Immunological and Microbiological perspectives on Irritable Bowel Syndrome

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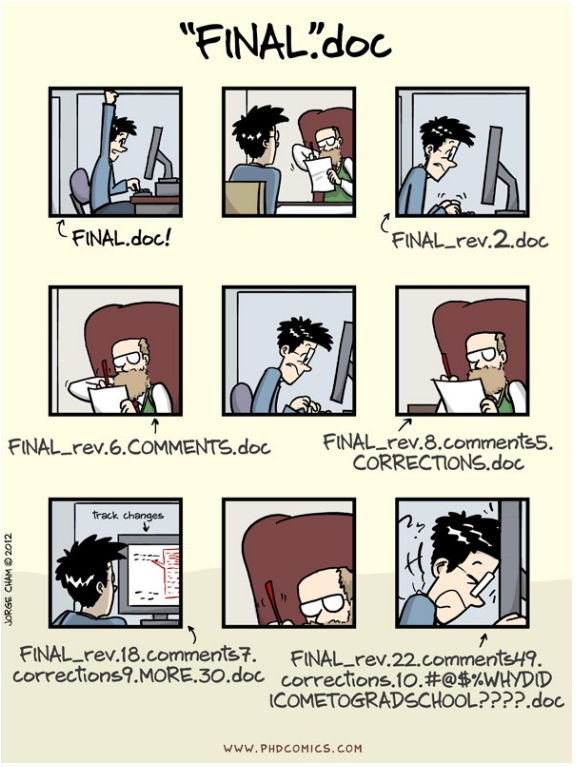
This thesis is dedicated to all my family and friends who have supported me so much over the years – here's to you!

"This was a triumph.
I'm making a note here:
HUGE SUCCESS.
It's hard to overstate
my satisfaction."

- GLaDOS



Abstract

Irritable bowel syndrome affects ~11% of the population in the Western world and is characterised by altered bowel habits and abdominal pain. The range of additional symptoms between subjects makes groups of IBS patients heterogeneous. Increased immune activity, altered gut microbiota and diet are implicated in symptom generation though the mechanisms are poorly understood. Moreover, gut microbiota and immune activity interplay in relation to symptoms requires elucidation and while dietary intervention is effective in some patients its impact on gut microbiota is unclear. Most likely, all patients do not share the same symptom generating mechanisms, and thus better means to stratify patients for both research and treatment is required.

This thesis aimed to demonstrate how gut microbiota, the immune system and their crosstalk result in symptom generation in IBS patients. Furthermore, we aimed to demonstrate how dietary intervention affects microbiota of the gut and if patient responsiveness to intervention therapy could be predicted by gut microbiota profiles.

This thesis demonstrates that a diet low in poorly absorbed carbohydrates (FODMAP) changes the gut microbiota composition and reduces beneficial bacteria in IBS patients. Moreover, the composition of gut microbiota can be used to discriminate patients whose IBS symptoms improved or not after a low FODMAP diet. Additionally, serum or mucosal cytokines cannot be used alone to diagnose IBS. However, a subset of immuno-active patients had comparatively raised serum levels of pro-inflammatory cytokines to healthy subjects and immuno-normal IBS patients, although no major associations between cytokines and symptoms were found. Further, IBS patients had an altered mucosal expression of genes associated with an innate antimicrobial response compared to healthy subjects. The antibacterial gene expression response profiles as well as faecal and mucosal bacterial profiles were different between immuno-active and immuno-normal IBS patients, but were not associated to symptoms.

In conclusion, a subset of IBS patients has altered immune activity, deemed by cytokine and innate antimicrobial response profiles, which do not seem to be associated with any specific symptom profile. Further, faecal microbial profiles may be used to identify responders to low FODMAP diet therapy but negative impact of the diet on beneficial bacteria requires further investigation. Thus, this thesis has identified novel subgroups of IBS patients based on underlying mechanisms which may guide development of innovative therapy options.

Keywords: IBS, Microbiota, Immune system, FODMAPs
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Populärvetenskaplig sammanfattning

Irritable bowel syndrome (IBS) är en vanlig funktionell tarmsjukdom som uppskattas påverka cirka 11% av befolkningen i västvärlden. IBS kännetecknas av buksmärta och avföringsrubbningar, men även andra symptom av varierande svårighetsgrad förekommer. Alla patienter med IBS upplever inte symtom på samma sätt och det är därför svårt att hitta en behandling som passar alla. Dessutom kan liknande symtom vara kopplade till olika underliggande faktorer t ex. ökad immunaktivitet, förändrad tarmflora eller av avvikande reaktion på födointag. Dock saknas detaljkunskap om hur dessa faktorer orsakar IBS och dess symtom.

Syftet med avhandlingen var att undersöka hur tarmfloran påverkas av IBS och en kostbehandling genom att jämföra patienter och friska individer och demonstrera huruvida immunaktivering och tarmfloran påverkar IBS symptom.

För att besvara dessa frågor, fick patienter och friska individer fylla i frågeformulär, genomgå fysiologiska mätningar och lämna blod samt avföring. Kolonbiopsier togs för att studera tarmfloran och uttrycket av inflammationsmarkörer. Statistiska metoder användes för att jämföra immunprofilen och tarmfloran mellan patienter och friska individer, eller mellan patienter som svarade respektive inte svarade på kostbehandling (kost med lågt innehåll av ofullständigt absorberbara kolhydater s.k. FODMAPs).

Vi visade att tarmflorans sammansättning förändrades, med minskad andel fördelaktiga bakterier efter kostbehandling, samt att tarmflorans sammansättning var annorlunda hos patienter som förbättrades av kostbehandlingen jämfört med patienter som inte svarade positivt. Vi visade också att cytokiner i serum och uttrycket av cytokiner i tjocktarmen i sig inte kan användas för att diagnostisera IBS. Även om vissa patienter hade förhöjda cytokinnivåer jämfört med friska individer kunde vi inte påvisa en koppling mellan immunaktivitet och IBS symtom. Vidare såg vi att gener som styr det antimikrobiella svaret hos individer var förändrade hos IBS patienter jämfört med friska individer, samt att genuttrycket och tarmfloran varierade mellan patienter med normal eller förhöjd immunaktivitet.

Sammanfattningsvis har vi påvisat avvikelser i immunförsvar och tarmflora hos patienter med IBS, och att tarmflorans sammansättning kan förutspå vem som svarar väl på kostbehandling. Våra fynd kan användas för att erbjuda individanpassad behandling för IBS patienter.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Multivariate modelling of faecal bacterial profiles of patients with IBS predicts responsiveness to a diet low in FODMAPs**

Bennet SMP, Böhn L, Störsrud S, Liljebo T, Collin L, Lindfors P, Törnblom H, Öhman L and Simrén M

Gut 2017 Apr 17. [Epub ahead of print].

II. **Global cytokine profiles and association with clinical characteristics in patients with irritable bowel syndrome**

Bennet SMP, Polster A, Törnblom H, Isaksson S, Capronnier S, Tessier A, Le Nevé B, Simrén M and Öhman L

Am J Gastroenterol 2016;111:1165-76.

III. **Systemic cytokines are elevated in a subset of patients with irritable bowel syndrome (IBS) but largely unrelated to symptom characteristics**

Bennet SMP, Palsson O, Whitehead WE, Barrow DA, Törnblom H, Öhman L, Simrén M and van Tilburg MAL

Submitted

IV. **Altered intestinal antibacterial gene expression response profile in irritable bowel syndrome is linked to bacterial composition and immune activation**

Bennet SMP#, Sundin J#, Magnusson MK, Strid H, Tap J, Derrien M, Le Nevé B, Doré J, Törnblom H, Simrén M* and Öhman L*

Submitted

Contents

Abbreviations

IBS	Irritable Bowel Syndrome
GI	Gastrointestinal
IBD	Inflammatory Bowel Diseases
CD	Crohn's disease
UC	Ulcerative Colitis
FBD	Functional bowel disorder
AGA	American Gastroenterological Association
QOL	Quality of life
BSF	Bristol Stool Form Scale
IBS-C	IBS with constipation
IBS-D	IBS with diarrhoea
IBS-M	Mixed IBS
IBS-U	Unsubtyped IBS
PI-IBS	Postinfectious IBS
SCFA	Short chain fatty acids
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
GOS	Galacto-oligosaccharides
TJP	Tight junction proteins
LPS	Lipopolysaccharides
IL	Interleukin
APC	Antigen presenting cells
Th	T helper
OATT	Oroanal Transit Time
HAD	Hospital Anxiety and Depression Scale
BSI	Brief Symptom Inventory Anxiety
IBS-SSS	IBS Severity Scoring System
PHQ-15	Patient Health Questionnaire 15
RPSQ	Recent Physical Symptoms Questionnaire
CMCQ	Comorbid Medical Conditions Questionnaire
PCA	Principal Component Analysis
HCA	Hierarchical Cluster Analysis
OPLA-DA	Orthogonal Partial Least Squares Discriminant Analysis
HS	Healthy subjects
DI	Dysbiosis Index

1

Introduction

This thesis describes an explorative investigation into irritable bowel syndrome (IBS) from an immunological and microbiological perspective. The effects of dietary intervention therapy on gut microbiota composition were also investigated. A prevalent method in this thesis is multivariate analysis, as its ability to analyse multiple variables simultaneously is of great benefit when working with many different variables from a heterogeneous group of patients. The outcome of such analysis in this thesis distinguished responders from non-responders to a diet intervention and identified a subset of IBS patients based upon markers of immune system activation. Additionally, the immune activation was found to be associated with an altered antimicrobial gene expression profile and gut bacteria composition. The studies of Papers 1-IV and how they are linked are summarized in **Figure 1**.

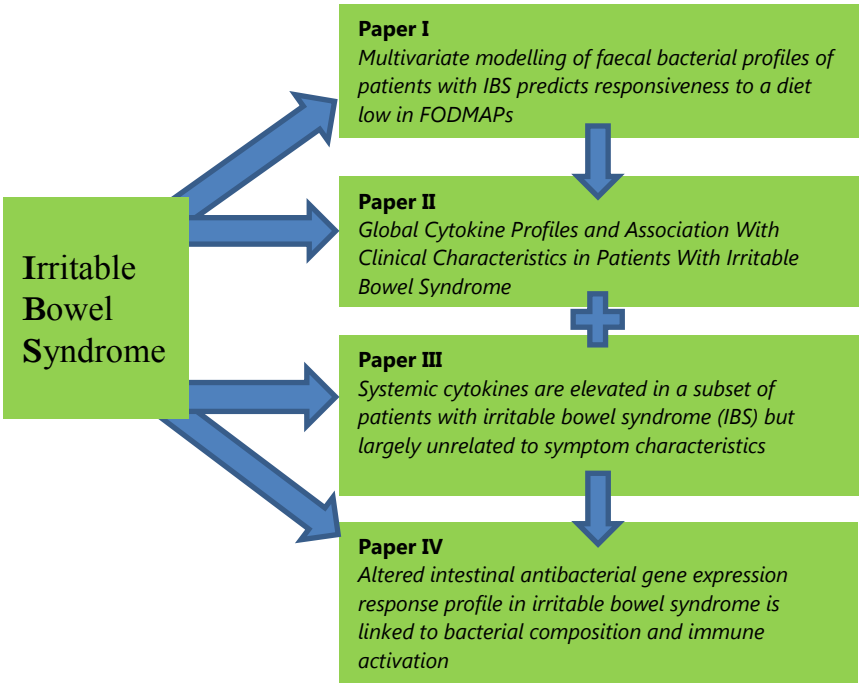

Figure 1. Flow chart overview of projects comprising this thesis. Paper I investigated the impact of two different dietary interventions on intestinal microbiota in IBS patients. Papers II and III used multivariate analysis to analyse the serum and mucosal cytokine profiles of two different IBS patient cohorts compared to healthy subjects. Paper IV assessed the antimicrobial gene expression profiles of IBS patients and healthy subjects, as well as the recently identified subsets of IBS patients based upon immune activity.

The Gastrointestinal Tract

The organ system known as the gastrointestinal (GI) tract is intricate and well-orchestrated towards its primary function of breaking down and transporting ingested food and liquids from mouth to anus while simultaneously absorbing nutrients and removing waste[1]. This 30 foot (9.1m) multi-tissue, multifaceted tract can be subdivided into the upper GI tract, including the mouth, oesophagus, stomach and duodenum, and

the lower GI tract, including most of the small intestine and all of the large intestine. For the most of us, this complex collaboration of organs works harmoniously together along the faeces production line and causes no bother. Perhaps there might be the odd passing of gas, or uncommon bout of constipation or diarrhoea for numerous reasons, but these are often infrequent events for a healthy individual. Some people however are more unfortunate and these infrequent occurrences become so common that they begin to impact their daily life. On top of that, other symptoms such as pain might occur and exacerbate the experience. When a noticeable enough amount of people are affected in a similar manner with similar problems then the human compulsion to name things is enacted and a formal name is defined.

History of IBS

Since a quote is something found in most theses then these stating that, “bad digestion is at the root of all evil” and “death sits in the bowels.” as supposedly said by Hippocrates, the father of medicine (400 B.C.) is most relevant here. During the years since, a constellation of symptoms including bloating, altered bowel habits (looser or harder stool) and, importantly, abdominal pain to mention the main three, have been given a number of different terms. Names like Irritable Colitis, Spastic Colon, Mucous Colitis, Nervous Stomach and Intestinal Neurosis are but a few which have not stood the test of time. To see why these names have not prevailed one has to understand that each have failed to describe to a sufficient degree what patients with these symptoms are experiencing. Colitis for example means disease pertaining to the colon as characterised by inflammation; neither acute nor chronic inflammation is observed in these patients but instead in those afflicted with the better defined Inflammatory Bowel Diseases (IBD) of Crohn's disease (CD)[2] and

Ulcerative Colitis (UC)[3]. Moreover, the colon is a commonly investigated section of the digestive tract due predominantly to its ease of access and location in the region where patients report bother; however, referral to only the colon neglects sections such as the 20 foot (6m) length of small intestine demonstrated to be not completely free of blame in symptom generation in some patients[4,5]. Conversely, while the stomach is indeed part of the GI tract, the polysemic nature of the word and the lack of a fine sensory network in the abdomen mean an individual may say stomach not to refer to the organ but instead to the abdominal area of concern. Although a pedantic argument it nevertheless highlights the difficulty in selecting the right nomenclature for a disease or syndrome, particularly one with no clear aetiology[6]. The use of the word spastic has mainly been linked to abdominal pain / cramps and refers to the increased spasms or motility of the bowel muscles. This clenching can be reported as belly cramps and may generate diarrhoea or constipation in some patients since spasms can also delay the passage of stool; yet studies showing a decreased motility in some patients[7] render the term spastic colon inaccurate. Finally, although words such as nervous or neurosis refer to how stress and anxiety can trigger or exacerbate symptoms, this may only be true for some patients and not for others reporting symptom occurrence through ingestion of certain foods or arising after a bout of gastroenteritis or infection[8,9].

Considering the history, it seems wise to thus state that as of writing this thesis the most prevalent name for this group of symptoms, as coined in 1950 by Philip W. Brown, is Irritable Bowel Syndrome, or IBS for short[10]. Currently the most encapsulating name, IBS uses the medical definition of irritable i.e. to be abnormally sensitive, to describe the condition of both the small and large intestine, collectively known as the bowels of an individual. With no universal trigger identified as of yet, the

diagnosis of IBS is thus symptom based and the term syndrome is used to describe the group of symptoms that together are characteristic of the irritable bowel.

