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IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF IRRITABLE BOWEL SYNDROME (IBS) RISK GENES AND VARIANTS

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Identification and functional characterization of irritable bowel syndrome (IBS) risk genes and variants

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To my dearest family

谨以此书献给我最亲爱的家人们

Thanks for your continued love, support and encouragements

感谢你们一直以来的爱，支持和鼓励

ABSTRACT

As one of the most prevalent functional gastrointestinal disorders (FGIDs), irritable bowel syndrome (IBS) affects more than 10% of the general population worldwide with a higher prevalence in women. The primary clinical manifestation of IBS is chronic abdominal pain or discomfort associated with changes in stool frequency and appearance. IBS is the second leading cause of work absenteeism after colds and has remarkable effects on the socio-economic system. The pathophysiology of IBS has not been fully clarified yet, including various peripheral and central mechanisms.

From the late 1980s, genetic predisposition to IBS has been demonstrated by family and twin studies. Several candidate genes have been linked to IBS susceptibility including *TNFSF15*, *NPSR1*, *SCN5A*, *TRPM8,* and *SI.* Moreover, a few underpowered genome-wide association studies (GWAS) have been performed to investigate IBS genetics in population-based cohorts. However, to date, no unequivocal genetic factor has been confirmed yet.

In this thesis, we aim to identify risk genes and variants associated with IBS and to characterize their functional roles. The first part focuses on the role of genetic variations in the sucrase-isomaltase (*SI*) gene and IBS susceptibility. In the second part, the hypothesis-free GWAS approaches are implemented to detect IBS risk genes and variants in large-scale powered cohorts.

In **Paper I**, we have exploited a two-step computational strategy to study the prevalence of *SI* rare pathogenic variants (SI-RPVs) in 2207 tertiary IBS patients. The prevalence of selected SI-RPVs in all IBS patients is 3.99%, which is significantly higher than the reference population (P=0.00049). This study has provided supporting evidence that links carrying SI-RPVs to increased risk of IBS.

Paper II has investigated the effects of *SI* functional variants in the response to dietary intervention in IBS patients. The genotypes of *SI* hypomorphic variants were obtained for a group of IBS-D patients previously treated with a low FODMAP diet in a clinical trial. After stratifying IBS patients into carriers and non-carriers of *SI* hypomorphic variants, we have observed significantly lower efficiency of low FODMAP diet in carriers compared to non-carriers (P=0.031). These findings suggest that *SI* genotype data may contribute to identifying individuals with higher chances to benefit from such dietary interventions.

In **Paper III**, we have performed a GWAS of self-reported IBS exploiting the large population-based UK Biobank. After quality control, the association analysis has been carried

out in 9,576 IBS patients and 336,499 controls via logistic regression. Genome-wide significant signals have been identified on chromosome 9q31.2, and sex-stratified analysis suggests this locus is female-specific. This finding has been further supported by replication evidence from analyses in a pooled cohort with multi-national tertiary IBS cases and controls and a Swedish population-based cohort.

In the end, IBS GWAS and their meta-analyses have been performed in large-scale multi-national tertiary IBS cases and controls from European countries and the US in **Paper IV**. We have identified two novel genome-wide significant loci in IBS-D meta-analyses, and the results from functional annotation and PheWAS screening have suggested the association of these loci with altered metabolic and immune activities as well as psychiatric conditions. Ion channel biology was also highlighted as plausible pathways linked to IBS.

Taken together, this thesis has provided new insight that improves current understanding of genetic predisposition to IBS. In the long run, the discovery of IBS predisposing genes and variants may have a significant impact on IBS management, since it is expected to allow patients stratification and therefore increase the specificity and efficacy of treatment.

LIST OF SCIENTIFIC PAPERS

I. Koldo Garcia-Etxebarria*, **Tenghao Zheng***, Ferdinando Bonfiglio, Luis Bujanda, Aldona Dlugosz, Greger Lindberg, Peter T Schmidt, Pontus Karling, Bodil Ohlsson, Magnus Simren, Susanna Walter, Gerardo Nardone, Rosario Cuomo, Paolo Usai-Satta, Francesca Galeazzi, Matteo Neri, Piero Portincasa, Massimo Bellini, Giovanni Barbara, Daisy Jonkers, Shanti Eswaran, William D Chey, Purna Kashyap, Lin Chang, Emeran A. Mayer, Mira M Wouters, Guy Boeckxstaens, Michael Camilleri, Andre Franke, Mauro D'Amato.
Increased prevalence of rare sucrase-isomaltase (SI) pathogenic variants in irritable bowel syndrome patients.
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Reduced efficacy of low FODMAPs diet in patients with IBS-D carrying sucrase-isomaltase (SI) hypomorphic variants.
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* Contributed equally

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Direct repression of anoctamin 1 (ANO1) gene transcription by Gli proteins.
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LIST OF ABBREVIATIONS

5-HT3	5-hydroxytryptamine receptor 3
5-HT4	5-hydroxytryptamine receptor 4
AAM	Age at menarche
ACO1	Aconitase 1
ANO3	Anoctamin 3
ANS	Autonomic nervous system
BAM	Bile acids malabsorption
BAMSE	Children, Allergy, Milieu, Stockholm, Epidemiology
BMI	Body mass index
C4	7α-hydroxy-4-cholesten-3-one
CACNA1A	Calcium Voltage-Gated Channel Subunit Alpha1 A
CACNA1E	Calcium Voltage-Gated Channel Subunit Alpha1 E
CADD	Combined Annotation-Dependent Depletion
CI	Confidence interval
CLCA1	Calcium-Activated Chloride Channel Protein 1
CLCA2	Calcium-Activated Chloride Channel Protein 2
CLCA4	Calcium-Activated Chloride Channel Protein 4
CNGA4	Cyclic nucleotide-gated channel alpha 4
CNS	Central nervous system
CRF	Corticotropin releasing factor
CSID	Congenital sucrase-isomaltase deficiency
DNA	Deoxyribonucleic acid
EALs	Early adverse life events
ELP1	Elongator complex protein 1
EMR	electronic medical records
ENS	Enteric nervous system
eQTL	Expression quantitative trait loci
ExAC	Exome Aggregation Consortium reference
FAM229B	Family With Sequence Similarity 229 Member B
FC	Functional constipation
FGF19	Fibroblast growth factor 19
FoCus	Food Chain Plus cohort

FODMAPs	Fermentable oligosaccharides, monosaccharides, and disaccharides and polyols
GI	Gastrointestinal
GRID2IP	Grid2 Interacting Protein
GVQW1	GVQW Motif Containing 1
GWAS	Genome-Wide Association Study
HPA	Hypothalamic-pituitary-adrenal
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IBS-C	Constipation-predominant irritable bowel syndrome
IBS-D	Diarrhea-predominant irritable bowel syndrome
IBS-M	Mixed-type irritable bowel syndrome
IBS-U	Unclassified irritable bowel syndrome
ICCs	Interstitial cells of Cajal
ICD	International Classification of Diseases
KCNK2	Potassium channel subfamily K member 2
KCNMB2	Potassium Calcium-Activated Channel Subfamily M Regulatory Beta Subunit 2
KDELR2	KDEL Endoplasmic Reticulum Protein Retention Receptor 2
LAMA4	Laminin Subunit Alpha 4
LCT	Lactase
LD	Linkage disequilibrium
LINGO2	Leucine Rich Repeat And Ig Domain Containing 2
LSM14A	MRNA Processing Body Assembly Factor
MAF	Minor allele frequency
MARCKS	Myristoylated Alanine Rich Protein Kinase C Substrate
M-CAP	Mendelian Clinically Applicable Pathogenicity
mNICE	modified guidance from the National Institute for Health and Care Excellence
NCGS	Nonceliac gluten sensitivity
NDUFB6	NADH:Ubiquinone Oxidoreductase Subunit B6
NFBC1966	Northern Finland Birth Cohort study 1966
NPS	Neuropeptide S
NPSR1	Neuropeptide S Receptor 1

P-body	mRNA processing body
PCs	Principal components
PheWAS	Phenome Wide Association Studies
PopGen	Population-based recruitment of patients and controls for the analysis of complex genotype-phenotype relationships
PRS	Polygenic risk score
QC	Quality control
RFPL4B	Ret Finger Protein Like 4B
SALT	Screening Across the Lifespan Twin
SCFAs	Short chain fatty acids
SCN5A	Nav 1.5 voltage-dependent sodium channel
SHIP-Trend	Study of Health in Pomerania
SI	Sucrase-Isomaltase
SI-RPVs	SI rare pathogenic variants
SMCs	Smooth muscle cells
SNP	Single nucleotide polymorphism
TGR5	G-protein-coupled bile acid receptor 1
TNFSF15	Tumor necrosis factor ligand superfamily member 15
TNF-α	Tumor necrosis factor-α
TRPA1	Transient Receptor Potential Cation Channel Subfamily A Member 1
TRPM8	Transient receptor potential cation channel subfamily M member 8
TRPV3	Transient Receptor Potential Cation Channel Subfamily V Member 3
TUBE1	Tubulin Epsilon 1
UKB	UK biobank
US	The United States of America
WISP3	WNT1-Inducible-Signaling Pathway Protein 3

1 BACKGROUND

1.1 FUNCTIONAL GASTROINTESTINAL DISORDERS

The functional gastrointestinal disorders (FGIDs) are described as a heterogeneous group of chronic functional conditions of the gastrointestinal (GI) system, as their symptoms manifest in the absence of identifiable structural or biochemical abnormalities.(1,2) The term "functional" generally refers to abnormal activities in the body such as changes in gut motility or visceral hypersensitivity. However, negative (non-disease) results are usually obtained from routine serological, imaging and endoscopic examinations.

The definition of FGIDs has been changing over time, from non-organic diseases, psychiatric comorbidities to disorders of gut-brain interaction.(1) They encompass disorders with symptoms related to GI motility, visceral hypersensitivity, altered immune activities, dysbiosis, and changes in the central nervous system processing.(2) Recent studies have proposed that the FGIDs phenotypes result from the complex interactions between genetic, environmental, psychological and physiological factors.(3,4) The pathophysiology of FGIDs is illustrated in a biopsychosocial model as shown in **Figure 1**.

FGIDs are the most common reasons for referral to a gastroenterologist worldwide, and many people do not consult a physician for their GI symptoms.(5) Despite the high prevalence, the etiology of FGIDs has not been clarified yet. To date, diagnosis and classification of FGIDs are established based on patients' symptoms. The current most widely accepted diagnostic criteria for FGIDs are from Rome Foundation. Since the late 1980s, expert researchers and clinicians worldwide have gathered and assessed the characteristics, diagnostic and the therapeutic aspects of FGIDs. The Rome Foundation was set up in 1996, and since then, the foundation has played a key role in FGIDs research work.(1,2) Their collaborations have resulted in the criteria in FGIDs diagnosis, the so-called "Rome Criteria." The latest Rome IV criteria comprise 33 adult and 20 pediatric FGIDs, which are classified into eight domains according to their anatomic locations.(2) A detailed list of adult FGIDs in Rome IV classification is shown in **Figure 1.** Nevertheless, the Rome IV criteria have just been released and not widely utilized yet in scientific literatures. Rome III criteria are still "golden standards" in most FGIDs studies and are what we refer to in this thesis.

There are limitations to the application of Rome Criteria in clinical settings. First, the symptom-based criteria categorize individuals into patients and non-patient groups. Those who have similar symptoms are excluded if they do not fully meet the criteria. Second, Rome Criteria weight more on patients' symptoms, while other dimensions of the patients'

conditions (e.g., psychosocial, quality of life) are not fully taken into consideration. Therefore, a more integrated profile should be added to patients' clinical manifestation when using Rome Criteria in clinical cares. Despite the limitations, Rome Criteria are still valuable instruments for research proposes.

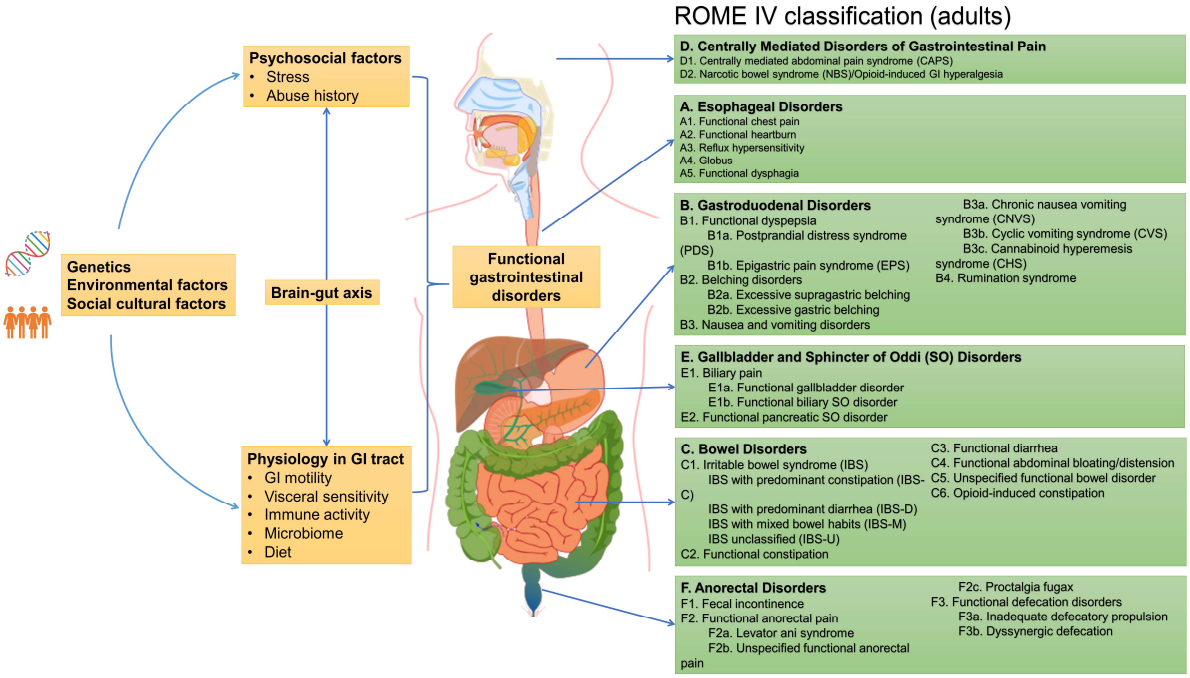

FIGURE 1. A biopsychosocial model for the conceptualization of FGIDs pathophysiology and Rome IV classification.

