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Effect of gluten and FODMAP elimination in celiac patients on gut inflammatory biomarkers

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Biotechnology

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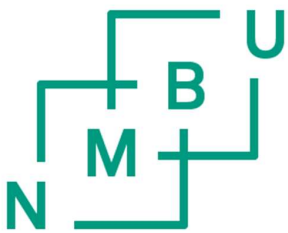

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ABSTRACT

Background: Reduced dietary intake of fermentable oligo-, di- and monosaccharides and polyols (FODMAPs) has been suggested as a strategy to improve gastrointestinal (GI) symptoms in people diagnosed with irritable bowel syndrome (IBS). In a Norwegian study of patients with celiac disease (CD), experiencing persistent GI symptoms similar to those observed in IBS-patients, reducing FODMAPs gave symptom relief. Based on the same study, the aim of the current master thesis was to investigate the effects of FODMAP restriction on fecal neutrophil gelatinase-associated lipocalin (NGAL); a potential biomarker of gut integrity and inflammation, and effects on the concentration of fecal short-chain fatty acids (SCFAs); metabolites of microbial fermentation. Also, the associations between GI symptoms and fecal NGAL and SCFA were investigated. Furthermore, the potential for using a Luminex-based method for measuring fecal NGAL, as an alternative to ELISA, was investigated.

Methods: The study, which followed a randomized parallel design, included adult CD patients on a gluten-free diet (GFD) having persistent GI symptoms. The participants were randomized to consume a low-FODMAP diet (LFD) in addition to their GFD (LFD-group, n=34) or to continue their regular GFD (controls, n=36) and were followed up for four weeks as out-patients at the clinic of Oslo University. Sampling of feces was performed at baseline and at 4-week follow-up. NGAL was measured in fecal extracts using ELISA, while fecal SCFAs were analysed using GC-FID technology. Also, a Luminex-based method was established, and compared to that of ELISA.

Results: Reducing the intake of FODMAPs did not affect fecal NGAL levels when comparing 4-week values between the LFD group and the controls using a linear regression model, controlling for baseline values. Similar linear regression models analysing the SCFAs indicated a significant effect of the intervention ($p<0.05$) for propionic acid and valeric acid, but the effects on both SFCAs were dependent on the baseline levels with a reduction only in those with initially high baseline levels. No differences between the groups were found for the remaining SCFAs. A Luminex microbead-based method for measuring NGAL in fecal samples was successfully established. Still, it showed higher concentrations than the ELISA method, and systematic proportional bias was detected when investigating the agreement between the methods. Several samples measured by the Luminex technology did not reach the limit of detection with ELISA.

Conclusion: Although the LFD reduced GI symptoms in CD patients with persistent GI symptoms, this was not reflected by changes in the inflammation associated biomarker NGAL indicating that the GI symptoms in the current study is not related to inflammation but may be caused by other FODMAP associated factors such as osmotic effects followed by mechanic stress. The effects of the FODMAP reduction on propionic acid and valeric acid also indicate possible changes in microbial communities which may affect the amounts of intestinal gasses produced. Further studies should explore the usefulness of NGAL as a biomarker of intestinal inflammation in CD and more directly explore the effects of LFD on intestinal gut microbiota.

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ABBREVIATIONS

4-PL	4-parameter logistics
AJ	Adherens junctions
ANCOVA	Analysis of covariance
APC	Antigen presenting cells
BA	Bland-Altman
BMI	Body mass index
CD	Celiac disease
CDK	Cyclin-dependent kinases
CI	Confidence interval
CRP	C-reactive protein
DGP	Deaminated gliadin peptide
ELISA	Enzyme-linked immunosorbent assay
EPI	Exocrine pancreatic insufficiency
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols
FT4	Free thyroxine
GALT	Gut-associated lymphoid tissue
GC	Gas chromatography
GFD	Gluten-free diet
GI	Gastrointestinal
GSRS-IBS	Gastrointestinal symptom sating scale – irritable bowel syndrome
HbA1c	Glycated haemoglobin
HLA	Human leukocyte antigen
IBD	Inflammatory bowl disease

IBS	Irritable bowl syndrom
IEC	Intestinal epithelial cell
IEL	Interepithelial lymphocytes
Ig	Immunoglobulin
IQR	Interquartile range
LCN2	NGAL gen
LFD	Low-FODMAP diet
LOA	Limit of agreement
MCV	Mean corpuscular volume
MFI	Mean fluorescence intensity
NCF	*Norsk cøliakiforening*
NGAL	Neutrophil gelatinase-associated lipocalin
RCT	Randomized controlled trial
SCC	Short-chain carbohydrates
SCFA	Short-chain fatty acids
SD	Standard deviation
TG2	Transglutaminase 2
TIBC	Total iron-binding capacity
TJ	Tight junction
TLR	Toll like receptors
TSH	Thyroid-stimulating hormone
tTG	Tissue transglutaminase
xMAP	Multi-analyte profiling of x analytes

1 INTRODUCTION

1.1 Anatomy and functionality of the GI system

The human gastrointestinal (GI) system is composed of a one-way continuous channel connected with accessory digestive organs to nourish, defend, and excrete waste products from our body (Liao et al., 2009). Most of the nutrition uptake takes place in the small intestine (**Figure 1.1a**), which is structured in three segments. Firstly, the initial C-shaped duodenum receives partly digested chyme from the stomach and further breaks it down mechanically and chemically while being supplied with pancreatic juice containing digestive enzymes (Fish & Burns, 2021). Then, the uptake of most of these catabolized macro- and micronutrients occurs in the jejunum and ileum segments (Patricia & Dhamoon, 2021) by absorbance across the semi-permeable gut wall as molecules and ions into the bloodstream (Matsumoto et al., 2015; Volk & Lacy, 2017).

The intestinal lining separates the host from its luminal environment by a single layer of intestinal epithelial cells (IECs) (Peterson & Artis, 2014). The majority of these IECs are the absorptive enterocytes, with their apical side being covered with ~3000 microvilli each, making up the brush border vital for the uptake (Fish & Burns, 2021). To prevent paracellular diffusion between the enterocytes, neighbouring enterocytes are connected with tight junctions (TJ) sealing the gut lining. (Khaleghi et al., 2016). Additionally, mucin-producing goblet cells, Paneth cells, microfold cells, and enteroendocrine cells are also essential for the intestinal lining to function as a physical, biochemical, and immunological barrier selectively absorbing nutrients and blocking other unwanted compounds out (Peterson & Artis, 2014). To ensure an effective nutritional uptake, the microvilli-covered enterocytes are curved on fingerlike villi structures **(Figure 1.1c)**, which again is curved on circular folds (plica) **(Figure 1.1b)**. This allows the small intestine to increase the absorbing surface area to impressively 400m^2 (Peterson & Artis, 2014; Thursby & Juge, 2017).

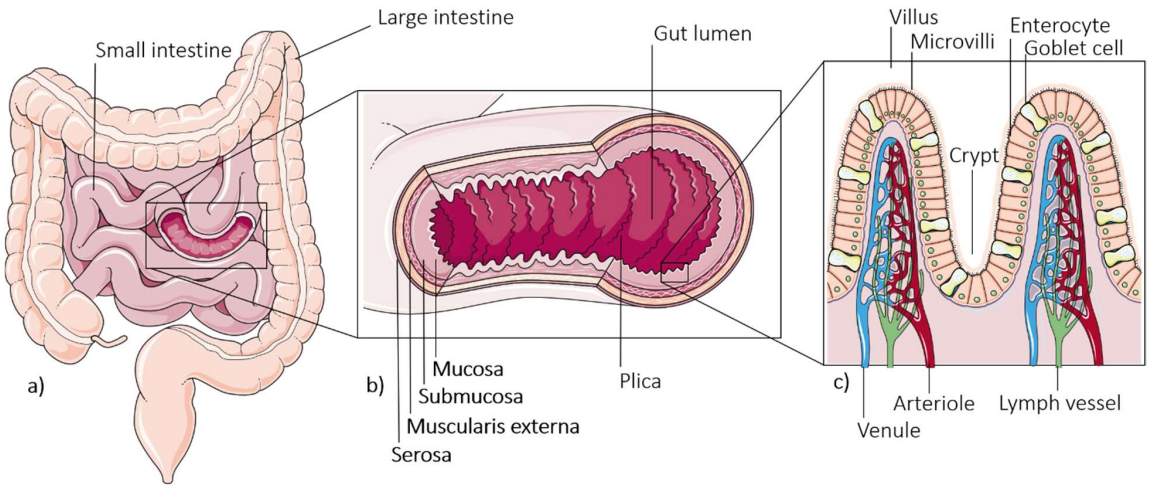

Figure 1.1: Structures of the small intestine. **a)** The small intestine is responsible for nutrition uptake by absorbance across the gut wall, **b)** which is composed of four layers: the mucosa, submucosa, muscularis externa, and serosa. Circular mucosal folds (plica) contain finger-like villi structures **c)** with invaginated crypts, enlarging the surface of the brush border (microvilli) to absorb nutrients effectively. This figure is based on an illustration from https://www.britannica.com/science/plica-circularis and further modified with elements from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/).

Once absorbed by the enterocytes, the underlying connective tissue (lamina propria) containing blood vessels allow nutrient transport into the circulation (Figure 1.1c). Underneath the lamina propria is a thin muscular layer (muscularis mucosa) contracting to fold the plicas. In total, these components make up the mucosa layer as the first of four main layers of the gut wall. Underneath the mucosa is the dense connective submucosa, following by a double muscle tissue consisting of longitudinal and circular muscle (muscularis externa), ensuring gut motility by contraction. Finally, a fibred tissue (serosa) separates the GI system from the abdominal cavity (Figure 1.1b).

Further down, the GI system follows the large intestine surrounding the small intestine with its ascending, transverse, descending, and sigmoid colon composition. Its primary function is to absorb water and electrolytes and eventually remove waste products through defecation (Azzouz & Sharma, 2021). The colon continues the small intestine by the caecum, which functions as a reservoir for commensal anaerobic bacteria by fermentation of complex carbohydrates undigestible by enzymes in the small intestine (Mowat & Agace, 2014). The diversity and richness of species increase dramatically after the small intestine and are assumed to be highest in the

caecum, ascending, and transverse colon. Colonocytes make up the majority of the intestinal layer in the colon, maintaining an anaerobic condition in the lumen due to the rapid metabolism of oxygen. This favours an environment for obligate aerobics, which tend to be the fermenting microbes. Their metabolism of dietary fibres results in end products such as beneficial short-chain fatty acids (SCFA) (Litvak et al., 2018) and gas such as H_2, CH_4, and CO_2. The GI tract finally terminates at the anus (Kim et al., 2008), defecating the metabolic products from microorganisms that are not absorbed by the colonocytes, in addition to other substances such as undigested foods and eliminated host components (Zubeldia-Varela et al., 2019).

With this dramatic exposure to the environment, the GI system needs to maintain a proper defence system balancing between tolerance and activation of immune responses (Valitutti & Fasano, 2019; Welcome, 2018). If this barrier somewhat breaches, early phases of an innate immune response are induced to aim for an intact and protective barrier (Mu et al., 2017).

1.2 Celiac disease (CD)

Celiac disease (CD) is a disease that resembles an autoimmune disease when the individual is exposed to gluten in the diet. In the presence of gluten, the players of the immune system are misguided to believe that the intestines have been infected, which will lead to the attack of healthy intestinal tissues resulting in villous degradation in the small intestine. The autoimmune response is causing the destruction of the intestinal villi (atrophy), leaving a much smaller surface available for nutrient uptake (Stamnaes & Sollid, 2015). Left untreated, CD can lead to additional severe health problems with a wide range of intestinal and extra-intestinal symptoms like diarrhoea, bloating, fatigue, weight loss, and anaemia. In untreated children, malabsorption of nutrients can impact growth and development, in addition to the symptoms seen in adults (Parzanese et al., 2017). When gluten is absent from the diet of an individual with CD, there will be no active immune responses against the IECs (Stamnaes & Sollid, 2015), and the pathological changes in the gut mucosa might heal. However, the small intestinal mucosa usually does not entirely restore to a fully healthy state (Sánchez et al., 2021). The onset of CD can be at any age but once diagnosed with CD, it is a lifelong disease only treatable with strict avoidance of gluten-containing food. Being gluten intolerant involves not only medical concerns but also social and economic concerns (Parzanese et al., 2017).

1.2.1 Prevalence and risk factors

CD is considered one of the most common food intolerances globally, with an estimated worldwide prevalence of 1% (Poddighe et al., 2019; Singh et al., 2018; Stein & Katz, 2017). Countries with predominant Caucasian populations in North America, Europe, North Africa, Northern India, and Australia tend to have a relatively higher prevalence (Gujral et al., 2012), and CD also occurs more frequently among women than men (Singh et al., 2018; Thomas et al., 2009). However, most undiagnosed cases, including other parts of the world, suggest a relatively higher prevalence than reported and raise the need for several population-based studies to improve the diagnostics (Gujral et al., 2012). The current incidence of CD in Norway is unknown. However, according to the Norwegian celiac disease association (NCF, 2020b), 1-2% of the Norwegian population is assumed to have CD, and the prevalence is increasing (King et al., 2020). Timing of gluten introduction, autoimmune disorders such as type 1 diabetes, genetic disorders, genetic predisposition, and prevalence of infections are highly related to CD development (Sarno et al., 2015). In addition to these genetic risk factors, the main environmental trigger of CD is exposure to gluten.

1.2.2 Gluten

Gluten collectively refers to diverse proteins found in the endosperm of the seeds in different grains such as wheat, barley, and rye (**Figure 1.2**) (Biesiekierski, 2017). They primarily function as storage proteins providing nutrients for plant growth (Wakasa & Takaiwa, 2013). However, their unique heat stable and binding properties have made gluten a major ingredient/component in bread and processed foods providing viscoelasticity. Gluten, therefore, plays a central role in the Western diet, with an average daily intake of 5-20 g (Biesiekierski, 2017). Wheat flour mainly consists of starch (~80%) and protein (~10%) (Shewry et al., 2013), in which gluten contributes to 85-90% of the total protein content. Wheat gluten consists of a mixture of two main types of polypeptides; glutenin and gliadin (Biesiekierski, 2017), and is very rich in repetitive glutamine- and proline-containing sequences (Wieser, 2007). This feature makes gluten highly resistant to digestive degradation (Shan et al., 2002) by resisting

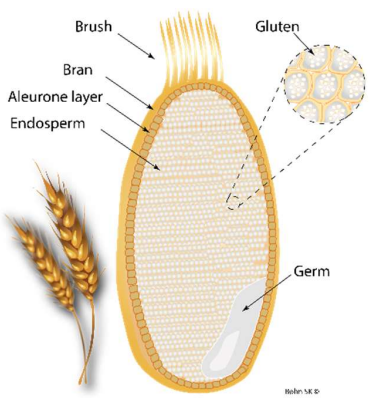

Figure 1.2: The main constituents of wheat grain. It is in the endosperm that we find gluten proteins. The figure has been used with permission.

hydrolysis mediated by several proteases in the GI tract (Balakireva & Zamyatnin, 2016). This incomplete degradation of gluten leaves a resistant fragment taken up by the enterocytes (Stamnaes & Sollid, 2015). For CD patients, gluten is the allergen causing the immune response giving severe health effects.

1.2.3 The mechanism behind CD pathology

CD is a severe systemic disease primarily affecting the small intestine (Maglio & Troncone, 2020), triggered by the ingestion of dietary gluten proteins (Parzanese et al., 2017). In the presence of gluten in the diet, CD resembles an autoimmune disease where the adaptive pro-inflammatory response is stimulated, and this pro-inflammatory condition is strictly dependent on the presence of gluten (Setty et al., 2015).

CD has a strong genetic factor and runs in families. People with a first-degree relative with CD disease (parent, child, sibling) have a 1 in 10 risks of developing CD (Al-Toma et al., 2019). One of the most important genetic risk factors for CD is the presence of HLA-DQ8 or HLA-DQ2, two genetic polymorphisms of the HLA-DQ molecule (Human Leukocyte Antigen, haplotype DQ) that serves to present antigens to the T-cells of the immune defence. It turns out that the HLA-DQ8 and -DQ2 are particularly well structured to bind the gluten peptides and that it is the gliadin part of the gluten molecule responsible for the immune response (Megiorni & Pizzuti, 2012).

For gliadin to induce an immune response, it must be presented to the T-cells on the HLA-DQ2/8 molecules. However, the gliadin molecule needs to be modified or deaminated to induce a negative charge necessary to bind in the groove of the HLA molecule (Sollid & Jabri, 2011; Sollid et al., 2012). The enzyme that performs this task is called Transglutaminase 2 (TG2) and is present in virtually all cells of the body. In untreated CD patients (still eating gluten in their diet), the level of TG2 is upregulated with an increased expression in the mucosa (Biagi et al., 2006; Iismaa et al., 2009). Ultimately a negative vicious circle is triggered with more deamination followed by more inflammatory responses. We know that anti-TG2 antibodies are essential in the pathology of CD, but their specific role remains unknown (Maglio & Troncone, 2020).

Autoantibodies are produced when B-cells that have specificity against TG2 recognize the TG2 in the TG2-gliadin complex and end up present gliadin on surface HLAs to gliadin specific T-cells. The help from the gliadin-specific T-cells will lead to clonal expansion and maturation of the

B-cell, leading to plasma cells secreting large quanta of anti-TG2 auto-antibodies (Martucciello et al., 2018). In the absence of gluten, the production of antibodies and autoantibodies will then be suppressed (Husby et al., 2020). However, there are still many knowledge gaps about the possible pathogenetic role of autoantibodies to TG2 in CD associated with the genesis of these antibodies. This involves the role of their specificities, their modulating ability toward TG2 enzymatic or non-enzymatic activities, and their biological effects (Maglio & Troncone, 2020).

Autoimmune diseases are due to a failure in tolerance of harmless and self. This type of failure is complex and caused by both genes and environmental causes. Suggestions of infectious agents for the development of CD are also highly discussed (Brown et al., 2018; Sánchez et al., 2021), in which they might, for instance, increase gut permeability as a possible contributing development factor (Moser et al., 2007). However, the exact causative factor(s) triggering the failure in tolerance remains unknown (Valitutti & Fasano, 2019). Therefore, there is much research in the gut health field to understand the mechanisms behind diseases like CD to improve treatment for easier diagnosing and adapting dietary advice to the different groups.

1.2.4 Diagnostics and treatment

In the presence of GI symptoms such as diarrhoea, weight loss, iron deficiency anaemia, and susceptibility to CD, a consultation for CD should be suggested (Sharma et al., 2020). The diagnosis of CD in adults is currently based on a combination of clinical, serological, and histopathologic consideration (Al-Toma et al., 2019). Due to the strong relation between the disease and an HLA-DQ2/8 genotype with ~95 and 5% of the CD patients (Leffler et al., 2015), respectively, positive genetic testing for either of these is required. However, due to the low specificity and the high prevalence in the population, this testing is only useful along with other tests (Kaukinen et al., 2002). A final removal and re-introduction of gluten in the diet is necessary to prove that gluten is the driver of the disease.

Due to the presence of IgA autoantibodies against TG2 in the serum of patients, TG2 is suggested as a specific and sensitive marker of the disease (Giersiepen et al., 2012; Werkstetter et al., 2017). Autoantibodies against TG2 are also present in CD patients' intestinal mucosa. They can be verified with IgA anti endomysial antibodies (EMA test), but this is a more resource-demanding test and is primarily reserved for children. IgG antibodies against deamidated gliadin peptides from gluten are also present in CD patients. In cases of IgA deficiency that frequently apply to these

patients, the serology evaluation is solely based on the result from the anti-DGP test (OUS, 2021). For those cases and adults in general, a biopsy confirming duodenal damage is required.

The classification of the biopsies is based on a modern Marsh classification (**Figure 1.3**) (Himalee Siriweera et al., 2016; Peña, 2015) considering the villi/crypt structure and the number of epithelial lymphocytes per 100 enterocytes. For the enterocytes in the duodenum and jejunum, the cut off for a normal count is <30/100 enterocytes and <40/100 enterocytes, respectively. The type 3 lesions are divided into three subtypes based on the severity of the villous atrophy, and CD patients are required a grade 3-4.

The mucosal damage is characterized as grades of:

- 0 = normal villi with normal lymphocyte count, indicating a normal small bowel.
- 1 = normal villi with increased lymphocyte count
- 2 = normal villi, crypt hyperplasia and increased lymphocyte count
- 3a = mild to moderate (partial) villous atrophy and increased lymphocyte count
- 3b = marked (subtotal) villous atrophy and increased lymphocyte count
- 3c = completely flat mucosa (total) villous atrophy and increased lymphocyte count
- 4 = completely damaged and eroded flat villi and increased lymphocyte count

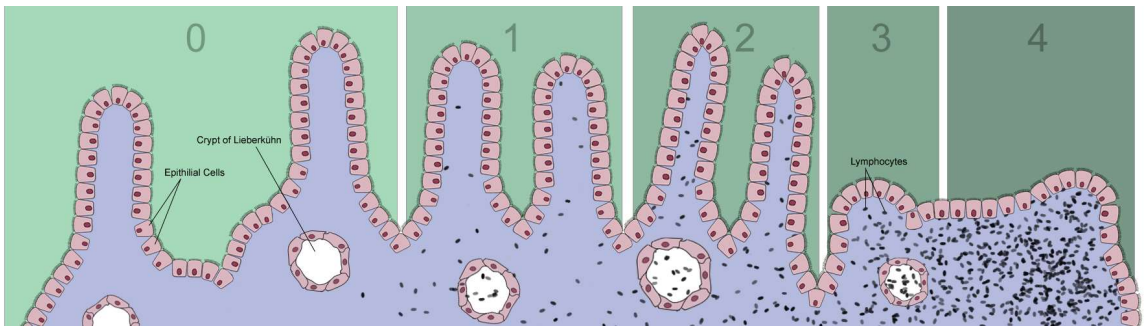

Figure 1.3: Illustration of the increasing destruction of epithelial cells, graded by Marsh classification from left to right: **0)** Normal villi structure *(Marsh grade 0)*, **1)** villi are still normal, but with infiltrative lymphocytes (Marsh grade 1), 2) additional crypt hyperplasia is seen *(Marsh grade 2)*, **3)** subdivisions of villous atrophy, seen in CD patients, classified either as mild, marked or completely atrophy *(Marsh grade 3a, 3b and 3c, respectively)*, and 4) villi are completely damaged and eroded flat (total atrophy) *(Marsh grade **4**)*. CD: celiac disease. The Figure is collected from Wikimedia https://upload.wikimedia.org/wikipedia/commons/9/9b/Coeliac_Disease.png and further modified.

The only treatment for CD so far is lifelong adherence to a gluten-free diet (GFD). The avoidance of gluten often leads to a lower intake of grains that are naturally rich in fibres. The intake of fibre in CD patients on a GFD diet has been shown to be lower than recommended. The daily recommended intake of fibres is 25 and 35 g for women and men, respectively (Helsedirektoratet, 2016). For comparison, CD patients have been reported to have a much lower intake, and for an American population is found to be around 20 and 24 g for women and men (Thompson et al., 2005). The low intake of fibres might be due to gluten-free food alternatives often being made with starches and/or refined flours (Wild et al., 2010), in which the outer layer of the grain containing the most fibre is removed (Penagini et al., 2013). Because fibre is a key nutrient for gut bacteria (Valdes et al., 2018), lifelong adherence to a GFD might not only lead to nutritional imbalance but also lead to shifts in the community of microbes in the gut called the microbiota.

1.3 Microbiota and the gut immune system

1.3.1 Gut leakage

The gut microbiota is of great importance to support the gut lining and is essential in regulating environmental factors that enter the body (Mu et al., 2017). Gut leakage is a term for increased intestinal permeability leading to the invasion of microbes and luminal components into the lamina propria (Camilleri et al., 2012). The lining of the essential epithelial cells combined with (secreted) protective factors such as mucins, antimicrobial molecules, immunoglobulins, and cytokines composes a defence barrier separating the host from the environment. A healthy intestinal lining obtains a selective permeability, absorbing essential nutritional factors while blocking the entry of other components (Mu et al., 2017). With a breach in the intestinal barrier, in which the TJs sealing the neighbouring epithelial cells are impaired, exterior gut containment such as food antigens, bacteria, and toxins might invade the mucosa beneath the leaking enterocytes. This might change the immune system from a tolerant mode to an inflammatory mode inducing a variety of immune components to be produced, causing local or systemic immune responses if further diffusing into the circulation (Camilleri et al., 2012).

A ‘leaky gut’ is associated with a dysbiosis in the microbiota homeostasis and might be caused by dietary changes, drugs, and infections (Le Barz et al., 2019). In addition, CD dysbiosis might be associated with abnormal TJs in which gliadin peptides might enter and expose the immune system

(Sánchez et al., 2021). Therefore, a possibility of reversing the 'leaky gut' might be considered by prebiotics with the production of proteins essential for TJs (Mu et al., 2017).

1.3.2 Metabolism, symbiosis, and tolerance

The gut microbiota is the community of all living microorganisms in the gut environment, with the majority being composed of bacteria (Valdes et al., 2018). The microbiota composition differs along the GI tract, causing different microbes to colonize specific environments depending on chemical and physical conditions (Thursby & Juge, 2017). This favours a symbiotic relationship between the colonizing bacteria and the host, and a selective immune tolerance to these beneficial microbes is established (Hills et al., 2019). The presence of bacteria is crucial for developing the immune system in which the microbiota 'train' the immune system to distinguish between harmless commensals and pathogens. In healthy individuals, the immune system tolerates and co-exist symbiotically with the commensal microbes by hosting and providing metabolic environments (harvesting energy from food) for the bacteria. Evidence of the importance of bacteria in developing tolerance and immunity is proven by germ-free mice having defects in the development of secondary lymphoid tissue (Macpherson & Harris, 2004). In return, the microbes serve beneficial protection against pathogens/colonization in which they compete with nutritional sources, produce antimicrobial molecules (Ducarmon et al., 2019), lower the pH by producing acids, inactivate toxins from foods, and during infections they react with inflammation responses (Hills et al., 2019).

The microbiota density increases along the GI system, and in the colon, the vast number of 10^{14} is estimated (Sender et al., 2016). Meeting lower oxygen levels in the colon, the microbiota is dominated by anaerobic bacteria fermenting undigestible foods from dietary sources not processed thoroughly in the small intestine (Mowat & Agace, 2014). In the metabolism of these complex carbohydrates (FODMAPs), SCFA, metabolites and certain gases are produced.

SCFA are organic volatile acids characterized by chains of 1-6 carbon atoms (Markowiak-Kopeć & Śliżewska, 2020) functioning as essential nutrients for the colonocytes (Siddiqui et al., 2017). In this way, the bacteria interact with the epithelial integrity, making sure lymphatic tissues hosting immune cells are growing (Sánchez et al., 2021). For instance, *Bifidobacterium* protects against colonization of pathogens by synthesizing acetate (Fukuda et al., 2011). Another major SCFA synthesized is butyrate, which helps to keep the intestinal immune system in a tolerant mode by

regulating T-cells (Sun et al., 2017). SCFA also contribute to lower the inflammation status epigenetically by inhibiting histone deacetylases, causing hyperacetylation of histones and in this way initiating anti-inflammatory gene activation in IECs (Woo & Alenghat, 2017) (Schilderink et al., 2013). In this way, the SCFAs is essential in maintaining intestinal and immune homeostasis.

Other essential metabolites synthesized by the colonic microbiota is beneficial vitamins taken up by the colonocytes such as vitamin K and B (LeBlanc et al., 2013). Further, in mice studies, it is found that some species contribute to the production and maintenance of the protective mucous layer (Martín et al., 2019; van der Lugt et al., 2019). Additionally, the properties of the intestine are the presence of protective and bacteria defeating essential IgA. These immunoglobulins are of great importance as a first line defence, maintaining gut homeostasis by binding and inhibiting pathogens present in the lumen, preventing them from affecting the gut barrier. IgAs are produced by the gut associated lymphoid tissue (GALT) and secreted by plasmacytes (Peterson et al., 2007). To sum up, these essential functions contribute to maintaining an active and tolerant mode in the small intestine. A change in the gut microbiota composition (dysbiosis) and its characteristics is therefore highly suggested to be connected with the pathogenesis of several diseases (Thursby & Juge, 2017).

1.3.3 Microbiota and CD

A specific CD microbiota signature is still not recognized. However there are still associations with CD to a change in the microbiota composition and function (Valitutti et al., 2019), as several of other chronic inflammatory diseases (Valitutti et al., 2019). Such autoimmune diseases are due to a failure in tolerance of harmless and self. This type of failure is complex and caused by both genes and environmental triggers. Viral or microbiological infections might have a possible causative role in triggering the failure of the immune responses and a lack of tolerance for the gliadin, leading to the development of CD (Sánchez et al., 2021). Antibodies against the reovirus are also higher in CD patients than in control, according to a study by Bouziat (2017). Additionally, discussed risk factors related to CD development connects to how the immune system is treated in the early years during the main development (Sánchez et al., 2021).

Influential factors of the intestinal CD microbiota composition starts in early life, being factors such as the genetic background (considering the HLA genotype), type of birth delivery, nutritional factors as new-born and in early age (Azad et al., 2013; Dominguez-Bello et al., 2010) and

exposure to antibiotics (Dydensborg Sander et al., 2019). Exposure to microbes at an early age seems to be decisive for which bacteria colonize the intestine and hence help to develop the immune system. Microbe exposure throughout the early age will make the diversity until it is stabilized by mainly the phylum of *Firmicutes* and *Bacteroidetes* (Rinninella et al., 2019). By the age of three years, the diversity and composition resemble the ones in adults (Yatsunenko et al., 2012). Additionally, the 'hygiene hypothesis' might be linked to the increased prevalence of CD, considering an under stimuli of infectious exposure to 'train' the immune system sufficiently during early life (Lerner & Matthias, 2015).

Suggestions that infections might play a crucial role in the development of CD is also recently known, but any exact pathogenetic pathway is not figured out (Bouziat et al., 2017). Considering viruses, different types have impacted the immune system in different ways, such as promoting apoptosis of enterocytes or immune responses to food antigens such as gliadin peptides (Bouziat et al., 2017). Suppose the mucosal immunity is disturbed either due to a breach in the gut epithelial linings and/or pathogenetic and invasive microbes. In that case, the immune response in the gut goes from a tolerant mode to an active inflammatory mode. Then the food antigens and commensals might induce inflammation in terms of allergy and hypersensitivity (Steele et al., 2012).

Among CD patients, the GFD itself might also impact the gut microbiota composition. Wheat is one of the primary sources of fructans (Whelan et al., 2011). As fructans are contributing with beneficial prebiotic effects by being an essential energy source for commensal microbes (Jackson, 2010), the lack of fructans intake during a GFD might cause dysbiosis for CD patients (Sánchez et al., 2021). To sum up, the microbiota specific for CD patients is somewhat different from healthy individuals. Still, the individual variations make it challenging to state a specific microbiota-signature applying to CD patients (with persistent GI symptoms) in general (Kverka & Tlaskalova-Hogenova, 2017).

1.4 Irritable Bowel syndrome (IBS) and fermentable oligo-, di-, monosaccharides and polyols (FODMAPs)

1.4.1 IBS

Despite a strict GFD, CD patients experience ongoing GI symptoms such as loose stool, diarrhea, constipation, nausea, bloating and abdominal pain. Additional extra intestinal symptoms such as fatigue, depression, and joint pain are common among CD patients (Stasi et al., 2016). These symptoms overlap with those in irritable bowel syndrome (IBS) (Usai-Satta et al., 2020), and according to different studies, IBS-like symptoms are strongly related with CD patients (Domżał-Magrowska et al., 2016; Gembe & Comba, 2020), and it is found to occur as frequently as 38% among CD patients (Sainsbury et al., 2013). Whether CD patients predisposes to the IBS description, or it 'accidently' has been given to the same individual is not fully understood (Saha, 2014).