Irritable bowel syndrome is regarded as a functional bowel disorder (FBD)[11] of the lower GI tract by which there is a disorder to the proper functioning of the bowel without any apparent structural or biochemical anomaly relating to, or arising in a bodily organ. Despite IBS and UC being both maladies of the bowel with unknown aetiologies, symptoms in UC can be attributed to organic structural abnormalities in the form of ulcers and inflammation along the colon or rectum. In IBS however, different underlying abnormalities for example in intestinal permeability[12], factors of the immune system[13], gut microbiota[14,15], as well as sensitivity to dietary components[16] have been linked to symptoms, but none of them are present universally in all patients.

Diagnosis of IBS

Believed by many clinicians, and to a lesser degree experts, to be a diagnosis by exclusion, IBS is often diagnosed only after an exhaustive battery of expensive and time consuming tests have been performed[17,18]. These tests are implemented in the effort to catch or exclude serious organic diseases such as IBD, infectious diarrhoea or colorectal cancer or those masquerading as IBS due to similar symptoms such as coeliac disease[19]. In 1997 the American Gastroenterological Association (AGA) provided guidelines including over 15 different examinations to assist physicians in their clinical understanding, diagnosis, and management for IBS [20]. Although the practice of subjecting patients to a wide repertoire of tests in the worry of missing a potentially life threatening diagnosis may provide peace of mind, to err on the side of caution might be considered

extensive[21] considering how poorly characterised the degree to which the diagnostic certainty of IBS is improved by this[22].

Despite the prevalence of diagnosing IBS through exclusion, guidelines for a positive symptom-based diagnosis of IBS are available and have been disseminated since 1978 in the form of the Manning criteria[23]. Ever evolving, the Manning criteria was succeeded by the ROME criteria released in 1989[24] with the latest fourth edition being released in 2016[25]. The ROME criteria working groups are multinational which aimed to develop a means to select patients for both therapeutic and diagnostic trials. Nowadays the criteria are being recommended as a diagnostic tool in clinical practice, together with the observation for "red flags" such as family history of colon cancer, weight loss or blood in the stool, in order to reduce the number of testing required for a diagnosis of IBS. See **Box 1** for the ROME III criteria[11] as predominantly used in this thesis, and **Box 2** for the current ROME IV criteria[25].

Box 1. ROME III diagnostic criteria* for IBS

Recurrent abdominal pain or discomfort** at least 3 days per month in the last 3 months associated with 2or more of the following:

1. Improvement with defecation
2. Onset associated with a change in frequency of stool
3. Onset associated with a change in form (appearance) of stool

*Criteria fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis.

**Discomfort means an uncomfortable sensation not described as pain. In pathophysiology research and clinical trials, a pain/discomfort frequency of at least 2 days a week during screening evaluation for subject eligibility.

(Longstreth et al. 2006)

Box 2. ROME IV diagnostic criteria[α] for IBS
Recurrent abdominal pain, on average, at least 1 day per week in the last 3 months, associated with 2 or more of the following criteria:

1. Related to defecation
2. Associated with a change in frequency of stool
3. Associated with a change in form (appearance) of stool

[α]Criteria fulfilled for the last 3 months with symptom onset at least 6 months before diagnosis.

(Lacy et al. 2016)

Review of the different iterations of the ROME criteria over the years have shown that the sensitivity (the probability that a test will indicate 'disease' among those with the disease) and specificity (the fraction of those without disease who will have a negative test result) of the criteria for diagnosing IBS has been consistently ~65% and ~98% respectively[26,27].

The global prevalence of IBS is often stated as being 11% as demonstrated in a review and meta-analysis from 2012 covering population-based studies from 1947–2011[28]. However, this 11% could be debated considering the lack of data from regions such as Africa and that the mean prevalence between countries where data is available can differ from 1.1% in France and Iran to as much as 35.5% in Mexico as demonstrated in a recent 2017 literature review in which the authors suggest focusing instead on reliable regional estimates of IBS prevalence[29]. Nevertheless IBS is the most prevalent GI disorder with onset occurring in the majority of patients before the age of 45[30] and being more common in females than males in an approximate two to one ratio[28]. The reason for the higher prevalence in females is a topic of conjecture; however, what has been demonstrated is that IBS affects

females and males differently. Regarding physiological abnormalities, females with IBS report higher visceral hypersensitivity[31] and slower transit time compared to males with IBS[7,32]. The difference in psychological wellbeing, quality of life (QOL) and coping abilities between female and male patients with IBS and how they are affected by the syndrome has also been assessed through clinical questionnaires, yet conflicting data calls for further research on the topic[33-36]. The level of anxiety and anxiety specifically focused on the happenings of a patients' own gut have been demonstrated to be higher in females compared to males with IBS[33]. Finally, while IBS is not life threatening *per se*, it has the power to severely impact the QOL of an individual[37]. Females have reported a lower QOL[33,38], but not all studies come to the same conclusion[39]. This detrimental impact on QOL is made quite clear through the findings of two independent studies which showed that in exchange for "perfect health" some patients would give up 15.1 years of their life[40], while others would accept a 1% risk of sudden death from a hypothetical medication if the chance of curing their IBS was 99%[41]. Regardless of gender, being afflicted with IBS often requires the patient to say goodbye to the way of life they were used to and instead one of malaise, planning around their dysfunctional bowels, continuously being mindful to foods which might set off another bout of symptoms and aware of where the nearest toilet is[37,42-44].

Economic Impact of IBS

Due in part to its chronicity, the burden of IBS is not limited to the individual patient but puts strain on clinics through high time requirements and costs. In America the annual direct medical costs were estimated in 2000 to be between $1.7 billion and $10 billion[45,46], with costs being estimated to reach $131 million per year in Ontario, Canada alone[47]. Although the annual spending of the NHS budget in 1995 on IBS was only 0.1%, this still equated to £45.6 million with no account taken for the personal spending of the patients on medication or other approaches to manage the symptoms[48]. Interestingly, aside from the drain on resources, IBS was assigned the least amount of research funds ($8.2 million) in the fiscal year of 2000 by the National Institutes of Health (NIH) compared to the $218.6 million allotted to the research of chronic liver disease and cirrhosis[44]. While IBS may not garner the highest amount of research money, and public knowledge is lacking[49,50], research prevails through groups around the globe aiming to understand the black box that is IBS.

Subgroups of IBS

When tackling a heterogeneous syndrome like IBS, a common practice is to identify groups of patients who share similar symptoms or other abnormalities thought to be pertinent to the diagnosis. By doing this, clinicians may find it easier to prescribe therapies or medications to alleviate the symptom or rectify what might be altered. Researchers however, can attempt to identify the underlying cause of the symptom through potential identification of physiological or mechanistic alterations shared by the patients[6]. In the case of IBS the easiest and least invasive means to subtype is based on bowel habits. The first step in this

method is for the patient to record the frequency of their bowel movements over a given period whilst grading the stool using the Bristol Stool Form Scale (BSF) which ranges from 1 “separate hard lumps” to 7 “completely liquid”[11]. Based on having a more or less than 25% occurrence of hard and lumpy or soft and watery stool, this well-established practice places patients into one of four subtypes, IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D), mixed IBS (IBS-M) (both loose and hard stools), and when there is insufficient abnormality in the stool consistency to be subtyped as IBS-C, D or M the patient is unsubtyped IBS (IBS-U)[11]. This method of subtyping is widely practiced and prescription of laxatives or bulking agents, and antidiarrhoeal agents can be effective treatments for constipation and diarrhoea respectively, while on the research side, focusing on a single subtype such as IBS-D has provided some interesting insights into potential shared underlying causes and physiological abnormalities[51]. However, there are potential problems incurred when focusing on a single patient group subtyped according to the predominant bowel habit. The stool consistency of nearly 80% of IBS patients has been demonstrated to fluctuate between loose and hard naturally over time with the underlying cause for this still requiring elucidation, but it has not been linked to stool modifying medication[52]. The clinical impact of this, as suggested by the authors, is that stool modifying medications should be prescribed in an “as needed” dose rather than fixed[52]. Regarding the research aspect, since the underlying mechanism for diarrhoea in IBS can be differing, including the osmotic effect of specific foods which draw water into the bowel[53], or increased colonic bile acid exposure[54], if not considered by the researcher this heterogeneity even among IBS-D patients may impact findings. While the current method of subtyping IBS patients according to bowel habits is not without its benefits, subgrouping based on pathophysiology

or symptom pathogenesis[55] may be a better choice allowing for more targeted treatment for a subset of patients[56,57]. These means of grouping patients have been investigated, from the conventional[15,58-61] to the alternative[62] but still as yet, none have achieved as much global use as the frequency and form of stool[11]. Many factors can influence the generation or severity of symptoms of IBS as depicted in **Figure 2**.

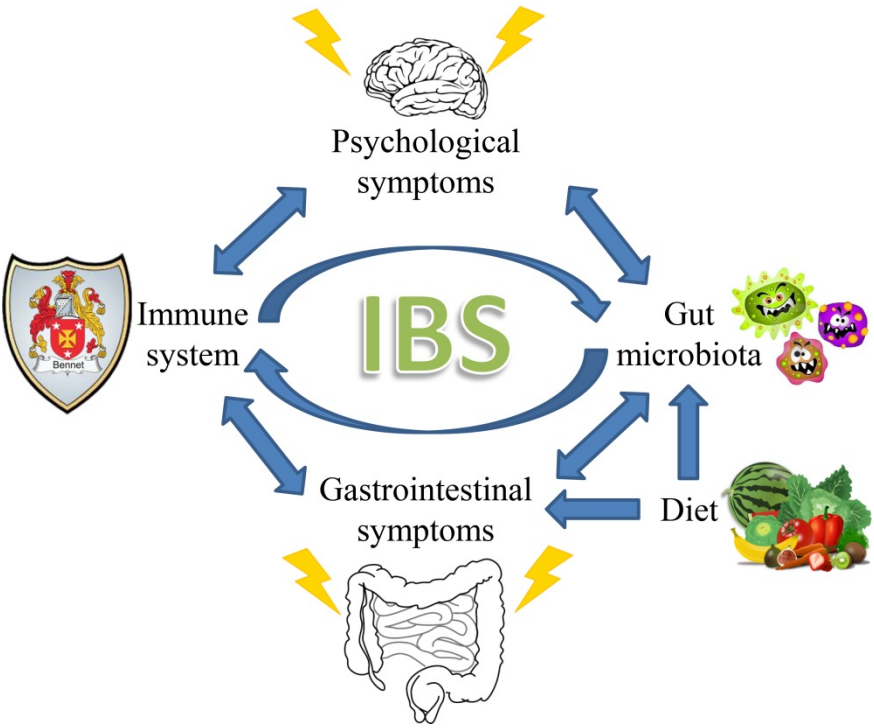

Figure 2: Overview of the many factors covered in this thesis which can impact the onset or severity of IBS in patients. Diet can in itself cause gastrointestinal symptoms but can also impact the gut microbiota composition. Similarly while the gut microbiota can impact the gut and the brain, it is also linked to the immune system and even its proper development. Finally, factors of the immune system can be implicated in many aspects of IBS.

Postinfectious IBS

An additional means to differentiate patients with IBS is based upon the abruptness of symptom onset. Gastroenteritis is an inflammation of the stomach and intestines and normally involves vomiting and diarrhoea with potential stomach pain, headache and even fever. These symptoms are typically caused by a viral, bacterial or protozoan infection and while a full recovery occurs for most, in a few cases the symptoms persists leading to that person eventually fulfilling the criteria for a diagnosis of IBS. If credence is given by the patient that their IBS symptoms developed after an illness, as approximately 6-17% of patients do[63], then their IBS is referred to as being postinfectious IBS (PI-IBS)[64,65]. While symptoms and history are enough for this diagnosis, a positive bacterial stool culture for pathogenic species such as *Salmonella enteritidis*, *Camplylobacter jejuni*, *Escherichia coli* and *Shigella flexneri*[66,67] makes for a more credible diagnosis. Compared to IBS with unknown aetiology, studying PI-IBS may seem more inviting considering that findings might be traced back to the comparatively more elucidated background of a preceding infection, yet PI-IBS involves its own points to consider such as risk factors including the severity of the initial illness, female gender, psychosocial difficulties at the time of the infection, and genetic predisposition[68]. Nevertheless, the occurrence of IBS symptoms after infection and the link to alteration of the normal microbiota[69], makes PI-IBS strong evidence for the involvement of microbiota alterations in the pathophysiology of IBS. The investigation of microbiota and its role in IBS is fairly recent yet since the late 1990's and concurrently when the ROME criteria was introduced, interest in this topic of research has been ever expanding[70].

Gut microbiota in IBS

The role of the GI tract is far from limited to digestion considering that it is in itself is a microenvironment playing host to multitude of microorganisms (**Figure 3**). This microbiota includes viruses, fungi, archaea, bacteria, bacterial phages, protozoa and in some unfortunate people, worms, with the complete genetic content referred to as the microbiome. Focusing on bacteria, the culture based techniques available for bacterial detection was limited in the 1970's and thus many microbial species remained undiscovered[71]. One study estimated that as many as 400 different bacterial species may inhabit the healthy colon, but that as few as 20 species had the most abundance[72]. Over the years, the use of culture-independent techniques such as DNA sequencing[73], fluorescence in situ hybridization (FISH)[74] and more recently massively parallel shotgun sequencing (high-throughput sequencing technologies)[75] have helped to characterize over 1000 species of the GI tract alone[76] with each individual person harbouring at least 160 species[77]. The first step when analysing the composition of gut microbiota is to choose the material. Since it has been demonstrated that the there is a separation between mucosal- and faecal-associated microbiota, both samples would be ideal, but this is often not feasible[78]. Faecal samples are the easiest to obtain and are thus used prevalently as well as seem to be a proxy of mucosal microbiota[14]. However, mucosal biopsies can be taken from various locations of the GI tract to provide a more site specific view of the mucosal adherent species. The next step is the method of analysis. A common method used in the identification and classification of bacteria focuses on the DNA gene coding for 16S ribosomal RNA known as the 16S rRNA gene.

