAlindon ME, Leeds JS, Skilling J, Sanders DS (2009) The Role of Serum Chromogranin A in Diarrhoea Predominant Irritable Boweø Syndrome. J Gastrointestin Liver Dis 18: 23-26.
81. Valeur J, Milde AM, Helle KB, Berstad A (2008) Low serum chromogranin A in pateints with self-reported food hypersensitivity. Scand J Gastroenterol 43: 1403-1404.
82. Kema IP, Schellings AMJ, Hoppenbrouwers CJM, Rutgers HM, de Vries EGE, Muskiet FAJ (1993) High performance liquid chromatographic profiling of tryptophan and related indoles in body fluids and tissues of carcinoid patients. Clinica Chimica Acta 221: 143-158.
83. Sasa S, Blank CL (1979) Simultaneous determination of norepinephrine, dopamine, and serotonin in brain tissue by high-pressure liquid chromatography with electrochemical detection. Anal Chim Acta 104: 29-45.
84. Danaceau JP, Anderson GM, McMahon WM, Couch DJ (2003) A liquid chromatographic-tandem mass spectrometric method for the analysis of serotonin and related indoles in human whole blood. Journal of analytical toxicology 27: 440-444.
85. Bose D, Durgbanshi A, Capella-Peiró ME, Gil-Agustí M, Esteve-Romero J, Carda-Broch S, Martinavarro-Domínguez A (2004) Micellar liquid chromatography determination of some biogenic amines with electrochemical detection. J Pharm Biomed Anal 36: 357-363.
86. Locker KL, Morrison D, Watt AP (2001) Quantitative determination of L-775,606, a selective 5-hydroxytryptamine 1D agonist, in rat plasma using automated sample preparation and detection by liquid chromatography-tandem mass spectrometry. J Chromatogr B 750: 13-23.
87. Johnson RD, Lewis RJ, Canfield DV, Blank CL (2004) Accurate assignment of ethanol origin in postmortem urine: liquid chromatographic-mass spectrometric determination of serotonin metabolites. J Chromatogr B 805: 223-234.
88. Huang T, Kissinger P (1996) Liquid Chromatographic Determination of Serotonin in Homogenized Dog Intestine and Rat Brain Tissue Using a 2 mm i.d. PEEK Column. Curr Sep 14: 114-119.
89. Araujo P, Couillard F, Leirnes E, Ask K, Bøkevoll A, Frøyland L (2006) Experimental design considerations in quantification experiments by using the internal standard

technique: cholesterol determination by gas chromatography as a case study. J Chromato A 1121: 99-105.
90. Bjørkkjær T, Brunborg LA, Arslan G, Lind RA, Brun JG, Valen M, Klementsen B, Berstad A, Frøyland L (2004) Reduced joint pain after short-term duodenal administration of seal oil in patients with inflammatory bowel disease: comparison with soy oil. Scand J Gastroenterol 39: 1088-1094.
91. Arslan G, Brunborg LA, Frøyland L, Brun JG, Valen M, Berstad A (2002) Effects of duodenal seal oil administration in patients with inflammatory bowel disease. Lipids 37: 935-940.
92. Riccabona M, Rossipal E (1993) Sonographic findings in celiac disease. J Pediatr Gastroenterol Nutr 17: 198-200.
93. Gilja OH, Hausken T, Odegaard S, Berstad A (1995) Monitorig postprandial size of the proximal stomach by ultrasonography. J Ultrasound Med 14: 81-89.
94. Berstad A, Hausken T, Gilja OH, Hveem K, Nesje LB, Ødegaard S (1996) Ultrasonography of the human stomach. Scand J Gastroenterol 31: 75-82.
95. Nylund K, Ødegaard S, Hausken T, Folvik G, Lied GA, Viola I, Hauser H, Gilja OH (2009) Sonography of the small intestine. World J Gastroenterol 15: 1319-1330.
96. NRRK http://www.nrrk.no/modules/module_123/proxy.asp?D=2&C=634&I=2668
97. http://www.nrrk.no/modules/module_123/proxy.asp?D=2&C=634&I=2668. Nasjonalt revmatologisk rehabiliterings- og kompetansesenter.
98. Talley NJ, Verlinden M, Jones M (2001) Quality of life in functional dyspepsia: responsiveness of the Nepean Dyspepsia Index and development of a new 10-item short form. Aliment Pharmacol Ther 15: 207-216.