IBS is a functional GI disorder with chronical abdominal pain or discomfort and changed bowel experiences (Ng et al., 2018). The IBS pathogenesis is poorly understood and with the lack of available biomarkers it is challenging to diagnose (Barbara & Stanghellini, 2009), however there are many possible mechanisms suggested for IBS development such as visceral hypersensitivity, microbiota dysfunction or low-grade inflammation (Holtmann et al., 2016). As IBS patients have an increased inflammatory status when compared to healthy control individuals (Ford & Talley, 2011; Marshall et al., 2004; Shulman et al., 2014; Vazquez-Roque et al., 2012) finding mucosal inflammation at both a microscopic and molecular level, IBS is considered a low-grade inflammation.

The presence of this low-grade inflammation overlaps with inflammatory bowel disease (IBD) (Lazaridis & Germanidis, 2018) including ulcerative colitis and Chron's disease (Zhang & Li, 2014), and has a central role among GI disorders. There are many overlaps in symptoms, possible mechanisms, and treatments for these patient groups. The reasons of CD patients experience persistent IBS-like symptoms are still not fully covered, meanwhile the presence of FODMAPs have been strongly suggested to play a role among IBS patients (Staudacher & Whelan, 2017).

1.4.2 FODMAPs

FODMAPs is an acronym for fermentable oligo-, di-, monosaccharides and polyols, and describes a collection of short-chain carbohydrates (SCCs) found naturally in many foods (**Table 1.1**) (Syed

& Iswara, 2021). Mainly monosaccharides can be actively absorbed by the small intestine while disaccharides, such as lactose, are hydrolysed by digestive enzymes, and polyols (sugar alcohols) might be passively absorbed (Shepherd et al., 2013). The SCCs that are not completely digested and absorbed due to their resistance to degradation by the digestive enzymes move slowly further to the colon. In this way, they cause an osmotic effect attracting water to the gut lumen, which may cause dehydration, diarrheal conditions (Barrett et al., 2010), and also create a higher pressure in the gut experienced as abdominal pain (Staudacher & Whelan, 2017). When migrating to the colon, nondigestible oligosaccharides such as fructo-oligosaccharides and galacto-oligosaccharides are degraded as metabolites by microbial fermentation (**Figure 1.4**) (Shepherd et al., 2013). This causes the production of gases, such as CH_4, H_2 and CO_2 e.g., which may be experienced as discomfort and flatulence symptoms for the patients (Ong et al., 2010). Along with the osmotically effect stretching and bloating mechanoreceptors in the intestinal wall (Hewawasam et al., 2018), these oligosaccharides are also known as the main triggers of IBS symptoms (Shepherd et al., 2013).

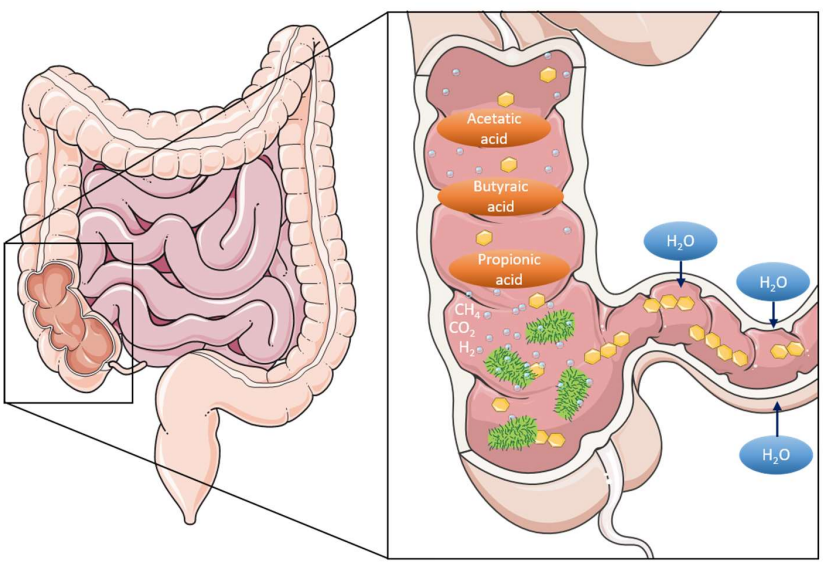

Figure 1.4: Illustration of the bacterial fermentation of FODMAPs in the colon. As SCC of the different FODMAP types are not completely digested in the small intestine, they enter the colon still intact. Due to the osmotic effect, water fluxes into the gut lumen and further down the GI system. When entering the colon, the SCC are digested through fermenting microbiota, causing a production of gas (H2, CO2 and CH4) and SCFA (such as acetic, butyric, and propionic acids). As the osmotic activity and production of gas expands the colonic lumen, GI symptoms might be experienced. FODMAP: fermentable oligo-, di-, mono-saccharides and polyols, SCC: short-chain carbohydrates, GI: gastrointestinal, SCFA, short-chain fatty acid. This figure is based on an illustration from https://www.nature.com/articles/nrgastro.2013.259 and figure elements are taken from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/) and Adobe Stock (https://stock.adobe.com/hu/license-terms/).

The low-FODMAP diet (LFD) was developed by researchers at Monash University in Australia in the early 2000s as a dietary therapy for managing IBS symptoms (Varney et al., 2017). Studies that have tested this dietary therapy show that the LFD tend to reduce GI symptoms for as many as ¾ of normal IBS patients (CD patients or not) (NCF, 2020a; Staudacher & Whelan, 2017). Further, it has been shown that the LFD can reduce certain biomarkers of inflammation in IBS patients reported to diet (Hustoft et al., 2017). Implementing an LFD includes three phases. Firstly, the initial FODMAP restriction of 2-6 weeks. Secondly, a structural reintroduction of individual FODMAPs to map which foods are tolerated and which triggers symptoms, and finally, a phase of tailoring a personal diet by re-introducing the tolerated FODMAP-rich foods to the regular diet (Whelan et al., 2018). Since FODMAPs naturally occur in many, a complete exclusion of FODMAPs from the diet is impossible. Therefore, the LFD is based on restricting the intake of FODMAPs by replacing certain FODMAP-rich foods with FODMAP-low foods. Low-FODMAP dietary items are based on extensive food composition data and defined 'cut-off values' used to classify foods as LFD (Varney et al., 2017). By selecting food items with a low FODMAP content, it is possible to reduce the amount of FODMAPs in the diet drastically.

Table 1.1: Common food sources for different FODMAP types in a descending order considering the short carbohydrate chains (SCCs) (Shepherd et al., 2013).

FODMAP	**Oligosaccharides** Fructans and galactans	**Disaccharides** Lactose	**Monosaccharides** Fructose*	**Polyols** Sorbitol, mannitol, maltitol and xylitol
Common food sources	Wheat, barley, rye, onion, leek, white part of spring onion, garlic, shallots, artichokes, beetroot, fennel, peas, chicory, pistachio, cashews, legumes, lentils, and chickpeas	Milk, custard, ice cream, and yoghurt	Apples, pears, mangoes, cherries, watermelon, asparagus, sugar snap peas, honey, high-fructose corn syrup	Apples, pears, apricots, cherries, nectarines, peaches, plums, watermelon, mushrooms, cauliflower, artificially sweetened chewing gum and confectionery

*Fructose is in excess of glucose (free fructose). FODMAP: fermentable oligo-, di-, monosaccharides and polyols.

There are no known histological criteria for IBS, therefore, symptom-based criteria are currently used to evaluate IBS diagnosis (Sood & Ford, 2018). As this provides self-reported GI symptoms it contributes to a lack of validation and struggles predicting IBS, and therefore, there are several

weaknesses with the current IBS evaluation (Kim et al., 2017). Accessing inflammation or gut leakage through biomarkers would allow for the implementation of personalized healthcare strategies and to identify new tools for the prevention, screening, and treatment of diseases. The development of potential biomarkers is therefore highly relevant (Mu et al., 2017).

1.5 Biomarkers for gut health

1.5.1 NGAL as an inflammatory biomarker

Human neutrophile gelatinase-associated lipocalin (NGAL) is a small glycoprotein (24 kDa) in the Lipocalin superfamily coded by the gene LCN2 (Thorsvik et al., 2017b) and is involved in iron homeostasis, inflammation and microbial infections (Zollner et al., 2020). NGAL proteins compete with bacteria by sequestering iron bound to siderophores (**Figure 1.5**), which are compounds secreted by the bacteria to capture iron (Bao et al., 2010). The complex of NGAL, siderophores and iron is shown to be transported into the cells by endocytosis (Chakraborty et al., 2012) and further into the bloodstream circulating as a complex to supply the cells with iron (Bao et al., 2010). As iron is an important nutritional factor for the bacteria, the lack of accessible iron due to the NGAL sequestering of the siderophores will prevent further bacterial protein synthesis (Nielsen et al., 1996). With this antibacterial effect on the bacteria by inhibiting growth, NGAL contributes to defeating infections and is considered part of the innate immunity (Goetz et al., 2002).

NGAL is mainly expressed in neutrophilic granulocytes and inflamed epithelium in the colon and released by the granules into the gut lumen as a response to inflammation and infections, causing the NGAL levels to be rapidly elevated (Thorsvik et al., 2017a). NGAL might also be released by epithelial cells during inflammatory conditions after being synthesised when epithelial cells are stimulated by cytokines (Chakraborty et al., 2012), where T-cells release the cytokines as a response to APC presenting antigens from bacteria. NGAL might further be detected in feces (Thorsvik et al., 2017a). As NGAL proteins are relatively stable to proteases and expressed in the colonic epithelium, NGAL is suggested as a promising non-invasive biomarker for IBD (Zinkevich et al., 2014; Zollner et al., 2020). Currently, calprotectin is the commonly used biomarker to reflect acute intestinal inflammation by neutrophilic release for IBD patients (Zollner et al., 2020). As calprotectin and NGAL are highly and positive correlating when measured with ELISA (rho = 0.82 and 0.87) (Thorsvik et al., 2016; Zollner et al., 2020), along with the additional

epithelial localization of NGAL in different from calprotectin, NGAL might provide supplementary useful information about the inflammatory process (Thorsvik et al., 2016). Therefore, NGAL is an attractive candidate to function as an IBD inflammation biomarker and potentially for IBS as well due to the high sensitivity of low-grade inflammation.

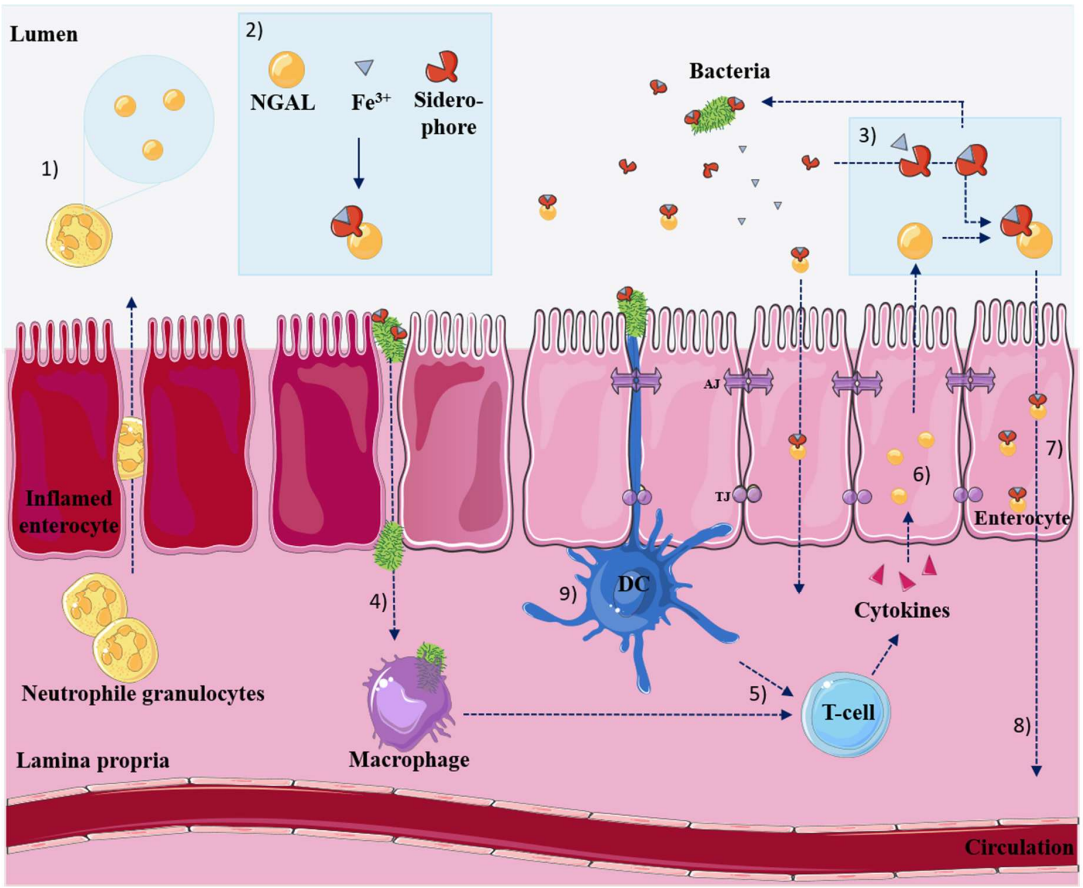

Figure 1.5: Illustration of a suggested mechanism for neutrophil gelatinase-associated lipocalin (NGAL) competing with iron to prevent bacterial growth. **1)** During inflammation of the intestinal enterocytes and infections of enteric pathogens, the epithelial layer becomes permeable, and NGAL containing neutrophilic granulocytes are released into the lumen. **2)** NGAL enables to bind siderophores, **3)** which is released by bacteria to capture iron. **4)** Bacteria penetrating the epithelial layer is met macrophages or other APC and is further presented to T-cells **5)** being activated and inducing the release of cytokines. **6)** These further trigger NGAL synthesis and release into the gut lumen. **7)** NGAL bound to siderophores and iron might be taken up by the enterocytes, **8)** and further into the circulation. The NGAL secretion during normal conditions (without inflammation) remains unknown. Low NGAL levels during inflammation might be due to a continuous expression or **9)** might be initiated by DC activating T-cells. NGAL: neutrophil-associated gelatinase lipocalin, APC: antigen-presenting cells, DC: dendritic cells, TJ: tight junctions, AJ: adherence junctions. The figure is based on a previous master's thesis (Aspholm, 2020), and figure elements are taken from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/) and Adobe Stock (https://stock.adobe.com/hu/license-terms/).

1.5.2 SCFA as an indirect biomarker of microbiota

SCFA are produced in the colon through microbial fermentation providing several health benefits to the host by maintaining intestinal barrier integrity, stimulating mucus production, and contribute with other anti-inflammatory factors (Markowiak-Kopeć & Śliżewska, 2020), as mentioned in section 1.3.2. By measuring the qualitative and quantitative factors of the host' SCFA, one can indicate the composition of the microbiota in the gut. As an imbalance of the intestinal microbiome is indicated to occur for IBS patients, even without any consistency in the altering, SCFA might function as a biomarker for intestinal dysbiosis and various intestinal diseases such as IBS (Farup et al., 2016).

1.6 Method validation for biomarker measurements

Biological substances present in our tissues, cells and fluidics such as circulatory drugs or fecal proteins are part of different biological homeostasis. Their concentrations are therefore of great interest when investigating pathophysiological conditions (Aydin, 2015). Biomarkers are, therefore, highly relevant as diagnostic tools and should be validated through both biologically and analytically aspects (Amaravadi, 2016). Furthermore, the sample holding the biomarker is also essential as the stability of the material and analyte is critical for avoiding an underestimation (Hewitt et al., 2012).

To reach a reliable diagnostic tool using biomarkers, the method measuring the analyte of interest should be verified. Unless a specific, accurate and precise assay is already measuring the true value of the analyte concentration, multiple initial phases of testing and failing are required (Amur et al., 2015). Therefore, reaching a uniform standard for each biomarker is challenging but necessary to reach the level where it can function as a patient measurement.

As new technology develops, biomarker measurements should adjust and take advantage of new available methods, and a conversion from one method to another should be performed with great attention. To successfully translate a biomarker measurement from one method to another, it is important that achieve the best optimal measurement by certain criteria considering both analytical and biological aspects (Amaravadi, 2016). Traditional immunoassays are being challenged by novel multiplex technology with the potential to measure hundreds of analytes simultaneously (Luminex Corporation, 2018), motivating for a method validation suggesting that the newest

method is providing several strengths considering the validation parameters (Anderson et al., 2015). Benefits with such newer methods, in which ELISA are being challenged by multiplex technology such as Luminex, includes the ability to use small volumes, measure up to 500 analytes at the same time for less labour and also provide a higher dynamic range (Table 1.2) (Invitrogen, 2016).

Table 1.2: Overview of essential benefits with xMAP multiplexing assays (Invitrogen, 2016)

Time savings	Luminex systems allow testing of up to 500 analytes in a single sample. In the same time it takes to set up one ELISA, multiple analytes can be measured, significantly reducing labour time.
Smaller sample volume	depending on the expression levels, assays require 50 µL of sample or less for multiplex assays, while still obtaining accurate results for all analytes.
Broad dynamic range	provides the ability to reliably detect proteins across a broad concentration range.
High throughput	the Luminex system automatically reads up to 96 samples from a conventional microtiter plate. Combined with the ability to read up to 500 analytes per sample, this provides a high-throughput path to data collection

ELISA: Enzyme-linked immunoassay.

2 AIM OF THE STUDY

This master thesis reports on secondary endpoints of a randomized clinical trial (RCT) which was led by investigators at Oslo University Hospital and University of Oslo and conducted from October 2018 to June 2019. The study was designed to investigate the primary endpoint of whether removal of FODMAPs can relieve GI symptoms in CD patients with persistent IBS-like symptoms despite following a strict gluten free diet. Preliminary results indicate that the reduction in FODMAPs reduced the GI symptoms (van Megen, F. et al., unpublished results). In the current master thesis, the effects of the intervention on secondary endpoints have been investigated, including effects on fecal biomarkers like NGAL and SCFA. The study is focusing on two major aspects. Firstly, to investigate whether removal of FODMAPS impacts biomarkers of gut health and secondly to establish and validate a multiplex method.

The specific aims of the current master thesis are to:

1. Investigate whether removal of FODMAPs impacts gut health in CD patients with persistent GI symptoms,
    I. by measuring the inflammation biomarker NGAL in feces.
    II. by measuring levels of SCFAs, which are metabolites produced by the microbiota.

2. Establish a multiplex method for measuring the inflammation biomarker NGAL using Luminex-based technology (antibody coupled MagPlex microspheres) and validate the method against ELISA using correlation and agreement analysis (Bland-Altman).

3 MATERIAL AND METHODS

3.1 The CD-FODMAP study design

CD-FODMAP study is a chosen short title for the RCT; 'Effect of LFD in well treated celiac patients with persistent GI symptoms - a randomized controlled trial'. In this RCT, which followed a randomized parallel design with two groups, Norwegian adult CD patients having persistent GI symptoms were invited to join the study and followed up as out-patients at the clinic of Oslo University. A cross-over design was not possible since it would be impossible to de-educate the intervention group after being introduced to and following the LFD. The control group was instructed to follow a GFD as normal, while the intervention group was instructed to follow a GFD *and* LFD. The participants were measured, and biomaterial was collected at baseline and after 4 weeks following the different diets. All patients were offered diet advisory visits at the clinic after the treatment in which a FODMAP re-introduction was instructed to the intervention group, while the control group was offered introduction to the LFD.

Inclusion criteria for the study population were determined in advance of the primary endpoint of the CD-FODMAP study (investigating a possible effect of the LFD on GI symptoms), with the purpose to avoid factors disturbing the study population. A web-based recruitment was performed as most CD patients in Norway are registered as members of the Norwegian celiac disease association along with the unique situation of social media platforms such as Facebook and other relevant channels. The recruitment was also directed to participants who had completed the primary study, in which patients scoring high on reported outcomes measuring GI symptoms were invited by email to participate in the current study. Potential participants between 18 and 75 years old were then contacted by telephone for a pre-screening verifying that they were biopsy-diagnosed for at least one year and had followed a strict gluten-free diet for at least one year. Further, they were invited at the study centre for a screening to be qualified for participation. Confirming a current inactive disease status was done by the serologic diagnostic markers IgA antibodies against tissue-TG2 and IgG antibodies against deaminated gliadin peptide (DGP). A negative test was verified by obtaining values under the reference limit at <4 U/mL and <20 Units, respectively. In addition to a normal serology, a normal duodenal biopsy (obtained by gastroscopy after the screening before baseline) was assessed to consider the histopathologic Marsh score, with a cut off score for inclusion being equal to or less than 1. As the final inclusion criteria, persistent GI symptoms necessary for trial attendance was a GSRS-IBS score equal to or above 30.

As illustrated in **Figure 3.1**, fecal samples was collected first at baseline (in addition to nutritional data and questionnaire-based physiological measurements to obtain information about their daily diet, quality of life and fatigue). Then the participants were allocated in either the control or the intervention group by randomization. The diet adherence was registered by a questionary halfway in the intervention. At follow-up, the same questionnaires answered at baseline and second fecal samples were again collected, in which the latter provide the main research material for answering the aims in this current master thesis.

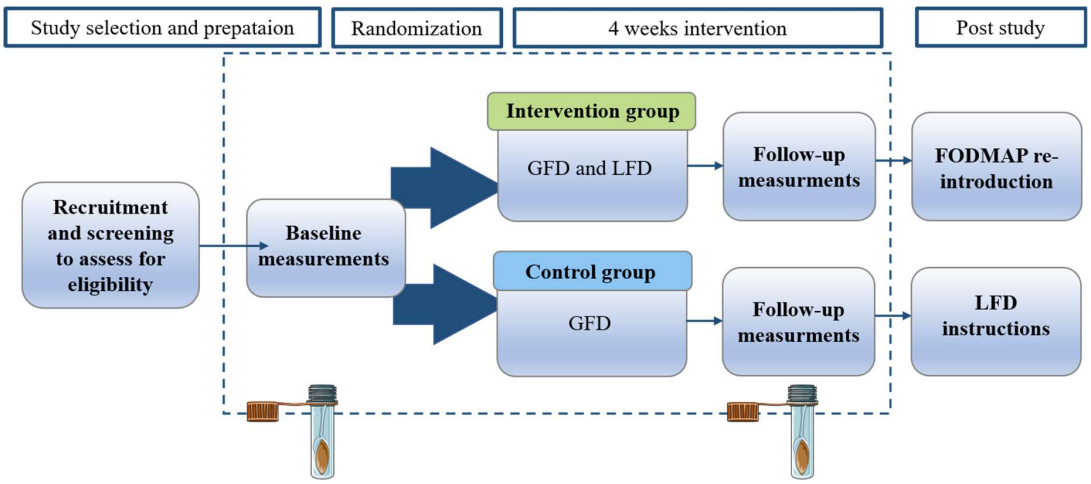

Figure 3.1: Schematic flow chart of the CD-FODMAP study design showing the progress through the phases of the parallel randomized trial. Celiac patients with persistent gastrointestinal symptoms were recruited to the study. Patient measurements were performed at screening to assess the recruited participants for trial eligibility, in advance of the group allocation (at baseline), and after the intervention (follow-up measurements). A re-introduction of FODMAPS was performed for a subgroup of the patients. However, the current master project focus on the effects of the reduction of FODMAPs only (dotted square). CD: celiac disease, FODMAP: fermentable oligo-, di-, mono-saccharides and polyols, GFD: gluten-free diet, LFD: low-FODMAP diet. Figure elements are taken from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/) and Adobe Stock (https://stock.adobe.com/hu/license-terms/).

3.2 Ethics and approvals

Ethical approval for the CD FODMAP study was obtained from the Regional Committees for Medical and Health Research Ethics (REC Id number 2018/1055) (**Attachment – E.1**) as well as from the data protection officials at Oslo University Hospital and Akershus University Hospital. The study is registered in the National Institutes of Health Clinical Trials (www.ClinicalTrials.gov; Identifier: NCT03678935). Subjects gave written informed consent. All approvals from REK and letter of informed consent are attached in **Attachment E.1, E.2, E.3** and **E.4.**

The intervention period was limited to 4 weeks, in which the intervention group was encouraged to limit certain types of foods containing high FODMAP levels. As this restriction only was applied over a short time it was considered as no harm for the participants. There is also little risk associated with gastroscopy and blood samples taken. When the intervention period was terminated, the control group was offered instructions to follow the LFD by individual guidance from a clinical dietitian. The intervention group was offered a supervised re-introduction of FODMAPs to detect their individual tolerations of different types and portions of FODMAP rich foods.

3.3 Intervention and sampling

The intervention in this master project is defined as the 4 weeks long diet-exposed period from baseline to follow-up, providing the fecal samples for the NGAL and SCFA measurements investigated in this study. During the intervention, the control group was instructed to maintain a regular GFD as normal, while the intervention group was encouraged to maintain their daily diet and change as few factors as possible except the intended replacement of high-FODMAP containing foods with low-FODMAP containing foods.

The fecal samples were collected by the participants at two timepoints; in advance to and after the intervention referred to as the baseline and follow-up samples, respectively. The sample equipment for the fecal collection was handed out to the patients at screening and delivered at screening or baseline. While they were asked to collect the fecal samples a few days in advance of the baseline, we know that several patients also collected the feces material several weeks in advance. The study lasted over many months, so the period between screening and baseline was ranging over a period up to 8 weeks, and in addition the intervention started on different dates and seasons for the

participants. However, everyone had exactly 4 weeks between baseline visit and follow-up (plus minus 1-2 days). The patients were encouraged to collect fecal material enough to fill up half the sample tubes (13 mL tubes assembled with a spoon cap).

3.4 Preparation of samples

The patients were required to collect the fresh fecal material, temporarily store them in their freezer and transporting them in a specially made freezing element in Styrofoam boxes for the delivering to the study centre at OUS. Here, they were then stored in -80°C until the transportation to NMBU which was performed in Styrofoam boxes with dry ice by car and directly put in -80°C at NMBU until further aliquoting. The fecal aliquoting procedure was based on a method recommended from Unger-Vetlesen Institute, Lovisenberg Diaconal Hospital in Oslo, and the aliquoting was performed in advance of the current master thesis.

At least one day before the aliquoting procedure the samples were transferred from -80°C to -20°C ensuring a gradually thawing process allowing the fecal lump to reach a thoroughly soften consistence, simplifying the cutting process. Each sample was thawed and transferred from the sample tubes and distributed into 6 new aliquots where three of the aliquots were weighted and dedicated for NGAL detection with ELISA (50-100 mg), GC-FID analysis of SCFA (minimum of 0,6 g aliquots), and DNA extraction for 16S/RMS sequencing (app. 0,2 g). The latter aliquot is not included in this master thesis, and the remaining three aliquots were stored as backup material in 1,5 mL Eppendorf tubes containing as much material as possible.

The aliquoting procedure was performed on a paper covered bench in a fume hood to avoid smell and contamination. One sample tube at a time were taken out of -20°C and put on ice whenever possible to prevent analyte. When the fecal lump was thawed enough to detach the sample tube, the sample spoon was gently pulled out of the tube with a tweezer and placed on a new piece of non-absorbent paper. In some cases, it was necessary to hold the sample tube by hand and warming it for some minutes for it to detach the sample tube. For the sample tubes that did not contain a spoon, a lab spatula was used to get the feces out of the tube. Further the feces lump was cut into aliquots of different sizes with a disposable scalpel blade. If observed, undigested foods such as large seeds, corn kernels and red peppers, were excluded as insoluble substances before transferring to corresponding Eppendorf tubes. When all aliquots from one sample were made, the

original sample tube (if anything left) and Eppendorf aliquots was brought back to -20°C for storage. Washing procedures before aliquoting a new sample was performed by washing the tweezer and spatula used with soap and hot water. In addition, it was sprayed with 70% ethanol and treated with DNA decontamination reagent. At the end of the day, original sample tubes were brought back to -80°C.

The aliquots were treated slightly different as part of the analysing preparations, in which the procedures described in section 3.5.1 applies for the aliquots assigned for NGAL measurement with ELISA (which also was analysed by Luminex after the ELISA measurement). For the SCFA analysis procedure described in 3.5.2, the aliquots were appointed 600 µL STAR buffer and resuspended by 'vortexing' before being sent to Lovisenberg Hospital for GC-FID to quantify and identify the SCFA. For the method evaluation described in section 3.5.3, a selection of the already measured ELISA samples was used, and therefore the sample selection was treated as equally to the ELISA preparation procedure as possible.

3.5 Biomarker measurements

As mentioned in section 1.5.1, ELISA is a suggested method for measuring fecal NGAL, as it is a commonly used assay and has several kits developed intended for this measurement. A possible effect on NGAL as a gut inflammation biomarker might reveal relations between the LFD and inflammation status in the CD patients with persistent GI symptoms. GC is the predominant method used to detect SCFA, which is of interest when investigating the SCFA composition to uncover knowledge of the metabolism to the microbiota as SCFA is a product of bacterial fermentation. This master thesis lastly also includes NGAL measurement with the multiplexing tool Luminex to develop and enhance the measurement of fecal NGAL as newer technology is available.

3.5.1 Quantifying fecal NGAL with ELISA sandwich assay

The ELISA sandwich assay is one of several enzymatic immunoassays based on specific antibody-antigen interactions. With the antibodies in excess, the antibody-antigen equilibrium forces to favour a complex ("sandwiching the antigen") in which conjugated detection antibodies are reflecting the analyte of interest with high sensitivity when the signal density is measured (Shcherbakova, 2007). In addition to simplicity, reagents stability and economic benefits, this

makes the ELISA sandwich assay a popular detection and quantification system applying to many different substances such as proteins in a biological liquid sample. In this case the concentration of fecal NGAL is measured. Materials, instruments, and software used are listed in **Attachment A, B and C**.

Preparation of fecal samples for NGAL detection by ELISA

As illustrated in **Figure 3.2,** the fecal samples were prepared by a suspension (Figure 3.2, element 2-6) and dilution (Figure 3.2, element 6-8) process in order to measure fecal NGAL by ELISA.

The feces suspension procedure was based on recommendations for fecal Lipocalin 2 (Chassaing et al., 2012) with some modifications. The fecal aliquots (of 50-100 mg) were transferred from -80°C to -20°C one day in advance of the suspension, ensuring a gradually thawing. This was beneficial to reach a more evenly softened consistent of the fecal material and helped speeding up the dissolving process. Frozen samples were put on ice whenever possible to prevent analyte degradation, except of the reconstitution step when dissolving the unprepared samples in a room temperature adjusted buffer solution (PBS with 0.1 % Tween20). Buffer amounts complementing the respective aliquot weights were added to reach a concentration of 100 mg feces per mL. To dissolve and achieve a visible evaluated homogenous feces solution, a mixer mill at speed 60 was used for 20 minutes in a combination with manually 'vortexing' before and after the mixing to accelerate the dissolving process. Followed by centrifugation for 10 minutes at 12,000 rpm and 4°C, clear supernatants were collected. In some cases, a secondary centrifugation at 13,000 rpm was necessary to achieve clear supernatants, often due to a fat rich layer releasing undissolved particles into the supernatant. Samples from same individuals were prepared the same day, and the supernatants were stored in the freezer at -20°C until the dilution process.

The sample preparations were finished by being diluted and transferred to a PCR plate used as a temporarily container, referred to as the 'sample plate'. First, the undiluted supernatants were thawed on ice and possibly being warmed by hands and 'vortexed' in order to speed up the thawing process. When all crystals were gone the undiluted supernatants were diluted into PBS, and to adjust for variations of the dilutions all samples were first diluted 1:10 before further being diluted to the final 1:100 dilution. Dilution tests were performed in advance to minimize the "matrix effect" and for the NGAL concentration to fall within the standard curve (scaling from 9,7625 pg/mL to 5000 pg/mL). To ensure a homogeneous solution, the samples were 'vortexed'

thoroughly before each dilution. Further, 120 µL supernatants were transferred from Eppendorf tubes to the 'sample plate allowing a simultaneously transfer directly to the ELISA assay plate later during the sample addition in the assay procedure. At the same time, other controls ensuring a similar treatment of the different solutions were added. The plate was sealed and stored in -20°C until the ELISA procedure. The fecal samples were exposed to a total of 4 freeze-thaw cycles (Figure 3.2) counting from fresh sample was collected to the sample was measured with ELISA.

