Universiteit Antwerpen

Faculty of Medicine

Pathophysiological mechanisms in irritable bowel syndrome: the search for novel cellular and volatile biomarkers

Pathofysiologische mechanismen in het prikkelbare darmsyndroom: de zoektocht naar nieuwe cellulaire en volatiele biomerkers

Proefschrift voorgelegd tot het behalen van de graad van doctor in de medische wetenschappen aan de Universiteit Antwerpen te verdedigen door:

Kathleen Van Malderen

Promotoren:
Prof. Dr. Heiko De Schepper
Prof. Dr. Vito Sabato
Prof. Dr. Benedicte De Winter

Antwerpen, 2023

The research described in this thesis was performed with the help of Research Foundation Flanders, PhD fellowship fundamental research (1144819N), and the University of Antwerp, BOF-TOP grant (35018).

Table of contents

Abbreviations

ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AMP	Antimicrobial peptides
ATP	Adenosine triphosphate
AUC	Area under the curve
AVP	Arginine vasopressin
BAM	Bile acid malabsorption
BSA	Bovine serum albumin
-C	Constipation
CBT	Cognitive behaviour therapy
CCL	Chemokine ligand
CD	Crohn's disease
CRF	Corticotropin releasing factor
CRH	Corticotropin releasing hormone
-D	Diarrhoea
DGBI	Disorders of the gut brain interaction
EAL	Early adverse life event
EMDR	Eye movement desensitisation and reprocessing
E-nose	Electronic nose
EPS	Epigastric pain syndrome
FAIMS	Field asymmetric ion mobility spectrometry
FcεRI	High affinity IgE receptor
FD	Functional dyspepsia
FFQ	Food frequency questionnaire
FGF	Fibroblast growth factor
FGID	Functional gastrointestinal disorder
FMO	Fluorescence minus one
FODMAP	Fermentable oligo-, di-, monosaccharides, and polyols
GA	Gentamicin amphotericin B
GBA	Gut brain axis
GC-MS	Gas chromatography – mass spectrometry
GI	Gastrointestinal
GP	General practitioner
HADS	Hospital anxiety and depression score
HAPC	High amplitude propagating contractions
HC	Healthy control
HPA	Hypothalamic–pituitary–adrenal
IAP	Intestinal alkaline phosphatase
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IMS	Ion mobility spectrometry
Lasso	Logistic least absolute shrinkage and selection operator
LOOCV	Leave-one-out cross-validation
LPS	lipopolysaccharides
-M	Mixed
MC	Mast cell
MD	Medical doctor
MIP	Macrophage inflammation protein
MRGPR	Mas-related gene receptor
MRI	Magnetic resonance imaging
NICE	National institute of clinical excellence
NCGS	Non-coeliac gluten sensitivity
NGF	Nerve growth factor
PB	Breath volatile
PCA	Principal component analysis
PDS	Postprandial distress syndrome
PF	Faecal volatile
PI	Post-infectious
PVN	Paraventricular nucleus
QOL	Quality of life
RBPA	Red blood loss per anum
RIP	Reactant ion peak
ROC	Receiver operating characteristic
SCF	Stem cell factor
SCFA	Short chain fatty acid
SD	Standard deviation
SF	Short form
SIBO	Small intestinal bacterial overgrowth
SIFT-MS	Selective ion flow tube - mass spectrometry
SNRI	Selective serotonin noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
SSS	Symptom severity index
TCA	Tricyclic antidepressant
TNF	Tumour necrosis factor
-U	Unspecified
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
VOC	Volatile organic compound
VSI	Visceral sensitivity index
5-HT	Serotonin

Chapter 1 Introduction

Partly based on:

Kindt S, Louis H, De Schepper H, Arts J, Caenepeel P, De Looze D, Gerkens A, Holvoet T, Latour P, Mahler T, Mokaddem F, Nullens S, Piessevaux H, Poortmans P, Rasschaert G, Surmont M, Vafa H, **Van Malderen K**, Vanuytsel T, Wuestenberghs F, Tack J. Belgian consensus on irritable bowel syndrome. *Acta Gastro-Enterologica Belgica* (2022) Volume 85:360-82

Van Malderen K, De Winter BY, De Man JG, De Schepper HU, Lamote K. Volatomics in inflammatory bowel disease and irritable bowel syndrome. *EBioMedicine* (2020) Volume 54:102725

1.1 Irritable bowel syndrome

1.1.1 Disorders of the gut brain interaction

Throughout history humans have been fascinated by the gut and intestinal physiology. Proper gut functioning is thought to be required for general well-being and a disturbance of its normal function is often linked to embarrassment and shame. Bowel behaviour is closely related to stress and emotions which becomes apparent when looking at old sayings: 'finding it hard to swallow', 'butterflies in my stomach', 'having a gut feeling'. The thought, smell, touch, or sight of intestinal content and faeces can provoke intense emotional responses, nausea, or even vomiting. All to illustrate that more than any other organ system in the human body, gut function is closely related to the brain.[1]

A disturbance of this interaction between the brain and the gut can give rise to disorders of the gut-brain interaction (DGBI), formerly known as functional gastrointestinal disorders (FGID). A functional disorder is a medical condition that impairs normal functioning in the absence of structural abnormalities which can explain the symptoms.[2] This contrasts with somatic disorders (in which structural abnormalities cause the symptoms) or psychosomatic disorders (in which symptoms are caused by psychological illnesses). DGBIs can affect every part of the gastrointestinal tract from the oesophagus to the anorectum. Two of the most well-known DGBIs are functional dyspepsia (FD) and irritable bowel syndrome (IBS).

Functional dyspepsia causes gastroduodenal symptoms and can be divided into two subtypes based on the dominant symptom pattern. The epigastric pain syndrome (EPS) is

characterised by epigastric pain or burning. The Postprandial distress syndrome (PDS) is characterised by postprandial fullness or early satiation.[3]

Irritable bowel syndrome is characterised by abdominal pain and an altered bowel habit. However, patients often describe other symptoms like bloating, abdominal distention, mucus in the stools, urgency, flatulence, and comorbid dyspepsia.[4] The following introduction will focus on IBS since this is the subject of this thesis.

1.1.2 Epidemiology

IBS is a highly prevalent disorder affecting 4% - 11% of the population worldwide.[5–7] It is prevalent across all ages and is most frequently diagnosed in the young adult.[8,9]

The prevalence of IBS globally is 67% higher in women compared to men. However, the female-to-male ratio varies greatly across the world from 3:1 in Western populations to 1:1 in Nigeria.[10] Women typically have more severe symptoms, a lower quality of life, more non-pain associated symptoms like bloating and constipation, and more extraintestinal symptoms. Many women report a link between symptoms and the menstrual cycle with increased intensity around the menses (which can make it difficult to differentiate from endometriosis). Abdominal pain scores are similar in men and women; however, men more frequently report diarrhoea while women more frequently report constipation.[10]

Sex hormones play an important role in these differences. They are linked to stress responses in the central and autonomic nervous system and can influence intestinal function.[11]

Fluctuations in sex hormone levels can also explain the variability of symptom severity throughout the menstrual cycle.

Another contributing factor to the differences between women and men can be found in stress and early adverse life events (EALs) which are known to be associated with the development of IBS later in life. Women are more likely to experience these EALs.[11] Furthermore, research has shown that the female and male brain react differently to EALs with changes not only in the structure and function of core regions of the salience network, but also in their anatomical network centrality.[12] These differences between women and men demonstrate the importance of taking a patient's sex into account not only in research but also in clinical practice, since it can influence both the clinical presentation and the response to treatment.

1.1.3 Rome criteria

IBS is defined by the Rome criteria which evaluate the presence of key symptoms combined with a temporal component. The Rome criteria are frequently updated based on current knowledge (table 1.1) and currently the Rome IV criteria are used.[13]

Table 1.1: Evolution of the Rome criteria[5]

	Rome III	Rome IV
In use since	2006	2016
Abdominal pain or discomfort	Abdominal discomfort (≥ 3 days per month)	Abdominal pain (≥ 1 day per week)
Altered bowel habit (≥ 2 criteria positive)	Improvement after defaecation	Relation to defaecation
	Change in frequency	Change in frequency
	Change in consistency	Change in consistency
Temporal component	Started ≥ 6 months ago	Started ≥ 6 months ago
Percentage fulfilling the criteria	10%	4%

The Rome IV criteria are more stringent compared to the Rome III criteria causing a decrease in the amount of people fulfilling the Rome criteria.