99. Berstad A, Arslan G, Folvik G (2000) Reationship between intestinal permeability and calprotectin concentration in gut lavage fluid. Scand J Gastroenterol 1: 64-69.
100. Lie O, Lambertsen G (1991) Fatty acid composition of glycerolphospholipids in seven tisues of cod (*Gadus morhua*), determined by combined high-performance liquid chromatography and gas chromatography. J Chromatogr 565: 119-129.
101. Hamre K, Næss T, Espe M, Holm JC, Lie Ø (2001) A formulated diet for Atlantic halibut (Hippoglossus hippoglossus, L.) larvae. Aquaculture Nutr 7: 123-132.
102. Schmedes A, Hølmer G (1989) A new thiobarbituric acid (TBARS) method for determining free malondialdehyde (MDA) and hydroperoxides selectively as a measure of lipid peroxidation. JAOCS 66: 813-817.
103. Morken MH, Berstad AE, Nysæter G, Berstad A (2007) Intestinal gas in plain abdominal radiographs does not correlate with symptoms after lactulose challenge. Eur J Gastroenterol Hepatol 19: 589-593.
104. Nöll GN (1996) High-performance liquid chromatographic analysis of retinal and retinol isomers. J Chromatogr A 721: 247-259.
105. CEN (2000) Foodstuffs - Determination of vitamin E by high performance liquid chromatograpy - Measurement of aplha-, beta-, gamma-, and delta-tocopherols (prEN 12822).
106. Doehlert DH (1970) Uniform Shell Designs. Appl Stat 19: 231-239.
107. Drossman DA, Li Z, Andruzzi E, Temple R, Talley NJ, Thompson WG, Whitehead WE, Janssens J, Funch-Jensen P, Carazziari E, Richter JE, Koch GG (1993) U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. Dig Dis Sci 38: 1569-1580.
108. Camilleri M (2001) Management of the irritable bowel syndrome. Gastroenterol 120: 652-668.
109. Chang L, Heitkemper MM (2002) Gender differences in irritable bowel syndrome. Gastroenterol 123: 1686-1701.

110. Drover JW (2007) Gastric versus postpyloric feeding. Gastrointest Endosc Clin N Am 17: 765-775.
111. Bjørkkjær T, Brun JG, Valen M, Arslan G, Lind R, Brunborg LA, Berstad A, Frøyland L (2006) Short-term duodenal seal oil administration normalised n-6 to n-3 fatty acid ratio in rectal mucosa and ameliorated bodily pain in patients with inflammatory bowel disease. Lipids Health Dis 5.
112. Bjørkkjær T, Araujo P, Madland TM, Berstad A, Frøyland L (2009) A randomized double blind comparison of short-term duodenally administrated whale and seal blubber oils in patients with inflammatory bowel disease and joint pain. Prostaglandins Leukot Essent Fatty Acids 81: 425-432.
113. Gregersen K, Lind R, Bjørkkjær T, Frøyland L, Berstad A (2008) Effects of seal oil on meal-induced symptoms and gastric accommodation in patients with subjective food hypersensitivity: A pilot study. Clin med: Gastroenterol 1: 33-41.
114. Madland TM, Bjørkkjær T, Brunborg LA, Frøyland L, Berstad A, Brun JG (2006) Subjective improvement in patients with psoriatic arthritis after short-term oral treatment with seal oil. A pilot study with double blind comparison to soy oil. J Rheumatol 33: 307-310.
115. Brunborg LA, Madland TM, Lind RA, Arslan G, Berstad A, Frøyland L (2008) Effects of short-term oral administration of dietary marine oils in patients with inflammatory bowel disease and joint pain: A pilot study comparing seal oil and cod liver oil. Clin Nutr 27: 614-622.
116. Calder PC (2008) Polyunsaturated fatty acids, inflammatory processes and inflammatory bowel diseases. Mol Nutr Food Res 52: 885-897.
117. Feagan BG, Sandborn WJ, Mittmann U, Bar-Meir S, D'Haens G, Bradette M, Cohen A, Dallaire C, Ponich TP, McDonald JW, Hébuterne X, Paré P, Klvana P, Niv Y, Ardizzone S, Alexeeva O, Rostom A, Kiudelis G, Spleiss J, Gilgen D, Vandervoort MK, Wong CJ, Zou GY, Donner A, Rutgeerts P (2008) Omega-3 free fatty acids for the maintenance of remission in Crohn disease: the EPIC Randomized Controlled Trials. JAMA 299: 1690-1697.