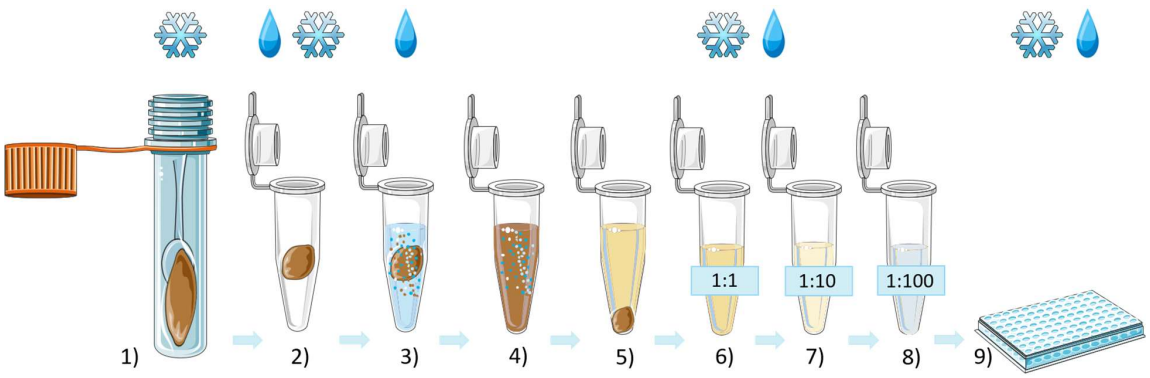

Figure 3.2: Fecal sample preparations including freezing and thawing steps (illustrated with a snowflake and raindrop, respectively). **1)** The sample collection was performed in a sample tube by the participants, and further treated in Eppendorf tubes with first **2)** being distributed into smaller aliquots. Then the suspension process included **3)** a reconstitution in PBS with 0.1% Tween20, **4)** dissolution into a homogeneous solution, and **5)** centrifugation to access the supernatant. The further dilution process starting with **6)** clear undiluted supernatant was diluted in PBS into **8)** the final dilution at 1:100, by **7)** an extra dilution step at 1:100, and **9)** was added to a PCR plate for storage until the assay procedure. PCR: polymerase chain reaction. Figure elements are taken from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/) and Adobe Stock (https://stock.adobe.com/hu/license-terms/).

Detecting NGAL by ELISA sandwich assay

Fecal NGAL was quantified using the procedure obtained from the ELISA kit (Human lipocalin-2/NGAL Duoset ELISA purchased from R&D system, Catalogue number DY 1757) with some modifications.

In advance of the samples being added from the 'sample plate and analysed, a 96-well microtiter plate was prepared as illustrated in **Figure 3.3, element 1.** First, each well in the ELISA plate was *coated* with 100 µL reconstituted and diluted primary antibody (capture antibody) (Rat Anti-Human Lipocalin-2), further referred to as capture antibody, and incubated in room temperature overnight. Then the wells were manually washed in 300 µL PBS three times with a complete removal of wash buffer at each step leaving the capture antibodies covering the polystyrene surface of the wells. The wash buffer was removed by inverting and blotting the plate against clean paper towels. Then the wells were *blocked* with 300 µL reagent diluent containing PBS with 1% BSA to prevent unspecific binding and incubated at 4°C overnight.

The assay procedure includes the step 2-5 in Figure 3.3, where standards with known NGAL concentrations and biological samples with unknown NGAL concentrations are added and detected. First, a new washing procedure was repeated, this time automatically by a microplate wash machine in PBS with 0.1% Tween20 and were now ready for sample and standard addition. The 'sample plate' with samples, standards, and controls were thawed on ice, and 100 µL of each were gently transferred to the ELISA plate to avoid contamination. Then the plate was covered with an adhesive strip and incubated at room temperature for 2 hours with gently 'vortexing' (300 rpm.). When performing the following washing procedure, the plate was first washed manually to avoid clogging the washing machine, and then the washing machine was used for additionally three washes. Then 100 µL reconstituted and diluted detection antibody (Biotinylated Goat Anti-Human Lipocalin-2) was added, and the plate was sealed in advance of an incubation of 2 hours at room temperature, followed by an automatic washing procedure. 100 µL Streptavidin-HRP B was added in advance of a new sealing and 20 minutes incubation at room temperature avoiding direct light exposure by covering with aluminium foil. Following a new automatically washing procedure, 100 µL substrate solution including colour reagent A and B was added, and a new 20-minute incubation at room temperature avoiding light was done. Finally, 50 µL stop solution was added, and the plate was gently tapped to ensure a thorough mixing before the plate was read.

The plate reader instrument was set to 450 nm, and the concentration was calculated out of the mean absorbance from the standard and sample duplicates, as a measure of the optical density immediately. CV values above 20 was set as a cut off considering sufficient deviation between the different absorbance measured between the duplicates. To control for internal plate differences, an identical sample was added to each plate to serve as an internal control. As a negative control for the capture antibody, one well was not appointed this, but further being treated with the same procedure including a known NGAL concentration to verify the function of the capture antibody. This was done to check for unspecific binding and prevent false negative results. To correct for optical imperfections in the plate, a wavelength correction was set to 540 nm. To correct for imperfections due to the sample and standard backgrounds, the signals from the respective background buffers PBS and reagent diluent was subtracted from the measured concentrations of the sample and standards. For each plate, the standard concentrations measured was used to create a standard curve by the Softmax Pro 6.5 software which was transformed into a 4-parameter logistic (4-PL) curve (**Attachment D**). Further, this standard curve was again used to calculate the NGAL concentration in the fecal supernatants which also were multiplied by the dilution factor of 100 followed by weight correction of the first aliquot weight to reach the NGAL concentrations unit of ng/g.

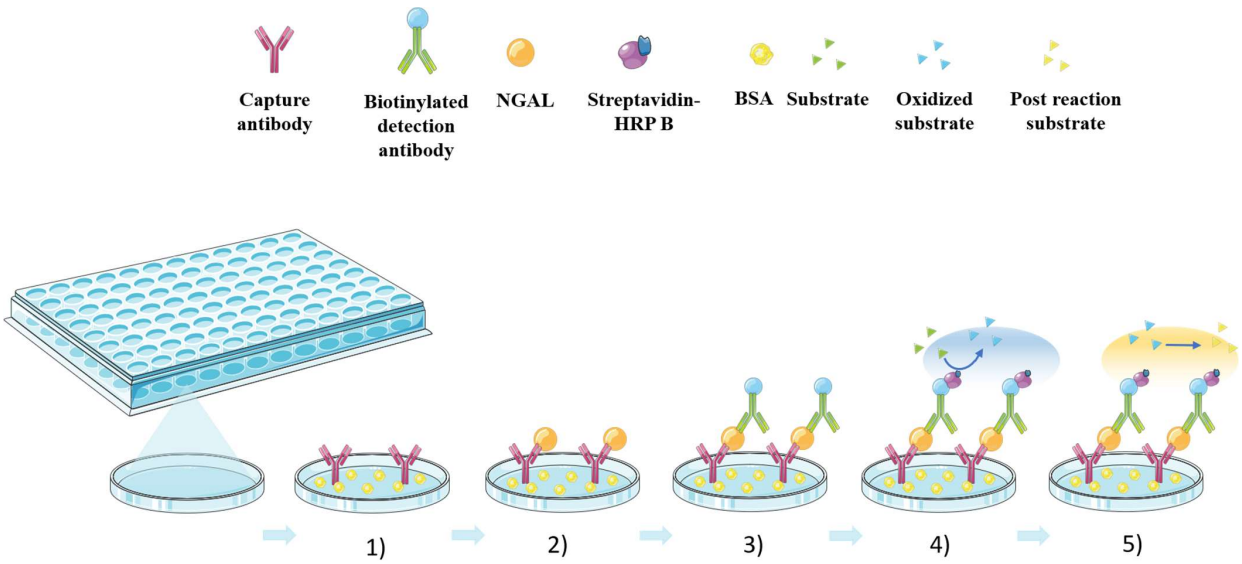

Figure 3.3: Illustration of ELISA sandwich assay detecting fecal NGAL. The figure illustrates two antibody-antigen reactions, and the washing steps are not illustrated for simplicity. **1)** Capture antibody (pink) binds the plate surface, and Bovine Serum Albumin (BSA) binds remaining surfaces functioning as a blocking protein (yellow) to avoid unspecific binding. **2)** Neutrophil gelatinase-associated lipocalin (NGAL) from the biological sample (orange) attaches the capture antibody, and **3)** detection antibody (green) which is biotinylated (blue) binds the NGAL, forming a "sandwich" complex. **4)** Streptavidin-Horseradish Peroxidase B (streptavidin-HRP B) is added, and the streptavidin binds to the biotin. When substrate solution is added tetrametylbenzidine (TMB) is oxidized by the HRP enzyme causing a blue colour reaction. **5)** When the stop solution is added, the reaction causes a colour change by forming a yellow stable derivative. Then the optical density in each well is measured with an absorbance plate reader at 450 nm. ELISA: enzyme-linked immunoassay, NGAL: neutrophil gelatinase-associated lipocalin. The figure is inspired by the principle assay image at R&D Systems (https://www.rndsystems.com/products/quantikine-elisa-kit-measuring-high-molecular-weight-adiponectin), and figure elements are collected and modified from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/).

3.5.2 Quantifying fecal SCFA with GC-FID

SCFA was analysed by the senior engineer at Unger-Vetlesen Institute at Lovisenberg Diaconal Hospital in Oslo. Unfortunately, due to the COVID19 epidemic the master student was not allowed to participate during the analysis but a thorough description of all steps of the procedure was given by the engineer.

For the analysis of SCFA levels, gas chromatography (GC) with flame ionization detector (FID) was used. In advance if the GC-FID analysis, the fecal sample was purified and made more suitable for the measurement process. During the preparation, 0.5 g of fecal material obtained from one aliquot was dissolved and homogenized. First, distilled water containing 3 mmol/L 2-methylbutanoic acid (C5H10O2, Merck) was added to function as an internal standard for quantification of the fatty acids in the fecal samples. This is possible since it obtains similar properties as the relevant SCFA but without being present in human feces. Then 0.5 mmol/L sulfuric acid (H2SO4, VWR) was added with the purpose to lower the pH of the sample so that the fatty acids was protonated (on a less polar form). This would increase their volatility and prevent them from binding to particles in the sample.

In short, 2.5 ml of the homogenate was vacuum distilled to obtain a cleaner and more stable product, as suggested by the method of Zijlstra et al. (1977) further modified by Høverstad et al. (1984). Gas chromatography (Agilent 6850, CA, USA) with capillary column (HP-FFA WAX column, part number: 19091F-112E serial number: USE400345H, Agilent J&W GC Columns, CA, USA) were used to separate relatively stable fatty acids in the pure distillate. Further, FID and internal standardization were used to detected and quantify the separated fatty acids. Eight SCFA were analysed: acetic acid, propionic acid, n-butyric acid, i-butyric acid, n-valeric acid, i-valeric acid, n-caproic acid and i-caproic acid, in which ‘n’ indicates unbranched conformation and ’i’ indicates branched conformation. Further in this thesis, the results are expressed in mmol/kg wet weight feces, and SCFA with unbranched conformation is referred to without prefix (‘n-‘), while the branched conformation is referred to as ‘iso’ variants.

3.5.3 Establishing quantification of fecal NGAL with Luminex and validating the method

The Luminex assaying is based on the concept of immunomagnetic MagPlex microspheres, carboxylated polystyrene microparticles (or microbeads), further referred to as “beads”. These beads are colour coded and used for separation of multiple analytes simultaneously within the

same (biological) sample and in the same assay run, typically called multi-plexing (Luminex Corporation, 2021). In this thesis, the Luminex assay has a single-plex design with fecal NGAL being the only analyte of interest. The detection system is based on flow cytometry principles using different laser beams to detect and quantify the analytes of interest (**Figure 3.4).**

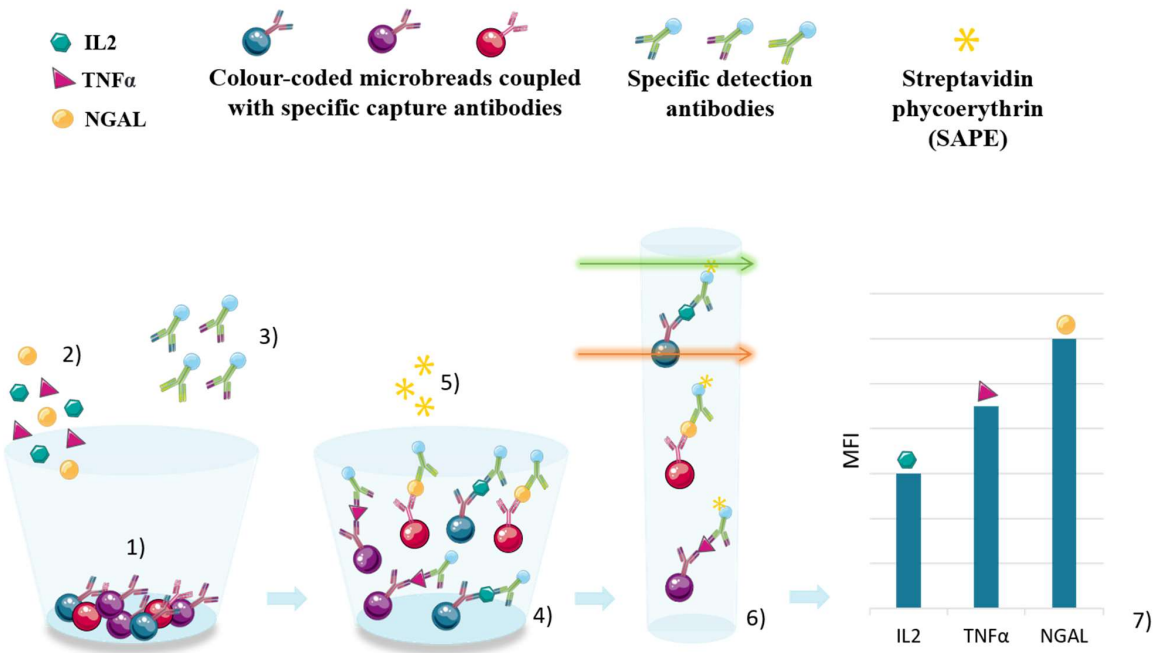

Figure 3.4: The Luminex assay principle overview. 1) Colour-coded microbeads are pre-coated with capture antibody specific for the analyte of interest. 2) The biologic sample containing analytes is added, and the specific antibodies bind to the analytes of interest. 3) Biotinylated detection antibodies with analyte specificity are added, 4) forming an antibody-antigen 'sandwich'. 5) Phycoerythrin conjugated streptavidin (SAPE) is added and binds to the biotin. 6) Beads are sent through a flow-based detection instrument (Luminex 200) and exposed to two laser beams. One laser of 635 nm wavelength (red) is classifying/identifying the bead based on the internal colour and determining the analyte. The other laser of 532 nm (green) is quantifying the binding events determining the magnitude of the PE-derived signal directly reflecting the amount of analyte bound. 7) Further the analyte calculation is provided by software (not shown). The figure is inspired by the principle assay overview at R&D Systems (https://resources.rndsystems.com/images/site/ca_luminex_may%202015_1488_pr.pdf), and figure elements are collected and modified from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/).

Materials, instruments, and software are listed in **Attachment A, B and C.** The same standard (*Recombinant Human Lipocalin-2*), primary antibody (*Human Lipocalin-2 rat IgG)* referred to as capture antibody and secondary antibody (*Human Lipocalin-2 Biotinylated Antibody)* referred to as detection antibody were used for both methods. A secondary phycoerythrin (PE)-conjugated antibody (*PE anti-rat IgG2b Antibody*) was additionally used as detection antibody only for Luminex during coupling confirmation. The Luminex procedure for development of xMAP proteomic assays is based on two parts in which the antibody coupling and coupling confirmation makes up the first part, and the second part is a sandwich immunoassay. The first part is not obligate in order to run the assay analysis, but aims to develop and establish a protocol for the antibody coupling to the magnetic beads by testing optimal conditions regarding the concentration of antibodies (Luminex Corporation, 2018). Furthermore, one can basically make a bead 'pool' containing the desired amount of coupled beads depending on the consumption and durability. If this analyte specific bead 'pool' is wanted to be included in a multiplex-panel later, necessary analyses considering cross reactivity must be performed. For the first part, we developed a procedure obtained from a combination of the Luminex antibody coupling kit (xMAP® Antibody Coupling Kit User Manual purchased from Luminex Corporation, Catalogue number 40-50016) and the xMAP® Cookbook (Luminex Corporation, 2018). For the sandwich assay, the procedure is only based on the xMAP® Cookbook only.

Preparation of fecal samples for NGAL detection by Luminex

As mentioned, the fecal samples dedicated to Luminex was based on a random selection of the supernatants already measured by ELISA, and therefore prepared the same way. Optimizing the matrix with respect to the instrument for Luminex was performed by investigating the same selection of dilution ratios as for ELISA, and 1: 100 was found as the highest detection signal within the standard curve. According to procedure recommendations, PBS 1% BSA was as a diluent buffer used for Luminex dedicated samples, in different from ELISA using PBS only. Since laboratory capacity made it difficult to run both assays in parallel the same day, the different dilution step required an extra freeze-thaw cycle for the Luminex assigned samples.

Antibody coupling

To establish a specific 'bead pool' containing beads coupled with NGAL specific antibodies, the optimal amount of capture antibody per million beads were suggested to be determined through a titration experiment. The beads were first "activated" to enable antibody binding. To homogenize

the bead solution and prevent the beads from aggregating, the beads were resuspended by 'vortexing' and sonicating for ~20 seconds, a routine frequently used throughout the whole procedure before working with the beads. 1 million beads (80 µL stock solution) were dispensed for each titration as recommended for training purpose. The beads were washed for a total of two times repeating the manually washing procedure as follows: first, pelleting the beads using a magnetic tube separator by allowing the bead solution to sit for 2 minutes, removing the supernatant carefully, and under a non-magnetic exposure, 500 µL activation buffer provided from the antibody coupling kit, were added. After the washing procedure, the beads were again pelleted, and resuspended (and left) in 480 µL activation buffer.

To favour the binding reaction with the capture antibody, the beads were already modified with surficial carboxyl groups when provided. By adding 10 µL each of reconstituted EDC and Sulfo-NHS, the surficial carboxylic acid formed a semi stable NHS ester intermediate during a 20-minute incubation with end-over-end mixing on a rotator at 20 rpm. The reaction was protected from direct light by being wrapped in aluminium folie whenever possible throughout the procedure. The beads were washed as described for a total of three washes. Then the beads were pelleted and appointed reconstituted and diluted capture antibody reaching a total volume of 500 µL, with the desired titration concentrations of 1, 2 and 5 µg antibody per million beads (**Figure 3.6a**). The titration was following the recommendation of the coupling guidelines from Luminex starting at 5 µg and titrating down. When adding the antibodies to the bead pellet, the amide group (more specifically the primary amine) on the lysine rich antibodies replaced the semi stable intermediate through a dehydration reaction, finally binding the carboxyl group covalently to the bead. After an overnight rotating incubation, the antibody coupled beads were now referred to as "activated". Another washing procedure using 500 µL wash buffer provided from the antibody coupling kit containing proteins blocking unreacted sites was performed a total of three times to help minimizing non-specific binding of the beads. Then the coupled and blocked beads were incubated at 4°C overnight.

For each antibody coupling procedure, a bead count was performed both manually using a counting chamber device (haemocytometer) and automatically by a cell counter, determining the number of beads recovering after the coupling and washing steps. When manually counting, 10 µL
1:10 diluted bead solution was loaded into the hemacytometer, and all 9 squares of 3x3 grid (**Figure 3.5**) was counted using a microscope.

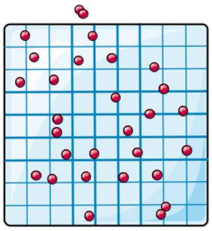

Figure 3.5: A haemocytometer with 3x3 grids was used to manually count the recovery of the 1 million beads (red) after the coupling and washing steps. Figure elements are collected and modified from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/)

Coupling confirmation

A successfully bead coupling is confirmed by enumerating a sufficiently recovery of the coupled beads and by assessing the efficiency of the coupling reaction. Confirming the coupling reaction was done by reacting the capture antibodies with phycoerythrin (PE)-conjugated detection antibodies (**Figure 3.6b**), and analysing this complex through a flow-based detection instrument (Luminex 200). Reaching a concentration of 50 beads/µL, the beads were diluted to a total volume of 1000 µL in PBS 1% BSA for each titration level. 50 µL bead solution was added to each well of a 96 well low binding microtiter plate followed by addition of 50 µL PE-conjugated detection antibodies being added in a dilution series of 1:2, starting at 4 µg/mL. The plate was covered and incubated for 30 minutes on an orbital plate shaker (moving horizontally) and washed using 100 µL PBS 1% BSA by mixing with gently tapping on all sides. With the plate placed and clipped on a handheld magnetic plate separator for 2 minutes, the beads were remained in the wells while the liquid were in one movement rapidly and forcefully inverted over a biohazard receptacle and blotted against paper towels, to ensure the liquid was removed from the wells. A total of two washes were performed. Then the beads were sent through the detection instrument (Luminex 200), and since the intensity of the fluorescent signal of the reaction is directly proportional with the amount of capture antibody coupled to the bead surface, the relative *amount* of the successful coupling reactions was reflected (Luminex Corporation, 2018). Importantly, to verify the *functionality* of the capture antibody, the final sandwich procedure is essential.

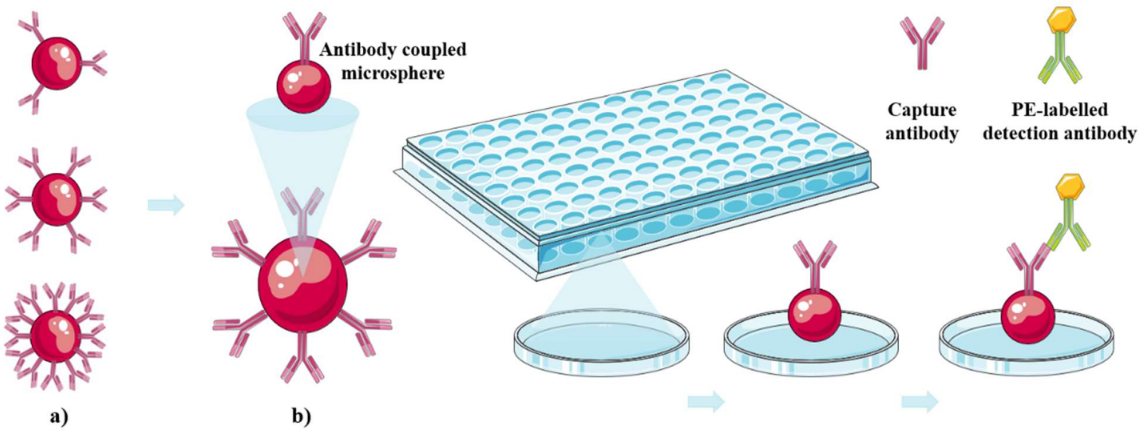

Figure 3.6: Antibody coupling titration levels and coupling confirmation **a)** Illustrating antibody coupled (activated) beads at titration levels of 1 µg, 2 µg and 5 µg/per million beads, and **b)** confirming the coupling directly by detection with PE-labelled antibodies reflecting the amount of capture antibodies. PE, phycoerythrin. The figure is inspired by the principle assay overview at R&D Systems (https://resources.rndsystems.com/images/site/ca_luminex_may%202015_1488_pr.pdf), and figure elements are collected and modified from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/).

Optimizing the plate washing procedure

By replacing the established manual procedure with an automatically washing machine (HydroSpeed Microplate Washer), the plate washing procedure could be more efficiently by saving time, achieve a more reproducible method and avoid hands to hands variation. An additional bead coupling experiment was therefore set up in parallel, investigating whether using the automatically washing technology would impact the coupling results. While the main titration experiment was performed as previously described, the additional experiment was performed simultaneously except of the last washing procedure. The automatically machine had a different approach considering washing detergent using PBS + 0,1% Tween20 instead of PBS 1% BSA as was used for the manual plate washing procedure. Also, the automatic washing machine was programmed to different cycles of aspirating/dispensing the liquid from the wells in which all steps were performed while the beads were magnetically forced against the well bottom instead of being resuspended, due to a magnetic bead separator keeping inserted in the machine throughout the entire washing procedure.

Validating the established bead 'pool' using a Luminex sandwich assay

The second part of the Luminex assay procedure is the sandwich assay providing the validation of the fecal NGAL measurement. The bead 'pool' of the 2-µg titration level was used based on the titration results. Based on calculation from the bead recovery, the bead solution was prepared using PBS 1% BSA to obtain 50 beads/mL. Following the 'vortexing' and sonication routine dispersing the beads, 50 µL of the bead working solution were added to each well of a 96-well flat bottom microtiter plate **(Figure 3.7)** with low binding affinity, yielding ~2500 beads per well. Then, 50 µL of the analyte solutions were added in the respective wells for the standard dilution series (starting at a concentration of 10 000 pg/mL with a 1:2 dilution series), and the 1:100 diluted fecal samples. The plate was gently tapped to mix the reactions, covered, and incubated on an orbital plate shaker with horizontal shaking at 800 rpm for 30 minutes at room temperature.

Controls were used for the plate:

- PBS 1% BSA was used as background for the standard and samples *(since the samples were diluted in this buffer in different from the ELISA diluting in PBS).*
- A negative control for the detection antibody
- Same internal controls as used for the ELISA

Following incubation, a manually washing procedure to avoid unclogging the washing machine using 100 µL of PBS 1% BSA was performed for two washes. The wells were left in 50 µL PBS 1% BSA, and 50 µL of biotinylated detection antibody was added using the same concentration for all wells, of 4 µg/mL. The reaction was mixed by gently tapping and followed by another 30 minutes incubation under the same conditions as the previous incubation. Next followed the automatically washing procedure for a total of three washing cycles and resuspending the beads in 50 µL PBS 1% BSA. Then 50 µL the reporter conjugate, Streptavidin-Phycoerythrin (SAPE), were added to each well followed by another similar incubation. After another automatically washing procedure and leaving the wells in 100 µL PBS 1 % BSA, the plate was analysed using the Luminex200 xMAP® instrument. The identifying and quantifying of the analyte were obtain by the colour coded beads and binding events, respectively by being exposed to laser beams. The laser detection was further calculating the concentration based on a 9-point standard curve, by the Bio-Plex Manager™ software.

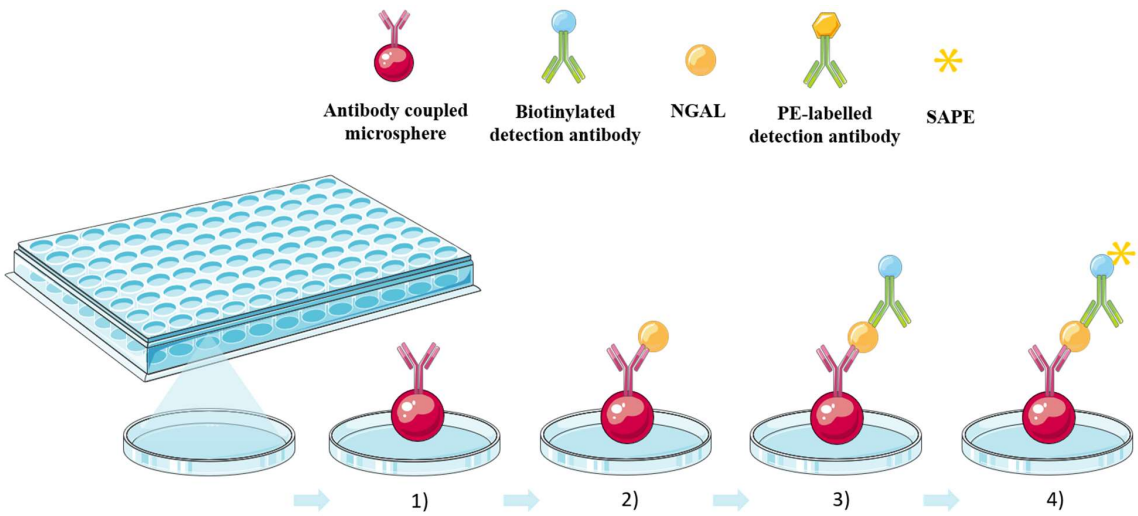

Figure 3.7: Illustrating the principles of Luminex sandwich assay detecting fecal neutrophil gelatinase associated lipocalin (NGAL). The figure illustrates one antibody-antigen reaction and does not include washing steps for simplicity. 1) Antibody coated beads are added to the wells, and Bovine Serum Albumin (BSA) binds remaining bead surfaces functioning as a blocking protein (not shown) to avoid unspecific binding. 2) NGAL from the biological sample (orange) attaches the capture antibody, and 3) detection antibody (green) which is biotinylated (blue) binds the NGAL, forming a 'sandwich' complex. 4) The photoreactive reporter conjugate, Streptavidin-R-phycoerythrin (SAPE) (yellow), is added, and the streptavidin binds to the biotin. Under exposure of laser beams, the SAPE is reflecting median fluorescence intensity (MFI) signals for each well by the Luminex xMAP instrument. The beams identify the colour-specific beads and quantify the NGAL attached as the mean principle of the analysis (not shown). NGAL: neutrophil gelatinase-associated lipocalin, MFI: median fluorescence intensity. Further the MFI signal was converted into concentration (not shown). The figure is inspired by the principle assay overview at R&D Systems (https://resources.rndsystems.com/images/site/ca_luminex_may%202015_1488_pr.pdf), and figure elements are collected and modified from Servier Medical Art Creative Commons Attribution Licence (https://creativecommons.org/licenses/by/3.0/).

3.6 Statistics

The investigation objects from this thesis were obtained form already collected data during the primary outcomes in the CD-FODMAP study. Importantly, in order to avoid bias, the statistics of the master project was conducted using concealment of group allocation until the primary results were obtained. Analysing these data was done using different statistical approaches. In advance of each analysis, it was checked whether assumptions required for the specific statistical method was met or not. One exception was made for the regression model, which was performed independent of the assumption of a linear relationship between baseline and follow-up values, since it is still not wrong to adjust for baseline values according to several studies (de Boer et al., 2015; J et al., 2018; Peterson et al., 2017).

Parametric and non-parametric methods were used depending on whether the data population was normal distributed or not. Normality was evaluated both visually by a normal distribution quantile-quantile (QQ) plot, a histogram with skewness and kurtosis values, and by a Shapiro-Wilk normality test. Applicable for all statistics is that significant p-values are defined as less than 0.05 ($\alpha = 0.05$), and plotted significant p-values are assigned with the symbol '*'. All p-values are written with 3 decimals, with exception of situations where crucial decimals are necessary to show a significance. To assure that the results reported are not based on a few values affecting them a lot, influential values were evaluated by inspecting the cook's distance and by checking whether exclusion of these potential high influential values affected the conclusion a lot or not.

All data is processed using R version 4.0.2 and RStudio version 1.3.1093 (**Attachment B**). To perform statistical analysis and visual presentations of the of these data, different R packages was used (**Table 3.1**).

