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UNDERSTANDING THE INTERPLAY BETWEEN GUT MICROBIOTA, GUT FUNCTION AND HOST GENES IN THE GENERATION OF GASTROINTESTINAL SYMPTOMS AND DISEASE

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Understanding the interplay between gut microbiota, gut function and host genes in the generation of gastrointestinal symptoms and disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my mother!

The kindest mom ever whom I owe my entire life to.

To Arash!

The embodiment of unconditional love and everlasting support.

To Ilya!

My world and endless joy of life.

ABSTRACT

Irritable bowel syndrome (IBS) is a multifactorial and complex disease categorized in the functional gastrointestinal disorders (FGIDs) with an intricate pathogenesis yet far from understood. The prevalence and burden of the FGIDs has urged the need to improve our understanding of them and recent findings have led us to realize that there are biochemical and structural alterations playing roles in their etiology. This thesis has concentrated on a number of factors including altered intestinal motility, pain perception, genetic predisposition, and gut microbiota in order to obtain a better understanding of the interplay between these factors in the generation of gastrointestinal (GI) symptoms. To achieve this, we have carried out five studies exploiting data from a Swedish data-rich general population-based cohort, namely PopCol, and some other cohorts.

In the **first study**, to investigate the link between gut microbiota and gut transit time (as an objective means to quantify GI functional abnormalities) we studied the association between different indices of fecal microbiota composition with stool consistency and stool frequency (as surrogates for gut transit time) in the PopCol cohort. The obtained results provide more support for the already reported association between gut microbiota and stool consistency and also revealed an even stronger association between the composition of fecal microbiota and stool frequency.

To study the association between one of the most common symptoms of FGIDs, i.e. abdominal pain, and gut microbiota in the **second study**, data of 159 individuals from PopCol cohort (including 52 individuals who reported pain) was inspected. Results indicated an association between fecal microbiota composition and abdominal pain occurrence as well as its frequency, duration, and severity. Also, we could provide more evidence for the negative association of *Prevotella* with pain in the general population.

In the **third study**, to investigate the genetic biology of stool frequency, we exploited data from two general population-based cohorts, PopCol and LifeLines-Deep, and carried out a genome-wide association study (GWAS) followed by a meta-analysis. Gene set enrichment analysis was performed on the resulting gene list. Although, possibly due to limited sample size, none of the association tests revealed genome-wide significant results, we could identify excellent functional candidate genes and more interestingly, the results from the post-GWAS analysis suggested xenobiotic metabolism and ion channel activity as two plausible underlying mechanisms for regulation of the stool frequency. This result pointing at the link between ion channel activity and bowel function in addition to the results of another study which revealed that 2.2% of IBS patients carry a mutation in a voltage-gated channel gene (*SCN5A*), led us to the **fourth study**. In this study, 14 single nucleotide polymorphisms (SNPs) spread over *TRPM8* (a gene involved in GI-related ion channel activity) were investigated in association to IBS and its subtypes in a cohort of IBS cases and controls followed by a meta-analysis of the results of this study and the GWAS of IBS (already published by our lab). Subsequently, PopCol data was used to study the association between *TRPM8* genotype variants and stool consistency. Logistic regression analysis revealed significant associations between different variants of *TRPM8* gene and

predisposition to IBS, which was restricted to the constipation-related subtypes of IBS (IBS-C and IBS-M). This result was confirmed by the meta-analysis. Moreover, a negative correlation between all IBS-C/M predisposing risk alleles and stool consistency was obtained from investigating the PopCol cohort. Finally, considering the importance of genes and diet in the susceptibility to IBS, in a *nutrigenetic* approach, we studied the sucrase-isomaltase (*SI*) gene variants (congenital defective form of this gene results from rare mutations and is characterized by abdominal pain, diarrhea, and bloating) for their potential relevance to IBS in the fifth study. To do this, the four most known congenital sucrase-isomaltase deficiency (CSID) mutations in addition to a *SI* coding SNP (p.Val15Phe) were screened in a multicenter cohort of IBS cases and controls. The effect of this SNP on the function of SI was also inspected *in vitro*. Finally, we analyzed p.Val15Phe genotype in association to fecal microbiota and stool frequency in PopCol cohort. Our results indicated that the four CSID mutations and the common variant were more common in patients than asymptomatic controls. The *in vitro* study indicated 35% reduced enzymatic activity for the SI protein with 15Phe compared to 15Val. Investigating PopCol samples, 15Phe copies correlated with stool frequency and the abundance of fecal *Parabacteroides*.

In summary, in this thesis we succeeded in providing additional strong evidence for the importance of human genes (*TRPM8* and *SI*) in the development of IBS and its symptoms. Moreover, we demonstrated a link between some of the important IBS symptoms, i.e. abdominal pain and altered stool frequency with gut microbiota composition in the general population. Taken together, this thesis contributes to a better understanding of the interplay between several factors in the generation of GI symptoms. The information we report here may contribute to translational opportunities for the stratification and eventual management of individuals with IBS and other FGIDs.

LIST OF SCIENTIFIC PAPERS

I. **Hadizadeh F**, Walter S, Belheouane M, Bonfiglio F, Heinsen FA, Andreasson A, Agreus L, Engstrand L, Baines JF, Rafter J, Franke A, D'Amato M. Stool frequency is associated with gut microbiota composition. Gut 2017;66(3):559-560.

II. **Hadizadeh F**, Bonfiglio F, Belheouane M, Vallier M, Sauer S, Bang C, Bujanda L, Andreasson A, Agreus L, Engstrand L, Talley NJ, Rafter J, Baines JF, Walter S, Franke A, D'Amato M. Faecal microbiota composition associates with abdominal pain in the general population. Gut 2017 (accessed on 1 Aug 2017). doi: 10.1136/gutjnl-2017-314792.

III. Jankipersadsing SA*, **Hadizadeh F***, Bonder MJ, Tigchelaar EF, Deelen P, Fu J, Andreasson A, Agreus L, Walter S, Wijmenga C, Hysi P, D'Amato M, Zhernakova A. A GWAS meta-analysis suggests roles for xenobiotic metabolism and ion channel activity in the biology of stool frequency. Gut 2017; 66(4): 756–758.

IV. Henström M, **Hadizadeh F**, Beyder A, Bonfiglio F, Zheng T, Assadi G, Rafter J, Bujanda L, Agreus L, Andreasson A, Dlugosz A, Lindberg G, Schmidt PT, Karling P, Ohlsson B, Talley NJ, Simren M, Walter S, Wouters M, Farrugia G, D'Amato M. TRPM8 polymorphisms associated with increased risk of IBS-C and IBS-M. Gut 2017; 66(9): 1725–1727.

V. Henström M*, Diekmann L*, Bonfiglio F[#], **Hadizadeh F**[#], Kuech EM[#], von Köckritz-Blickwede M, Thingholm LB, Zheng T, Assadi G, Dierks C, Heine M, Philipp U, Distl O, Money ME, Belheouane M, Heinsen FA, Rafter J, Nardone G, Cuomo R, Usai-Satta P, Galeazzi F, Neri M, Walter S, Simrén M, Karling P, Ohlsson B, Schmidt PT, Lindberg G, Dlugosz A, Agreus L, Andreasson A, Mayer E, Baines JF, Engstrand L, Portincasa P, Bellini M, Stanghellini V, Barbara G, Chang L, Camilleri M, Franke A, Naim HY, D'Amato M. Functional variants in the sucrase-isomaltase gene associate with increased risk of irritable bowel syndrome. Gut 2016 (accessed on 21 Nov 2016) doi: 10.1136/gutjnl-2016-312456.

*Shared first authorship

[#]Shared second authorship

LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Assadi G, Saleh R, **Hadizadeh F**, Vesterlund L, Bonfiglio F, Halfvarson J, Törkvist L, Eriksson AS, Harris HE, Sundberg E, D'Amato M. LACC1 polymorphisms in inflammatory bowel disease and juvenile idiopathic arthritis. Genes and Immunity 2016;17(4):261-4.

II. Bonfiglio F, Henström M, Nag A, **Hadizadeh F**, Zheng T, Cenit MC, Tigchelaar E, Williams F, Reznichenko A, Ek WE, Rivera NV, Homuth G, Aghdassi AA, Kacprowski T, Männikkö M, Karhunen V, Bujanda L, Rafter J, Wijmenga C, Ronkainen J, Hysi P, Zhernakova A, D'Amato M. A GWAS meta-analysis from five population-based cohorts implicates ion channel genes in the pathogenesis of irritable bowel syndrome. (Submitted manuscript)

III. **Hadizadeh F**, Belheouane M, Walter S, Andreasson A, Agreus L, Engstrand L, John F. Baines JF, Rafter J, Franke A, D'Amato M. A Genome Wide Association Study of fecal microbiota composition in a Swedish population-based cohort. (Manuscript)

CONTENTS

LIST OF ABBREVIATIONS

AR	Antibiotic resistance
BSFS	Bristol stool form scale
CADD	Combined Annotation Dependent Depletion
CAZymes	Carbohydrate-active enzymes
ccf	Commensal colonization factors
CD	Crohn's disease
CgA	Fecal chromogranin A
CS	Cesarean section
CSID	Congenital sucrose-isomaltase deficiency
DZt	Dizygotic twins
FBDs	Functional bowel disorders
FDR	False discovery rate
FGIDs	Functional gastrointestinal disorders
FODMAPs	Fermentable oligo-, di-, and monosaccharides and polyols
GI	Gastrointestinal
GO	Gene Ontology
GSEA	Gene set enrichment analysis
GWAS	Genome-wide association study
GWS	Genome-wide significant
HIT	Human intestinal tract
HMP	Human microbiome project
IBS	Irritable bowel syndrome
IBS-A	IBS with alternating constipation and diarrhea
IBS-C	Constipation-predominant IBS
IBS-D	Diarrhea-predominant IBS
IBS-M	Mixed IBS
IBS-U	Unclassified IBS
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LD	Linkage disequilibrium

LLD	LifeLines-Deep
MDI	Microbial dysbiosis index
MetaHIT	Metagenomics of the human intestinal tract
MTC	Multiple test correction
MZt	Monozygotic twins
OTU	Operational taxonomic unit
PC	Principal component /Principal coordinate
PCA	Principal component analysis
PCo	Principal coordinate
PCoA	Principal coordinate analysis
PD	Phylogenetic diversity
PI-IBS	Post-infectious IBS
PopCol	Population-based colonoscopy cohort
QOL	Quality of life
SALT	Screening across the Lifespan Twin study
SCFAs	Short-chain fatty acids
SI	Sucrase-isomaltase
SNP	Single nucleotide polymorphism
SO	Sphincter of oddi
Spp.	Species
SVS	SNP and variation suite
TNF	Tumor necrosis factor

1 FUNCTIONAL GASTROINTESTINAL DISORDERS

Functional gastrointestinal disorders (FGIDs), conceptualized as disorders of the gut-brain interaction, are the most common gastrointestinal-related disorders worldwide (1, 2). FGIDs are defined as illness experiences by sufferers, which are mainly diagnosed by symptoms and can affect all people regardless of gender, age, race, etc. although their prevalence could be influenced by these factors (3). The etiology of FGIDs is not well-characterized yet but impairment in the physiological factors such as gut motility, immune system, microbiota composition and diet in a complex interaction with psycho-social factors as well as the genetic background has been implicated in the development of the vast spectrum of these diseases (1). The classification of FGIDs in adults based on ROME IV (2016) has been illustrated in figure 1A (1, 2). Among these six categories, functional bowel disorders (FBDs) are one of the most prevalent ones.

FBDs encompass a wide range of chronic gastrointestinal (GI) disorders defined by common symptoms of abdominal pain, distention, bloating and alterations in bowel movements and in adults are categorized into six different classes (figure 1A). Except for the last class, namely Opioid-induced constipation which can create the symptoms similar to the functional constipation but has a specific etiology and is distinct from the other FBDs, there is big overlap between the other classes (figure 1B). In the definition of all these classes of FBDs, four different criteria of *chronicity*, *frequency*, *current activity* and *lack of physiologic abnormality* have been highlighted. In other words, a disorder could be labeled as an FBD if it has been presented for at least 1 day per week (on average) within the last three months, continued for at least 6 months at the time of presentation and no pathologic origin could be identified for it through diagnostic examinations (3).

1.1 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is the most common FGID (4) with different clinical appearances. Based on ROME IV, this disorder is characterized by ‘recurrent abdominal pain’ accompanied by at least two of the following three criteria:

1. In relation to defecation
2. In association to an alteration in stool frequency
3. In association to an alteration in stool consistency (appearance of the stool)

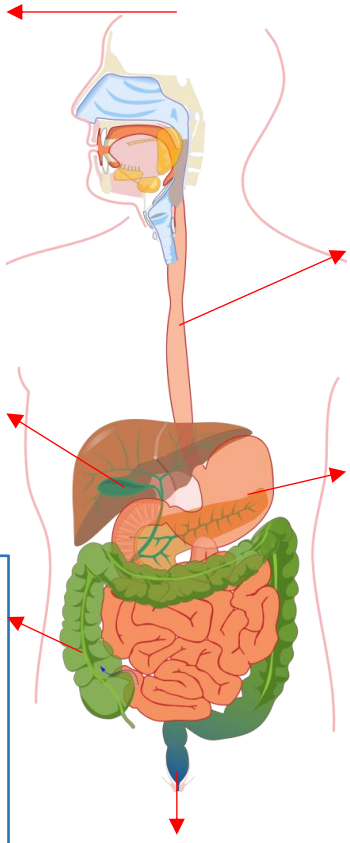
Also, to be diagnosed as an IBS sufferer, the patient should not have any alarming signs including more than 10% unintended weight loss in 3 months, lower GI bleeding (in the absence of hemorrhoids or anal fissures), and a positive family history of colon cancer (3).
However, it should be noted that ROME IV is the most updated version of the ROME criteria and although it will be a valuable tool for upcoming research, all of the so-far published investigations, including the papers in this thesis, have exploited the older versions of II and III. Noteworthy, the present definition has been stated based on a few amendments to ROME III criteria such as omission of ‘discomfort’ from the definition, modification of the phrase ‘improvement with defecation’ to ‘relation to defecation’ and elimination of the word ‘onset’ from the second and third IBS criteria.

D. Centrally Mediated Disorders of Gastrointestinal Pain
D1. Centrally mediated abdominal pain syndrome
D2. Narcotic bowel syndrome/ Opioid-induced GI hyperalgesia

A

A. Esophageal Disorders
A1. Functional chest pain
A2. Functional heartburn
A3. Reflux hypersensitivity
A4. Globus
A5. Functional dysphagia

E. Gallbladder and Sphincter of Oddi (SO) Disorders
E1. Biliary pain
E1a. Functional gallbladder disorder
E1b. Functional biliary SO disorder
E2. Functional pancreatic SO disorder



B. Gastroduodenal Disorders
B1. Functional dyspepsia
B1a. Postprandial distress syndrome
B1b. Epigastric pain syndrome
B2. Belching disorders
B2a. Excessive supragastric belching
B2b. Excessive gastric belching
B3. Nausea and vomiting disorders
B3a. Chronic nausea vomiting syndrome
B3b. Cyclic vomiting syndrome
B3c. Cannabinoid hyperemesis syndrome
B4. Rumination syndrome

C. Bowel Disorders
C1. Irritable bowel syndrome
IBS with predominant constipation
IBS with predominant diarrhea
IBS with mixed bowel habits
IBS unclassified
C2. Functional constipation
C3. Functional diarrhea
C4. Functional abdominal bloating/distension
C5. Unspecified functional bowel disorder
C6. Opioid-induced constipation

F. Anorectal Disorders
F1. Fecal incontinence
F2. Functional anorectal pain
F2a. Levator ani syndrome
F2b. Unspecified functional anorectal pain
F2c. Proctalgia fugax
F3. Functional defecation disorders
F3a. Inadequate defecatory propulsion
F3b. Dyssynergic defecation

B

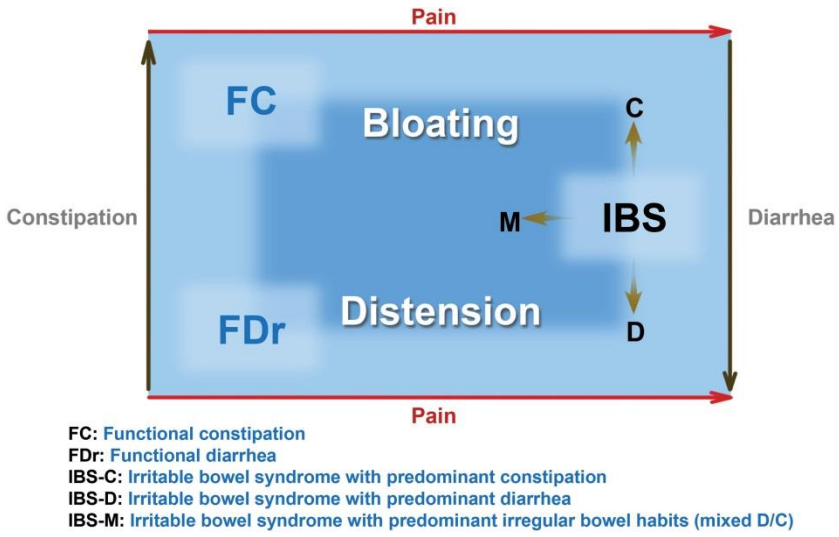


Figure 1.A. Classification of functional gastrointestinal disorders in adults (1).
B. FBDs' conceptual framework. This figure displays the existing overlap between five categories of FBDs. Reprinted from Gastroenterology, 150/6, Lacy BE *et al.*, Bowel Disorders, 1393–1407, Copyright (2016), with permission from Elsevier.

Furthermore, the frequency of the appearance of the symptoms has been changed from ‘at least 3 days per month’ to ‘at least 1 day per week during the past 3 months’ (5, 6).

Moreover, it should be acknowledged that the consensus-based ROME criteria have been developed to compensate for the lack of any reliable diagnostic biomarker or test and have considerable limitations to be used by clinicians in their daily practice. In other words, meeting the criteria is especially useful/valuable for selecting the study samples for the clinical research and the symptom-based criteria do not provide a high positive predictive value (ROME III: 45.2%, 95%CI 41.1-49.4) to aid physicians in making a positive diagnosis of IBS (7). On the other hand, the unclear pathophysiological nature of this disorder, directs physicians towards symptom suppressive treatments which are mainly based on a trial and error approach and do not result in a persistent cure (8). To overcome these limitations and given the considerable burden of IBS on the different levels of patient, society and the health system (described later), movement toward an individualized diagnosis and treatment for this disorder looks inevitable.

1.1.1 IBS classification

Trying to expand the knowledge of the potential underlying mechanisms of the disease as well as providing clinicians and researchers with a better tool for a more effective diagnosis/treatment and a more reliable case recruitment, IBS has been categorized into four different classes (figure 2). This classification is according to the stool consistency of the patients defined based on the Bristol stool form scale (BSFS) for the days with alteration in the stool form. Indeed, this classification is more reliable if patients suffer from the symptom (abnormal bowel habits) on at least four days a month (3, 6).

- IBS with predominant constipation (IBS-C): a patient is categorized in IBS-C class if his stool is hard (1 or 2 on BSFS) in more than 25% of the defecations. Moreover, less than 25% of his bowel movements should be loose (6 or 7 on BSFS).

- IBS with predominant diarrhea (IBS-D): a patient is classified as suffering from IBS-D if more than 25% of his defecations are loose (6 or 7 on BSFS) and less than 25% of them are hard (1 or 2 on BSFS).

- IBS with mixed bowel habits (IBS-M): IBS patients with > 25% of loose bowel movement (6 or 7 on BSFS) and > 25% of hard (1 or 2 on BSFS) are considered suffering from mixed IBS.

- IBS unclassified (IBS-U): If

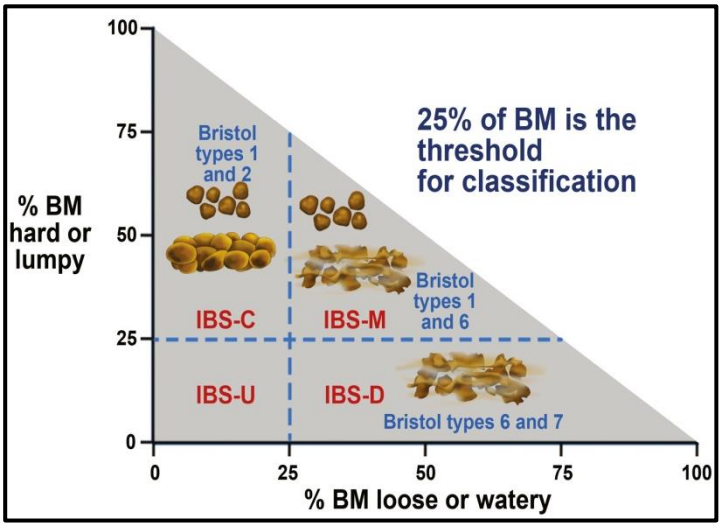


Figure 2. Subtypes of IBS.
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the bowel habits cannot be classified into any of the above-mentioned 3 categories while the patient has diagnostic criteria for IBS, IBS-U term is applied.

1.1.2 IBS epidemiology and burden

A meta-analysis published in 2012 on roughly 261,000 individuals from 80 studies reported a world-wide prevalence of 11.2% (95%CI: 9.8%-12.8%) for IBS (figure 3) (9). However, another review article published in 2017 with a bigger sample size (> 288,000) could not provide a single world-wide prevalence rate due to the vast variation in the methodological approaches. In this study, the mean prevalence of IBS among different countries was reported between 35.5% (in Mexico) to 1.1% (in France and Iran) (10). Also, the incidence of IBS has been reported to be 1.35% in the United States (11) and 1.5% in the United Kingdom (12) (both over 12 years follow-up). In general, the prevalence of IBS is considered higher in women and in younger adults (less than 50 years) (9). However, classifying IBS into three categories of mild (40% of cases), moderate (35% of cases) and severe (25% of cases), changes these estimations to some extent. For example, in the mild version of the disease, the prevalence of IBS is equal between men and women while it is much more common in women when it comes to the severe type of the disease. Also, the mild version of IBS is more prevalent among older people while younger individuals suffer more from the severe form, and the moderate version of the disease is equally common in both age categories (1).