1.2 IRRITABLE BOWEL SYNDROME

1.2.1 Clinical characteristics

Irritable bowel syndrome (IBS) is one of FGIDs with high population prevalence, with main clinical manifestations of chronic abdominal pain associated with bloating, gas, constipation or diarrhea.(6) IBS is defined as recurrent abdominal pain or discomfort (more than 3 days/month in >3 months) based on Rome III criteria, together with at least two accompanying symptoms: 1) symptom remission after defecation, 2) symptom onset links to changes in bowel movements, 3) symptom onset associated with stool form changes.(1) Specific subtypes of IBS are established on patients' bowel habits and the predominant pattern, including IBS-D (diarrhea), IBS-C (constipation), IBS-M (alternating diarrhea and constipation) or IBS-U (unclassified), as described in **Figure 2**. Given that IBS symptoms

can also be present in other GI diseases (primarily inflammatory bowel diseases and celiac disease), IBS often remains a diagnosis of exclusion in routine clinical practice.

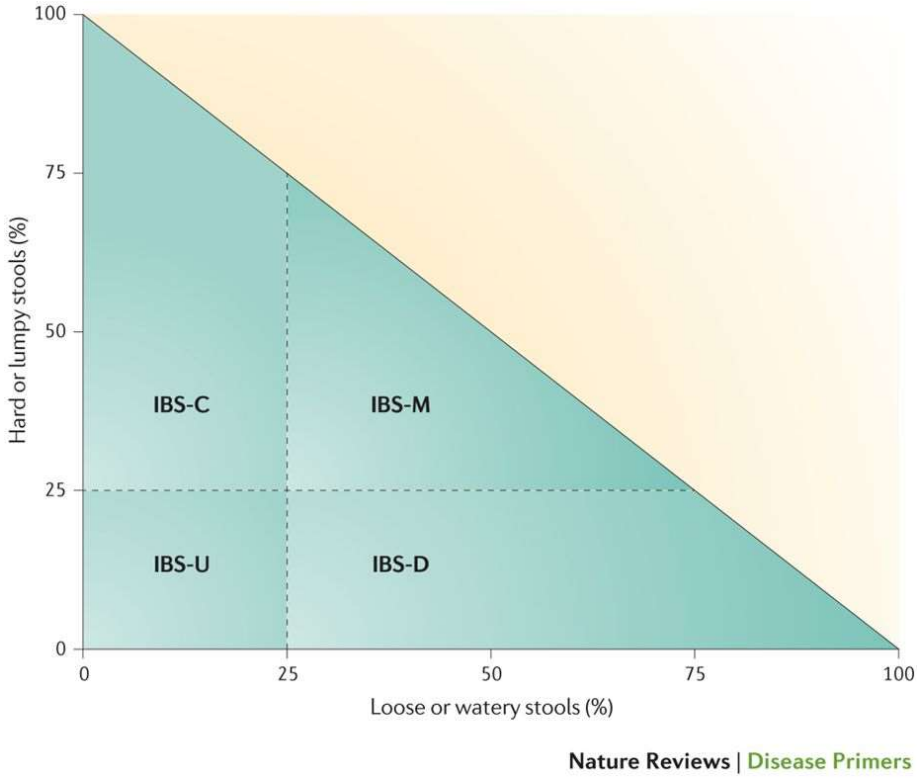

FIGURE 2. A two-dimensional figure illustrates the definition of IBS subtypes. According to the frequency of lumpy and watery stools, IBS patients can be classified into four subgroups: IBS-D (diarrhea), IBS-C (constipation), IBS-M (alternating diarrhea and constipation) or IBS-U (unclassified). Reprinted with permission from Enck, P. *et al.*, Nat Rev Dis Primers. 2016 Mar 24;2:16014. Copyright © 2016, Springer Nature.

Although IBS is not a life-threatening condition, it seriously impacts patients' quality of life and has significant impacts on the health and socio-economic system. As a chronic condition, IBS symptoms affect many patients for more than ten years,(7) hence IBS accounts for a large proportion of primary care and gastroenterology practice.(8,9) As the second leading cause of work absenteeism after colds, IBS costs translate approximately into 1600 Euros per patient per year, for an estimated 0.5% of the annual national healthcare budget in the US.(10)

1.2.2 Epidemiology of IBS

The prevalence of IBS ranges between 10% and 25% in individual community surveys,(8,11–17) **Figure 3** shows the detailed population IBS prevalence worldwide.(18) A

meta-analysis has demonstrated a global IBS prevalence of 11.2% (95% CI: 9.8–12.8),(19,20) while another recent literature review from the Rome Foundation working team described a significant degree of heterogeneity of IBS prevalence among different countries, ranging from 5.8% in the Middle East/Africa to 17.5% in Latin America.(21) The epidemiological data from most African countries and many Asian countries are not yet available, which may attribute to the inadequate attention paid to functional disorders. On the other hand, it is also noteworthy that the reported IBS prevalence in some developing countries is higher than developed countries, this may be due to the poorer life condition and a higher incidence of infectious diarrhea (mainly in tropical countries), their milder types could be misdiagnosed as IBS.

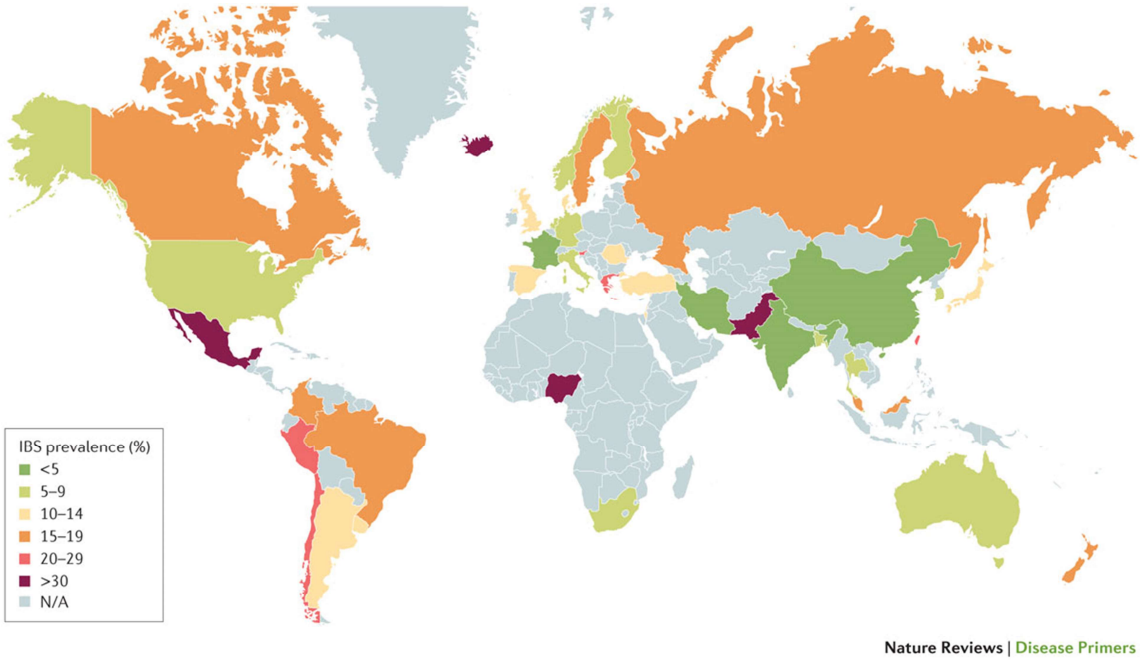

FIGURE 3. IBS prevalence in population studies around the world. Reprinted with permission from Enck, P. *et al.*, Nat Rev Dis Primers. 2016 Mar 24;2:16014. Copyright © 2016, Springer Nature.

A few factors have been demonstrated to be associated with IBS, including sex, age, socioeconomic status, and family clustering. In most studies, IBS rates were reported to be higher in women than men,(9) and a meta-analysis estimated a 67% increase of odds in females.(20) While the specific mechanisms accounting for sex differences in IBS remain to be fully understood. Individuals from all age groups can be affected by IBS. Pooled analyses showed that IBS prevalence decreased with increasing age, especially in the age group above 50 years' old, but none of the difference was statistically significant.(20,22) Another study

revealed milder abdominal pain in older IBS patients, but worse quality of life was found compared with younger groups.(23) Some studies have reported IBS is more frequent in individuals with lower socioeconomic status,(13) although other independent studies failed in replicating this observation. Increased risk of IBS has been reported in individuals with a family history.(24,25) Genetic, environmental factors, as well as social learning, have been considered to play a role in the IBS family aggregations. The genetic predisposition of IBS will be discussed in detail in **Section 1.3**.

Recent studies have demonstrated the association between GI infections and IBS. The estimated odds ratio for developing IBS is 5.86 (95% CI: 3.60–9.54) in individuals after their gastroenteritis,(26) The mechanism of post-infectious IBS is not yet clarified, low-grade intestinal inflammation and increased infiltration of mast cells may involve in the generation of GI symptoms. However, there is no consensus on whether infections from specific pathogens are linked to IBS.

1.2.3 Current understanding of IBS pathophysiology

Although the etiology of IBS is still poorly understood, there are extensive studies on the roles of central and peripheral mechanisms in IBS pathophysiology.(27–29) There is accumulating evidence suggesting the involvement of intestinal immunity, disordered gut-brain communication, visceral hypersensitivity and dysbiosis in the generation of IBS symptoms in different individuals.(30,31) However, given the heterogeneity of IBS phenotypes and their different response rates to treatments, there may be no uniform mechanism for all IBS patients even when they share the same clinical manifestations.(30) In this section, some of the well-documented potential mechanisms and aetiological factors will be discussed in detail.

Brain-gut axis: For a long time, IBS has been known as a brain-gut disorder as the central nervous system can influence GI functions (e.g., GI motility, intestinal permeability, immune activity, secretion, and microbiota composition) through the autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axis. However, in about half of the IBS patients, their GI symptoms originate from the gut rather than the brain, as the psychological conditions occur after the diagnosis of IBS.(32) A gut-to-brain pathway is also supported by the fact that signal processing in the CNS can be affected by the use of probiotics.(33) Some of the above intestinal peripheral alterations may lead to the structural and functional changes in the brain, which further suggests a bi-directional regulatory network (**Figure 4**). Moreover, the brain plays a vital role in the central processing of interoceptive information from

peripheral sensory receptors. Some psychosocial disturbances may modify the normal way of central processing and amplify the sensory information (such as visceral pain). In a study testing coping model of catastrophizing in IBS patients, catastrophizing was found to be strongly associated with severity of pain syndrome.(34)

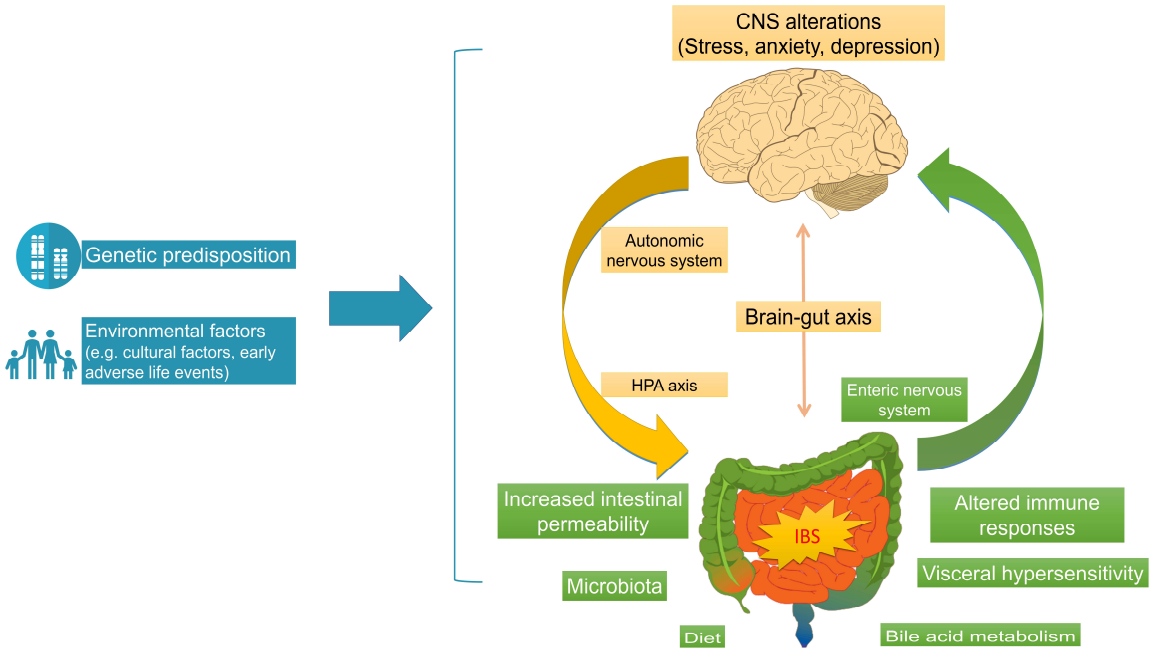

FIGURE 4. A schematic diagram to summarize the current understanding of IBS pathophysiology. Some key central and peripheral mechanisms, genetic and environmental factors are highlighted in IBS development.