Table 3.1: Essential R packages used for statistical analysis

R package	Brief description
readxl	to read excel files
openexlsx	to transfer a table from R to excel
stats	to calculate median and standard deviation
Tidyverse	for data manipulation and visualization
Rcompanion	to calculate median of groups
Rstatix	to access pipe-friendly R functions for easy statistical analysis
Dplyr	to manipulate data frames
Ggpubr	to create publication ready plots
Ggplot2	to create data visualizations through plots
Blandr	for Bland-Altman method comparison
blandAltmanLeh	to make Bland-Altman plots
Corrr	to calculate correlations
Corrplot	tor visualization of correlation matrix

R packages contain fundamental functions that are usable when coding with the programming language R. They include reusable R functions, the documentation that describes how to use them, and sample data.

Since this master project is based on the secondary endpoints of the RCT, the sample power is calculated based on unpublished results from the ongoing clinical trial for the primary endpoints of the RCT and not this master thesis' aims specifically. Calculation based on the primary endpoints found that clinically significant difference in symptom score was present when group means are and 29,5 (and standard deviation 11). With a power of 80 % and a significance level of 0,05 we will need 31 patients in each group. To account for a 15% drop out (type I error rate), the aim was to include 72 individuals, which was successfully met hence the study population of 70 participants was completing the intervention.

For the method validation as described in aim 2, an intentional selection of the provided samples was made. This was based on a power estimation performed in advance of the experiment to define the number of samples necessary to include for the validation to show a true correlation between the methods. According to an online sample size calculator provided by Lippincott Williams & Wilkins (Hulley et al., 2013) it was necessary to include at least 19 samples to gain a true correlation with an expected coefficient of 0.6. However, since the validation precision depends

on the amount of observed data the sample selection was increased as much as the assay performance allowed considering limitational factors such as available wells in the plate and reagent amounts. Therefore, the population was scaled up and based on a selection of 35 samples instead of 19 (out of 140 available samples). The majority of this new population represented a good distribution of both groups (control group (n = 19) and intervention group (n = 16)) and both timepoints (baseline (n = 14) and follow-up (n = 21)). Since the method evaluation does not have the purpose to detect a treatment effect (or any other possible effect), the ranging was performed independent on the group and sample timepoint. However, both times points for a sample (paired samples) were represented for 10 of the 35 samples. Additionally, samples at the end of the range of concentrations obtained from the ELISA measurements (meaning the highest and lowest concentrations) was more frequently selected with the purpose to investigate how the two comparable methods behaved for the most extreme values observed. This was favourable in specific to be able to evaluate the existence of a proportional bias between the methods. With this sample power and selection, the population was considered a successful randomization representing the whole concentration spectra with respect of the already measurements from ELISA.

3.6.1 Statistics used for thesis aim I

When analysing the NGAL (measured by ELISA) and SCFA results, three types of analyses was performed with the purpose of answering three different questions:

(1) Were the changes observed in NGAL/SCFAs from baseline to follow-up different between the control and intervention group?
(2) Was there any change in NGAL/SCFAs from baseline to follow-up within any of the diet groups?
(3) Was the change observed in NGAL/SCFAs related to the changes observed in GSRS-IBS?

Question 1

Question 1 was answered by applying linear regression models where you test for any difference between the diet groups at follow-up, while adjusting for the baseline values. The advantage of using such model (as compared to analysis of follow-up values only, or analysis of change values) is that each participants follow-up value gets adjusted to their respective baseline values, and that the analysis results will be unaffected by any baseline differences that might occur between the groups (Vickers & Altman, 2001).

In our specific models, the (dependent) response variable was the NGAL/SCFAs concentration at follow-up ($Y_{follow\text{-}up}$), while the NGAL/SCFAs concentrations at baseline ($Y_{baseline}$) and diet group (Group) was included as (independent) explanatory variables. Initially the model also included the interaction between baseline $Y_{baseline}$ and Group. The Group variable was coded 0 for the control group and 1 for the intervention group. The setup for this initial model can be seen in **Equation 1**, where a visualization of the model estimates and regression lines can be seen in **Figure 3.8a**.

Equation 1:

$$Y_{follow-up} = \beta_0 + (\beta_1 * Y_{baseline}) + (\beta_2 * Group) + (\beta_3 * Y_{baseline} * Group) + \varepsilon$$

$$where\ \varepsilon \sim N(0, \sigma^2)\ and\ indepedent.$$

When applying the dummy-coding for Group (control=0, intervention=1) in Equation 1, β_0 represents the intercept for the control group, $\beta_0+\beta_2$ represents the intercept for the intervention group, β_1 represents the slope for the control group, and $\beta_1+\beta_3$ represents the slop for the intervention group. When fitting this model however, we were mostly interested in whether the slope for the two groups were different or not, which is represented by the interaction term between $Y_{baseline}$ and Group (the β_3 estimate). Interaction means here that the relationship between $Y_{baseline}$ and $Y_{follow\text{-}up}$ were the same for both diet groups. In other words, that the difference in NGAL/SCFA between the diet groups at follow-up was dependent of the NGAL/SCFA baseline values. A β_3 estimate of for example 10 ng/g would mean that the intervention group had slope that was 10 ng/g steeper than the control group.

In cases where the interaction term was found to account for an insignificant amount of the variation in NGAL/SCFAs follow-up values, it was excluded from the model. In this way, we were no longer modelling a different slope for each group, but rather a common slope. The setup for this reduced model can be seen in **Equation 2**, where a visualization of the model estimates and regression lines can be seen in **Figure 3.8b**. This type of reduced model, where the interaction term is not included, is often referred to as an analysis of covariance (ANCOVA) model.

Equation 2:

$$Y_{follow-up} = \beta_0 + \beta_1 * Y_{baseline} + \beta_2 * Group$$

$$where\ \varepsilon \sim\ N(0, \sigma^2)\ and\ indepedent.$$

When applying the dummy-coding for Group (control=0, intervention=1) in Equation 2, β_0 represents the intercept for the control group, $\beta_0+\beta_2$ represents the intercept for the intervention group and β_1 represents the common slope. In this model, we are mostly interested in the difference between the intercepts for the two groups, as represented by the β_2 estimate. A β_2 estimate of for example (-) 20 ng/g would mean that the intervention group had follow-up values that on average were 20 ng/g (lower) higher at follow-up when compared to the control group, when adjusting for the baseline values.

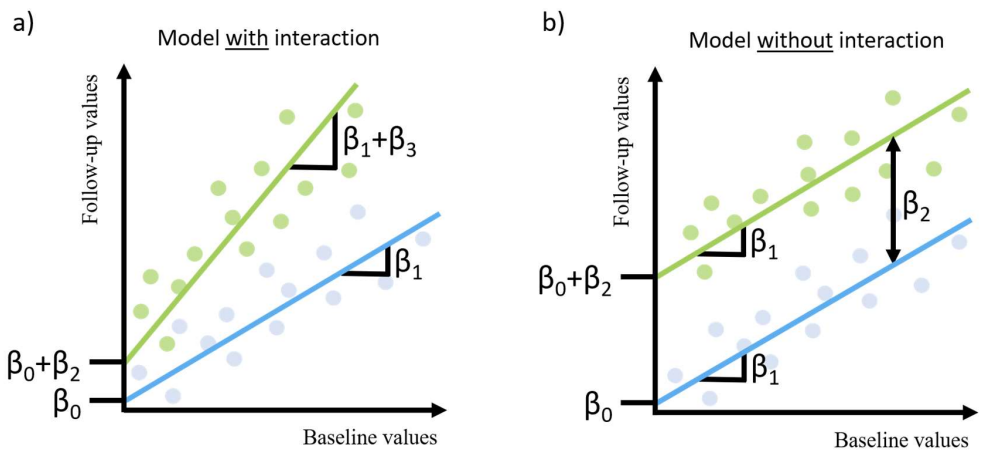

Figure 3.8: Illustration of model plots with the linear regression model testing whether the control and intervention group differed at follow-up. **a)** Initially, the model was fitted with the interaction term (β_3) between the baseline values for NGAL and the diet group. This model was used to explain the variation in the follow-up NGAL values (outcome variable) when the difference in NGAL between the diet groups were dependent on the NGAL baseline values. **b)** Contrarily, when the relationship between baseline and follow-up values for NGAL were independent on the baseline values, the model was fitted without the interaction term (model was reduced), forcing the lines to parallel with similar slopes (β_1) and consistent differences between the diet groups (β_2).
For each fitted model, the assumption of normally distributed error terms was evaluated both visually by quantile-quantile (QQ) plots and histograms of the residual distributions, in addition to Shapiro-Wilk normality test and skewness and kurtosis values of the residual distributions. The assumption of constant variance of the error terms was evaluated by residuals versus fitted values plots, by using Levene's test to assess the equality of residual variance between the two diet groups and by boxplot of the residuals in each diet group.

These results (see section 4.2 and 4.3) are presented by reporting from tables and figures:

- The overall model is presented in tables.
- When referring to the effect estimates (β_0, β_1, β_2 and β_3) for all fitted linear regression models, tables are used.
- Additional plots where the regression lines for each group are drawn with each individual's response, further referred to as model plots, are used for visualization of the fitted linear regression models and presented with p-values in figures.

Question 2 was answered by applying paired T-tests within each diet group, evaluating the change values for each patient from baseline to follow-up.

In difference to the regression analysis, paired T-tests does not adjust for baseline values. For each paired T-test, box plots for each group at each timepoint was made, visualizing the median, Q1, Q3 and whiskers. The change values of NGAL/SCFA concentrations are visualized as connected lines between the timepoints for each sample, and p-values are provided for all T-tests performed. The change values are calculated by the follow-up value minus the baseline value and presented with the Greek delta symbol (Δ).

Question 3 was answered by testing the correlation between the change values to the explorative analyses.

To find potential correlation between different variables, correlation tests were performed. Due to limitations of time capacity normality was not checked, and the data are treated as non-parametric data. Therefore, Spearman's correlation coefficient is used, presented with the correlation coefficient rho (and R in the figures and tables). Rho values are written with 2 decimals. The correlation coefficients range from the lower limit of -1 to the upper limit of 1, with 0 indicating no correlation. The correlations are stronger the closer the correlation coefficient is to the endpoint limits, with a perfect positive (or negative) correlation at 1 (or -1). According to (Mukaka, 2012), the different types of positive correlations are defined as neglected correlation (0 – 0.3), weak correlation (0.3 – 0.5), moderate correlation (0.5 – 0.7), high correlation (0.7 – 0.9) and very high correlation (0.9 – 1). The respective relation of correlation type and correlation coefficient intervals applies for negative correlation with coefficients in the interval from 0 to -1. All correlations are performed on change values from baseline to follow-up and visualized with scatterplots.

3.6.2 Statistics used for theses aim II

Concentrations of fecal NGAL was measured with two quantitative methods, Luminex and ELISA, to validate the Luminex-based method against ELISA. Since the ELISA method was based on a development kit and not considered an 'industry gold standard', ELISA was not used as a 'reference' method providing the 'true values'. Instead, the mean of the two methods was used as the closest estimate of the 'true values', as suggested by Bland and Altman (1986). The strategy to evaluate whether there was a concordance between the measurement methods was to first perform correlation analysis and paired statistics (Wilcoxon signed rank test). Furthermore, the degree of agreement was evaluated by an approach proposed by Bland and Altman which is widely used to detect and quantify a possible systematic bias between the methods.

Spearman's correlation between the two methods was run to assess the degree of relation between the paired measurements, and as in 'question 3' section 3.6.1, the correlation coefficient is represented as rho (and 'R' in figures and tables). This was done to assess the relationship between the methods as a preliminary indicator of the agreement. However, it should be noted that a high correlation does not imply whether there was a good agreement, since they only evaluate the linear association of the sets of observations (Giavarina, 2015). As a non-parametric alternative for T-testing since the values were not normally distributed, Wilcoxon rank sum test was used to investigate whether there was a difference between the absolute values of the two methods.

The degree of agreement between the methods was evaluated by first plotting the difference between the methods (Luminex - ELISA) on the y-axis, against the estimated "true values" on the x-axis using the average of the two methods. From this so-called Bland and Altman (BA) plot the agreement range (meaning the interval between the limits of agreement) was calculated based on the standard deviation of the differences, which was used in order to identify a possibly bias. The upper limit of agreement was calculated by the mean difference + 1.96 * standard deviation, while the lower limit of agreement was calculated by the mean difference - 1.96 * standard deviation. This interval was then catching 95% of the differences between the methods (Bland & Altman, 1986). In general, even though these limits define the intervals of agreement, they do not provide information of whether they are acceptable or not (Giavarina, 2015).

By inspection of the BA plot, a systematic bias between the measurements could be easily observed based on the mean differences of the methods. To further quantify the potential bias was

done by calculating the confidence interval of the mean difference. If the line of equality, visualized as the x-axis at zero, found place inside the confidence interval of the mean difference, the bias would not be considered significant. However, if such a disagreement was found, the question of whether there was a systematically fixed or *proportional* bias in the measurements needed to be answered. A *fixed* bias would mean that one method estimates constantly higher (or lower) values than the other, while a *proportional* bias would occur when a method estimates higher (or lower) values by an amount that is proportional to the level of the measured variable (Ludbrook, 1997). In other words, a proportional bias means that the methods do not *equally* agree through the range of measurements. This would be the case if one method overestimated for higher values (while underestimating for lower values).

4 RESULTS

4.1 Basic characteristics of the study population

4.1.1 Population selection

The study population was representative of Norwegian adult celiac patients (age 18-75) with persistent GI symptoms. As illustrated in the flow chart shown in **Figure 4.1**, a total of 894 patients were invited by email to the study. Of these, 253 accepted the invitation to attend and responded with the GSRS-IBS questionnaire. Of the recruited participants, 88 were excluded due to a total GSRS-IBS score lower than the predefined cut-off (<30). After the screening, additional 90 recruited participants were removed due to specific reasons for exclusion. These were, among other things, medical conditions such as exposal of immunosuppressive, acid neutralizing, NSAIDs, serious disease or pregnancy/breastfeeding. It was also due to conditions mismatching with the criteria to prove trial attendance, such as initiating the LFD in advance of baseline or lack of motivation. Of the 75 participants who completed the baseline visit, additional five participants were excluded due to various exclusion criteria. Detailed exclusion justifications at each selection step can be seen in Figure 4.1. In the end, 70 participants completed the intervention.

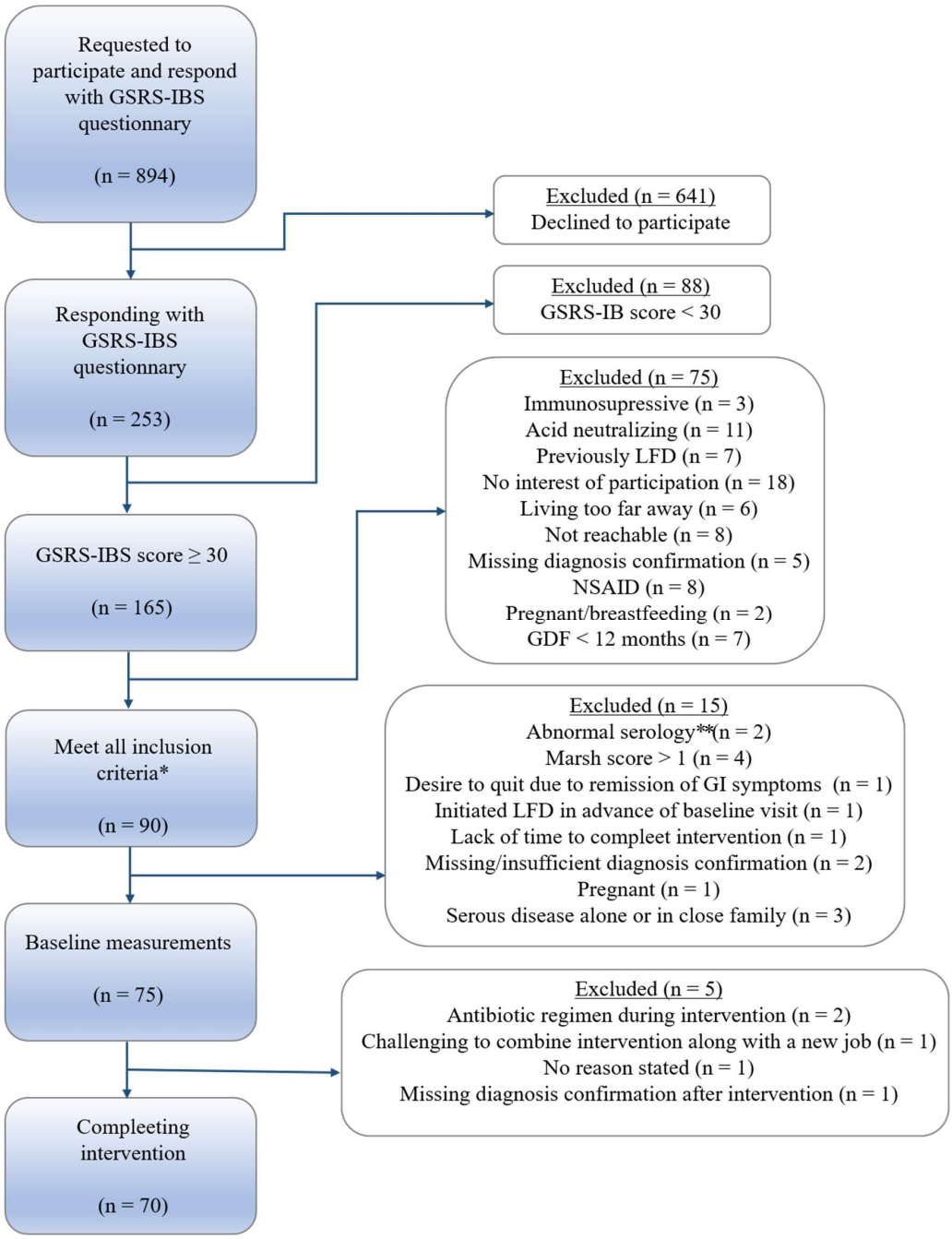

Figure 4.1: Schematic flow chart of the population selection process with inclusion and exclusion of participants in the CD-FODMAP study. GSRS: gastrointestinal symptom rating scale, IBS: irritable bowel syndrome, LFD: low-FODMAP diet, NSAID: non-steroidal anti-inflammatory drugs, GFD: gluten-free diet, TG2: transglutaminase 2, GI: gastrointestinal. *Inclusion criteria is defined as 17-75 years, was treated with GFD at least for 12 months and complied with a strict GFD adherence. **Abnormal serology in means of anti TG2 proven.

4.1.2 Demographic and clinical baseline characteristics

Table 4.1 lists the lifestyle, diet, and diagnostic characteristic for the study population at baseline and shows the distribution of the population between the control and intervention group. The average age for the control and intervention group was 44 and 45 years, respectively, with age ranging from 19 to 71 years. Most of the participants were women (over 80%) for both groups, and all participants were European. The median BMI was 25 for both groups, which is the lowest limit of the cut-off, indicating overweight (25.0-29.9). It should be noted that the BMI alone is not sufficient to provide information about how weight impacts the individual's health (WHO, 2021) and that it is only used as an indication of obesity at the population level. Considering education level, the majority had finished high school or had a lower or higher university/college degree. For the control group, the distribution of these levels is very even, while for the intervention group, most participants had some form of university/college degree. Under half of the participants in each group reported allergy or intolerance. Considering the diagnostics of CD, the distribution in the two groups were relatively equal, where most of the participants had an HLA DQ 2 type. Most of the population (over 70%) for both groups had a Marsh score of 0, while 10 and 9 participants in the control and intervention group respectively had a Mars score corresponding to more than 25 IEL per 100 enterocytes. The median duration of the CD disease was 9 and 11 years for the control and intervention group, respectively. As described earlier, all participants had a total GSRS-IBS score of ≥ 30 at screening. Since this being 38 and 36 for the control and intervention group, respectively, the groups represent a similar mean of the symptom scores. It should be mentioned that the total GSRS-IBS score at baseline was below 30 for some individuals, although they had scores above or equal to 30 at screening.

Baseline characteristics of clinical variables also seemed to be evenly distributed in the two groups, as shown in **Table 4.2.** This indicates successful randomization for these variables as well. According to the blood cell counts, all values at baseline were in a healthy range according to their respective reference values (OUS, 2016). This was also the case for the clinical biomarkers involved in metabolic processes and the immune system biomarkers. Those participants that were infected by H. pylori were treated before the intervention.

Table 4.1: Lifestyle, diet, and diagnostic characteristic at baseline (n = 70).

	Control group (n = 36)	Intervention group (n = 34)
Female/male	29/7 (81/19)	30/4 (88/12)
Age (years)	44 ±15	45 ±13
European	36 (100)	34 (100)
Body mass index (kg/m^2)	25 [5]	25 [5]
BMI category		
Underweight (< 18.5)	0 (0)	1 (3)
Normal weight (18.5 – 24.9)	17 (50)	16 (47)
Overweight (25.0 – 29.9)	13 (38)	13 (38)
Obese (> 30.0)	5 (15)	4 (12)
Education level		
Primary school	1 (3)	1 (3)
High school	13 (36)	5 (15)
University/college, lower degree	12 (33)	14 (41)
University/college, higher degree	10 (28)	14 (41)
Allergy/intolerance	14 (39)	17 (50)
CD diagnostics		
IgA anti-tTG (10^3 U/L)	0.5 [0.9]	0.5 [0.9]
IgG anti-DGP (Units)	2.5 [0.6]	2.5 [0]
HLA DQ 2	27 (82) [2]	28 (88)[1]
HLA DQ 8	6 (18) [2]	4 (12) [1]
Marsh score 0	26 (72)	25 (74)
Marsh score 1	10 (28)	9 (26)
Duration of CD (years)	9 [10]	11 [9]
GSRS-IBS total score	38 (14)	36 (12)

Data is presented as n (%), mean ±SD or as median [IQR]. For all variables, there are tested whether each group has a normal distribution of the data or not. If at least one group has not normality, both groups are presented with median to be more comparable. Despite normality, some variables are presented as median [IQR] to be comparable with not normal values in the other group. Among the demographic variables, these are the BMI (for the intervention group) and total GSRS-IBS score (for the control group). The questionnaires (GSRS) are presented with mean ±SD but are not checked for normality because the method is based on the mean independently of normality. SD: standard deviation, IQR: interquartile range, BMI: body mass index, CD: celiac disease, DGP: deaminated gliadin peptide, HLA: human leukocyte antigen, GSRS: gastrointestinal symptom rating scale, IBS: inflammatory bowel syndrome. [1] n = 32. [2] n = 33.

Table 4.2: Clinical baseline characteristics (n = 70).

	Control group (n = 36)		Intervention group (n = 34)	
Cell numbers				
MCV (fL)	90	[4]	91	[4]
Platelets (x10^9/L)	253	[70]	245	[70]
Leukocytes (x10^9/L)	7	[2]	6	[2]
Neutrophilic granulocytes (x10^9/L)	4	[2]	4	[2]
Lymphocytes (x10^9/L)	2	±1	2	±1
Monocytes (x10^9/L)	0.5	[0]	0.5	[0]
Eosinophilic (x10^9/L)	0.1	[0]	0.1	[0]
Basophilic (x10^9/L)	0	[0]	0	[0]
Clinical biomarkers				
Haemoglobin (g/dL)	14	±1	14	±1
P bilirubin (µmol/L)	7	[4]	6	[4]
P creatinine (µmol/L)	64	±10	65	±10
CDK EPI creatinine (mL/min/1,73 m2)	104	±16	101	±16
Homocysteine (µmol/L)	11	[3]	12	[3]
HbA1c (µmol/L)	33	[4]	34	[4]
Albumin (g/L)	46	[4]	46	[4]
TSH (x10^{-3} IU/L)	1.2	[1]	1.7	[1]
FT4 (pmol/L)	16	[4]	15	[4]
Biomarkers of the immune system				
CRP (mg/L)	0.75	[2]	0.45	[2]
IgA (x10^3 U/L)	0.5	[1]	0.5	[1]
IgG (x10^3 U/L)	2.5	[1]	2.5	[1]
Heliobacter pylori (positive)	5	(14)	2	(14)

Data is presented as n (%), mean ±SD or as median [IQR]. Decimals are presented exceptionally for crucial values. For all variables, there are tested whether each group has a normal distribution of the data or not. If at least one group has no normality, both groups are presented with a median to be more comparable. Despite normality, some variables are presented as median [IQR] to be comparable with not normal values in the other group. Among the clinical variable are FT4 (the intervention group) and MCV, platelets, neutrophilic granulocytes, transferrin, TIBC, potassium and HbA1c (for the control group).
SD: standard deviation, IQR: interquartile range, MCV: mean corpuscular volume, CDK: cyclin-dependent kinases, EPI: exocrine pancreatic insufficiency, HbA1c: glycated haemoglobin, TSH: thyroid-stimulating hormone, FT4: free thyroxine, CRP: C-reactive protein, IgA: immunoglobulin A, IgG: immunoglobulin G.

As shown in **Table 4.3**, the blood micronutrients and iron homeostasis were evenly distributed in the two groups at baseline, and according to reference values, these biomarkers are within their normal range for the population overall (OUS, 2016).

In total, considering the lifestyle, diet, and diagnostic characteristic at baseline, the randomization was considered successful as the variation for the different baseline variables of the 70 participants had an even distribution between the two groups.

Table 4.3: Blood micronutrients and iron homeostasis biomarkers at baseline (n = 70).

	Control group (n = 36)	Intervention group (n = 34)
Blood micronutrients		
Sodium (mmol/L)	140 [2]	140 [2]
Potassium (mmol/L)	3.8 [0]	3.8 [0]
Calcium free (mmol/L)	1.25 [0]	1.27 [0]
Calcium free pH (mmol/L)	1.23 [0]	1.23 [0]
Magnesium (mmol/L)	0.86 [0]	0.85 [0]
B12 (pmol/L)	421 [241]	370 [229]
Folic acid B9 (nmol/L)	17 [13]	15 [6]
Vitamin D (nmol/L)	72 ±20	71 ±24
Iron homeostasis biomarkers		
Iron (µmol/L)	17 ±5	17 ±5
Ferritin (µg/L)	80 [110]	85 [70]
Transferrin (g/L)	2.5 [1]	2.6 [0]
TIBC (µmol/L)	62 [15]	65 [10]
Transferrin saturation (g/L)	0.27 ±0.1	0.27 ±0.09

Data are presented as n (%), mean ±SD or as median [IQR]. For reference values with crucial decimals, data are presented with either one or two decimals. For all variables, there are tested whether each group has a normal distribution of the data or not. If at least one group has not normality, both groups are presented with a median to be more comparable.
SD: standard deviation, IQR: interquartile range, B12: vitamin B type 12, B9: vitamin B type 9, TIBC: total iron-binding capacity.

4.2 Effects of LFD on fecal NGAL

In IBS patients, several studies have illustrated increased inflammatory status when compared to healthy control individuals (Ng et al., 2018). Further, it has been shown that certain markers of inflammation were reduced in IBS patients when following an LFD (Hustoft et al., 2017). Our hypothesis was therefore that CD patients with persistent GI symptoms might be experiencing a similar state of low-grade intestinal inflammation, and that reduced GI symptoms following an LFD could be partially explained through a reduced state of inflammation. The first sub-aim of this thesis was therefore to investigate whether removal of FODMAPs from the diet impacts gut health by means of the highly sensitive intestinal inflammation marker NGAL, which was measured in fecal samples by ELISA. Due to technical circumstances during the ELISA procedure, one individual in the intervention group was excluded from the NGAL analysis.

4.2.1 Effect on NGAL concentrations between the diet groups

A linear regression model was fitted to test whether the LFD led to a change in NGAL concentrations that were different from the control group. The response variable in this model was the NGAL concentration at follow-up, while the NGAL concentrations at baseline and diet group was included as explanatory variables. Initially the model also included the interaction between baseline NGAL and group (see Equation 1 in section 3.6.1). After finding that the interaction between baseline NGAL and group accounted for an insignificant amount of the variation in NGAL follow-up values (p-value=0.724), the interaction term was excluded from the model.

The model fitted without the interaction term (see Equation 2 in section 3.6.1) significantly explained 31 % (R^2=0.31) of the variation in NGAL at follow-up ($p<0.001$). The effect estimates from the reduced model (β_0, β_1 and β_2) are summarized in **Table 4.4**, and **Figure 4.2** displays the model plot, where the regression lines for each diet group are drawn, together with each individual's response.

The intercepts for the control and intervention group were 114.6 (β_0) and 123.1 (β_0+ β_2) ng/g feces respectively meaning that NGAL levels at follow-up was on average 8.5 ng/g feces higher in the intervention group compared to the control group. This difference was however not significant as the β_2 estimate had a p-value of 0.741. From this we conclude that the effect of the LFD diet was not different from the effect of the control diet. By visually inspecting the model

plot, this is reasonable as the within-group variation is large compared to the between-group difference.

Table 4.4: Effect estimates (β0, β1 and β2) and corresponding p-values from the fitted linear regression model for NGAL

Parameter	Estimate	p-value
β_0	114.6	0.000
β_1 (NGAL baseline)	0.5416	0.000
β2 (Diet group	8.529	0.741

The model was fitted without the interaction between diet group and baseline NGAL values. The control group was used as reference group in the model, meaning that β_0 represents the intercept for the control group, $\beta_2+\beta_2$ represents the intercept for the intervention group and β_2 represents the difference between groups.

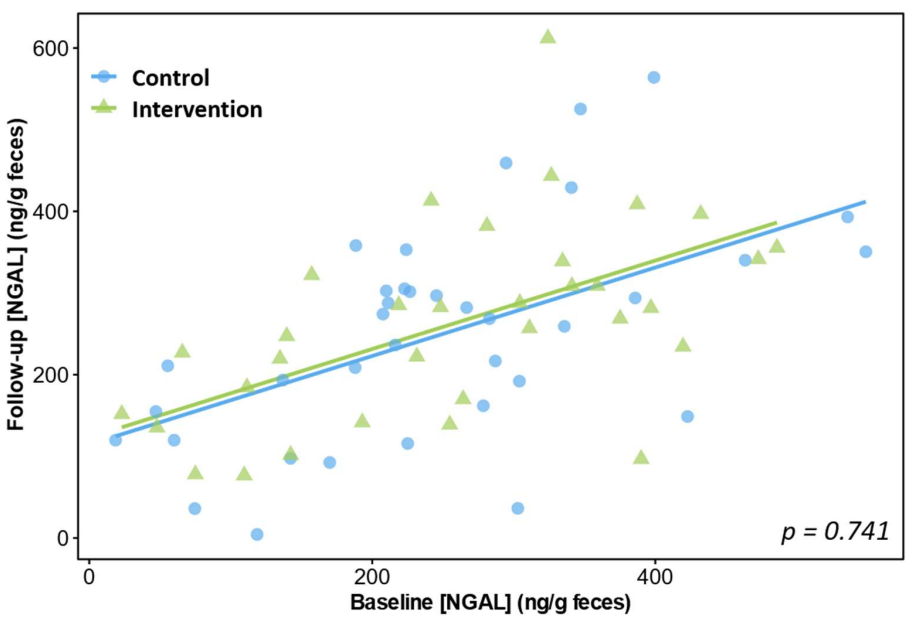

Figure 4.2: Model plot of NGAL concentrations (ng/g feces) at baseline and follow-up for each diet group. The control group (n = 36) (blue colour) and intervention group (n = 33) (green colour) has a regression line fitted to the observed values. The estimated intercept is not significantly different between the groups (p = 0.741).

4.2.2 Effect on NGAL concentrations within the diet groups

In addition to the regression analysis investigating the group effect at follow-up, paired T-tests were performed to investigate whether the lack of an intervention effect was due to similar changes within each of the groups or to similar lack of changes. **Figure 4.3** shows the NGAL values at baseline and follow-up in each diet group, where the connecting lines visualize the change in NGAL concentration for each participant.

The within group analyses revealed that the groups were similar in that there was no significant change from baseline to follow-up in neither group (p=0.994 for the control group and p=0.863 for the intervention group). By visually inspecting the boxplot, there are no clear trend or specific response pattern observed from baseline to follow-up within neither group.

To sum up, there was not found any significant effect of the intervention on fecal NGAL which implies that the reduction of FODMAPs from baseline to follow-up does not impact NGAL in this study population.