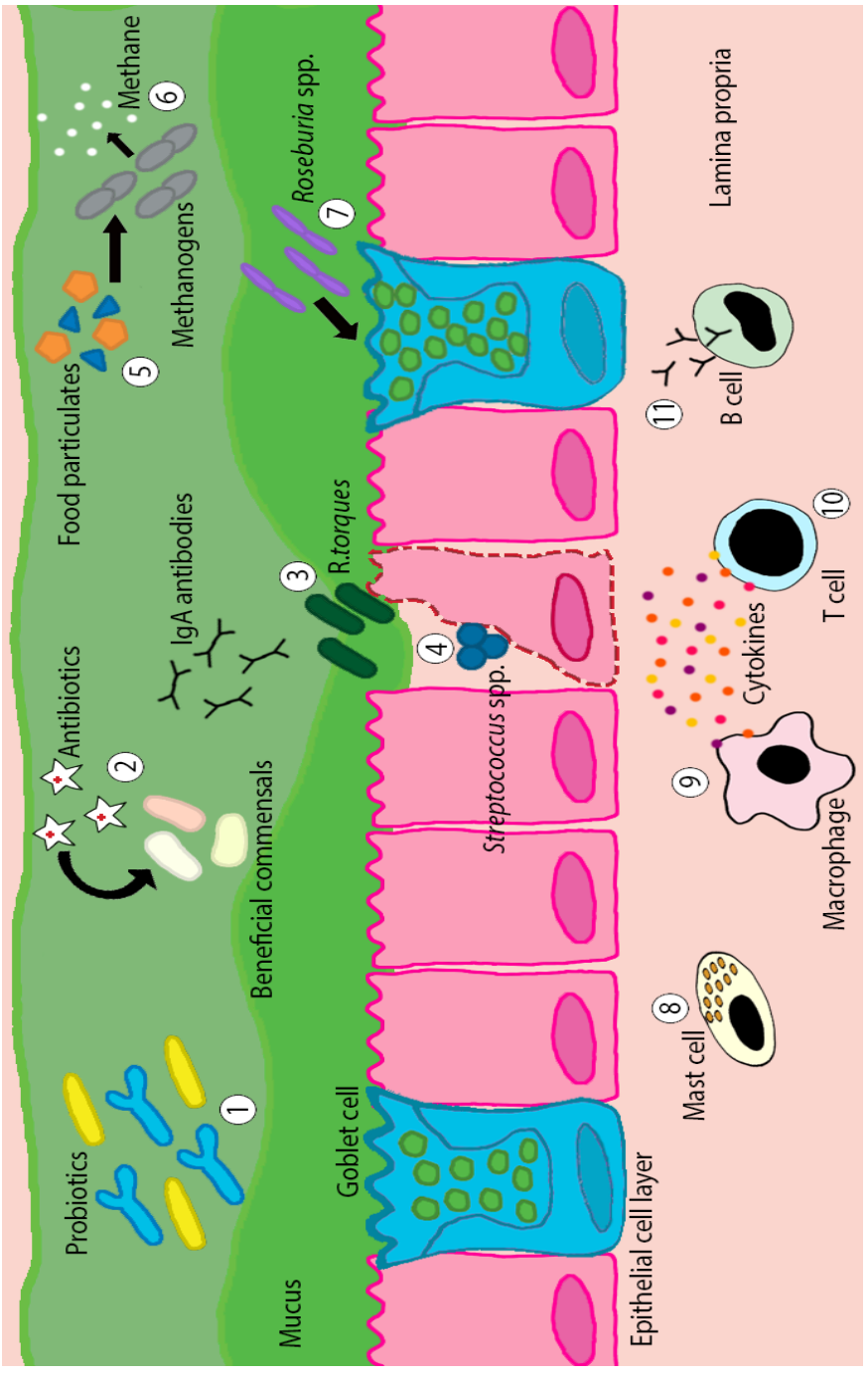
Probiotics
1
Antibiotics
2
Beneficial commensals
IgA antibodies
3
R.torques
4
Streptococcus spp.
Food particulates
5
Methanogens
6
Methane
Roseburia spp.
7
Mucus
Goblet cell
Epithelial cell layer
8
Mast cell
9
Cytokines
Macrophage
10
T cell
11
B cell
Lamina propria

Figure 3. Illustration of host and microbiota interactions in the gut of IBS patients.

Often recorded at lower levels in IBS patients, therapeutic administration of probiotic species such as *Bifidobacterium spp.* and *Lactobacillus spp.* have been shown to have positive effects on symptoms of IBS through their anti-inflammatory metabolites (1). Antibiotic use can have potential side effects such as depleting levels of beneficial commensal gut microbiota thus opening niches for non-specific species to establish themselves (2). Species such as *R.gnavus* and R.*torques* are mucin degraders which may breach the mucus barrier allowing for potential pathogenic infiltration (3). Potential inflammation causing species including *Streptococcus* spp. or Staphylococcus aureus may enter into the epithelial layer and provoke an immune response (4). Diet plays a role in gut microbiota composition since nutrients not absorbed by the host become energy for both beneficial and non-beneficial gut microbiota (5). Found to be increased in IBS patients, the non-beneficial gut microbiota Methanogens produce methane which has been shown to slow down gut transit, potentially leading to constipation (6). Beneficial species such as *Roseburia* spp. produce butyrate, known to help to maintain normal intestinal barrier function through regulation of colon epithelial mucin gene MUC2, a primary component of mucus (7). A potential intestinal dysbiosis of IBS patients may lead to, or be the result of, an altered activity of the mucosal immune system. Although still under debate, increased density of activated mast cells in the mucosa might provoke symptoms (8). Altered macrophage density or function in IBS patients has been suggested leading to a hampered recognition of pathogenic microbiota (9). Possibly, an increased presence or activation of T cells may contribute to symptom generation (10). Also, higher levels of flagellin specific antibodies, as reported in IBS patients, sugests an increased B cell activity (11)[70].

This gene is found in all prokaryotic organisms and has several functions including acting as a scaffold which defines the positions of the ribosomal proteins[79]. Due to its slow rate of evolution, the 16S sRNA gene has several highly conserved regions which can be targeted for polymerase chain reaction (PCR) amplification using universal primers[80]. Once the conserved regions have been targeted, the nine hypervariable regions of the gene (V1-V9) allow for species identification[81] and eventual creation of a bacterial profile can be made for an individual. The current dogma for the ratio of bacteria cells to human body cells is 10:1 sometimes 100:1 from the widely referenced D.C Savage paper and progenitor paper by T.D. Luckey of the 1970's[82,83]. However a revision performed in 2016 calculated that the number of bacteria cells is $3.8 \cdot 10^{13}$ in a 1:1 ratio to the human cells numbering $3.0 \cdot 10^{13}$, though this ratio estimation is dependent on the inclusion or not of the non-nucleated red blood cells[84]. These $3.8 \cdot 10^{13}$ bacteria calling the human body home can be grouped into one of over 50 respective phyla[85] of which 29 have culturable representatives[86]. Although ten phyla have been discovered in the gut[87] the majority of the bacteria are classified as either Firmicutes or Bacteroidetes and are termed Gram-positive or Gram-negative respectively due to the composition of their cell wall structure[87,88].

In a less Linnaean manner, bacteria can be grouped into three categories, commensals, pathogens and beneficial bacteria (**Figure 3**). Commensal bacteria are those "who eat at the same table" as us and generally cause no harm but serve no direct benefit either. Some exceptional species such as *Bacteroides thetaiotaomicron* modulates the expression of genes involved in nutrient absorption[89]. However, the majority of commensals help passively through filling distinct colonization niches[90] and outcompeting for resources so that pathogenic "bad bacteria" cannot gain and sustain an important foothold. These pathogenic bacteria could be

divided into two categories, those which are pathogenic by nature and begin to cause harm once they have entered the host e.g. *Vibrio cholerae* and those which are potentially pathogenic. Potentially pathogenic bacteria e.g. *Clostridium difficile* are similar to commensals, since at low abundance they cause no harm, but if overgrowth occurs and numbers increase past a certain threshold then their activity can become malicious and cause problems for the host[91]. Infectious enteritis and diarrhoea are associated with *C.difficile* infection with a risk of developing PI-IBS once the infection has been treated, but estimates of the risk are contradictory[92,93]. Finally, there are species of bacteria which are beneficial for the host and are thus "good bacteria" or "probiotics" included in genera such as *Bifidobacterium* and *Lactobacillus*. These bacteria may secrete inhibitory substances, known as bacteriocins, which have a similar effect to narrow spectrum antibiotics[94,95], or produce metabolites such as lactic acid and short chain fatty acids (SCFAs) e.g. butyrate, which not only inhibits growth of pathogenic bacteria by lowering the pH of the surrounding tissue, but also 'feed' the epithelium of the gut and even aid in its repair[96-98] (**Figure 3**). Such probiotics are ingested by many individuals for their potential beneficial effects. Research has been performed and has shown some positive effects of probiotics on the symptoms of IBS[99-101], though this is not always the case[102-107] suggesting that they may only benefit a subgroup of patients. Generally, diversity helps to support health and minimize pathogenic takeover[108] meaning that the microenvironment of the gut should have broad species richness with an even representation of each species in the community, known as α-diversity.

A healthy gut ecosystem composition or 'microbial profile' is advertised as having high diversity yet balance and thus also being in a state of 'eubiosis' (Greek eu = good/healthy, bios = life) meaning that there is a healthy balance of the good bacteria and pathogens in the GI tract. However, the actual constituents of what make a microbial profile 'healthy' are still under investigation. Efforts are ongoing and the Human Microbiome Project for example found that species like *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* were common in the gut among healthy individuals as previously demonstrated[89]. However, while more homogenous than the oral or skin microbial profiles, those of the gut of healthy individuals are not identical[109,110]. Moreover, it has even been claimed that based on the composition of our gut microbial profiles, we all belong to one of three enterotypes[111] whereby either *Bacteroides, Prevotella* or *Ruminococcus* are the enriched genus, but this is still a topic of further investigation[112]. The previously mentioned studies included only healthy subjects yet considering the intersubject variability, this approach can only get you so far before the question is asked as to what is potentially keeping the GI tract of these subjects healthy. While relatively stable, large changes in the healthy microbiota composition may lead to a permanent imbalance known as dysbiosis (Greek dys = bad, bios = life)[113,114]. Coined by the Russian zoologist and Nobel Prize laureate Élie Metchnikoff, dysbiosis is defined as an imbalance of the microbiota of the GI tract associated with an increase in pathogenic species and subsequent decrease in beneficial species. This shift in the communities, generally after some form of perturbation, is suggested to be associated with conditions such as obesity[115], diabetes[116], metabolic syndrome[117], cardiovascular disease[118], and IBD[119]. It has also been demonstrated that symptoms of IBS may manifest when a disruption to this ecosystem occurs [120,121].

A change in microbial composition is identifiable, yet it needs to be put into context by which the alterations need to be attributed to either the healthy or non-healthy state. This can be achieved by comparing profiles of healthy subjects to those who are not healthy, e.g. IBS patients. Although a relatively new field of research in IBS, there are an ever increasing number of studies investigating the composition of intestinal microbiota in IBS[15,70,78,122,123]. However, these studies can provide inconsistent results regarding the abundance of certain bacteria.

Good bacteria

Examples of inconsistency in findings from studies performing microbial analysis in IBS are the probiotic genera *Lactobacillus* and *Bifidobacterium* (**Figure 3**). While one might expect these beneficial bacteria to be lower in patients compared to healthy subjects as has been demonstrated in some studies[123-127], several studies have found an increase[122,123,125,128-131] or even no change[125]. The reduced levels of butyrate producing bacteria including *Eubacterium*, Faecalibacterium and *Roseburia* spp.[123,127,132,133] may potentially be an ancillary cause for IBS symptoms in some patients since inhibition of potentially pathogenic species such as *Campylobacter* spp., *Salmonella* spp., *Shigella* spp. and *E. coli* [134] is then hampered.

Detrimental bacteria

Several genera and species known to have detrimental characteristics have been identified to be increased in IBS and might in part cause or exacerbate symptoms in some patients (**Figure 3**). One such feature is the ability to degrade mucin[122,135], a family of high molecular weight, heavily glycosylated proteins that form the protective mucus barrier resting over the epithelium of the respiratory and gastrointestinal tracts[136].

Increased abundance of bacteria with this ability such as *Ruminococcus* spp. and *Akkermansia* spp. as well as phylotypes of Clostridium Group XIVa related to *R. gnavus* and *R. torques* have been found in patients[122,123,137] to the degree that Rajilić-Stojanović et al. suggests them to be markers of IBS[122]. Degradation of the mucus barrier might allow for the potential infiltration of pathogenic bacteria such as *Streptococcus* spp. or *Staphylococcus aureus* demonstrated to be increased in patients[122,123,138] into the tissue. Increased *Streptococcus* spp. being of particular interest considering that it has been shown to have a positive correlation with the pro-inflammatory cytokine interleukin 6 (IL-6)[139]. Additionally, *Dorea* a species capable to produce formic acid has also been found to be associated with IBS in children[137]. Finally, a branch of archaea called Methanogens because they convert hydrogen to methane, have been demonstrated to be increased in IBS patients, and especially in those with constipation predominance[140]. Although thought to be inert[141], methane has been demonstrated to reduce transit time[142] and might be one explanation for constipation in IBS-C[143,144].

Dissimilarity in microbial profiles irrespective of being healthy or not is likely caused by the many factors known to influence which bacteria are residing in the gut. One study found 69 clinical and questionnaire-based covariates which associated to microbiota composition with stool form, self assed through the BSF, emerging as the top feature covarying with faecal microbiome composition[145]. However, of these covariates, only seven percent accounted for the variations in the microbiome with the study suggesting genetics as having a significant role[145]. Research investigating the role of genetics on microbial composition in the gut is ongoing[146,147] but because the field is relatively new, studies are lacking with even fewer focusing on genetics and microbiota in relation to IBS[148,149].

Two factors which are however more researched and demonstrate abilities to alter gut microbiota are diet[150] and medications, specifically, antibiotics[151,152].

Medication use in IBS

Neither medications in general, nor specific antibiotics were investigated during the course of this PhD project, but it is clear that they must be considered in any study looking at the gut microbiota (**Figure 3**). Antibiotics are either narrow-spectrum, effective against one specific family of bacteria, or broad-spectrum, which targets a wider range of bacteria. Irrespective of type, antibiotics will attack indiscriminately both pathogens and commensals alike and can thus cause dysbiosis[151,153] which may potentially lead to symptoms of IBS[154,155]. The findings showing reduction in IBS symptoms through the use of non-absorbable antibiotics such as neomycin[156] and rifaximin[157] support the influence microbiota has on gut wellbeing and how the restoration of intestinal microbial eubiosis may help some patients with IBS.

Diet and IBS

As a fact most of the food you eat is broken down and absorbed by the body. Generally, we are adapted to getting sustenance from a meal with our range of enzymes, e.g. amylases used in the breakdown of starch to sugars, and secretions e.g. bile for fat breakdown. However, there are foods which contain poorly absorbed carbohydrates, fermentable carbohydrates including oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs), or which we cannot breakdown such as non-digestible carbohydrates e.g. fibre which become a food source for our microscopic intestinal passengers (**Figure 3**).

Some of these foods not absorbed by the host are referred to as prebiotics and could be likened to fertilizer which promotes the growth of many favourable species of bacteria already living in the colon. Considering the suggested dysbiosis occurring in some IBS patients the use of prebiotics to rectify the imbalance might potentially be a solution for some patients. However, there have been few studies and fewer randomized controlled trials evaluating the efficacy of prebiotics on reducing the symptoms of IBS and those which have been performed are conflicting. The positive effects of prebiotics on symptoms of IBS have been demonstrated such as reducing anxiety, bloating and lowering flatulence[158-162]; however other studies showed no effect or have found the very opposite occurring whereby prebiotics counteractively intensify bloating and flatulence[158].

Diet is a factor which many IBS patients are cautious with in their everyday lives (**Figure 2**). Approximately two thirds of patients associate the intake of food as instigating or exacerbating their symptoms as has been consistently demonstrated[163-165]. A higher degree of food-related symptoms appeared to be predictable in patients of the female sex, as well as in those with anxiety[163]. As previously mentioned, prebiotics actually cause symptoms in some patients and this might be because some of them, namely fructans and galacto-oligosachairdes (GOS), are also FODMAPs. An overview of what FODMAPs are, which foods they can be found in as well as how they escape absorption by the host is given in **Table 1**.