Psychological factors: Coexisting psychological conditions (particularly anxiety, and depression) have been well-documented to exacerbate IBS symptoms.(35,36) In IBS patients, a higher level of corticotropin releasing factor (CRF) was found to associate with excessive stress.(37) However, the therapeutic attempts of using CRF receptor antagonists failed in restoring colonic transits in female IBS-D patients.(38) Studies also suggest the association of early adverse life events (EALs) history with IBS susceptibility.(39) The imaging studies of the brain have shown associations of structural and functional alterations with EALs,(40) which can affect brain activities in IBS patients.(41) EALs have also been reported to affect gene expression regulation in brain via DNA methylation,(42–44) which may, together with alterations in brain networks, affect the risk of IBS development.

Epithelial barrier: The intestinal epithelium serves as an interface for complex interactions between the intestinal environment, microbiota, and the gut mucosa. The mechanisms for gut

mucosal barrier dysfunction remain unknown, while many factors are considered to play a role including genetic variations, infections, altered microbiota composition, and food allergies.(45) Increased intestinal permeability has been known to be an important contributing factor to IBS, which could result in low-grade inflammation and altered immune activities in the intestinal mucosa.(45) Electron microscopic studies have identified enlarged epithelial cell spaces in gut mucosa biopsies from IBS-D patients.(46) Other structural defects of the gut barrier in IBS patients were also observed.(47) Besides, the role of tight junction proteins have been highlighted in increased intestinal permeability, the protein expression levels of zonula occludens-1 and occludin were found lower in IBS patients compared with controls.(48) A recent study has also demonstrated that miRNAs (miR-16 and miR-125b) can affect barrier function in IBS-D patients by regulating the expression of tight junction proteins claudin-2 and cingulin.(49)

Altered immune response: Low-grade mucosal inflammation has been demonstrated to contribute to the generation of IBS symptoms from numerous studies.(50) Increased immune activities (such as increased inflammatory cells and levels of inflammatory markers) in the gut have been detected in IBS patients, pointing to an immune-mediated mechanism in at least subsets of IBS.(51) IBS-like symptoms can be manifested in around 33% of inflammatory bowel disease (IBD) patients in remission, which supports the role of the intestinal immunity in IBS pathophysiology.(52) However, negative results were obtained when testing the therapeutic effects of an anti-inflammatory agent (mesalazine) in IBS patients from two clinical trials.(53,54) Several studies have linked mast cells to the low-grade immune activation in IBS, as higher amounts of mast cells and their mediators (protease, histamine) were found in colonic biopsies from IBS patients than control samples.(55,56) Moreover, IBS patients also showed higher serum levels of interleukin-6, interleukin-1-β and tumor necrosis factor-α (TNF-α) compared with healthy controls.(57) Of note, a recent study has highlighted the role of self-maintaining gut macrophages in intestinal homeostasis. Depletion of these macrophages may lead to several GI abnormalities including reduced intestinal motility and loss of enteric neurons, which further support the link of altered GI immune activities to the generation of IBS symptoms.(58)

Bile acid malabsorption: Bile acids are substances synthesized in the liver and stored in the gallbladder, which are primarily responsible for digestion of fat in the small intestine. They recirculate between the liver and small intestine, and normally only a small portion will escape the circulation and enter the colon. Bile acids malabsorption (BAM) will result in excess amounts of bile acids entering the colon, and lead to GI symptoms.(59) A systematic

review reported a pooled prevalence of 28.1% (95% CI: 22.6–34%) for BAM in IBS-D.(60) Increased levels of serum C4 (7α-Hydroxy-4-cholesten-3-one, a product in bile acid synthesis) and fibroblast growth factor 19 (FGF19) have been linked to altered colonic transit time in IBS.(61,62) Also, genetic polymorphism in *TGR5* (G-protein-coupled bile acid receptor 1) gene was shown to affect the regulation of colonic transit time in IBS-D patients.(63)

Microbiota: The GI microbiota inhabits the entire digestive tract and includes around 400 species. The commensal microbiota has a complex impact on human health, playing a vital role in the development of the intestinal immune system.(64,65) The gut microbiota composition is affected by many factors, above all, for instance diet and the use of antibiotics.(66,67) Although the associations between microbiota profiles and IBS have been extensively investigated and altered microbiota diversity has been highlighted,(65) the causative role of individual taxa and/or species is still unclear in IBS pathophysiology. Recent studies have shown significant differences in fecal microbiota composition between IBS patients and controls, and among IBS subtypes.(68–73) However, these studies were performed in small cohorts, and therefore require replication in independent larger surveys. Fecal samples are commonly used in microbiota researches as they are easily obtainable, but the location information is missing comparing with mucosal biopsies.

1.2.4 Food components in IBS

Dietary factors have been demonstrated to be involved in the development of IBS by several mechanisms. Firstly, the poorly absorbed components in the diet, particularly fermentable oligosaccharides, monosaccharides, disaccharides and polyols (FODMAPs), could result in GI symptoms due to their osmotic effect and colonic fermentation.(74) Secondly, some molecules in the food components (e.g., bioactive food molecules) and products from digestion may activate the receptors on GI tracts. These receptors include taste, nutrient, and fatty acids, and their activation will result in the release of various neurotransmitters and hormones affecting gut function. Studies also reveal that some food can activate mast cell directly and induce immune activities in the gut.(75) Last but not least, the interplays between diet and gut microbiota composition also play a vital role in IBS pathogenesis. Dietary changes have been reported to influence the gut microbial composition.(66) The dysbiosis in the gut can affect GI functions and involve in IBS pathogenesis in many ways, some of which have been discussed in **Section 1.2.3**. The complex network between the brain-gut axis and microbiota may be affected by diet-induced dysbiosis, and thus influence ENS function, gut motility, and sensation.(33)

Many IBS sufferers believe certain foods trigger their symptoms, and avoiding such foods is a common strategy they often self-implement.(76) Although food is complex and dietary components vary significantly from person to person, researchers have attempted to identify specific food components that induce GI symptoms. Once the role of any specific component in IBS pathogenesis is well clarified, dietary interventions can be designed to target certain subgroups of IBS patients. Several food components have been proposed to associate with IBS including carbohydrates, proteins and bioactive food chemicals.(74,77) Dietary proteins, especially gluten in wheat, may involve in the pathophysiology of IBS. Gluten has been implicated as the key contributing factor in celiac disease.(78) While nonceliac gluten sensitivity (NCGS) has also reported in other individuals and a gluten-free diet has shown to be beneficial for their IBS-like symptoms.(79)

Latest researches have focused on poorly absorbed carbohydrates of relevance to IBS. Certain types of carbohydrates cannot be digested (oligosaccharides and non-starch polysaccharides) or slowly digested (fructose and polyols) in the small intestine. Moreover, the capability of carbohydrates digestion in GI tract could be affected by lack of hydrolases or reduced enzymatic activities, such as lactose intolerance caused by *LCT* (lactase) gene variations. Genetic variations in *SI* gene can result in congenital sucrase-isomaltase deficiency (CSID) which leads to malabsorption of sucrose and starch and a series of GI symptoms. The association of *SI* variants with IBS will be discussed in **Section 1.3.1** and **Paper I & II**. The accumulation of all these mal-absorbed carbohydrates (FODMAPs) in the small intestine can cause increased water retention due to their osmotic effects.(80,81) Moreover, their colonic fermentation by gut microbiota may lead to overproduction of gas and the production of SCFAs. Both pain sensation and intestinal motility can be affected, leading to the generation of IBS symptoms.(82–84) An overview summarizing the role of FODMAPs in IBS pathogenesis is shown in **Figure 5**.

Several strategies have been designed to target specific dietary alterations, such as lactose- or fructose-restricted diet. Their specific contents and nutritional risks have been summarized in a review article, there is lack of evidence for their long-term beneficial effects in IBS patients.(74) An Australian group has first proposed the low FODMAP diet in 2004 which recommends restricting intake of multiple mal-absorbed carbohydrates.(85) Since then, the supporting evidence for its benefits in IBS management has been accumulating and it has been recommended for IBS patients as a promising therapeutic approach.(86–88) There is evidence supporting that limiting dietary intake of FODMAPs helps the remission of symptoms in IBS patients.(89–91)

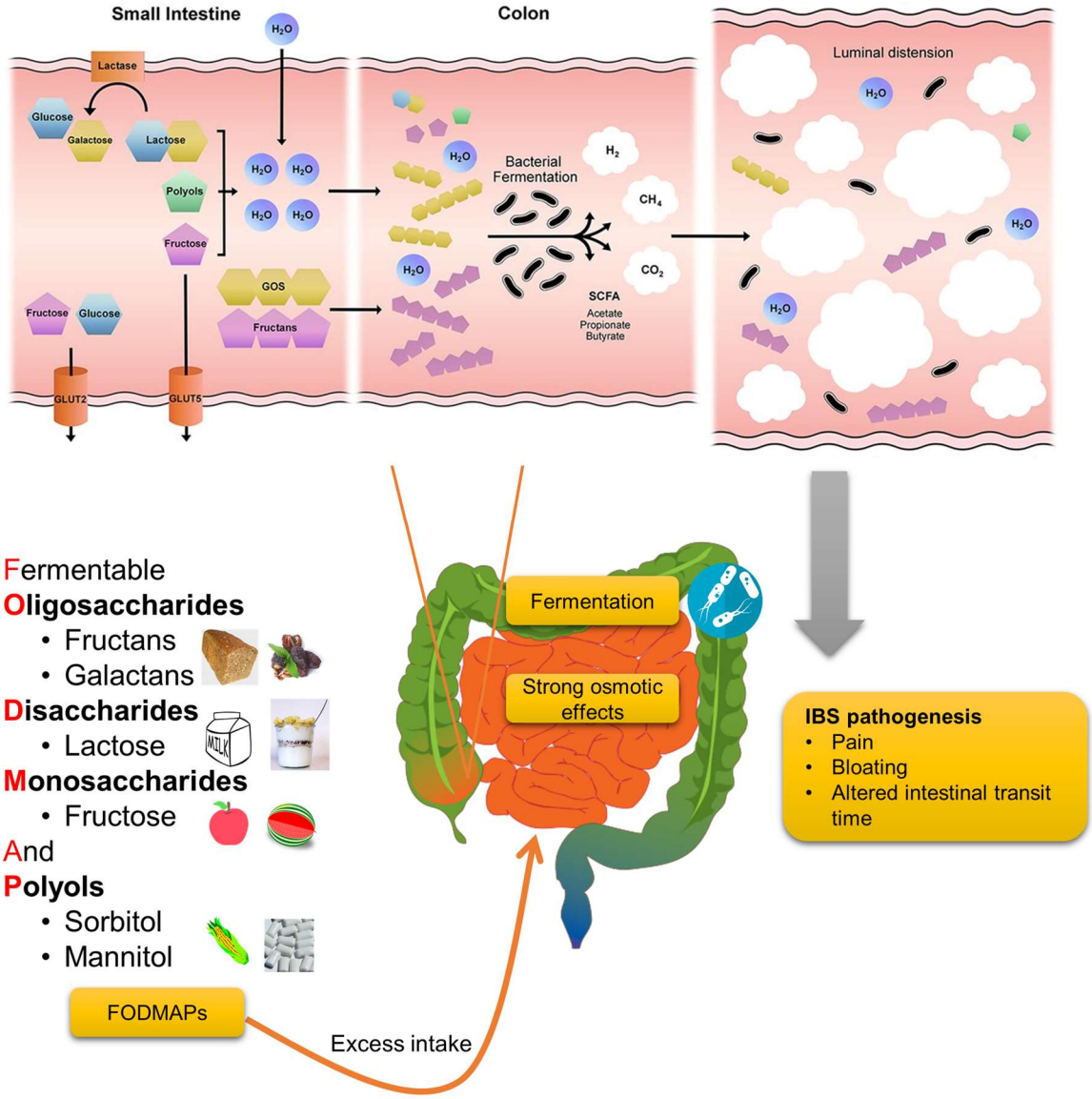

FIGURE 5. The description of FODMAPs contents and the proposed mechanism for their involvements in IBS pathogenesis. The schematic diagram which shows gut lumen on the top has been reused with permission from Staudacher HM. *et al*. Gut. 2017 Aug;66(8):1517-1527. Copyright © 2017, BMJ Publishing Group Ltd.

Moreover, a recent review article has shown that 50-80% of IBS patients benefit from low FODMAP diet for their GI symptoms.(92) A clinical trial has compared low FODMAP diet with modified guidance from the National Institute for Health and Care Excellence (mNICE) diet in IBS-D patients and reported a significantly higher symptom relief rate in low FODMAP diet treated group.(93) However, another study has reported no significant difference in the efficacy of IBS treatment between low FODMAP diet and other traditional

dietary practice.(94) There is also criticism for low FODMAP diet including potential deficiency of calcium intake(90) and altered microbiota composition in patients.(90,95) Further high-quality studies are warranted to validate the therapeutic effects of low FODMAP diet in IBS patients.

1.2.5 Therapeutic intervention

Multiple mechanisms have been implicated in IBS pathogenesis. The IBS phenotypes encompass subgroups with different predominant symptoms, the incomplete pathophysiological picture has added the difficulties in designing an overall therapeutic strategy that fits all IBS patients. So far, the therapeutic options for IBS have been limited, hampered by the poor understanding of IBS pathogenesis. Most of the interventions aim at remission of symptoms and sometimes may result in an unsatisfactory endpoint. An integrated management approach has been proposed, which incorporates diet, drugs, education, and psychotherapy.(96)

Apart from the dietary intervention being discussed in **Section 1.2.4**, pharmacotherapy is usually applied to relieve GI symptoms in IBS (primarily altered intestinal transit time and visceral pain). The proposed pharmaceuticals for IBS cover a wide range of drug species, including antispasmodic drugs (e.g., dicyclomine),(97) intestinal motility accelerants (e.g., lubiprostone(98) and 5-HT4 receptor agonist prucalopride(99)), antidiarrheals (e.g., loperamide(100) and 5-HT3 receptor antagonists alosetron(101)), and probiotics.(102–104) Clinical trials have evaluated the efficacy of these drugs in IBS treatment. Many of them reported low quality of evidence, as summarized in a review article.(31)

Genetic studies in IBS may open new gates for therapeutic interventions in at least a subset of IBS patients. The best example for their applications is in the candidate gene study of ion channel gene *SCN5A* (described in **Section 1.3.1**). It has been demonstrated that a chronic IBS-C female patient with a functional-damaging *SCN5A* mutation responded well with the administration of mexiletine, a compound known to rescue Nav 1.5 expression defects, as shown in **Figure 6**.