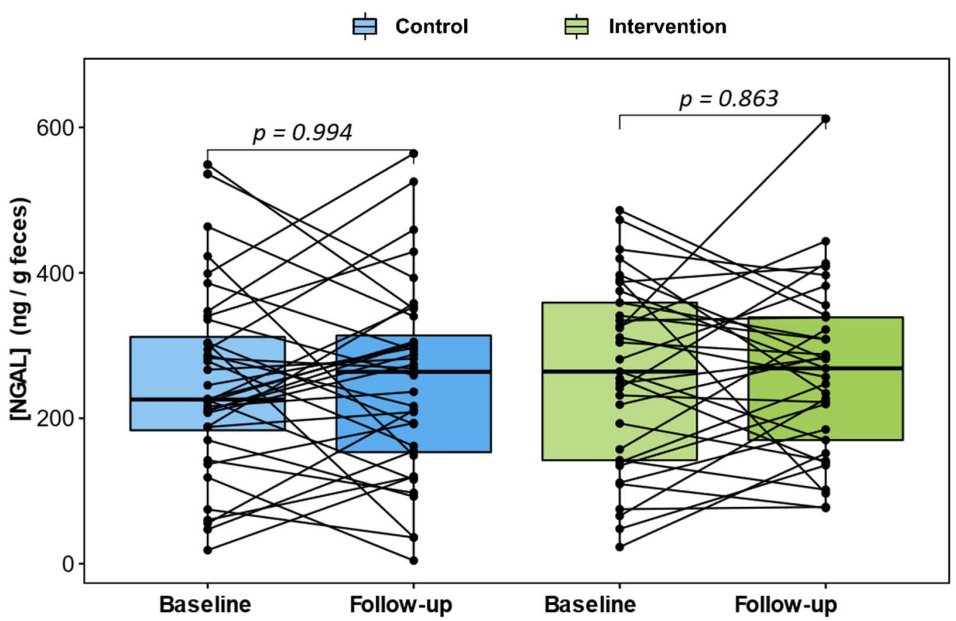

Figure 4.3: Boxplot of NGAL concentrations (ng/g feces) for the control group (blue) (n = 36) and intervention group (green) (n = 33) at baseline and follow-up after 4 weeks. The p-values are from ANCOVA and paired T-tests on normal distributed data. NGAL: neutrophil gelatinase-associated lipocalin, ANCOVA: analysis of covariance.

4.3 Effects of LFD diet on fecal SCFA

As introduced in section 1.3, the gut microbiota might be influenced when changing the diet since SCFAs are products of fermenting bacteria when metabolizing undigestible fibres. Therefore, we investigated the effects of FODMAPs restriction on the levels of SCFAs (total SCFA, acetic, propionic, butyric, iso-butyric, valeric, iso-valeric and caproic acid) as an indirect investigation of the microbiota composition and/or function. Since iso-caproic acid was present in only 1 participant in the intervention group at baseline, no statistical test was performed to test the intervention effects on this SCFA. Further, since there were relatively few observations of caproic acid above the detection limits, statistics were not performed on this SCFA. Four individuals (two in the control group and two in the intervention group) were excluded from the SCFA analysis due to too small sample size.

4.3.1 Effect on SCFA concentrations between the diet groups

As for NGAL, a linear regression model was fitted to test whether the LFD led to a change in SCFA concentrations that were different from the control group. **Figure 4.4** shows plots of the individual baseline and follow-up values for total and individual SCFA concentrations with separate regression lines for each of the groups. The difference between the groups was tested for total and individual SCFA (total SCFA, acetic, propionic, butyric, iso-butyric, valeric and iso-valeric) fitting separate linear regression models. The response variable in each model was the SCFA concentration at follow-up, while the SCFA concentrations at baseline, diet group and their interaction were initially included as explanatory variables (see Equation 1 in section 3.6.1). The effect estimates and p-values from the fitted linear regression models with coefficients of determination for the overall models for total and single SCFAs are summarized in **Table 4.5.**

For propionic and valeric acid significant interactions were found between baseline and diet group (p=0.009 and p=0.003, respectively) and the interaction parameter; diet*baseline, was therefore included in the model resulting in effect estimates for β_0, β_1, β_2 and β_3. This is also visualized in the plots showing that the difference between the groups is dependent on the baseline values (Figure 4.4 f and g). For total SCFA, acetic, butyric, iso-butyric and iso-valeric, no significant interaction was found between the SCFA baseline values and group. The interaction term was therefore excluded from the model for these SCFA resulting in reduced regression models (ANCOVA) with estimates for β_0, β_1 and β_2.

The results from the linear regression models indicate a significant reduction of the intervention (group effects – β_2, $p<0.05$) for propionic acid (Figure 4.4f) and valeric acid (Figure 4.4g) but the effects on both these SFCAs are dependent on the baseline levels. For the SCFA that did not have any significant interaction (total SCFA, acetic, butyric, iso-butyric and iso-valeric) also lacked an effect of the LFD intervention.

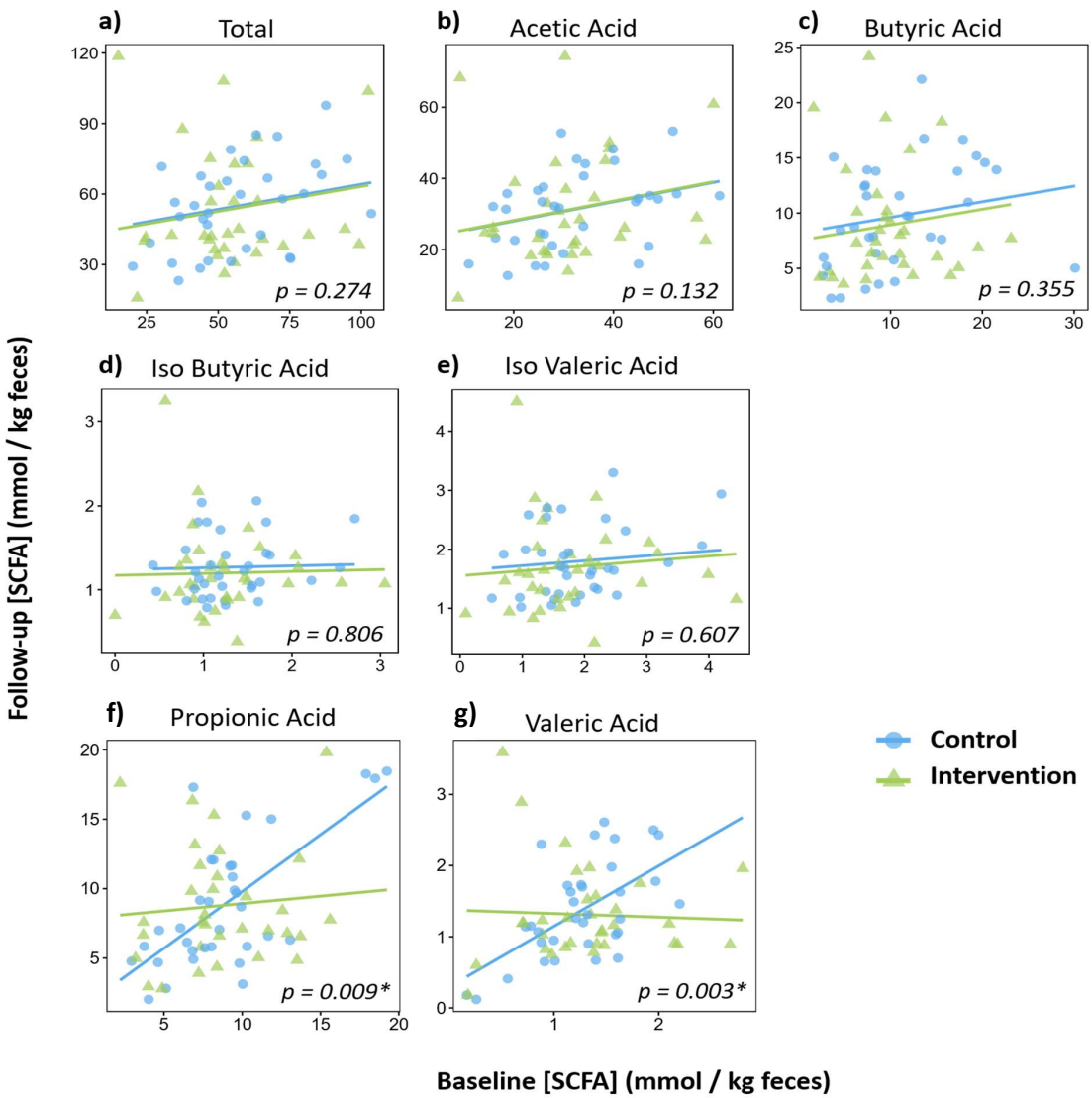

Figure 4.4: Model plots over total and individual SCFA concentrations (mmol/kg feces). Significant p-values are stated with '*'. Significant p-values for propionic and valeric is based on the *interaction*, and the other SCFA variables on the *overall model* forcing the slopes to parallel.

It should be noted that one individual had a particularly deviating change in SCFAs and its potential influential effect on the model was checked. While having high influence on the interaction for propionic and valeric acid, the interaction was found significant both with and without the individual (p=0.043 and p=0.007, respectively). The interactions were therefore considered robust.

Table 4.5: Effect estimates and p-values from the fitted linear regression models with ccoefficients of determination for the overall models for total and single SCFAs.

	Model parameterization			Overall model	
SCFA	Parameter	Estimate	p-value	R^2	p-value
Total SCFA	β_0	42.83	0.000	0.04	0.274
	β_1 (SCFA baseline)	0.21	0.114		
	β_2 (Diet group)	-0.89	0.870		
Acetic acid	β_0	22.55	0.000	0.06	0.132
	β_1 (SCFA baseline)	0.27	0.045		
	β_2 (Diet group)	0.20	0.950		
Butyric acid	β_0	8.19	0.000		
	β_1 (SCFA baseline)	0.14	0.201	0.03	0.355
	β_2 (Diet group)	-0.68	0.585		
Iso-butyric	β_0	1.24	0.000		
	β_1 (SCFA baseline)	0.02	0.822	0.01	0.806
	β_2 (Diet group)	-0.07	0.548		
Iso-valeric	β_0	1.63	0.000		
	β_1 (SCFA baseline)	0.08	0.410	0.02	0.607
	β_2 (Diet group)	-0.09	0.616		
Propionic acid§	β_0	1.61	0.349		
	β_1 (SCFA baseline)	0.82	0.000	0.26	<0.001*
	β_2 (Diet group)	6.26	0.015*		
	β_3 (Diet group*SCFA baseline)	-0.71	0.009*		
Valeric acid§	β_0	0.30	0.344		
	β_1 (SCFA baseline)	0.85	0.001	0.18	0.006*
	β_2 (Diet group)	1.08	0.010*		
	β_3 (Diet group*SCFA baseline)	-0.90	0.003*		

Effect estimates and p-values from the fitted linear regression models for total SCFA. For the first six mentions, model was fitted without the interaction between diet group and baseline SCFA values. The control group was used as reference for all estimates. Significant p-values are stated with '*'. §: The interaction term was included in the model (the β_3 estimate).

Exploring the group effects on valeric acid

The significant interaction between baseline values and diet group for valeric acid means that the interaction accounts for a significant amount of the variation in the follow-up values of valeric acid. The fact that the intervention group has a slope that is -0.90 mmol/kg feces less steep than the control group (as the β_3 estimate is -0.90) looks reasonably when visual inspecting the plot (**Figure 4.4g**). Interestingly, for the intervention group, it seems like there are several observations of lower follow-up values among values that start with a high baseline value compared to the individuals starting with a lower baseline value. This might indicate that the significant interaction (significant difference in slope between the groups) can be explained by a reduction in valeric acid among patients with higher baseline values.

The impact of the baseline levels for the group effects of valeric acid were explored further. We observed that all individuals in the intervention group with baseline values above 1.4 had negative changes (reduction) in valeric acid. Therefore, analyses were therefore performed separately for those with low baseline values (≤1.4 mmol/kg feces) and high baseline values (>1.4 mmol/kg feces). As illustrated by **Figure 4.5**, a significant difference between the groups was found only among the participants that had high baseline levels (p=0.012, Figure 4.5b).

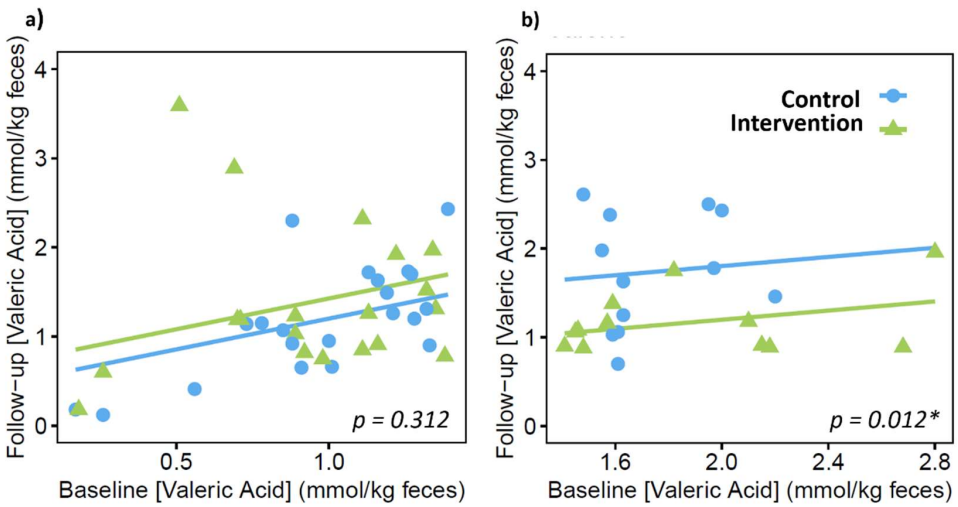

Figure 4.5: Regression model plots of valeric acid for the control (blue) and intervention group (green) for **a)** lower baseline values (≤1.4 mmol/kg feces) to **b)** higher baseline values (>1.4 mmol/kg feces) to visualize that the significant effects might be explained for higher baseline values only as there is a significant effect for baseline values above 1.4 (p=0.012).

Exploring the group effects on propionic acid

As described in section 3.6.1 the significant interaction for propionic acid means that the interaction accounts for a significant amount of the variation in the follow-up values of propionic acid. The fact that the intervention group has a slope that is -0.71 mmol/kg feces less steep than the control group (as the β_3 estimate is -0.71) looks reasonably when visual inspecting the plot (Figure 4.4f). As for valeric acid, we found that high baseline values were mostly associated with negative changes. However, we could not identify a clear cut off point for assessing intervention effect for high and low baseline levels.

To sum up it seems like the LFD intervention has led to a reduction for the intervention group compared to the control group for valeric and propionic acid. However, the trend seems to only apply for the patients starting with a higher concentration of these acids at baseline.

4.3.2 Effect on SCFA concentrations within the diet groups

In order to explore the lack of effects of the intervention for total SCFA, acetic, butyric, iso-butyric, and iso-valeric, paired T-tests were performed to investigate whether the lack of an effect was due to similar changes within each of the groups or to similar lack of changes. The boxplot for the different SCFA **(Figure 4.6)** shows the total and individual concentrations for each participant at baseline and follow-up. Paired T-tests within each group confirm a lack of significant effects from baseline to follow-up within both the groups. With the change values visualized by the connecting lines, this looks reasonable due to no observed trends of the differences between the measurements within each group by visually inspecting the boxplots.

To sum up, the paired T-tests performed within each group indicate that the lack of a differential effect of the intervention for total SCFA, acetic, butyric, iso-butyric, and iso-valeric acid in the regression analysis, is due to a similar lack of changes in both groups.

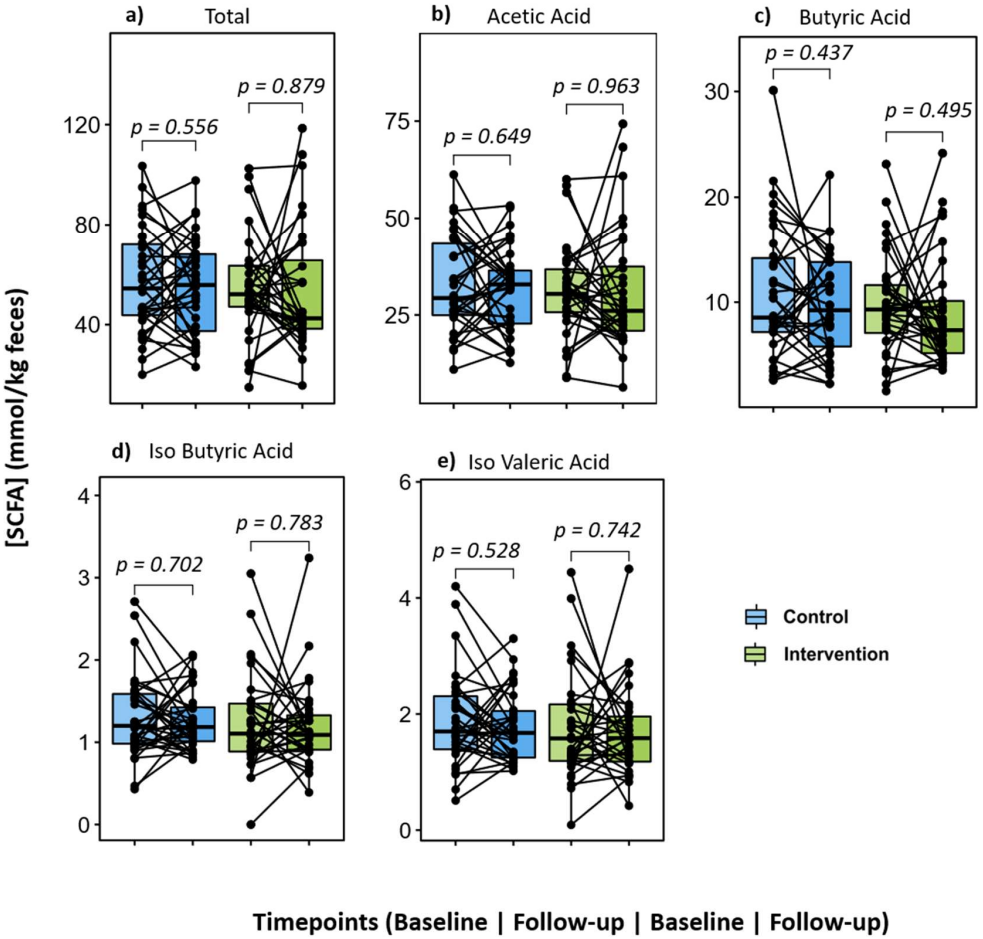

Figure 4.6: Boxplots of the total and individual SCFA concentrations (mmol/kg feces) from baseline to follow-up of the intervention group (n = 32) (blue) and control group (n = 34) (green). **a)** Total SCFA, **b)** acetic acid, **c)** butyric acid, **d)** iso-butyric acid and **e)** iso-valeric acid. The concentrations are mmol SCFA per kg feces. The p-values are from paired T-tests on change values.

4.4 Explorative analysis – Associations between changes in NGAL, SCFA and GI symptoms

4.4.1 Relation between NGAL and SCFA

Although we initially hypothesized that reduction in dietary FODMAPs would induce changes in NGAL and the microbial production of SFCAs only minor effects were found for valeric and propionic acid and no effects were found for NGAL. However, due to the unsystematic changes in both NGAL and many of the SFCAs in both the LFD and control group we explored associations between changes in these parameters for the total study population, regardless of group. No significant correlations were however detected between changes in NGAL and changes in total SCFA, nor individual SCFAs (data not shown).

4.4.2 Relation between NGAL and GI symptoms

As mentioned under 'Aims of the study', chapter 2, our collaborators who has investigated the primary endpoints of the CD-FODMAP study found a reduction in GI symptoms by the LFD. Since NGAL has been suggested as a biomarker for inflammation we explored possible associations between NGAL and GI symptoms, assessed by GSRS-IBS scores.

Spearman's correlation was applied to assess the relationship between change in the NGAL concentrations from baseline to follow-up and change in GSRS-IBS scores from baseline to follow-up. No correlations were found between changes in NGAL and changes in the total GSRS-IBS score. When investigating changes in NGAL against the changes in GSRS-IBS sub symptoms **(Figure 4.7)**, for most sub-symptoms there were no correlations. However, there was a significant positive correlation between NGAL and constipation ($p=0.0497$) for the whole population independently on group with increased NGAL concentrations associated with increased constipation symptoms. It should however be noted that this correlation was negligible with a rho of 0.24.

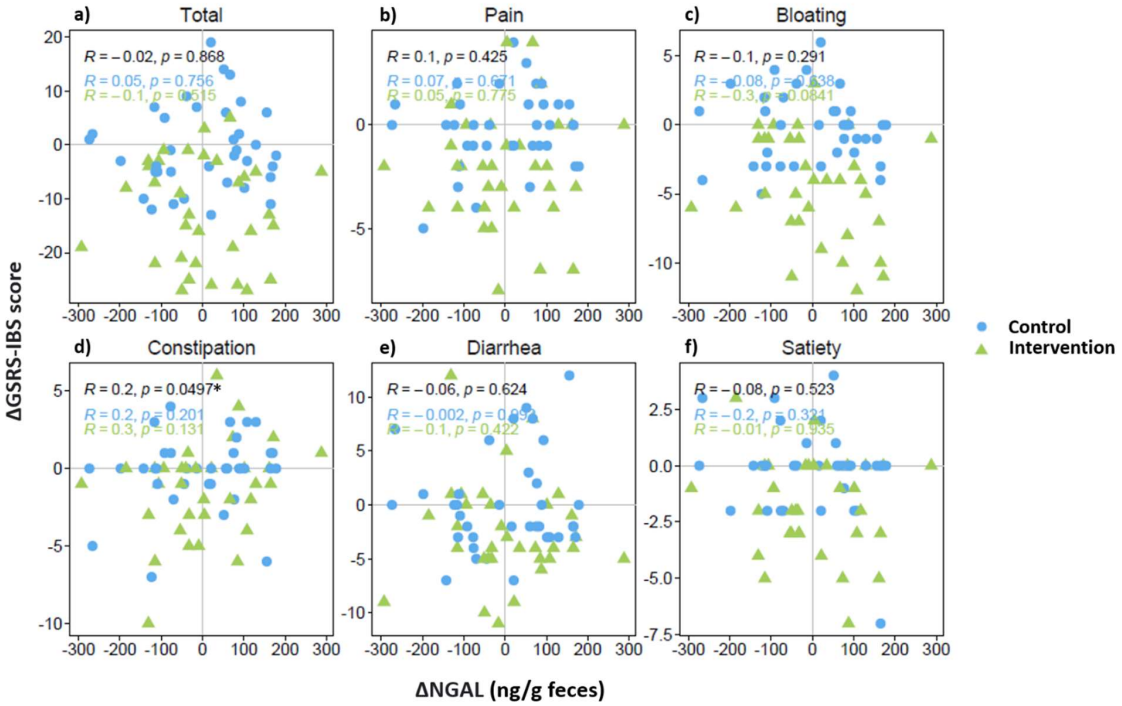

Figure 4.7: Scatterplots showing the correlation between changes in NGAL and changes in GI symptoms from baseline to follow-up. **a)** Total GSRS-IBS score, **b)** pain, **c)** bloating, d) constipation, **e)** diarrhoea and **f)** satiety. Both total GSRS-IBS score and each GSRS-IBS sub symptom represents the GI symptoms. Each plot presents the correlation for the control group (blue) and the intervention group (green) in addition to the total correlation independent of group (black). Deviation presentation of the plot is that the significant p-value is exceptionally given with more decimals as due to a crucial decimal. Also, the p-value is not assigned with the indicational

4.4.3 Relation between changes in SCFA and changes GI symptoms

With the purpose to investigate if a change in the SCFA can explain other relations such as changes in symptoms, correlations tests were performed among all combinations of SCFA and GI symptoms (**Table 4.6).** No significant correlations were found with one exception for propionic acid and satiety (rho=-0.249) (p=0.044) (marked in red).

Table 4.6: Correlation between changes in SCFA concentrations and GSRS-IBS scores.

[SCFA] (mmol/kg feces)	ΔTotal GSRS-IBS	ΔPain	ΔBloating	ΔConstipation	ΔDiarrhea	ΔSatiety
ΔTotal SCFA	R = -0.023 p = 0.852	R = -0.061 p = 0.624	R = 0.112 p = 0.371	R = 0.049 p = 0.698	R = -0.091 p = 0.466	R = -0.113 p = 0.367
ΔAcetic acid	R = -0.041 p = 0.741	R = -0.069 p = 0.584	R = 0.088 p = 0.481	R = 0.017 p = 0.892	R = -0.102 p = 0.417	R = -0.102 p = 0.416
ΔPropionic acid	R = -0.043 p = 0.729	R = -0.050 p = 0.690	R = 0.065 p = 0.604	R = 0.102 p = 0.415	R = -0.045 p = 0.719	**R = -0.249** **p = 0.044**
ΔButyric acid	R = 0.026 p = 0.838	R = -0.042 p = 0.738	R = 0.084 p = 0.505	R = 0.014 p = 0.914	R = -0.020 p = 0.873	R = -0.051 p = 0.685
ΔIso butyric acid	R = 0.065 p = 0.603	R = -0.051 p = 0.685	R = 0.109 p = 0.384	R = 0.124 p = 0.322	R = 0.060 p = 0.632	R = -0.035 p = 0.783
ΔValeric acid	R = 0.018 p = 0.888	R = -0.101 p = 0.422	R = 0.134 p = 0.282	R = 0.105 p = 0.403	R = 0.002 p = 0.989	R = -0.016 p = 0.901
ΔIso valeric acid	R = 0.078 p = 0.536	R = -0.040 p = 0.748	R = 0.081 p = 0.518	R = 0.119 p = 0.339	R = 0.084 p = 0.504	R = -0.003 p = 0.979
ΔCapronic acid	R = -0.109 p = 0.383	R = -0.202 p = 0.103	R = 0.048 p = 0.702	R = -0.102 p = 0.417	R = -0.068 p = 0.588	R = 0.121 p = 0.334
ΔIso capronic acid	R = -0.148 p = 0.237	R = -0.079 p = 0.528	R = -0.201 p = 0.106	R = -0.096 p = 0.443	R = -0.148 p = 0.235	R = 0.067 p = 0.593

Data is presented with Spearman's correlation coefficient and p-value. Significant correlation is found between propionic acid and satiety. **Δ:** change. R = rho.

The correlations were also investigated by visual inspection of scatter plots, plotting the changes in SCFA against the change in symptoms. The correlation between changes in propionic acid changes in symptoms is shown in **Figure 4.8**, while the plots are not shown for the remaining of the SFCAs. The negative correlation indicates that an increased propionic concentration is related with a decrease in satiety. However, rho-value of -0.249 indicates that the negative correlation should be judged as negligible.

To sum up, for the majority of SCFA and symptoms no associations were found when correlating the changes in these variables with each other. A significant but neglectable correlation was found between increase in propionic acid and decrease in satiety.

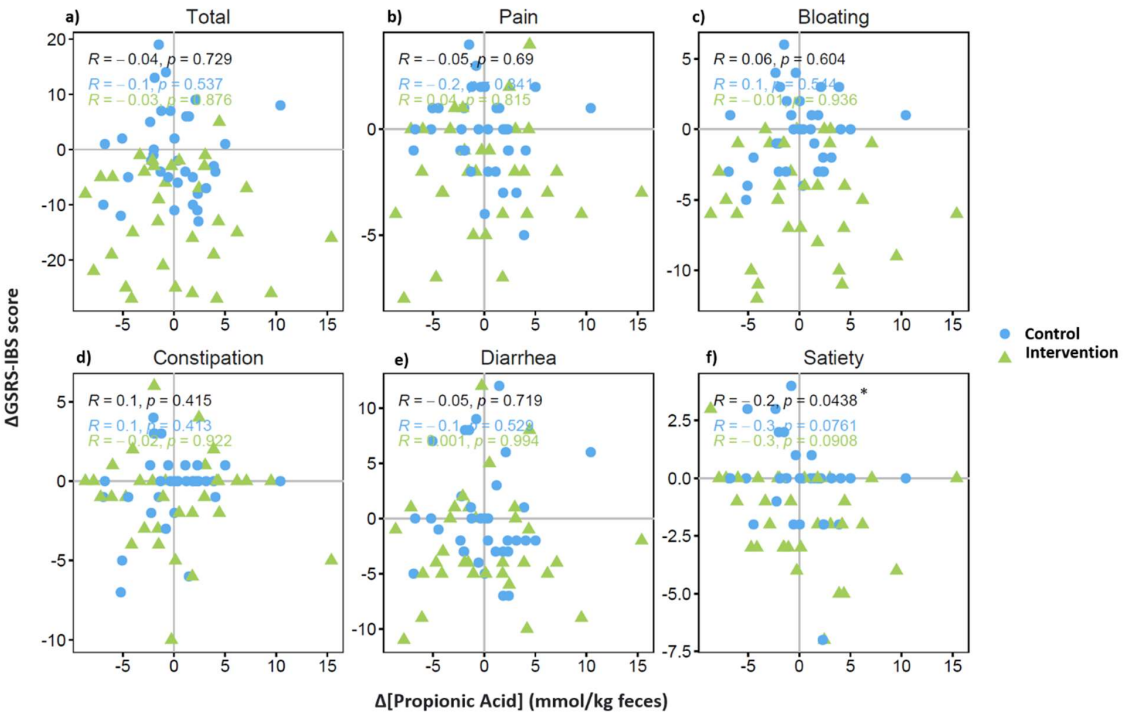

Figure 4.8: Scatterplots for correlations between changes in propionic acid and GSRS-IBS. **a)** Total GSRS-IBS score, **b)** pain, **c)** bloating, **d)** constipation, **e)** diarrhoea and **f)** satiety. Since no variables were checked for normality, all data are presented with Spearman's correlation coefficient. Correlations are given over all (black) and correlations for the control and intervention group when studying the groups independently (blue and green, respectively). Deviation presentation of the plot is that the significant p-value is exceptionally given with more decimals as due to a crucial decimal. Also, the p-value is not assigned with the indicational symbol '*', and 'R' indicates rho.

4.5 Development of an antibody-based multiplex method for quantification of fecal NGAL, validated against ELISA

The antibody-based Luminex technology allows for simultaneously detecting multiple biomarkers in the same sample. Depending on the system design, it is possible to detect up to 500 different biomarkers. The use of such multiple biomarker panel may not only reduce labour and costs but can also offer other benefits such as higher sensitivity and fewer methodological challenges compared with single-analyte traditional immunoassays such as ELISA. As NGAL is a relatively new gut inflammatory biomarker, this motivated to establish a Luminex-based method to measure fecal NGAL for the potential to be measured as part of a larger analyte panel. The development of a multiplex method for fecal NGAL detection was composed of two parts; 1) establishing the method by making a 'pool' of antibody-coated microbeads that can detect NGAL and 2) comparing the results obtained by Luminex to that of ELISA.

4.5.1 Establishment of the Luminex-based method using fecal NGAL

To successfully establish the Luminex-based method for NGAL detection, it is necessary to couple microbeads to NGAL specific antibodies (referred to as capture antibodies) and find the optimal concentration of antibodies and optimize other conditions that can be important for the successful establishment of the method. Matrix effects and impact of washing procedures were therefore investigated as part of the establishment of the method.

Determining optimal capture antibody concentrations for bead coupling

Figure 4.9 shows the result of the titration experiment to find the optimal amount of capture antibody for the bead 'pool'. The various concentrations tested were 5 µg (yellow), 2 µg (blue) and 1 µg (green) of NGAL specific capture antibodies (monoclonal rat IgG) per million beads, at different concentrations PE-conjugated detection antibodies (polyclonal goat IgG). According to the xMAP® Cookbook (Luminex Corporation, 2018), the optimal coupling efficiency is achieved when the mean fluorescence intensity (MFI) signal becomes saturated right above 10 000 (MFI). While the 5-µg titration level exceeded the threshold for optimal binding with saturating above 18 000, the 1 and 2 µg/million beads titrations both gave similar flattening of the curve just above 10 000 and were thus considered to be successful for further use.