118. Cleland LG, James MJ, Proudman SM (2006) Fish oil: what the prescriber needs to know. Arthritis Res Ther 8: 202-210.
119. Fried M, Feinle C (2002) The role of fat and cholecystokinin in functional dyspepsia. Gut 51: i54-57.
120. Feinle C, Meier O, Otto B, D'Amato M, Fried M (2001) Role of duodenal lipid and cholecystokinin A receptors in the pathophysiology of functional dyspepsia. Gut 48: 347-355.
121. Araujo P, Frøyland L (2006) Optimisation of an extraction method for the determination of prostaglandin E2 in plasma using experimental design and liquid chromatography tandem mass spectrometry. J Chromatography B 830: 212-217.
122. Benninga MA, Mayer EA (2009) The Power of Placebo in Pediatric Functional Gastrointestinal Disease. Gastroenterol 137: 1207-1210.
123. Saps M, Youssef N, Miranda A, Nurko S, Hyman P, Cocjin J, Di Lorenzo C (2009) Multicenter, randomized, placebo-controlled trial of amitriptyline in children with functional gastrointestinal disorders. Gastroenterol 137: 1261-1269.
124. Stock JL, Shinjo K, Burkhardt J, Roach M, Taniguchi K, Ishikawa T, Kim H-S, Flannery PJ, Coffman TM, McNeish JD, Audoly LP (2001) The prostaglandin E2 EP1 receptor mediates pain perception and regulates blood pressure. J Clin Invest 107: 325-331.
125. Spiller R, Jenkins D, Thornley J, Hebden J, Wright T, Skinner M, Neal K (2000) Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut

permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. Gut 47: 804-811.
126. Clarke G, Fitzgerald P, Hennessy AA, Cassidy EM, Quigley EM, Ross P, Stanton C, Cryan JF, Dinan TG (2010) Marked elevations in pro-inflammatory polyunsaturated fatty acid metabolites in females with irritable bowel syndrome. J Lipid Res 51: 1186-1192.
127. Lillestøl K, Helgeland LI, Lied AG, Florvaag E, Valeur J, Lind R, Berstad A (2010) Indications of 'atopic bowel' in patients with self-reported food hypersensitivity. Aliment Pharmacol Ther 31: 1112-1122.
128. Lied AG, Vogelsang P, Berstad A, Appel S (2011) Dendritic cell populations in patients with self-reported food hypersensitivity. Int J Gen Med 4: 389-396.
129. Meeus M, Nijs J (2007) Central sensitization: a biopsychosocial explanation for chronic widespread pain in patients with fibromyalgia and chronic fatigue syndrome. Clin Rheumatol 26: 465-473.
130. Neal KR, Hebden J, Spiller R (1997) Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients. BMJ 314: 779-782.
131. Luyer MD, Greve JW, Hadfoune M, Jacobs JA, Dejong CH, Buurman WA (2005) Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve. J Exp Med 202: 1023-1029.
132. Aerssens J, Hillsley K, Peeters PJ, de Hoogt R, Stanisz A, Lin JH, Van den Wyngaert I, Göhlmann HW, Grundy D, Stead RH, Coulie B (2007) Alterations in the brain-gut axis underlying visceral chemosensitivity in Nippostrongylus brasiliensis-infected mice. Gastroenterol 132: 1375-1387.
133. Lied AG, Lillestøl K, Valeur J, Berstad A (2010) Intestinal B cell-activating factor (BAFF): an indicator of non-IgE-mediated hypersensitivity reactions to food? Aliment Pharmacol Ther Epub ahead of print.
134. Collins SM, Bercik P (2009) The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. Gastroenterol 136: 2003-2014.
135. Kinross JM, Darzi AW, Nicholson JK (2011) Gut microbiome-host interactions in health and disease. Gen Med 3: 14-25.
136. O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM, Cryan JF, Dinan TG (2009) Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 65: 263-267.
137. Spiller R, Garsed K (2009) Postinfectious irritable bowel syndrome. Gastroenterol 136: 1979-1988.
138. Lin HC (2004) Small intestinal bacterial overgrowth: a framework for understanding irritable bowel syndrome. JAMA 292: 852-858.
139. Pimentel M, Chow EJ, Lin HC (2000) Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. Am J Gastroenterol 95: 3503-3506.
140. Strocchi A, Furne J, Ellis C, Levitt MD (1994) Methanogens outcompete sulphate reducing bacteria for H2 in the human colon. Gut 35: 1098-1101.