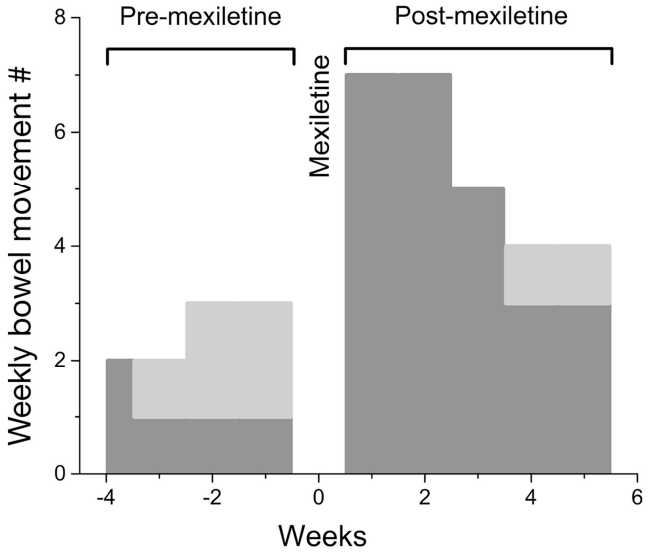

FIGURE 6. Treatment with mexiletine improved stool frequencies in an IBS-C patient carrying an *SCN5A* mutation. Complete spontaneous and small hard bowel movements are shown in dark and light grey shading, respectively. Reprinted with permission from Beyder et al., Gastroenterology. 2014 Jun; 146(7): 1659–1668. Copyright © 2014, Elsevier.

1.3 GENETIC PREDISPOSITION TO IBS

Genetic predisposition to IBS has been poorly investigated, although a heritable component has been demonstrated by a series of family and twin studies.(105–107) The heritability estimates of IBS reported from twin studies range from 0-57% (108–112) as described in **Table 1.** Despite the large variation, IBS heritability has been demonstrated in two large cohort twin studies (N=12,700 in Norway and N=16,961 in Sweden).

TABLE 1. Summary of twin studies in IBS.

Author (year)	Cohort	Number of study twins	Heritability (%)
Morris-Yates et al. (1998)(108)	Australian Twin Study	688	57
Mohammed et al. (2005)(109)	British Twin Study	4,480	0
Bengtson et al. (2006)(112)	Norwegian Twin Study	12,700	48
Lembo et al. (2007)(110)	Minnesota Twin Study	986	22

Svedberg et al. (2008)(111)	Swedish Twin Study	16,961	25

Since the late 1980s, the epidemiological studies have demonstrated an increased risk of IBS among relatives of patients. The evidence of IBS familial aggregation is summarized in **Table 2.** Among them, the strongest evidence is shown in a large Swedish national study with more than 50,000 individuals where increased IBS risk has been found among first-, second- and third-degree relatives. Of note, a higher odd ratio for IBS was observed in closer kinship with IBS patients, e.g. an OR of 1.90 in parent, 1.27 in niece/nephew and 1.11 in cousins.(113)

TABLE 2. Summary of familial aggregation studies in IBS. Reformulated from Makker, J. *et al.* (2015). Genetic epidemiology of irritable bowel syndrome. World J Gastroenterol. Oct 28, 2015; 21(40): 11353-11361.(114) Copyright ©The Author(s) 2015. Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license (https://creativecommons.org/licenses/by-nc/4.0/)

Authors (Year)	Size of study cohort	Findings
Whorwell et al. (1986)(115)	100	Family history of IBS has been detected in one-third of the studied IBS patients.
Levy et al. (2000)(116)	631	Children with IBS parents have more health care visits for their GI symptoms
Locke et al. (2000)(24)	643	Higher prevalence of IBS was reported in individuals whose first-degree relatives were with a history of bowel symptoms.
Kalantar et al. (2003)(117)	355	Relatives of IBS patients' parents reported a higher IBS prevalence than relatives of IBS patients' spouses.
Saito et al. (2008)(118)	202	Higher prevalence of IBS was observed in IBS patients' relatives (21%) than controls (4%).
Saito et al. (2010)(119)	477	50% of IBS cases reported family history.
Waehrens et al. (2015)(113)	51,952	The IBS risk is increased in first-, second- and even third-degree relatives.

It is believed that IBS is a complex genetic disorder with multiple factors being involved. The majority of IBS phenotypes may result from interactions between the genetic susceptibility background and environmental contributors. Moreover, IBS phenotypes may cover both complex genetic conditions and rare monogenic forms, and this implies different strategic approaches need to be adopted to identify causative factors in IBS genetic predisposition.

1.3.1 Candidate gene approaches

Numerous studies have explored the genetic predisposition to IBS in the past years, mostly based on candidate gene approaches and concentrating on single biological pathways such as serotonin signaling pathways due to the connection between the brain-gut axis and IBS.(120) Other genes involved in the control of intestinal immune activities, bile acid metabolism, and secretion have also been investigated. Some 60 genes or more have been tested over the years for their potential to contribute to the genetic predisposition of IBS and its clinical subtypes. However, these studies are mostly performed on small sample size and lacked replication in independent cohorts. Hence they may be of value from a historical perspective but poorly indicative of true genetic findings. The only exceptions may be represented by a few genes recently been reported by our research group to affect IBS risk across several independent cohorts such as *NPSR1*, *TNFSF15*, *SCN5A*, *TRPM8,* and *SI*.(121–126)

NPSR1: *NPSR1* encodes for neuropeptide S (NPS) receptor, which belongs to the G protein-coupled receptor family. The NPSR1-NPS system is known to play a role in the HPA axis, modulating central signaling processing.(127) NPS-NPSR1 signaling pathway and *NPSR1* polymorphisms have been reported to be involved in the pathogenesis of a few conditions including asthma, IBD, rheumatoid arthritis, and panic disorders.(128–131) Our group has investigated *NPSR1* polymorphism and its correlation with GI functions in IBS patients and identified several *NPSR1* variants significantly associated with GI motility and sensation.(121) Physiological data from animal models were consistent with the findings that NPS receptors had noticeable effects on GI motility in mice.(132) Furthermore, another study from our group has demonstrated that *NPSR1* polymorphisms also associated with recurrent abdominal pain in 1744 children from the Swedish birth cohort BAMSE.(124)

TNFSF15: The *TNFSF15* (tumor necrosis factor ligand superfamily member 15) gene was initially described as a genetic risk factor associated with Crohn's disease.(133) Its genetic polymorphisms were later found to be involved in the pathogenesis of other conditions including leprosy and spondyloarthritis.(134–136) In order to investigate the role of immune-related genes and their polymorphisms in the pathophysiology of IBS,(51,137) our group has

selected 30 risk loci from Crohn's disease associations and tested their associations with IBS in two independent Swedish and American cohorts. Among all the variants, SNP rs4263839 in the *TNFSF15* gene was significantly associated with IBS risk ($P=2.2\times10^{-5}$), an even stronger signal was shown in individuals with constipation-predominant IBS ($P=8.7\times10^{-7}$).(122) These findings were later replicated by a UK study,(138) a study in a US/Canada cohort(139) and a meta-analysis,(140) suggesting *TNFSF15* may be a true IBS genetic factor.

SCN5A: Ion channels represent potential pathophysiologic and therapeutic targets in IBS because they are directly involved in both GI motility and visceral pain.(141) In collaboration with the Mayo Clinic, we tested the hypothesis that ion channelopathies might be involved in IBS pathophysiology by screening patients for *SCN5A* (Nav 1.5 voltage-dependent sodium channel) mutations. These mutations are often found in Brugada syndrome patients who report bowel symptoms more often than the general population.(123) The results showed that rare *SCN5A* mutations were present in 2.2% of IBS subjects from a cohort including 584 IBS patients and 1380 asymptomatic controls, and the majority of these (77%) were demonstrated to be functionally disruptive. Moreover, both common and rare variants in *SCN5A* gene were found to be associated with IBS risk in our IBS GWAS of a Swedish general population cohort(142) and tertiary IBS case-control cohorts from three European countries and the US, which further confirmed the correlations between *SCN5A* variants and IBS genetic predisposition. *SCN5A* findings support the notion that there may be subsets of IBS patients with rare genetic abnormalities, hence studies on these genetic variants may provide novel therapeutic targets and personalized treatment options for a subset of IBS phenotypes. More recently, another study has replicated the *SCN5A* findings in an IBS case-control cohort from US. *SCN5A* mutations were present in 2% of IBS patients (N=252) but none of the healthy controls (N=377).(143)

TRPM8: In order to further explore the role of ion channel genes in IBS pathophysiology, we selected 27 ion channel genes contributing to GI motility and sensory function as additional candidates to affect IBS risk. Among these, nominal association signals were detected in our previous IBS GWAS(142) for four channels, namely the transient receptor potential channels *TRPV3* and *TRPM8*, and the calcium voltage-gated channels *CACNA1A* and *CACNA1E*, which were selected for replication. In a Swedish multicenter study of Rome III defined IBS patients (N=386) together with asymptomatic healthy controls (N=357), several SNPs in the promoter region of *TRPM8* gene showed significant replications. Furthermore, subtype

analyses revealed that *TRPM8* SNPs affect IBS risk exclusively in the IBS-C patients, and correlate with harder stools in a general population sample.(125)

Sucrase-isomaltase (SI): Congenital sucrase-isomaltase deficiency (CSID) is a rare genetic condition caused by malabsorption of carbohydrates. It is characterized primarily by diarrhea associated with bloating, gas and pain, which shares clinical symptoms with diarrhea-predominant IBS forms. The SI enzyme is a disaccharidase that hydrolyzes sucrose (and starch) into glucose and fructose, and its functional defects lead to increased amounts of undigested carbohydrates in the colon, with luminal osmotic changes, fermentation and the generation of bowel symptoms and diarrhea. CSID manifestations vary in severity from patient to patient,(144) and CSID patients misdiagnosed with IBS have also been reported.(145,146) Hence, we hypothesized that *SI* dysfunctional polymorphisms may associate with IBS susceptibility, and tested four CSID mutations in three independent tertiary IBS case-control cohorts from Sweden, Italy, and the USA. We detected a suggestive association of carrying a CSID mutation with increased IBS risk (p=0.074, OR=1.84). In addition, we also demonstrated a relatively common variant in *SI* gene (rs9290264, p.Val15Phe) that was associated with reduced SI enzymatic activity in vitro. Its 15Phe variant was also linked to increased risks of IBS, especially IBS subtypes with diarrhea (IBS-D and IBS-M combined p=0.00012, OR=1.36). These findings may contribute to novel strategies for stratification and individualized treatment in IBS patients.(126)

More recently, we have investigated the associations between the 15Phe variant of rs9290264, carbohydrate consumption and microbiota composition in two general population cohorts from Germany, PopGen (N=639) and FoCus (N=759).(147) The prevalence of IBS in 15Phe carriers (3.69%) was significantly higher than in non-carriers (1.84%). After stratifying the individuals based on their daily consumption of starch, the strongest association between 15Phe and IBS susceptibility was detected in the group of individuals with low intake of daily starch (IBS prevalence 7.8% in carriers vs. 1.9% in non-carriers; P=0.029, OR=4.17). Moreover, the analysis of microbiota data from fecal samples of IBS patients reported an increased abundance of *Blautia* compared with controls (P=0.00035). After stratification by Val15Phe genotypes, we only observed the significantly increased abundance of *Blautia* in 15Phe-carrier IBS group (P=0.00041) but not in non-carriers. This study provides evidence that links the complex interaction between *SI* variants, carbohydrates intake and gut microbiota to IBS risk.

1.3.2 Genome-wide association studies for IBS

Genome-wide association studies (GWAS) and their meta-analyses are powerful hypothesis-free approaches for identifying polygenetic risk factors in complex disease.(148) Although the conventional methodology of GWAS has been well established, to date, very few GWAS efforts have been made to investigate IBS genetic predisposition. It is believed that the genetic susceptibility in the majority of IBS phenotypes is composed of a combination of genetic effects from low-penetrance common variants. Unequivocal IBS risk loci can thus only be identified through the analysis of exceptionally large sample sizes, likely coming from multinational global efforts.

Recently, our group has proposed that a powerful approach to gene-hunting efforts in IBS may come from the study of general populations and biobank-scale samples exploiting the existing genotypic data and phenotypic information, resulting in a considerable gain in sample size and homogeneity.(107) Informative phenotypic data in the general population cohorts including Rome-criteria from questionnaires and International Classification of Diseases (ICD) codes from electronic medical records (EMR) can be applied to identify IBS cases and asymptomatic controls.

The very first pilot IBS GWAS has been performed by our group on genotype data from 5466 singletons (534 cases and 4932 controls) from the Swedish population-based Screening Across the Lifespan Twin (SALT) study, which includes questionnaires modules of gastrointestinal symptoms similar to Rome II criteria. Replication of findings confirmed evidence of a risk locus on chromosome 7p22.1 in 1,718 IBS cases and 1,793 healthy controls from 6 independent international cohorts.(142) This study provided experimental/methodological supports to our hypothesis that general population cohorts are ideal data sources for large-scale genetic studies in IBS.

We then expanded the GWAS study in other four European population-based cohorts (LifeLines-DEEP, SHIP-Trend, TwinsUK, and NFBC1966) adapting the similar approach,(149) resulting in a total of 1335 IBS cases and 9768 controls in meta-analysis. We have identified seven additional genomic regions, mapping to 64 suggestive genes associated with IBS risk. Ion channel biology has been highlighted as a plausible pathway linked to IBS by functional annotation of all mapped genes.