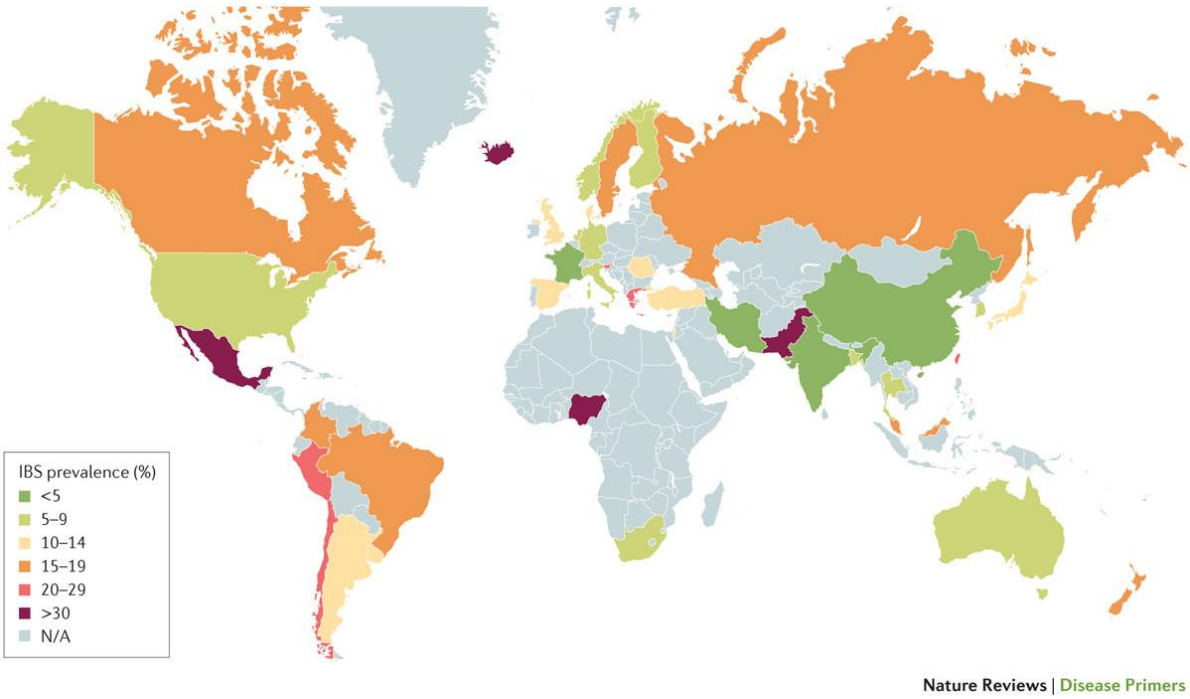


Figure 3. IBS prevalence in population studies around the world. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Disease Primers] (Enck P *et al*. Irritable bowel syndrome. Nat Rev Dis Primers 2016;2:16014), copyright (2016).

On the other hand, IBS has a considerable impact on the work productivity as well as the quality of life (QOL) of the sufferers, accompanied by a substantial economic burden on the health system resources (13, 14). Intriguingly, a review article in 2014 has demonstrated that

IBS patients are ready to give up between 10 to 15 years of their residual life expectancy (on average) in order to get rid of IBS immediately (15).

1.1.3 IBS pathophysiology

IBS is a multifactorial and complex disorder with convoluted underlying pathogenesis yet far from understood. The current knowledge implies a lack of any overt structural or organic abnormalities in this disorder and IBS, as well as the other FGIDs, is traditionally called 'functional'. However, the importance of these diseases with regards to their prevalence and burden has urged the need to improve our understanding of them and recent findings have led us to admit that there are biochemical and structural alterations which are playing a role in the development of at least a subset of these disorders, particularly in IBS (6, 16, 17) (figure 4).

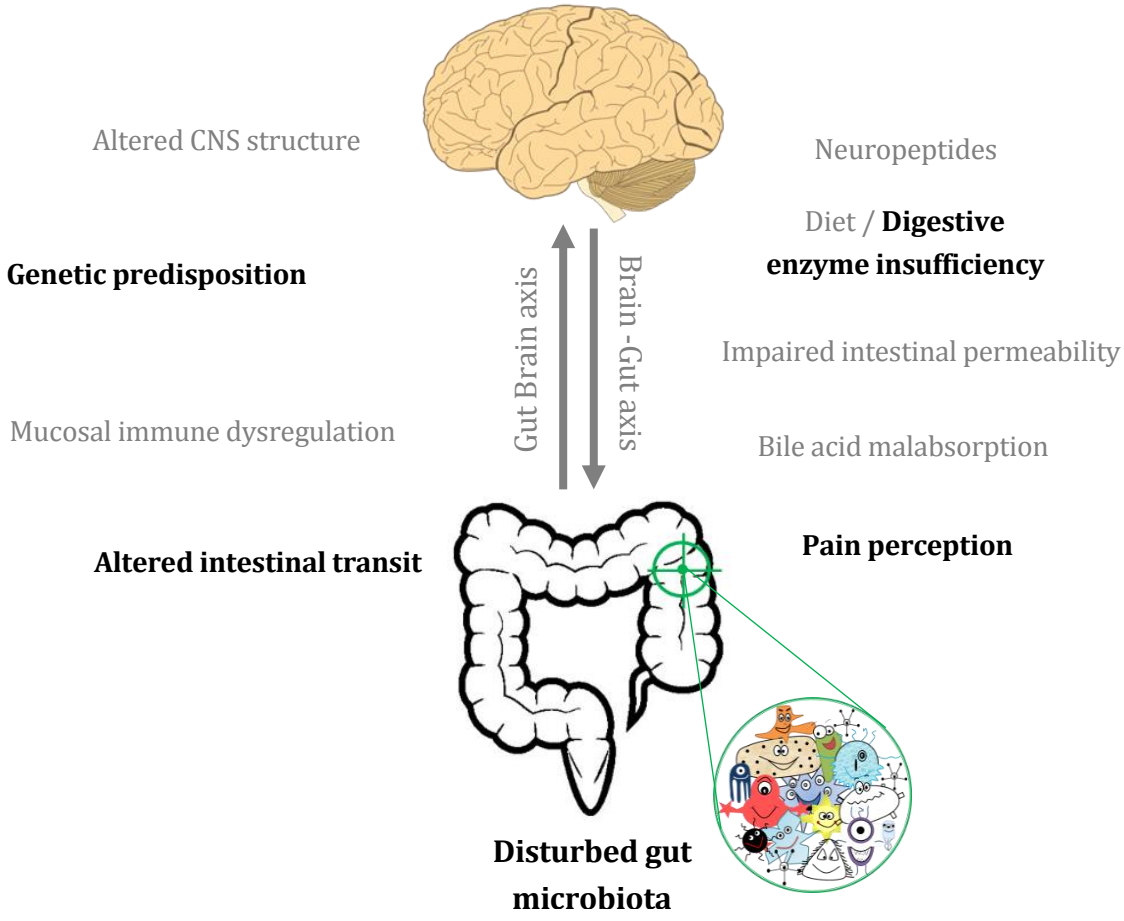


Figure 4. Pathophysiological factors proposed to be implicated in the development of IBS. Factors in the bold font have been studied in this thesis project.

Impaired intestinal permeability is one of the potential underlying pathophysiologies with support from several bodies of evidence. Although this abnormal situation is implicated mainly in the commencement of IBS-D and specifically post-infectious IBS, it could be considered as a possible mechanism in other classes of IBS too. Based on the present evidence, food and food allergy, bile acids, microbiota, genetic and epigenetic predisposition

probably through different mechanisms such as alteration of gene expression and protein translation of tight junctions can increase the intestinal permeability. It has been demonstrated that impaired intestinal permeability is linked to immune system activation, pain, and diarrhea which are common symptoms of IBS (18, 19).

Mucosal immune dysregulation is another mechanism postulated to play a role in the development of FGIDs (17). The main cells involved in the mucosal immune system are T cells, mononuclear phagocytes (including monocytes, macrophages, and dendritic cells), innate lymphoid cells, and other innate immune cells. Dysregulation of the mucosal immune system has been implicated in many of the GI disorders but could be specifically important in FGIDs. Among them, the pathway related to activation of mast cells by IL-4, secreted locally by T cells (T-helper 2), is of particular interest (17). Activation of the mast cells, through different mechanisms, could eventually result in the impaired intestinal permeability and visceral hypersensitivity (20, 21). The activity and number of mast cells have been shown to be increased in IBS patients (22).

Bile acid malabsorption has been recorded in more than 25% of IBS-D patients. Bile acids are produced by the liver and excreted into the small intestine to help absorption of dietary fats. As part of the enterohepatic circulation, they should be reabsorbed in the last section of the small intestine and if this step is defective (for any reason), the bile acids reach the colon and through various mechanisms such as acceleration of colonic transit could result in different IBS symptoms including abdominal pain, diarrhea, and bloating (19, 23).

Altered CNS structure could be another key player in the development of IBS which has been investigated in different studies (24-27). However, the results are not so conclusive yet which could be related to the heterogeneity and inadequacy of the samples. Probably the most prevalent finding in the brain of the IBS patient so far is cortical thinning which has constantly been reported in different segments of the brain of the patients in comparison to the controls (24-27). Also, interestingly, a recent study has found association between CNS structure and specific microbiota in a subset of IBS patients (28)

Neuropeptides are other potential players in the generation of IBS. Different neuropeptides have been demonstrated to be associated with FGIDs and IBS, among them one can mention gastrin, motilin, pancreatic polypeptide (29), ghrelin(30), leptin(31), substance P (32), neuropeptide Y(33) and so forth, which have different mechanisms to induce IBS, of which activation of mast cells could be a prominent candidate.

Brain-Gut axis or Gut-Brain axis Although IBS has been conventionally considered as a brain-gut axis disorder, in up to 50% of the patients it has emerged as a gut to brain pathway illness. It means that it could start with a psychological distress accompanied by an abnormal stress response in a genetically vulnerable individual and result in alteration of intestinal permeability and consequently and through a chain of events end in IBS or the trigger could be from the gut such as an alteration in the gut microbiota composition (like in post-infectious IBS). This could then initiate a cascade of events such as releasing of the inflammatory mediators which in the end could engage the central nervous system and complete the puzzle of IBS symptoms (6, 19). Nevertheless, due to the importance of gut microbiota in the

development of FGIDs and particularly IBS, some researchers have coined the name of the implicated pathway as 'microbiota-gut-brain axis' (34, 35).

Apart from what is discussed above, there are several additional important factors probably playing a key role in the development of IBS such as pain sensation, genetic predisposition, gut microbiota, diet and digestive enzyme deficiency, and altered intestinal transit which potentially through interactions with each other could result in different FGIDs such as IBS. Since my thesis has focused on these factors, I will describe them in more detail.

1.1.3.1 Pain perception

Abdominal pain is a major complaint in many diseases including organic and functional disorders and is the main symptom in IBS where lack of pain precludes the diagnosis (6). However, in recent years and with more knowledge of the unpleasant impact of visceral pain on the different life aspects of FGID sufferers, including depression, anxiety (36), sleep dysfunction and disturbed QOL (37), researchers are more and more eager to understand the underlying causes/stimuli contributing to pain perception in these patients. Abdominal visceral pain originates from activation of abdominal nociceptors which can be triggered by several stimuli such as inflammation, stretching, immune mediators, ischemia, bacterial products and so forth [32]. Having a closer look at these stimuli indicates gut microbiota as a key player which could directly or indirectly be involved in the initiation or exacerbation of abdominal pain. Probably one of the first studies that demonstrated an association between gut microbiota and abdominal pain was a randomized clinical trial which claimed that a specific strain of *Lactobacillus* could relieve pain as well as flatulence in IBS patients (38). In the same manner, another study in 2009 exhibited a significant effect for *Bacillus coagulants* GBI-30, 6086 probiotics in relieving the abdominal discomfort and bloating in IBS patients (39). The influence of probiotics on diminishing functional abdominal pain has been supported by various clinical trials in children as well (40-43). Moreover, in an intriguing mouse model study published in 2008, Amaral and his colleagues tried to examine whether the presence of microbiota is necessary for the generation of inflammatory pain. Their investigation demonstrated that inflammatory pain (inducible by different stimuli such as lipopolysaccharide, chemokine, TNF-α, IL-1β and so forth) was reduced in germ-free mice (44). Another interesting study in the murine model could support the causative role of microbiota in the development of IBS symptoms by revealing that the hypersensitivity to colonic distension is transferable from the fecal microbiota of IBS sufferers to the germ-free rats (45). Finally, in another study taking advantage of germ-free mice in the assessment of visceral hypersensitivity as well as gene expression in the spinal cord, researchers found that in the lumbosacral spinal cord of the germ-free mice, expression of TLR1-5,7,9, 12 as well as IL-6,10, TNF-α and IL-1(α,β) were significantly increased compared to the controls. In addition, germ-free mice compared to control group had increased visceral hypersensitivity at the different levels of pressure (46). It could be helpful to remember that FGIDs symptoms (mainly abdominal pain and discomfort) are positively associated with visceral hypersensitivity (47). It is known that the gut microbiota is able to impact the nervous system of the host but exactly how is yet to be clarified. An interesting mechanism has been elaborated for this in a key publication in Nature. In this outstanding study in the murine model, through different steps, the researchers demonstrated that pain could be induced directly by bacteria through activation of sensory neurons which are involved in the

modulation of inflammation. In this study, it was shown that pain sensation is associated with a load of bacteria and not activation or even presence of the immune system and can take place by two alternate pathways. One pathway is related to the binding of a specific pattern recognition receptor called FPR1 with bacterial formylpeptide (discovered in *Staphylococcus aureus*) and the other one concerns activation of pore-forming toxin α-hemolysin by ADAM10 enzyme which results in rapid calcium flux and subsequently, induction of action potentials in the nociceptors (48). Although abdominal pain and visceral hypersensitivity are considered among definition criteria and major distinguishing features of IBS (35), their association with different etiological factors of IBS could be confounded with numerous variables present in the already sufferers of IBS. To have a more solid and clear understanding of the existence of any relation or association between these factors such as abdominal pain and gut microbiota, it could be helpful to exclude the confounders as much as possible. Exploiting general population cohorts instead of case/controls is one means to achieve this. However, this kind of data is not easily available and only one study on a small sample size of 15 has been published so far. In this longitudinal research (7 weeks follow-up) on Finnish healthy individuals, two methods of qPCR and HIT (Human intestinal tract) chip were applied to study the gut bacteria as well as archaea. In addition to a significant correlation between abdominal pain and bloating with gut microbiota, their intriguing finding was the negative correlation observed between abdominal pain and *Bifidobacterium* (49). We also had the opportunity of having access to a well-designed data-enriched cohort, so-called PopCol, that is Population-based colonoscopy cohort (described in detail later) and we aimed to study the association between abdominal pain and gut microbiota in a bigger sample size of the general population.

1.1.3.2. Altered intestinal transit

Impaired gut motility is associated with many functional GI symptoms such as bloating and distension and is classified among the most important mechanisms implicated in the development of IBS (19, 50). In addition, intestinal transit time is considered as a solid and objective method to quantify GI functional abnormalities and is used as an endophenotype for some functional disorders like IBS (51). Transit time is measured by different methods and techniques including radiologic, magnet tracking, and scintigraphic methods, that could be costly, invasive and require special equipment (52-54), which turned them into almost useless approaches for general population based studies. In 1990, O'Donnell and his colleagues for the first time showed that mean stool consistency recorded by patients is in good correlation with whole gut transit time (r=-0.77) (55). Thereafter, this association was confirmed by other investigations (56, 57) and also stool frequency, another feature of bowel habit, was revealed to be correlated with gut transit time, although to a lesser extent (58-61). Therefore, these two features turned into reliable surrogate markers for assessment of the colonic transit time. Stool consistency is measured by using the Bristol Stool Form Scale (figure 5) which is a medical aid to classify the form of feces into seven categories; from type 1 (severe constipation) to type 7 (watery diarrhea) (57, 62). Over the years, it has been disclosed that gut transit time could be altered by many different factors including food and its features (e.g. volume, acidity, osmolarity, fat content, etc.), hormones, medications (such as opioids, narcotics, analgesics, antispasmodics, and prokinetics), severe pain, pregnancy, neuropathies such as diabetes-induced neuropathy, body posture and even stress (63-67).

Two plexuses of Meisner and Auerbach belonging to and regulated by the enteric and autonomic nervous systems, respectively, modulate the motility of the GI tract. The autonomic nervous system, in turn, is controlled by different mechanisms including the level of serotonin and its receptors and it has been demonstrated that serotonin could directly regulate the gut motility (50, 68). Intriguingly, different studies have recently discovered that gut microbiota is strongly involved in the regulation of the biosynthesis and concentration of serotonin in the gut lumen (69, 70). Nonetheless, this is not the only reason to make us believe in the importance of gut microbiota and its probable bidirectional association with gut motility. Many years ago, the observation of up to 10 times larger cecum in germ-free rats compared to conventional rats in addition to delayed gastric emptying in them suggested a strong role for the microbiota in the development of normal gut motility (71-74). Furthermore, these findings were consolidated when conventionalized germ-free animals, restored their physical function (75, 76). On the other hand, it is believed that a healthy gut motility, in turn, could exclude the pathogenic microorganisms and contribute to the formation of a healthy gut microbiota(77).

In 2008, an interesting publication from Jeffrey Gordon's lab revealed that gut microbiota through short-chain fatty acids (SCFAs) and one of their receptors (Gpr41) can modulate gut motility and change intestinal transit rate (78). Later on, in 2013, a paper from the Bäckhed lab suggested another mechanism for modulation of the intestinal transit time by gut microbiota. They showed that increased level of GLP1 in the animal model could result in a slower transit time. GLP1 is a hormone which is regulated by SCFAs produced by the gut microbiota (79). On the other hand, very recently, Vandeputte *et al.* hypothesized that faster transit rate contributes to variation in the colon ecosystem. To investigate that, they applied BSFS as a surrogate for transit time and studied it in association to different markers of microbiota composition in 53 healthy women. They found that stool consistency is strongly associated with microbial richness, i.e. looser stool was associated with fewer observed species and Chao1 (two parameters of richness). Moreover, looking at each individual's microbiota growth potential, they found that shorter transit time is probably linked to the higher abundances of fast-growing species (80). Part of these results were replicated in another study by Tigchelaar *et al.* which was done on a bigger sample size (n=1126) and in both men and women using averaged 7-day records of BSFS as the measurement for transit time (81). However, stool frequency, as another surrogate index for colonic transit time had not yet been investigated in relation to microbiota composition. We hypothesized that there is an

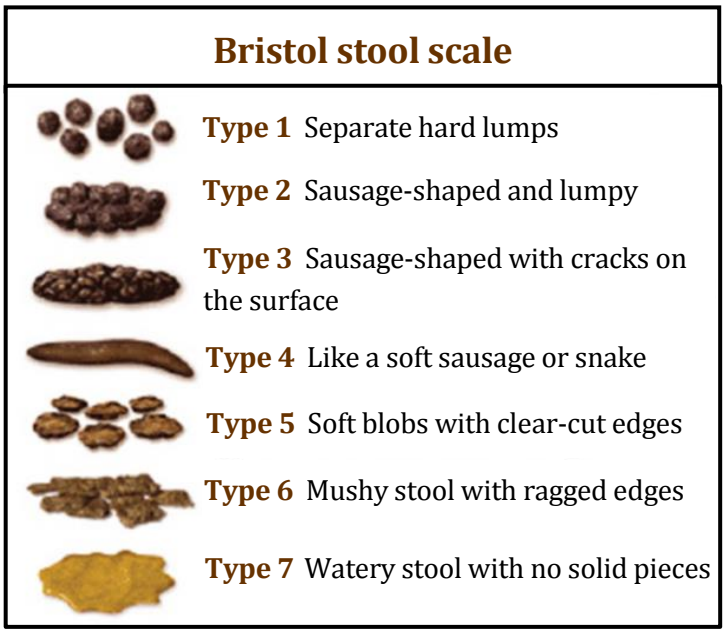


Figure 5. Bristol stool chart.
Photo from Wikipedia, labeled for reuse.

association between gut microbiota and stool frequency and were enthusiastic to examine it in the PopCol cohort.
Surprisingly, another very important factor that potentially shapes and regulates all the structures and functions of the body, genetics, had not yet been investigated well here. Although limited numbers of genotype analysis have nominated a few genes associated with gut transit time (51, 82, 83) and defecation (84), no hypothesis-free genetic study had yet been accomplished. Regarding the significance of the impaired gut motility in the development of gut symptoms, a genome-wide study approach for hunting the genes or loci which are associated with the regulation of transit time could be of great importance for understanding the underlying pathogenesis of bowel functional disease and optimistically finding new treatments for them (83). Therefore, we aimed to undertake a genome-wide association analysis to study one of the surrogate markers of transit time, stool frequency, in two well-characterized population-based cohorts.

1.1.3.3 Genetic predisposition

For the first time in 1998 in a twin study from the Australian Twin Registry, it was suggested that genetics could substantially contribute to the development of functional bowel disorders (85). Shortly after that, different twin studies were performed to investigate the heritability component of FBDs and particularly IBS (86-89). Although these studies overall supported the idea of the existence of a genetic predisposition for IBS, the results were somehow conflicting (90). However, all of these studies have been conducted on first-degree kins and spouses (to distinguish between the effects of environment and genetics in adults), except for a prominent study from Sweden published in 2015. In this investigation, first-, second- and third-degree relatives, as well as spouses, were taken into consideration for the calculation of the odds ratio for IBS development. In this study, they observed that the risk of IBS is significantly higher in all the relatives (the closer, the higher) as well as in spouses and concluded that both genetic and environmental factors are important in the development of IBS (91). Becoming aware of the importance of a genetic background in IBS generation, the next step would be the identification of the involved genes and pathways. Thus far, no unequivocal IBS risk gene has been recognized and the genetic studies are limited to one original genome-wide assocaition study (GWAS) paper and mostly underpowered, candidate-gene investigations.
IBS, similar to many other common diseases (e.g. hypertension, diabetes, obesity and so forth), is considered a ‘complex genetic disorder’, that is a multifactorial genetic disorder in which many genes and genetic variants are involved in its development, each with a small contribution to the increase of the risk. These categories of disease are still considered as heritable and like what we see in IBS they aggregate more in some families but most of the time we cannot see the Mendelian pattern of transmission in the families (92). However, there might be a few exceptions and we could probably discover some highly penetrant genetic variants which could be mainly implicated as the cause of the disease in a subset of the patients. This is of great importance because it could potentially result in stratification of patients based on their genetic predisposing factor and one could then take advantage of individualized medicine for them. One good example for this is the specific mutation discovered in the *SCN5A* gene (a sodium ion channel gene) through collaboration between Mayo Clinic and our lab from Karolinska Institute. This gene (shown to explain the

symptoms in more than 2% of mainly IBS-C cases) is potentially involved in gut motility. Interestingly, Mexiletine, a medication effective for the improvement of the sodium channel defects, could ameliorate the symptoms in a patient with severe IBS-C carrying a loss-of-function mutation (82). In addition to the 10 deleterious mutations in this gene, a few common *SCN5A* variants (single nucleotide polymorphisms (SNPs)) showed association with IBS risk in the only IBS GWAS published so far with a decent samples size (described later) as well (93).