141. Thompson L, Spiller RC (1995) Impact of polyunsaturated fatty acids on human colonic bacterial metabolism: an *in vitro* and *in vivo* study. Br J Nutr 74: 733-741.
142. Bomba A, Nemcova R, Gancarcikova S, Herich R, Guba P, Mudronova D (2002) Improvement of the probiotic effect of micro-organisms by their combination with

maltodextrins, fructo-oligosaccharides and polyunsaturated fatty acids. Br J Nutr 88: S95-99.
143. Pachikian BD, Neyrinck AM, Portois L, De Backer FC, Sohet FM, Hacquebard M, Carpentier YA, Cani PD, Delzenne NM (2011) Involvement of gut microbial fermentation in the metabolic alterations occurring in n-3 polyunsaturated fatty acids-depleted mice. Nutr Metabol 8: 44-54.
144. Gibson GR, Macfarlane GT, Cummings JH (1993) Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34: 437-439.
145. Lichtenberger LM (1995) The hydrophobic barrier properties of gastrointestinal mucus. Ann Rev Physiol 57: 565-583.
146. Biesiekierski JR, Newnham ED, Irving PM, Barrett JS, Haines M, Doecke JD, Shepherd SJ, Muir JG, Gibson PR (2011) Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double blind randomized placebo-controlled trial. Am J Gastroenterol 106: 508-514.
147. Morken MH, Valeur J, Norin E, Midtvedt T, Nysæter G, Berstad A (2009) Antibiotic or bacterial therapy in post-giardiasis irritable bowel syndrome. Scan J Gastroenterol 44: 1296-1303.
148. Berstad A, Undseth R, Lind R, Valeur J (2012) Functional bowel symptoms, fibromyalgia and fatigue: A food-induced triad? Scand J Gastroenterol.
149. Solakivi T, Kaukinen K, Kunnas T, Lehtimäki T, Mäki M, Nikkari ST (2011) Serum fatty acid profile in subjects with irritable bowel syndrome. Scan J Gastroenterol 46: 299-303.
150. Frank L, Kleinman L, Rentz A, Ciesla G, Kim JJ, Zacker C (2002) Health-related quality of life associated with irritable bowel syndrome: comparison with other chronic diseases. Clin Ther 24: 675-689.
151. Gralnek IM, Hays RD, Kilbourne A, Naliboff B, Mayer EA (2000) The impact of irritable bowel syndrome on health-related quality of life. Gastroenterol 119: 654-660.
152. Gregersen K, Lind R, Valeur J, Bjørkkjær T, Berstad A, Lied AG (2010) Duodenal administered seal oil for patients with food hypersensitivity: an explorative open pilot study. Int J Gen Med 3: 383-392.
153. Brosschot JF (2002) Cognitive-emotional sensitization and somatic health complaints. Scan J Psychol 43: 113-121.
154. Frost K, Frank E, Maibach E (1997) Relative risk in the news media: a quantification of misrepresentation. Am J Public Health 87: 842-845.
155. Zeilhofer HU (2007) Prostanoids in nociception and pain. Biochem Pharma 73: 165-174.
156. Wallace DJ, Hallequa DS (2004) Fibromyalgia: the gastrointestinal link. Curr Pain Headache Rep 8: 364-368.
157. Wilhelmsen I, Berstad A (2004) Reduced relapse rate in duodenal ulcer disease leads to normalization of psychological distress: twelve-year follow-up. Scand J Gastroenterol 39: 717-721.
158. El-Salhy M, Lomholt-Beck B, Hausken T (2010) Chromogranin A as a possible tool in the diagnosis of irritable bowel syndrome. Scan J Gastroenterol 45: 1435-1439.
159. Dunlop SP, Jenkins D, Neal K, Spiller R (2003) Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS. Gastroenterology 125: 1651-1659.
160. Helle KB (2010) Chromogranins A and B and secretogranin II as prohormones for regulatory peptides from the diffuse neuroendocrine system. Results Probl Cell Differ 50: 21-44.
161. Shooshtarizadeh P, Zhang D, Chich JF, Gasnier C, Schneider F, Haïkel Y, Aunis D, Metz-Boutigue MH (2009) The antimicrobial peptides derived from

chromogranin/secretogranin family, new actors of innate immunity. Regul Pept: Article in Press.
162. Khan WI, Ghia JE (2010) Gut hormones: emerging role in immune activation and inflammation. Clin Exp Immunol: Epub ahead of print.