Despite previous GWAS efforts in IBS, so far, no genome-wide significant locus has been identified. It may be due to the limited statistical power of current studies. For example, to obtain a statistical power larger than 80%, around 10,000 cases and 10,000 controls are

needed to detect a genome-wide significant association ($P<5.0\times10^{-8}$) for a variant with 20% minor allele frequency (MAF) and genotype relative risk 1.15 (calculated by GAS Power Calculator, https://csg.sph.umich.edu/abecasis/gas_power_calculator/index.html). Powerful GWASs with an adequate sample size of IBS are expected to discover true unequivocal IBS risk factors. Meanwhile, clinically-relevant well-characterized IBS cases and controls are the most suitable material for validation and replication purposes in genetic studies. The large-scale GWA studies with these samples will rely on international collaborations from multicenter studies.

In summary, there are significant challenges in identifying IBS risk genes and variants. Given the heterogeneity of the IBS phenotype, various strategies may be necessary including candidate gene approaches and GWAS on large-scale cohorts to capture rare and common risk variants, respectively. Genetic research in IBS may contribute to the identification of pathophysiological mechanisms, a molecular re-classification of this condition, and hence ultimately provide novel therapeutic targets.

2 AIM OF THE THESIS

2.1 OVERALL AIMS

The overarching aim included in this thesis is to identify predisposing genes and risk variants of IBS and to characterize their functional roles. The ultimate goal of our IBS genetic studies is to identify important physiological pathways being involved in IBS pathogenesis, which will contribute to revealing novel targets for therapeutic development.

2.2 SPECIFIC RESEARCH QUESTIONS

In the first two studies of the thesis, we have adopted a candidate gene approach to further investigate the pathogenic role of functional variants of the *SI* gene in IBS patients. As described in **Section 1.3.1**, our previous study has demonstrated the significant associations between *SI* dysfunctional (hypomorphic) variants and increased risk of IBS. In this thesis, our further efforts were aimed at addressing the following research questions:

- Is IBS risk affected also by hypomorphic *SI* variants other than rare CSID mutations? (**Paper I**)
- Does *SI* genotype (hypomorphic variants carriership) affect the response to a low FODMAP diet in IBS patients? (**Paper II**)

In the later large-scale studies, we have exploited a hypothesis-free GWAS approach to answer the research question:

- Can true (genome-wide significant) IBS risk loci and genes be identified by applying large scale GWAS approaches in population-based and case-control cohorts? (**Paper III & IV**)

3 MATERIALS AND METHODS

The data source of all cohorts and detailed description of methodology in each study have been included in the constitute papers of the thesis. **Figure 7** shows an overview of the research questions and the overall framework.

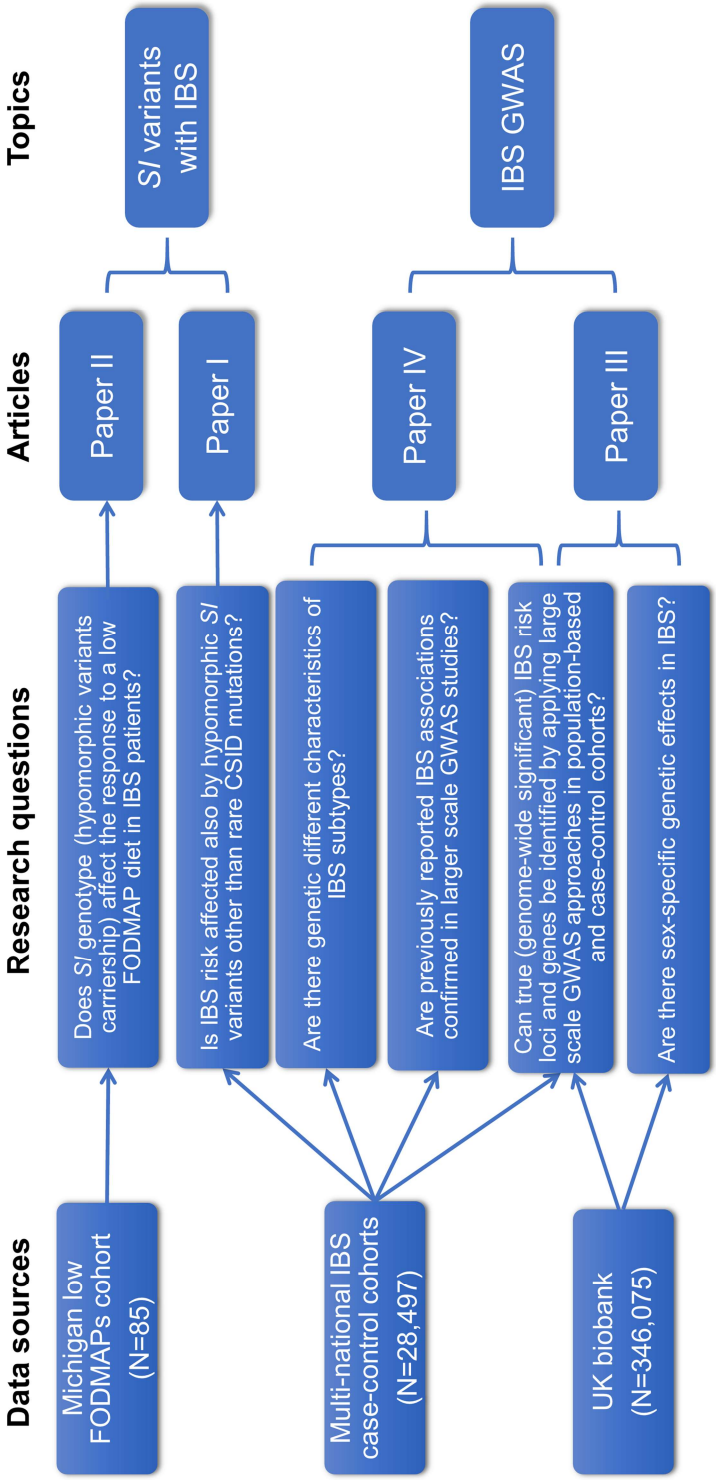

FIGURE 7. A conceptual framework describing the research questions addressed by this thesis, including data sources in each study, corresponding constitute papers, and research topics.

4 RESULTS AND DISCUSSION

4.1 *SI* RARE PATHOGENIC VARIANTS AND IBS SUSCEPTIBILITY

Mutations on the *SI* gene are reported to affect enzymatic activities and lead to congenital sucrase-isomaltase deficiency (CSID).(150,151) Given that CSID shares similar manifestations with IBS-D (primarily abdominal pain and diarrhea), some milder types of CSID can be misdiagnosed with IBS. In a recent study, we have investigated the pathogenic mechanism of *SI* functional variants in IBS symptom's generation and demonstrated one common variant, as well as four mutations, are associated with increased risk of IBS.(126) In order to investigate the prevalence of other rare functional variants in IBS patients, we have implemented a two-step computational strategy in **Paper I** to first identify *SI* rare pathogenic variants (SI-RPVs) and then test their associations with IBS risk exploiting genotype data of 2,207 IBS patients from multi-national tertiary centers.

The working flow for SI-RPVs' selection is summarized in a schematic diagram as shown in **Figure 8**.

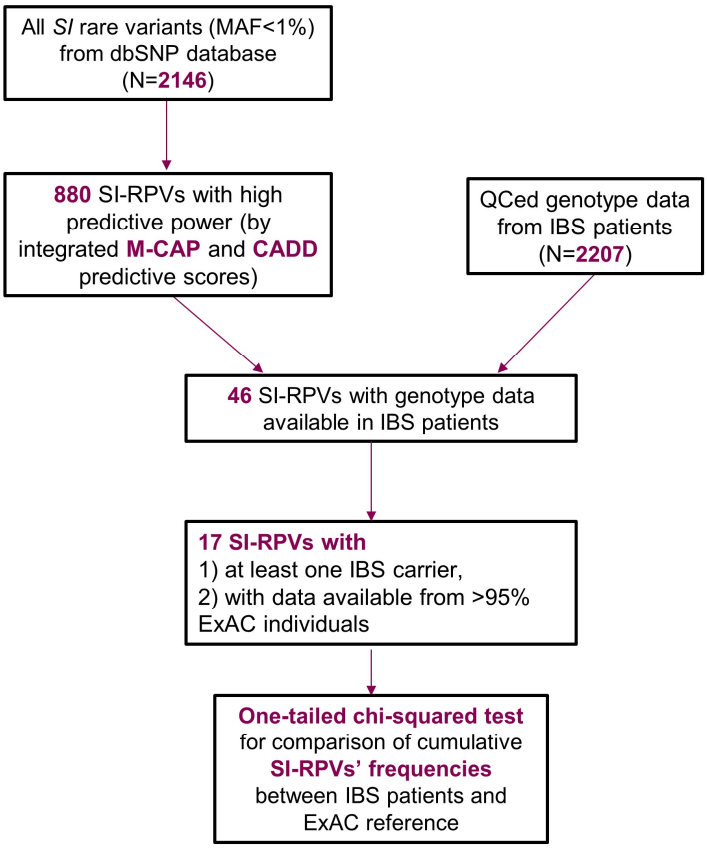

FIGURE 8. A schematic diagram to demonstrate the computational strategy and results of SI-RPVs' selection.

A total 2,146 rare variants (MAF<1%) within the *SI* gene region were identified after screening the dbSNP database. Among them, 880 variants were predicted to be pathogenic based on their Mendelian Clinically Applicable Pathogenicity (M-CAP) and Combined Annotation-Dependent Depletion (CADD) scores. QCed genotype data was available for 46 such variants in IBS patients. And 17 SI-RPVs could be tested based on the possibility of at least one IBS patients and available reference data from ancestry-matched The Exome Aggregation Consortium (ExAC).

We identified 88 risk allele carriers out of 2207 IBS patients for the 17 selected SI-RPVs. A higher frequency was observed for most SI-RPVs when comparing IBS patients with the ExAC reference panel. And the cumulative χ^2 test revealed that their overall prevalence in IBS patients (3.99%) is significantly higher than the controls (ancestry-matched ExAC reference population, P=0.00049, OR=1.45). Subtype stratified analyses in IBS patients showed consistent associations in IBS-C (prevalence 4.51%, P=0.0055, OR=1.65) and IBS-D (prevalence 4.20%, P=0.0045, OR=1.53).

This study represents a significant follow-up to our previous work on *SI* functional variants in IBS patients,(126) in which the prevalence of 2.1% for the four most common CSID mutations was reported. We have detected a higher prevalence of SI-RPVs in a large group of tertiary IBS patients compared to reference allele frequencies from the general population, which further supports the association of *SI* rare and dysfunctional mutations with IBS susceptibility.

The reference population from ExAC has been exploited as our source of controls, which constitutes one of the limitations in our study as these individuals were not screened for IBS symptoms. Given the high prevalence of IBS in the general population, there may be individuals with GI symptoms being included in the controls. However, a worse scenario, in this case, is a type II error, which would mean we underestimate the genetic risk of SI-RPVs in IBS patients.

Furthermore, we also evaluated the genetic risk effect of other SI-RPVs without a risk allele carrier in IBS patients. One million times' simulation has been run to randomly sample the reference population with the same size of IBS patients (N=2,207), and we have calculated the number of carriers for all 46 SI-RPVs in each simulation. **Figure 9** shows the simulation results including the distribution of numbers of carriers in controls. We have observed a significantly low P-value (P=0.005713) for obtaining the same carriers' number as in IBS patients (N=88) in control samples.

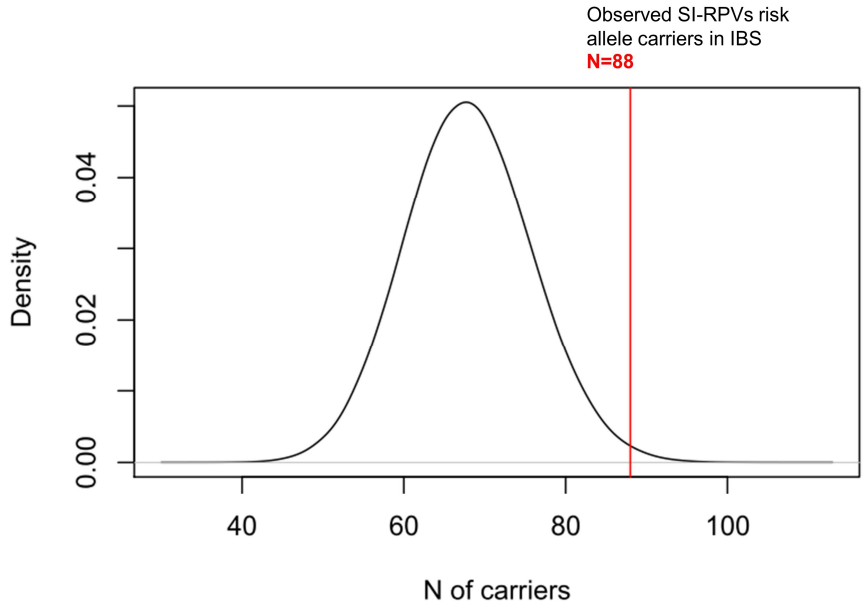

FIGURE 9. Distribution of the number of SI-RPVs carriers from one million simulations of sampling control population (N=2,207). The red line represents the number of SI-RPVs carriers in IBS patients (N=88). Reformulated from original work of Koldo Garcia Etxebarria.

To sum up, our study has provided supporting evidence that carrying any of SI-RPVs could affect IBS susceptibility and lead to 45% higher odds of getting IBS than an ethnically matched reference population.

4.2 *SI* GENOTYPE AFFECTS RESPONSE TO A LOW FODMAP DIET IN IBS PATIENTS

The pathogenic role of *SI* functional variants in IBS susceptibility has been documented in previous studies and **Paper I**.(126) Carrying *SI* risk allele(s) could affect the function of SI enzyme, leading to accumulation of undigested sucrose and starch hydrolysis products (disaccharides) in the gut lumen, and their osmotic effect and fermentation may result in bowel symptoms (e.g., diarrhea, pain). These findings may contribute to better stratification of IBS patients based on their *SI* genotype for improved therapeutic strategies (e.g., specific dietary intervention).