Therefore, to have a comprehensive understanding of the genetic background of the complex disease like IBS, a combination of different approaches such as family-based studies, hypothesis-free GWA studies, candidate gene approaches and eventually fine-mapping sequencing studies could be helpful.

However, evidently, compared to other complex diseases, less effort has been made in the discovery of the genetic background of IBS thus far and most of these efforts have been limited to candidate gene studies conducted in small case-control cohorts (8). In addition, most of the genes with suggestive mechanisms in the development of IBS, are not replicated and validated by other studies, except for a few genes like *TNSFS15* which has been constantly shown to be associated with IBS in several studies from different countries (Sweden/US (94), Canada (95), and the UK (96)). A selection of the genes suggested to play a role in the generation of IBS in different studies, has been gathered in table 1. It should be noted that each of these genes is implicated in the development of the disease in merely 1-5% of sufferers and according to the definition of common variants, they have only a small contribution (97). Unsurprisingly, genes related to the serotonin pathways are among the most common investigated/proposed genes related to IBS pathophysiology, which could possibly be related to the renowned gut–brain pathway (51, 98).

As was mentioned earlier, also one original GWAS paper has been published so far. Except for that, only one pilot study exploiting a small sample size (n(case): 172, n(control): almost 1400) from an Australian cohort has been published. In that study two genome-wide significant loci on chromosomes 4 and 10 including one and four genes respectively, were identified (99).

The more comprehensive GWAS included 534 IBS cases and close to 5000 controls from a Swedish general population cohort. Subsequently, suggestive-threshold significant results were replicated in a total sample of 1793/1718 (cases/controls) from cohorts of Sweden, Belgium, Italy, Germany, Greece and the USA followed by a meta-analysis. Intriguingly, one locus on chromosome 7 including the two genes *KDELR2* and *GRID2IP* were shown to be associated with IBS in the GWAS (suggestive threshold) as well as in the replication study and meta-analysis. Furthermore, GWAS results were screened for the previously reported IBS risk genes. Out of 31 studied genes, 16 were shown to be nominally significant with the most convincing results for *IL1R1* and *SCN5A* respectively. *TNSFS15* was also shown to be nominally significant in this GWA study, although the p-value was not so strong (p=0.046) (93). Given the promising results regarding *SCN5A*, channelopathies could be considered as potential underlying abnormalities in IBS. To test this hypothesis, 27 genes coding for various GI-related ion channels were selected from this GWA study accomplished in our lab. Our aim then was to further investigate the involvement of these ion channel genes in IBS.

Table 1. Selection of the genes suggested to play a role in the generation of IBS in the different studies

Gene name	**Gene function**	**IBS type**	**Ref**
ADRA2A	Adrenergic pathway	IBS-C, IBS-D	(100, 101)
ADRA2C	Adrenergic pathway	IBS-C	(100)
BDNF	Psychiatric	IBS	(92)
CCK1R	Gastrointestinal transit	IBS-C/ IBS-M	(102, 103)
CCK2R	Gastrointestinal transit	IBS-C (only in women)	(102)
CDC42	Cell cycle regulation and likely epithelial barrier function	IBS-C	(96)
CNR1	Psychiatric genes (endocannabinoid system)	IBS	(104)
COMT	Psychiatric	IBS-C	(92)
DDC	Serotonin pathway	IBS-C, IBS-M	(92, 105)
FAAH (C385A)	Gastrointestinal transit	IBS-D, IBS-M	(106)
FGF2	Inflammatory/Immune pathway genes	IBS	(99)
FGFR4	Gastrointestinal transit	IBS-D	(107)
GNβ3	Inflammatory/Immune pathway genes	IBS	(108, 109)
GPBAR1	Accelerated colonic transit possibly through impaired bile acid metabolism and function	IBS	(110)
GRID2IP	Gastrointestinal transit and host-microbiota interactions	IBS	(93)
HTR2A	Serotonin pathway genes	IBS-D	(92, 105)
HTR3A	Serotonin pathway genes	IBS-D	(92, 105, 111)
HTR3B	Serotonin pathway genes	IBS	(92, 105)
HTR3C	Serotonin pathway genes	IBS-D	(92, 105)
HTR3E	Serotonin pathway genes	IBS-D	(92, 105)
HTR4	Serotonin pathway genes	IBS-D, IBS-C	(92, 105)
HTR7	Serotonin pathway genes	IBS-C	(92, 105)
IL1R	Inflammatory/Immune pathway genes	IBS	(112)
IL4	Inflammatory/Immune pathway genes	IBS	(113)

IL6	Inflammatory/Immune pathway genes	PI-IBS, IBS	(112, 114)
IL8	Inflammatory/Immune pathway genes	IBS	(115)
IL10	Inflammatory/Immune pathway genes	IBS	(115-117)
KDELR2	Mediates the retrograde transportation of the proteins to the endoplasmic reticulum	IBS	(93)
KLB	Colon transit time (possibly through impaired bile acid metabolism and function)	IBS-D	(107)
NUDT6	Inflammatory/Immune pathway genes	IBS	(99)
NXPH1	Unknown mechanisms	IBS-D	(96)
NPSR1	Is candidate to be associated with IBS through two mechanisms of altered colonic transit and/or epithelial barrier dysfunction	IBS	(51, 118)
PRDM1	Barrier function and permeability	IBS-D	(119)
PCDH15	Barrier function and permeability	IBS	(99)
SCN5A	Voltage-gated sodium channel	IBS (mainly IBS-C)	(82)
SLC6A4 (5-HTTLPR)	Serotonin pathway genes	IBS-C	(100, 109)
TDO2	Serotonin pathway genes	IBS	(92, 105)
TLR9	Inflammatory/Immune pathway genes	PI-IBS	(120)
TNFa	Inflammatory/Immune pathway genes	IBS	(112, 117)
TNFSF15	Altered immune function	IBS, IBS-C, IBS-D, IBS-A	(94-96)
TPH1	Serotonin pathway genes	IBS-D	(121)
TPH2	Serotonin pathway genes	IBS-M	(92, 105)

IBS-A: IBS with alternating constipation and diarrhea, IBS-C: Constipation-predominant IBS, IBS-D: Diarrhea-predominant IBS, IBS-M: Mixed IBS, PI-IBS: Post-infectious IBS.

1.1.3.4 Diet/digestive enzyme deficiency

Diet is probably the most well-known contributing factor to IBS which is mainly reported by the patients. Many IBS sufferers have a list of foods which they believe exacerbate their symptoms, and therefore often try to avoid them (122, 123).

FODMAPs, which stands for ‘fermentable oligo-, di-, and monosaccharides and polyols’, are good examples repetitively identified as possible triggers for the IBS symptom development. FODMAPs are short-chain carbohydrates that, if not properly digested and absorbed, can induce accumulation of excessive luminal fluid due to their osmotic activity, and are also rapidly fermented by gas-producing colonic bacteria. This can together lead to IBS symptoms including distention, bloating and abdominal pain. FODMAPs exist in various foods and trigger symptoms in a subset of people while most people can tolerate them well. Different

factors are implicated in the symptom generation by FODMAPs including the absence or deficiency of the luminal or brush border enzymes, absence or hypoactivity of the epithelial transporters, dysbiosis and altered fermentation rate (124, 125).

Lactose intolerance classified in 'IBS-like disorders' is one example of a brush border enzyme (lactase) deficiency which is characterized by diarrhea, flatus, abdominal pain and distension. Lactase persistence (persistence of the lactase activity after the weaning phase) is dominantly inherited and has a strong genetic background. However, in addition to the expression level of lactase, other factors such as gut microbiota, GI motility and the level of GI tract sensitivity to the gas accumulation play important roles in the induction of the IBS-like symptoms (126, 127).

Sucrase-isomaltase (SI) is another intestinal brush border enzyme which is key to the degradation of the daily digested sugars (sucrose) and starch (128) and its deficiency could cause the accumulation of unabsorbed carbohydrates in the colon leading to a variety of symptoms commonly reported by IBS-D patients (abdominal pain, bloating, and osmotic diarrhea). CSID (congenital sucrase-isomaltase deficiency) is the congenital form of SI deficiency and is generated due to the harboring of two defective alleles of the gene which results in a serious reduction of the enzyme activity or even a complete abolishment of it (129, 130). However, although CSID is usually diagnosed in infancy, there are reports of diagnosis in adults who have been misdiagnosed with IBS due to the milder symptoms (131, 132). Therefore, it could be speculated that alterations in the enzyme activity secondary to the milder (heterozygotic) functional genetic variations could contribute to IBS predisposition. In paper V, through a series of independent experiments, we test the potential relevance of *SI* gene variants for IBS, stool frequency, and gut microbiota composition.

1.1.3.5 Gut Microbiota

A major focus of my Ph.D. project was the gut microbiota. So herein I will discuss it in more detail.

Human individuals are born with a few passive mother-to-child transferred bacteria which co-develop with them from birth until death (133). The human gut microbiota is a very complex ecosystem, which has co-evolved with the host and imparts immense effects on her/his physiology. This effect is so large that it has been postulated that there are several axes in the body in which the gut microbiota is at one end, including gut-liver axis (134), gut-immune system axis (135), gut-heart axis (136), gut-muscle axis (137) gut-brain axis (138, 139) and even gut-renal axis (140, 141). For decades, it was believed that the human gut microbiota outnumbers the host cells by 10 fold. However, recently it has been shown that this was due to a misestimation of the body's cell number as well as the volume of the colon and therefore the correct ratio seems to be 3.8 to 3 (bacteria to human cells) which is still remarkable because it means that we are more bacteria (56%) than human! (142, 143) (figure 6). In the same manner, the total weight of the bacteria living in and on the human body is estimated to be 200 gr (it was miscalculated as 2 kg before). However, although the gene number of the microbiota has been estimated/calculated differently in different attempts (144, 145), it seems that microbial gene content outnumbers the human genes by a factor of 100 (at least) which

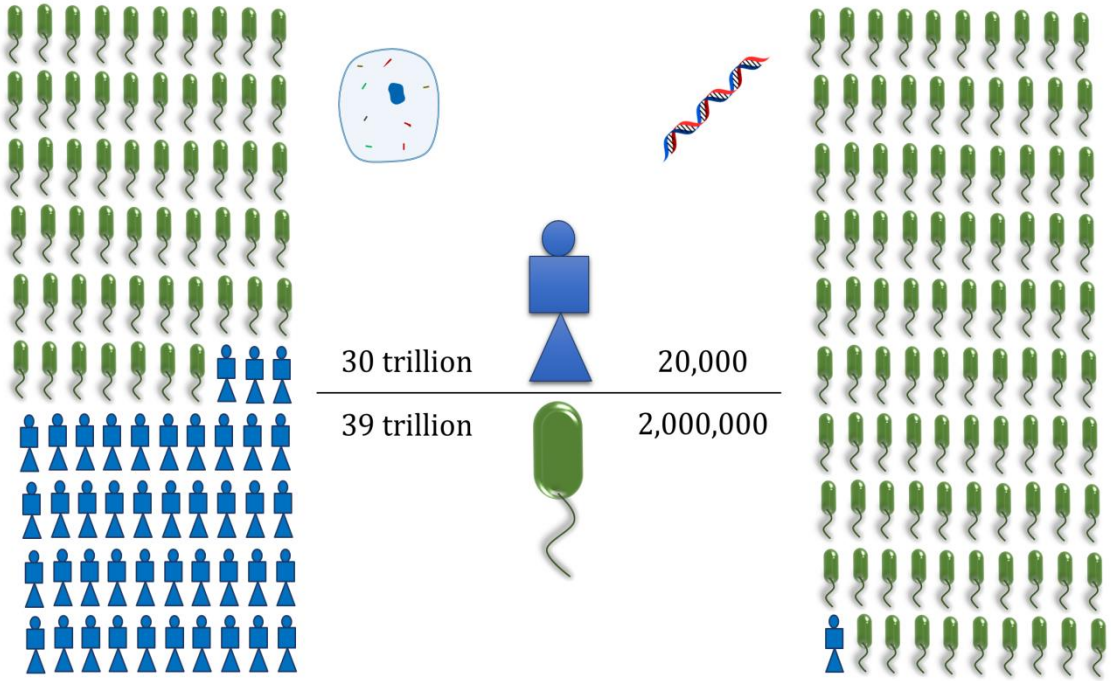


Figure 6. The ratio between number of human cells to the number of gut microbiota (left) and the number of human genes to the number of gut microbial genes (right).

led to the coining of the term 'the *second human genome*' (146). Nevertheless, it is very important to note that unlike our own genome, our microbiome (all microbes and their genes (147)) is possible to be re-shaped or even cultivated (138, 139) which could be considered as a big opportunity for future medical interventions. Another interesting aspect that should be taken into consideration is that although we, as humans, are very similar to each other regarding our genome (almost 99.9% similarity) our microbiomes are very different (roughly 10% similarity) (148) which could be a positive point regarding individualized medicine.

Noteworthy, the human microbiota encompasses bacteria, as well as, archaea, eukaryotes (mainly yeasts) and viruses (mainly phages) but since bacteria prodigiously outnumber the other domains, scientists sometimes refer to the whole microbiota as the bacteria. Obviously this amount of bacteria is not distributed equally over the GI tract and regardless of the mouth which is considered as the second dirtiest place in the body, there is a density gradient for bacteria from the stomach to the anus (figure 7) (142). Therefore, when we talk about the gut microbiota we mostly refer to the bacteria colonizing our colon. In 2011, Arumugam and Raes introduced a new concept to the world of microbiota, i.e. enterotype. Deep profiling of metagenomics fecal sample data from 39 healthy individuals revealed that human gut microbiota can be clustered into three discrete groups which were dominated by *Bacteroides*, *Ruminococcus* and *Prevotella* respectively (149). However, these enterotypes could not be replicated in all subsequent studies which different methodological factors including clustering approaches, operational taxonomic unit (OTU)-picking methods, read depth and 16S rRNA primers could be the explanation (150). Moreover, some studies even have claimed that enterotype distribution is spectrum-wise and there is a gradient instead of discrete groups similar to blood groups (151).

Until a couple of years ago, the gut microbiota was considered as a forgotten organ (152) but nowadays its importance is so obvious that scientists have started to call it the second brain of the human (138, 139), the second liver (153) and as mentioned before the second genome (146). Gut microbiota's impacts on the host body can be categorized into two different classes of beneficial effects which arise from the healthy gut microbiota and the harmful effects which are secondary to the dysbiosis. Achieving a better comprehension of a 'healthy gut microbiota' could be the first step in better understanding of the substantial effects of gut microbiota on host health.

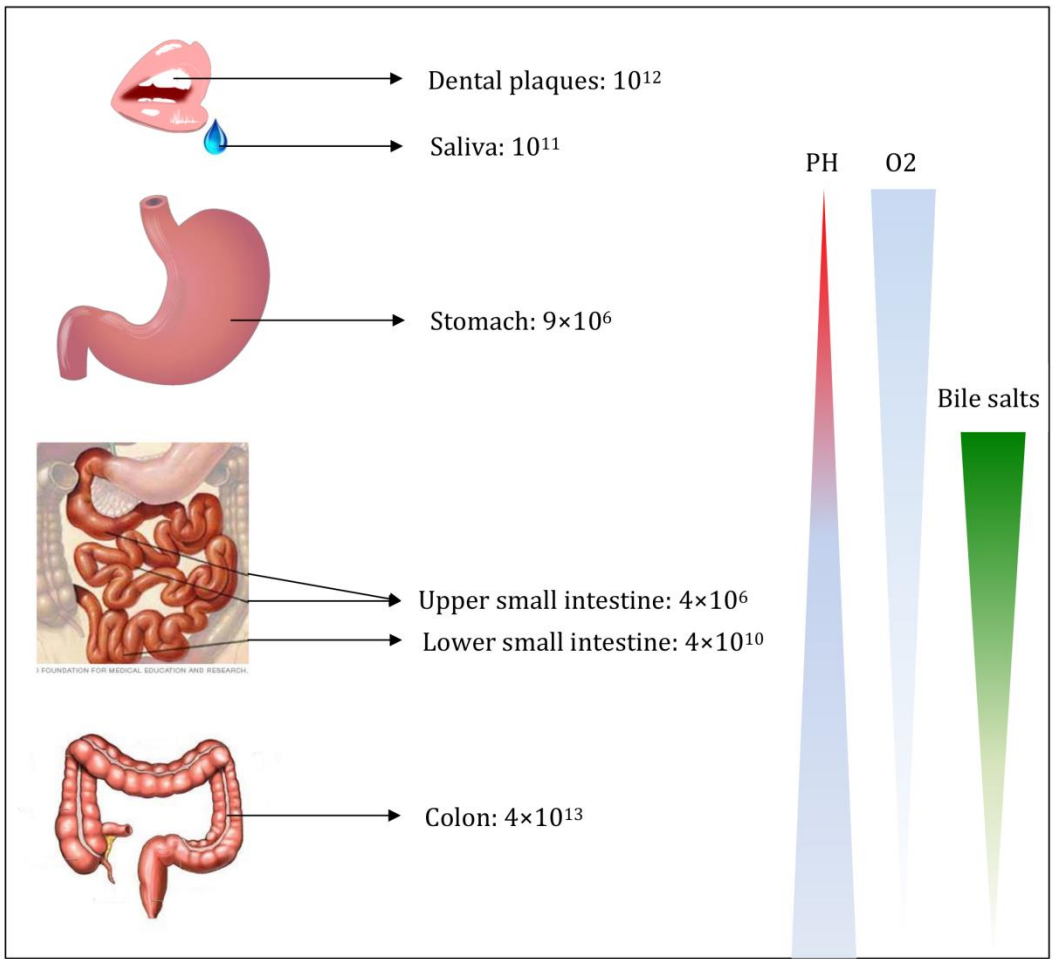


Figure 7. Bacterial density in each section of GI tract, calculated based on the concentration of bacteria and volume (Sender, revised, 2016).

I. Healthy gut microbiota

A literature review shows that there is no unique consensus definition for a 'healthy gut' yet. The European metagenomics of the human intestinal tract (MetaHIT) as well as the human microbiome project (HMP) from the US are among the first large-scale sequencing projects (n=124, and 242, respectively) accomplished to describe the healthy gut's as well as other organ's microbiota (figure 8).

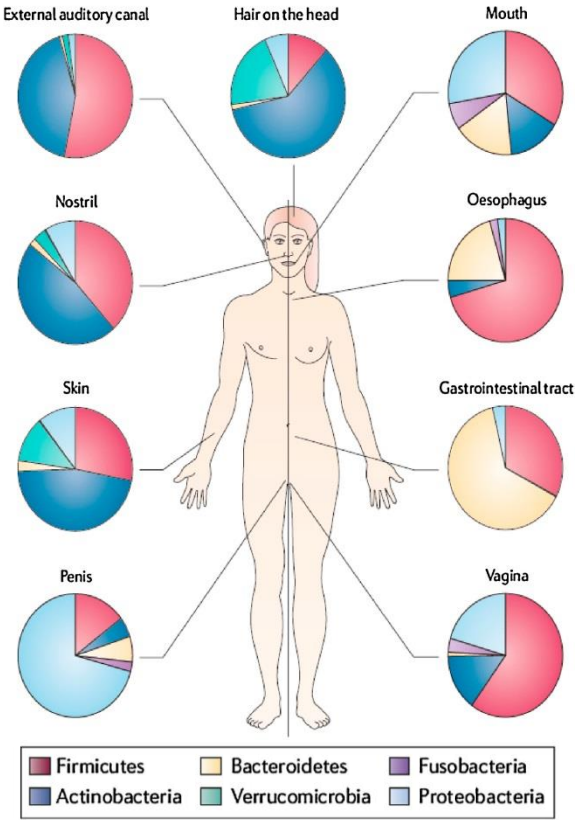


Figure 8. Different phyla inhabiting different body sites. Reprinted from Advances in Medical Sciences, 62/2, Blum HE, The human microbiome, Pages No. 414–420, Copyright (2017), with permission from Elsevier.

However, these remarkable studies followed by considerable amounts of other investigations indicated that the microbiota composition is very complex and varies significantly between and even within individuals (over time). Noteworthy, it has been known that each individual is colonized by more than 1,000 species which obviously originate from only a few phyla. However, although almost all the studies have a consensus over the two major bacterial phyla being *Firmicutes* and *Bacteroidetes* (154, 155) a Dutch cohort, called LifeLines-DEEP (n=1135), showed that agreement even over this level of information is not easily achievable by revealing *Firmicutes* and *Actinobacteria* as the two dominant phyla in the study participants (156).

Intriguingly, a Cell publication in 2013, has indicated that the functionally active part of the gut microbiota is predominant in *Firmicutes* and depleted in *Bacteroidetes*, so it could be concluded that the *Firmicutes* phylum is the functional subset of the gut microbiota although *Bacteroidetes* is usually the more abundant phylum (157, 158). In addition to the number of different bacteria that harbor our gut, how evenly they are distributed, how phylogenetically close they are, how many genes they have and how many of them are active in the body, are other very important factors that should be taken into consideration (155). In general, it seems that high diversity, more stability and probably a decent ratio between so-called good and bad bacteria (such as *Faecalibacterium prausnitzii* and *Roseburia* versus *Escherichia* and *Fusobacterium*), are among a few facts that we know so far about a healthy gut microbiota.

Having proven the high variability of the healthy gut microbiota, the next step would be to understand the physiological factors contributing to program this normal variation as well as the factors potentially able to disturb it and push it towards dysbiosis.

I.I. Intrinsic host factors

Age. Before delivery, during it and immediately afterward, the infant's GI tract is colonized by a low diversity microbiota originating from mother, diet, and environment (133, 159). When starting the solid food, the gut is gradually inhabited with more diverse microbiota and when established (from around 18 months to three years old), this compositional structure remains highly resilient (although one study has shown that adolescent's gut microbiota differs from adults' (160)). Again in elderly (after 70) the diversity decreases. These age

categories have been shown to be accompanied by changes in the ratio between *Firmicutes* and *Bacteroidetes* being 0.4, 10.9 and 0.6 for infants, adults and elderly subjects, respectively (161). However, another interesting study performed on three groups of people aged 99 to 104 years (centenarians), 63 to 76 years and 25 to 40 years, revealed that only in centenarians was the gut microbiota altered significantly but the composition in young adults and seventy-year-old people was quite similar (162). Moreover, age has been recorded as being among the 18 non-redundant variables associated with microbiota composition that resulted from one of the most comprehensive analyses carried out so far (163) and this result has been confirmed by another general population-based important study in the field (156). Finally, in a key study investigating the different genetic and non-genetic factors involved in the shaping of gut microbiota structure, age was revealed to be responsible for almost 5% of all the variations (164).