Table 1: Overview of FODMAPs (Fermentable Oligosaccharides, Disaccharides, Monosaccharides And Polyols)

FODMAP		Mechanism through which they escape small bowel absorption	Examples of food items with high FODMAP content
Oligosaccharides	Fructans, Galacto-oligosaccharides (GOS)	No small intestinal hydrolysis	Grains: wheat, rye, barley Vegetables: onion, leek, garlic, peas, artichoke Fruit: nectarines, watermelon Legumes: beans, lentils Nuts: pistachio, cashews
Disaccharides	Lactose	Hypolactasia in 10-95%; lactose maldigestion	Dairy: milk, ice cream, custard
Monosaccharides	Free fructose, i.e. fructose in excess of glucose	Slow active absorption; poor in 30-60%	Fruit: apples, pears, cherries, mangoes, watermelon Vegetables: asparagus, sugar snap peas, artichoke Sweeteners: high-fructose corn syrup, honey
Polyols	Sorbitol, mannitol, lactitol, erythritol, maltitol, xylitol, isomalt	Slow passive absorption; variable between individuals	Fruit: apples, apricots, blackberries, nectarines, pears, plums, peaches Vegetables: cauliflower, mushrooms Artificial sweeteners: sorbitol, mannitol, isomalt, xylitol

Fructans and GOS are examples of oligosaccharides and are found in large quantity in peaches, onion and lentils to name a few (**Table 1**). They are not able to be broken down in the GI tract due to our lack of enzymes, so they remain unabsorbed and reach the large intestine[166]. Disaccharides are found in table sugar (Sucrose), barley (Maltose), corn syrup (Isomaltose), and food additives (Trehalose), with the one most likely heard of being found in dairy products e.g. milk and ice-cream (Lactose) (**Table 1**). Absorption of disaccharides is not normally a problem; however the deficiency of lactase in some people leads to lactose maldigestion leading to lactose entering the colon. As the most basic unit of carbohydrates the absorption of monosaccharides such as glucose and galactose is effective in the small intestine. Fructose however, as found in fruit, honey and most root vegetables, has a varied absorption ranging from 5-50g absorbed per meal under normal conditions[167] (**Table 1**). The highest absorption rate is achieved through the facilitated uptake by glucose, though if fructose is in excess of glucose, this process becomes saturated and a malabsorption occurs, which may lead to symptoms associated with IBS[168]. The "P" in FODMAP standing for polyols are sugar alcohols, whereby part of their chemical structure resembles sugar and part resembles alcohol. They occur naturally in some fruit and vegetables such as apples (Sorbitol) and cauliflower (Mannitol), but are typically manufactured for commercial use (**Table 1**). Found also in processed foods and products, Maltitol, Lactitol, Xylitol and Erythritol will make your chewing-gum and other oral hygiene products palatable, as well as sugar free sweets and many weight loss snacks. Only about one-third of polyols are taken up by the body with absorption being generally slow and very dependent on the individual and type of polyol. For healthy individuals the consumption of FODMAP-rich foods does not cause any noticeable upsets (with the

exception of ingestion of large amounts of polyols which have a laxative effect[169,170]). However when a person is susceptible then symptoms can be generated[166].

As FODMAPs enter the large intestine two processes occur which can result in the build-up of gas and the influx of water into the colon. Fermentation of the carbohydrates by bacteria is possible due to their wide repertoire of enzymes which far outnumbers our own personal assortment[171]. Aside from creating 5-10% of our energy requirements[172] bacterial fermentation creates gases such as hydrogen which has been demonstrated to be produced in higher quantities in some IBS patients[141] as well methane, demonstrated to be associated with constipation[142]. This gas production causes distention of the bowel giving the feeling of being bloated and may coincide with pain and flatulence[16,173]. Another mechanism occurring which FODMAPs can cause problems is osmosis whereby liquids flowing from a high concentration i.e. the tissue of the gut to a low concentration i.e. the lumen over a semi-permeable membrane. Beginning in the small intestine, once water has been drawn into the intestine the consistency of stool will become looser and an affected individual might start to experience diarrhoea[16,53,174].

Dietary management and interventions have for a long time been one of the basic treatment options for patients with IBS. The relative ease in implementation make it appealing, but few randomized controlled studies of dietary therapy for IBS have been performed[175-177] and long-term effects of restriction diets are currently unknown. Dietary recommendations from the British Dietetic Association[178] and National Institute for Health and Clinical Excellence (NICE)[179], include first-line dietary suggestions, which most likely only moderately impact the gut microbiota[180,181]. The low FODMAP diet is however a strict restriction

diet, which promotes restricted consumption of FODMAP-rich foods. FODMAPs can be removed completely or can be re-introduced overtime to identify which specific FODMAP might be the culprit, since not all FODMAPs are created equal and might affect patients differently. This dietary approach is relatively new and the safety of a restriction diet is still unknown, with this diet having been shown to impact the gut microbiota composition[182,183]. In a low FODMAP diet, while the food you are removing might be problematic, some are still classified as prebiotics. Thus, the patient is removing the food source for beneficial bacteria, causing their starvation and decrease, which has been found in previous studies[177,184]. However, if this has a long-term negative impact in the gut health or promotes dysbiosis which could lead to something more severe is unknown. The problem with any dietary therapy is that it is very difficult to predict who might respond or not. This may often result in a patient following an arduous regime of food restriction, which negatively impacts their nutritional intake and gut wellbeing, yet does nothing for their IBS symptoms.

With the knowledge that the microbiota composition is susceptible to many different factors, these factors should be taken into consideration when trying to identify the underlying cause for the altered microbial composition as suggested in IBS patients (**Figure 3**). Although they can sometimes be problematic, our dependence on gut bacteria is well documented[185,186], to the degree that some regard the microbiota as a neglected organ[187]. Work on germ free mice i.e. mice, which have been raised to be completely devoid of any microbes[188], has demonstrated that the lack of bacteria leads to a range of physiological alterations. The sterile milieu of a germ free mouse leads to development issues such as altered amount[189] and physiology[190] of mucus, altered gastrointestinal physiology[191], different brain development and behaviour[192], as well as an

immature immune system[193], to name a few when comparing to healthy mice with gut microbes. The results of a germ free upbringing have of course not been tested in humans, but one could speculate that if an event were to occur which affected the normal development of the microbiota, such as antibiotics early in life, then this may hamper the proper maturation of the immature immune system[194,195], which must be able to both tolerate food antigens and commensal bacteria, but also mount a response against pathogens[196,197].

Barrier of the gastrointestinal tract

Considering that GI tract is in effect constantly exposed to the outside environment defence is needed. The major first line of defence on the human body is the formidable multi-layer barrier of the skin; a physical barrier created by epithelial cells found also lining the respiratory, urinogenital and gastrointestinal tracts. Along these tracts this wall is not solid and is instead semi-permeable and in the GI tract allows for the absorption of water, nutrients and electrolytes from the lumen into the blood[198]. The epithelial barrier is but a single layer of cells held together by tight junction proteins (TJP). Aberrations in the expression of TJP have been reported in IBS patients[199,200] and previous studies indicate an altered intestinal permeability[58,66,201], but conflicting findings exist[202]. The basis of this alteration in permeability is as yet not completely elucidated, although it has been suggested that lipopolysaccharides (LPS) found on the outer membrane of gram-negative bacteria stimulate enhanced intestinal permeability[203]. Although permeability was not investigated in this thesis *per se*, much like medication, it is a factor which must be considered since alteration in mucosal permeability may facilitate microbial translocation into the underlying mucosal tissue, as well as

food particles and toxins, whereby a local immune activation may subsequently ensue[204] leading to symptom generation[205].

Immune activity in IBS

The immune system is complex and in essence is an organism's protective means to minimise damage from toxic insults and stop itself from being taken over by invading pathogens. Divided into two subsystems, the innate immune system provides immediate (within seconds to minutes) defence against infection while the adaptive immune system is slower (after 4-7 days) provides a stronger more targeted immune response adapted to the type of pathogen. White blood cells, also called leukocytes are soldiers of the immune system with various categories, not all covered in this thesis, who have specialized roles in the defence of the host. Studies have suggested that a low grade immune activation is occurring in a subgroup of IBS patients[206,207].

Antigen recognition

Considering that the human body is a complex number of systems linked to each other in diverse ways, the link between the immune system and the microbiota is important and how friend and foe is identified is critical in keeping balance. There are many different mechanisms and pathways in which the innate immune system keeps a homeostatic balance between the host and the microbiota[208] and one of them is though Toll-like receptors (TLRs)[209].

"Das ist ja toll!" was once shouted out in 1985 by German researcher Christiane Nüsslein-Volhard as the Toll gene was, for the first time, associated with host defence. As of now Toll-like receptors come in 14 different varieties with only TLR1 to TLR10 found in humans. TLRs recognize pathogen-associated molecular patterns (PAMPs) such as

structures found on bacteria or viruses, their RNA or DNA or even large structures like flagellin used in bacterial movement. Expressed on macrophages and epithelial cells, as well as other cell types, TLRs have been demonstrated to be altered in IBS compared to healthy subjects[210]. Specifically, toll-like receptors 2, 4 and 5 have higher[211-213] while TLR7 and TLR8 lower[211] expression in colonic biopsies from patients.

Once unwanted microbes have been recognized, signalling cascades begin and lead to an antimicrobial response involving, among others, such components as antimicrobial peptides[214,215]. This crosstalk between the immune system and gut microbiota has been investigated in IBS and has been suggested to be altered[216], but the implications and cause is not fully understood[121]. Moreover, the impact the antibacterial response can have on the gut bacteria is still unclear as well as the degree to which the gut bacteria can modulate the immune systems[217].

Cytokines, signalling proteins of the immune system

A growing number of studies have investigated the immune system of patients with IBS through the measurement of systemic and local cytokines, including chemokines and pro- and anti-inflammatory cytokines (**Table 2**). Cytokines can be thought of as protein signals or messages produced by cells to communicate with other cells. They have a range of functions, but generally induce the activation, proliferation or movement towards a site of infection, inflammation or trauma of immune cells. Pro-inflammatory means that the cytokine pushes the immune system into a more active state and is predominantly associated with infection and inflammation. Regarding findings pertaining to IBS, studies have tended to demonstrate higher levels of circulating plasma/serum IL-1β, IL-6, IL-8 and tumour necrosis factor alpha (TNFα)[206,210,218-222], yet

contradictory findings have also been observed [223]. However studies have demonstrated that psychological conditions such as stress and depression can be associated with an increase in these cytokines[218,219,224,225] which is something to keep in mind considering that these symptoms are often found in patients with IBS[226]. Anti-inflammatory cytokines serve to quench the fire of the immune system and supress the activity of pro-inflammatory cells so the actions of the cells do not cause too much collateral damage to healthy tissue. Although there are several cytokines, the predominant anti-inflammatory cytokines are TGFβ and IL-10. In IBS, IL-10 has been investigated, yet its systemic abundance in patients compared to healthy subjects is as yet debatable[210,218,219,222,227]. Several pro- and anti-inflammatory cytokines were investigated during this PhD project (**Table 2**).

Investigating the status of the intestinal immune system is more invasive and involves taking a mucosal biopsy. Techniques for mucosal analysis vary and include immunohistochemistry, protein analysis or gene expression analysis[228] to name a few, each suitable in its own way for the hypothesis of the study performed. Mucosal expression of pro-inflammatory cytokines in the gut of IBS patients is less well researched compared to analyses of systemic cytokines. However, the results presented thus far demonstrate a lack of augmented gene expression of IL-1β, IL-6 and TNFα in IBS patients compared to healthy subjects[223,229]. Unlike pro-inflammatory cytokines, the data on mucosal expression of IL-10 in IBS is more consistent and a lower IL-10 expression in patients compared to healthy subjects has been demonstrated[223,229].

Table 2: Overview of cytokines analysed in this thesis

Cytokine	Main Source	Main Targets	Main Function
IL-1β	Macrophages	Macrophages and T cells	Fever, T cell and macrophage activation
IL-2	T cells	T cells	T cell proliferation
IL-4	T cells and Mast cells	B cells and T cells	Mediates antibody-driven responses
IL-5	T cells and Mast cells	Eosinophils	Eosinophil growth
IL-6	T cells and Macrophages	T cells and B Cells	Fever, T and B cell growth and differentiation
IL-8	Macrophages	Neutrophils and other granulocytes	Induces chemotaxis and phagocytosis
IL-10	T, B and DC cells	Macrophages, T, B and DC cells	Immune suppression
IL-12p70	Macrophages	T cells, NK cells	Activation of NK cells
IL-13	T cells	B cells and Mast Cells	Mediates antibody-driven responses
IL-17A	Th17 cells	Mucosal tissues, epithelial and endothelial cells	Induces cytokine production by epithelial cells
TNFα	Macrophage, NK and T cells	Neutrophils, macrophages, endothelial cells	Pro inflammatory, endothelial activation
IFNγ	T and NK cells	Macrophages and NK cells	Promotes NK cell activity

Cells of the immune system

Cells of the innate immune system are on the front line of defence, and provide immediate protection against infection. Macrophages for example patrol just below the epithelial barrier in the tissue called the lamina propria (**Figure 3**). They engulf pathogens and damaged/dying cells and subsequently break them down. There are reportedly several active forms of macrophage[230]. For example, the M1 "killer" macrophage is activated by the cytokine interferon gamma (IFNγ) as well as LPS. M1 macrophages secrete high levels of IL-12 which not only activates natural killer cells (NK-cells) of the innate immune system but also induces the differentiation of T-cells into T helper (Th) 1 lymphocytes of the adaptive immune system[231] (**Figure 3**). The M2 "repair" macrophages, activated by IL-4 and IL-13 primarily promote wound healing, tissue remodelling and attract the regulatory T cells (Treg), Th2, eosinophil and basophil cells[231]. Studies investigating specific M1 and M2 macrophage populations in IBS are lacking however general macrophage abundance in IBS are conflicting whereby they have been both demonstrated to be decreased[232], as well as increased[66,233] in patients.

Upon activation mast cells, part of the innate immune system, which are important in the defence against parasites such as worms, yet also involved in allergic reactions, release an array of biologically active substances including histamine, serotonin and proteases. In the context of IBS, mast cell tissue infiltration was associated with the frequency of abdominal bloating[234] while their proximity to nerve fibres which might become stimulated after granule release might evoke visceral pain[13] (**Figure 3**).

T helper cells denoted as cluster of differentiation (CD) 4^+ cells are one arm of the adaptive immune system and differentiate into subtypes known as Th1, Th2, Th17, T Follicular Helper (Tfh) Cells and Regulatory T cells (Tregs)[235]. Professional antigen presenting cells (APCs) are able to take up an antigen, break it down and present fragments of it on its surface through the major histocompatibility complex II (MHC II) to activate $CD4^+$ T cells. One of the professionals are the dendritic cells (DC)[235], on which very little research relating to IBS has been performed. So far, one study demonstrated an increase of $CD103^+$ DCs in the colonic mucosa of IBS patients which subsequently stimulated $CD4^+$ T helper cells[236] to secrete IL-4.