To fulfil the Rome IV criteria a patient must have abdominal pain, at least one day a week, related to the defaecation, this can be both an increase or decrease in pain.[5,6] Furthermore, patients also need to have a change in stool consistency and/or frequency. Stool consistency can be assessed with the help of stool form scales like the Bristol stool scale (figure 1.1).[14] Constipation, diarrhoea, or a combination of both can occur. Based on the dominant stool pattern patients are divided into subtypes: diarrhoea (IBS-D), constipation (IBS-C), and mixed (IBS-M). When the dominant stool pattern does not fall into one of these categories, patients are classified as unspecified (IBS-U).[5]

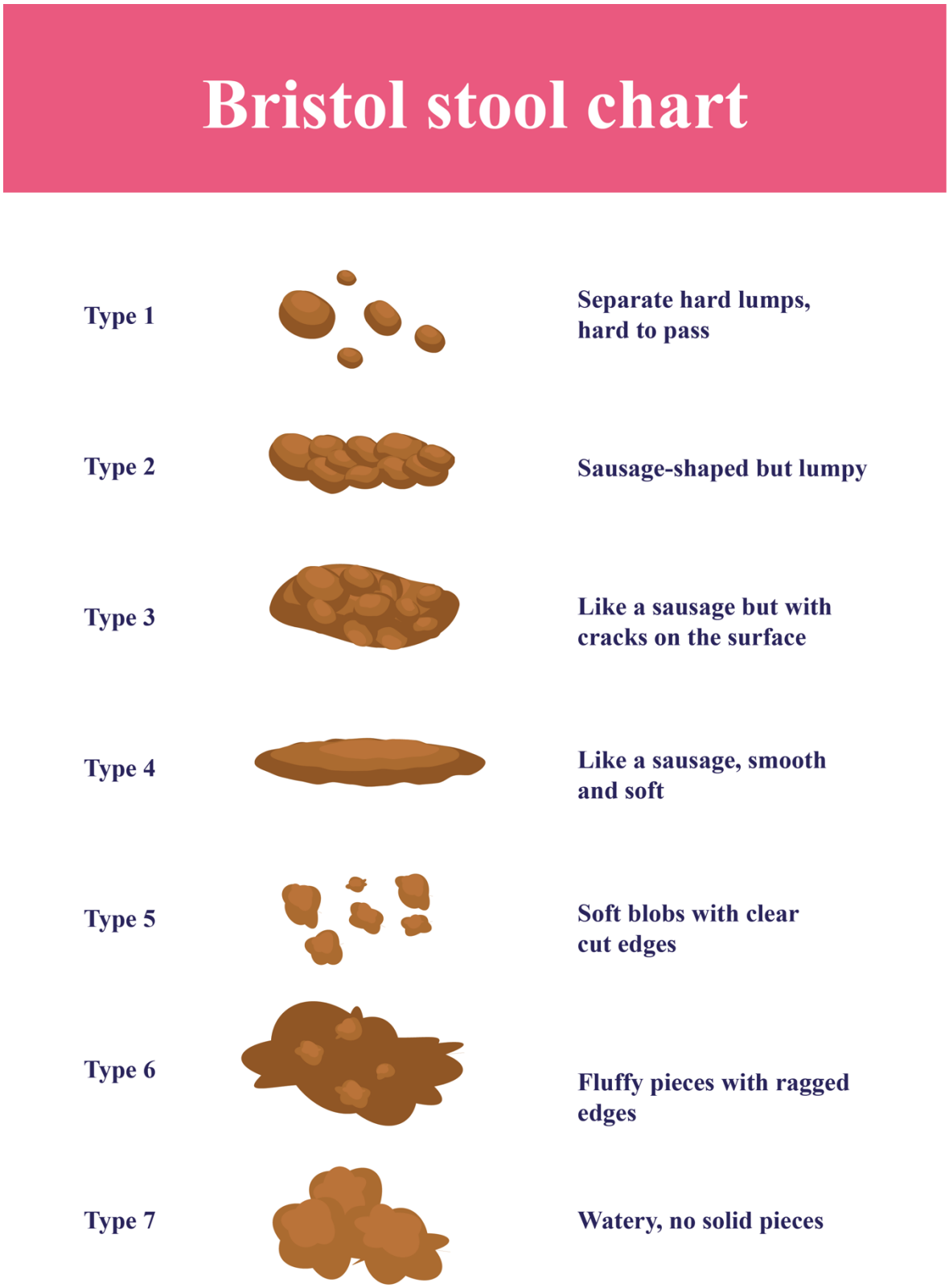


Figure 1.1: Bristol stool chart. Figure created by Bjorn Lauwerijs for this PhD and used with consent

1.1.4 Impact on daily life

The impact of IBS on daily functioning and quality of life (QOL) is often underestimated. Gralnek *et al.* assessed QOL with the SF-36 which contains eight subscales (physical functioning; role limitations physical; bodily pain; general health; emotional well-being; role limitations emotional; energy/fatigue; and social functioning), each scoring between 0 and 100 with a higher score indicating a better QOL. They found that IBS patients had a considerably poorer QOL compared to the general population but also compared to people suffering from other diseases like gastroesophageal reflux (except for the subscale physical functioning), diabetes (except for the subscales general health and physical functioning), or even end-stage renal disease (for the subscales physical functioning, role limitations physical, and general health).[15–18] QOL is not influenced by a patients subtype but it is affected by symptom severity.[15] A recent extensive systematic review from Shorey *et al.* looked into the influence of IBS on daily living.[8] They identified four themes: physical, psychological, and social consequences; impact on working life; dealing with the disease; sources of support and support needs. IBS is an unpredictable disorder and the combination of symptoms and the influence on daily life can cause additional stress. Considering that stress can initiate and exacerbate symptoms, patients can easily fall into a vicious cycle. For this reason, stress management, self-care, and emotional well-being should be prioritised in the management of IBS.

1.2 Pathophysiological mechanisms in irritable bowel syndrome

The exact aetiology of IBS is still largely unknown, but patients often report an infectious, traumatic, or stressful event preceding the onset of symptoms. The underlying pathophysiology is multifactorial, and involves increased intestinal permeability, dysmotility, intestinal dysbiosis, food hypersensitivity, visceral hypersensitivity, brain-gut axis dysregulation, inflammation, genetics, and psychological stress (figure 1.2).[19–21b]

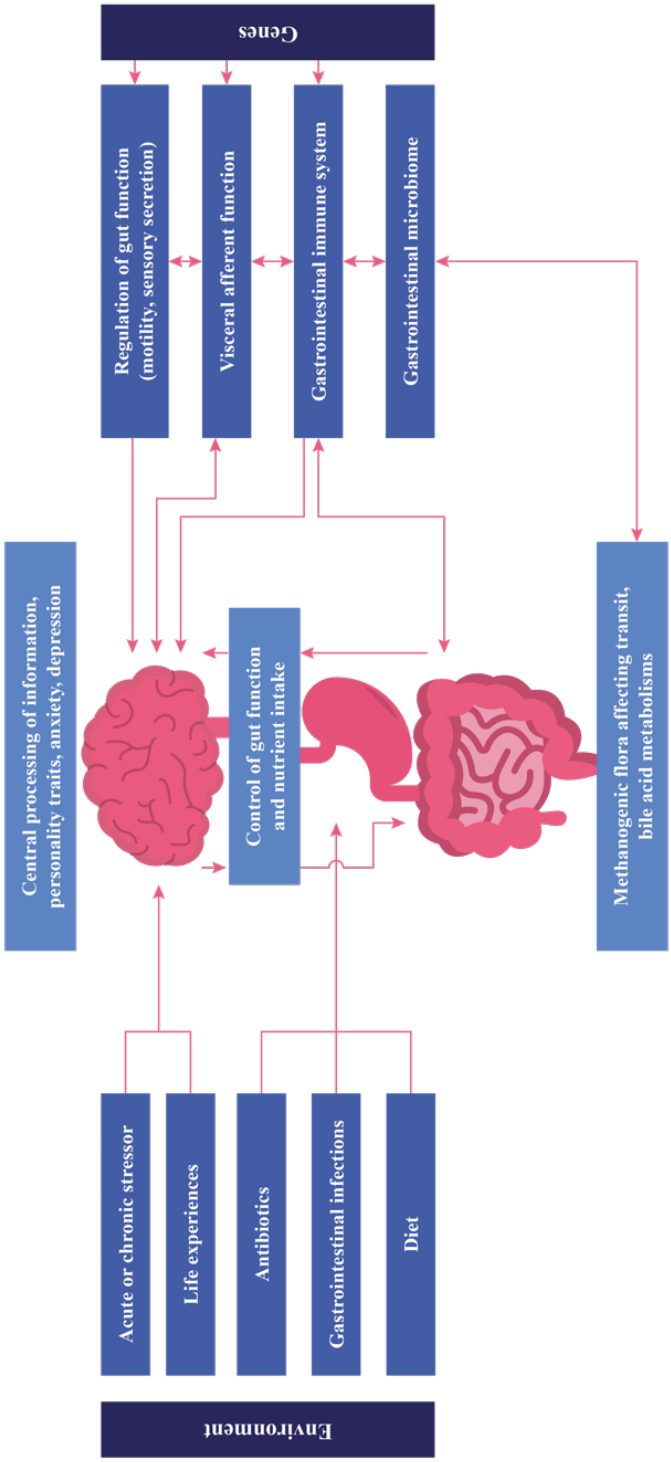


Figure 1.2: Pathophysiology of irritable bowel syndrome; Adapted from Holtmann et al., 2016[22]