163. Uribe A, Alam M, Johansson O, Midtvedt T, Theodorsson E (1994) Microflora modulates endocrine cells in the gastrointestinal mucosa of the rat Gastroenterology 107: 1259-1269.
164. Valeur J, Morken MH, Norin E, Midtvedt T, Berstad A (2010) Intestinal fermentation in patients with self-reported food hypersensitivity: painful, but protective? Clin Exp Gastroenterol 3: 65-70.
165. Dlugosz A, Törnblom H, Mohammadian G, Morgan G, Veress B, Edvinsson B, Sandström G, Lindberg G (2010) Chlamydia trachomatis antigens in enteroendocrine cells and macrophages of the small bowel in patients with severe irritable bowel syndrome. BMJ Gastroenterol 10: 1-9.
166. Binimelis J, Webb SM, Monés J, Serrano J, Casamitjana R, Elena M, Peinado MA, Vilardell F, De Leiva A (1987) Circulating immunoreactive somatostatin in gastrointestinal diseases. Decrease after vagotomy and enhancement in active ulcerative colitis, irritable bowel syndrome, and duodenal ulcer. Scand J Gastroenterol 22: 931-937.
167. Berutti A, Dogliotti L, Mosca A, Tarabuzzi R, Torta M, Mari M, Gorzegno G, Fontana D, Angeli A (2001) Effects of the somatostatin analog lanreotide on the circulating levels of chromogranin-A, prostate-specific antigen, and insulin-like growth factor-1 in advanced prostate cancer patients. The Prostate 47: 205-211.
168. Öhman L, Stridsberg M, Isaksson S, Jerlstad P, Simren M (2012) Altered Levels of Fecal Chromogranins and Secretogranins in IBS: Relevance for Pathophysiology and Symptoms. Am J Gastroenterol 107: 440-447.
169. Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL (2004) Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. Gastroenterology 126: 1657-1664.
170. Gilkin RJ (2005) The spectrum of irritable bowel syndrome: A clinical reviw. Clin Ther 27: 1696-1709.
171. Lundgren O (1998) 5-Hydroxytryptamine, enterotoxins, and intestinal fluid secretion. Gastroenterology 115: 1009-1012.
172. Turvill JL, Connor P, Farthing MJ (2000) The inhibition of cholera toxin-induced 5-HT release by the 5-HT(3) receptor antagonist, granisetron, in the rat. Br J Pharmacol 130: 1031-1036.
173. Sanger GJ (1990) New antiemetic drugs. Can J Physiol Pharmacol 68: 314-324.
174. McCormick D (2002) Carcinoid tumors and syndrome. Gastroenterol Nurs 25: 105-111.
175. El-Salhy M, Danielsson A, Stenling R, Grimelius L (1997) Colonic endocrine cells in inflammatory bowel disease. J Internal Med 242: 413-419.
176. Grundy D (2006) Serotonin and sensory signalling from the gastrointestinal lumen. J Physiol 575: 1-2.
177. Atkinson W, Lockhart S, Whorwell PJ, Keevil B, Houghton LA (2006) Altered 5-hydroxytryptamine signaling in patients with constipation- and diarrhea-predominant irritable bowel syndrome. Gastroenterology 130: 34-43.
178. Dunlop SP, Coleman NS, Blackshaw E, Perkins AC, Singh G, Marsden CA, Spiller RC (2005) Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome. Clin Gastroenterol Hepatol 3: 349-357.

179. Houghton LA, Atkinson W, Whitaker RP, Whorwell PJ, Rimmer MJ (2003) Increased platelet depleted plasma 5-hydroxytryptamine concentration following meal ingestion in symptomatic female subjects with diarrhoea predominant irritable bowel syndrome. Gut 52: 663-670.
180. Wheatcroft J, Wakelin D, Smith A, Mahoney CR, Mawe G, Spiller R (2005) Enterochromaffin cell hyperplasia and decreased serotonin transporter in a mouse model of postinfectious bowel dysfunction. Neurogastroenetrol Motil 17: 863-870.
181. Wang SH, Dong L, Luo JY, Gong J, Li L, Lu XL, Han SP (2007) Decreased expression of serotonin in the jejunum and increased numbers of mast cells in the terminal ileum in patients with irritable bowel syndrome. World J Gastroenterol 13: 6041-6047.
182. Arslan G, Kahrs GE, Lind R, Frøyland L, Florvaag E, Berstad A (2004) Patients with subjective food hypersensitivity: the value of analyzing intestinal permeability and inflammation markers in gut lavage fluid. Digestion 70: 26-35.