Th1 cells instigate the cell-mediated immune response primarily against intracellular bacteria. Their predominantly secreted cytokine is IFNγ which target macrophages and the $CD8^+$ cytotoxic T cells, increasing their killing ability and proliferation, respectively. Th2 cells drive what is known as the humoral immune system, so named because it involves substances found in the bodily fluids, or humors as they were once called by Hippocrates. Cytokines produced by Th2 cells include IL-4, IL-5 and IL-13, which among other things help control against parasitic infection and promote responses mediated by granulocytes e.g. mast cells and are required for the switching of B cells to produce the IgE class of antibody (**Figure 3**). Th17 is another important subset, defined by their production of IL-17. This third class of $CD4^+$ T cell induces local epithelial cells to produce chemokines that mediate the recruitment of neutrophils to infected tissues[237]. The fourth of the cardinal T helper cell subgroups are the Tfh cells which secrete cytokines characteristic of Th1 and Th2 cells. Their main role is in the activation of B cells, allowing them to differentiate, class switch and proliferate. Finally, there are the

immunosuppressive Tregs ($CD4^+CD25^+$) which secrete anti-inflammatory IL-10 and TGF-β which inhibits the activity of the DCs but also seem to have direct effect of effector T cells.

The other branch of the T cells are the cytotoxic $CD8^+$ T cells tasked with limiting internal cellular infection by viruses and bacteria as well as controlling protozoan infection. In order to remove the infected cells without causing healthy tissue destruction the mechanisms employed by the cytotoxic T cell has to be powerful and accurately targeted for specific elimination. Each nucleated cell in the body express on their surface the major histocompatibility complex I (MHC I) on which peptide fragments of proteins from within the cell are displayed. If the peptide fragment presented by MHC I indicates that the host cell is infected then the cytotoxic T cell will act. By programming infected cells to undergo apoptosis, cytotoxic T cells keep the contents of the cell contained without spilling out into the surrounding tissue both sparing the surrounding cells and minimising additional infection. Due to their destructive abilities $CD8^+$ T cells require more co-stimulation to become activated than the $CD4^+$ T cells. The simplest means is by a mature DC, however in the majority of viral infections the additional help from $CD4^+$ T effector cells is required.

Research in IBS determining the specific abundance of differentiated Th1, Th2, Th17 and Tfh cell populations are lacking, nevertheless one study has investigated Tregs and found comparable frequencies between patients and healthy subjects[238]. By using the primary T helper cell and cytotoxic T cell surface molecules as markers, several studies have found an increased abundance and or frequency of activated $CD4^+$ and $CD8^+$ T cells [64,66,234,239-242] in IBS patients compared to healthy subjects. Still, one contradictory study depicted T cells to be decreased in patients[232].

As mentioned, specific T helper cell subgroup studies in IBS are lacking, but the estimation of which population might be more abundant can be roughly elucidated through measuring the level and proportions of circulating cytokines or expression of precursor genes[243]. The levels of individual markers are an indication of the activity of immune cells and can also be interpreted to identify if there is a more Th1, Th2 or even another T helper response occurring as mentioned earlier in the this thesis.

The complexity of IBS as a multifactorial disease

Having made it to the end of this introduction, even having it limited to topics relevant to the PhD project, the complexity of irritable bowel syndrome is something which makes research a difficult task (**Figure 3**). There are always attempts to tease out patients with similar aspects which might be linked to a symptom. When such a group is identified then targeted treatment can be administered and we move one step closer to solving the mystery of irritable bowel syndrome.

Aim

This overall aim of this thesis was to demonstrate how gut microbiota, systemic and intestinal immunity as well as the crosstalk between the two results in symptom generation in patients with IBS. Furthermore, we aimed to demonstrate how dietary intervention affects bacteria of the gut and if patient responsiveness to intervention therapy could be predicted by gut bacteria profiles.

Specific aims:

- To determine how differing diets impacted gut bacteria and if bacterial profiles predict intervention response.

- To determine if immune activity based on cytokine measurements differed between IBS patients and healthy subjects and to establish if immune activity was associated with the severity or pattern of IBS symptoms.

- To determine whether antibacterial gene expression of immune activity defined IBS patients, differed compared to that of healthy subjects, and if antibacterial profiles reflected gut microbiota composition and IBS symptoms.

2

Patient cohorts, Materials and Methods

This section serves to provide an overview of the cohorts (**Table 3**) and materials and methods (**Table 5**) used in this thesis, as well as a description and rational for the methods employed. All studies were approved by the Swedish Regional Ethical Review Board at the University of Gothenburg Paper I (12/08/2013; Dnr 619-13); Paper II and IV (25/01/2010; Dnr 731-09), or the Institutional Review Board of the University of North Carolina; Paper III (NCT: 01072903)

Cohort of Paper I

This multicentre study recruited patients with irritable bowel syndrome from the gastroenterology outpatient clinics of Sahlgrenska University Hospital, Gothenburg; Karolinska University Hospital, Stockholm; and Sabbatsbergs Hospital, Stockholm, Sweden. A total of 61 IBS patients

were included in Paper I (**Table 3**). Of these patients, 30 had been following a traditional IBS diet and 31 had been following a low FODMAP diet for four weeks[244]. Faecal samples were collected once during the screening period and once during the last week of the diet intervention.

Table 3: Demographics of cohorts included in Papers I-IV

		Paper I	Paper II	Paper III	Paper IV
IBS	Total	61	173	246	31
	Sex (F/M)	(51/10)	(119/54)	(190/56)	(16/15)
	Age*	46 (29–57)	30 (24–43)	33 (25–45)	32 (25–44)
	IBS-C	17	41	35	6
	IBS-D	16	69	51	18
	IBS-M	28	63	160	7
Healthy	Total	n/a	58	21	16
	Sex (F/M)	n/a	(36/22)	(21/0)	(9/6)
	Age*	n/a	27 (25–34)	30 (27–44)	27(24–30)

Abbreviations:
IBS-C = Constipation predominant IBS (F/M) = Females/Males
IBS-D = Diarrhoea predominant IBS
IBS-M = Mixed loose and hard stools IBS
*Data shown as median (25–75th percentile)

A traditional IBS diet has emphasis on how and when to eat rather than on what foods to ingest. Examples of the advice received by a patient are found in **Box 3**.

Box 3. Examples of advice given in traditional IBS dietary advice

- Eat small, frequent meals.
- Peel and divide foods into pieces.
- Chew thoroughly.
- Boil food rather than fry.
- Reduce fatty and spicy foods, legumes, onions, coffee and alcohol.
- Avoid carbonated beverages and sweeteners that end with –ol.
- Fibre intake should be evenly distributed over the day.

(McKenzie YA et al. 2016)

The low FODMAP diet might be considered more extreme than the traditional IBS diet since it involves the restriction of food items with high FODMAP contents. Examples of foods to avoid and foods which can be eaten while on a low FODMAP diet are shown in **Table 4**.

Table 4: Examples of FODMAPs advice

Avoid ✗	Okay to eat ✓
Apples, pears	Blueberries, raspberries
Apricots, plums	Citrus fruits, banana
Beans, lentils	Celery, lettuce, carrot
Cabbage, cauliflower	Olives, potatoes
Onions, beans	Spinach, zucchini
Milk products	Lactose-free milk products
Wheat, barley, rye	Oats, gluten-free, spelt
Pasta	Rice, polenta

Cohorts of Papers II

Patients of this cohort were recruited through the outpatient clinic of Sahlgrenska University Hospital, Gothenburg, Sweden. Healthy subjects were volunteers with no prior history of GI disorders or current bowel symptoms. A total of 173 IBS patients and 58 healthy subjects were included (**Table 3**). From this cohort, serum was collected from 144 patients and 42 healthy subjects, while sigmoid colon mucosal biopsies were collected in 109 patients and 36 healthy subjects.

Cohort of Paper III

The 247 IBS patients in this American cohort were recruited by physician referrals or advertisements at the Center for Functional Gastrointestinal and Motility Disorders, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. Twenty-one healthy subjects were recruited by advertisement and were paid for their participation (**Table 3**).

Cohort of Paper IV

Patients of this cohort were recruited through the outpatient clinic of Sahlgrenska University Hospital, Gothenburg, Sweden. Healthy subjects were volunteers with no prior history of GI disorders or current bowel symptoms. A total of 31 patients and 16 healthy subjects were included, from which a sigmoid colon biopsy and a faecal sample was collected from each individual. An additional 12 patients with inflammatory bowel disease (IBD) with active inflammation were recruited at the endoscopy units at Sahlgrenska University Hospital, Gothenburg and Södra Älvsborgs Hospital, Borås, Sweden.

Table 5: Overview of methods used in each study of this thesis

		Paper I	Paper II	Paper III	Paper IV
Physical assessments	Rectal barostat		X	X	
	Oroanal Transit Time		X		
	Motility Index				
	Bristol stool form	X	X	X	X
Questionnaire assessment	Hospital Anxiety and Depression Scale		X		
	Brief Symptom Inventory Anxiety and Depression			X	
	Food Diary	X			
	IBS Severiry Scoring System	X	X	X	X
	Patient Health Questionnaire 15		X		
	Recent Physical Symptoms Questionaire			X	
	Comorbid Medical Conditions Questionnaire			X	
	Catastrophizing			X	
Laboratory Analyses	Serum protein immunoassay		X	X	
	Polymerase chain reaction		X	X	
	Microbial analysis	X			X

Clinical Analysis

Although characterised by altered bowel habits and abdominal pain, the pattern of symptoms experienced by patients with IBS can vary. A strong aspect of this thesis is that the cohorts used, were very well characterised. Both healthy subjects, but primarily patients completed a battery of questionnaires and underwent various clinical assessments with the aim

to accumulate as much relevant symptom data as possible. The databases were thus veritable "smorgasbords" of measurements with each recording having a potential link to the expression level of a gene, amount of a protein in the serum or abundance of a bacterial species in the gut to name a few.

Physical assessments

Colorectal sensitivity testing

To measure colorectal sensitivity, a barostat was used, which in essence is an electronic pump which incrementally inflates a balloon positioned in the rectum or colon of the subject to pre-defined pressures (mm Hg). The subject is then asked to report when they first feel a sensation that the balloon is inflating, that they have a desire to defecate, an urge to defecate, discomfort and finally pain. The recorded balloon pressures at these points are thus the sensory thresholds, displayed in mmHg[245,246].

Oroanal Transit Time (OATT)

Subjects ingested 10 radiopaque markers daily for six days ending the week with an overnight fast. The number of remaining markers in the gut were then counted using fluoroscopy on the seventh day; the number of markers divided by ten gives the oroanal transit time in days. Subjects who retained more markers had a slower transit time than those with fewer rings still remaining[7].

Motility Index

These indices were calculated after measuring phasic contractions measured with manometry using a combined balloon-manometry catheter in the fasting state to obtain a baseline (BMI), during balloon distention (DMI) and recovery (RMI) and 30 minutes post-meal (PMI)[245].

Bristol stool form (BSF)

Commonly during a one week period, subjects record the form of their stool by grading it on a scale from 1 to 7 whereby 1 = separate hard lumps and 7 = completely liquid[11]. After, patients were characterised as having IBS with constipation (IBS-C) or IBS with diarrhoea (IBS-D). Patients with IBS with mixed loose and hard stools (IBS-M) and those who had unsubtyped IBS (IBS-U) were combined into one group (IBS-nonCnonD)[11].

Questionnaire assessment

Anxiety and Depression measurement

Hospital Anxiety and Depression Scale (HAD)

Seven questions each for anxiety and depression with each question answered on a Likert scale (0-3)[247].

Brief Symptom Inventory Anxiety (BSI-A) and Depression (BSI-D)

On a scale ranging from "not at all" to "extremely", the amount of psychological distress 18 symptoms caused a subject during the past week is assessed[248].

Food Diary

A four day food diary was completed by all patients of this study once during the screening period and once during the last week of the 28-day intervention. The average daily intakes were calculated DIETIST XP V.3.1 (Kostdata.se, Stockholm, Sweden) for energy, monosaccharides, lactose, dietary fibres, and FODMAPs as described in detail in Böhn *et al.*[175]

IBS Severity Scoring System (IBS-SSS)

Used to assess the perceived severity of abdominal distention, abdominal pain and its frequency, dissatisfaction with bowel habits and the interference of IBS symptoms with daily life; this five question method is widely used and places patients into subgroups of mild (75-175), moderate (176-300) or severe (>300) IBS symptoms. A 50 point reduction is considered as a clinically significant improvement in symptom severity as was used to measure the effectiveness of the dietary interventions, identifying patients with a 50 point reduction as responders to the therapy[249].

Patient Health Questionnaire 15 (PHQ-15)

A means to assess perceived severity of 15 different somatic symptoms using a scale ranging from 0 (not bothered at all) to 2 (bothered a lot) for each symptom[250].

Recent Physical Symptoms Questionnaire (RPSQ)

A measure of the psychological tendency to report any of the 26 non-gastrointestinal physical symptoms that are significantly more common in IBS patients compared to healthy subjects, with a higher frequency than 'never or only once' in the past month[251].

Comorbid Medical Conditions Questionnaire (CMCQ)

Provides an index of the subject's number of medical comorbidities from 0 to 16 non-gastrointestinal diagnoses as diagnosed by a physician[251].

Catastrophizing

Six items from the Coping Strategies Scale[252] was used to gauge how subjects expressed feelings of hopelessness and the expectation that pain (if they are patients) will worsen.

Laboratory Analyses

Serum

In the process of making cheese, the solid curds separate from the liquid known as 'whey'. In a similar manner, once blood has been under centrifugation and the red and white blood cells, the platelets as well as the clotting factors have formed a pellet at the bottom of the tube, the left over liquid with a yellow tint is known as serum as from the Latin for whey. Serum allows for a systemic view of the immune system, whereby levels of circulating markers of immunity can be assessed. The fairly simple collection process makes serum analysis common within research as compared to taking a biopsy and in some cases, study subject dependent, easier than obtaining a faecal sample.

In the first study investigating the immune system in IBS, venous blood samples from healthy subjects and IBS patients were collected in 9ml tubes without additives. Serum was extracted after the samples were centrifuged at room temperature. Once aliquoted into separate tubes, the serum was frozen until further analysis using the Meso Scale Discovery (MSD) array (MSD SCALE DISCOVERY, Rockville, MD). The MSD platform is relatively new immunoassay using electrochemiluminescence for the detection of a broad range of targets. Its sensitivity and range out performs previously used methods such as Luminex® and so was implemented for this study due to the very low levels of systemic cytokines in patients with IBS. The assay used covered cytokine markers for T-helper 1, Th2 and Th17 responses. The cytokines covered were thus IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, interferon gamma (IFN-γ), and tumour necrosis factor alpha (TNFα)[253]. Considering the exploratory nature of the study, this broad range was thus chosen to

elucidate which, if any, of the distinct pathways was driving symptom generation in IBS.

In the second study of immune activity in IBS, the serum cytokine levels were analysed using a different method. During the period between waking up and breakfast, blood was collected in serum-separating tubes from healthy subjects and IBS patients. Within two hours of collection, the samples were left to stand for 30 minutes and then spun at 3,000 rpm for 10 minutes at room temperature. Once serum was extracted, it was frozen at -80°C until analysis was performed. Previous studies have demonstrated alterations in the serum levels of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα and anti-inflammatory IL-10 between healthy subjects and IBS patients and were thus focused on in this study[254,255]. The serum was analysed using a high sensitivity multiplex assays (Bio-Plex 200, Bio-Rad, Hercules CA, using FMAP reagents from R&D Systems, Minneapolis, MN). Measurements which were under the detection limit threshold were set as the respective detection limit threshold.

Mucosal biopsy

In this thesis mucosal biopsies were collected from the unprepared colon of IBS patient i.e. there were no laxatives given to the patient since this can interfere with the faecal and mucosal adherent bacterial composition. From IBD patients rectal biopsies were collected. Taking a biopsy is required for the local analysis of expression of genes of interest as well as the abundance of mucosal adherent bacteria.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a commonly used method for the quantification of how much a gene is expressed at the RNA level. The amplification of cDNA prepared from RNA in homogenised tissue is performed though polymerase reactions.