There may be potential nutrigenetic effects in terms of response to dietary intervention. Because a low FODMAP diet has been shown to exert beneficial effects in IBS patients, we elect to study *SI* genotype in relation to this therapeutic approach. Considering sucrose and

starch intakes are not restricted according to the standard low FODMAP diet, we hypothesize the individuals carrying effective *SI* variants would not benefit from a low FODMAP diet as much as non-carriers. To test our hypothesis, **in Paper II** we exploited the IBS-D cohort (N=85) which was included in a published clinical trial comparing the efficacy between low FODMAP and mNICE diets.(93)

A similar strategy has been adopted as described in **Paper I**, we identified three SI-RPVs and the common *SI* variant Val15Phe (rs9290264) whose genotype data was available for IBS-D patients previously being included in a low FODMAP dietary intervention trial.

IBS patients were treated with two different dietary interventions in the original study, low FODMAP diet (N=46) and mNICE diet (N=39). Their responses to dietary treatment were represented by overall symptom relief (≥50%) and pain response (≥30% reduction in abdominal pain score). We stratified the IBS patients according to their *SI* genotypes and performed an age/sex/BMI/Race-adjusted one-tailed logistic regression analysis to compare the endpoints between carriers and non-carriers.

In low FODMAP diet-treated individuals, *SI* hypomorphic variants carriers reported a significant lower symptom relief rate than non-carriers (P=0.0308 and OR=4.66, **Figure 10**). Although no significant result was obtained in pain response, we observed a similar trend in the comparison (47.8% in carriers vs. 52.2% in non-carriers). We have also compared both endpoints in mNICE diet group, although the analyses showed no significant difference, lower response rates were detected in *SI* hypomorphic carriers compared with non-carriers from all comparisons.

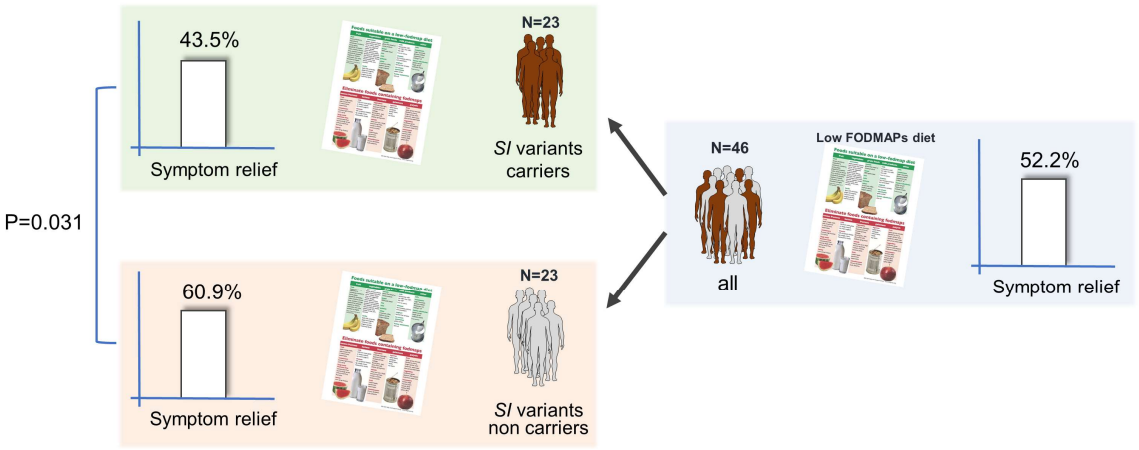

FIGURE 10. Symptom relief rates after treatment with low FODMAP diet in IBS-D patients stratified based on their genotypes of *SI* hypomorphic variants (carriers vs. non-carriers).

To further explore whether the number of *SI* hypomorphic variants is also relevant to the response to low FODMAP diet (as carrying more *SI* hypomorphic variants may lead to a higher reduction of SI enzymatic activity), we stratified the IBS-D patients according to the number of *SI* hypomorphic variants they carried. The efficacy of the diet in terms of symptom relief and pain response was evaluated in three subgroups of IBS-D patients: double *SI* carriers based on the genotype of *SI* hypomorphic variants, single carriers and non-carriers. We first performed the analysis combining all IBS-D patients from two diet treatments (low FODMAP and mNICE), as shown in **Figure 11**, the age/sex/BMI/Race-adjusted one-tailed logistic regression analysis revealed a significant decrease of positive response rates to symptom relief as the *SI* hypomorphic copy numbers increase (P=0.0039). We also observed a similar trend in pain response although with a non-significant P-value (P=0.092).

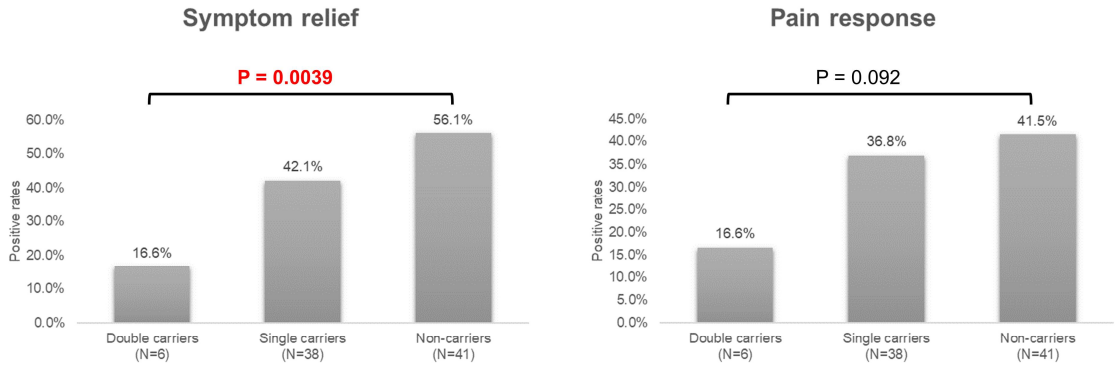

FIGURE 11. The associations between copy numbers of *SI* hypomorphic variants and response rate to endpoints (symptom relief and pain response) in all IBS-D patients combining two diet groups (low FODMAP and mNICE). Statistical analyses were performed by one-tailed logistic regression adjusting for gender, age, BMI and race groups.

This study represents a significant step forward in the analysis of *SI* gene's role in IBS management. The importance of carrying *SI* hypomorphic variants in relation to IBS risk have already been demonstrated in **Paper I**. Now **Paper II** also demonstrated that *SI* genotype can be informative when it comes to expected individual response to a low FODMAP diet. This opens up new lines of investigation, and it promises to provide opportunities for improving the efficacy and specificity of dietary interventions based on patients' genotype (personalizing therapy). However, this study has been performed in a small cohort and the reduced SI enzymatic activities have not been validated in IBS-D

patients by biopsies and experimental measurements, follow-up studies with large sample size are warranted.

In summary, we have shown that the efficacy of low FODMAP diet treatment was reduced in IBS patients carrying *SI* hypomorphic variants. Our findings suggest that screening for *SI* dysfunctional variants may be relative to inform patients' stratification and improve the efficacy of dietary intervention in IBS patients.

4.3 IBS RISK FACTORS IDENTIFIED VIA GWAS IN LARGE COHORTS

We have discussed genetic predisposition to IBS in **Section 1.3**. Powered genetic studies with adequate sample size are lacking, and no genome-wide significant signal has been identified prior to this thesis. In **Paper III** and **Paper IV**, we explored IBS GWAS to identify genuine IBS risk factors through the analyses of large population-based and case-control cohorts.

4.3.1 The female-specific IBS locus on chromosome 9q31.2

UK Biobank (UKB) is a large population-based cohort from the UK with genotype data and rich phenotype information (demographics and health-related data) available for around 500,000 individuals. In **Paper III**, we have exploited this resource for a GWAS comparing participants reporting a doctor's diagnosis of IBS with the remainder of the cohort.

After quality control (QC) per sample and per marker, association analysis was performed on 7,287,191 high-quality SNP markers from a total 9,576 IBS cases and 336,499 controls using logistic regression correcting for gender, age, genotyping array and top 10 PCs (principal components). We identified a genome-wide significant locus on chromosome 9q31.2 (tag SNP rs10512344, $P=3.57\times10^{-8}$) and 13 suggestive loci ($P<5.0\times10^{-6}$), harbouring a total of 93 genes based on physical and regulatory elements mapping (**Figure 12**).

Interestingly, the 9q31.2 locus was previously associated with age at menarche (AAM) in other GWASs.(152,153) Given the epidemiological evidence that IBS is more prevalent in females than males,(21) we further investigated the potential sex differences of the genetic effect of 9q31.2 association. Sex-stratified analysis showed a striking result, in that the 9q31.2 finding appeared to be female-specific (rs10512344, $P=4.29\times10^{-10}$) and no association was found in the male subset (rs10512344, P=0.79) (**Figure 12)**.

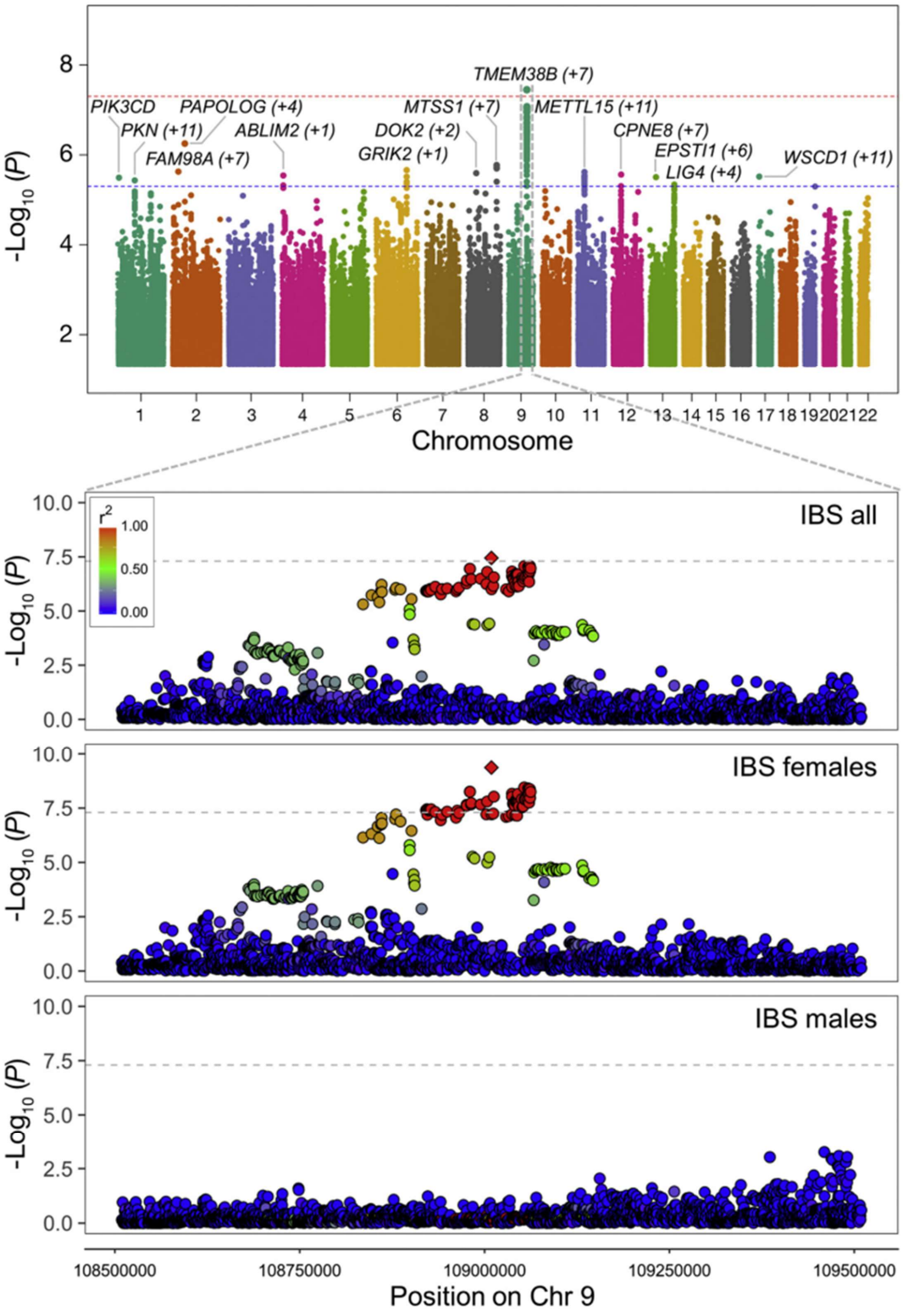

FIGURE 12. The Manhattan plot of IBS GWAS in UK Biobank and regional plots for the genome-wide significant locus 9q31.2 (all samples and sex-stratified). In the Manhattan plot, genome-wide significant ($P=5.0\times10^{-8}$) and suggestive ($P=5.0\times10^{-6}$) thresholds are shown by horizontal dashed lines (red and blue, respectively). Each suggestive locus ($P<5.0\times10^{-6}$) is highlighted and labelled by the closest gene mapped to the locus, and the number of additional mapped genes is shown in the following bracket. In the regional plots, the dash

lines represent the genome-wide significant threshold ($P=5.0\times10^{-8}$), and the color labels of each variant show their degrees of linkage disequilibrium (r^2) with tag SNP rs10512344. Reprinted with permission from **Paper III.**(154) Copyright © 2018, Elsevier.

The fact that 9q31.2 locus is associated with both AAM and female IBS raise the question of whether the associations are independent. A recent study in UKB demonstrated that early AAM is associated with IBS.(155) We applied three parallel approaches to address this question using full AAM summary statistics from a GWAS meta-analysis,(152) where rs10156597 is the most significant 9q31.2 marker associated with AAM ($P=4.29\times10^{-10}$, Beta=0.245). First, we performed a haplotype analysis combining rs10156597 and rs10512344 (tag SNP of IBS): the two markers show very low linkage disequilibrium (r2=0.04) and the haplotype was significantly associated with IBS only at the presence of risk allele "C" in rs10512344 (**Figure 13A**). Second, no reduction of the association signals was detected when conditioning on AAM (**Figure 13B**). Last, approximate Bayes factor colocalization analysis demonstrated that IBS and AAM were associated with 9q31.2 locus via different casual genetic risk factors (posterior probability H_3=99.98%).