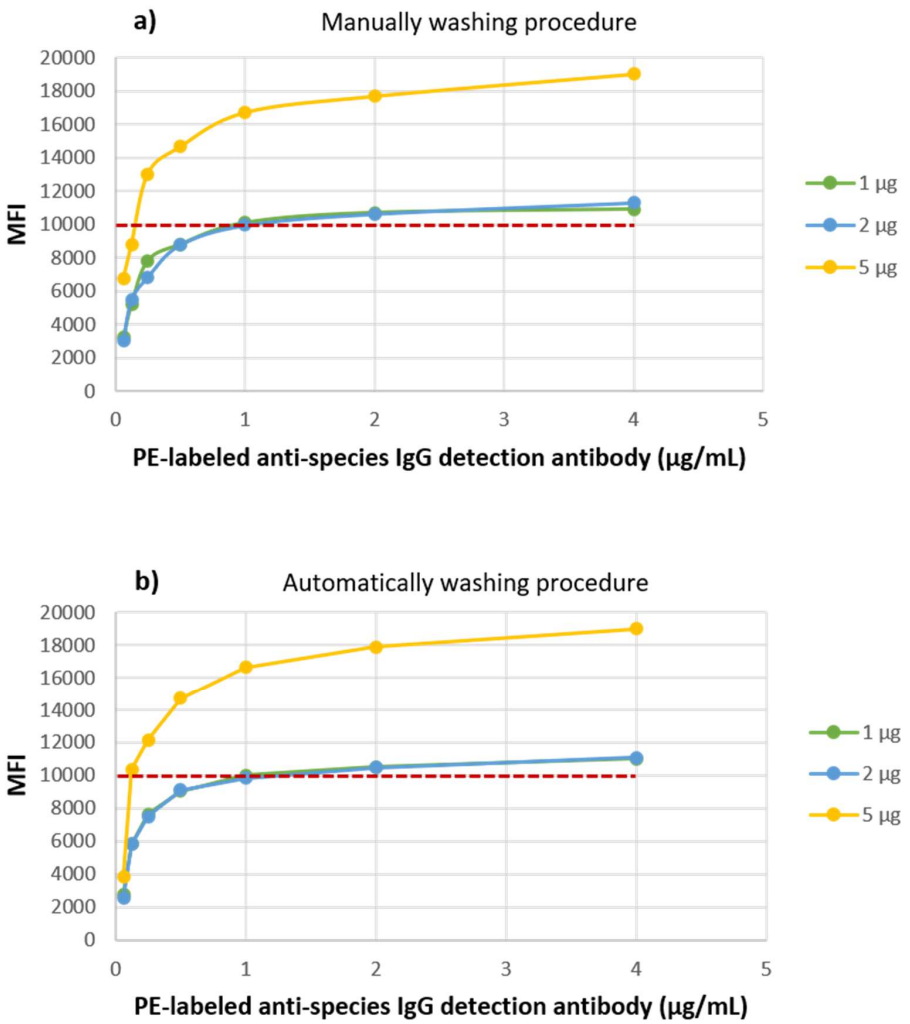

Figure 4.9: Scatterplots of antibody coupling titration experiments using different approachoes **a)** Scatterplot of the titration experiment using 1 µg (green), 2 µg (blue) and 5 µg (yellow) capture antibody per million beads. The optimal MFI with a threshold of 10 000 indicating saturation (red dotted line) was reached using all selected concentrations, in which the 1 µg and 2 µg amount of capture antibody reflected an almost similar MFI saturating slightly above 10 000. With the purpose to investigate whether an automatically washing procedure (using PBS + 0,1% Tween20 as a washing detergent) had any impact on the results, **b)** a similar titration experiment using the automatic washing procedure was set up in parallel to compare with the results obtained by using the manually washing procedure (using PBS as washing detergent).

The recovery of the beads was tested for the three bead 'pools' that were coupled with 1, 2 or 5 µg/million beads (**Table 4.7**). The recovery was best for the bead 'pool' coupled with 2 µg/million beads, with a recovery of ~88%, indicating a loss of ~122 000 beads. Therefore, the bead 'pool' with 2 µg capture antibody/million beads was selected for further use.

1 million beads are recommended to be coupled for training purposes, while for the assay run, it is recommended not to couple less than 2.5 million beads (Luminex Corporation, 2018). When coupling new beads for the assay run, the MFI was not sufficient for unknown reasons, also mentioned in section 5.1.3, and the 'training beads' of 1 million beads were used for the validation. Since a bead recovery may become difficult when coupling down to 1 million beads (Luminex Corporation, 2018), it would be understandable not to reach a full 90% recovery as the protocol normally would yield. However, 88% recovery is considered acceptable.

Table 4.7: Recovery of beads following the coupling procedure.

Coupling reaction	Amount of capture antibody per million beads	Recovered beads (count)	Percent recovery
a	1 µg	7.22×10^5	~72%
b	2 µg	8.78×10^5	~88%
c	5 µg	8.67×10^5	~87%

1 million beads were used for each coupling reaction (a-c). The number of recovered beads was counted using a haemocytometer.

Minimizing 'matrix effect' in the fecal samples

As feces is a complex matrix, substances other than the analyte of interest might compete with NGAL by binding the NGAL specific antibodies or somehow interfere and disturb the specific binding. As this might cause underestimation when detecting NGAL, a dilution series of several samples were performed to determine whether this phenomenon was present in the current assay as observed for the ELISA. Therefore, several dilution ratios were tested to find the optimal dilution that gave the highest NGAL response and lowest 'matrix effect', or inhibition (Zhou et al., 2017).

Feces supernatants from six different patients were selected for the experiment. Each sample was diluted in ratios of 1:1, 1:2, 1:10, 1:50, 1:100 and 1:500. The standard curve covered a concentration range from 78.125 pg/mL to 5 000 pg/mL, and ratios appearing within the standard curve was 1:50, 1:100 and 1:500. Among these dilution ratios, 1:100 dilution gave the highest detected concentration for most samples after correcting for the dilution factor. Also, the median of the concentrations measured with this ratio was closest to the median of the standard curve and thus lying in the steepest part of the standard curve, which is considered optimal. Based on these factors, 1:100 was evaluated as providing the best dilution to detect NGAL using the bead 'pool' developed in the current project and therefore used to dilute all fecal samples for comparing against the ELISA method.

Improvement of plate washing procedure

The plate washing procedure is a step that can potentially introduce technical variations for the Luminex-based method. Therefore, the results from two experiments using i) an automatic washing machine and ii) a manual washing procedure were compared. The number of washing cycles was similar, but the detergents used were slightly different. The results for the titration experiment (section 3.5.3) after the two washing procedures were approximately identical (Figure 4.9) judged by visually inspecting the curves for each assay performance. It seems that it is indifferent whether the manual or automatic washing procedure is used.

4.5.2 Comparing the Luminex based results to that of ELISA for NGAL detection

The NGAL concentrations from the established Luminex-based method were compared to the values obtained by the ELISA method from R&D. We selected 35 samples from the CD-FODMAP study to compare NGAL concentration measured with Luminex to that of ELISA. The samples were selected to reflect a broad concentration range from low concentrations to the highest concentrations based on the results from the ELISA measurements. Due to technical difficulties during the Luminex procedure, two individuals were excluded from the analysis.

When investigating paired statistics performed by the Wilcoxon signed-rank test, the absolute NGAL concentration from the two different methods was compared. A high significant difference ($p<0.001$) between these paired samples of the methods was found. Due to the positive mean difference of 715 concentration units between the methods, when subtracting the ELISA method from the Luminex-based method, the Luminex-based method is constantly measuring higher

values than ELISA. This indicates that the Luminex-based method estimates higher NGAL concentrations than the ELISA method (data not shown). However, this paired statistic does not tell whether this average of difference is constant or not.

The relationship between the NGAL concentrations measured by Luminex and ELISA were also studied by simple regression analysis. When comparing the methods directly, there is a high interclass correlation (rho=0.82) ($p<0.001$) between the different methods indicating a strong relation between the assays. This is supported by visual inspection of the correlation plot (**Figure 4.10)**, in which it seems like lower NGAL concentrations measured with Luminex are also measured as lower NGAL concentrations with ELISA and vice versa. However, the scales for the different methods have dramatically different ranges, in which the ELISA method has detected NGAL concentrations in the interval of 36-612 ng/g feces, while the Luminex measurements were in the interval of 60-3637 ng/g feces. Therefore, the scales are barely overlapping in scale range and Luminex measures in general *higher* concentrations than the ELISA method. When log transforming the values (Figure 4.10b) to obtain more similar ranges, the same strong correlation as illustrated in the correlation plot in Figure 4.10a appears.

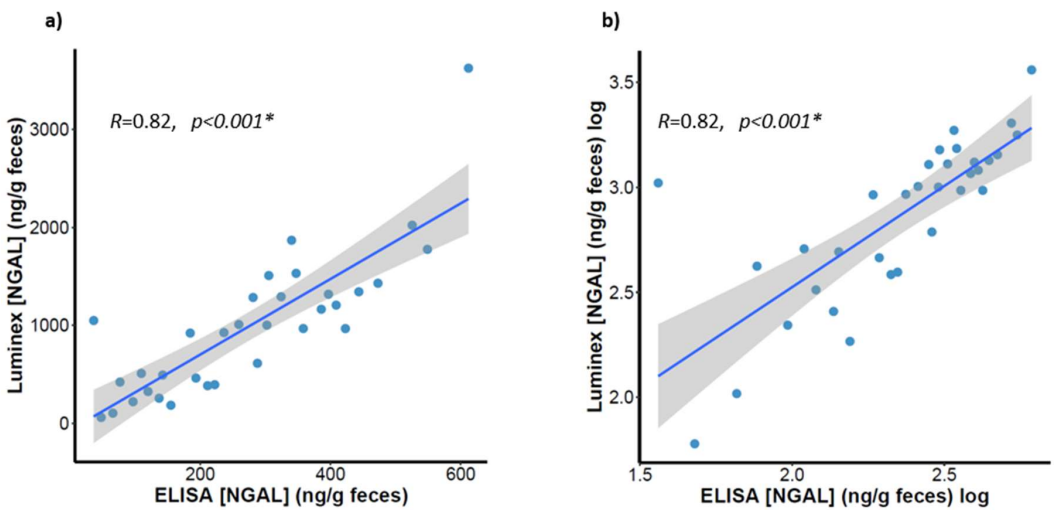

Figure 4.10: Correlation plot of NGAL concentrations measured with ELISA and Luminex-based method on **a)** non-transformed data and **b)** log-transformed data. Significant p-values are stated with '*'. Rho is indicated by 'R'.

When assessing the agreement between the methods proposed by Bland & Altman (BA) (1986), the BA plot **(Figure 4.11)** is provided. Since the values are not normally distributed (either when log-transformed), we cannot state the expectation to find 95% of the differences between the limits of agreement. By visual inspection of the plot, the points have a clear trend in starting narrow and widening out to the right as the magnitude of the measurement increases, meaning that it seems to be a clear increase of difference between the methods for higher values measured. Furthermore, as the distribution of dots is not horizontally above the line of equality (the light blue x-axis at zero difference), there seems not only to be a *systematic* bias but a *systematic proportional* bias. This indicates that there is a relation between the average difference and the magnitude, and was confirmed by the correlation stated a *significant* bias (rho=0.94, $p<0.001$) (Figure 4.11) stating that the methods do not agree *equally* through the range of measurements.

To conclude this, there is a good correlation between the methods. Most importantly, the proportional bias occurring during the method validation stating that the Luminex and ELISA not equally agreed implies that it is not indifferent which method is selected for measuring fecal NGAL. Also, the mean difference of 715 units between the methods cannot function as a simple and directly converting factor between the assays as they do not agree equally and cannot de directly compared against each other or used indifferently.

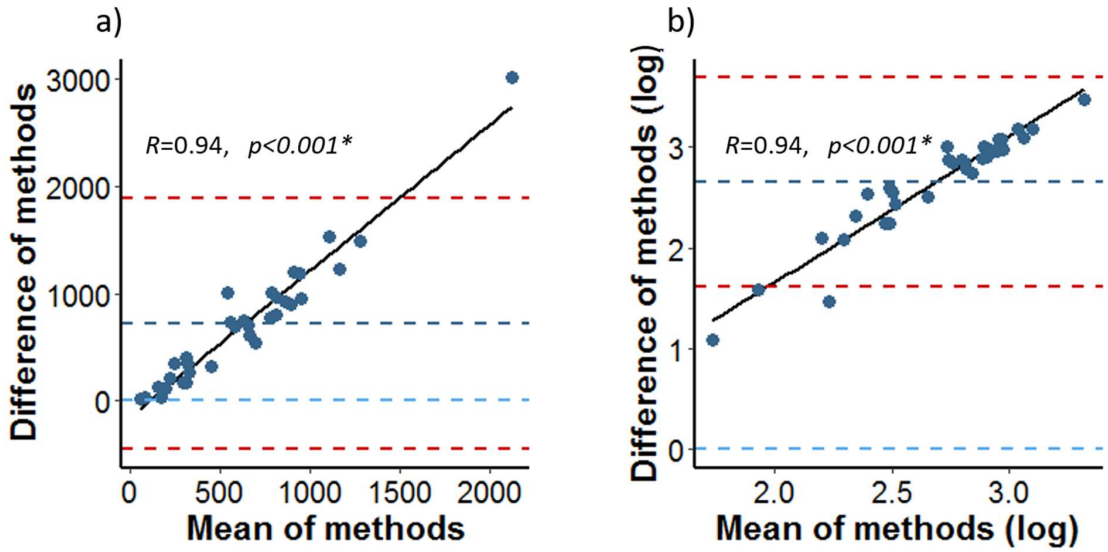

Figure 4.11: Bland-Altman plots (BA plots) illustrating the agreement between Luminex and ELISA by plotting the differences from the methods against the mean of the methods on paired samples (n = 33). The line of equality is constant at 0 (light blue). The higher mean of methods, the higher becomes the difference of the methods, indicating a proportional systemic bias and as visualized by the regression line. A 95% confidence interval is used to determine the upper and lower limits of agreement (red dotted lines) and is calculated by adding (or subtracting) 1.96 x the standard deviation to the average value of the difference between the methods. **a)** BA plot for non-transformed data and **b)** BA plot when values are log-transformed since normal distribution for the difference values between the methods is not verified according to statistical tests (Shapiro-Wilk) and histogram. Significant p-values are stated with '*'. Rho is indicated by 'R'. ELISA: enxzyme-linked immunoassay, BA: Bland-Altman.

5 DISCUSSION

This master study is a sub-project within a study that found GI symptoms relief following an LFD in CD patients that experienced persistent GI symptoms. No effects were found on the gut-inflammation biomarker NGAL, but effects were found for a reduction in two SCFAs. Also, a Luminex-based method for measuring NGAL in fecal samples was successfully established.

The work conducted in this thesis is important because there is little knowledge on how a FODMAP reduction affects the gut health in CD patients. Also, the establishment of the Luminex-based method may be useful for diagnostic and other clinical use in the future, not only in CD patients but also in other populations.

The discussion consists of two separate sections discussing the methods used in this thesis (6.1) and the discussion of the results (6.2).

5.1 DISCUSSION OF METHODS

5.1.1 Quantifying fecal NGAL measured with ELISA

Feces is a heterogeneous and complex material composed of metabolic products from microorganisms in addition to other substances such as undigested foods or eliminated host components (Zubeldia-Varela et al., 2019). This makes feces a challenging matrix to work with compared to other biological homogenous fluids. During the aliquoting procedure of the fecal material, insoluble substances such as seeds, corn, and other undigested foods were eliminated when observed. Still, there was a chance of smaller particles hiding in the matrix, as this only was visually evaluated. Depending on the weight of these substances and their affinity to bind NGAL, they might have impacted the NGAL concentrations measured. An underestimation would occur when correcting for the weight by including the unwanted large substances (or several smaller substances) and due to the possible lack of assessable NGAL due to their possible affinity. It is uncertain whether this was of importance when considering the NGAL representability for the fecal aliquot.

Considering the fecal composition, the wet mass was not corrected for when measuring NGAL. Therefore, the NGAL concentration might vary depending on the feces consistency, as a more moisture fecal sample might unintentionally dilute the NGAL concentrations, causing an

underestimation. The water content in feces for healthy individuals are ~75% but varies with dietary intake of food and fluid (Rose et al., 2015), such as intake of non-degradable fibre, which might cause water to flux into the lumen (Eastwood, 1973). In addition to the digestive challenges of insoluble foods, the water content also depends on the gut absorption ability. During active CD and destruction of the brush boarder, a poor absorption might cause an imbalance in absorptive and secretory enterocytes, inflammatory responses inducing a secretion to the lumen, as well as an osmotic effect increasing the gut lumen water content. As this patient population did not have an active CD status, the fecal consistency would primarily be associated with the intake. Also, depending on the reabsorption by the colon, the water content in the feces will vary (Field, 2003). As a solution to this, freeze-drying the feces material would leave the dry weight only, as performed by previous studies (Lewis et al., 2016; Reygner et al., 2020). However, this technique requires advanced lab equipment and is, in general, a resource-demanding process, and was therefore not implemented in the lab performance during this master thesis project. Thus, the water content in the fecal samples could impact the NGAL measurements in this master project.

As the daily water content for patients varies depending on their dietary intake, this might have caused varying NGAL concentrations from one day to another for the same patient. Therefore, such individual variations question whether the collected fecal sample is a sufficient representation of the NGAL levels for each patient. In addition to the measured part not being completely homogenous, it is also questionable how representable the measured aliquot is to the collected sample and again to the whole defecation. By homogenizing the entire sample collected, this could have been prevented. As this would require a complete thawing of the sample and was therefore not prioritized to prevent a possible analyte degradation. The homogenization of the matrix was also evaluated visually, and we can therefore not guarantee a completely homogenous sample. This is a weakness in the method considering representability, and it is uncertain how great importance this has for the final measurement result, as it would depend on the distribution of NGAL in the sample. However, as part of diagnostic procedures measuring calprotectin as a biomarker for IBD, an even smaller amount of the sample compared to what was used in this study is sufficient (Bühlmann, 2018). Suppose this applies to NGAL as well, which is highly correlated with calprotectin, as mentioned in section 1.5.1, it could mean that it would not delimit the NGAL representation for a patient significantly.

Despite feces being a challenging matrix with uncertainties connected to the representation of the fecal biomarker, it is suggested to be more specific for intestinal inflammation compared to serum biomarkers as serum might be elevated due to non-GI reasons. Another reason why fecal samples are preferred is because of the direct contact with the intestine, which might improve the ability to reflect the degree of gut inflammation (Lopez et al., 2017).

Whether one defecation during the day represented the individuals' NGAL level for the representable period of time is also questionable. Therefore, the biomarker's stability is critical, not being degraded by bacteria, proteases or other relevant degradation secretions during the digestion, one concern shared by a study measuring calprotectin for IBD patients (Bjarnason, 2017). Whether NGAL might function as an invasive biomarker to quantify the neutrophil flux into the intestine, require that NGAL is not degraded and that the fecal measurement is representative.

In the search for an optimal fecal matrix with the least inhibition effects, the dilution of 1:100 (data not shown) was tested and found. However, as seen during both the ELISA and Luminex dilution testing, this optimal dilution might vary between different samples. The variation of water mass might be an explanatory reason for different samples peaking for different dilutions in which very watery samples might be less compact and have less inhibition factors interfering with NGAL compared to other more concentrated fecal samples. To improve the selection of the dilution ratio giving the least inhibition, one could 'spike' the samples as suggested by Thermo Fisher Scientific (2017). This is performed by adding the same high and known concentration to the different dilutions, investigating which dilution is detecting the highest analytes amount, and maximizing the signal-to-noise ratio (Thermo Fisher Scientific, 2017).

5.1.2 Fecal SCFA

Fecal measurement of SCFA faced the same challenges as the measurement of fecal NGAL discussed in 5.1.1. Considering the homogenization of the matrix, inhibition effects and lack of water mass correcting, a possible underestimation must be considered as an insecurity during the sample preparation before the further analysis. The further GC-FID analysis of the SCFA provided high sensitivity. As described in section 3.5.2, the SCFA were protonated with the addition of sulfuric acid, increasing their volatility and preventing binding to unwanted sample particles. With the time-consuming separation technique using vacuum distillation, there was a high possibility of

losing volatile acids (Scortichini et al., 2020), which again might increase the likelihood of an underestimation.

Considering underestimation, it should be noted that only about 5% of the SCFA produced by bacteria are excreted in feces (Primec et al., 2017). The remaining SCFA produced in the colon are mainly absorbed in the mucosa (Parada Venegas et al., 2019). Therefore, this weakens the representation of the SCFA concentration in the fecal samples compared to the actual concentration in the intestine. Nevertheless, fecal SCFA provides a useful reflection and indication of the gut's relative microbiota composition and production of SCFA.

5.1.3 Method establishment and validation of Luminex using fecal NGAL

As part of the establishment, the coupling efficiency was evaluated before proceeding to the validation. The same fecal supernatants were used in both methods to compare the measurements themselves and limit possibly disturbances from the samples.

The titration experiment was done to find the quantity of the capture antibody necessary to promote optimal binging of the desired target molecule (NGAL). A good coupling of capture antibodies was determined by the dose-dependent curve typically saturating around 10 000 MFI when reaching its sensitivity goals. Unexpectedly both bead 'pools' with 1 µg and 2 µg capture antibody/million beads saturated at MFI signals slightly above 10 000, obtaining almost identical curves. As different quantities of capture antibody were expected to reflect different MFI signals, it was suggested that both quantities accidentally were the same due to improper scaling possible connected with pipetting error during the reagent reconstitution or dilution. Troubleshooting was performed to investigate this condition but will not be explained any further. A new bead coupling was repeated two times (results not shown), and for unknown reasons, they were not successful. The amount of 5 µg obtaining a saturation at 18 000 MFI could have been used for the validation but was eliminated as the recommendations were based on a saturation of MFI signals *slightly* above 10 000, and we considered establishing it as a method for future analysis in the lab. Also, the sensitivity of the coupling confirmation is rather increased using less amount of both capture antibodies and detection antibodies (as long as it exceeds the threshold) (Luminex Corporation, 2018). Even though the true quantities of the potential bead 'pool' candidates of 1 µg and 2 µg remain unknown, the bead 'pool' intentionally assigned with 2 µg capture antibody/million beads was selected.

As the same PE-conjugated detection antibody amount was used for all three titration levels of capture antibody, it should not have impacted the interpretation of the results since the curves were saturated relatively similarly. Therefore, it was not further tested which amount that was the most optimal one, and the concentration of 4 µg/mL was used. However, a suggestion from the xMAP Cookbook is that the sensitivity might be improved as the antibodies are reduced.

During the serial dilution investigating the most sensitive detectable dilution, only half of the tested dilutions appeared within the standard curve, decreasing from 5000 pg/mL, in which of the least diluted samples (1:1, 1:2 and 1:10) the concentration occurred above the standard curve. As higher concentrations than 5000 pg/mL were not tested, the detected values of these samples were estimated based on extrapolation of the standard curve by the Luminex software. However, when correcting for the dilutions to investigate a potential inhibition effect, all these three dilution ratios seemed to obtain potential inhibition effects and were not accepted as potential candidates for the final dilution. As mentioned as a possibility for the ELISA method considering the inhibition, 'spiking' the samples to determine which dilution ratio detects the highest concentration is also a suggestion applying for the Luminex-based method. Therefore, it is not likely to think that a higher concentration of the standard curve would have provided a higher NGAL detection for the least diluted samples, but this remains unknown. This limitation could have been avoided by determining the top and bottom signal of the standard curve in advance of the dilution testing and then selecting a more representable concentration range for the standard curve.

The MFI signal will be saturated at some point, determining the upper dynamic range of the instrument considering the dynamic range. As the validation was using 10 000 µg/mL as the highest range of the standard curve, and this was still within the instrument's dynamic range, the dynamic range is most likely compatible measuring higher concentrations than this. Again, this could have been done before the dilution testing, potentially finding another optimal dilution ratio than 1:100 and then optimizing the best effective biological range with respect to the instrument (Luminex Corporation, 2018). The MFI range could have been determined by investigating how low the standard curve would go as well.

Conditions associated with the samples and reagents for the different methods were minimized to decrease other potentially influential differences affecting the measuring. The main differences for

the sample treatment for the two methods were extra freeze-thaw cycle for the samples dedicated to Luminex and the addition of 50 µL versus 100 µL in the plate assay for the Luminex and ELISA, respectively. Reagents and instruments differed as well, such as SAPE being added for Luminex instead of the HRP-Streptavidin-B as the detection antibody conjugated signal. However, as such differences might be considered a part of the different procedures, this was appropriate for the method comparison.

Limitations of the method establishment in this master project was that it was not designed to investigate certain common validation parameters. Different levels of precision are found by measuring the same sample multiple times within the same run, laboratory and between laboratories. Also, to validate the method's robustness, the assay capacity to remain unaffected by small variations such as incubation times and pH variations are tested (WHO, 2006), but due to time limitation and scope in this master thesis, this was not performed.

5.1.4 Strengths and imitations in the CD-FODMAP study design

The CD-FODMAP study was designed as an RCT with a parallel design with a control and intervention group. The control group was maintaining a gluten-free diet as normal, controlling the background diet for the intervention. A strength in this study design was the randomization, reducing selection bias and prevention of disturbance by randomly distributing the population in each group (Karanicolas et al., 2010). Noticeably considering the distribution was the uneven representation of gender, in which more than 80% of the study population was women. This is reasonable as autoimmune diseases tend to have a higher prevalence among women than men (with ~80%) (Fairweather & Rose, 2004). However, this might be considered a limitation in which this study is representative primarily of women.

Considering limitations in this RCT, a *cross-over* design was carefully considered but was not possible as the participants could not be de-educated regarding the LFD instructions. Furthermore, a double-blinded study was neither possible as the diet-based treatment demanded educational consciousness of both participants and the clinic. Regarding the inclusion criteria with a high GSRS-IBS score, there was an unintentional improvement from the screening to the start of the intervention at baseline. What caused this symptom reduction is not clear, but a possible explanation might be an increased awareness of GFD adherence.

5.1.5 Challenges with patient measurements

A potential challenge with these RCTs was the lack of diet adherence among the participants. The LFD adherence was evaluated using two approaches. First, a self-reported score between 1-100 was used as a simple measurement in which 100 indicated 100% compliance. An average compliance score of 94% indicated 'good' compliance, but as this effort of compliance was subjectively evaluated, the margins of error in this measure should be considered. Additionally, dietary data were recorded. The mean intake of FODMAPs in the LFD was reduced from 20 g to 8 g from baseline to follow-up for the intervention group. Contrarily the control group remained 'stable' with a level of FODMAP of ~20 g. Although this also was a 'good' indication that the participants followed the LFD, it should be noticed that diet registration is not always accurate. Also, the FODMAP database used is mainly based on Australian foods (as the LFD was developed here), causing insecurity in the registrations as FODMAP content in foods can vary between different countries (Varney et al., 2017). Also, there were significant variations in how much each participant in the intervention group reduced their FODMAP intake. This might be explained by natural variations as people with higher FODMAP intake at baseline have a greater potential for FODMAP reduction. Some participants in the LFD group who had low intake already at baseline even reported a slightly increased FODMAP intake from baseline to follow-up, which might be considered a measurement weakness. However, this was attempted to be avoided by including the baseline values as a covariate during the statistical analysis.

Diet-based interventions demand an active role from the participants considering this lifestyle adaptation. As CD patients are already used to adhering to a special diet, it is thinkable that this could be advantageous for the intervention group when adapting an additional diet. This is because they might be used to reading the list of ingredients and often making some meals from scratch. At the same time, there will also be an extra burden of dealing with two diets simultaneously, which might lead to a deficient diet and increased stress. This underscores the importance of good guidance from the study centre.

Another concern of patient measurements is the chance of dropouts throughout the trial. It can result in missing values and may cause both systematic and unpredictable bias in RCTs in the same way as lack of compliance (Porta et al., 2007). This is further discussed in section 5.1.6.

5.1.6 Statistical considerations

In this RCT, linear regression models with adjustment for baseline levels were selected as the longitudinal statistical analysing method. This adjustment is considered an attractive possibility as it, to some degree, protects the estimated group effects to be caused by characteristics of the populations instead of the LFD (J et al., 2018; Roberts & Torgerson, 1999). However, there is an ongoing debate on whether the baseline values of the outcome variables should be adjusted for or not. This will not be discussed further in this thesis, but as several studies support the adjustment (de Boer et al., 2015; J et al., 2018; Peterson et al., 2017), the approach was used in this study.

For the data set and statistics considering NGAL and SCFA measurements, one individual was noticed by having a remarkable combination of very low baseline values and high follow-up values compared to the other individuals. When further investigating other measurements connected with this individual, no deviating information was found to help predict this deviating behaviour. There were, therefore, no reasons for excluding this individual. As this individual seemed to affect the interaction, the statistical analyses were tested with and without this individual, stating that the significant results were robust also without this individual.

For the data set and statistics considering the NGAL measurements, one individual was excluded after completing the intervention due to extremely low NGAL concentrations measured below the standard curve range. Whether these values were ‘true values’ explained by biological variations or caused by unintentional errors during the method performance remains unknown. However, instead of extrapolating the standard curve and due to time capacity preventing extra lab trials, these measurements were treated as missing values. They were, therefore, excluded from the population, which was relatively large and consequently robust enough not to be affected by this removal.

5.2 DISCUSSION OF RESULTS

5.2.1 Effects of LFD on NGAL concentrations

While our collaborators found that the LFD led to a decrease in GI symptoms in CD patients with persistent GI symptoms (van Megen, F. et al., unpublished results) the current study finds no effect of the LFD intervention on NGAL concentrations.

Assuming that NGAL is a good biomarker of gut leakage and gut inflammation, the lack of an effect on NGAL in the current study may imply that improvements in the low-grade inflammation is not a contributing factor to the relief in GI symptoms. Also, the lack of correlations between changes in total or individual GI symptoms and changes in NGAL supports a lack of dependency between the GI symptoms and low-grade inflammation for this population.

The improvement in GI symptoms is therefore assumed to be due to other problematic aspects of FODMAPs. One aspect of FODMAP intake that can cause discomfort and flatulence symptoms is associated with the gases that are produced when the FODMAPs are metabolized by bacteria (Ong et al., 2010). This aspect may be independent of inflammation and/or effects on gut integrity. Another aspect of FODMAP that can lead to pain and discomfort is the possibility that the undigestible FODMAPs can create an osmotic effect pulling water into the intestine to create a higher mechanical pressure. This osmotic ability may, however, be dependent on the type of FODMAP (Murray et al., 2014).

While the effects of the LFD on GI symptoms in the CD-FODMAP study is in line with another study in CD patients with persistent GI symptoms (Roncoroni et al., 2018), to the best of our knowledge, we find no studies that have investigated the effect of LFD on gut inflammatory biomarkers among CD patients with persistent GI symptoms. Thus, whether LFD affects gut integrity and/or inflammation in this patient group awaits further investigation.

The lack of an effect of LFD on NGAL in the current study may also be related to an insufficient change in FODMAP during the intervention. While the mean reduction of total FODMAPs in the intervention group decreased from 20 to 8 g this change might not be sufficient to impact NGAL levels. As previously mentioned in section 1.3.3, wheat is a major FODMAP source in specific for fructans (Whelan et al., 2018), therefore CD patients on a GFD often have reduced intake of fructans, and possibly other FODMAPs, which may imply that the baseline levels of FODMAPs

were too low to have generated conditions that would impact NGAL levels. According to a review by Melini & Melini (2019), most studies in CD patients show that they struggle to maintain fibre intake after initiating a GFD (Wild et al., 2010).