1.2.1 Disturbed motility

Early on IBS was called ‘spastic colon’ because it was believed that symptoms were caused by an aberrant motility. At present it is known that changes in motility are only a piece of the puzzle. Researchers have spent a lot of time trying to find a specific abnormality in the myoelectrical and motor pattern in the intestine of IBS patients. Studies have shown an increased frequency of high amplitude propagating contractions (HAPC) in the colon of non-constipated IBS patients.[23,24] These HAPC are also associated with pain episodes.[23–25] IBS patients have a longer and more pronounced postprandial myoelectrical and motor response compared to healthy controls, and their gut seems to respond stronger to normal physiological stimuli like food but also stress in general.[23,24] Transit times play an important role in stool consistency and patient subtype.[24,26]

Apart from a disturbed colorectal motility there is evidence of abnormal small bowel and gastroesophageal motility. However, since there are large inter- and intraindividual variations it has been proven difficult to detect consistent and characteristic changes. When looking at the small bowel motility, the time between migrating motor complexes is shorter in IBS-D and longer in IBS-C, and there is an increased frequency of clustered activity.[24,27,28] A subset of patients with IBS has a lower pressure in the lower oesophageal sphincter or delayed gastric emptying. However, these gastric and small intestinal changes might be related more to comorbid upper-GI symptoms/disorders than to IBS itself.[24]

1.2.2 Increased intestinal permeability

The intestinal barrier serves as a defence layer against pathogens and antigens travelling through the gut. At the same time, it helps maintain homeostasis via the uptake of nutrients and water. To be able to execute all these tasks the intestinal barrier evolved into a complex system including immune, physical, and biochemical components.[29]

1.2.2.1 *Normal intestinal barrier*

The intestinal barrier consists of four main layers[29,30]: GI secretions and microbiota products at the luminal site, the mucus layer, the epithelial layer, and immune cells with their mediators.

1.2.2.1.1 Gastrointestinal secretions and products produced by the gut microbiota

Numerous gastrointestinal secretions (e.g. bile salts, defensins and other antimicrobial peptides (AMP), intestinal alkaline phosphatase) and microbial metabolites (e.g. bacteriocin) circulate in the intestinal lumen preventing colonisation with pathogens.

1.2.2.1.2 Mucus layer

The mucus layer is the first physical barrier and consists of an inner layer which is firmly attached to the epithelial cells, and an outer layer which is thicker but also looser and less firmly attached. The inner layer protects the epithelial cells from direct contact with bacteria by not allowing them to penetrate. The outer layer houses commensal bacteria preventing pathogens to penetrate into the underlying layers. These layers consist mainly out of water and glycoproteins (mucins) and to a lesser degree out of electrolytes, antibodies, and nucleic acids.[29,30]

1.2.2.1.3 Epithelial layer

The epithelium is a single layer of cells acting as highly selective barrier. On the one hand, it allows translocation of water, electrolytes, and nutrients into the systemic circulation. On the other hand, it prevents passage of foreign antigens, microorganisms, and toxins. Transport through the barrier is mediated by trans- or paracellular transport.[29,30]

The integrity of epithelial cells is amongst others secured by the tight junctions forming an important layer that can be tightly regulated.

1.2.2.1.4 Immune cells and their secretions

Closest to the gut lumen we can find Paneth cells which are specialised secretory cells in the epithelium secreting antimicrobial peptides. The most abundant antimicrobial peptide in the gut is α-defensin which is active against both gram-positive and gram-negative bacteria. Immune cells like for example dendritic cells, macrophages, plasma cells, T cells, B cells, and mast cells in the mucosa and lamina propria form a protective layer through release of immunoglobulins, cytokines, and other immunomodulators.[29,30]

1.2.2.2 *Barrier dysfunction in IBS*

Disruption of the intestinal barrier causes increased permeability which in turn can cause local or systemic inflammation. In IBS, disruption of the barrier is thought to be linked to visceral hypersensitivity through exposure of the submucosal neuronal and immune system to luminal pathogens, antigens, and other mediators.[29] A systematic review by Hanning *et al.* stated that increased permeability is present in 37% - 62% of patients with IBS-D and 16% - 50% of patients with post-infectious symptoms. The prevalence of barrier disruption

in IBS-C and IBS-M patients has not been extensively studied.[29] Furthermore, the underlying mechanisms to barrier dysfunction are not fully understood. Mast cell activation, changes in the microbiota, diet, and mediators like vasoactive intestinal polypeptide, serotonin, serine proteases, and cysteine proteases are believed to play a role.[29]

1.2.3 Food hypersensitivity

Over 60% of patients report a clear link between symptoms and food intake, and dietary changes are often used as a therapeutic option.[31] Adverse food reactions can be non-immunologically mediated or immunologically mediated. The former includes direct effects of pharmacologically active components (e.g. caffeine, tyramine, FODMAPs) and enzyme deficiencies (e.g. lactose or fructose intolerance). 20 - 65% of IBS patients attribute their symptoms to immunological reactions or food allergies, however, these reactions are uncommon with approximately 5% of the general population having a food allergy.[32] While IgE-mediated symptoms might play a role in a subgroup of patients, they are unlikely to explain symptoms in the majority of patients.

A group of foods frequently associated with IBS are the fermentable oligo-, di-, monosaccharides, and polyols (FODMAPs). FODMAPs are poorly absorbed in the small intestine causing an osmotic effect with subsequent increased water content in the lumen. When these FODMAPs arrive in the colon they are fermented by the microbiota causing gas production. IBS patients will experience symptoms earlier than healthy people because of the gut hypersensitivity.[31] An exclusion diet low in FODMAPs is currently widely used in the treatment of IBS.[33]

Despite having negative serology for coeliac disease some patients report an alleviation of symptoms when avoiding gluten in the diet. There are multiple possible explanations for this. First, it is possible that the patient reacts to the fructan in wheat products, which is a FODMAP, and not the gluten. When patients have a fructan hypersensitivity they often also experience symptoms upon ingestion of rye and barley.[33] A second mechanism is non-coeliac gluten sensitivity (NCGS), a condition in which patients experience symptoms after gluten ingestion despite negative coeliac serology and exclusion of wheat allergy. In this case, symptoms will disappear completely under a strict gluten-free diet. The pathophysiology of NCGS is still largely undetermined but it has been suggested that these patients react to other proteins than gluten.[34]

Other food hypersensitivities and diets like a low-histamine diet or IgG based avoidance currently do not have sufficient evidence to be used in clinical practice.[33]

1.2.4 Inflammation

IBS is increasingly viewed as a chronic low-grade inflammatory disorder. Some studies have shown an increase in pro-inflammatory cytokines, mainly in IBS-D patients, or a decrease in anti-inflammatory cytokines.[21,35] Studies looking at inflammatory cells like mast cells (MCs), eosinophils, and lymphocytes have conflicting results with some of them showing increased numbers and activation of these cells while others report no change.[22] It is, however, unclear if all these changes reflect genuine pathophysiological mechanisms, if they are a consequence of IBS, or if they are random associations.[22] Furthermore, research has not been able to demonstrate a significant association between cytokine levels or cell counts and symptoms.[21]

A potential cause of the low-grade inflammation is a previous gastrointestinal infection leading to post-infectious IBS (PI-IBS). Approximately 10% of patients experiencing a gastrointestinal infection will develop PI-IBS.[36] However, this incidence can vary depending on the severity of infection demonstrating that the degree of gut inflammation has an influence on the risk of developing IBS (protozoal/parasitic infection > bacterial infection > viral infection).[37] Other risk factors are female sex, high somatisation, antibiotics during infection, and psychological comorbidities.[36,37] Research on the prognosis of PI-IBS has shown contradicting results with some stating PI-IBS has a better prognosis while others do not see any differences when comparing these patients to non-PI-IBS. In general, patients with PI-IBS are more likely to have a diarrhoea predominant subtype with increased stool frequency.[36,38]

Apart from the lasting low-grade inflammation, a gastrointestinal infection can also increase permeability, change serotonin metabolism, and alter the microbiota, which may also contribute to the development of IBS.[36] A recent study by Aguilera-Lizarraga *et al.* in mice demonstrated that a bacterial infection and bacterial toxins can trigger an immune response which leads to the production of dietary-antigen specific IgE-antibodies limited to the gut. Afterwards, when these specific dietary antigens are ingested, mast cells are activated in an IgE-dependent mechanism and elicit visceral pain through sensitised histamine-1 receptors on sensory nerve endings.