Gender. For years, although gender was usually considered among the covariates in microbiota studies, there was not a solid scientific background for this. In an investigation of subjects from four countries of France, Germany, Italy, and Sweden in 2006, a gender effect was only reported for a specific bacterial group (165). Another study in five northern European countries, could not find any grouping for colonic microbiota according to the gender of the hosts (166). However, in an attempt to investigate the association between obesity as a function of gender with gut microbiota composition, Haro *et al*. observed that gut microbiota probably is different between the genders (167). Almost reassuringly, this finding was confirmed by two recent Science publications (156, 163). More interestingly, in Zhernakova's paper, gender, as well as age, was shown to be correlated with microbial functional richness in addition to the composition and diversity (156). Also, in the Wang *et al.* study sex was shown to be responsible for close to 2% of the variation in the gut microbiota composition (164).

Fecal chromogranin A (CgA). CgA is another factor recently shown to be negatively associated with gut microbiota composition (156). CgA is secreted from neurons, the endocrine system and immune cells (under stress) and is considered as an indicator for the activation of the neuroendocrine system. Interestingly, in this study, 126 host factors (intrinsic and extrinsic) have been studied and CgA demonstrated the strongest association with different microbial indices including several taxa in which their total abundances account for more than 50% of the gut microbial composition. However, it should be noted that in the same study, CgA was shown to be negatively associated with the amount of fruit and vegetable intake in the studied population so any conclusion should be made with caution.

Genes. From the very beginning of the 20th century, twin studies suggested a heritable origin for microbiota, indicating that host genetics could play an essential role in the formation of the gut microbiota composition (168). Since then, additional heritability studies, as well as candidate gene studies in both murine models and human settings, have supported this hypothesis. A selection of heritability studies and candidate gene studies are summarized in tables 2 and 3. Finally, very recently a few well- designed GWAS have confirmed this (table 4).

Table 2. Selection of the microbiota heritability studies.

Study	Sample	Sequencing method	Results
Zoetendal *et al.* (2001) (168)	50 adults younger than 60 y (genetically related people were living separately)	Denaturing gradient gel electrophoresis	Fecal microbiota profiles of MZt were more similar compared to unrelated people. Bacterial profiles of spouses were not significantly more similar than unrelated individuals. The more genetically related, the more similar bacterial profile.
Stewart *et al*. (2005) (169)	13 MZt, 7 DZt and 12 unrelated control pairs. median age: 23 m (4 m to 10 y)	Temporal temperature gradient gel electrophoresis	Fecal profiles of the MZt showed the highest level of similarity in addition to the significant difference with DZt and controls.
Turnbaugh *et al.* (2009) (170)	31 MZt and 23 DZt (21–32 y/ all females and concordant for BMI) and 46 of their mothers (where available)	16S rRNA sequencing (gene's full-length) plus pyrosequencing (V2 and V6)	Gut microbiota of MZt was not significantly more similar than DZt. People from the same family shared a more similar bacterial profile compared to unrelated individuals (not correlated with the physical living distances).
Goodrich *et al.* (2014) (171)	171 MZt, 245 DZt, 2 unknown zygosities, 143 single individuals from a twin (20 men, the rest women) from TwinsUK registry	16S rRNA (V4)	Fecal microbiota was more similar within twin pairs than controls. Comparing MZt and DZt, MZt had more similar microbiota only when testing unweighted Unifrac. About 5% of the taxa were shown to have heritability and *Christensenellaceae*, a family associated with low BMI, was the most heritable taxon.
Goodrich *et al.* (2016) (172)	472 MZt and 418 DZt from TwinsUK registry	16S rRNA (V4)	They expanded their initial twin study and detected 8.8% of the taxa to be heritable.
Xie *et al.* (2016) (173)	69 MZt and 181 DZt females from TwinsUK registry	Whole-metagenome shotgun sequencing	In addition to supporting many of the previous results, they inferred that the high similarity in microbiome variations within twins reduces slowly over years of separate living.

DZt: Dizygotic twins, MZt: Monozygotic twins, m: months, y: years old.

Table 3. Selection of the microbiota candidate gene studies*.

Gene name	Gene function	Description
Nucleotide-binding, oligomerization domain 2 (*NOD2*)	Mediates the host response to the bacterial peptidoglycan and is implicated in susceptibility to the Crohn's disease.	• In one study in Nod2-deficient mice, the load of commensal bacteria was increased in these mice while their ability to prevent their GI tract from colonization by pathogenic bacteria was decreased (174). • Another murine model study in addition to supporting these results indicated the substantial influence of *nod2* on the early development of the intestinal microbiota in Crohn's disease (CD) patients (175). • In a cross-sectional analysis of the human mucosal (ileal) samples, *NOD2* genotype (and IBD phenotype) was linked to a shift in the relative abundance of the *Clostridium coccoides* and *Eubacterium rectale* groups (176). • A significant association between an increase in the relative abundance of *Enterobacteriaceae* and *NOD2* risk allele counts was demonstrated in an investigation on 474 individuals from three independent cohorts (151).
Fucosyltransferase 2 (*FUT2*)	Is responsible for the presence of ABO histo-blood group antigens found on the GI mucosa and secretions	• Comparing the healthy subjects and CD patients revealed that *FUT2* genotype can explain some differences in the composition and diversity of the gut microbiota (177). • Two years later these results were replicated in another study using mouse model and it was shown that microbial diversity of *Fut2*$^{-}$ mice is diminished (76). • An investigation on 14 non-secretor and 57 secretor adult humans, confirmed the previous results by demonstrating lower species richness in non-secretors compared to the secretors (178). • Study on 35 healthy individuals including 27 secretors and 6 non-secretors, supported the hypothesis of significant difference between bacterial taxa of secretors and not secretors. However, in this study non-secretors had higher α-diversity at the taxonomic higher levels of phylum, class, and order. Also, this study claimed a bigger effect for being non-secretor compared to blood groups (179).
Human leukocyte antigen (*HLA*)-*DQ*	Recognizes and presents foreign antigens to the immune cells and its mutations predisposed the carrier to the Coeliac disease (CoD)	• A study on 20 newborns whom at least one of the first-degree relatives suffered from CoD, showed that this gene can influence the gut microbiota composition by inducing changes in the abundances of different bacteria (180). • Those results were supported by another study which in addition, demonstrated that high genetic risk of suffering from CoD is accompanied by considerably higher proportions of *Firmicutes* and *Proteobacteria* and lower abundances of *Actinobacteria* (181).
Immunity-related GTPase M (*IRGM*)	Involved in the regulation of autophagy	This gene which is among a few genes recognized to affect gut microbiota composition in the healthy population is the only one shown to be associated with the presence of an enterotype (*Prevotella*-predominant

		enterotype) (182).
lactase (*LCT*)	Translates to the lactase (an enzyme for hydrolyzing lactose in the GI tract)	• In one of the first microbiota-GWA studies (n=93), this gene was shown to be associated with the GI abundances of *Bifidobacterium* (183). This interesting result has been supported by another investigation on 1,126 twin pairs (172) and two other big GWA studies (164, 184), in one at the genome-wide significance threshold level (184)**.**

*There are other genes that have been shown to be involved in the formation of gut microbiota composition. Among them, one can refer to *IgA* (185), *MEFV* (186), *NLRP6* (187), *ABO* (188), *RELMβ* (189), and *DEFA5* (190) which except for *ABO* and *RELMβ*, all are involved in the immune system related functions.

CD: Crohn's disease, CoD: Coeliac disease.

Table 4. Microbiota genome-wide association studies (GWAS).

Study	**Sample**	**Sequencing method**	**Results**
Benson *et al.* (2010) (191)	645 mice	16S rRNA (V1-V2)	64 core measurable microbiotas were studied in association to 530 SNPs. This study revealed 18 host-associated QTLs (quantitative trait loci) correlated with the abundances of some specific microbiota. It also revealed that the gut microbiota composition could be a polygenic trait. Moreover, some of the QTLs showed pleiotropic effects on the microbiota signature. They also reported phyla of *Actinobacteria*, *Erysipilotrichi*, and *Epsilon* to be QTL associated.
Blekhman *et al.* (2015) (183)	Discovery cohort: 93 from HMP Replication: 984 from the TwinsUK	Whole-genome sequencing	In this study, 83 significant associations were reported (at the suggestive significance threshold of 1.16E-5). A significant association between the first stool microbiome principal coordinate and host genetic principal component was reported. An SNP in *LCT* gene was shown to be linked to the *Bifidobacterium* abundance.
Davenport *et al.* (2015) (192)	91 and 93 (summer and winter, respectively) from Hutterites	16S rRNA (V4)	α-diversity and bacterial relative abundance were studied in this GWAS. Running a classic GWA analysis and applying the genome-wide significance threshold, they did not find any significant result. But changing the significance level (q-value=0.2), at least in each sample, one bacteria was shown to be significantly associated with at least one SNP. *Akkermansia* (previously shown to be obesity-related) was associated with an SNP close to *PLD1* gene (also shown to

			be associated with BMI). Performing gene set enrichment analysis (GSEA), they found some pathways including immune processes related to summer samples and metabolic processes enriched in winter samples.
Goodrich *et al.* (2016) (172)	1126 including 472 MZt and 418 DZt from TwinsUK	16S rRNA (V4)	Bacterial taxa and β-diversity indices were investigated as the study traits. No study-wide significant signal was detected. Testing the taxa level, 28 loci, and studying β-diversity indices, 3 loci showed genome-wide significant (GWS) associations. Strongest associations were reported for the gene *SLIT3* with the taxa *unclassified Clostridiaceae*. Also, *LCT* gene was revealed to be in strong linkage disequilibrium (LD) with the gene *R3HDM1* (including a SNP with the strongest association with *Bifidobacterium*).
Wang *et al.* (2016) (164)	Discovery cohort: 1,812 individuals from two German cohorts Replication: 371 German individuals	16S rRNA (V1-V2)	In this study bacterial taxa and β diversity indices were studied. Investigating β-diversity measures (PCoA based on Bray-Curtis and Jaccard), and bacterial taxa, 42 and 40 GWS associated loci respectively were reported. Among them, the most interesting result was related to the locus including *VDR* gene which encodes vitamin D receptor. This gene was significantly associated with β-diversity indices as well and vitamin D response appeared in the results of the GSE analysis. In this study, the results for three genes of *FUT2*, *NOD2*, and *LCT* were replicated too. Also, they indicated that genetic factors account for roughly 10% of the gut microbiota variations (almost equal to all known non-genetic factors).
Bonder *et al.* (2016) (184)	Discovery cohort: 984 Replication: 530 Dutch individuals	Whole genome sequencing	In addition to bacterial taxa, functional gene categories and bacterial pathways were studied. In this study, 9 GWS associated loci were detected in relation to 8 taxa of which the most significant associations were reported with genus *Blautia* and Species *Bacteroides xylanisolvens* (*Blautia* has shown heritability in the Goodrich *et al.* study (2016) too). Moreover, 33 GWS loci in association with the other two traits were

			reported, without any overlap with the results from bacterial taxa. Also, in addition to the replication of the association between *LCT* and *Bifidobacterium*, they suggested that an interaction between a specific genotype and milk consumption could play a role in the determination of the *Bifidobacterium* abundance.
Turpin *et al.* (2016) (193)	Discovery cohort: 1098 Replication: 463 (from US and Israel)	16S rRNA (V4)	α-diversity, microbial dysbiosis index (MDI) and bacterial taxa were three study traits. They could not find any GWS association for either α-diversity or MDI. However, 58 loci significantly associated with bacterial taxa, of which 6 remained significant after multiple test correction. Also, they could replicate the association of 4 loci (containing *UBR3*, *CNTN6*, *DMRTB1* and *SALL3* genes) with *Rikenellaceae*, *Faecalibacterium*, *Lachnospira*, and *Eubacterium*, respectively, in the replications.

DZt: Dizygotic twins, LCT: Lactase, LD: Linkage disequilibrium, MDI: Microbial dysbiosis index, MZt: Monozygotic twins, PCoA: Principal coordinate analysis, GSEA: gene set enrichment analysis, GWAS: genome-wide association study, GWS: Genome-wide significant.

Although at least the last four GWAS have exploited decent sample sizes, surprisingly most of the signals were only reported in one study and very few overlaps are detectable between the results (194)(the best result we have so far is the signal related to the *LCT* gene which has constantly been shown to be associated with *Bifidobacterium*). This observation has been linked to the high complexity of the trait. It means that a large number of genes and loci are involved in the formation of the trait and each gene/locus contributes to a very small fraction of the variations (according to the definition of complex genetic traits) which makes it very difficult for it to be identified or replicated in different studies (195). Moreover, when the effect sizes are very small, differences in methodology and experimental protocols applied in the different studies could easily outweigh the underlying effects. In one study from the Rob Knight lab, it was observed that individuals from different microbiota study cohorts, primarily clustered by the studies which showed that any difference in the study methods including primer selection for 16S studies, DNA extraction techniques (one of the largest sources of bias (196)), sequencing platform, and bioinformatics pipelines could introduce systematic biases into these studies (197).

I.II. Extrinsic host factors

I.II.I. Mother induced

-*Method of delivery*. Infants born through natural vaginal delivery within a few days after birth are mainly colonized by microbiota which is similar to their mothers' vaginal microbes (mainly *Lactobacillus*, *Prevotella*, or *Sneathia* spp.). However, gut microbiota of infants born through cesarean section (CS) more resembles the commensal skin bacteria (dominated by

Corynebacterium, *Staphylococcus*, and *Propionibacterium* spp.) (198). An interesting study which followed vaginally and CS delivered babies for two years, revealed that microbial diversity of CS born children was significantly diminished. Moreover, the abundance and diversity of *Bacteroidetes* phylum were lower in these children compared to vaginally delivered babies (199). However, the long-term effect of the delivery method is not supported by adult studies and is not clear yet (154). It is worth mentioning that gut microbiota of pregnant mothers alters from the first trimester to the third. The first-trimester microbiota is quite similar to non-pregnant women but the third-trimester microbiota has less *Faecalibacterium* (butyrate producer with anti-inflammatory effects) and more *Proteobacteria* and *Actinobacteria* and shows decreased richness which is strongly correlated with inflammation and energy loss. However, interestingly, the children's microbiota composition is more similar to the microbiota composition of mothers in the first trimester (200).

- *Method of infant feeding*. Mothers' breast milk contains particular oligosaccharides including indigestible sugars which are completely unique to humans and can be directly consumed by the infant's gut microbiota (mainly *Bifidobacterium* and also *Bacteroides*). During the breastfeeding process, the type and amount of the carbohydrate content of the breast milk changes which could be reflected in the alterations of the gut microbiota composition (154, 201).

In summary, breastfeeding in contrast to formula has been shown to affect gut microbiota in at least three ways:

1. Breast milk includes viable bacteria (10^2 to 10^4 per ml), which means at least 10^5 bacteria per day for exclusively breastfed infants. Hence, breast milk, on its own, can be considered as a probiotic (202, 203).
2. Breast milk can be considered as a prebiotic as well. Oligosaccharides are one of the key components of the breast milk which can reach the colon and thereby contribute to the development of selective gut microbiota (204). So, regarding these two items, breast milk can be referred to as a synbiotic!
3. Human milk also includes some immunological compounds like Lactoferrin and IgA. So in the end, through interaction with pathogens and helping colonization of the commensal bacteria (by preventing colonization and attachment of non-commensal ones), breast milk can contribute to shaping a normal microbiota (205).

I.II.II. Lifestyle-related factors

- *Diet.* Diet is obviously the most well-studied factor associated with gut microbiota composition and probably the most important one. The long-term effects of diet have been studied vastly and it has been demonstrated that diets full of fruit and fiber are associated with higher microbial diversity and a predominance of *Prevotella* over *Bacteroides*. In contrast, Western diets including high amounts of fat and/or sugar and low amounts of fiber, result in diminishing the SCFA producing *Firmicutes* and increasing enteric pathogens (206). In addition, in the last couple of years, two papers published in Science and Nature have revealed the importance of short-term diet on the alteration of gut microbiota composition (207, 208). Unsurprisingly, the significant association between specific diets and gut microbiota composition has been further confirmed by another two

key studies in the field (156, 163). In one of these studies, a negative association was revealed between all the Western-style foods and the microbiota diversity. Noteworthy, in this investigation, they could not find any association between carbohydrates and *Prevotella* to replicate previous results (156). However, in the Wang *et al.* study carried out on a decent sample size from two German cohorts, diet was shown to be associated with the gut microbiota signature but it merely could explain roughly 6% of all the variations (164).

- *Exercise.* There are multiple, mainly animal studies, examining the effect of exercise on the structure of the gut microbiota. Human studies seemingly are more focused on comparing athletes with controls. For instance, one study in 2014 showed that athletes in comparison to control group had a more diverse gut microbiota structure which partly could be related to their different diet (high amount of protein consumption) (209). Moreover, another very recent Gut publication comparing professional athletes with sedentary controls has revealed that even SCFA (produced by gut microbiota), are increased in the athletes which is indicative of the importance of exercise at the levels of metagenomics and metabolomics in addition to the compositional level (210).

I.II.III. Environmental factors

- *Geography.* There are a couple of studies concentrating on the differences in gut microbiota composition based on geographical region. One study accomplished in the Jeffrey Gordon lab (2012), demonstrated that there are significant differences in the microbial composition of people residing in the US (metropolitan areas) compared to individuals living in the Amazonas of Venezuela and rural Malawian communities (211). Also, additional studies have indicated that people living in western countries harbor different microbiota compared to individuals living in under-developed countries (212, 213). These differences can be explained by differences in the genetic background and lifestyle including diet and hygiene. Moreover, a very interesting study on 1020 healthy individuals from 23 populations has displayed geographical latitude to be correlated with the abundances of *Firmicutes* (positively) and *Bacteroidetes* (negatively). The motivating hypothesis for this study originated from the observation of an increasing ratio between *Firmicutes* and *Bacteroidetes* in obesity and increasing of the body mass in the colder climates as an adaptation mechanism (214). Finally, another intriguing study on the residents of an absolutely isolated village in South America revealed the most diverse bacterial ecosystem ever observed in a human population. In addition, the genetic function of their microbiome showed the highest diversity (in fecal and skin samples). This became even more interesting when they found the functional antibiotic resistance (AR) genes among the microbiome of this population while they didn't have any known exposure to antibiotics. This could be interpreted that those functional AR genes are a feature of the human genome regardless of its exposure to synthetic antibiotics (213)

- *Having Siblings*. There are a few studies that have compared gut microbiota of infants with and without older siblings. Results in this field are still preliminary and contradictory, i.e. while one study has reported a decrease in the measurements of α-diversity in the infants with older siblings (215), these indices were shown to be increased in another study (216). Also, in the presence of controversies, it seems that not being the first child can result in having a more beneficial microbiota such as *Bifidobacterium* (217).

- *Pet owning.* There is no consensus in this area either. While Laursen *et al*. did not find a remarkable effect for furred pets on the infants' gut microbiota composition (216), Azad showed that infants born in houses with pets have an increased gut microbiota richness and diversity (215).

I.III. Microbial factors

Although it potentially looks very important, thus far very little is known about the role of microbial factors in the stability of host-microbial composition. A key study in 2013, has defined a genetic locus, namely commensal colonization factors (*ccf*) which possibly could play a role. This locus harbors genes coding for polysaccharide utilization and has been shown to be conserved among different species of *Bacteroides* genus (one of the most prevalent genera established in the colon). This locus was discovered subsequent to the interesting observation of the resistance of germ-free mice mono-colonized with a single *Bacteroides* species to colonization by the same species while it was not the case for another species. Deletion of the genes mapped to the *ccf* loci in the murine model resulted in defective colonization and these genes were shown to be necessary for the *Bacteroides* re-colonization after an induced microbiome disruption. Moreover, different independent studies confirmed a difference for the physical colonization location of the wild-type bacteria compared to the *ccf*-mutant harboring species in a way that only wild-type bacteria could reside within the intestinal crypts. Interestingly, crypt-associated species have been shown to be able to persist in the presence of antibiotic treatment (with the capacity of later repopulating the GI tract) which indicates the importance of this locus for the resilience and stability of *Bacteroides* in the complex composition of the gut microbiota (218).

II. Dysbiosis

In the next section, I will shortly describe some of the known factors implicated in disturbing the healthy composition of the gut microbiota and pushing it toward dysbiosis.

Antibiotics and other xenometabolites. Thus far, a couple of dozen of medications have been shown to play a substrate role for microbial enzymes in the process of metabolism (158, 219). All of these xenometabolites which could target both human cells and microbes (antibiotics) could potentially change the microbial composition. Short-term and long-term effects of antibiotics on alteration of gut microbiota composition have been discussed for years (220, 221). However, it should be pointed out that different bacteria react differently to exposure to different antimicrobials. For instance, the response of *F. Prausnitzii* to ampicillin and ciprofloxacin is increasing (by 4.5 fold) and decreasing, respectively (157). In recent years, the influence of host-cell targeted drugs has been studied as well. Among these, one can refer to metformin (222), proton pump inhibitors (223, 224) and rifaximin (225, 226). Moreover, in a very interesting study, Maurice *et al.* revealed that antibiotics and other xenobiotics could change the gene expression of the active phyla (primarily *Firmicutes*) of the gut microbiota in addition to its structure and diversity (157).

- *Smoking.* It has been elucidated that gut microbiota composition in humans is affected by smoking. Seemingly most of the investigations in this area are concentrated on ex-smokers. In this regard, two studies have shown that gut microbiota of individuals who ceased smoking

is characterized by more *Firmicutes* and *Actinobacteria* and less *Bacteroidetes* and *Proteobacteria* (16, 227). Also, in one of these studies smoke quitting was associated with a more diverse microbiota composition (16). Zhernakova *et al.* studied the association between gut microbiota with being smoker or ex-smoker as well as having a smoker father or mother (during pregnancy). All these parameters were associated with microbiota diversity although the results were not very strong (156). In addition, it has been demonstrated that slightly more than 2% of the variation in the gut microbiota composition results from smoking (164).

- *Alcohol.* Alcohol dependency can be associated with altered gut microbiota composition and function (228). Moreover, it has been demonstrated that the presence of alcohol could increase the growth of Gram-negative bacteria which can result in the accumulation of endotoxin and acetaldehyde in the colon. In this situation, acetaldehyde through some alterations in tight junctions could increase the intestinal permeability and result in the absorption of more endotoxin. Subsequently, this alcohol-induced bacterial endotoxin could be transferred to the liver as well as blood circulation which results in inflammation in the liver and many other organs (229). The mild association between microbiota composition and alcohol-containing products has also been demonstrated in Zhernakova's publication (156).