To validate the female-specific findings in 9q31.2 locus, we further tested the association in independent follow-up cohorts, including a multi-national tertiary IBS case-control cohort (2045 cases and 7955 controls) and a Swedish Population-based colonoscopy study (Popcol, N=249). In line with the results in UKB, the significant association was replicated for tag SNP rs10512344 (P=0.015, Beta=0.383) in female IBS-C patients from case-control cohort. A consistent finding was also observed in females Popcol participants, as the risk allele C of rs10512344 was associated with harder stools. (P=0.0012, Beta=-1.105) There was no significant association in analyses of male samples from both follow-up cohorts.

A

rs10512344	rs10156597	OR	*P*	FREQ
G	A	1.00	-	0.682
G	T (AAM EA)	0.98	.250	0.277
C (IBS EA)	T (AAM EA)	1.24	4.55E-07	0.036
C (IBS EA)	A	1.45	2.43E-03	0.005

B

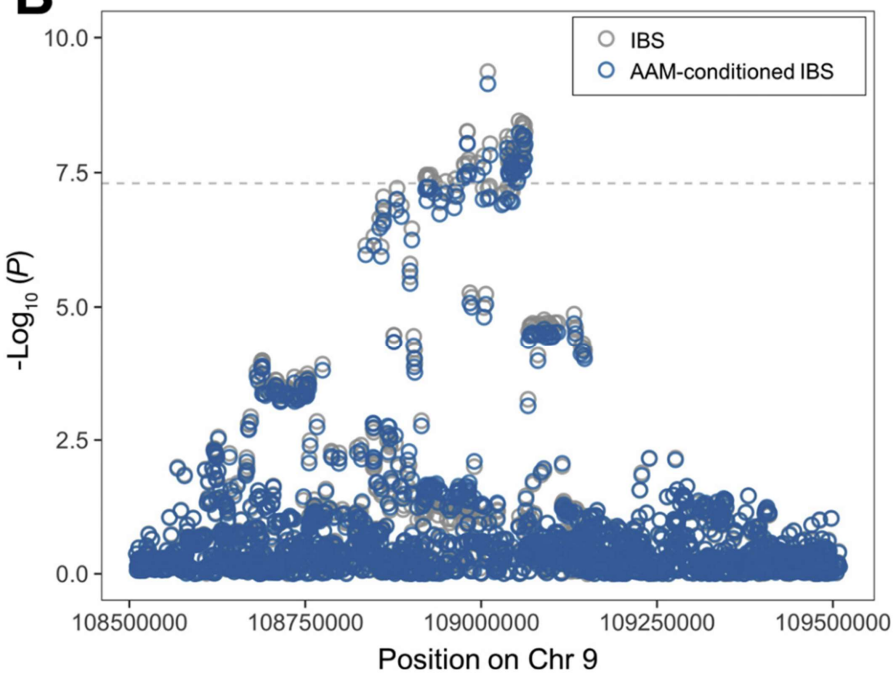

FIGURE 13. Haplotype association and conditional analysis suggest IBS genetic effects in 9q31.2 locus are independent of AAM. **A.** Summary statistics from haplotype association analysis between AAM and IBS tag SNPs. **B.** Regional plot of associations in 9q31.2 locus after conditioning on AAM summary statistics. The horizontal dash line represents the genome-wide significant threshold ($P=5.0\times10^{-8}$). Reprinted with permission from **Paper III.**(154) Copyright © 2018, Elsevier.

In this study, we performed a GWAS of IBS in UKB cohort including 9,576 cases and 336,499 controls, which is so far the only reasonable powered study to explore IBS genetics. We have identified the first genome-wide significant locus on chromosome 9q31.2, together with other 13 suggestive loci. The main strengths of this study include the large sample size, ideal replication materials (tertiary IBS case-control cohorts) and stringent QC pipeline on genotype data. While the IBS cases were defined by self-reported diagnosis in this study, the lack of direct clinical evidence constitutes the major limitation. Despite this, the application of self-reported traits enables us to gain a remarkable sample size for IBS genetic studies.

Our results have demonstrated that AAM and IBS associations are due to independent genetic effects, although both were located within 9q31.2 genomic region. Several traits have previously been linked to this locus including BMI, male' voice breaking and waist circumference. Interestingly, sex hormones are known to play a key role in pathophysiological mechanisms of almost all these traits. Moreover, sex is also associated with different IBS prevalence, predominant clinical signs, and responses to treatment.(156,157) Females have been shown to report slower transit time than males as well as more frequent constipation episodes,(158,159) which is consistent with our results in follow-up replication analyses whether the risk allele of rs10512344 was associated with IBS-C and harder stools. Sex hormones may be involved in the regulation of the brain-gut axis, affecting intestinal functions (motility, sensory, permeability, and immune activities) directly or through other hormones.(160,161) The GI symptoms in females have reported to vary according to the menstrual cycle.(160,162) Furthermore, exacerbated bowel symptoms have been observed in female IBS patients during their menstrual period compared with healthy controls.(163,164)

Eight genes were mapped to the 9q31.2 locus based on chromatin interaction data. Among them, we proposed *ELP1* (elongator complex protein 1, or *IKBKAP*) as the most likely causative gene within the locus. Autonomic dysfunction has been linked to IBS,(167) and mutations in the *ELP1* gene, notably, lead to familial dysautonomia, an autonomic nervous system condition affecting the neuron's development in sensory, sympathetic and parasympathetic nerves. Familial dysautonomia patients usually suffer from impaired pain sensitivity, altered intestinal motility, and temperature sensation.(165) Moreover, delayed AAM and premenstrual symptoms are often manifested in female familial dysautonomia patients.(166)

4.3.2 Two genome-wide significant loci associated with IBS-D

Well-characterized IBS patients diagnosed at specialized (neuro)gastroenterology clinics represent the best material to study IBS genetics and validate previous genetic findings linked to IBS. Individual cohorts from clinics are certainly underpowered for large-scale GWAS studies. Through multinational collaborations, our group has gathered IBS material from more than 20 tertiary centers from Europe and North America which enabled us to perform an unprecedented GWAS meta-analysis in tertiary IBS cases and controls (**Paper IV)**.

We have applied a robust GWAS pipeline to perform quality control and imputation on the original genotype data, resulting in 5,387,366 high-quality markers for 2,304 IBS cases and

14,614 controls from European pooled dataset, and 5,162,024 markers for 1,077 IBS cases and 10,502 controls from US dataset, respectively. The individual GWASs were carried out via a linear mixed model adjusting for sex, age and top 10 PCs, followed by the Z-score based meta-analysis.

The meta-analyses results revealed a total of 38 suggestive loci linked to IBS and its subtypes. Two genome-wide significant loci were identified in IBS-D meta-analysis, one on chromosome 9p21 (tag SNP rs10970019, $P=4.9\times10^{-8}$) and the other on 19q13.11 (tag SNP rs1260633, $P=4.1\times10^{-8}$). Of note, the most significant locus on chromosome 6q21 in IBS-C meta-analysis also showed an association close to genome-wide significance (tag SNP rs74742584, $P=5.69\times10^{-8}$). The Manhattan plots of GWAS meta-analyses are shown in **Figure 14**.

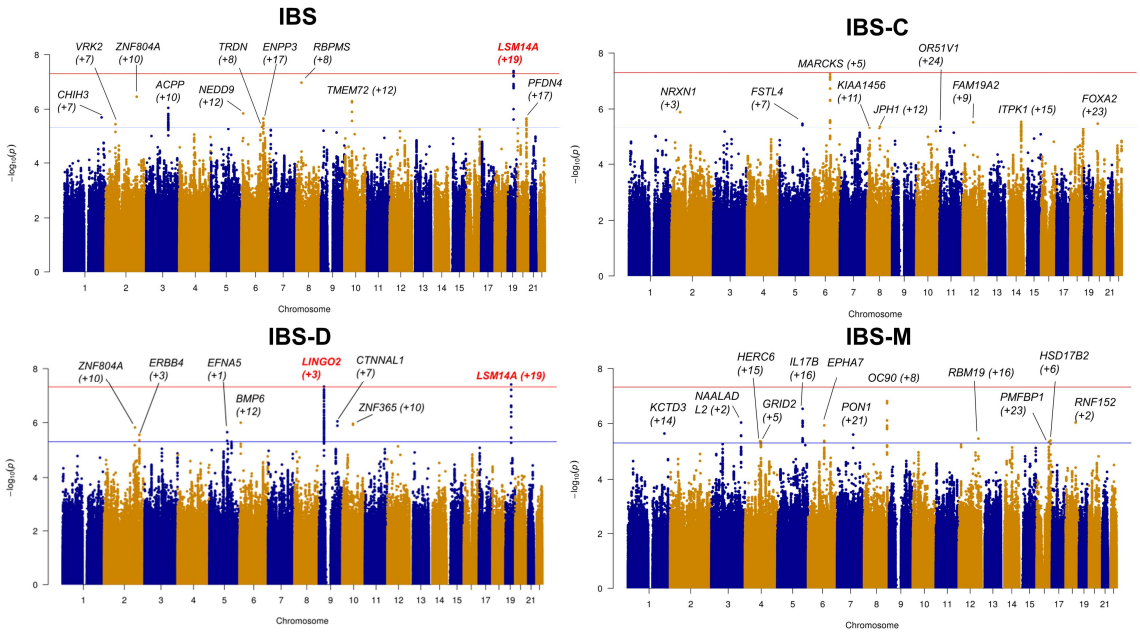

FIGURE 14. Manhattan plots summarizing GWAS meta-analyses results of IBS and each subtype. Genome-wide significant ($P=5.0\times10^{-8}$) and suggestive ($P=5.0\times10^{-6}$) thresholds are shown by horizontal lines (red and blue, respectively). Genome-wide significant loci are highlighted as red and bold. The closest mapping gene to the lead SNP is reported for each association signal, the number of additional mapped genes from the same locus is shown in brackets.

Functional annotation (via FUMA) of identified suggestive loci mapped 138 genes via physical locations and regulatory elements (eQTL and chromatin interaction) in meta-analysis of IBS-ALL (and 108 genes in IBS-C, 72 in IBS-D, 131 in IBS-M respectively).

Gene-sets enrichment analyses highlighted several pathways associated with IBS or their subgroups, including ion channel activities (IBS-C & IBS-M). A summary of significantly enriched pathways from each meta-analysis is presented in **Figure 15.** The most significant enriched pathways were from IBS-C meta-analysis, including sensory perception of chemical stimulus (adjusted $P=2.02\times10^{-14}$), olfactory receptor activity (adjusted $P=1.31\times10^{-11}$) and G protein coupled receptor activity (adjusted $P=2.82\times10^{-9}$).

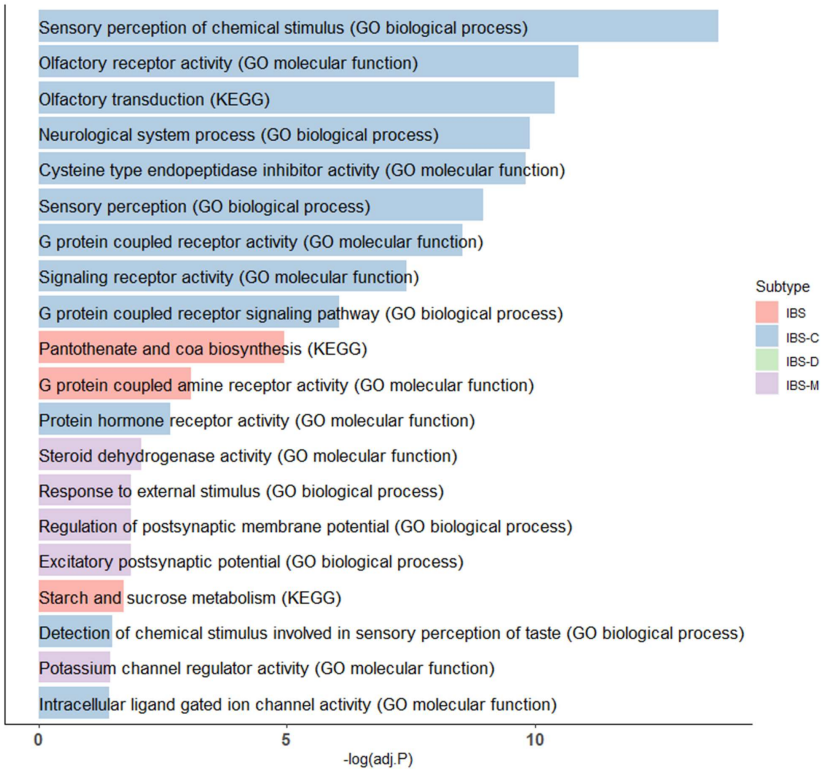

FIGURE 15. Significant findings in gene-sets enrichment analyses by mapping genes from each IBS meta-analyses (all samples and each subtype).

The genome-wide significant loci in IBS-D meta-analysis have not been previously described. *LSM14A* (MRNA Processing Body Assembly Factor) gene was physically mapped to locus 19q13.11. Other 19 genes were mapped to the same locus via their associations with eQTL or chromatin interaction. The tag SNP rs10970019 for another genome-wide significant locus 9p21 was located at an intergenic region. Thus no physical mapping gene was identified. *LINGO2, ACO1, NDUFB6, and GVQW1* genes were mapped via regulatory elements. The near genome-wide significant locus 6q21 associated with IBS-C contained six mapped genes (*MARCKS, TUBE1, WISP3, LAMA4, RFPL4B, and FAM229B*). In order to gain insight into the putative biology and align these associations, we searched the GWAS

Catalog and other repositories for evidence or associations of these genes with other conditions (PheWAS). The results are summarized in **Figure 16**, candidate genes who were mostly associated also to metabolic, immunological and psychiatric domains.