1.2.5 Intestinal dysbiosis

Another pathway receiving increasing attention is the gut microbiota. The gut microbiota evolves continuously and forms an intricate and mutually beneficial relationship with its

host. It consists of bacteria, archaea, and eukarya and has an influence on gut physiology, absorption of nutrients, and development of the immune system.[39] There is a large variability of the microbiota between individuals and a normal, healthy microbiota is characterised by high bacterial diversity and stability over time.[40] Several gastrointestinal disorders like inflammatory bowel disease, colorectal cancer, and coeliac disease are associated with dysbiosis (a disturbed microbiota), with reduced diversity and stability.[40–43]

Various studies showed an aberrant microbiota in IBS, but a unique pattern associated with IBS has not been identified. Recurrent observations in studies are an increase of relative abundance of the *Firmicutes*, mainly *Clostridium* and *Ruminococcaceae*, a decrease in *Bacteroides* (except IBS-D), and a depletion of *Bifidobacteria*.[40,44,45]

The microbiota are strongly influenced by exogenous factors, in particular the diet. A diet high in proteins and animal fat is associated with an enterotype rich in *Bacteroides*. A diet with a high carbohydrate content is associated with an enterotype rich in *Prevotella*.[40] Short term changes in the diet only have a limited effect on the microbiota with no changes in a patients enterotype.[46,47] However, adaptations of the diet for more than a few days can already cause detectable changes in the microbiota.[40] The microbiota vice versa has an important role in the digestion of food with the production of metabolites. These metabolites can influence IBS symptoms both directly and indirectly (table 1.2). Therefore, the potential therapeutic effect of diet changes is heavily influenced by the microbiota of the patient.[40]

Table 1.2: Influence of microbiota on food digestion[40,48,49]

Gut microbial activity	Produced metabolites	Influence on IBS symptoms
Degradation of undigested proteins and carbohydrates	Sugars	Direct: Bloating, flatulence, increased osmotic load
	Oligosaccharides	Direct: Bloating, flatulence, increased osmotic load
	Peptides amino acids	Indirect: Improvement of barrier function
Amino acid and monosaccharide fermentation	Short chain fatty acids (SCFAs)	Direct: Increased osmotic load Indirect: Improvement of barrier function, improvement of immune function
	Lactate	Indirect: Afferent nerve activation
	Succinate	Indirect: Afferent nerve activation
	Ethanol	Indirect: Disturbance of barrier function, cell damage
	H_2	Direct: Bloating and flatulence
	CO_2	Direct: Bloating and flatulence
	Amines	Indirect: Cell damage
	NH_3	Indirect: Cell damage
	Phenols	Indirect: Cell damage
	Indoles	Indirect: Cell damage
	Thiols	Indirect: Cell damage
Hydrogen disposal	CH_4	Direct: Slower transit Indirect: Decreased serotonin levels
	H_2S	Indirect: Disturbance of barrier function, disturbance of immune function, cell damage
	Acetate	Indirect: Afferent nerve activation
Bile acid transformation	Deconjugated and secondary bile acids	Direct: Accelerated transit Indirect: Disturbance of barrier function, disturbance of immune function, cell damage

1.2.6 Brain-gut axis dysregulation and psychological stress

The gut-brain axis or GBA is a bidirectional pathway of communication between the central and enteric nervous system, linking emotional and cognitive centres in the brain with peripheral intestinal functions.[50] Its role is to monitor and regulate gut functions like immune activation, enteric reflexes, intestinal permeability, and entero-endocrine signalling.[50] In IBS this bidirectional communication is disturbed.[50]

Studies looking at the function of the central nervous system have identified several changes in IBS such as a reduced inhibitory feedback on the emotional arousal network, which is important for the autonomic modulation of the gastrointestinal function; an increased activity after visceral stimulation; an aberrant central processing of sensory information; a heightened awareness for gastrointestinal stimuli with accompanying reduced activity of the inhibitory response; an increased susceptibility to stressors.[22,51] Local inflammatory processes in the gut can alter this central processing of information and influence visceral afferents through the production of pro-inflammatory cytokines.[22]

Another mechanism playing a role in brain-gut axis dysregulation is the occurrence of early adverse life events (EALs). EALs are traumatic experiences during childhood which can include physical violence, sexual abuse, emotional abuse, household mental illness, or injury. Approximately 75% of IBS patients have experienced some form of EAL. Persons experiencing EALs have a twofold higher risk of developing IBS at a later age.[52] A higher number of EALs, EALs with a larger impact, or more fear at the time of the EAL correlate with a higher risk to develop IBS and more severe symptoms.[52–54] Given the tremendous influence that EALs have, not only on developing IBS but also on symptom severity,

adequate counselling of children experiencing these EALs is paramount to prevent future disease and associated health care costs.

There has been a lot of attention for the role of the brain-gut axis and the interplay between IBS and psychological disorders. Psychological comorbidities are prevalent in IBS patients with 44% suffering from anxiety and 36% from depression.[55] There is a complex interplay with stress exacerbating symptoms and excessive symptoms causing further stress. Psychological disorders are a risk factor for developing IBS. However, an American study also demonstrated that 23% of patients with anxiety and 40% of patients with depression developed these disorders after the onset of IBS.[22,56] This further validates the bidirectional interaction between gut and brain.

1.2.7 Visceral hypersensitivity

Visceral afferents can be activated by chemical, mechanical, or local luminal stimuli. Furthermore, the gut also contains silent nociceptors which are only activated when tissue injury occurs after which they spontaneously activate and develop mechanosensitivity. Activation of these silent nociceptors can contribute to chronic visceral hypersensitivity through both peripheral and central nervous system mechanisms.[57]

The occurrence of visceral hypersensitivity is often multifactorial and can develop either through a greater sensitivity of visceral afferent pathways (peripheral sensitisation) or through central amplification of visceral afferent input in the spinal cord or brain (central sensitisation).[21,57,58]

Peripheral sensitisation of sensory nerves develops when these nerves are activated by, for example, products of the gut microbiota, mediators released by epithelial cells or immune cells, alterations in gene expression, or alterations in the second messenger system.[21,57,59] For example, previous research by our group examined the role of histamine 1 and histamine 4 receptors. These receptors are activated by histamine produced by mast cells. Antagonists of these receptors reduce visceral hypersensitivity and have an antinociceptive effect.[60]

In central sensitisation the nervous system is in a continuous state of hyperreactivity resulting in decreased sensory thresholds and therefore increased sensitivity to stimuli. Non-painful stimuli can be experienced as painful (allodynia), or painful stimuli can be experienced more severe (hyperalgesia).[21,58] Central sensitisation is triggered by an increased nociceptive input, for example after periods of increased visceral stimulation like with gastroenteritis. Brain regions involved in pain perception are the primary and secondary somatosensory cortex, the anterior cingulate cortex, prefrontal cortex, insular cortex, amygdala, thalamus, cerebellum, and the periaqueductal grey matter.[61] Functional magnetic resonance imaging (MRI) in IBS patients has demonstrated changes in these regions. For example, a recent review by Yu *et al.* described an inactivation of the insular cortex, prefrontal cortex and anterior cingulate cortex.[62]

Compared to healthy controls, a subset of IBS patients also experiences a higher sensitivity to somatic stimuli like thermal, cold pressor, or ischemic stimuli, but not to somatic mechanical stimuli. This could be because mechanical stimuli activate non-nociceptive mechanoreceptors in the cerebral cortex which can be inhibited by the chronic visceral nociceptive input in IBS patients.[57]

1.2.8 Genetics

Studies have shown familial clustering of IBS suggesting some degree of heritability. Still, environmental factors also have an important influence on the development of IBS.[21] Over the years, research has found several genetic variations in patients with IBS. Both rare single gene abnormalities and complex polygenic conditions, combinations of common variants, have been found. In these polygenic conditions each variant contributes a small risk with their combination being sufficient to cause IBS.[63] Genes involved in serotonin synthesis and reuptake, mucosal immune activation and inflammation, neuropeptide signalling, nociception, bile acid synthesis, and intestinal secretions have been implicated.[63,64] Identifying the genes involved could help gain further insight in the pathophysiological mechanisms underlying IBS.