- *Stress.* Although most of the studies involved in the investigation of the gut-brain axis are interested in the effect of microbes on stress and controlling this by bacteria-containing products such as probiotics, there is evidence, originating from several studies, demonstrating a negative effect of stress on the gut microbiota composition (230-232). This effect could be mediated through some stress-induced modifications in the GI physiology and function such as alterations in the gut motility, inhibition of gastric acid secretion, and induction of the growth rate of Gram-negative bacteria (by norepinephrine). The overall impact of stress on gut microbiota composition has been summarized in a review article as an increase in the abundance of potentially pathogenic bacteria (e.g. *Escherichia coli*) and at the same time a decrease in the abundance of advantageous bacteria (such as *Lactobacilli* and *Bifidobacterium*) (233).

III. Beneficial effects of the gut microbiota for host health

Most of the gut microbiota are either harmless or beneficial for the host body. They have interactions with the body and influence it in different ways.

Probably the most known influence of gut microbiota is its effect on the digestive system since most of the dietary fibers are not digestible by the enzymes of the small intestine (234), in contrast to gut microbiota which is a surprisingly rich source of enzymes that can digest these non-digestible carbohydrates.

Digestion of low-digestible carbohydrates happens through fementation by Carbohydrate-active enzymes (CAZymes), encoded by gut microbiome, which results in energy in the form of ATP and SCFAs as the end products of this fermentation process. SCFAs have been demonstrated to have numerous beneficial effects on the host body (235). Acetate, propionate, butyrate, isobutyrate, 2-methyl propionate, valerate, isovalerate and hexanoate are different SCFAs where the first three, are the most important ones. SCFAs (mainly butyrate) are the main energy source for colonocytes which keep them healthy and protect them from

colorectal cancer (236). *Faecalibacterium*, *Eubacterium*, *Roseburia*, and *F. prausnitzii* are among the bacteria known as SCFA producers (200, 237). Gut microbiota also has an extensive metabolic interaction with the host (238). It plays an important role in the regulation of glucose and cholesterol metabolism, i.e. it reduces plasma levels of glucose and cholesterol through different mechanisms and in this way could promote cardiovascular health (239). Another important impact of gut microbiota on the host physiology is akin to its effects on the functional structure of the GI tract including its influences on the tissue regeneration, gut barrier integrity, and morphogenesis of the GI vascular system (240). Gut microbiota also owns the interesting capacity of regulating the host immune homeostasis. Germ-free studies have shed light on the importance of the gut microbiota in the development of the host immune system. Both innate and adaptive immune systems have co-evolved with gut microbiota and these microorganisms play a special role in the maturation of gut-associated lymphoid tissue (240, 241). Furthermore, gut bacteria can protect the host against entero-pathogenic microbes and finally help to have a normal immune system/function (155). Gut microbiota is also essential for the homeostasis of some other tissues of which homeostasis of bone mass through regulation of osteoclastogenesis is notable (242). Production of vitamin B12, vitamin K, folate, biotin, thiamine, and riboflavin, mainly done by *Bifidobacterium*, is another important role played by microbiota for the host. They also facilitate the absorption of dietary fats as well as fat-soluble vitamins via their effects on the bile acids (238). Bile acid signaling is a pathway through which the gut-liver axis works (243) and the gut microbiota plays a pivotal role in the homeostasis of the host's bile acid pool through its different bile salt related enzymes (244). Another important everyday job done by microbiota is detoxification of harmful bioactive compounds in the host body. More than 40 bioactive compounds (either diet- or therapeutic- derived) have been recognized that need direct metabolic alteration by the bacteria in the body, among them xenobiotics (245). In relation to this, the gut microbiota plays an essential role in drug metabolism. Probably one of the most important reasons for the observed differences between individuals in response to drug therapy, which now is redirecting therapies toward personalized medicine, is the variation of gut microbiota composition among different individuals (246). Gut microbiota also has been shown to have an interaction with many host pathways, including the endocannabinoid system (247).

IV. Dysbiosis induced effects

Considering the immense interaction between gut microbes and the host, as well as the important roles played by these organisms, it is obvious that disturbance of this relationship, termed dysbiosis (248), may result in a plethora of pathological states/diseases in the host. A literature review shows that dysbiosis is associated with an emerging list of diseases which can be classified into two different categories of 'extra-intestinal' and 'gastro-intestinal' disorders.

IV.I. Extra-intestinal diseases

The mechanisms underlying many of these diseases are not crystal clear yet. However, abnormal intestinal permeability and microbial effects on epigenetic regulations are among the mechanisms possibly involved in the development of many of them. (249-251).

- Allergy (252, 253)
- Ankylosing spondylitis (254)
- Asthma (255)
- Atherosclerosis and thrombosis risk (256)
- Atopic dermatitis (257)
- Autism (258)
- Cardiovascular disorders(256, 259)
- Cystic fibrosis (260, 261)
- Depression (262)
- Diabetes[I and II] (263, 264)
- Multiple sclerosis (265, 266)
- Neurodevelopmental disorders (267)
- Obesity (170)
- Parkinson's disease (268, 269)
- Psoriasis, Psoriatic arthritis (270)
- Psychiatric and neurodegenerative disorders (256)
- Renal disease (271)
- Rheumatoid arthritis (272)

IV.II. Gastro-intestinal disorders

Numerous GI diseases have been recognized as being associated with an imbalanced relationship between gut microbiota and host. Among the important ones, I refer to:

- Alcoholic and nonalcoholic fatty liver disease (273, 274)
- Celiac disease (275)
- Cholesterol gallstones (276)
- Colon polyps (277)
- Colorectal cancer (278, 279)
- Infantile colic (280)
- Inflammatory bowel disease (281)
- Irritable bowel syndrome (282)
- Liver cirrhosis (283, 284)
- Mucositis and diarrhea (285)
- Necrotizing enterocolitis (286)
- Nonalcoholic steatohepatitis (287)
- Pouchitis (288)

Sections I to IV have been summarized in figure 9.

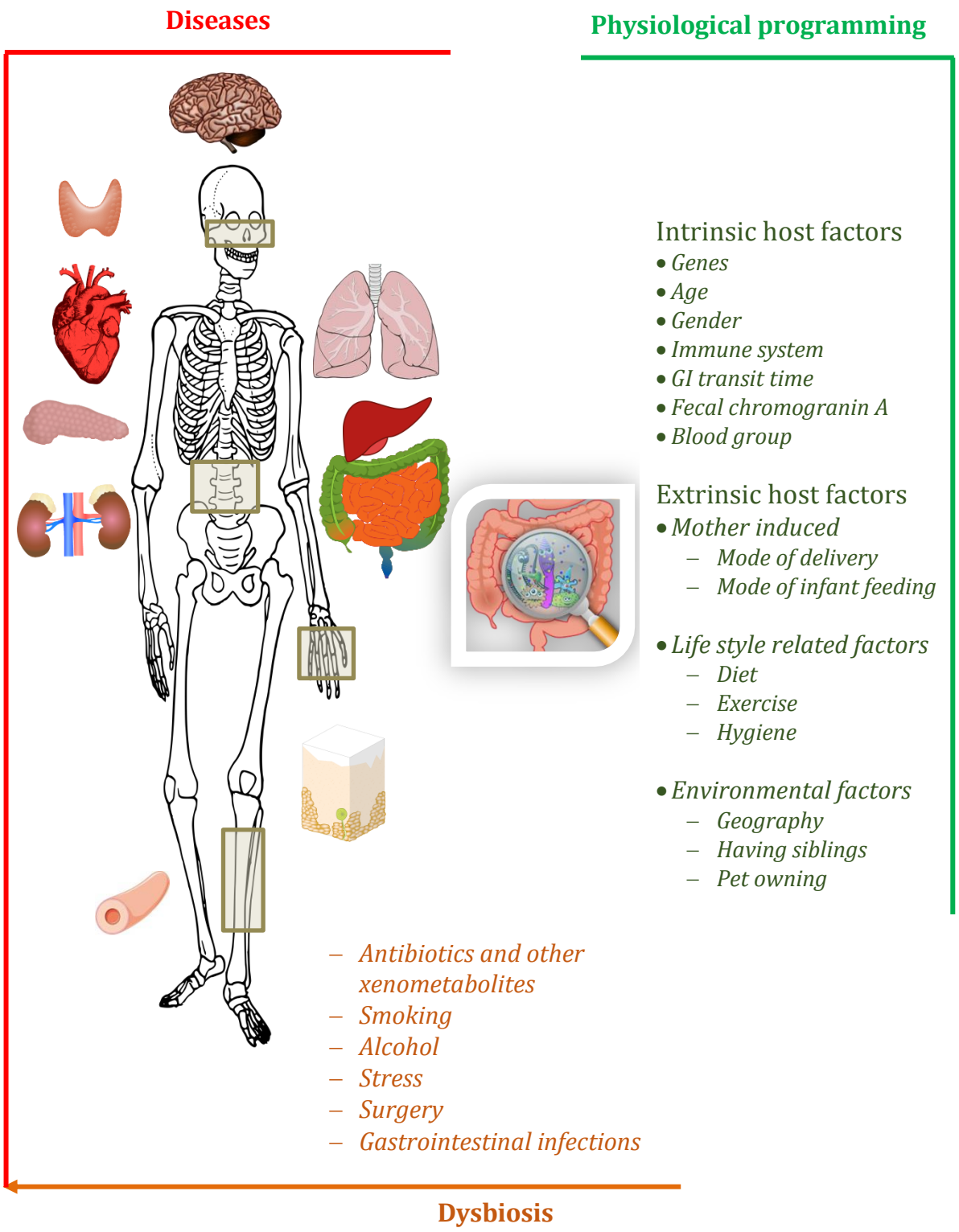


Figure 9. An overview of the physiological programming of a healthy gut, dysbiosis inducing factors and subsequent intra- and extra- GI disorders.

IV.II.I. Gut microbiota in IBS

From a couple of years ago, an emerging number of studies have been published discussing the association between gut microbiota and IBS. They have been accomplished on fecal or mucosal samples from colon or small intestine. Different and sometimes contradicting results

with very low reproducibility have been reported from the different studies so far (table 5). For example, in a few studies comparing small intestinal bacteria of IBS patients with controls, one showed no difference (289) while another revealed different alterations (290).
There is even no agreement regarding the overall changes in the total diversity and different publications have reported decreases, increases or no changes (282, 291-295). This amount of discordance could be related to several different factors. Patient characterization is one of the important factors discussed earlier. The small sample size of most studies could be another factor (292). Considering the complexity of the gut microbiota (comprising roughly 1000 different species), and assuming a need for 10 subjects per variable to have an acceptable power, a 'back of the envelope' calculation displays a very far-reaching sample size (196). Also, it should be noted that gut microbes are very location-specific (296) and thus samples from different sections of the lower GI tract should be compared with caution.
Results of a selection of the studies concerning dysbiosis in IBS have been presentd in table 5. Having a closer look at this table led us to admit that with the present information, we can only claim that IBS is accompanied with microbial alterations. What exactly these changes are and whether these alterations are causal or only a simple association remains to be elucidated (154).

To sum up, the importance of microbiota is not to be ignored and should seriously be taken into consideration although with caution.

Table 5. Dysbiosis in IBS

Study	**N**	**Sample**	**Microbial changes in IBS**
Si JM *et al.* (2004) (297)	IBS: 25 Ctrl: 25	Stool	*Bifidobacterium* ↓, *Enterobacteriaceae* ↑
Malinen *et al.* (2005) (298)	IBS: 27 Ctrl: 22	Stool	IBS-C: *Veillonella* spp.↑ IBS-D: *Lactobacillus* spp.↓ IBS: *Clostridium coccoides* subgroup ↓ *Bifidobacterium catenulatum* group ↓
Mättö *et al.* (2005) (299)	IBS: 26 Ctrl: 25	Stool	*Coliform bacteria*↑ (slightly), aerob:anaerob ratio↑
Maukonen *et al.* (2006) (300)	IBS: 24 Ctrl: 16	Stool	IBS-C: *Clostridium coccoides-Eubacterium rectale* group ↓
Sobieszczańska *et al.* (2007) (301)	IBS: 44 Ctrl: 34	Stool	*Enteroaggregative E. coli* strains ↑
Kerckhoffs *et al.* (2009) (302)	IBS: 41 Ctrl: 26	Stool, Duodenal mucosa	*Bifidobacterium catenulatum* ↓ (in both samples)
Krogius-Kurikka *et al.* (2009) (303)	IBS: 10 Ctrl: 23	Stool	IBS-D: *Proteobacteria* ↑, *Firmicutes* (Particularly *Lachnospiraceae* family) ↑ *Actinobacteria* ↓, *Bacteroidetes* ↓
Lyra *et al.* (2009) (304)	IBS: 20 Ctrl: 15	Stool	IBS-D: *Ruminococcus torques* ↑, *Clostridium thermosuccinogenes* ↓ IBS-M: *Ruminococcus torques* ↓, *Clostridium thermosuccinogenes* ↑ IBS-C: *Ruminococcus bromii*-Like ↑
Tana *et al.* (2010) (305)	IBS: 26 Ctrl: 26	Stool	*Veillonella* ↑ *Lactobacillus* ↑
Carroll IM *et al.*	IBS: 10	Stool,	IBS-D: Aerobic bacteria ↓ (in stool)

(2010) (306)	Ctrl: 10	Colonic mucosa	*Lactobacillus* spp. ↑ (in stool)
Noor *et al.* (2010) (307)	IBS: 11 Ctrl: 22	Stool	*Bacteroides vulgatus* ↓, *Bacteroides ovatus* ↓, *Bacteroides uniformis* ↓, *Parabacteroides* ↓
Kerckhoffs *et al.* (2011) (308)	IBS: 37 Ctrl: 20	Stool, Small intestin mucosa	*Pseudomonas aeruginosa* ↑ (in both samples)
Ponnusamy *et al.* (2011) (293)	IBS: 11 Ctrl: 8	Stool	Diversity of total bacteria ↑ *Bacteroidetes* ↑, *lactobacilli* ↑ *Bifidobacterium* ↓, *Clostridium coccoides* ↓
Rajilić-Stojanović *et al.* (2011) (309)	IBS: 62 Ctrl: 42	Stool	*Firmicutes*:*Bacteroidetes* ratio ↑ *Dorea* ↑, *Ruminococcus* ↑, *Clostridium* ↑ *Bacteroidetes*↓, *Bifidobacterium* ↓ *Faecalibacterium* ↓, *Methanogens* (when present) ↓
Parkes *et al.* (2012) (295)	IBS: 47 Ctrl: 26	Rectal biopsies	Total mucosa-associated bacteria ↑ *Bacteroides*↑ , *Eubacterium rectale-Clostridium coccoides*↑ IBS-D: *Bifidobacterium* ↓
Jeffery *et al.* (2012) (310)	IBS: 37 Ctrl: 20	Stool	*Firmicutes*-associated taxa ↑ *Bacteroidetes*-related taxa ↓
Jalanka-Tuovinen *et al.* (2014) (311)	IBS: 37 Ctrl: 20	Stool	PI-IBS and IBS-D: Several members of *Bacteroidetes* phylum ↑ uncultured *Clostridia* ↓
Rangel *et al.* (2015) (312)	IBS: 33 Ctrl: 16	Stool, Mucosal biopsies	*Actinobacteria*↑, *Bacteroidetes*↓, *Bacilli*↑, *Clostridium* clusters, *Proteobacteria*↑ (in stool)
Chung *et al.* (2016) (290)	IBS: 28 Ctrl: 19	Stool, Jejunal mucosa	*Veillonellaceae*↑ (in stool) *Prevotellaceae*↑, *Mycobacteriaceae* ↓, *Neisseriaceae* ↓ (in jejunal mucosa) *Firmicutes*:*Bacteroidetes* ratio ↑ (in stool) *Firmicutes*:*Actinobacter* ratio ↑ (in jejunal mucosa)
Ganji *et al.* (2016) (313)	IBS: 80 Ctrl: 50	Stool	*Citrobacter* ↑, *Lactobacilli* ↑, *Actinomycetes* ↑
Gobert *et al.* (2016) (314)	IBS: 33 Ctrl: 58	Stool	IBS-C: *Bacteroides* ↓, *Roseburia*↓, *Eubacterium rectale* ↓, *Bifidobacterium*↓ and an increase of *Enterobacteriaceae* ↑, *Desulfovibrio* ↑ *Akkermansia muciniphila* ↑
Tap *et al.* (2017) (315)	IBS: 110 Ctrl: 39	Stool, Mucosal biopsy	Enterotype dominated by *Bacteroides*↑

Ctrl: Controls, IBS: Irritable bowel syndrome cases, IBS-C: Constipation-predominant IBS, IBS-D: Diarrhea-predominant IBS, IBS-M: Mixed IBS, PI-IBS: Post-infectious IBS.

2 AIMS OF THE THESIS

The overall aim of this thesis is to contribute to understanding the interplay between gut microbiota, gut function and human genes in the generation of gastrointestinal symptoms.

More specifically the aims are:

- To determine how the gut microbiota influence gastrointestinal function/symptoms in the general population in relation to physiological traits and symptoms such as stool frequency, stool consistency, and pain (papers I and II).

- To characterize the association between human genes and gastrointestinal function (quantified by stool frequency as a surrogate marker for transit time) (paper III).

- To characterize how host genes, gut microbiota and gastrointestinal function are interconnected in the development of IBS (papers IV and V).

3 MATERIALS AND METHODS

3.1 Study population

The main cohort exploited in this thesis project was the Population-based colonoscopy cohort (PopCol). This cohort has been exploited in all of the constituent papers of this thesis project and was the only sample source for the first and second papers. This cohort is described below.

For the third paper, in addition to PopCol, a Dutch cohort, namely LifeLines-DEEP (described in detailed before (316)) was used.

In the fourth publication, GWAS association results from TwinGene (part of the Screening across the Lifespan Twin study, SALT) were inspected, and replication of selected genes was performed in a Swedish case-control cohort. The SALT cohort encompasses 45,750 Swedish twins with extensive epidemiological data including GI symptoms, of which 11,326, mostly dizygotic twins, have GWAS genotyping data and were recruited in our study. This cohort has been described in another publication from our lab (93). The case-control cohort is a Swedish multicenter cohort including IBS patients (Rome III definition) and asymptomatic healthy controls described in detail in the supplementary material of the fourth paper. The PopCol cohort also was exploited in this study.

For the fifth paper, the PopCol cohort, IBS cases and controls from the Swedish multicenter study, as well as sequencing data (of the *SI* gene) from seven IBS-D cases and one asymptomatic relative from four unrelated families, were used. The latter has been described in detail in the supplementary material of the fifth paper.

3.1.1 Population-based Colonoscopy cohort (PopCol)

For this cohort, 3347 Swedish adults (aged 18–70 years) were randomly selected, of which 1244 accepted an evaluation by a gastroenterologist and subsequently, 745 volunteers agreed to undergo an Ileo-colonoscopy. Based on the assumption that the gut microbiota is mainly established during early infancy, persons born abroad were excluded. Likewise, people with any organic gastrointestinal disorders such as inflammatory bowel disease or celiac disease were excluded and offered standard treatment.

Two subsamples of 264 and 204 subjects kept a detailed daily record of their bowel habits over 7 and 14 days respectively, of which 153 kept both (six years in between, on average). Moreover, close to the time of diary recording, subjects were asked to donate a fecal sample in addition to giving blood samples in the beginning of the study. At the end, we had different subsamples with different layers of data available from the first and second runs of data gathering which were used for the different studies (317).

3.2 16S sequencing and data processing

Two batches of fecal samples were collected over two time periods (from 2000 to 2012 with roughly 6 years in between samplings) and all the samples were stored at -80°C. EasyMAG NucliSens kits (Biomerieux) and Qiagen QIAamp DNA Stool Mini Kits, according to

manufacturer's instructions, were used for DNA extraction of the batches and extracted DNA was used at IKMB facility (Kiel, Germany) for amplicon sequencing.

The V1-V2 hypervariable region from 16S ribosomal DNA was amplified using universal 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 338R (5'-TGCTGCCTCCCGTAGGAGT-3') primers and sequenced using MiSeq next-generation sequencing platform (Illumina. the USA).

For data processing and quality control, raw paired-end reads of the coupled V1-V2 regions were merged using the fastq_mergepairs function of Usearch (v7.0.1090) (318). maxdiffs, minlen, minovlen, minmergelen, and maxmergelen were considered as 2, 200, 150, 270, and 330, respectively. fastq_filter function (maxee: 0.1) from the same software was used to quality filter FASTQ reads and convert them to FASTA. Chimeric sequences were specified using the UCHIME_ref function of the usearch (v7.0.1090) software (319) applying the SILVA gold database (320) as the reference. To remove homopolymers longer than 8 sequences, screen.seqs function was applied from Mothur (321). Sequences were classified applying classify.seqs command of mothur software, by using Silva bacterial reference and SILVA bacterial taxonomy files with a bootstrap value of 80% and applying 1000 iterations for the calculation of the bootstrap confidence score. Sequences related to chloroplasts, mitochondria, unknown, archea and eukaryotes were removed using remove.lineage function and the remaining sequences were aligned to the SILVA alignment database using align.seqs command implementing k-mer alignment procedure and non- aligned sequences were trimmed manually. Extra columns generated by alignment command were removed using the filter.seqs function and pre.cluster command was used for removing sequences that are likely due to sequencing errors. Then data was rarefied to 10,000 sequences per sample by subsample command. For defining OTUs, the dist.seqs command was used to make a distance matrix and reads were grouped using the average neighbour algorithm into OTUs (with 97% similarity) applying the cluster.split command (splitmethod=classify, taxlevel=4) and classify.otu (label=0.03) command was applied to get a consensus taxonomy for each OTU.

3.3 Genotyping and quality control

PopCol data genotyping was carried out using the Illumina HumanOmniExpressExome-8v1 array containing 951,123 SNPs at the SNP&SEQ Technology Platform at Uppsala University, following the manufacturer's protocol. SNPs with minor allele frequency and call rate smaller than 1% and 98% respectively, were excluded. Tests for the Hardy-Weinberg equilibrium were performed by the Fisher's exact test to confirm ($P > 10E-7$) allelic equilibrium at each SNP site. Sample with call rate < 0.95 or genotype-phenotype sex mismatch were excluded. Related individuals were filtered out when genomic relatedness was > 0.15. Principal component analysis (PCA) was applied to control for population stratification. All the quality assurance steps were performed in SNP and Variation Suite (SVS) v8.3.3 (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com).