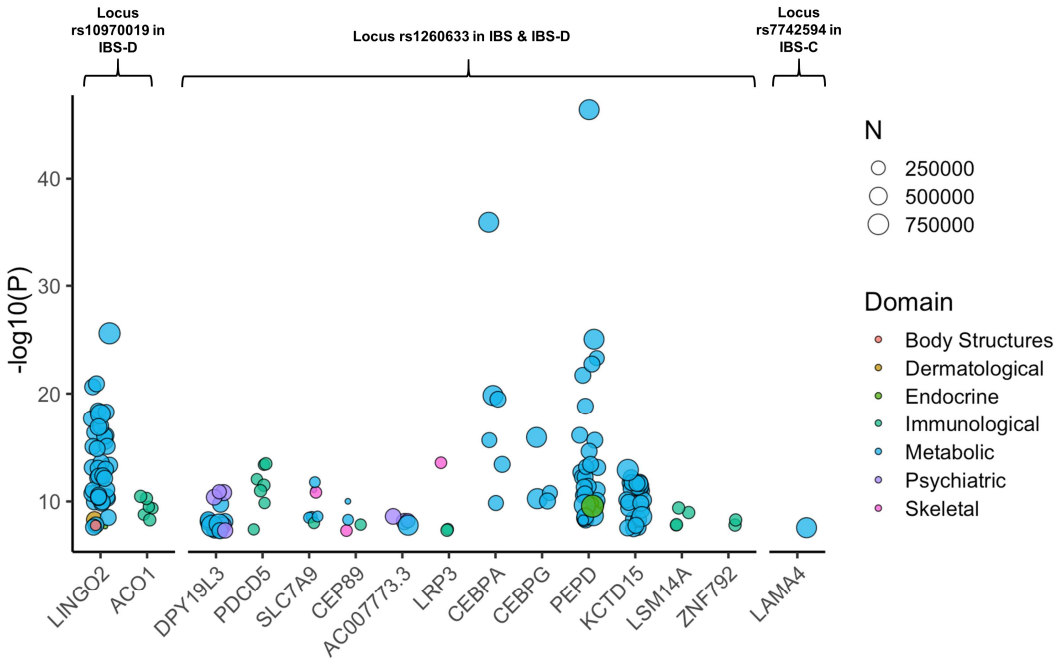

FIGURE 16. PheWAS associations of top significant loci and their mapped genes in IBS meta-analyses. Only the genome-wide significant associations ($P<5.0\times10^{-8}$) are shown. N represents the sample size of each publish GWAS.

As this is the largest case-control genetic study of IBS so far, we sought to assess previously reported associations in our GWAS meta-analyses. Out of the 15 tested genes, some evidence of replication was detected for 12 genes including *TNFSF15*, *NPSR1, SI* (**Paper I & II**), *SCN5A* and *KDELR2/GRID2IP* locus. The female-specific locus 9q31.2 being highlighted in **Paper III** was also investigated in this study via sex-stratified meta-analyses. We observed a similar trend for the tag SNP rs10512344, as a significant association was detected in female IBS-C meta-analysis (P=0.034), but not in analysis with male IBS-C patients (P=0.25). This finding is as expected given that the majority of tertiary IBS case-control samples have already been exploited as replication material in **Paper III**.

These GWAS meta-analyses represent the current largest efforts in the study of IBS susceptibility in well-characterized tertiary IBS patients, including a total 3,381 IBS cases and 25,116 controls. Different from our GWAS study in **Paper III** focusing on self-reported IBS (lack of direct clinical diagnoses), this study aims to capture IBS genetic risks exploiting

smaller numbers but well-characterized IBS phenotypes. A robust GWAS pipeline has been implemented on the multinational data sources to control for potential population stratifications including using the linear mixed model in the association tests. Among the limitations of the study, it is the fact that controls samples have not been recruited at the same sites as cases and IBS symptoms were not screened.

Two novel genome-wide significant loci have been identified to associate with IBS-D, the tag SNP rs1260633 of locus 19q13.11 is situated in gene *LSM14A*, which encodes molecules as a component of the mRNA processing body (P-body). *LSM14A* has been reported to be involved in antiviral responses and IFN pathways.(168) Of note, *LSM14A* is also associated with chronic inflammatory diseases (including Crohn's disease and Ulcerative colitis),(169) which suggests it may contribute to GI symptoms by disturbing antiviral immune activities in the gut. No physical mapping gene has been identified for the other locus 9p21. Interestingly, genes mapped to this locus based on chromatin-interactions have been reported to associate with immune activities (*NDUFB6, ACO1*) and neuroticism (*LINGO2*). This finding is noteworthy as neuroticism has been demonstrated to be psychiatric comorbidity of IBS and affect IBS risk.(170–172) PheWAS results including all genes mapped to genome-wide significant loci indicate that they may contribute to the generation of IBS symptoms via altered metabolic and immune activities, and/or psychiatric conditions.

This study also provides initial evidence of the genetic differences among IBS subtypes. The association patterns from meta-analyses results are entirely different between IBS-C and IBS-D, with no shared identified risk loci or mapped genes. The downstream functional annotation also suggests different biological pathways in the two IBS subtypes. Ion channel activities have been significantly enriched with IBS-C mapped genes. Instead, PheWAS results have linked disordered metabolic and immune activities to IBS-D. These findings may help elucidate pathophysiological mechanisms among IBS subtypes.

4.3.3 Ion channel activities as plausible pathways contributing to IBS risk

One of the most striking results to emerge is that ion channel pathways have been highlighted in gene-sets enrichment analyses from both **Paper III & IV**. Ion channels are membrane proteins presenting in all excitable cells that respond to signals and control ion flows across the cell membrane. These proteins are widely expressed across the gut, particularly in interstitial cells of Cajal (ICCs) and smooth muscle cells (SMCs), playing vital roles in controlling intestinal motility, sensation and fluid secretion.(141,173) Mutations of ion

channel genes may lead to malfunctions of these transmembrane molecules (channelopathies) and contribute to the IBS pathogenesis.(174)

The role of genetic variations in ion channel genes in IBS susceptibility is discussed in **Section 1.3.1,** in which genetic polymorphism in *SCN5A* and *TRPM8* have been demonstrated to associate with IBS risk. Remarkably, there is additional evidence supporting the role of ion channel activities in IBS pathophysiology including the results from the two previous GWAS and meta-analyses of Rome criteria defined IBS(149) and stool frequency(175), respectively. Our results further support these observations, highlight the link of ion channel activities to IBS pathophysiology, and pinpoint genes being involved in ion channel activities (*CLCA1*, *CLCA2*, *CLCA4*, *ANO3*, *TRPA1*, *CNGA4*, *KCNK2,* and *KCNMB2*) as important candidates warranting independent follow-ups.

5 CONCLUDING REMARKS

This thesis contributes to improving our understanding of genetic predisposition to IBS and adds new knowledge to IBS pathophysiology. In these genetic studies, we have evaluated and validated some predisposing genes and risk variants linked to IBS susceptibility, and identified some novel IBS risk factors warranting further investigation.

Back to the specific research questions in **Section 2.2**, this thesis attempts to provide preliminary answers:

Is IBS risk affected also by hypomorphic *SI* variants other than rare CSID mutations?

In **Paper I**, after screening the genotypes of SI-RPVs in 2207 tertiary IBS patients, we have observed a prevalence 3.99% of SI-RPVs in all IBS patients (and 4.51% in IBS-C, 4.20% in IBS-D respectively), which are significantly higher than the reference population (2.78 % in ExAC). The odds of getting IBS is 45% higher in SI-RPVs carriers than non-carriers.

Does *SI* genotype (hypomorphic variants carriership) affect the response to a low FODMAP diet in IBS patients?

Paper II has evaluated the response rates in a group of IBS-D patients treated with low FODMAP or mNICE diet after stratification based on genotype data for *SI* hypomorphic variants. Our results have demonstrated that carrying *SI* hypomorphic variants reduces by 3-4 folds the chances of benefiting from a low FODMAP diet.

Can true (genome-wide significant) IBS risk loci and genes be identified by applying large scale GWAS approaches in population-based and case-control cohorts?

We have exploited a population-based cohort in the UK in **Paper III** and multi-national tertiary IBS cases and controls in **Paper IV** to study IBS genetics.

In **Paper III**, we have identified a female-specific genome-wide significant association at chromosome 9q31.2. This finding has been consolidated by the replication evidence in tertiary IBS case-control cohorts and a Swedish population-based cohort (Popcol). Two more genome-wide association loci have been detected in **Paper IV** from the IBS-D meta-analysis combining European and US datasets. Of note, the ion channel activities have been highlighted in both studies from the gene-sets enrichment analyses, which is in line with a series of our previous findings that link ion channel biology to IBS. Follow-up studies in independent cohorts are needed to confirm these findings and clarify their biological mechanisms in IBS pathophysiology.

Additionally, **Paper III** and **Paper IV** have also addressed the following research questions:

Are there sex-specific genetic effects in IBS?

The sex-stratified analyses of the genome-wide significant locus 9q31.2 in **Paper III** revealed that the association was absent in males and entirely derived from the female group. This female-specific locus may be involved in IBS pathogenesis via the action of sex hormones, which could partially account for the different IBS prevalence and clinical manifestations between female and male groups.

Are there different genetic characteristics of IBS subtypes?

In **Paper IV**, we have provided preliminary evidence for the genetic comparisons between IBS-C and IBS-D. No common suggestive locus or mapped gene was identified from the tertiary IBS GWAS meta-analyses. Moreover, functional annotation of their GWAS results has highlighted different biological pathways, suggesting different genetic architecture and underlying molecular mechanisms in the two IBS subgroups.

Are previously reported IBS associations confirmed in larger scale GWAS studies?

We have inspected the association signals for previously reported IBS risk genes and loci in **Paper IV**. 12 out of 15 tested genes have been validated in the GWAS meta-analyses exploiting tertiary IBS cases and controls including *TNFSF15*, *NPSR1, SI*, *SCN5A,* and *KDELR2/GRID2IP* locus. The supporting evidence may help prioritize candidate genes for investigating their causative role in IBS pathophysiology.

To sum up, this thesis has validated the role of *SI* functional variants in IBS susceptibility and identified new genetic factors predisposing to IBS. These results may contribute to the identification of pathophysiological mechanisms that can help explain the etiology of IBS and may ultimately provide novel therapeutic targets.

The current definition of IBS may correspond to a constellation of various conditions. Even within the same IBS phenotype, the underlying molecular mechanism can vary significantly from patient to patient. Our genetic studies may contribute to a better classification system for IBS patients in two ways. On the one hand, for a small subset of IBS patients, their GI symptoms may be accounted for by the dysfunctional variations in single genes, such as channelopathies (*SCN5A*, *TRPM8*) and carbohydrate malabsorption (*SI*). These “organic” conditions can be treated with specific interventions including ion channel blockers for channelopathies or dietary intervention for carbohydrate malabsorption (one successful

example of channelopathies treatment has already been discussed in **Section 1.2.5**). On the other hand, the majority of IBS phenotypes result from the interactions between environmental factors and the polygenetic background. Large-scale genetic studies are powerful tools to capture their polygenetic risk factors, contributing to a better stratification of IBS patients based on their polygenic risk scores (PRS) and pathways from biological annotations. **Figure 17** shows the application of two genetic approaches in re-classification and designing personal therapeutic strategies for IBS patients.

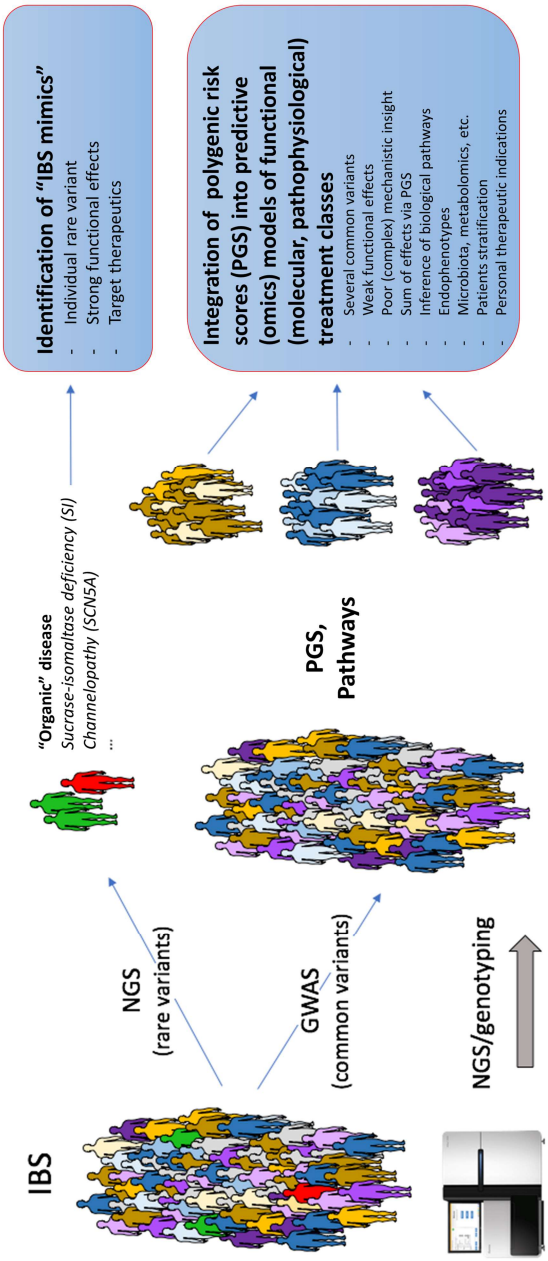

FIGURE 17. Potential interpretation of genetic information (obtained in this thesis and elsewhere) for patients stratification and precision medicine in IBS. Original work designed by Mauro D'Amato, reformulated and print with permission.

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