While there are no studies investigating dietary impacts on fecal NGAL in CD patients, results from other studies on IBS patients might offer some relevant results. In a study performed on inactive and active IBD, IBS patients and healthy controls NGAL levels were elevated among those with active IBD patients compared with inactive IBD patients and a trend for higher NGAL levels were found in IBS patients compared with healthy controls (Thorsvik et al., 2017b). Those results support that the NGAL levels among IBS patients are not as high as during inflammatory conditions. The median baseline levels in the current study were 259 ng/g feces which is lower than the concentrations (~400 ng/g) found for IBS in the study by Thorsvik, suggesting that the baseline inflammation levels are too low in the CD-FODMAP to achieve an effect of the intervention.

The inclusion criteria considering the GI symptoms was a GSRS-IBS score of 30 or above. However, after the screening (before the first baseline measurements), several patients had a reduction of the symptoms, resulting in a score below 30. This might be due to the effect of awareness, or that they intentionally started to avoid FODMAP rich foods before baseline unintentionally. There is therefore a probability of an NGAL reduction connected with this initial reduction, in which might have explained the lack of effect on NGAL as this could cause an underestimation of the true NGAL concentration among the population at baseline, causing that it would be harder to detect a change in such low NGAL values in the first place. However, since the current study compared the effects of the LFD with that of a control, the ‘awareness-factor’ should potentially be similar in both groups and therefore controlled for.

Finally, the lack of an effect on NGAL from the LFD intervention might be due to lack of statistical power due to both lower potential for an effect and large variations in the NGAL results. Also as discussed in section 5.1.1, there are yet unsolved challenged using feces for biomarker analysis that could have introduced technical variations in the results. In particular, the unknown amount of water mass in feces might have impacted the total amounts. In line with this suggestion a negligible correlation was found between NGAL and constipation for the total population. A firmer stool during constipation might cause higher NGAL simply due to higher concentrations

due to less water content in stool, as discussed in section 5.1.1. Also during constipation, the bacteria could be allowed to interact with the epithelium for a longer period which could cause NGAL levels to increase. However, a study among IBS patients showed less serum NGAL among the constipation dominant subtype than those having other stool subtypes -diarrhoea, and -mix and healthy controls (Jones et al., 2014) which challenges our interpretation. However, serum and fecal NGAL might reflect the intestinal inflammation differently as suggested by Nielsen et al performing a study on IBS patients (Nielsen et al., 1999).

Possible solutions to control for the technical impact of stool consistencies could be to control for measured dry mass by using freeze-drying methods or standardize the NGAL values against total protein in feces. Also, the Bristol stool scale (Blake et al., 2016) could be used as a crude way of standardizing the results.

5.2.2 Effects of LFD on SCFA concentrations

For the majority of the SCFA the current study found no effect on LFD. However, significant effects of the diet were found for valeric and propionic acid depending on the baseline level. Furthermore, we found that among those individuals that had a high baseline level, there was a significant reduction in valeric and propionic acid after LFD.

After thorough review of the scientific literature, and to the best of our knowledge, no other papers were found reporting on effects of FODMAP-restrictions in CD patients with persistent IBS symptoms. We did, however, find two studies investigating LFD in IBS patients (Hustoft et al., 2017; Staudacher et al., 2016). While these studies also showed a reduction in SFCAs after LFD the reduction was not seen for the same SCFAs as found in the CD-FODMAP study. Taken together this might imply that the LFD limit the FODMAP substrates for bacteria causing a lack in SCFA production. On the contrary, two other studies on IBS patients did not find any significant effects on SCFA after an LFD (Halmos et al., 2015; Staudacher et al., 2012), supporting the lack of effects on most of the individual SCFA and the total SCFA in the CD-FODMAP study. The lack of effects on the SFCAs could also be related to an insufficient change in FODMAP during the intervention as discussed for NGAL in the previous section. De Palma et al. reported that changing into a GFD, in itself, could cause a decrease in beneficial gut bacteria and impact the SCFA production (De Palma et al., 2009).

The relatively large individual variations in SFCAs may also hide a potential effect of the LFD. Also, the day-to-day variation in diet impacts the microbiota and the SFCAs produced (Havenaar, 2011). This might weaken the use of SCFA as a biomarker for gut health. Another concern is that 95% of the total SFCAs produced is absorbed by the colonocytes leaving only 5% for potential detection. Therefore the SFCAs measured in stool samples do not quantitively represent the total amount of SCFA produced in the intestine (Siddiqui et al., 2017). Such underestimation of the SCFA could therefore explain the poor effect on SCFA as a response to the LFD. Also, the absorption rates for the different SCFAs might vary disturbing the 'true' ratio between the SFCAs.

In the current project, the reduction in valeric acid was observed for those individuals in the LFD group that had high concentration of valeric acid at baseline. The other relevant studies that found effects of LFD on SFCAs did however not confirm the reduction in valeric acid (Halmos et al., 2015; Hustoft et al., 2017; Staudacher et al., 2012; Staudacher et al., 2016). A possible explanation for discrepancy between our study and the other trials could be because the individuals in the CD-FODMAP study have an initially higher capacity of producing valeric acid due to particular high capacity of the microbiota to produce this SCFA. Following similar argument, the individuals with lower concentrations at baseline therefore have less potential to lower their valeric acid levels by converting to an LFD. Although valeric acid is not a very well-studied SCFA, it is normally found at low concentrations and is associated with certain health benefits such as contributing to stimulation of epithelial growth (Onrust et al., 2018).

A reduction in propionic acid was also found after the LFD in the current study. Similar as for valeric acid the effect applied mostly for individuals with higher concentrations at baseline. A review by Sun et al supports the higher potential for changes in propionic acid among IBS patients having initially higher levels Sun et al. (2019). Noticeably the number of individuals representing the high baseline values for propionic acid in the LFD group were fewer than for valeric acid.

A reduction in valeric acid and propionic acid could therefore have potential consequences in disturbing the intestinal barrier, particularly if the adherence to an LFD lasts over time. However, as we did not find reduction in other SCFA this concern could be limited if these SCFAs sufficiently act as maintainers of the epithelial integrity (Parada Venegas et al., 2019).

The reduction in valeric and propionic acid is likely to be caused by microbial changes induced by the elimination of FODMAPs that usually supplies the producers of these SCFAs. It is possible that a lack of propionic- and valeric acid production could potentially prompt GI symptoms or inflammatory conditions if absent over a longer period. However, this remains to be investigated. The concern for an unbeneficial effect of an LFD on the microbiota has been a concern among dieticians applying the LFD strategy for GI problems. Therefore it is recommended that the food restricting period should be limited to avoid a negative long-term effect of the gut microbiota (Staudacher & Whelan, 2017).

To summarize, based on the modest changes in SCFAs in the current study, the changes induced by the LFD for 4 weeks seems not to have evoked particularly large negative effects. It could be, however, that the intervention period was too short to manifest the negative potential consequences of the LFD.

5.2.3 Relation between NGAL and SCFA

Because SCFAs function to maintain the gut integrity protecting against infections (Parada Venegas et al., 2019), a reduction in SCFA could be promoting inflammatory conditions. Since NGAL, as an inflammatory biomaker, would increase during inflammation (Thorsvik et al., 2017a), a negative correlation between the changes in these biomarkers would be expected. However no correlation was found. According to our knowledge, there exist no current literature considering the relationship between NGAL and SCFAs indicating the need for future studies investigating the relationship between those biomarkers. A lack of a negative correlation could also be due to the large individual variations observed for both types of biomarkers.

5.2.4 Relation between SCFA and GI symptoms

The absent relation between the majority of SCFA and GI symptoms is in line with the findings from a study by Valeur et al. (2016) reporting on a study with IBS patients. However, we observed a negative correlation between propionic acid and satiety. The correlation was however negligible, and we cannot explain the result in context with other studies as propionic acid is suggested to be central in the regulation of appetite hormones and highly related with satiety (Adam et al., 2016). Therefore, we would have expected a positive rather than a negative correlation between propionic acid and satiety. However since we found no correlation for the more problematic GI symptoms it is likely that the correlation with satiety is not so important in this context.

5.2.5 Method establishment and validation of Luminex using fecal NGAL

The Luminex-based method for detecting NGAL in fecal samples was successfully established but we found a relatively large mean difference between the Luminex-based method and the ELISA method where the Luminex-based method measured higher NGAL concentrations in average than the ELISA. The mean difference of 715 units between the methods indicates that the methods are not directly comparable. In fact, the scales for each method considering NGAL concentrations were barely overlapping. Other studies confirm the same higher average measurements with Luminex (Çetin et al., 2018) but to the best of our knowledge, we did not find any published work on multiplex-based detection of NGAL in feces for the comparison to that of ELISA. A PubMed search, date 04.06.2021, using the following combination of keywords: 'Luminex AND ELISA AND NGAL' revealed 7 hits that were not relevant after examining the abstract and/or full paper.

However, the high correlation between the methods indicates that the methods are comparable when used to rank the samples depending on whether they have a high or low concentration value. With the intention to measure the same analyte in the same matrix with similar conditions, such as reagents, high correlations are also expected (Elshal & McCoy, 2006). As correlation does not provide information about any agreement between the methods and only states whether they relate or not, it is therefore often misused as a comparison tool (Giavarina, 2015).

It is important to note that a high correlation between the methods does not necessarily imply that the agreement is good. We therefore investigated the agreement between the methods using the Bland-Altman method and found a significant correlation between the difference and magnitude of the measurements, referred to as a proportional bias. The difference between the methods increased more with higher concentrations. If the Luminex-based method had shown systematically higher values across all concentrations it could have been possible to use a converting factor to compare the results from the two assays directly. However, with a present proportional bias, such as identified in the current project, it is not possible to convert the values using a simple factor.

When inspecting the BA plot, the ELISA method seems to deviate more from the Luminex-based method with higher values. In other words, the Luminex-based method enables the measurement of NGAL in feces matrix that is out of the upper threshold for the ELISA method A possible

explanation for an underestimation using the ELISA method could be associated with an inhibition matrix effect applying more to ELISA than the Luminex-based method. Also, many of the samples measured above the Luminex-based method's detection limit were not detected by the ELISA method. Taken together, the results suggest a higher sensitivity for the Luminex based technology for detecting NGAL in feces. It is possible that the bead technology is better in terms of avoiding potential inhibitory matrix effects enabling to detect analytes at higher concentrations which is reasonable as the different methods use different fluidics and optics for the NGAL detection.

Another possible explanation for the lower detected concentrations by ELISA assay may be related to the enzyme amplification step, which might cause the results not always to be linear (Baker et al., 2012). On the other hand, Luminex uses fluorescence as a reporting system, and it is more likely that this system provides a larger degree of linearity than the ELISA assay based on colorimetric substrates that are changed by enzymes. Another difference in the procedures that might explain some of the difference between the methods is the use of a blocking agent (BSA) in the Luminex-based procedure (Luminex Corporation, 2018). This might have impacted the assay as the blocking agents in this serum containing buffer might have reduced non-specific bindings of extraneous proteins in the feces matrix, possibly increasing the binding specificity. Whether this solely can explain the dramatic difference of the NGAL quantification in the methods is unlikely as the PBS 1% BSA was used during other steps in the ELISA procedure.

Although the Luminex-based method seems to detect NGAL in the sample successfully, it cannot be directly compared to the results obtained by ELISA. Also, the Luminex technology is associated with problems regarding cross-reactivities that must be considered if the newly developed anti-NGAL-coated beads should be used as part of a multi-plex assay design along with beads coated with antibodies against other analytes (Elshal & McCoy, 2006). This type of cross-reactivity might include that the antibodies react to other analytes, other cross-species antibodies (Kellar & Douglass, 2003), or act as interfering substances referred to as the ‘matrix effect’ (Ryska, 2015).

The differences in detected NGAL concentrations by the two methods can either be interpreted as an overestimation of NGAL values by the Luminex-based method or that the ELISA method is underestimating the values since we do not know the ‘true’ NGAL values in the sample. When methods are validated, it is common to use a method that produces what is recognized as a ‘true’ measure and used as a ‘golden standard’. When such a golden standard does not exist, Bland and

Altman recommend using the mean of the two methods to represent the closest estimate of the 'true values' (Bland & Altman, 2010). An alternative approach would be to assume that the ELISA represents the golden standard and use this instead in the BA plots (Krouwer, 2008). As an alternative approach, we used the ELISA method as a reference for the agreement analysis and again, it was still found a clear proportional bias. Therefore, in this case, there was found a relationship between the differences and magnitude independent of whether the reference approach was used, indicating that the Luminex-based method was measuring higher values. However, this approach is questionable as using the gold standard might be misleading since it will always relate the difference of the methods to the magnitude of the concentrations also measured in cases when there is none (Bland & Altman, 1995).

Although the Luminex-based method was successfully established for NGAL detection in fecal samples, further optimization of the method might be needed. Also, an experiment should be performed to test the reproducibility of the method.

6 CONCLUSION

The current study investigated effects of a LFD intervention on fecal biomarkers in CD patients with GI problems, which had previously been shown to relieve their GI symptoms after 4 weeks on the intervention.

No significant intervention effect was found for the inflammation associated biomarker NGAL, implying that the reduced GI problems were not reflected by changes in NGAL. This result might indicate that the GI symptoms in the current CD population is not related to inflammation but may be caused by other FODMAP associated factors such as osmotic effects followed by mechanic stress. The effect of the LFD intervention was also investigated on the fecal concentrations of SCFAs. For the majority of SCFAs no effects were found but a reducing effect on propionic acid and valeric acid for those with initially high levels at baseline indicate a possible impact of microbial changes which may affect the amounts of intestinal gasses produced and thereby reduced GI problems. However, the effects of the LFD on the microbiota composition remains to be investigated.

As part of the current study a Luminex-based single-plex for the measurement of NGAL in feces was successfully established. The single-plex could potentially be used as part of a multiplex-panel to measure NGAL in concert with other relevant fecal biomarkers. However, further experiments must be performed for a complete validation of the method and test the reproducibility as well as test for cross-reactivity when used as part of a multiplex-panel.

7 FUTURE PERSPECTIVES

Although we did not find effects of the LFD on NGAL in the current CD population further studies should explore the usefulness of NGAL as a biomarker of intestinal inflammation across disease severity. It is likely that newly diagnosed CD patients have a higher NGAL level before complying to a GFD. As a highly sensitive inflammation biomarker NGAL could possibly supplement the currently used TG2 providing further information about the inflammation status and be included as an indicator of gluten adherence. In particular it would be interesting to measure the response in NGAL when gluten is re-introduced during the diagnostic examination phases of CD.

The finding that the LFD impacted some SCFA might indicate effects on the microbiota. We are currently profiling the microbiota in the fecal samples from the CD-FODMAP study using 16S rRNA sequencing analyses to identify the bacteria and their relative abundances. Hopefully, this will increase our knowledge on how the microbiota composition relates to GI symptoms, inflammation and how FODMAP intake affects this interaction. Also, it might be possible to identify microbial signatures that can predict the GI responses to FODMAPs. In addition to characterizing the bacterial content in feces there are also other microorganisms that might be important for the FODMAP-GI connection, such as fungal species.

It is important to continue the search for relevant fecal biomarkers as indicators of gut health conditions. The development of a sensitive Luminex-based method to detect NGAL could be used as part of a multiplex panel enabling detection of multiple fecal biomarkers in the same sample. Such a panel could be useful for diagnostics and clinical research in the future. This would require establishment of Luminex-beads coupled with the other biomarker specific antibodies. Prior to introducing and accepting a new method used in analytical contexts such as for diagnostic purposes, validation parameters characterizing the quality of the measurement such as precision, robustness and reproducibility must be highly evaluated. as the ELISA method is suspected to lack sensitivity, Finally, it would be interesting and relevant to compare the sensitivity and specificity between Luminex- and ELISA- based methods for diagnostic purposes as the current study found the proportional bias when examining the agreement between the methods.

Technical issues with analyte measurement using fecal samples must be addressed. There are challenges with the fecal matrix which urge for improvement. Methodological optimizations such as correcting for wet mass or determination of total protein and normalizing of the values might offer some solution to the problem.

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ATTACHMENTS

Attachment A – Materials

Table A.1: List over materials used for NGAL measurement by ELISA and Luminex

Product name	Prod. No.	Supplier
ELISA		
Human lipocalin-2/NGAL Duoset ELISA:	DY1757	R & D systems Europe, Abingdon, UK
Capture Antibody (Rat Anti-Human Lipocalin-2)	*844864*	
Detection Antibody (Biotinylated Goat Anti-Human Lipocalin-2)	*844865*	
Standard (Recombinat Human Lipocalin-2)	*842273*	
Streptavidin-HRP B	*893975*	
Substrare Reagent Pack	DY999	R & D systems Europe, Abingdon, UK
Stop Solution 2N Sulfuric Acid	DY994	R & D systems Europe, Abingdon, UK
Reagent Diluent Concentrate 2	DY995	R & D systems Europe, Abingdon, UK
96-well EIA/RIA Plate	3590	Corning, USA
ELISA Plate Sealers	DY992	R & D systems Europe, Abingdon, UK
Luminex		
xMAP® Antibody Coupling Kit	40-50016	Luminex Corporation, Austin, USA
EDC Reagent	*11-40144 *(ThermoSci #77149)*	
Sulfo-NHS	*11-25169*	
Activation Buffer (100 mM NaH_2PO_4, pH 6.2)	*11-25171*	
Wash buffer	*11-25167*	
1.5 mL tubes	*11-00277*	
Disposable pipettes	*11-00321*	
MagPlex® Microsphere (Region 61)	MC10061-YY	Luminex Corporation, Austin, USA

Standard (Recombinat Human Lipocalin-2) from Human lipocalin-2/NGAL Duoset ELISA #DY1757	842273	R & D systems Europe, Abingdon, UK
Capture antibody (Human Lipocalin2/NGAL Antibody)	MAB17571R100	R & D systems Europe, Abingdon, UK
Detection antibody (Human Lipocalin-2/NGAL Biotinylated Antibody)	BAF1757	R & D systems Europe, Abingdon, UK
Detection antibody (PE anti-rat IgG2b Antibody)	408213	BioLegend Way, San Diego, USA
Bovine Serum Albumin (35 % BSA in DPBS)	A7979	Sigma-Aldrich, Oslo, Norway
Bio-Plex® Handheld Magnetic Washer	171020100	Bio-Rad Laboratories, USA
HydroFlex™ Insert Plate Carrier Smart2 MBS 96	30054611 04	TECAN, Austria
Nunc™ F96 MicroWell™ Black Polystyrene Plate	236105	NUNC
Bio-Plex Calibration Kit	171203060	Bio-Rad Laboratories, USA
Bio-Plex Validation Kit	171203001	Bio-Rad Laboratories, USA
Programmable rotator	Multi RS-60	BioSan
Automated Cell/Bead counter	Countess II	Life Technologies, USA
Glasstic™ Slide 10 with haemocytometer type-grid	87144	KOVA® International, USA
DYNAL® Bead Separator 16 position rack	Unknown	Invitrogen™
Materials applying for ELISA and Luminex		
Tween®20	P1379	Sigma-Aldrich, Oslo, Norway
Delbecco's Phosphate Buffered Saline (PBS)	L0615-1000	Biowest, France
Sodium phosphate dibasic dodecahydrate	71650	Sigma-Aldrich, Oslo, Norway
Sodium phosphate monobasic monohydrate	S9638	Sigma-Aldrich, Oslo, Norway
Sodium chloride	106404	Merck KGaA, Germany
Adapter for 5 reaction vials 1.5 and 2.0 ml	22.008.0005	Retsch, Germany
96-well PCR plate	72.1979.102	SARSTEDT AG & Co. KG, Germany
Adhesive PCR Seal	95.1993	SARSTEDT AG & Co. KG, Germany

*The EDC reagent is a Thermo Scientific® product and is manufactured for Luminex® for use in this kit.

Attachment B – Instruments

Table B: List over instruments used for NGAL measurement by ELISA and Luminex

Instrument	Modell	Supplier
Mixer mill	MM2	Retsch, Germany
Microplate washer	HydroSpeed™	TECAN, Austria
Plate reader SpectraMax	M2	Molecular Devices
Bio-Plex® 200 System With HTF	171000205	Bio-Rad Laboratories, USA
Ultrasonic cleaner	BRANSONIC® 200	VWR International

Attachment C – Software

Tabell C.1: Overwiev of softwares used for analysing NGAL with ELISa and Luminex

Software	Reference
Softmax Pro 6.5	Molecular Devices (2015). *Softmax Pro* (Version 6.5). Software. Accessible from: https://softmax-pro.software.informer.com/6.5/ (read: 08.06.2021)
R	The R Foundation for Statistical Computing (2020). *R* (Version 4.0.2). Software. Accessible from: https://www.r-project.org/ (read: 08.06.2021)
RStudio	RStudio Inc. (2009-2020). *RStudio* (Version 1.3.1093). Accessible from: https://www.rstudio.com/products/rstudio/older-versions/ (read: 08.06.2021)
Bio-Plex Manager™ Software	Bio-Plex Manager Software *(version 6.2)*. Software. Accessible from: https://www.bio-rad.com/en-no/product/bio-plex-manager-software-standard-edition?ID=5846e84e-03a7-4599-a8ae-7ba5dd2c7684 (read: 08.06.2021)

Attachment D – 4-PL standard curve for NGAL ELISA

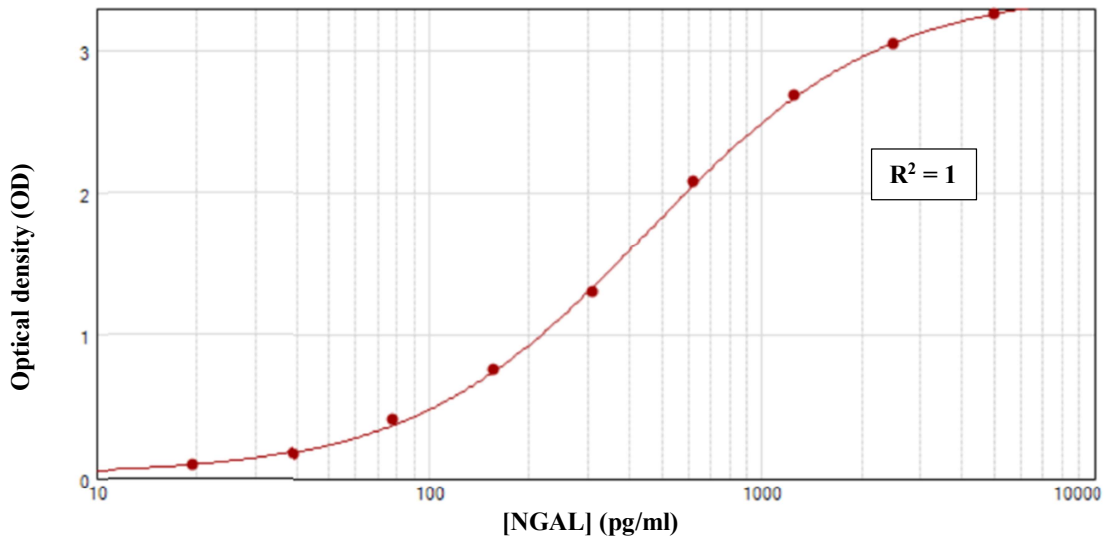

Figure D.1: 4-parameter logistic (4-PL) curve fit for NGAL ELISA prepared using the software Softmax pro 6.5. Measured NGAL concentration [NGAL] (pg/mL) is plotted against optical density (OD). R-square (R2) = 1.

Attachment E – Information, approval and questionnaires regarding the CD-FODMAP study

The following attachments include:

Attachment E1 – Approval from REK

Attachment E2 – Protocol for REK

Attachment E3 – Statement of consent

Attachment E4 – Collaboration agreement

Attachment E5 – Questionnaire regarding the GSRS-IBS score

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst	Silje Hansen	22845514	09.01.2019	2018/1055 REK sør-øst B
			Deres dato:	**Deres referanse:**
			27.09.2018	

Vår referanse må oppgis ved alle henvendelser

Knut E. A. Lundin
Oslo universitetssykehus HF

2018/1055 Effekt av FODMAP reduksjon på symptomer hos cøliakipasienter på glutenfri kost

Forskningsansvarlig: Oslo universitetssykehus HF
Prosjektleder: Knut E. A. Lundin

Vi viser til søknad om prosjektendring datert 27.09.2018 for ovennevnte forskningsprosjekt. Søknaden er behandlet av leder for REK sør-øst B på fullmakt, med hjemmel i helseforskningsloven § 11.

Endringene innebærer:
1. Lagt inn et egen screening besøk.
2. Presiserer at studien vil bli publisert men at deltagerne ikke kan identifiseres i publikasjonene.
3. Vil gi deltagerne et gavekort på kr 500 som takk for deltakelse i studien.

Prosjektleder skriver:
1. Et eget screening besøk før man gjør endoskopi sikrer at vi ikke gjør unødige undersøkelser. Ved screeningbesøket får studiedeltakerne ytterligere informasjon om studien og hva som kommer til å foregå på de 4 ulike oppmøtene. Samtykke innhentes. Det gjøres en legeundersøkelse med blodtrykk, puls, lytte på hjerte og lunge og kjenne på magen. Deretter utføres en 24-timers recal for å kartlegge kostholdet, og til slutt tas en blodprøve med orienterende prøver, vitamin- og mineralstatus og cøliaki-serologi. Serologi med anti-transglutaminase IgA skal være innenfor referanseområde for å starte intervensjonen.

2. Setningen om publisering er tatt med etter generelle innspill fra personvernombudet på OUS.

3. Vi informerer studiedeltakerne om at de kan søke om reiserefusjon gjennom Pasientreiser. Deltakerne får dekket reiseutgifter etter gjeldende regler for Helse Sør-Øst. Gavekortet på 500,- gis som en takk til deltakelse i studien.

Vurdering
Leder i REK sør-øst komité B har vurdert de omsøkte endringene, og har ingen forskningsetiske innvendinger til endringene slik de er beskrevet i skjema for prosjektendring.

Vedtak
REK har gjort en forskningsetisk vurdering av endringene i prosjektet, og godkjenner prosjektet slik det nå foreligger, jf. helseforskningsloven § 11.

Vi gjør samtidig oppmerksom på at etter ny personopplysningslov må det også foreligge et behandlingsgrunnlag etter personvernforordningen. Det må forankres i egen institusjon.

Tillatelsen er gitt under forutsetning av at prosjektendringen gjennomføres slik det er beskrevet i

prosjektendringsmeldingen og endringsprotokoll, og de bestemmelser som følger av helseforskningsloven med forskrifter.

Klageadgang
REKs vedtak kan påklages, jf. forvaltningslovens § 28 flg. Eventuell klage sendes til REK sør-øst B. Klagefristen er tre uker fra mottak av dette brevet. Dersom vedtaket opprettholdes av REK sør-øst B, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering, jf. forskningsetikkloven § 10 og helseforskningsloven § 10.

Vi ber om at alle henvendelser sendes inn med korrekt skjema via vår saksportal: http://helseforskning.etikkom.no. Dersom det ikke finnes passende skjema kan henvendelsen rettes på e-post til: post@helseforskning.etikkom.no.

Vennligst oppgi vårt referansenummer i korrespondansen.

Med vennlig hilsen

Knut W. Ruyter
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REK sør-øst

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1. Project title

Effect of FODMAP restriction on persistent GI-symptoms in coeliac patients

2. Introduction

The project targets a major disease group in Norway where there is an unmet and unresolved clinical need. The project is very patient-oriented and will contribute to clinical tools in management of coeliac disease. The research will immediately benefit the patient group.

Coeliac disease is a common condition, which affects at least 1-2 % of the population and the prevalence is increasing [2]. Many coeliacs worldwide are undiagnosed. This also applies in Norway, where Norwegian Coeliac Disease Association (NCF) has ca. 10 000 members, while it is expected that at least 50-100 000 people in the Norwegian population have the disease. There is no good disease registry on how many people have been diagnosed. Coeliac disease is characterised by small intestine is damage by an inflammatory process in the mucosa [2, 3]. This inflammation triggered by wheat gluten and similar proteins of rye and barley. Detection of tottering changes in tissue samples taken with gastroscopy, blood tests examining serum antibodies to the enzyme Transglutaminase 2 (TG2) or to Deamidated gliadin peptides (DGP) is necessary to detect the disease. It is important that these tests are taken while the patient eats gluten, because they will normalize on a gluten free diet. Follow-up of the patients is either done by serology or by serology and biopsy. Mucosal recovery is a treatment goal, persistent inflammation predisposes to malignant disease [4]. Serology is less sensitive than biopsy in this matter.

The symptoms of coeliac disease are only partially explained by the small bowel injury [2]. Micronutrient deficiency such as iron, folic acid and vitamin D as well as osteoporosis is common at the time of diagnosis. These deficiencies are not obligate nor typically for coeliac disease as they also exist in other diseases. Discomfort from stomach with flatulence, bloating, diarrhoea or constipation is very common in the disease but does not affect everyone. A feeling of constant tiredness and difficulty concentrating called "foggy brain" is also common. In addition to these characters is a plethora of associated diseases such as hypothyroidism, diabetes, infertility, arthritis and skin lesions. Patients with coeliac disease have increased mortality because of a certain cancer hazard. They are prone to lymphomas and adenocarcinomas, but the risk is reduced with treatment by gluten free diet [2].

2.1 Needs description

Treatment of coeliac disease

The treatment of coeliac disease is today a lifelong gluten free diet, although alternative treatment options are being developed [5]. A gluten free diet means a diet devoid of wheat, rye, barley and closely related cereals. This treatment provides in most cases good results. Monitoring and teaching by a clinical dietician are particularly important for coeliacs, and this practice group is invaluable. However, patients are dependent of gastroenterologists` and GP`s awareness of the disease, and there is a continuous effort to improve this knowledge.

Although treatment with gluten free diet usually gives good results, there are many patients who have continued ailments from their stomach or with their general health after they have started a diet [6-10]. This could be due to either failure to adapt to a really strict diet (compliance problems due to lack of knowledge or lack of motivation), or it could be symptoms compatible with, and caused by factors giving, what is referred to as “irritable bowel syndrome” (IBS). Data from US suggest this to affect 10-20 % of coeliac patients [8], whereas a recent Finnish study found that 23 % of long-term well-treated coeliac patients still had persistent gastrointestinal symptoms [10].

There have been no studies on persistent gastro-intestinal symptoms in celiac patients from Norway, but with our broad clinical experience from the patient group, we believe that the numbers

are at least at this level in Norway. We specifically aim at investigating the impact of FODMAPon this frequent condition with persistent symptoms in coeliac disease patients on a gluten free diet.

The FODMAP concept

The term is short for Fermentable Oligo-, Di-, Monosaccharides And Polyols (FODMAP); dietary carbohydrates and other substances that are relatively resistant to intestinal digestion and absorbtion and as a result they pass on to the large bowel where they meet a rich bacterial flora able to ferment these substances. FODMAP rich food includes certain cereals, fruits, vegetables and legumes, see figure 1.