Target genotyping of the *TRPM8* and *SI* loci in the Swedish case-control cohort has been described in the supplementary materials of paper IV and V, respectively.

3.4 Statistical analysis

3.4.1 Paper I.

At the initiation of this study, no other study had been published to examine the association between surrogates of the transit time (stool frequency and consistency) and gut microbiota. However, before submission of our findings, two other papers (both examining only the stool consistency) were published in Gut (80, 81). Therefore, we decided to replicate their findings and expand them to include another surrogate of GI transit time, namely stool frequency. Thus, paper I includes only the methods and results relevant to those two studies. However, in addition to them, we have done some other analyses which have resulted in significant results (at the level of β-diversity measurements) and they will be presented here.

In this study, after exclusion of the outliers, 69 individuals (48 women and 21 men, aged 55.6±10.33) with available microbiota and fecal characteristics data were included in the study. Mean daily stool frequency and mean BSFS were calculated for all the participants.

Moreover, the four different α-diversity measurements of observed species and Chao1 (as measurements of species richness), Faith's phylogenetic diversity (PD, an index for phylogenetic diversity) and Shannon–Weaver entropy (as a measure of evenness), all at the level of OTU with 97% sequence identity (species-level), were calculated applying two R packages of 'Vegan' and 'Phyloseq'.

Distance matrices of Bray-Curtis (a statistics method used to quantify the compositional dissimilarity between two paired sites, based on the relative abundances of the studied taxa) and Jaccard (calculated based on the presence of a single taxa in paired samples) as well as Unweighted and Weighted Unifrac (β-diversity indices based on phylogenetic structure, and phylogenetic structure weighted by OTU abundances, respectively) were calculated using R packages of 'Vegan', 'phyloseq' and 'Picante'. Adonis test (permutations=1000) was used to study the association between these matrices with 'mean stool frequency' and 'mean BSFS' after applying Cailliez correction for the negative eigenvalues (R package: 'ade4'), adjusting for age and gender.

Subsequently, taking advantage of the '*PCoA'* function of the 'Ape' package, the inter-individual relationships based on each distance matrix was calculated through principal coordinate decomposition analysis. Two first principal coordinates (for each matrix) were chosen to study in relation to the study variables of mean stool frequency and consistency with Spearman's correlation test.

In addition, the ratios between dominant phyla (*Bacteroidetes*:*Firmicutes*) and genera (*Bacteroides*:*unclassified Ruminococcaceae*) were calculated and their correlation with the study variables was assessed (Spearman's correlation). This test was also used to investigate the relationship between stool frequency and consistency with the first 10 genera.

3.4.2 Paper II.

For this study, 159 individuals (96 female and 63 male, aged 59.1±10.70) from the PopCol cohort with 16S sequencing data and daily recordings of abdominal pain (in a 14 days diary) collected over the same period (7.41±7.91 days in between) were included. Based on the diary information including number of episodes as well as the duration and intensity of each episode (score 1 for light pain, 2 for moderate pain and 3 for intense, unbearable pain), three variables of mean pain episodes per day, mean pain duration per episode and mean pain severity per episode were calculated (figure 10). Accordingly, 52 individuals reported at least one episode of pain (assigned to the case group), whereas the other 107 persons did not report any pain even though they had filled the other sections of their diaries (assigned to the control group). Principal-coordinate analysis (PCoA) (based on Bray–Curtis and Jaccard dissimilarity matrices) was accomplished at both levels of genus and OTU 97%. The first three principal coordinates (related to each matrix and at both levels) were compared between cases and controls and studied in association to the three pain characteristics. P-values were corrected for multiple testing using the false discovery rate (FDR) method of Benjamini-Hochberg (applying FDR q-value threshold of < 0.1). Subsequently, study samples were classified into three clusters, namely enterotypes (149) according to their microbiota profiles at the genus level using R packages of 'cluster' and 'clusterSim' (for determination of the optimal number of clusters) following the tutorial available on http://enterotyping.embl.de/enterotypes.html. To further characterize the bacterial taxa potentially playing a role in the pain development, we took a closer look at the specific taxa previously associated with abdominal symptoms in the animal models and clinical studies. Abundances of 12 genera (*Bacteroides*, *unclassified Ruminococcaceae*, *Butyricicoccus*, *Prevotella*, *Faecalibacterium*, *Streptococcus*, *Bifidobacterium Blautia*, *Akkermansia*, *Lactobacillus*, *Alistipes*, and *Enterobacter*) were

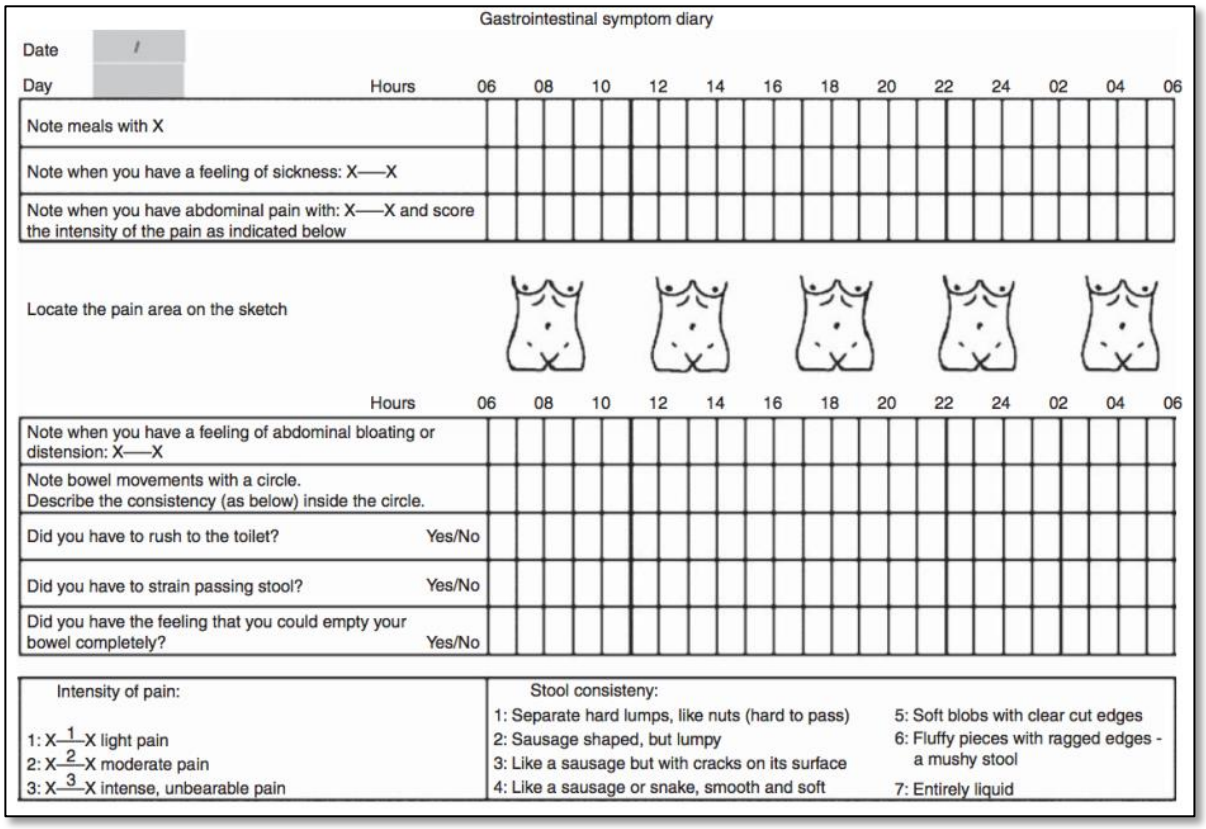


Figure 10. PopCol diary. Participants kept records for 7 consecutive days.

compared between cases and controls with Wilcoxon rank-sum test and p-values were corrected for multiple testing. Thereafter, *multipatt* function (func='IndVal.g') of the 'indicspecies' package was applied to accomplish an indicator value analysis on the core microbiota at the genus and species (OTU 97%) levels. Core microbiota included all the genera and species-level OTUs presented in at least 25% of the samples. To ensure that the results have been obtained independent of the IBS status, the analyses were repeated after exclusion of the 18 individuals whose questionnaire data were compatible with IBS diagnosis (according to Rome III criteria).

3.4.3 Paper III.

In total, 1546 participants from LifeLines-Deep (LLD) cohort (including 897 women (58%) and 649 men; mean age of 44 years (range 18–86)) and 264 individuals from PopCol cohort (encompassing 158 women (60%) and 106 men; mean age of 54 years (range 22–71)) were included in the study. Mean stool frequency per day was calculated for all the individuals based on the daily records kept by both populations for seven consecutive days. Genotyping data from both cohorts were imputed with IMPUTE2 (322) using the Genome of the Netherlands as the reference (323). Association testing was carried out within each cohort using linear regression tests under an additive genetic model (frequentist=1) adjusting for age and gender in SNPTEST (324) followed by a meta-analysis (fixed-effect model) with META (method 1) according to the instructions available at https://mathgen.stats.ox.ac.uk/genetics_software/meta/meta.html.

Genes mapped to the top-10 loci resulting from the meta-analysis were subjected to further investigation through Gene Network co-expression analysis (http://www.genenetwork.nl/genenetwork/). Moreover, a suggestive threshold of $p < 5E\text{-}05$ was considered as the significance level and associated loci were defined with 250 kb window surrounding the lead

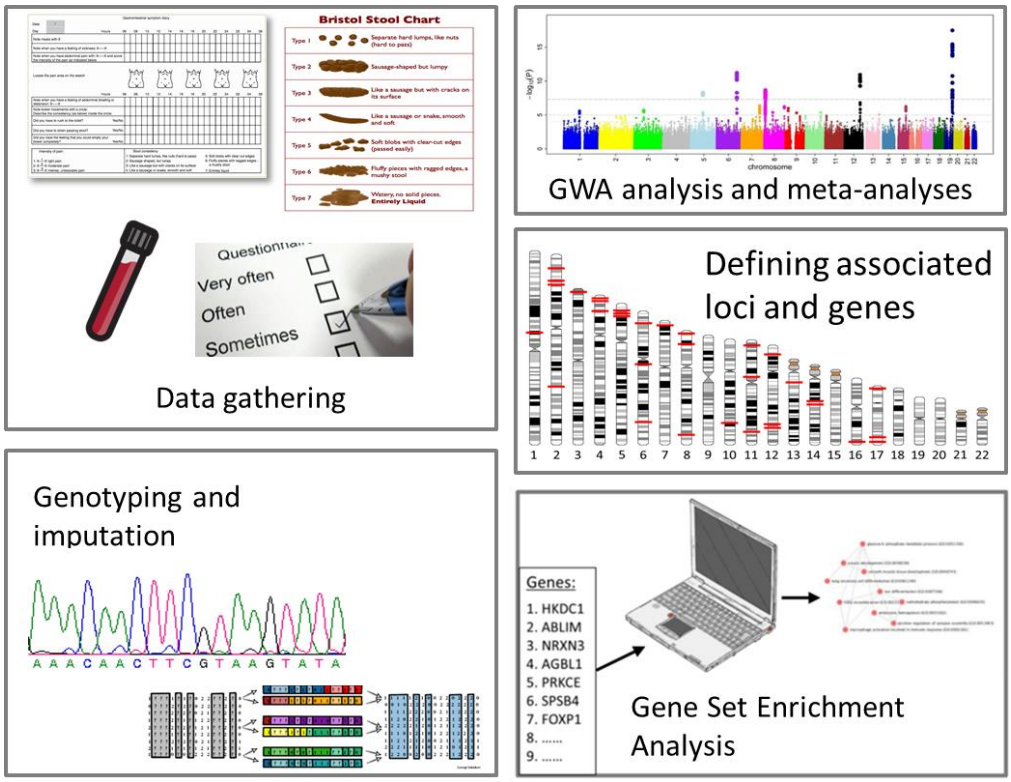


Figure 11. An overview of methods applied in this study

SNPs. All the genes mapped to these loci were included in a gene set enrichment analysis (http://129.125.135.180:8080/Gene-Network/pathway.html) and p < 1E-04 was defined as the significant threshold for the gene ontology pathways. An overview of the methods applied in this study has been provided in figure 11.

3.4.4 Paper IV.

In total, 27 genes with GI-linked ion channel activity were inspected in the results from the GWA study of IBS accomplished previously by our group (93). They included gene families of *ASIC* (3 genes), *CACNA1* (5 genes), *HCN* (1 gene) and *KCN* (4 genes), *P2RX* (3 genes), *SCN* (4 genes), *TRP* (6 genes) and *ANO* (1 gene). Significantly associated genes were subjected to a replication analysis in an independent cohort of IBS cases and controls. For this, a logistic regression analysis under an additive genetic model adjusted for gender was accomplished with SVS (v.8.3.4) on 386 IBS cases and 357 controls, followed by performing an analysis in each IBS subtype (IBS-C, IBS-D, and IBS-M defined based on Rome III criteria). Subsequently, a meta-analysis of GWAS and replication results was implemented in SVS using a fixed-effect model weighted by inverse-variance. Cochran's Q test was used to test the heterogeneity of study results. Using the PopCol cohort, data from 120 IBS-free individuals were exploited to study the correlation between *TRPM8* genotype and average BSFS scores by means of Spearman's rank test in SPSS (v.22.0.0.0). *In silico* prediction of transcription factor affinity change was carried out using sTRAP tool (325). To do this, a sequence including a window of 10 nucleotides around the two SNPs of interest mapping to the promoter of the *TRPM8* was selected and used as input for the analysis. In addition, the Genevestigator search engine was used to identify genes co-expressed with *TRPM8*. Probe signals with a correlation coefficient r > 0.25 in the Perturbation dataset (326) were filtered out. Transcription factors with a significant difference in the binding affinity and *TRPM8* co-expressed gene lists were subjected to gene set enrichment analysis to screen the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (http://www.genome.jp/kegg/) database and the Gene Ontology (GO) terms of 'Biological Process', 'Molecular Function' and 'Mammalian Phenotype' with the Enrichr web-based software (327). All the steps have been briefly summarized in figure 12.

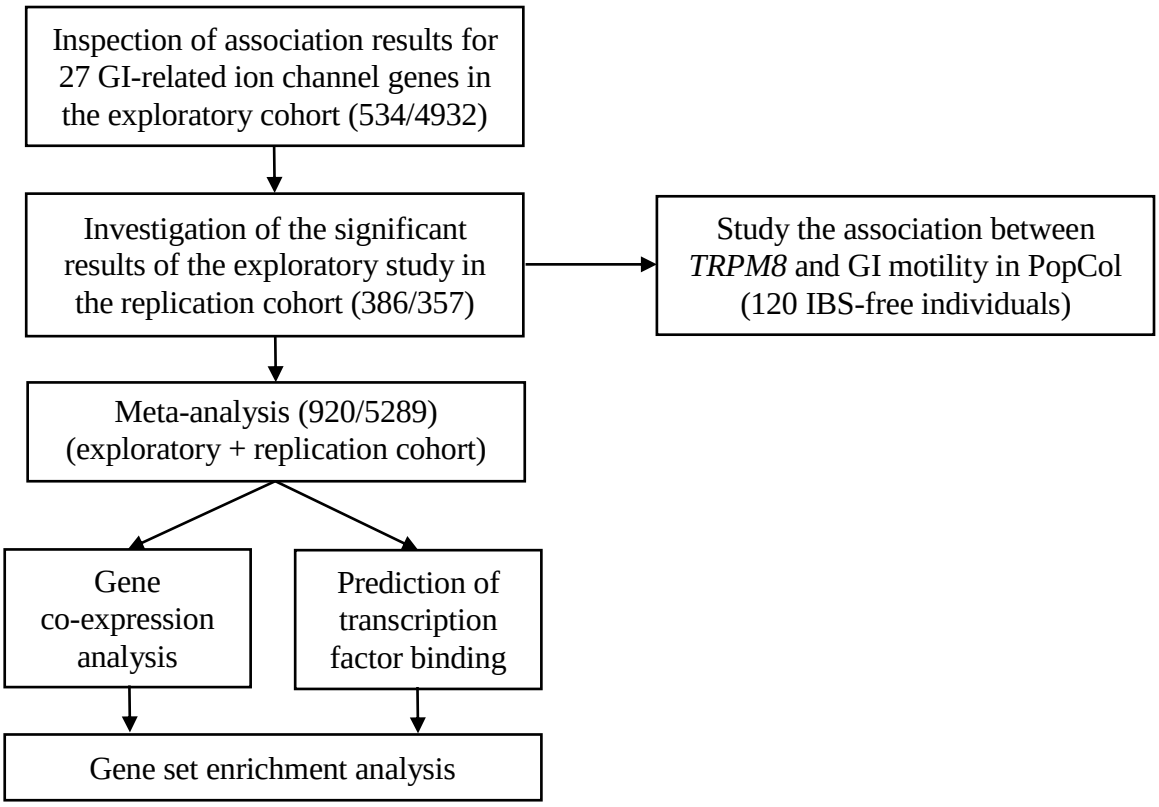


Figure 12. Flowchart of the study steps.
(number of cases/number of controls)

3.4.5 Paper V.

Since the methodology of paper V has been very comprehensively described in the paper's text and supplementary material, it is not elaborated on here.

4 RESULTS AND DISCUSSION

4.1 Paper I.

To investigate the link between gut microbiota and gut transit time, as an objective means to quantify GI functional abnormalities, we studied the association between different indices of fecal microbiota composition with stool consistency and stool frequency (as surrogates for gut transit time) in the PopCol cohort. The mean BSFS score and mean daily stool frequency of the participants were 3.91 (range 1.5–6) and 1.38 (range 0.57–3), respectively, and these two measures were significantly correlated (Spearman's p=0.007, r=0.32). The microbial composition of each individual at two levels of phylum and genus is shown in figures 13 and 14. Examining α-diversity, a negative correlation was detected between stool consistency and all four α-diversity indices. Of interest, studying the mean stool frequency resulted in even more significant and stronger correlations with all the α-diversity measurements (figure 15).

At the level of β-diversity analysis and with applying Adonis test, only unweighted unifrac showed association (positive) with daily mean stool frequency (p=0.0079, R2=0.024). Testing for the interaction with age and gender, decreased the p-value slightly (p=0.0039). No significant association was observed regarding stool consistency. Moreover, testing the correlation between study variables and the first principal coordinate calculated based on weighted and unweighted unifrac as well as Bray-Curtis diversity indices (account for 15%, 10% and 8.2% of variations, respectively) yielded significant results (figure 16).

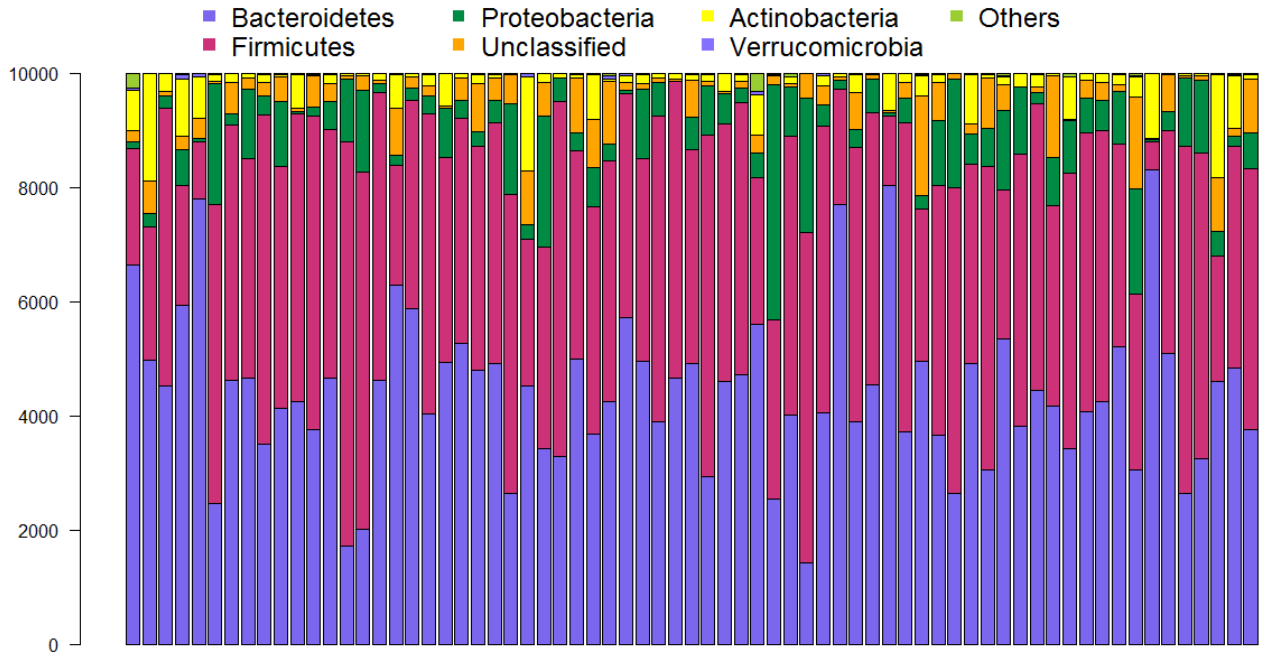


Figure 13. The microbial phyla across the study individuals (n=61).

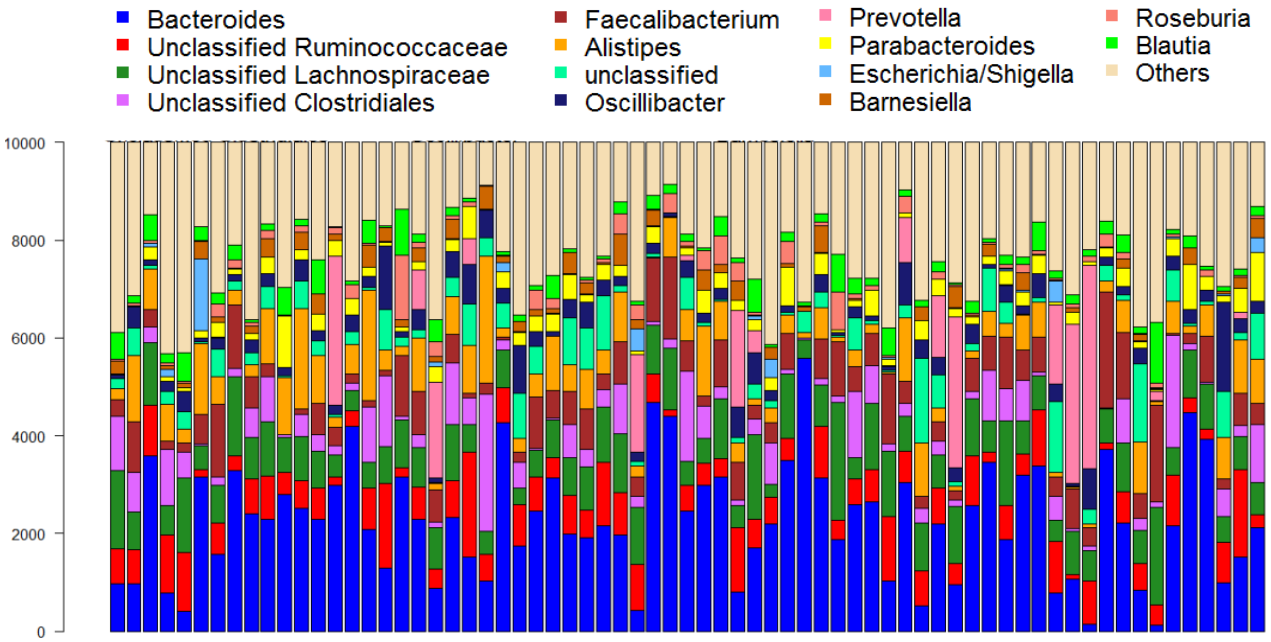


Figure14. The microbial genera across the study individuals (n=61).