A study by Eijsbouts *et al.* identified six genetic susceptibility loci of which four are also associated with anxiety and mood disorders.[65] This suggests shared pathogenic pathways with psychological disorders rather than them causing IBS or vice versa.

Next to genetics, epigenetics are believed to play an even bigger role in the pathophysiology of IBS.[66] Epigenetics are molecular changes which can mediate environmental effects on central and peripheral function. They alter gene expression without alterations to the underlying DNA sequence and are key in normal development, cell function, and differentiation. Examples of epigenetic changes are DNA methylation changes, histone modifications, and differential expression of non-coding RNA like microRNA or long non-coding RNA.[66] Epigenetics are influenced by environmental factors like diet and the microbiota, both of which also play a role in IBS. As shown in figure 1.3, when looking at

the pathophysiology of IBS it is important to take this complex interplay between genetic, epigenetic, environmental, and peripheral factors into account.[66]

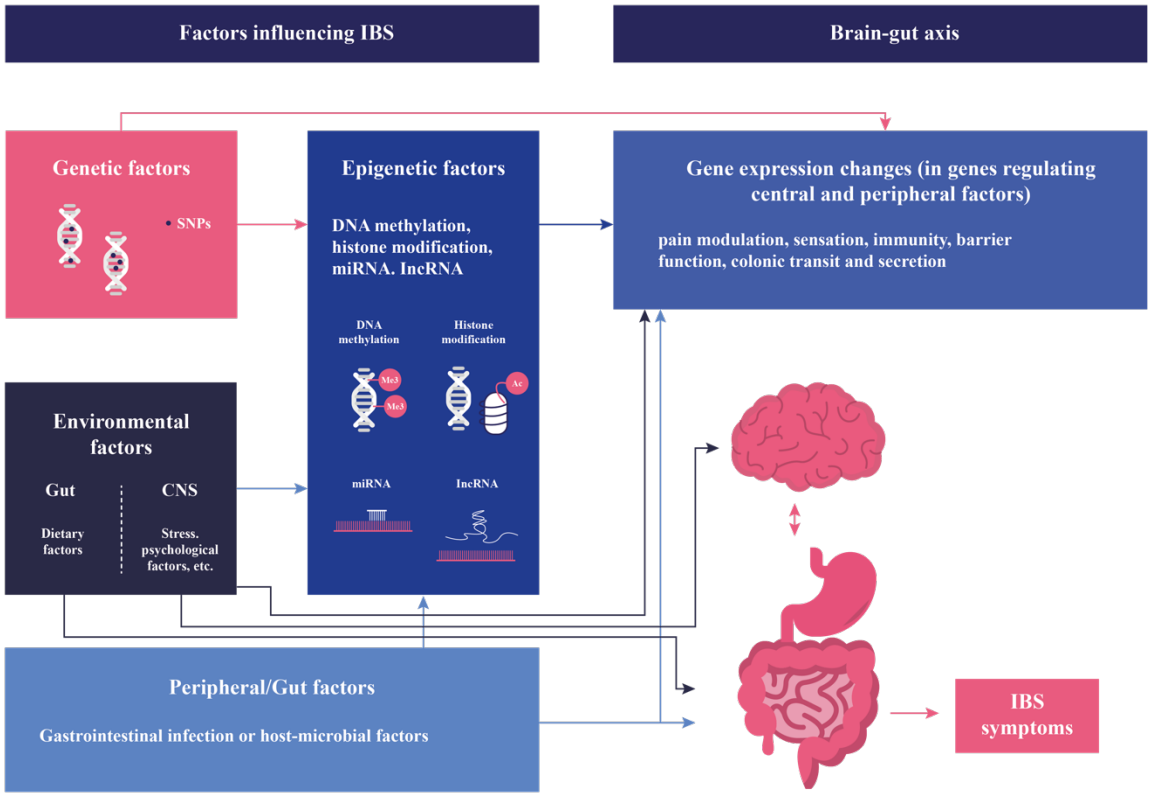


Figure 1.3: Genetic, epigenetic, environmental factors, and peripheral factors in IBS. Adapted from Mahurkar-Joshi *et al.*[66]

1.3 Diagnosis of irritable bowel syndrome

1.3.1 Using a positive diagnostic strategy

Currently, there is no diagnostic test available for IBS. Therefore, a positive diagnosis should be made based on a careful history, physical examination, and limited diagnostic testing based on a patient's individual case.[67] Afterwards, careful follow-up is crucial to detect any changes in symptom patterns.[5] Furthermore, empathic communication, explaining the

diagnostic process, and likelihood of negative testing beforehand facilitates patient acceptance of a positive diagnosis and promotes a positive treatment outcome.[68]

A Danish study comparing a diagnostic strategy of exclusion (analyses of blood, stool samples for intestinal parasites, and sigmoidoscopy with biopsies) and a positive strategy (analyses of blood cell count and C-reactive protein) found a similar effect on symptoms, patient satisfaction, and use of healthcare resources after one year. Furthermore, a positive diagnostic strategy had a lower direct cost further supporting its use.[69,70]

The first step in diagnosing IBS is a thorough history. A patient should have abdominal pain, changed bowel habits, and a temporal association between them. Constipation, diarrhoea, or a combination of both can occur.[5] Stool form scales like the Bristol stool scale can be used to determine stool consistency and document evolution over time.[14] Supportive symptoms for IBS are bloating, abdominal distention, mucus in the stools, urgency, and exacerbation after food ingestion. Concomitant diseases like fibromyalgia, dyspareunia, or mental illnesses are also frequently present in IBS.[5]

The absence of red flag symptoms in addition to the presence of traditional symptoms increases the predictive value in diagnosing IBS. Red flag symptoms like fever, weight loss, blood in the stools, nocturnal symptoms, start of symptoms at an older age, or a family history of colorectal cancer should always warrant more extensive examinations like a colonoscopy.[5,71–74]

The second step is a physical examination with evaluation of both the abdomen, peri-anal region, and pelvic floor which can direct the diagnosis towards other differential diagnosis

like malignancies (abdominal mass), inflammatory bowel disease (fistulas), or pelvic floor dysfunction (paradoxical contraction of the pelvic floor when straining).[5]

Diagnostic criteria like the Rome IV criteria offer some structure and direction but do have their limitations. Since other diseases (for example inflammatory bowel disease, coeliac disease, microscopic colitis, small intestinal bacterial overgrowth) can present similarly to IBS, some limited testing is required to make an accurate diagnosis.

A first line approach for undiagnosed patients should include a full blood count (to detect anaemia or leucocytosis), C-reactive protein, and faecal calprotectin (to exclude inflammatory bowel disease).[5,72,74,75] Standard serologic testing for coeliac disease should be considered in a diarrhoea predominant phenotype.[75,76]

Additional testing can be performed based on a suggestive clinical history or after failing empirical therapy. Examples of additional tests are thyroid function blood tests, upper gastrointestinal endoscopy with duodenal biopsies, colonoscopy, stool analysis for bacteria, parasites or ova, breath testing for carbohydrate malabsorption, assessment of pelvic floor function (anal manometry, balloon expulsion), or evaluation of bile acid malabsorption (scintigraphic evaluation, postprandial serum C4, or empirical therapy).[5,72,77]

1.4 Treatment of irritable bowel syndrome

Treatment of IBS is focused on the predominant symptom (abdominal pain, constipation, or diarrhoea) and can consist of dietary, pharmacological, and/or psychotherapy.[78–81] Patients are also encouraged to make lifestyle changes with sufficient sleep, relaxation, and physical exercise. Research has shown that increased physical activity has a positive effect on IBS symptoms, and comorbid mood disorders.[82] Figure 1.4 demonstrates a schematic representation of the Belgian consensus on the management of IBS.