Figure 1. Examples of foods rich in FODMAP. Photo: Øistein Horgmo

As a consequence gas may be produced and water-flux to the bowel is increased. This is a physiological process [11]. In patients with IBS this physiological effect is coupled to visceral hypersensitivity leading to patient discomfort that in many cases can be very substantial [12, 13], as illustrated in Figure 2

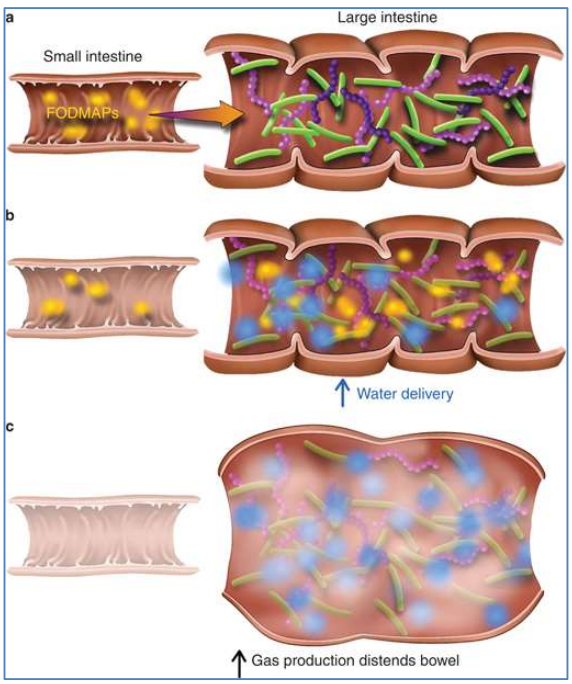

Figure 2. FODMAPs are poorly absorbed in the small intestine and can cause gas production and abdominal symptoms (with permission from [1])

Thus, FODMAP restriction has been proposed as a treatment for IBS and has been documented to be efficient in the majority of patients [14-17] In a recent study from Sweden, the effect of FODMAP reduction in IBS patients was seen, but not superior to alternative, standard treatment [18]. In clinical practice, FODMAP restriction is performed under the guidance of a clinical dietician. The first step is usually to record the patient's diet, exclude all food items with high FODMAP content from diet, followed by a structured re-introduction again under close guidance of a clinical dietician. FODMAP restriction done by patients themselves after written or online guidance lacks scientific support. FODMAP restriction has been included in the most recent NICE guidelines (NICE guideline CG61Irritable bowel syndrome in adults www.nice.org.uk).

A low FODMAP diet may also have impact on faecal microbiota. A recent study in IBS patients indicates that the responsiveness to a low FODMAP diet may be predicted by the microbiota profiles[19].

Although FODMAP reduction may play a role in patients with diet treated coeliac disease, there are no published studies addressing this topic. In fact, there is a huge knowledge gap and unmet clinical need for studying FODMAP in other patient populations than those suffering from IBS.

3. Hypotheses, aims and objectives

The overall aim is to improve dietary treatment of coeliac patients with persistent gastrointestinal symptoms in spite of a strict gluten free diet.

The project has two main objectives:

A) To investigate the prevalence of persistent gastrointestinal symptoms and assess the amount of FODMAP in the diet of coeliacs on a strict gluten free diet. This will be done with a web-based quest back tool in collaboration with the Norwegian Coeliac Association.

B) To perform a randomized, controlled trial in coeliac disease patients with gastrointestinal symptoms, comparing a low FODMAP glutenfree approach to an ordinary gluten free diet. This part of the project will also validate questionnaires and biomarkers for the assessment of gluten-free diet adherence.

In addition, the present project will provide unique biological materials for biobank and ongoing projects in our coeliac group. This is basic and translational research and is beyond the scope of this application.

The target audience is adult, Norwegian patients who have been diagnosed with coeliac disease, but despite strict gluten free diet experience considerable gastrointestinal symptoms.

The project is aimed directly at patients being treated in our public health system. The project is closely related to activities at OUH Rikshospitalet and the University of Oslo (Centre for Immune Regulation and KG Jebsen Centre for Coeliac Disease Research). It is appropriate to cooperate with other hospitals in the South-East Region for patient recruitment. Norwegian Coeliac Disease Association (NCF) will also be an important partner through their newsletter, website and Facebook group. We have recruited patients in other studies in this way so that we know this is a good approach [20-25].

4. Project methodology

4.1. Project design, method selection and analyses

Study A: Prevalence of persistent GI-symptoms in coeliac disease

Setting

We have a rather unique situation in Norway as the majority of patients with coeliac disease are or have been members of the NCF, and communication electronically and on social media like Facebook is widespread. This will give us the opportunity to perform an online survey as, in fact, has been done in Canada [26]. However, in the mentioned study they did not record on going symptoms. Persistent symptoms has more recently been evaluated in a Finnish study using direct patient contact and written formulas [10]. We propose to use a web-based technology to interact with as many coeliac disease patients as possible.

Design
A national cross-sectional study exploring prevalence of persistent symptoms in treated coeliac patients.

Patients
Members of NCF will receive e-mail invitation to participate in the online survey, as well open invitations in relevant social media that target the coeliac disease population. This method gives the opportunity to include a large sample, and since we aim to obtain the number of participants needed for study B, the number of participants in this part of the project will be unlimited within the following inclusion criteria:

- Biopsy verified coeliac disease patient, 18-75 years of age
- Strict gluten free diet for at least 12 months

NCF has approximately 10 000 members. We expect a 30 % response rate, and aim to include 3000 patients in this study.

Methods
We will use web-based questionnaires to survey gastrointestinal symptoms, health related quality of life and diet adherence by questionnaire.

For recording of gastrointestinal symptoms we will use a published and validated questionnaire that was originally developed for IBS purposes, the Gastrointestinal Symptom Rating Scale, IBS version (GSRS-IBS) [27]. We, and others, have found that this form is very well suited to evaluate the burden of disease and symptom level for IBS-like symptoms in treated coeliac disease, and it is suitable for measuring the effect of interventions [28]. The questionnaire is translated to Norwegian. FODMAP intake will be estimated by use of a questionnaire developed by Hatlebakk & co-workers [29].

In the current project we will employ recently developed disease specific quality of life forms. First, the Coeliac Disease Questionnaire (CDQ), a disease specific health related quality of life measure for adults with coeliac disease [30]. The form is translated into Norwegian, but has not been tested. Second, the Coeliac Symptom Index (CSI) was published by our collaborator in US, professor Dan Leffler at Harvard University, Boston [31]. This questionnaire is already translated and approved by the Harvard group. The project will include testing and validation of these disease specific tools for coeliac disease. Other relevant questions may add to the survey by NCF.

This study will also enable us to compare the collected information between CD patients with and without persistent GI-symptoms. Those patients who score high on the reported outcomes, will, as part of the survey, be invited to participate in study B.

Study B. A randomized controlled trial in patients with persistent GI-symptoms

Design
We will follow recommendations for design of clinical trials evaluating dietary interventions in patients with persistent functional gastrointestinal disorders [32]. A cross-over design has been carefully considered but is not possible as patients cannot be de-educated. A flow-chart of the study is given in Figure 3.

Patients
CD patients selected from study A, and willing to continue to study B

Inclusion criteria
- Coeliac patients (18-75 years) treated with GFD for at least 12 months
- Normal CD serology and duodenal biopsy (Marsh 0-1)
- Persistent gastrointestinal symptoms defined by GSRS-IBS score of 30 or more
- Strictly adherent to GFD
- Living less than 2 hours from study centre

Intervention
- Both groups will follow strict gluten free diet (GFD). The intervention group will in addition receive instructions on how to follow a FODMAP diet (LFD).

Endpoint
- Primary endpoint will be change in gastrointestinal symptoms measured by GSRS-IBS score.
- Secondary endpoints will be changes in biomarkers like serology and faecal microbiota

Adherence
Adherence to the diets will be monitored halfway in both groups. Assessment of dietary FODMAP and gluten intake will be obtained by 24-hour recall, which is a validated method for nutritional assessment in nutrition research [32]. Nutritional calculations will be done by the software Dietist Pro, which is implemented and used by dieticians at Oslo University Hospital, as well as in the other Nordic countries (www.kostdata.se/nb/dietist-net/dietist-net-pro).

Differentiated FODMAP calculations will be done by the software FoodWorks by our collaborators at the Monash University, Melbourne. To date, they are the only site that has a reliable FODMAP database based on food analysis.

Dietary assessment will also include evaluation of dietary adherence to the gluten-free diet by standardized dietician interview and by the coeliac disease adherence test (CDAT) [34]. There are few objective measures for diet adherence and the Norwegian translated CDAT version is not tested or implemented in Norwegian practice.

We will validate the CDAT questionnaire and recently suggested blood based tests for mucosal damage, the S-Intestinal Fatty Acid Binding Protein (S-IFABP) [35] and the faecal 33-mer peptide as a measure for gluten contamination [36]. Both these novel tests will be implemented at our clinical unit during 2018.

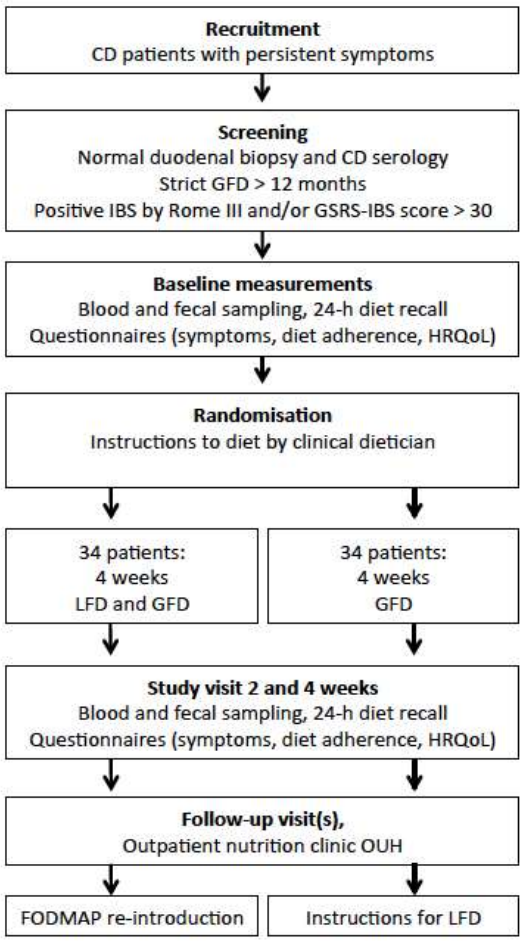

Figure 3 Flow-chart of study B.
LFD: Low FODMAP diet. GFD: Gluten free diet

All partisipants will be offered follow-up visits for re-introduction of FODMAPS (intervention group) or instructions for LFD (control group).

Power calculation:
The primary endpoint for measuring effect of the low FODMAP diet will be GSRS-IBS total symptom score. From previous studies with coeliac disease patients and non-coeliac gluten sensitivity persons we know that a clinical significant difference in symptom score is present when group means are 22,5 (SD 8) and 29,5 (SD 11), respectively (based on our unpublished results from on going clinical trial). With a power of 80 % and a significance level of 0,05 we will need 31 patients in each group. To account for 15 % drop out, we aim to include 72 individuals.

4.2. Participants, organization and collaborations

Project leader and main supervisor

Project leader, professor, dr. med. **Knut E. A. Lundin** is head of clinical education of medical students at Institute of Clinical Medicine, Faculty of Medicine, University of Oslo. He has a 20 % position as Consultant Gastroenterologist at Section for gastroenterology, Department of Transplantation Medicine, Oslo University Hospital. He has previously received funding from the Norwegian Research Council, the Extra Foundation as well as from Helse Sør-Øst. He has for the last 9 years been a senior faculty member of the Centre for Immune Regulation (CIR), led by

Professor Ludvig M. Sollid. CIR is a Centre of Excellence of the Norwegian Research Council, and is a FOCIS Center of Excellence. He is since 2016 group leader in KG Jebsen Centre for Coeliac Disease Research at the University of Oslo and Oslo University Hospital.

Lundin has published approximately 210 scientific papers including 120 original articles and 52 reviews and book chapters. His H-index is 50 and has 11,258 citations. He is main supervisor of Gry Skodje together with associate professor Christine Henriksen and Professor Marit Veierød. They have together supervised two clinical dietician students, who have completed the MSc thesis.

PhD student
We have received PhD grant for clinical dietician **Frida van Megen**. A few years ago, she finished her master degree in clinical nutrition: ”Sammenheng mellom inntak av FODMAPs og symptomer hos pasienter med inflammatorisk tarmsykdom i remisjonsfase som har irritabel tarm” with grade A. She experience with several of the methods to be used in the present study.

Co-workers
Associate professor and co-supervisor, PhD, MSc **Christine Henriksen** is a clinical dietician at Department of Nutrition, Institute of Basic Medical Science, University of Oslo. She has clinical experience and research experience with the patient group. She has published more than 20 scientific papers and is Editor in Norwegian Journal of Nutrition (**www.ntfe.no**). She has has an H-index of 11, has supervised 15 master students and is currently supervising two PhD students.

Clinical dietician **Gry Irene Skodje** has an MSc degree as a clinical dietician and is now employee at Unit for Clinical Nutrition at OUH Rikshospitalet. She has extensive clinical experience with patient group, she has both her own research and counselling experience on master level. She has been an Extra foundation PhD student and will complete her PhD degree during 2018.

Marit Veierød is Professor in Medical statistics at the Oslo Centre for Biostatistics and Epidemiology, Department of Biostatistics, Institute of Basic Medical Sciences, University of Oslo. She is also affiliated to the Department of Nutrition Research at the same institute. She has extensive supervising experience and is involved in numerous projects of clinical research. Lundin, Henriksen and Veirød have together supervised Skodje in her PhD project.

Jessica Biesiekierski is a current Post-doctoral Research Fellow with the Translational Research in Gastrointestinal Disorders (TARGID) group of KU Leuven in Belgium. In her PhD thesis from Monash University, Melbourne, Australia she investigated the effects of gluten and dietary carbohydrates in individuals who do not have coeliac disease. She has expertise in nutritional sciences, especially on large, randomised, double-blind placebo-controlled human dietary trials. She will help with the interpretation of the symptom assessment and FODMAP intake.

Eric de Muinck is a Post-doctoral Research Fellow with the Center for Ecological and Evolutionary Synthesis (CEES), a Norwegian Centre of of Excellence at the Department of Biosciences, University of Oslo. He has extensive experience in characterizing gut microbiotas and gut microbial ecology including cutting edge sequencing techniques (ref). The project leader has already co-authored paper with him[37].

The project will be done in collaboration with the Department of Gastroenterology at Haukeland University Hospital. There, Professor **Jan Gunnar Hatlebakk** is running an outpatient coeliac disease clinic where they also aim at investigating FODMAP restriction in coeliac disease patients. He will bring into the project his methods for assessing FODMAP intake by a short questionnaire.

In this project, we work closely with a research group at Monash University, Melbourne, Australia, with whom we already have published [38, 39]. This group is led by Professor **Peter Gibson** (clinical gastroenterologist) and Professor **Jane Muir** (clinical dietician). This group has pioneered the FODMAP concept, and will be responsible for the analyses of FODMAP intake from the diet.

We are at the moment running a large, collaborative project with this group, where we investigate the effect of gluten versus FODMAP challenge in individuals with self-perceived gluten sensitivity.

General secretary **Knut H. Peterson** in NCF will participate as the represent from the user group. He will be responsible for pre-testing the online questionnaire and patient support group contact.

Supportive infrastructure

The project will be run with infrastructure support already available within the project group. This includes

- Access to an endoscopy unit where the project leader has a 20 % position as a clinical gastroenterologist. The unit has a tradition for participating in studies like this for decades.
- Access to research engineers located at the endoscopy unit. These two engineers both have MSc degrees. They perform partly routine analysis, partly biobank tasks.
- Access to a study nurse located at the endoscopy unit. This nurse receives 1/3 of her salary from the Jebsen Centre where the principal investigator is group leader. She will aid in arranging patient appointments and in handling informed consent forms.
- Access to the locations at the Clinical dietician unit, Oslo University Hospital, Rikshospitalet for study visits. We will recruit at least two master students in Clinical nutrition.

KG Jebsen Centre for Coeliac Disease Research

The project will be performed within the recently started KG Jebsen Centre for Coeliac Disease Research (Centre director professor Ludvig M. Sollid, Professor Knut Lundin is one of five group leaders). This Centre was initiated in august 2016 and will be active till august 2020. Thus, it will cover the whole period for the current application. The Centre is located within the location of Institute of Immunology at OUH Rikshospitalet. The Centre has as its focus to study the immunobiology of coeliac disease, to improve diagnosis of coeliac disease and to investigate possibilities for non-dietary medical treatment options for coeliac disease. The current project not only fits very well within the clinical arm of the KG Jebsen Centre for Coeliac Disease Research but will also provide invaluable clinical material for the Centre's immunobiological activities. All clinical material in the current project will, with the informed consent of the participating patients, be deposited in the General biobank "Tarmsykdommer" where Lundin is the responsible clinician.

Clinical Dietician Unit at OUS

The project relies completely on access to an operable clinical dietician unit, the outpatient clinic (Ernæringspoliklinikken) at OUH Rikshospitalet. The study group is closely related to that clinic. One of the supervisors, Gry Skodje, has done the bulk of her PhD thesis work there. The unit is ideally situated at the Gaustad Campus; it is very close to the Jebsen Centre and the Endoscopy unit at the Department of Gastroenterology. Thus, all three units are located under the same roof.

4.3. Budget

The study will be done completely within the public health system and thus benefit from well-developed infrastructure. There will be no internal billing of hospital services as gastroscopy, blood tests or clinical consultations. The cost for Study Nurse is already covered by other sources (KG Jebsen Centre for Coeliac Disease Research). We here apply for the cost to a PhD student.

4.4 Plan for activities, visibility and dissemination

The time until the start of the project 01.06.2018 will be used for the completion of protocols and obtaining the necessary ethical approvals. The project is closely related to several of the projects we already have running in our group. Subproject with gluten provocation of treated coeliacs part of a South-East supported PhD project where PhD candidate will examine immune biological mechanisms of mucosal reaction to gluten. Since protocols are adjacent to each other, it is expected that all necessary approvals will be in place early. The following plan is submitted:

2018

- Completion of clinical report forms (CRF) for the two sub-studies
- Online Survey
- Planning and designing of the studies, application for REK, pilot testing, advertising for study participants
- Recruitment and clinical assessment of treated coeliacs with persistent symptoms
- Researcher training in the PhD education program

2019

- Recruitment, study conduct
- Analysis and writing subproject A
- Treatment and follow-up of patients in subproject B (expected to take the entire year)
- Researcher training in the doctoral program

2020

- Completion of subproject B
- Analysis of results and paper writing
- Writing frame report to the PhD degree, disputation

Suggested titles of the papers:

1. Prevalence of persistent gastrointestinal symptoms and FODMAP intake in patients with coeliac disease
2. Effect of FODMAP reduction in coeliac disease patients with gastrointestinal symptoms, a randomized, controlled trial
3. Validation of questionnaires and biomarkers for the assessment of gluten-free diet adherence

4.5. Plan for implementation

The findings in the study are expected to have immediate clinical impact that can be rapidly transformed to improve care of this large patient group. The project leader and the co-workers/supervisors (Skodje and Henriksen) all have active contact with local and national patient support groups. Lundin frequently holds talks at patient meetings, is a teacher at the Medical faculty at University of Oslo, and gives talks to clinicians. Skodje is member of the Scientific advisory board of the Norwegian Coeliac Disease Association. Henriksen is current editor of the Journal of the Norwegian Association of Clinical Dieticians. We will further communicate our findings in the Journal of the Norwegian Coeliac Disease Association ("Glutenfri").

5. User involvement

Participation of patients is of paramount importance for success in this project. As mentioned under chapter 4.5 we have broad contact with the patient group. The project will be done in close understanding and follow-up from representatives from the Norwegian Coeliac Disease Association. We have already established a Patient Advisory Board as part of our Jebsen Centre. We will seek to include the current project in the interaction with this Patient Advisory Board. Members of this board include the Secretary General of the NCF as well as the Director of Coeliac UK. For the current project, the representatives from the NCF: Knut H. Peterson will attend our bi-annual project meetings (PhD supervision will be regular and much more frequent).

6. Ethical considerations

The diet intervention is a short time restriction of a limited number of food items, and will do no harm to the participants. If the FODMAP intervention is superior to strict gluten free diet, the patients in the control group will be offered that treatment after the study is finished.

We will apply to Regional Ethical Committee (REK) for this project. We have a number of closely related applications that all have been approved by REK. We foresee that the current project also will be approved by REK. The project will further be posted on Helsenorge.no and

Clinicaltrials.gov. The biological material will be deposited in the approved general biobank "Tarmsykdommer" (REK ID number 2012/341).

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UiO : Universitetet i Oslo

Oslo universitetssykehus

FORESPØRSEL OM DELTAKELSE I FORSKNINGSPROSJEKTET

EFFEKT AV FODMAP REDUKSJON HOS PASIENTER MED CØLIAKI

Dette er et spørsmål til deg om å delta i et forskningsprosjekt for å undersøke effekten av diett med lavt innhold av FODMAP (Fermenterbare Oligo-, Di-, Monosakkarider og Polyoler) på mage-tarmplager hos pasienter med cøliaki som spiser glutenfri kost. Resultatene vil gi ny kunnskap om hvordan man kan tilpasse kostråd til cøliakere med vedvarende mage- og tarmplager, og bidra til at de kan få et bedre liv med færre plager.

HVA INNEBÆRER PROSJEKTET?

FODMAP er en type karbohydrater som fordøyes dårlig og passerer videre til tykktarmen der de nedbrytes av tarmbakteriene. Cøliakere med vedvarende mage- og tarmplager blir delt inn i to grupper der den ene gruppen vil få råd om å redusere inntaket av FODMAP, og den andre gruppen skal følge sitt vanlige glutenfrie kosthold. Diettene skal følges i fire uker og deltagerne skal registrere mage- og tarmplager før og etter diettperioden. Vi vil undersøke om det er forskjell på mage- og tarmplager mellom de to gruppene. Det vil også bli gjort en gastroskopi og en blodprøve, samt en avføring- og urinprøve ved to anledninger. Deltagerne må møte opp på studiesenteret i Oslo 3 ganger. Dato for besøkene blir tilpasset deltagernes ønsker. Utfylling av skjemaer underveis i de 4 ukene anslås til å ta maksimalt 10 minutter per uke.

Følgende helseopplysninger om deg vil bli lagret: mage-tarmplager, kosthold, livskvalitet. I tillegg til biopsisvar, blodprøvesvar, og sammensetning av bakterieflora i avføring. Samtykke til oppbevaring i biobanken gjøres på et eget skriv.

MULIGE FORDELER OG ULEMPER

Dersom du kommer i diettgruppen må du følge kostrådene som gis i fire uker. Dersom du har effekt av behandlingen kan du velge å fortsette med en individuell tilpasset kost, som gir deg færre mage-tarmplager. Dersom du kommer i gruppen som skal følge sitt vanlige kosthold, vil du likevel få tilbud om individuell kostbehandling når prosjektet er ferdig. All medisinsk informasjon som fremkommer som ledd i forskningen, vil bli vurdert av prosjektleder og klinikerne som deltar i forskningen. Der det er klinisk grunn til det, vil du bli kontaktet for å drøfte funnene. Resultatene vil gi ny kunnskap om hvordan man kan tilpasse kostråd til cøliakere med vedvarende mage- og tarmplager, og bidra til at de kan få et bedre liv med færre plager.

Noen deltagere vil kunne få ubehag under gastroskopi og blodprøvetagning. Alle prosedyrer blir utført av spesialtrenet personell som vil legge vekt på å minimalisere ubehaget for den enkelte deltager. Det blir ikke tatt prøver hvis man mistenker at dette kan gå utover pasientenes sikkerhet eller hvis pasienten er uvanlig besværet under prøvetakingen.

FRIVILLIG DELTAKELSE OG MULIGHET FOR Å TREKKE SITT SAMTYKKE

Det er frivillig å delta i prosjektet. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke. Dersom du trekker deg fra prosjektet, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner. Dersom du senere ønsker å trekke deg eller har spørsmål til prosjektet, kan du kontakte overlege, professor, dr. med. Knut E. A. Lundin, Gastroundersøkelse. OUS Rikshospitalet, tlf. 23 07 23 88, 2307 24 00 el. 23 07 00 00. Mail adresse knut.lundin@medisin.uio.no (NB ikke send sensitive opplysninger pr mail).

HVA SKJER MED INFORMASJONEN OM DEG?

Informasjonen som registreres om deg skal kun brukes slik som beskrevet i hensikten med studien. Du har rett til innsyn i hvilke opplysninger som er registrert om deg og rett til å få korrigert eventuelle feil i de opplysningene som er registrert.

Alle opplysninger om deg vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger gjennom en navneliste som oppbevares på et sikkert sted. Prosjektleder har ansvar for den daglige driften av forskningsprosjektet og at opplysninger om deg blir behandlet på en sikker måte. Informasjon om deg vil bli anonymisert eller slettet senest fem år etter prosjektslutt.

HVA SKJER MED PRØVER SOM BLIR TATT AV DEG?

Biologisk materiale vil bli lagret og analysert som beskrevet i hensikten med studien. Materiale vil bli lagret i en forskningsbiobank ved Oslo Universitetssykehus, og kan også bli brukt til videre forskning. Biobanken er tilrådd av Regional etisk komité for medisinsk og helsefaglig forskningsetikk, og godkjent av Helsedirektoratet. Biobanken drives i tråd med nasjonale og lokale retningslinjer. Overlege, professor, dr. med. Knut E. A. Lundin er ansvarshavende for forskningsbiobanken. Samtykke til oppbevaring i biobanken gjøres på et eget skriv.

FORSIKRING

Du har vanlige pasientrettigheter som ledd i din kontakt med helseinstitusjonen og eventuell søknad til Norsk pasientskadeerstatning kan sendes på vanlig måte. Det er ingen spesiell forsikring knyttet til prosjektene.

UTLEVERING AV OPPLYSNINGER TIL ANDRE

Hvis du sier ja til å delta i studiene, gir du også ditt samtykke til at prøver og avidentifiserte opplysninger utleveres til forskningsgrupper vi samarbeider med. Vi samarbeider hovedsakelig med forskningsgrupper innen EU, men samarbeidet kan også gjelde land med lover som ikke tilfredsstiller europeisk personvernlovgivning.

ØKONOMI

Prosjektet og biobanken er finansiert gjennom offentlige forskningsmidler. Slike midler tilføres forskningsgruppen fra Universitetet i Oslo, Helse Sør-Øst, Stiftelsen Kristian Gerhard Jebsen, fra EUs forskningsprogram, fra National Institute of Health, fra ExtraStiftelsen, fra forskningsstiftelsen Inven2 og fra en rekke andre finansieringskilder.

GODKJENNING

Prosjektet er godkjent av Regional komite for medisinsk og helsefaglig forskningsetikk, 2018/1055.

SAMTYKKE TIL DELTAKELSE I PROSJEKTET (SETT KRYSS)

Jeg samtykker i å delta i studien: Effekt av FODMAP reduksjon hos pasienter med cøliaki ☐

Sted og dato

Deltakers signatur

Deltakers navn med trykte bokstaver

JEG BEKREFTER Å HA GITT INFORMASJON OM STUDIEN ☐

(Signert, rolle i studien)

Attachment E.4

Samarbeidsprosjekt / laboratorietjenester ved Unger-Vetlesens Institutt (UVI)

Prosjekttittel
Effect of FODMAP restriction on persistent GI-symptoms in coeliac patients

Kontaktinformasjon	
Navn på institusjon/firma	**NMBU, Ås**
Navn på prosjektleder	**Siv Kjølsrud Bøhn og Anne Mari Herfindal**
Telefon	
E-post	siv.kjolsrud.bohn@nmbu.no anne.mari.herfindal@nmbu.no>

1. Prosjektet

Laboratorieanalyser ønskes utført av UVI: **SCFA**

Antall prøver: **ca 140**

Avtalt pris: kr. 300,-

Hvordan skal prøvemateriell lagres hos UVI?: **Kommer ferdig alliqvotert for analysen**

- ☐ Materialet oppbevares etter fullførte analyser, til dato: .....
- ☐ Materialet destrueres ved fullførte analyser
- ☐ Materialet føres tilbake til «bestiller» /eksisterende biobank
- ☐ Skal biobankmaterialet overføres til annen institusjon? Navn:

2. Fremdriftsplan

Ønsket oppstart:

uke 36/37

Fordeling av vitenskapelig meritt(er):

Laboratorieanalysen forutsetter medforfatterskap for forsker Jørgen Valeur i publikasjoner der resultatene brukes.

3. Vedleggsoversikt

Prosjektbeskrivelse	X
REK godkjenning	
Kontrakt/avtale med prosjektansvarlig institusjon	
Ev. andre vedlegg	

6. Underskrifter

Dato: 15.09.2020 Prosjektleder Signatur Siv Kjølsrud Bøhn

Dato: 1/7-20 UVI Signatur

Lovisenberg Diakonale Sykehus AS, Pb 4970 Nydalen, 0440 Oslo
Unger-Vetlesens Institutt
Tlf: 23 22 51 40, guhm@lds.no, Org nr: NO 965 985 166 MVA

THE GASTROINTESTINAL SYMPTOM RATING SCALE (GSRS) IRRITABLE BOWEL SYNDROME (IBS)-VERSJON

Les dette først:

Undersøkelsen inneholder spørsmål om hvordan du har følt deg og hvordan du har hatt det DE 3 SISTE DAGER. Sett kryss (X) ved det alternativet som passer best på deg og din situasjon.

1. Har du i løpet av de siste tre dagene vært plaget av MAGESMERTER?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
- ☐ Milde plager
- ☐ Moderate plager
- ☐ Ganske alvorlige plager
- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

2. Har du i løpet av den siste tre dagene vært plaget av SMERTER ELLER UBEHAG I MAGEN SOM GIR SEG NÅR DU HAR HATT AVFØRING?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
- ☐ Milde plager
- ☐ Moderate plager
- ☐ Ganske alvorlige plager
- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

3. Har du i løpet av den siste tre dagene vært plaget av OPPBLÅSTHET?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
- ☐ Milde plager
- ☐ Moderate plager
- ☐ Ganske alvorlige plager
- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

4. Har du i løpet av den siste tre dagene vært plaget av LUFTAVGANG?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
- ☐ Milde plager
- ☐ Moderate plager
- ☐ Ganske alvorlige plager
- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

5. Har du i løpet av den siste tre dagene vært plaget av FORSTOPPELSE (problemer med å tømme tarmen)?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
- ☐ Milde plager
- ☐ Moderate plager
- ☐ Ganske alvorlige plager
- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

6. Har du i løpet av den siste tre dagene vært plaget av DIARÉ (hyppig avføring)?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
- ☐ Milde plager
- ☐ Moderate plager
- ☐ Ganske alvorlige plager
- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

7. Har du i løpet av den siste tre dagene vært plaget av LØS AVFØRING?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
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- ☐ Moderate plager
- ☐ Ganske alvorlige plager
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- ☐ Meget alvorlige plager

8. Har du i løpet av de siste tre dagene vært plaget av HARD AVFØRING?

- ☐ Ingen plager i det hele tatt
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- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

9. Har du i løpet av den siste tre dagene vært plaget av TVINGENDE AVFØRINGSBEHOV (plutselig behov for å gå på toalettet for å tømme tarmen)?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
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- ☐ Meget alvorlige plager

10. Har du i løpet av de siste tre dagene vært plaget av en FØLELSE AV UFULLSTENDIG TØMMING AV TARMEN ETTER AVFØRING?

- ☐ Ingen plager i det hele tatt
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- ☐ Meget alvorlige plager

11. Har du i løpet av den siste tre dagene vært plaget av at du FØLER DEG METT LIKE ETTER AT DU HAR BEGYNT PÅ ET MÅLTID?

- ☐ Ingen plager i det hele tatt
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- ☐ Meget alvorlige plager

12. Har du i løpet av den siste tre dagene vært plaget av at du FØLER DEG METT SELV LENGE ETTER AT DU ER FERDIG MED Å SPISE?

- ☐ Ingen plager i det hele tatt
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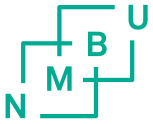
- ☐ Ganske alvorlige plager
- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

13. Har du i løpet av den siste tre dagene vært plaget av at MAGEN ER SYNLIG OPPBLÅST?

- ☐ Ingen plager i det hele tatt
- ☐ Ubetydelige plager
- ☐ Milde plager
- ☐ Moderate plager
- ☐ Ganske alvorlige plager
- ☐ Alvorlige plager
- ☐ Meget alvorlige plager

KONTROLLER AT ALLE SPØRSMÅLENE ER BESVART!

TAKK FOR DIN MEDVIRKNING.



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