Studying the ratio between dominant phyla and genera in our dataset revealed significant correlations with mean stool frequency (phyla p=0.017 r=0.29; genera p=0.0001 r=0.45). However, mean stool consistency did not show significant correlation ($p > 0.05$). After exclusion of 9 individuals with the diagnosis of IBS, similar results were obtained. Our results could contribute to accumulating evidence linking gut microbiota to stool consistency as a surrogate of intestinal transit time. Reassuringly, this finding has been supported by results of the recent population-level study on a remarkable sample size from Belgium and the Netherlands, in which stool consistency demonstrated the largest effect size among 69 different covariates explained to be associated with the variation in the microbiota composition (163).

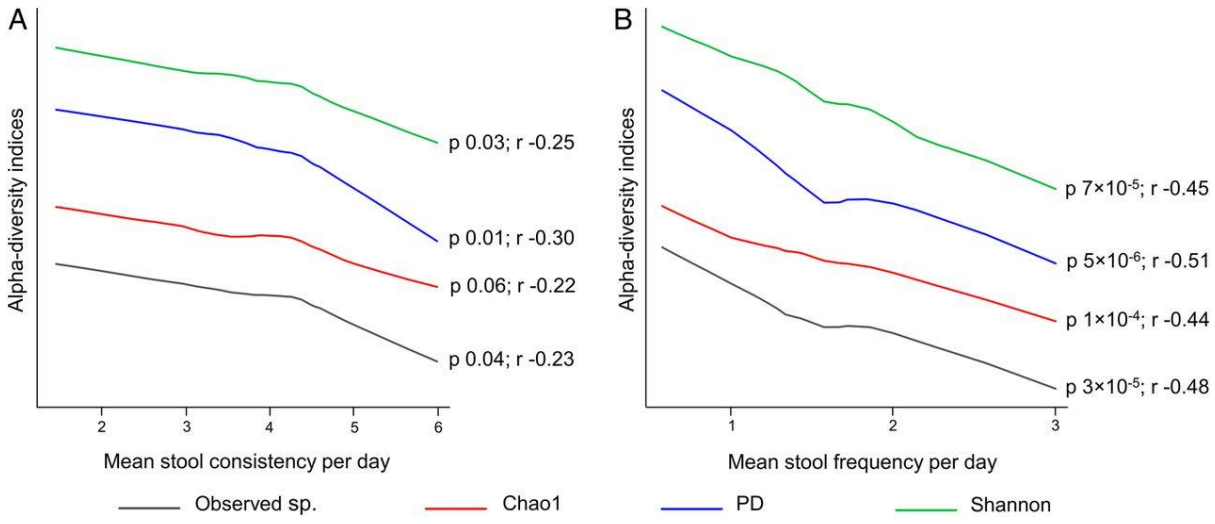
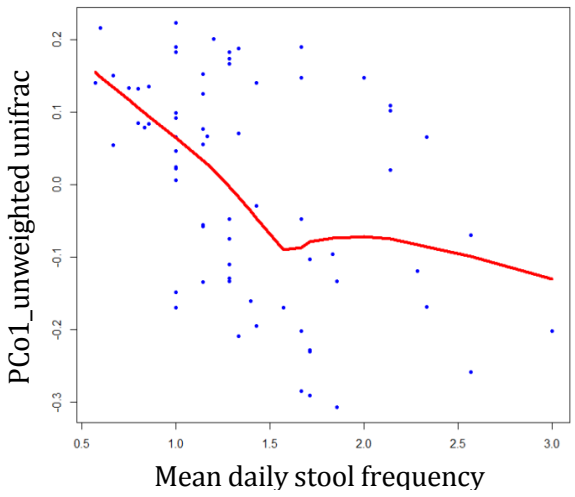
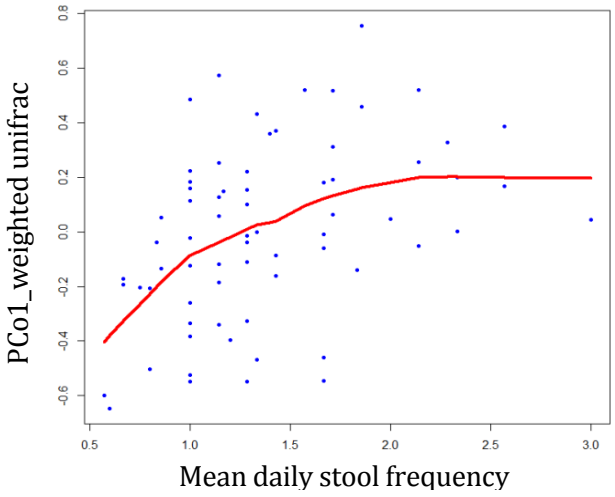


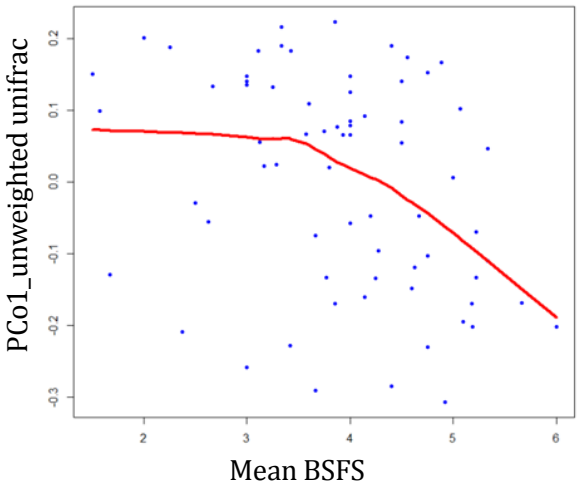
Figure 15. Correlation between different α-diversity indices and mean daily stool consistency and frequency. Stool consistency has been defined based on the BSFS. Reproduced from [Stool frequency is associated with gut microbiota composition, Hadizadeh F *et al.*, 66, 559-560, 2016] with permission from BMJ Publishing Group Ltd.



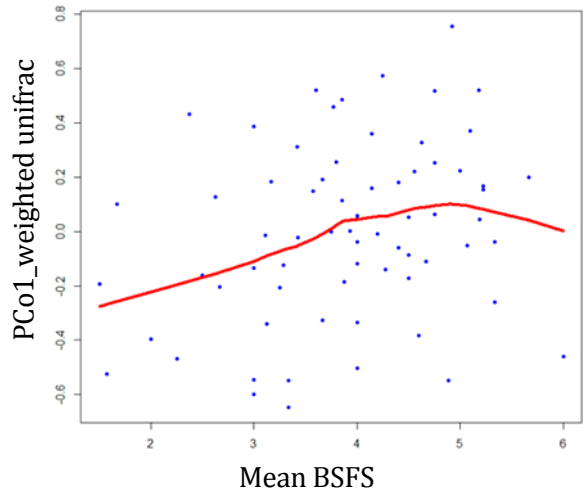
p-value = 7.74E-05 r = -0.45



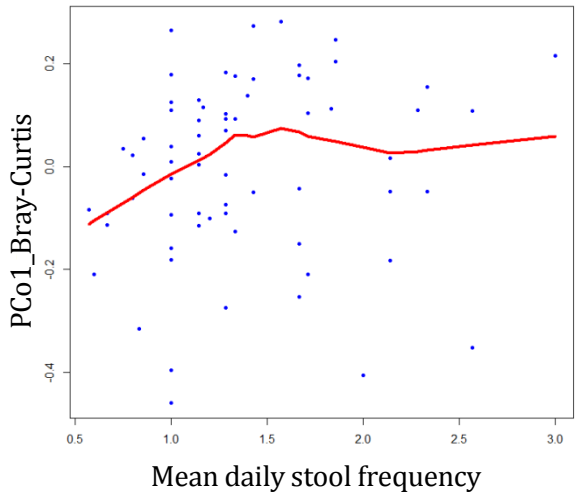
p-value = 4.89E-05 r = 0.46



p-value = 0.0033 r = -0.34



p-value = 0.024 r = 0.27



p-value = 0.03 r = 0.25

Figure 16. Correlation between β-diversity indices, stool frequency, and stool consistency. First principle coordinate (PCo1) of Principle coordinate analyses based on unweighted, weighted and Bray-Curtis diversity indices are plotted against Mean daily stool frequency and Mean BSFS (when significant) in different plots with their respective statistics.

Moreover, for the first time, we displayed that stool frequency as another surrogate of GI transit rate is associated with gut microbiota composition. This remarkable finding has also been supported later by another population-based metagenomics analysis on 1,135 Dutch participants which indicated both stool frequency and consistency among the top intrinsic factors associated with gut microbiota composition and interestingly, similar to our results, stool frequency showed a stronger association than stool consistency (156). Hypothetically, it could be a bidirectional association, and different factors can link microbiota composition to the GI transit time. Among them and in addition to what has already been discussed in the introduction, one can refer to the colon oxygen concentration which could be increased in the rapid transit state and possibly could be considered as a competitive advantage for aerobic bacteria over anaerobic ones. Intriguingly, in our study, we detected a negative association between the abundances of three anaerobic genera of *unclassified Ruminococcaceae*, *Alistipes*, and *Oscillobacter* with stool frequency. Also, a faster transit could be considered as a benefit for the fast-growing organisms which can prevail in the gut microbiota after the "washed out" situation resulting from diarrhea. On the other hand, slow transit time results in the very low nutrient content in the last segment of the colon which could result in increased abundance of the microbes with the ability to adapt to this situation (196, 311). Although the causal relationships and the direction of effects are yet far from understood, these findings are of importance because they may contribute to describing the potential role played by gut microbiota in host health maintenance and symptom generation.

4.2 Paper II.

To study the association between abdominal pain, the most important symptom of IBS, and gut microbiota, 52 individuals who reported pain (cases) and 107 controls were studied. The microbial composition of each individual at two levels of phylum and genus is shown in figures 17 and 18. On average, the number of pain episodes reported by the pain group was 0.30 times per day (range 0.07–1.57) in which each episode lasted for 2.46 hours (range 0.37–9 hours) with the mean intensity of 1.39 (range 1–2.1) that is categorized as light to moderated level. Applying Wilcoxon rank sum test, after multiple test correction (MTC), PCo1-Jaccard at the level of genera (q=0.017) and PCo3-Bray-Curtis, as well as PCo3-Jaccard at the level of OTU 97% (q=0.033 and 0.017, respectively), exhibited significant differences between cases and controls. Moreover, pain index scores for episode number, duration and severity appeared to be all significantly correlated (Spearman's) to different indices of β-diversity (figure 19). Subsequently, case and control groups were compared based on their enterotype distribution. Study samples were clustered into three enterotypes (enriched by *unclassified Ruminococcaceae*, *Prevotella*, and *Bacteroides*, respectively) and chi-square test demonstrated a significant difference between cases and controls (p=0.039) in which, the enterotype enriched by *Prevotella* was underrepresented in the pain group (21% vs 41% in controls), while cases were more likely to fit into the third enterotype (enriched by *Bacteroides)* (figure 20).

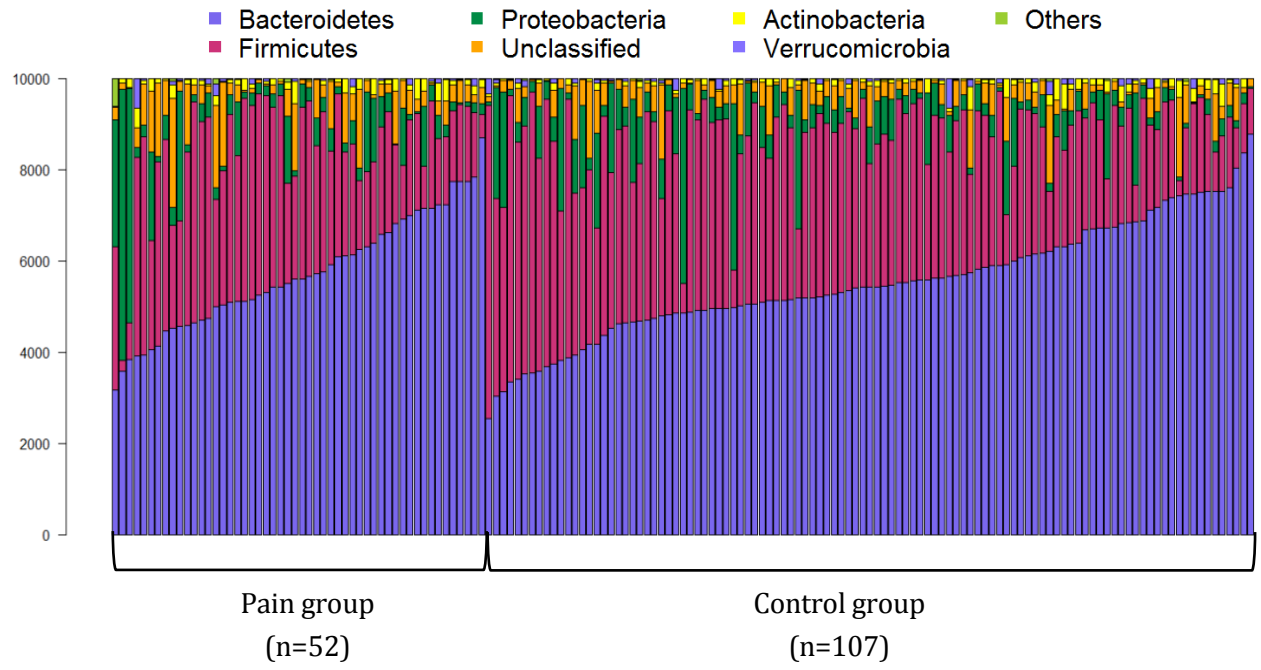


Figure 17. The microbial phyla across the cases and controls. The individuals are sorted by the abundance of *Bacteroidetes*.

These results remained constant after exclusion of the IBS cases (*Prevotella* 18% vs 40%; *Bacteroides* 46% vs 30% (case vs control); Pair-wise test p-value: 0.019).

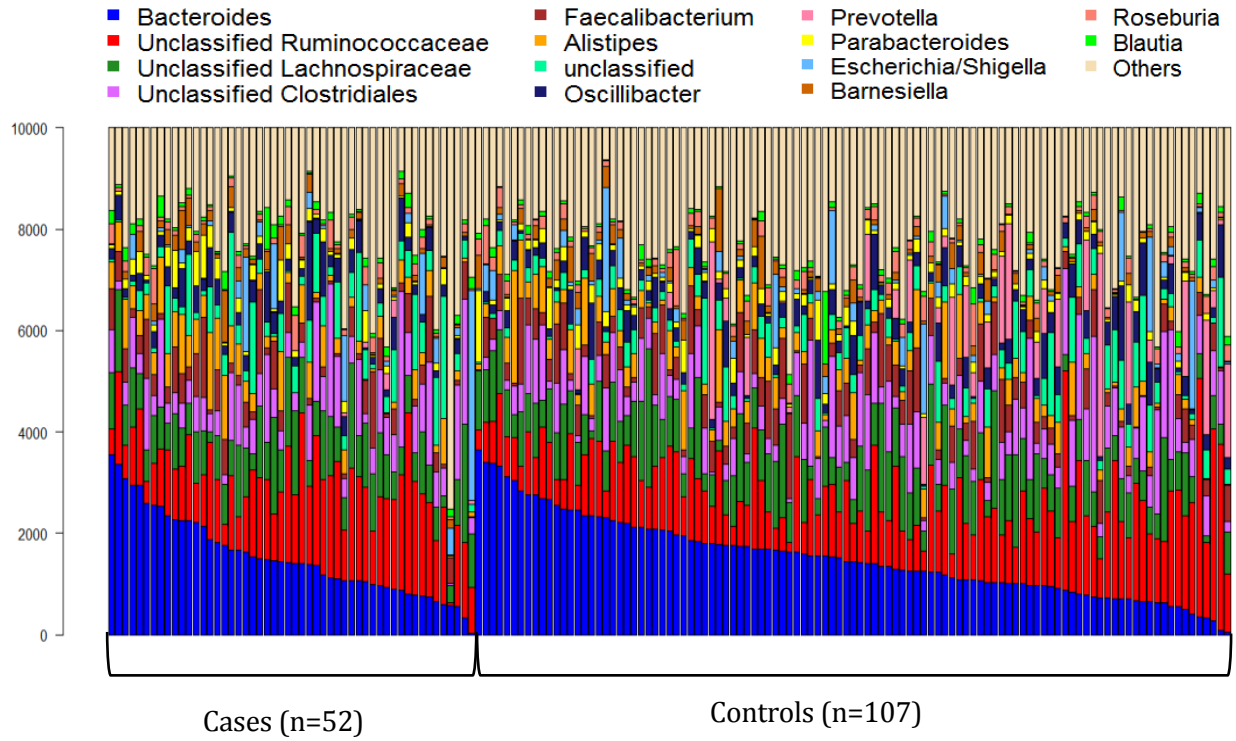


Figure 18. The microbial genera across the cases and controls. The individuals are sorted by the abundance of *Bacteroides*.

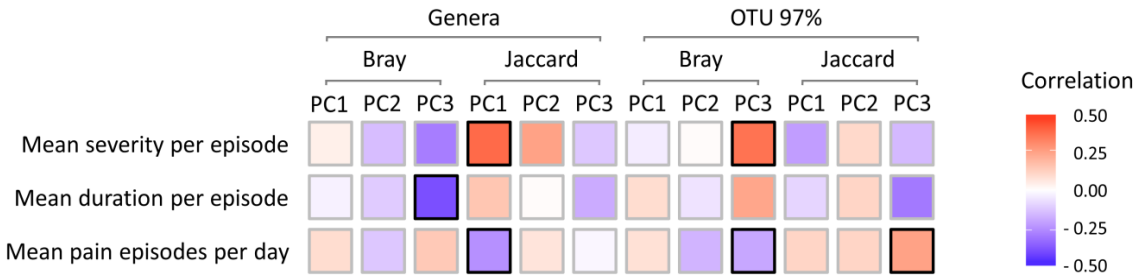


Figure 19. Fecal microbiota β-diversity associates with abdominal pain. Heat map of Spearman's correlation between pain indices and fecal microbiota β-diversity, based on principal coordinate analysis applied to Bray-Curtis and Jaccard matrices at the level of genera (Genera) and operational taxonomic units with 97% sequence similarity (OTU 97%). The first three principal coordinates (PC) are reported (PC1, PC2, and PC3) and significant correlations (false discovery rate < 0.1) are highlighted by a black frame.

When cases and controls were compared for the abundances of the taxa previously linked to the abdominal symptoms in animal models and clinical studies, Benjamini-Hochberg corrected significant differences were observed for *Prevotella* (decreased in cases, q=0.038), *Blautia* (increased in cases, q=0.045), *Streptococcus* (increased in cases, q=0.038) and *Lactobacillus* (increased in cases, q=0.038). Moreover, '*multipatt'* function of R package '*indicspecies'* represented *Prevotella* as the indicator of the no-pain group (q=0.016, stat=0.752). However, *Lactobacillus* which was observed to be an indicator for the pain group (p=0.0078, stat=0.64), after MTC was not significant anymore. Similarly, one OTU97% which belonged to the *Prevotella* genus and appeared as the indicator of the no-pain group (p=0.0013, stat=0.581) lost its significance after MTC.

Our study, at the general population level, could link the composition of the facal microbiota to the occurrence of abdominal pain as well as its characteristics including frequency, duration, and intensity. Also, we detected a negative association between *Prevotella* and pain which was supported by the difference in the distribution of enterotypes between cases and controls as well as by the indicator value analysis test. This finding in addition to the observation of an increase in the prevalence of the *Bacteroides* enterotype in the pain group parallels the observations previously made in IBS studies where the *Prevotella*-predominant enterotype was shown to be less common among the IBS patients while it was the other way around for the *Bacteroides* enterotype (315). This information, reported at the level of the general population, may contribute to translational opportunities for the identification and eventual treatment of individuals at risk for FGIDs and particularly IBS.