1.4.1 Patient education

Patient education and a strong physician-patient relationship have been proven to positively impact symptoms and improve quality of life.[83] However, research has shown that most IBS patients feel insufficiently informed and have misconceptions on the cause and appropriate treatment for IBS.[84,85] There is an important demand for information with an increasing role for internet-based resources. Internet users with IBS are mostly younger, generally more knowledgeable, and report moderate to severe symptoms.[86,87]

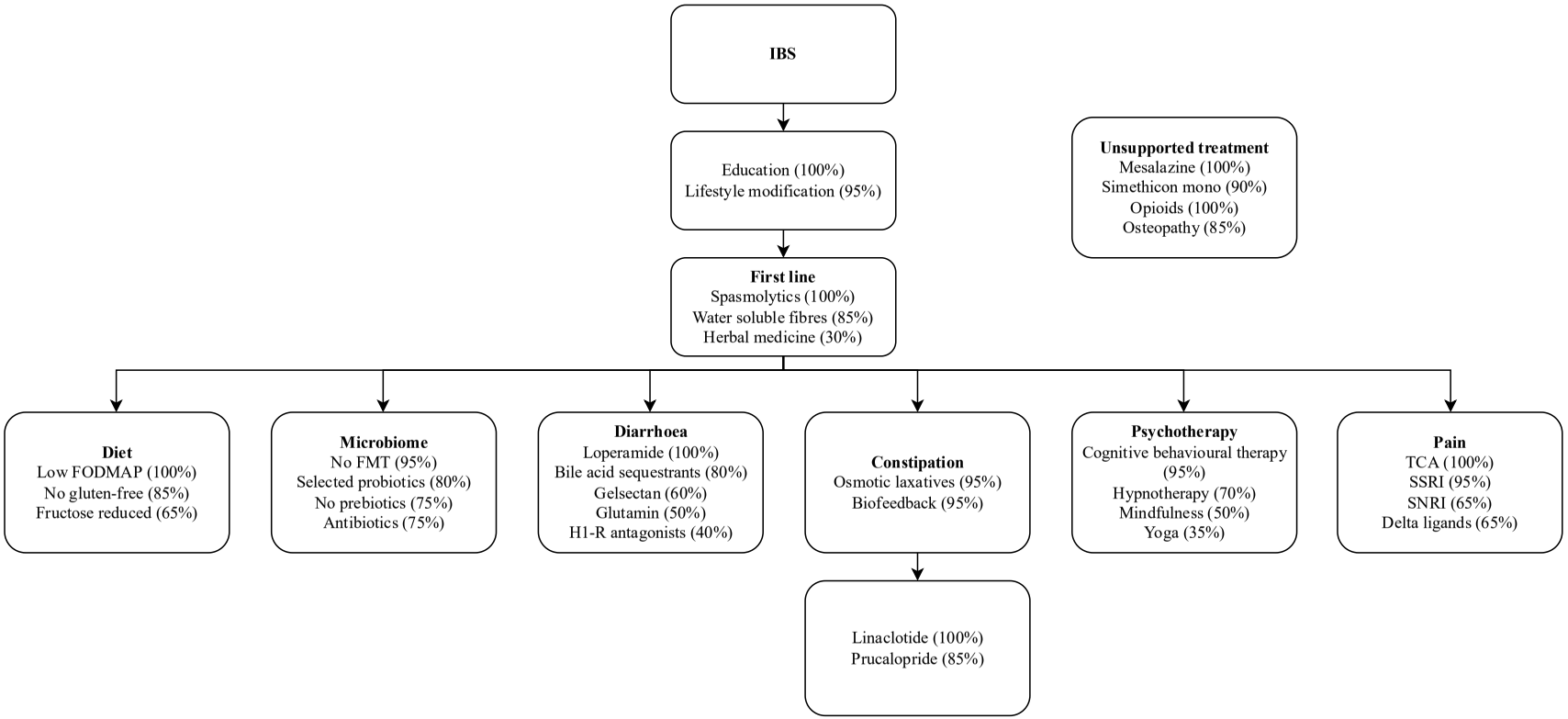


Figure 1.4: Schematic representation of the Belgian consensus on the management of IBS. The percentage of agreement is shown.
FMT = faecal microbiota transplantation; FODMAP = fermentable oligo-, di-, polysaccharides and polyols; SSRI = selective serotonin reuptake inhibitor; SNRI = serotonin and noradrenaline reuptake inhibitors; TCA = tricyclic antidepressant. Adapted from Kindt et al.[88]

1.4.2 Dietary therapy in IBS

Apart from general lifestyle recommendations, dietary management is often one of the first line approaches.

Dietary advice to increase fibre intake and the prescription of fibre as a bulking agent are frequently used in the treatment of both diarrhoea and constipation.[89] Fibres can be divided into two groups: soluble (e.g., psyllium, ispaghula) and insoluble (e.g., corn fibre, wheat bran). Studies have shown fibre to be effective in reducing global IBS symptoms and optimising stool consistency but with no effect on abdominal pain. It is recommended to only supplement soluble fibre since insoluble fibres can worsen symptoms.[89]

In 2008 the National Institute of Clinical Excellence (NICE) from the United Kingdom published guidelines on diagnosis and treatment of IBS. This guideline contains lifestyle and dietary recommendations which are now referred to as the NICE diet (table 1.3).[90]

Table 1.3: NICE diet

NICE recommendations
Have regular meals and take time to eat
Avoid missing meals or long periods between meals
Drink ≥8 cups of fluid per day (preferably water or other non-caffeinated drinks)
Limit tea and coffee to 3 cups per day
Limit alcohol and carbonated drinks
Limit intake of high-fibre food
Limit intake of resistant starch (often found in processed or pre-cooked meals)
Limit fresh fruit to 3 portions per day
Avoid sorbitol (if diarrhoea predominant)

The most extensive and well-known diet in the treatment of IBS is the low-FODMAP diet. As mentioned earlier, FODMAPs or fermentable oligo-, di-, monosaccharides, and polyols are poorly absorbed in the small bowel causing increased fluid content, through osmotic effects, and flatulency. A low-FODMAP diet consists of three phases. First, all FODMAPs are excluded from the diet for a period of six to eight weeks. Second, if sufficient symptom reduction is reached, the reintroduction phase will start. In the reintroduction phase, each FODMAP group will be gradually reintroduced to evaluate whether they provoke symptoms. Third, the final, personalised diet is composed with exclusion of the FODMAPs causing symptoms. Since it is a very restrictive diet from the start, it is important for patients to be coached by a trained dietician. Furthermore, because of its restrictive nature there have been concerns about the effect on nutritional intake, gut microbiota, and quality of life.[91]

A meta-analysis from 2021 from Van Lanen *et al.* demonstrated a moderate to large effect of the low-FODMAP diet on symptom severity and on quality of life compared to a control diet.[91] Studies looking into the effect of the low-FODMAP diet on gut microbiota found no effect on microbial diversity and density but there was a reduced abundance of *bifidobacteria* and an increase in bacteria associated with dysbiosis.[92] However, most studies followed patients over a short time period (3-8 weeks) only looking at the first restrictive phase.[92] One study by Harvie *et al*. followed patients also through the reintroduction phase and found no significant changes in microbiota composition.[93] Further research looking into the long term effect of the low-FODMAP diet on microbiota composition is needed to help assess its long term safety. Few studies have examined the nutritional side of the low-FODMAP diet. When followed correctly, the diet does not seem to have any negative effects on nutritional intake.[91]

1.4.3 Pharmacotherapy in IBS

Since IBS cannot be cured, pharmacotherapy is mainly focussed on the predominant symptom(s). However, treatment options directly working on underlying pathophysiological mechanisms are becoming increasingly important.