After a given number of cycles the fluorescence of the sample is able to be detected with that cycle number equitable to the amount of mRNA from the target gene of interest. This can then be compared between samples. In Paper II qRT-PCA was used with reference housekeeping genes 18S, POLR2A and RPLP0, of which the average expression was used to normalize the expression of the targeted gene sequence.

Human Antibacterial Response RT² Profiler PCR Arrays (Cat No.ID PAHS-148Z, Qiagen) were conducted on intestinal biopsies as previously described[256], to profile the expression of 84 key genes involved in innate immune response to microbes. B2M, GAPDH and HPRT1 were chosen as reference housekeeping genes.

Faecal samples

As mentioned, faecal samples are in general the easiest sample to obtain and can be used for the assessment and quantification of the gut microbiota. In this thesis two different methods were used for this analysis.

Gut bacterial analysis

Method I

The commercially available test, GA-map™ Dysbiosis Test[257] (Genetic Analysis AS, Oslo, Norway) was used. Briefly, the GA-map™ Dysbiosis Test[257] output is a bacterial profile and a Dysbiosis Index (DI) score. A DI >2 (maximum 5) indicates a bacteria composition that differs from a healthy reference group and are as such considered to be dysbiotic[257].

Method II

The microbial DNA was extracted from both the faecal and mucosal biopsy samples as previously described[14]. Briefly, the hypervariable 16S

rRNA regions (V5-V6) were amplified and analyzed using titanium chemistry on a 454 Life Sciences Genome Sequencer FLX instrument (Roche, Switzerland). Richness was assessed through the number of observed operational taxonomic units (OTUs) verified at the same sequence depth. The α-diversity was calculated using the square Shannon index using the vegan R package as previously described[14]

Data and statistical analysis

A cornerstone in scientific research is statistical analysis and is the means through which raw data is put into a meaningful context. The hope for a respectable probability value (*p*-value), typically <0.05, is at the forefront of any researchers mind. The *p*-value ranges from 0-1 and having a low *p*-value signifies that the null hypothesis i.e. the claim about a population e.g. "Serum levels of Interleukin 6 are the same in IBS patients compared to healthy subjects" is false. Statistics also involves mathematical models which are not unlike a scaled-down model of a skyscraper or a boat; these models could be used to test various factors such as strength and then predict how long the real world version would last. In mathematics, a statistical model is a suitable summary of the data collected and should summarise the data as close as possible i.e. 'be a good fit' while being as simple and easy to comprehend as possible. Since we cannot measure the Swedish or let alone global population of IBS patients, the best we can do is to take a sample and to make generalisations using a representative summary i.e. a statistical model.

Univariate analysis

One of the most common statistical analyses is that which focuses on comparing one variable (univariate) between two or more groups. Depending on if the data follows a normal (Gaussian) distribution (a bell

curve shape if the data was plotted on a graph) e.g. the height of people in a classroom, or not e.g. amount of pro-inflammatory protein in the blood, denotes which type of analysis should be performed i.e. parametric or non-parametric respectively[258]. Aside from plotting out the data on a graph which is often impractical, different methods can be used to quickly test if the data follows a specific distribution such as the Kolmogonov-Smirnov and Anderson-Darling or, as used in this thesis, the Shapiro-Wilk test[259]. The majority of data analysed in this thesis was non-parametric and thus the Mann-Whitney *U* test was used for comparing two groups e.g. IBS against healthy subjects, while the Kruskal-Wallis test was used to compare three or more groups, e.g. IBS-C against IBS-D against IBS-M. For correlations non-parametric Spearman's rank coefficient was used. If one variable increases with the other variable, then there is positive correlation, denoted as a value from 0 to 1. If a variable decreases while the other variable increases, then there is a negative correlation, denoted as a value from 0 to -1. Univariate statistical analysis was performed using both GraphPad Prism V.6.04 (GraphPad Software, California, USA) and SPSS statistical package, V.21.0 (SPSS, Chicago, Illinois, USA).

Multivariate analysis

As has been said countless times, groups of patients diagnosed with irritable bowel syndrome are heterogeneous, and the causes behind this have been touched upon in this thesis, but not the inherent problems this can have when performing research. While univariate analysis is often sufficient when groups are well-defined e.g. IBS-D, the degree to which the groups are defined might always be improved such as further defining IBS-D patients based on the cause of their diarrhoea e.g. infectious, dietary induced or malabsorption of bile. The multivariate analysis used

in this thesis could be said to take a different stance when comparing healthy subjects to IBS patients or subsets thereof. Instead of focusing on one variable at a time and investigating how it differs between two groups of many individuals, multivariate analysis takes multiple variables (X variables) and analyses their relationship not only between individuals of groups (Y variables) but the relationships of the variables being investigated. All multivariate analysis performed in this thesis was done using the SIMCA software (Version 14.1.3.0, copyright © MKS Data Analytics Solutions). Here we will now discuss the two methods of multivariate analysis used and the pros and cons of each.

Principal Component Analysis (PCA)

Principal Component Analysis is an unsupervised method, meaning no prior assumptions in regards to possible underlying variables or characteristics are made. This method removes dimensionality and can effectively define differing groups based upon the variables of the model. The model creates a score plot on which all subjects (Y variables) are positioned in relation to each other, based on the levels of each X variable (cytokine, bacteria etc.). In a PCA the R^2 parameter represents the goodness of the fit of the model. Used in Paper III, we investigated if patients with an increased immune activity could be identified using an unsupervised method indicating the presence of natural underlying differences, or if their prior identification was in part due to the supervised method. A loading plot was generated which may be superimposed over the score plot to better visualize which of the X variables (cytokines, bacteria etc.) are associated with the Y variables (IBS patients or healthy subjects). X variables localizing to a group of Y variables on the score plot are indicated to be found at higher levels in those patients/subjects.

Hierarchical Cluster Analysis (HCA)

Unsupervised "bottom-up" hierarchical clustering analysis (HCA) was performed in Paper III to identify clusters of study subjects with similar serum cytokine profiles.

Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA)

The OPLS-DA method is best used to identify inter-group predictive variation in the data along the X-axis and the intra-group differences along the Y-axis, providing the groups are explicitly defined. This supervised analysis is most useful for identifying discriminatory variables between two or more defined groups, and potentially used to predict which group a subject is part of. The Hotelling's T^2 is a multivariate form of Student's t-test implemented to define the normal area ellipse corresponding to either a 95% or 99% confidence limit. Respective to the other subjects, those falling outside of this ellipse are broadly defined as explainable outliers having a different profile of measured X variables. In this thesis a 95% confidence ellipse was used meaning any subjects outside of the ellipse were considered potential outliers which deviate from normality and can skew the model. However not all of these outliers are likely to be "real" outliers. The distance to the model (DmodX) plot indicates how well a subject fits the model with a high DmodX indicating a poor fit and denoting the subject as a weak outlier. If a subject did not fit the model and falls outside of the confidence ellipse, then it was considered a true outlier and was excluded. The R^2 parameter represents the goodness of the fit of the OPLS-DA while the Q^2 represents the internal cross-validation of the model. Although the best possible fit is $R^2=1$ and an optimal Q^2 is 0.7 or higher, when regarding biological variables an $R^2 \geq 0.5$ and Q^2 value ≥ 0.4 is considered satisfactory[260].

Although the graph provides a visual means to represent the data, the images can be misinterpreted since while the split (R^2) might be good (≥ 0.5), the cross validation (Q^2) might be poor (<0.4) indicating poor predictability. Finally, the difference between these two indices should ideally not exceed 0.2–0.3 since this indicates presence of many irrelevant model terms. The reliance of OPLS-DA on the defining of which group the subjects belong to and that it is trying to identify differences between the groups means that it is constrained *per se* by the supervised parameters set on the model by the user. Thus the model has been influenced by the user and is thus not an unmodified view of the data. Additionally, the identification of the immuno-active subset of patients by this method in this thesis is only possible by the inclusion of healthy subjects and thus cannot be used to discriminate patients with an increased immune activity from those with a 'normal' level of immune activity if they have not already been pre-defined. A loading plot was generated to identify which of the X variables (serum cytokines, bacteria etc.) had the most power regarding their ability to discriminate healthy subjects from IBS patients. X variables localizing further away from the center of the x-axis contribute more to the discrimination of the two groups. A loading scatter plot was generated which may be superimposed over the OPLS-DA to better visualize which cytokines are associated with the groups of the cohort, e.g. IBS patients or healthy subjects.

In order to aid the understanding of this statistical method an example is given in **Figure 4**. The levels of 12 different proteins have been measured in the serum of group one and group two and two scenarios are depicted (**Figure 4**). In the first scenario, the protein levels in subjects of group one and two are similar (**Figure 4a and b**). In **Figure 4a** we can see that there is a large overlap of the two groups of subjects indicating

that the protein profiles for each subject are similar. While the score plot gives a visual representation of the model, the R^2 and Q^2 indices are what should be consulted. In this scenario there is a poor split represented by the low R^2 (0.03) and very low cross validation Q^2 (-0.19), i.e. no possibility to successfully predict, based on the serum protein levels, if a new subject included in this model belongs to group one or two. In the loading plot, we can see that none of the measured proteins are found at higher levels in either group one or two as indicated by their vertical alignment along the center of the x-axis (**Figure 4b**). Scenario two depicts what happens when the serum protein levels in subjects of group one and two are different (**Figure 4c and d**). In the score plot (**Figure 4c**) we can see that there is a distinct split between the groups as confirmed by the high R^2 (0.7) and due to the high Q^2 (0.5) we can see that that this difference is consistent enough so that it can be used to predict subject group associations. Moreover, we can even see that group one is potentially comprised of two sub clusters considering the split along the Y-axis (**Figure 4c**). The loading plot shows that indeed subjects from group two have a different serum protein profile and have higher levels of proteins 1, 5, 6 and 7 compared to group one (**Figure 4d**). Although the other serum proteins are higher in group one, we can see that the two sub clusters are distinguished by having either higher levels of proteins 3, 4, and 9 or higher levels of 2, 10 and 11, respectively (**Figure 4d**).

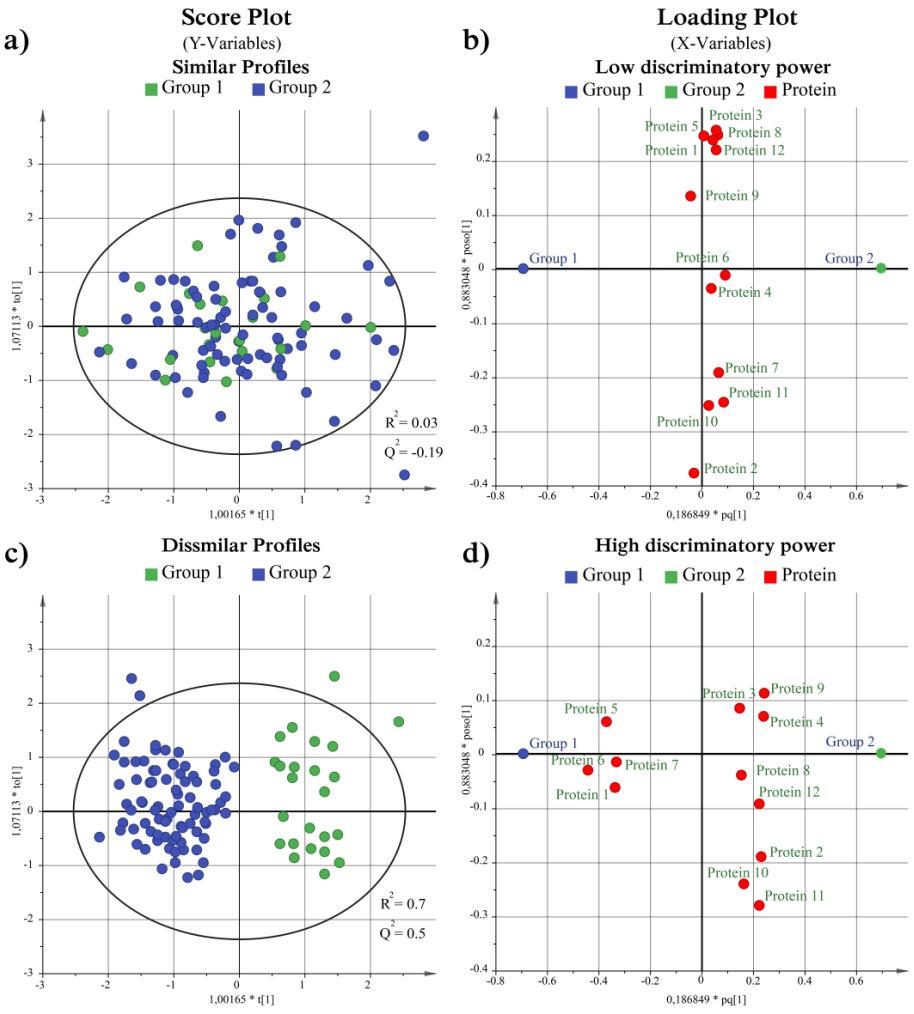

Figure 4. Example Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) models. OPLS-DA plots for two different scenarios in which subjects of group one (Green dots) and two (Blue dots) have similar or different serum protein levels (Red dots). a) OPLS-DA score plot of two groups with similar levels of serum proteins. b) Loading plot of 12 proteins measured in the serum of subjects of groups one and two. c) OPLS-DA plot of two groups with differing levels of serum proteins. d) Loading plot of 12 proteins measured in the serum of subjects of groups one and two.

3

Results and Discussion

The exploratory nature of this thesis means an extensive look into the immune system and gut microbiota in the frame of IBS has been performed and has resulted in a range of novel findings. This section of the thesis will present those identified as key findings of each study and simultaneously discuss their context in the field of IBS as well as implications and potential future use in the clinic.

Dietary impact on microbiota and symptoms of IBS

Faecal bacteria profiles of IBS patients whose symptoms improved after following a low FODMAP diet are different before the intervention as compared to profiles of patients who had no significant improvement in symptoms from being on the same diet.

The low FODMAP diet could be considered as arduous due to its requirement for the wide exclusion of foods (**Table 4**)[244]. Additionally, the long-term studies on the safety of its impact on gut bacteria are lacking. Multivariate analysis revealed that discrimination of responders

and non-responders to a low FODMAP diet, but not a traditional IBS diet, could be achieved based on gut bacterial profiles before intervention (**Figure 5**). The model for the patients following the traditional IBS diet was able to forcibly discriminate responders from non-responders, as indicated by the reasonable model R^2 indices of 0.46. However, the differences in bacterial profiles were not consistent enough as shown by an abysmal Q^2 of -0.04, meaning it could not be used to predict the response of a new patient (**Figure 5a**). Thus, the bacterial profiles of IBS patients could not be used to predict response to a traditional IBS diet intervention.

In the model for the patients following the low FODMAP diet, the bacterial profiles of responders and non-responders differed as indicated by the acceptable R^2 indices of 0.65. The differences between the bacterial profiles were significant and consistent enough as to achieve a high Q^2 of 0.54 (**Figure 5b**). Thus bacterial profiles of IBS patients could be used to reliably predict if a new patient would respond or not to a low FODMAP diet. Although the use of multivariate modelling suggests the prediction of intervention response for a patient, the practicality for clinical implication is quite low.