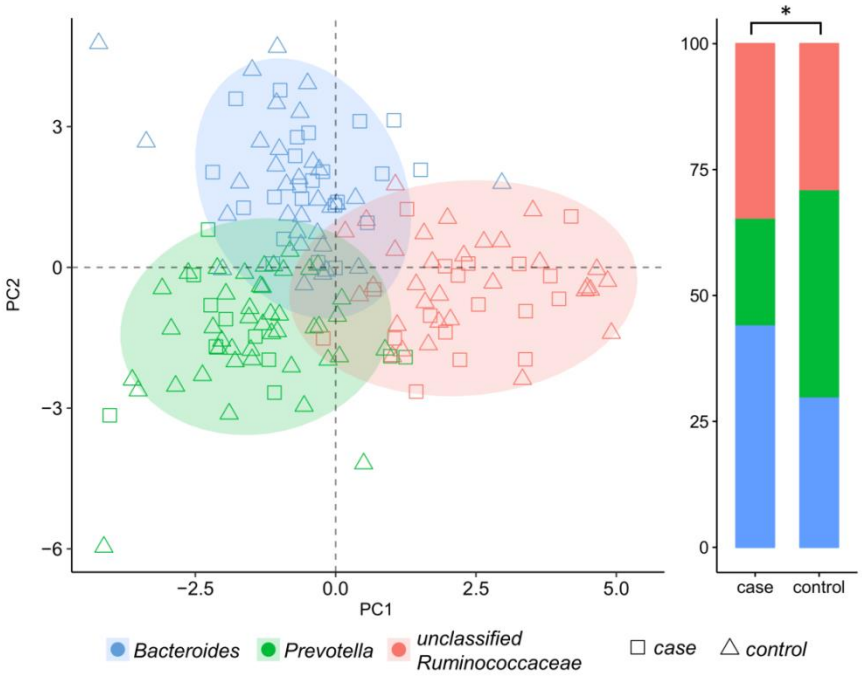


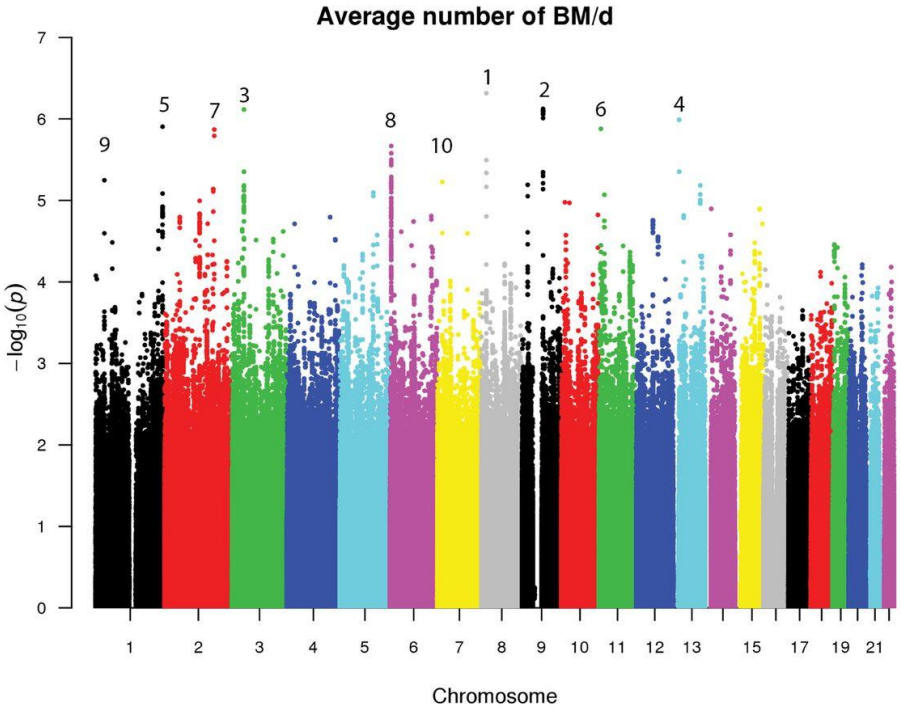
Figure 20. Fecal microbiota enterotype distribution differs between individuals with abdominal pain and controls. Principal component analysis (left) and relative distribution (right) of enterotypes according to the presence (case) or absence (control) of abdominal pain. Participants were classified into three enterotypes primarily characterized by *unclassified Ruminococcaceae*, *Prevotella* or *Bacteroides*. *p < 0.05.

4.3 Paper III.

To investigate the genetic biology of stool frequency as a surrogate for gut transit time, after SNP and sample quality control, 5,390,800 SNPs (matched with diary data) for 1,022 LLD and 259 PopCol individuals were studied. None of the association test results passed the genome-wide significant threshold (5E-08) which could possibly be related to the limited number of the study samples. However, we could detect a couple of signals (the best SNP p-value=4.8E-07) with excellent functional candidate genes mapping to them (figure 21). Among them is *ALDH1A1* gene which mapped to the second strongest signal (on chromosome 9, the best SNP p-value=7.5E-07). *ALDH1A1* gene is one of the family members of the aldehyde dehydrogenases. *ALDH1L1* is another member of this family which has been reported to be associated with human gut microbiota composition. The aldehyde dehydrogenase coded by this gene is involved in formate oxidation which is a fermentation product and plays the role of electron carrier between interspecies syntrophs (172). Furthermore, gene network co-expression analysis indicated a role for *ALDH1A1* as well as *CYP8B1* (mapped to the third top signal) in the cytochrome P450 metabolism of drugs and xenobiotics. *AHR*, a gene mapped to another top-10 locus, is a transcription factor that can modulate gene expression along the cytochrome P450 pathway. Also, interestingly, *CYB5R2* (cytochrome B5 reductase), another gene mapping to one of the top defined regions, has been shown to be involved in the biosynthesis of cholesterol and desaturation and elongation of fatty acids. Considering a suggestive threshold of p < 5E-05, resulted in 53 loci. In a broader pathway analysis, including all the genes mapped to these loci, the cellular component class

of the GO identified the sodium channel complex (p=6E-07), voltage-gated sodium channel complex (p=2E-05) and ion channel complex (p=6E-05) among the most enriched pathways (p < 1E-04). In a similar manner, voltage-gated sodium channel activity (p=2E-05) and ion channel activity (p=4E-05) were displayed among the significant pathways in the GO terms derived from molecular function.

The genetic contribution of xenobiotic and P450 metabolic pathways in the formation of human defecation patterns is not so surprising considering the known interactions linking diet, gut microbiota and pharmaceutical compounds, however, it has not been reported previously. Another remarkable finding of this study was related to the identification of the pathways linked to the voltage-gated sodium channels. Intriguingly, as described earlier in the introduction section, *SCN5A* gene has been implicated in the etiology of IBS in a subset of patients (mainly IBS-C) and is estimated to be potentially involved in gut motility (82). To conclude, we reported the results of the first GWAS of stool frequency in two coherent population-based cohorts from Sweden and the Netherlands and suggested possible candidate genes and biological pathways which could potentially be exploited by future investigations conducted on GI functions.



#	Lead SNP	Chr	BP	SNP	Assessed allele	P-value	Beta	se	Nearest gene	Other genes in 250kb region (protein coding)
1	rs62498365	8	17622887	C/T	T	4.8E-07	-0.38	0.08	MTUS1	SLC7A2, PDGFRL, FGL1, PCM1
2	rs13959	9	75545882	G/A	A	7.5E-07	0.20	0.04	ALDH1A1	ANXA1, TMC1
3	rs735320	3	42915878	C/T	T	7.7E-07	-0.27	0.05	CYP8B1	HHATL, NKTR, ZBTB47, CCDC13, HIGD1A, KLHL40, ZNF662, FAM198A, ACKR2, KRBOX1, POMGNT2
4	rs9550650	13	21035343	A/G	G	1.0E-06	-0.27	0.06	CRYL1	GJB6, IL17D, IFT88
5	rs9782914	1	241892235	T/C	C	1.2E-06	0.29	0.06	WDR64	FH, CHML, EXO1, KMO, OPN3
6	rs4090286	11	7644539	G/C	C	1.3E-06	0.30	0.06	PPFIBP2	CYB5R2, OR5P2, OR5P3, OLFML1, SYT9
7	rs115304436	2	178944137	G/A	A	1.3E-06	0.40	0.08	PDE11A	RBM45, OSBPL6
8	rs408709	6	4568772	C/T	T	2.1E-06	0.24	0.05	CDYL	
9	rs2377852	1	31901133	C/T	T	5.6E-06	-0.27	0.06	SERINC2	FABP3, AC114494.1, NKAIN1, SNRNP40, ZC-CHC17, TINAGL1, HCRTR1, PEF1, COL16A1
10	rs1979097	7	17582940	C/G	G	5.9E-06	0.19	0.04	AHR	SNX13

Figure 21. Above: Manhattan plot of the meta-analysis results of the LifeLines-Deep and PopCol GWA studies. X-axis: SNPs sorted according to their genomic positions. Y-axis: negative logarithm of each SNP's association p-value. Below: Table including the numbers of top-10 loci identified in the plot and their statistics in addition to their genomic positions, nearest gene, and the protein coding genes in 250kb loci around them.

4.4 Paper IV.

Following the third study (described above), to further investigating the association between GI-related ion channel genes and IBS, the association results of the IBS GWAS (already accomplished by our group) were inspected for a number of GI-related ion channel genes. Four genes, including two genes from the calcium voltage-gated channels family (*CACNA1A* and *CACNA1E*) and two genes from the transient receptor potential channels family (*TRPV3* and *TRPM8*) displayed nominally significant association with IBS. In total, 33 SNPs were selected from these loci for a replication study. The logistic regression analysis of the genotype data from these four genes uncovered a significant association between *TRPM8* and IBS. Subsequently, the meta-analysis of the 14 SNPs in this gene (*TRPM8*) from GWAS and the replication (case-control cohort), returned the strongest evidence of association at this locus with identical direction of the genetic risk effects in the two studies. Also, the Cochran's Q test confirmed that there was no statistical heterogeneity between the two studies' results ($p > 0.05$). Interestingly, performing the analysis in the IBS-subtypes revealed that the *TRPM8* variations were exclusively associated with the IBS-C and IBS-M (constipation-related sub-types) and combining data from these two categories yielded the strongest evidence. Seven SNPs out of 14 were present in the PopCol genotype data (including 6 predisposing and one protecting). Intriguingly, investigation of the association between BSFS scores and *TRPM8* genotypes, displayed a negative correlation between all IBS-C/M risk alleles and BSFS (consistent association between IBS-C/M risk alleles and harder stools) (figure 22).

Correlation between *TRPM8* genotype and BSFS scores

SNP	Test (minor) allele	Genetic risk effect in IBS	Allele frequency	P	Rho
rs10519356	T	predisposing	0,083	**2,9E-02**	-0,174
rs10166942	C	predisposing	0,208	**3,3E-02**	-0,169
rs1003757	A	predisposing	0,095	**3,5E-02**	-0,166
rs1003756	T	predisposing	0,095	**3,9E-02**	-0,162
rs6431648	A	predisposing	0,212	**7,7E-03**	-0,221
rs758277	T	protecting	0,417	**1,5E-02**	0,199
rs7577157	A	predisposing	0,107	**2,3E-02**	-0,184

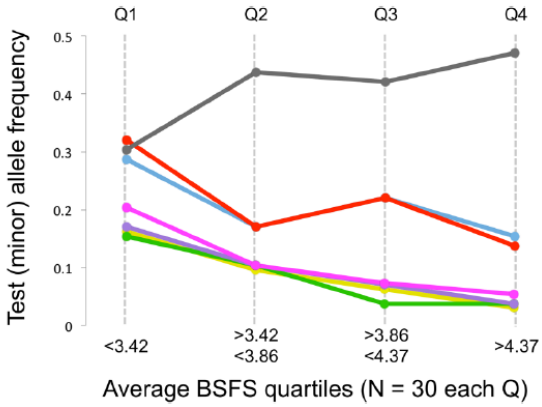


Figure 22. Correlation between *TRPM8* genotype and average BSFS scores. Left: Spearman's correlation statistics. Right: frequency of *TRPM8* alleles across BSFS quartile groups (alleles from each SNP are color-coded as in the table on the left).

Two SNPs with the strongest association with the IBS-C/M risk in the meta-analysis (rs10166942 and rs2362290) were mapped within the promoter region of the gene. *In silico* analysis predicted these variants to change the affinity of the transcription factor to their binding sites. Moreover, gene set enrichment analysis of the transcription factor pool affected by *TRPM8* promoter SNPs, resulted in different enriched pathways and GO terms (after MTC), among them "abnormal hepatobiliary system" was enriched as the top scoring mammalian phenotype. In the same manner, co-expression analysis revealed 26 *TRPM8* co-

expressed genes of which GSE analysis returned “bile secretion”, “bile acid and bile salt transport” as well as “bile acid metabolic process” as the main GO biological process terms.

These findings are potentially important. Functional dysregulation of transient receptor potential (TRP) channels has already been shown to associate with constipation and IBS (328). Furthermore, interestingly, it has been demonstrated that peppermint oil (containing the *TRPM8* activator menthol) could relieve IBS symptoms (63, 329). Our findings of the association between *TRPM8* SNPs with slower colonic transit, constipation, and IBS could contribute to the understanding of the pathophysiological mechanisms underlying IBS. On the other hand, the regulatory mechanisms for *TRPM8* expression are not well characterized yet and modifications of the bile acid synthesis and metabolism, which has been shown to be associated with constipation and IBS could be considered as a potential mechanism (330).

To sum up, for the first time we identified *TRPM8* polymorphisms to be associated with increased risk of IBS with constipation (IBS-C and IBS-M) and slower colonic transit time. These findings may contribute to identifying subsets of IBS patients and eventually result in the improvement of their diagnosis and clinical management.

4.5 Paper V.

In view of the speculated effects of specific foods in triggering IBS and the reports of IBS-like symptoms in patients with CSID (caused by *SI* mutations), we were motivated to investigate the possible role played by this enzyme (SI) in the development of IBS. To detect the *SI* variants with potential relevance to IBS development, four unrelated IBS-D families including seven postprandial IBS-D cases and one control (asymptomatic family member) were selected for sequencing of the *SI* gene. No new *SI* mutation was identified in this effort and all family members had two reference alleles of all common coding variants, except at two sites. Because together there are only three common missense SNPs in the *SI* gene, all three missense SNPs of p.Thr231Ala, p.Met1523Ile, and p.Val15Phe were investigated for their deleteriousness *in silico* (using CADD scores). While the first two were not classified as deleterious (bottom 90% of the variations ranked by deleteriousness), p.Val15Phe was ranked among the top 1% most deleterious amino acid substitutions in the human genome (similar to known CSID mutations). Interestingly, this SNP was detected in six cases out of the seven (in the family study) and was shown to cosegregate with IBS. Therefore, this SNP was selected for further studies and characterization. *In vitro* functional characterization of this coding SNP through comparison of the functional properties of 15Val and 15Phe, demonstrated 35% reduction of the total enzyme activity for the 15Phe. The next step was dedicated to the study of the association between four known CSID mutations (p.Val557Gly, p.Gly1073Asp, p.Arg1124Ter and p.Phe1745Cys) as well as the p.Val15Phe variant with IBS. Studying the association of the CSID mutations and IBS in a cohort of 1887 individuals from four independent IBS case-control cohorts showed a trend for IBS patients to have a higher odds of carrying one of the CSID mutations compared to the control group, which was consolidated by checking public data from a reference panel of > 31,000 sequenced data from the European Exome Aggregation Consortium. Moreover, a positive association between 15Phe and IBS was detected exploiting data from the four independent IBS case-control cohorts ($p=0.0030$, $OR=1.26$) with the best evidence of association with the diarrhea-linked subtypes ($p=0.00051$, $OR=1.34$ for the IBS-D/IBS-M combined) (logistic regression under

additive model adjusted for sex and enrollment center). Furthermore, these results (from the pooled analyses of all the cohorts) were strongly supported by a meta-analysis of associations from individual cohorts.

The next step was to investigate 15Phe in relation to bowel function, symptoms and microbiota composition in the general population. To do this, data related to 250 individuals from the PopCol cohort including 30 IBS cases was exploited. Although the sample size was small, a significant positive association between 15Phe and IBS risk was observed (p=0.045, OR=1.89) which again was stronger for IBS-D/IBS-M (p=0.013, OR=2.50). Combining case-control and PopCol data further strengthened the results particularly for the diarrhea-related subtypes (combination of IBS-D/IBS-M p=0.00012, OR=1.36). To study the association between carrying the risk allele and bowel function, a subset of 133 individuals with daily recordings of stool frequency was selected. A significant positive correlation was detected between mean stool frequency and the number of 15Phe copies (p=0.026, r=0.19). However, no significant association was observed in relation to stool consistency. To examine the potential association between fecal microbiota and the number of risk alleles, data from 136 individuals from the PopCol cohort with available 16S rRNA sequencing and genotyping data were included in the study. A core microbiota of 20 of the most abundant genera was selected and tested in association with the number of 15Phe copies using Pearson's correlation. A Bonferroni-corrected significant inverse correlation was observed between the abundance of *Parabacteroides* and the number of 15Phe copies (Figure 23).

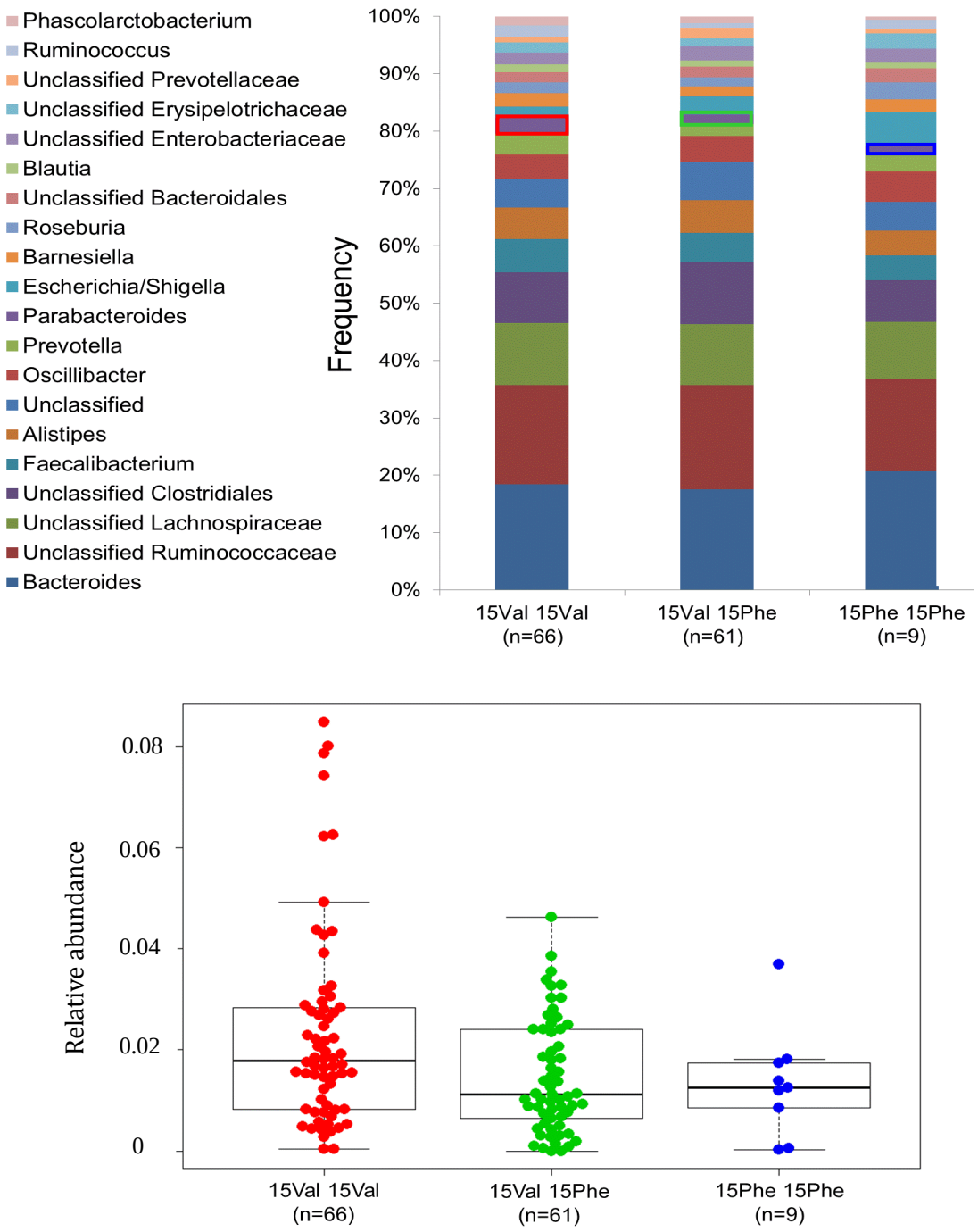


Figure 23. Fecal microbiota abundance of *Parabacteroides* correlates with p.Val15Phe genotype. Above: Stacked barplots show the frequency of 20 most abundant genera across three genotypes. Below: Beeswarm plots depict the relative abundance of *Parabacteroides* (sequence counts/10000) for PopCol individuals (dots) stratified according to genotype at the p.Val15Phe SNP site. The distribution of the data is reported as box plots for each genotype group.

In summary, this study demonstrated an association between genetic variation in the sucrase-isomaltase gene and predisposition to IBS which was detected principally in the diarrhea-related IBS. This notion is noteworthy because it may help identify a subgroup of IBS patients with genetic susceptibility to disaccharide maldigestion, which could eventually result in taking advantage of personalized medicine approaches to improve their management.

Moreover, as described in the introduction, some nutrients such as carbohydrates are believed to play a trigger role in many IBS patients. Intriguingly, our findings could provide a biological basis for this belief by displaying the potential interplay between defects in carbohydrate digestion enzymes, alterations in the GI transit rate and gut microbiota composition.

Finally, the pilot study using PopCol displayed an association between *SI* genotype variant p.Val15Phe and the fecal concentration of *Parabacteroides*. This is intriguing because, in agreement with this finding, the fecal abundance of this genus has been revealed to have a negative correlation with a higher intake of dietary carbohydrate (207) and in some studies *Parabacteroides* have been shown to be under-represented in IBS sufferers (307, 312). Thus, if confirmed by further studies, the inverse association between 15Phe copy number and *Parabacteroides* abundance may contribute to the identification of IBS patients with *SI* defects.

5 CONCLUDING REMARKS

The work presented in this thesis mainly concerns an attempt to further understand the interplay between gut microbiota, gut function and human genes in the generation of gastrointestinal symptoms.

In **paper I**, we provided more evidence for the association between gut microbiota and stool consistency and also for the first time displayed an association between fecal microbiota and stool frequency. Both of these fecal characteristics are considered as surrogates for GI transit time which in turn is used as an objective method to quantify GI functional abnormalities and has been shown to be associated with many GI functional symptoms. In **paper II,** we demonstrated an association between gut microbiota composition and abdominal pain occurrence as well as its frequency, duration, and severity. Also, we could provide more evidence for the negative association of *Prevotella* with pain in the general population. In **paper III**, for the first time, we could link human genes to GI function quantified by stool frequency in a genome-wide association study of two harmonized general population cohorts and suggested xenobiotic metabolism and ion channel activity as two plausible underlying mechanisms for the regulation of stool frequency. In **papers IV and V**, we identified two genes whose variants are associated with IBS and colonic transit rate. Of particular interest, these genes showed a stronger association with different subtypes of IBS (*TRPM8* variants showed a stronger association with IBS-C while *SI* SNPs were more linked to IBS-D) which suggests a possible genetic ground for stratification of IBS sufferers in the future. Also, **paper V**, through a combination of genetic results with phenotypic characteristics and microbiota-related findings could be considered as the first experimental evidence pointing to *nutrigenetic* mechanisms associated with IBS susceptibility and symptom generation.

The information resulting from this thesis project may contribute to translational opportunities for the stratification and eventual management of individuals with IBS and other FGIDs. Finally, the thesis was carried out based on a very ambitious aim and we have hopefully succeeded to some degree in shedding more light on the phenomenally complex interplay between the several different elements involved in the generation of GI symptoms.

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you and you will take care of it no matter what we have asked you for. Thank you very much and I love you.

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