1.4.3.1 *Treatment of diarrhoea*

A first option in the treatment of diarrhoea is loperamide. Loperamide is a peripheral acting opioid agonist inhibiting peristalsis and secretory activity. It improves stool consistency, but results on the effect on abdominal pain are inconsistent.[88,94]

A second option in the treatment of diarrhoea are bile acid sequestrants. Bile acid malabsorption (BAM) is a commonly overlooked mechanism causing chronic diarrhoea,

playing a role in up to 30% of the IBS population. BAM can be diagnosed with the help of a ^{75}Selenium-homotaurocholic acid test (^{75}SeHCAT) or measurement of bile acid content in a faecal sample. However, because of the limited availability of these tests, an empiric treatment with bile acid sequestrants like cholestyramine, is often used.[88,94]

The third option in the treatment of diarrhoea is antibiotics. They work either through their effect on small intestinal bacterial overgrowth (SIBO) or on colon dysbiosis. Apart from a positive effect on diarrhoea they have the ability to reduce bloating. The most extensively studied antibiotic is rifaximin, a nonabsorbable, broad spectrum oral antibiotic working against both aerobic and anaerobic bacteria.[88,94] However, it is an expensive product which is not reimbursed in Belgium, and often requires retreatment causing it to be rarely used in clinical practice. Keeping in line with antibiotics is the use of probiotics. Research on probiotics is inconsistent with some studies indicating a positive effect on stool consistency, abdominal pain, and bloating while other studies report no change or even an increase in symptoms. However, it is difficult to compare studies since there is a large variety in bacterial composition of these probiotics.[88,95] Positive effects do seem to be more pronounced in studies using multi-strain probiotics or combinations of different probiotics compared to mono-strain probiotics.[95,96]

Recently, drugs working more directly on underlying pathophysiological processes like ebastine and Gelsectan® have received increasing attention.[88] Ebastine is a histamine-1 receptor antagonist counteracting the effect of histamine release by mast cells on visceral hypersensitivity.[97] Its effect has been demonstrated in a small study by Wouters *et al.*[97], however a larger phase two study is still ongoing (NCT01908465). Aguilera-Lizarraga *et al.*

suggest that it might work best on patients with post-infectious IBS-D.[98,99] Gelsectan consists of xyloglucan, pea protein, tannins from grape seed extract, and xylo-oligosaccharides. Xyloglucan has a mucin-like molecular structure giving it mucoadhesive properties allowing it to form a physical barrier protecting the mucosa against damage from microorganisms, allergens, and proinflammatory compounds. Pea proteins and tannins also have mucoprotective properties while xylo-oligosaccharides are a prebiotic exerting a bifidogenic effect in the colon. Two randomised controlled trials demonstrated an improvement of intestinal barrier dysfunction resulting in less diarrhoea and pain.[100,101] However, more research is still needed.

1.4.3.2 *Treatment of constipation*

Laxatives, both osmotic (e.g., polyethylene glycol) and stimulating (e.g., bisacodyl, senna), are one of the first choices when treating constipation. Osmotic laxatives have a local effect in the gut and keep water in the lumen through osmosis making them a safe to use long-term treatment for constipation. Long term use of stimulating laxatives is not advised for two reasons. First, efficacy decreases over time and second, there is insufficient data available on the safety when using these laxatives for a longer period of time.[102–104]

Since osmotic laxatives usually only have a mild effect and stimulating laxatives cannot be used long-term, there was a need for additional pharmacotherapeutic options in the treatment of constipation. Two products are currently available in Belgium, linaclotide and prucalopride. Prucalopride is a highly selective $5HT_4$ receptor agonist stimulating motility throughout the entire GI tract making it an interesting alternative in patients with comorbid gastroparesis, functional dyspepsia, or reflux disease.[88,104–108] Linaclotide stimulates the

guanylate cyclase C receptor on enterocytes leading to the production of cGMP, which stimulates secretion of chloride and bicarbonate and reduces mechanosensitivity of colonic nociceptors. This gives linaclotide an advantage over prucalopride in the treatment of IBS since it has a positive effect on both constipation and abdominal pain.[107]

1.4.3.3 *Treatment of abdominal pain*

Abdominal pain is often one of the most difficult to treat symptoms in patients with IBS. First line pharmacotherapeutic treatment of abdominal pain are the antispasmodics, including otilonium bromide, mebeverine, peppermint oil, and butylhyoscine. They have a positive effect on abdominal pain compared to placebo, however, studies in IBS are heterogenous and often have methodological flaws.[88,98,109]

Central neuromodulators have the most evidence in treatment of abdominal pain in IBS. They do not work instantly and should be taken for at least four weeks before assessing their effect. Since they work centrally, they have a higher chance of side effects like sedation, however, these negative effects often diminish after a couple of weeks of treatment. Furthermore, product-specific side effects can even be used as an advantage like constipation with tricyclic antidepressants (TCA) and diarrhoea with selective serotonin reuptake inhibitors (SSRI).[81] Central neuromodulators are often known for their use as antidepressants causing apprehension in patients before starting these therapies because of the stigmata they might induce. Education of patients on the mechanism of action of these neuromodulators is essential to ensure therapy compliance. When using neuromodulators for pain, lower doses are used making it less likely they will affect a patient's mental health.[81,88]

Tricyclic antidepressants are the first-choice central neuromodulator and have the most evidence in IBS.[81] Nortriptyline is preferred in IBS-C and IBS-M because it causes less constipation than for example amitriptyline which is preferred in IBS-D. When anxiety, depression, or phobic features are prominent, SSRIs are preferred while tetracyclic antidepressants, like mirtazapine, are favoured when a patient has comorbid dyspeptic features or weight loss. When the side effects of TCA are too severe a switch to a selective noradrenaline reuptake inhibitor (SNRI) like duloxetine can be considered.

When central neuromodulators provide insufficient symptom relief, a dose augmentation should be considered. However, adding or switching to another central neuromodulator can be necessary. Delta ligand agents, like gabapentin or pregabalin, bind on voltage-gated calcium channels and can be useful when there is a neuropathic pain component, abdominal wall pain, or comorbid fibromyalgia. When fatigue or sleepiness are prominent, bupropion should be considered while atypical antipsychotics can be useful when patients have trouble sleeping, anxiety, or nausea.[81]

1.4.1 Psychotherapy in IBS

Research has shown the benefit of complementary psychological interventions in reducing disease burden, healthcare costs, and increasing coping and quality of life.[110–112] When choosing an appropriate therapy, it is important to consider barriers to care like travel distance, time availability, and financial abilities.[111]

All IBS patients can benefit from psychological treatment, not only patients with psychological symptoms or comorbid mental health diseases.[110] In contrast to classic

psychological treatments, behavioural treatments for IBS focus on symptom specific mechanisms and outcomes. Furthermore, a large study has shown a better response to cognitive behavioural therapy in patients with low anxiety levels.[113] Before starting any psychological treatment, it is important to identify barriers like poor health management, refusal to participate in psychotherapy, or low insight into the interaction between physical and emotional health.[110]

Different types of psychological therapy available in IBS treatment are contingency management (behaviour modification intervention which reinforces desired behaviours through incentives), cognitive behaviour therapy (via telephone, internet, minimal contact, self-administered, group, or face-to-face), hypnotherapy, stress management, and dynamic psychotherapy.[111,114] A meta-analysis by Black *et al*. showed that psychological treatments are superior to control conditions (watchful waiting, routine care, education, or support, and dietary or lifestyle advice). No significant differences between the different psychotherapy methods were found.[111] Furthermore, a recent meta-analysis showed a number needed to treat of 4 (4 patients need to be treated so that 1 patient can experience a positive effect).[115]

Cognitive behaviour therapy (CBT) is one of the most extensively studied and substantiated methods of the beforementioned.[111,116–119] CBT is a psychological treatment method focused on the way patients process information about their environment and to help them gain control and reduce symptoms. It works by modifying thinking patterns and identifying cognitive errors and faulty logic. This can help patients control their difficulties and change the way they behave and feel both emotionally and physically. In contrast to classic psychotherapy, CBT requires active participation and is more problem-focused, goal-

directed, and time-limited.[116] In the long-term CBT via the telephone seems to be the most beneficial.[111,119] CBT via the internet has the added advantage of easy accessibility and flexibility. Compared to one-to-one clinic-based psychotherapy CBT has a relatively low cost making it more affordable and easier to implement on a large scale.[117]

Hypnotherapy has also been used for the treatment of IBS. Hypnosis is a state of consciousness with focused attention and reduced peripheral awareness, in which patients are more receptive to suggestions. Studies have shown the beneficial effects of hypnotherapy, however, since blinding patients and therapists is not possible, bias is to be expected.[120] A study by Lindfors *et al.* demonstrated that a large proportion of patients is very satisfied with the results of hypnotherapy, which was associated with an improvement of quality of life and reduction of GI symptoms.[121] However, patients without improvement of GI symptoms are also satisfied with the treatment outcome suggesting that other factors also play a role in patient satisfaction.[121] In some countries like the United Kingdom and the Netherlands hypnotherapy is frequently used in the treatment of IBS patients. However, in Belgium patients are rarely referred which is largely due to the limited number of hypnotherapists specialised in somatic disorders like IBS.[88,120,121]

Advantages of psychological treatment over the use of drugs are their safety and lasting effects beyond the duration of treatment.[116] Limitations of psychological treatment are the need for longer treatment durations, the need for motivated patients, the lack of proper reimbursement, and the availability of specialised mental health professionals.[116] Possible adverse events associated with psychological treatment are treatment failure, worsened

symptoms, elevated distress levels, self-harm, or even suicide.[111] However, there is uncertainty on the causality of these adverse events, requiring more research.