However, another finding was that those who were defined as non-responders to the low FODMAP diet were consistently more dysbiotic than responders, both before and after the intervention (**Figure 6a and b**). Further evaluation is required, but if combined with other patient data such as from a dietary questionnaire, the dysbiosis index (DI) might be sufficient for patient selection for dietary intervention therapy.

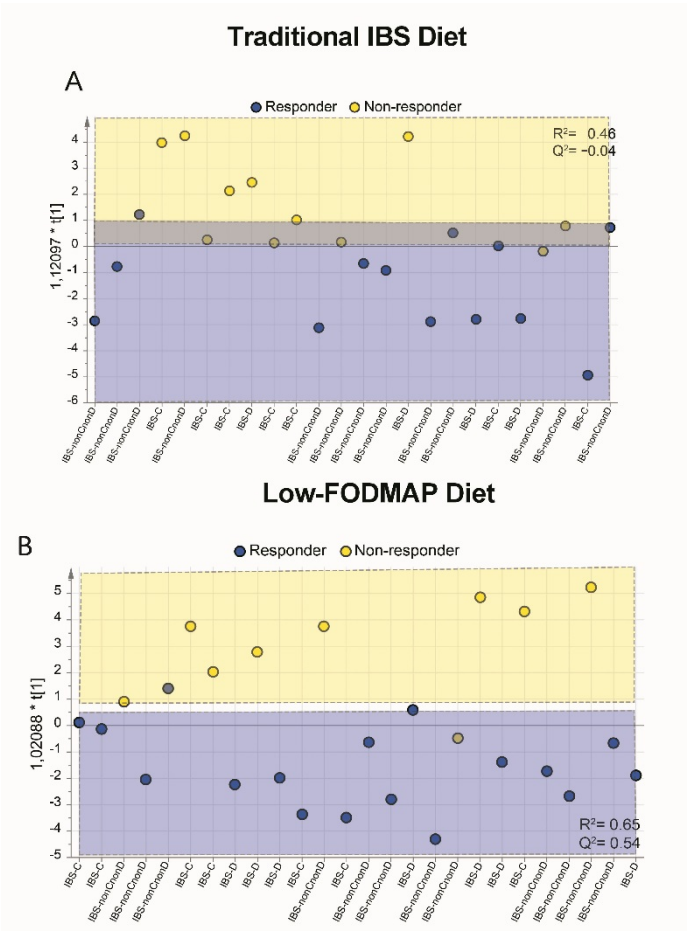

Figure 5: Bacterial profile analysis of non-responders and responders to traditional IBS or low FODMAP dietary intervention. The GA-map™ Dysbiosis Test was used to create bacterial profiles for each patient. Each individual patient is plotted along the X axis with class discriminations made between responders (blue dots) and non-responders (yellow dots) depicted along the Y axis. (A) Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) between responders and non-responders before traditional IBS dietary advice (n=24), R^2=0.46, Q^2=−0.04. (B) OPLS-DA between responders and non-responders before a low FODMAP diet (n=26), R^2=0.65, Q^2=0.54. Light yellow and light blue boxes have been used to depict where the majority (>90%) of each class are on each scatter plot. IBS-C, constipation-predominant IBS; IBS-D, diarrhoea-predominant IBS; IBS-nonCnonD, IBS with mixed loose and hard stools (IBS-M) or unsubtyped IBS (IBS-U)

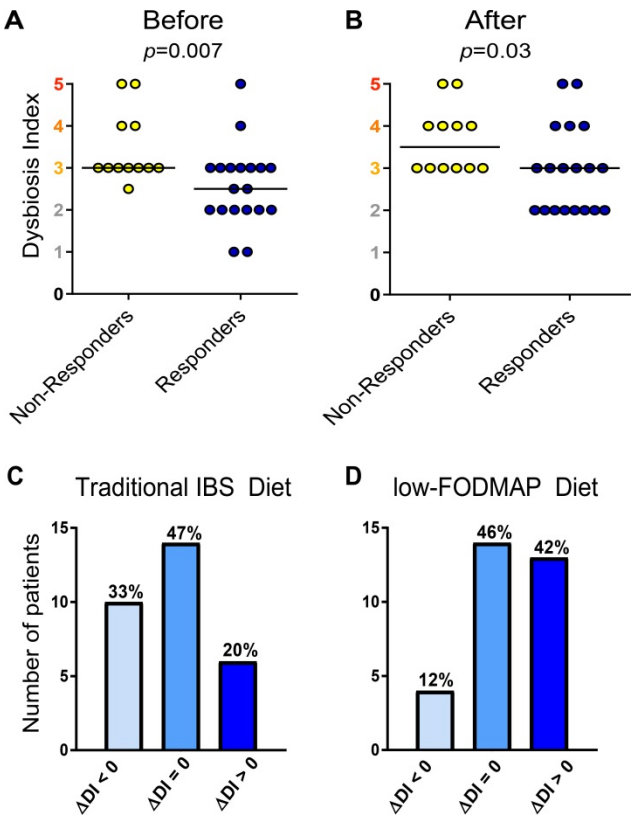

Figure 6: Dysbiosis analysis of dietary interventions. The GA-map™ Dysbiosis Test was used to create Dysbiosis Index (DI) scores for each patient. (A) Comparison of DI between non-responders and responders before a low FODMAP intervention. (B) Comparison of DI scores after the low FODMAP intervention. Grey coloured numbers signifies eubiosis, while yellow, orange and red indicate dysbiosis of increasing severity. (C) Change in DI scores from before to after the traditional IBS diet irrespective of intervention response status (D) Change in DI scores from before to after the low FODMAP

Although requiring validation, the findings from this study may be used to quicken the rate at which patients are placed on the right therapy for them. Instead of having to follow a dietary intervention to know the result, the outcome could be predicted allowing for the patient to either know whether starting the diet would be beneficial or if another course of action would be wise.

This study also demonstrated that a low FODMAP, but not a traditional IBS diet may have significant impact on faecal bacteria. This can be seen whereby, irrespective of response, almost half of the patients following the low FODMAP diet had an increase in their DI while a third of the patients following the traditional IBS diet had a decrease in their DI (**Figure 6c and d**).

Restriction of certain foods used by certain bacteria causes their starvation and their numbers dwindle in accordance. In the case of a low FODMAP diet, the foods excluded are those which are prevalently metabolised by beneficial species of bacteria e.g. *Bifidobacteria*. We saw this reduction in patients following the low FODMAP diet but not the traditional IBS diet (**Figure 7**) and thus may have contributed to the increased DI seen in the patients following the low FODMAP intervention. Bacteria are suggested to aid the functioning of the gut and maintain a good level of wellbeing with a reduction of these species being synonymous with dysbiosis. Not all species of *Bifidobacteria* have beneficial effects, but studies have shown that the supplementation of *Bifidobacterium animalis* DN-173 010 and *Bifidobacterium infantis* 35624 reduced IBS symptoms in some patients[106,261,262]. This suggests that perhaps not all patients experienced symptom improvement after following a low FODMAP diet due to the reduced effects from lowered beneficial *Bifidobacteria* abundance.

Although a reduction was observed in *Bifidobacteria* which has been demonstrated before[177,263], two things were not considered. The first is that there is a multitude of species of bacteria living in the gut, of which only a fraction were targeted for investigation in the GA-map™ analysis.

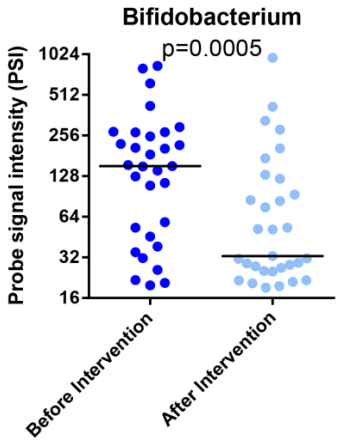

Figure 7: Abundance of *Bifidobacteria*. Faecal samples were collected from all patients before and after following the low FODMAP diet from which abundance of *Bifidobacteria* was recorded.

Due to this, other beneficial species of bacteria might not have been included and remained unaltered or increased but not documented. For example, a previous study performed by McIntosh *et al.* used 16S RNA profiling for colonic microbiome analysis of faeces from IBS patients following a diet either high or low in FODMAPs for three weeks. While the findings of their study largely corroborate findings of this thesis regarding microbial impact of a low FODMAP diet, they demonstrated a higher abundance of *Adlercreutzia* compared to baseline[263]. *Adlercreutzia* is a hydrogen gas consuming bacteria and might account for the reduced bloating and pain reported by their patients. Thus, the GA-map™ analysis which assesses a predefined set of bacteria might give a limited view on the potential unsafe view of a low FODMAP diet. The second is that the metabolic profile, or metabolome, was not taken into consideration. The significance of this is that although the abundance of some bacteria was reduced, the metabolic products which these species produce may not have actually altered in level. As previously mentioned, the bacteria in the gut have a very versatile enzymatic repertoire meaning

that it might be that some bacteria are still able to make the beneficial metabolites. In those people where symptoms improved the beneficial metabolic products might not have been impacted to such a large degree as in those who did not respond. The same study performed by McIntosh *et al.* also investigated the metabolic profiles, as measured in urine[263]. Patients following a low FODMAP diet experienced changes in their metabolome however, out of 29 candidate metabolites, only histamine was significantly reduced after a low FODMAP diet[263]. Histamine is an important signalling molecule released from mast cells which are in turn associated with abdominal pain in IBS[13]. The authors suggest two microbiologically pertinent pathways for mast cell activation involving SCFAs and mechanically induced degranulation through gas distention of the gut. While a reduction in gas production might occur from less fermentation occurring due to restricted FODMAP intake, SCFAs were not shown to be significantly reduced after a low FODMAP diet. Thus the underlying mechanism for symptom reduction in some patients after a low FODMAP diet still remains unclear.

The short term benefits of a diet restricting FODMAPs has been shown for some patients however its recommendation as therapy is often for a limited period. This is in part due to the demonstrated impact it has on the gut microbiota composition. The effects of the long term reduction of beneficial bacteria have not yet been studied. However, until so it is hypothesised that the dysbiotic state created might have detrimental ramifications directly to the host or by making the gut more susceptible for pathogenic species colonisation. Thus, investigation into how patients can retain the benefits of FODMAP restriction while maintaining eubiosis is required.

Immune system activity and impact on symptoms of IBS

IBS patients have an altered immune activity when compared to healthy subjects; however this is more prominent in a subset of patients and does not seem to play a direct role in the type or severity of symptoms experienced.

This thesis demonstrated that while IBS patients have altered serum and mucosal cytokine levels compared to healthy subjects, a group of patients and healthy subjects cannot be discriminated from each other based on their global or serum alone cytokine profiles (**Figure 8**). Interestingly, a subset of patients characterised by having an increase in immune activity, hence named 'immuno-active' were identified in the two separate cohorts indicating their potential prevalence among the IBS patient community. In Paper II, the immuno-active patients had higher serum levels of IL-6, IL-8 and a lower mucosal expression of IL-10, while in Paper III they had higher serum levels of all which were measured i.e. IL-1β, IL-6, IL-8 and TNFα, as well as IL-10 compared to healthy subjects, but also to IBS patients who had similar cytokine profiles as the healthy subjects, hence named 'immuno-normal'. Defining a subset of IBS patients based on their immune activity may be novel but it is in line with prior studies which speculate about such a group of patients[206,207,239,241,264]. Additionally, due to the heterogeneity between patients with the IBS diagnosis, it can be hypothesised that there is more than a single underlying mechanism for symptom generation and that at least one is related to provocation of the immune system. Although previous studies on the anti-inflammatory drug mesalazine had only partial success in symptom relief in IBS patients, the authors speculated that this may be due to their hypothesis that the majority of patients may not have

inflammation as a primary driver of their symptoms[56,57]. It could thus be hypothesized that only a subset of patents would benefit from such drugs. Anti-inflammatory therapy has yet to be studied as treatment for the immuno-active subset of patients we defined. However, the weak associations of cytokines and symptoms within the IBS cohorts and even in the immuno-active patients alone question the role of immune system activation on its own in symptom development and exacerbation in IBS. Thus, a future study might first identify an immuno-active subset of patients in a cohort and then attempt to abate their symptoms with an anti-inflammatory drug such as mesalazine. In this study neither of the pro-inflammatory cytokines IL-8 nor TNFα had higher expression in the mucosa compared to healthy subjects. This makes for the hypothesis that at least the mucosa of the sigmoidal colon might not be the location of the gut where the higher immune activity, as indicated by the higher serum cytokines, is originating. Potentially, the ascending colon or transverse colon is the source. However, the lower expression in the mucosa of IL-10 and marker for regulatory T cells, FOXP3, in IBS compared to healthy might hold importance for IBS, though it requires further elucidation.

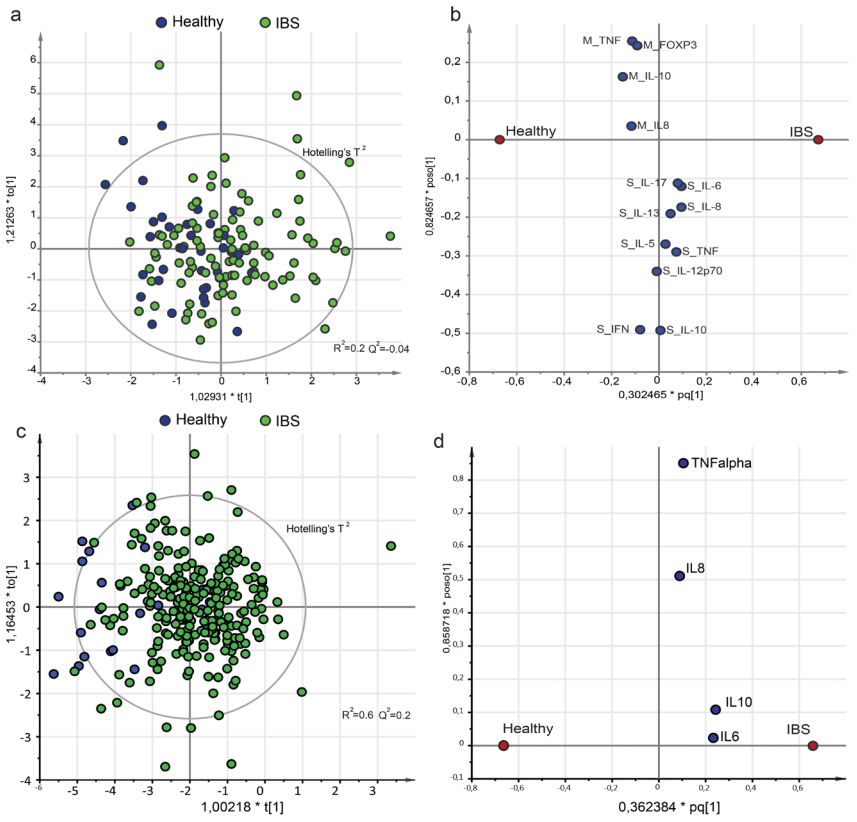

Figure 8: Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) of cytokine profiles of IBS patients (green dots) and healthy subjects (blue dots). a) OPLS-DA scatter plot showing the discrimination between IBS and healthy subjects based on all the analysed cytokines (n=13) from biopsies and serum samples. b) Loading scatter plot showing the relationship between the respective cytokines (Mucosal mRNA expression, with the prefix M, analysed with qRT-PCR and Serum levels, with the prefix S were analysed by MSD MULTI-ARRAY) and the study groups (IBS vs. healthy). c) OPLS-DA score scatter plot showing the discrimination between IBS patients and healthy subjects based on their serum cytokine profiles, comprising serum levels of IL-6, IL-8, TNFα and IL-10. d) The loading scatter plot showing the relationship between the IBS patients and healthy subjects and the respective cytokines.

Few previous studies have investigated immune activity in relation to symptoms of IBS and so this thesis brings forth valuable knowledge with moderate correlations of cytokines and symptoms demonstrated. Serum levels of pro-inflammatory TNFα correlating with mean stool form (BSF) and oroanal transit time, and serum IL-6 correlating with the average stool frequency corroborates with previous findings[206]. These two correlations suggesting higher levels of serum TNFα and IL-6 are found in patients with a bowel movement profile in line with that of IBS-D, a subgroup of IBS which has been previously demonstrated to have high immune activity based upon serum levels of pro-inflammatory cytokines[206,222,265]. However, the second study did not find any differences between any of the serum levels of the cytokines between the bowel habit based subgroups. Similarly, while IL-6 tended to be correlated with both anxiety and depression in the first study, no such correlations were found in the second.

It would have been interesting to directly combine and compare the serum cytokine level data of patients and even healthy subjects from both studies i.e. Swedish nationals to American nationals. The comparison would however be more suited to its own study investigating if the factors associated with each nationality or geographical location etc. gave different immunological profiles and if so how big discrepancy do they cause[266]. This was not performed in this thesis for such reasons though primarily due to the use of different cytokine assays and detection limits between the two cohorts. What can be performed though is to compare how differences between healthy subjects and patients were between the respective groups as performed in **Figure 9**. The first point to consider is that regardless of the cohort, there is a large overlap between the healthy subjects and patients, commonly seen among studies on cytokines in IBS[219,223]. The second point is the statistical significance found within the

American cohort. The disparity in the number of healthy subjects to patients might likely be the cause since when the sample size is large any small departure from the null hypothesis will very likely be detected by the test. Thus meaning that the significance might have little practical significance, in this case it would be unreasonable to measure the serum level of one cytokine and be sure that the subject did or did not have IBS. This is one of the additional points why multivariate analysis was performed since it is comparing the more encompassing cytokine profiles instead.

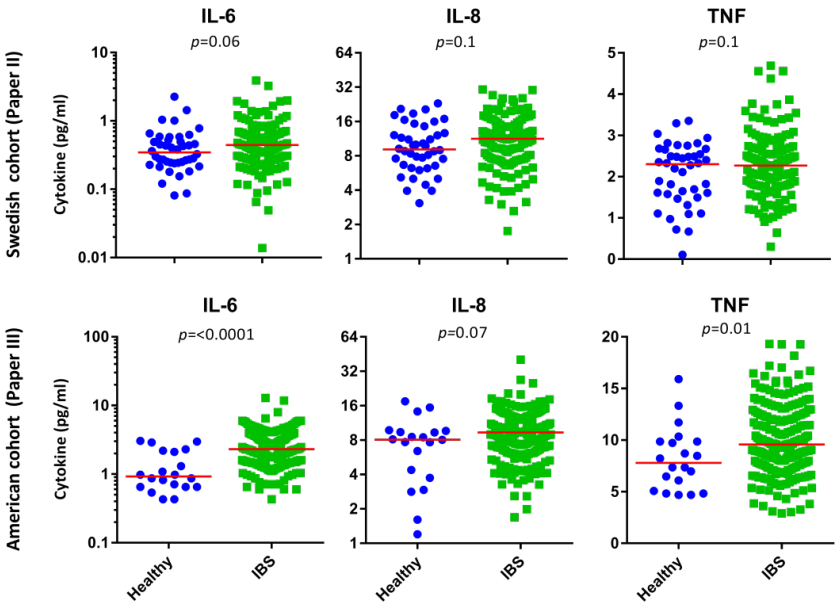

Figure 9: Overview of serum cytokine levels in IBS patients and healthy subjects from respective Swedish and American cohorts. Serum levels of pro-inflammatory cytokines IL-6, IL-8 and TNFα were compared in the serum of healthy subjects and IBS patients both within a Swedish cohort and American cohort.

The findings of the second study on immunity and IBS complement and expand on the findings of the first. The unsupervised cluster analysis of the second study strengthens the concept of an immuno-active subset within each IBS cohort, however several factors still need elucidation, such as what is the optimal size cohort to detect these patients or if there should be a cytokine cut off to identify patients who are immune-activated.

Host management of the gut microbiota in IBS

Antibacterial response gene expression profiles are altered in IBS patients compared to healthy subjects. Moreover, this difference in expression profiles is also found between clusters of immuno-normal and immuno-active IBS patients. These clusters also differ regarding the bacterial composition of faecal samples and mucosal biopsies.

This study demonstrated that IBS patients have altered antibacterial gene expression response profiles compared to healthy subjects and patients with inflammatory bowel disease (IBD). Although the majority (79%) of the genes responsible for antimicrobial recognition and response were similarly expressed in patients with IBS compared to healthy subjects, almost 20% of the total 84 genes were less expressed. The reason for this lowered expression is unclear but may be explainable. One hypothesis is that there is a problem in gene expression in IBS patients which leads to a hampering in microbial recognition. Another idea may be that in these IBS patients there is a lack of certain required bacteria which may regulate antimicrobial genes. Regardless, if there is a change in how the host controls bacteria residing in the gut then there may be a

destabilization of the microenvironment. In this case, IBS patients may be less able to recognize and appropriately respond to microbiota allowing for potentially pathogenic or opportunistic bacteria to thrive and lead to dysbiosis. Unlike in the dietary intervention study where GA-map™ was performed, the technique of 16S sequencing was applied in this study. The deeper analysis provides a unique view of the gut microbiota and allows for the potential identification of bacteria not targeted by the GA-map™ analysis. Both techniques give different views of the gut microbiota composition but in large provide the same information.

Taking the predefined immuno-normal and immuno-active IBS patients, this study attempted to identify a potential mechanism for the difference in immune activity between the two subsets. Interestingly, while not linked to symptoms, the immuno-normal and immuno-active IBS clusters showed different antibacterial gene expression profiles. Considering all IBS patients had a different antibacterial gene expression profile compared to healthy, it was thus expected that immuno-normal and immuno-active IBS patients would also differ from healthy subjects. However, it was interesting that it was the immuno-active patients which had a more similar profile to healthy subjects than the immuno-normal IBS patients (**Figure 10**). The two profiles were primarily differentiated through potential major upstream regulatory factors of TLR9 in immuno-normal IBS and TLR4 in immuno-active IBS. Toll-like receptor 9 recognizes bacterial CpG DNA motifs not present in mammalian DNA[267], while TLR4 recognizes LPS as previously mentioned to be associated with gram-negative bacteria[268].

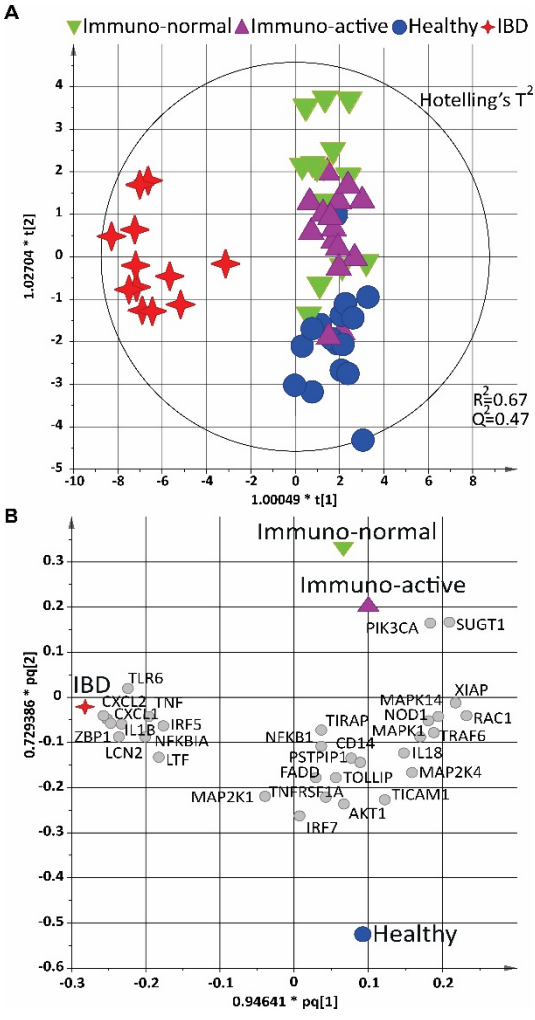

Figure 10: Comparison of antibacterial gene expression profiles of IBS patients subsets based to immune activity, healthy subjects and IBD patients with active inflammation. Mucosal mRNA antibacterial gene expression was analysed using PCR array. A) Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) score plot of immuno-normal IBS patients (green downturned triangles), immuno-active IBS patients (purple upturned triangles), healthy subjects (blue circles) and IBD patients (red stars) based on the mucosal expression of the most discriminatory antibacterial response genes (VIP > 0.07). B) The loading scatter plot showing the relationship between the IBS patients, healthy subjects, IBD patients and the respective genes.

Considering IBS has been proposed as being on a spectrum between healthy and IBD[269,270], it was thus interesting that irrespective of the IBS patients being immuno-active, they still had a distinct antibacterial gene expression profile compared to IBD patients with active inflammation. However, IBD patients in remission, with reduced severity or absence of symptoms have not been investigated. Thus it is unknown if their antibacterial gene expression profiles would be similar to IBD with active inflammation, one of the IBS subsets or healthy subjects.

Furthermore, the microbial analysis found separate faecal and mucosal bacterial compositions between the two IBS subsets. On one hand, the abundance of the *Paraprevotella* genus, of which some species produce antibacterial acids[271], was increased in immuno-normal IBS patients. On the other hand, the bacterial genus *Parabacteroides* genus, which contains species that can produce toxins (bacteriocins) that has similar effect as narrow spectrum antibiotics[94], was more abundant in immuno-active patients. It is thus suggested by these findings that differences in microbial composition profiles of immuno-normal and immuno-active IBS patients may partly be driven by or even result in alterations in their antibacterial responses.

Although the underlying cause for this alteration is to a large extent unknown this study provides new insight of a potential link between gut bacteria composition, immune activation and antibacterial gene expression. The underlying mechanisms and significance of these antibacterial gene expression alterations must be elucidated. However once done, the implications might provide an alternate means to subgroup patients and provide targeted therapy.

4

Conclusion and future perspectives

This PhD thesis was very much exploratory in nature. The results are in most ways novel and while they do help in providing some answers, they also bring further questions and findings to the field which should be investigated and validated.

For the first time, we have shown that the composition of the bacterial profiles of IBS patients who respond to a low FODMAP dietary intervention is different before intervention when compared to the IBS patients who do not respond. While in need of refinement, this thesis suggests the potential ability to identify responders to a low FODMAP diet through faecal bacterial profile multivariate analyses before intervention. Irrespective of responsiveness, we have demonstrated that the low FODMAP diet alters gut bacteria composition in a potentially detrimental way. A reduction in certain bacterial abundance after a low FODMAP diet is commonly reported in similar studies yet the long term ramifications are unknown. Interestingly none of these findings were seen in the patients following a traditional IBS diet.

In a future validation study, faecal samples would be collected and analysed according to this thesis i.e. through GA-map™ analysis. Only patients with a bacterial profile that was found to predict a positive response would be treated. The response rate could then be evaluated. Furthermore, future studies could perform a deeper microbial analysis than GA-map™ analysis to potentially find other microbial markers of a responding patient. Moreover, investigating the metabolite profiles of different samples should be priority since we are becoming familiar with what bacteria reside in the gut and which are affected, but knowledge of what they are producing is lacking. Considering that no one technique can measure the complete metabolome[272] and that different samples give different metabolomics insights, both faecal metabolites as well as urine should be investigated.

It is unclear why the bacteria profile of some patients means that they are likely respond to a low FODMAP diet but the underlying mechanism may be linked to the immune system. Considering a potential underlying mechanism for symptom generation is mast cell activation, it would be interesting to investigate if these responders also exhibit an immuno-active cytokine profile or if perhaps they have an altered antibacterial gene expression profile.

This thesis further demonstrated that within two large IBS cohorts, there was an alteration in the levels of several serum cytokines compared to healthy subjects. However, these measured cytokines can neither be used individually, nor together as a cytokine profile, to distinguish patients from healthy subjects. The identification of a subset of IBS patients with an increased immune activity became a more credible finding when a similar subset was also identified in a second cohort of IBS patients. Still,

only weak associations between serum and mucosal cytokine levels and symptoms were identified.

Thus, the future perspectives would be to focus on identifying the underlying mechanisms behind the increased immune activity in a subset of patients, and the potential relevance in the pathophysiology or even pathogenesis of IBS through interaction with factors not investigated in these studies. Furthermore, investigation needs to be performed longitudinally to establish if patients defined as immuno-active stay as immuno-active. Additionally, other tissues/samples/scans may be investigated to further characterise this subset. Finally, a randomized controlled trial could be performed on non-selected IBS patients to evaluate if immune activity predicts treatment response to anti-inflammatory or other therapies.

We have also demonstrated that IBS patients have an altered ability to recognise and deal with microbes in the gut, due to the demonstration of an altered expression of antibacterial genes compared to healthy subjects. Moreover, both faecal and mucosal bacterial profiles also differ between the immuno-normal and immuno-active IBS patients. These findings provide potential elucidation to the difference in immune activity by implicating gut microbiota as instigators for the higher levels of cytokines.

Moving forward with this study would involve the validation at the protein level of such antimicrobial products found within the mucosa. While gene expression provides one view, it does not provide an accurate representation of what is actually being produced. This type of confirmation, as well as validation in a large cohort is required.

While not life threatening irritable bowel syndrome is a life altering and debilitating syndrome for anyone to be afflicted with. Time consuming, costly and demanding IBS affects a large number of people yet no two patients experience the same IBS. Although different underlying mechanisms for symptom generation are proposed, our detailed understanding is lacking. Moreover, the means to definitively identify which mechanism is behind the symptoms of a patient requires elucidation. This thesis explored three aspects commonly associated with the severity of IBS, namely, the immune system, diet and the gut microbiota. We have demonstrated that diet has a direct impact on the composition of the gut microbiota and that modulation by diet can happen relatively quickly. For now the short term reduction in symptoms for some patients following a low FODMAP diet is the focus but it is time that the long term effects are investigated. This should be done in conjunction with metabolomics as the products of the gut microbiota are just as important as the bacteria themselves. The immune system has been suggested to be implicated in many ways in the pathogenesis of IBS. Although this thesis identified no strong direct influence of serum and mucosal cytokines on symptoms, there is nevertheless a subset of patients with an increased immune activity. These patients should be further investigated and may potentially help in the identification of indirect immune system IBS pathogenesis. While the immune system and the gut microbiota are inherently linked, the mechanisms behind their interplay and mutual modulation are unclear, let alone in the context of IBS. Thus, further investigation is required into the microbial-immunological crosstalk. Finally, while IBS is likely not a single disease, this thesis takes us closer to potentially identifying a novel subgroup of patients which may guide development of future mechanistically targeted therapy options.

5

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"Look at me still talking
when there's Science to do.
When I look out there,
it makes me GLaD I'm not you.
I've experiments to run.
There is research to be done.
On the people who are
still alive."

– GLaDOS

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