1.5 The Future of irritable bowel syndrome

In recent years our knowledge about IBS has risen exponentially. However, IBS is an extremely heterogenous disorder making it difficult to generalise new discoveries to the whole population. This slows down further development and translation of fundamental scientific knowledge into clinically applicable information like biomarkers or novel therapies. These difficulties lead to high health care costs and frustrated patients and health care professionals.

In this last part of the introduction, we will take a deeper look into two promising topics: mast cells and volatile organic compounds.

1.5.1 Biomarkers

According to the Biomarkers Definitions working group of the National Institute of Health, a biomarker is a medical characteristic which is an objective indication of the medical state that can be observed from outside the patient.[122] Biomarkers can be molecular, histologic, radiographic, or physiologic characteristics, corresponding to normal or pathological metabolic processes. They need to be measured accurately and results should be reproducible.[123,124]

Over the years several faecal, blood, mucosal, microbial, radiological, and genetic biomarkers have been proposed for IBS. However, the use of these biomarkers is heavily

dependent on the understanding of their behaviour in normal and pathological circumstances. A review by Camilleri *et al.* looked into potential biomarkers in IBS and evaluated their diagnostic utility, availability, invasiveness, and cost-effectiveness (table 1.4).[123] A major issue in biomarker development in IBS is the heterogeneity of the disorder. The applicability of specific biomarkers is therefore often limited to specific subgroups of IBS patients. Two biomarkers have shown a high diagnostic utility, faecal bile acids and colonic transit time. However, at the moment these tests are not widely available limiting their use in clinical practice.[123]

As it stands, there is insufficient scientific data to support a specific biomarker, which can be used in clinical practice to aid in the identification and the follow-up of IBS patients. Consequently, the diagnostic process is often cumbersome and is associated with high health care costs.[125]

Table 1.4: Potential biomarkers in irritable bowel syndrome

	Diagnostic utility	Predominant application	Availability	Invasiveness	Cost-effectiveness
Serum biomarkers					
Inflammatory: interleukins, cytokines	Low	IBS-D	Specialised clinics	Low	Moderate
Enteroendocrine: serotonin, chromogranin	Low	IBS-D	Specialised clinics	Low	Moderate
Faecal biomarkers					
Faecal bile acids	High	IBS-D	Referral labs and centre	Low	High
Soluble mediators: proteases, chromogranin, calprotectin	Low	IBS-D	Specialised clinics	Low	Low
Microbiome	Moderate	IBS-D, IBS-C	Specialised clinics	Low	Low
GI tract biomarkers					
Colonic transit	High	IBS-D, IBS-C	Specialised clinics	Low - moderate	Moderate
Visceral hypersensitivity	Low	IBS-D, IBS-C	Referral labs and centre	Moderate – high	Low
Permeability	Moderate	IBS-D	Referral labs and centre	Moderate – high	Moderate
Mucosal biomarkers: mast cells, B and T cells, miRNA	Low	IBS-D, IBS-C	Referral labs and centre	Moderate – high	Low
Neurological and psychological biomarkers					
Brain imaging	Moderate	IBS	Referral labs and centre	High	Low
Psychological markers	Moderate	IBS	Widely available	High	Low

Adapted from Camilleri *et al.*[123] GI = gastrointestinal; C = constipation; D = diarrhoea; IBS = irritable bowel syndrome; miRNA = micro-ribonucleic acid
Diagnostic utility: low = no validation of clinical significance; moderate = shows promise, but further validation is needed; high = validated and usable in clinical practice; Availability: widely available = high availability; specialised clinics = moderate availability, clinics with a special interest and focus in neurogastroenterology; referral labs and centre = low availability, only a few specific centra will offer testing for this biomarker

1.5.2 Mast cells

Mature mast cells (MCs) are heterogeneous, tissue-resident, long-lived, granulated cells which are particularly abundant in barrier sites.[19,126,127] MCs originate from CD34+ multipotent hematopoietic progenitors in the bone marrow. These immature progenitors migrate through the bloodstream to the tissues where they will mature. Depending on the tissue and specific microenvironment MCs will differentiate differently throughout the body.[127] In rodents, MCs can be classified based on their location, histochemical staining, mediator content, and reactivity to compounds resulting in two major subtypes: connective tissue MCs and mucosal MCs. Human MCs are classified based on the predominant protease: tryptase, chymase, or a combination of both. MC_T (tryptase) share characteristics with the rodent mucosal type and MC_{TC} (tryptase and chymase) with the connective tissue type.[127] MCs can be further characterised based on their cell surface markers and granule content. MCs are CD117 (c-kit or stem cell factor receptor) and CD203c positive cells, expressing a high affinity IgE receptor (FcεRI), and MRGPRX2.[126,127] MRGPRX2 is a mas-related gene receptor (MRGPR) mainly expressed on MC_{TC}, and in a lesser number on MC_T. MRGPR is a family of G protein-coupled receptors containing over 50 members in both humans and rodents. They are mainly expressed on nociceptive neurons and specialised immune cells. According to their function and structures the receptors are assigned to several subfamilies. MRGPRX 1-4 and MRGPR D to G are human receptors, while MRGPR A to G are rodent receptors. The human MRGPRX 1-4 can be paired to the rodent MRGPRA and MRGPRB receptors. Human receptors MRGPR D to G do not have rodent orthologs.[128]

Upon activation of MCs a variety of inflammatory mediators is released. MC mediators can be subdivided into two groups: preformed mediators present in granules, and de novo formed mediators (table 1.5). The latter can be further subdivided into newly formed lipid mediators derived from membrane lipids and mediators which are de novo synthesised following transcriptional activation depending on the type of stimuli.[19,127,129,130] Release of mediators by the MCs changes the MC microenvironment, in turn influencing MC function and activation, leading to a positive feedback loop.[130]

Table 1.5: Mediators produced by mast cells

Type of molecule	Example
Preformed in granules	
Biogenic amine	Histamine, serotonin, dopamine
Protease	Tryptase, chymase, carboxypeptidase A, cathepsin G
Lysosomal enzyme	B-hexosaminidase
Proteoglycan	Heparin, chondroitin sulphates
Other	TNF-α
Lipid mediators	
Eicosanoids	Prostaglandins and leukotrienes
De novo synthesized	
Cytokines	Interleukins, TNF-α, IFN-γ
Chemokines	CCL, MIP, MIF
Growth factors	VEGF, NGF, FGF
Others	Nitric oxide, CRF, VIP, ATP, substance P

TNF = tumour necrosis factor; IFN = interferon; CCL = chemokine ligand; MIP = macrophage inflammation protein; MIF = macrophage migration inhibitory factor; VEGF = vascular endothelial growth factor; NGF = nerve growth factor; FGF = fibroblast growth factor; CRF = corticotropin releasing factor; VIP = vasoactive intestinal peptide; ATP = adenosine triphosphatase [19,127,129,130]

1.5.2.1 *Mast cell activation*

MCs can be activated via the classical IgE-mediated pathway, which is crucial in the pathophysiology of allergic disorders. However, MCs can also be activated through an equally important IgE-independent activation mechanism, which can be triggered by a variety of substances such as cytokines, hormones, immunoglobulins, neuropeptides, and complement components.[131,132] MC activation can also be modulated by central or psychological pathways through the release of corticotropin releasing hormone.[133]

IgE-dependent activation happens through cross linking of the FcεRI by IgE. In short, a first exposure to allergens leads to the production of specific IgE antibodies (sIgE). In turn, these sIgE cause sensitisation of MCs by binding to the high affinity receptor FcεRI present on the membrane of MCs. A second exposure will lead to crosslinking of the membrane bound IgE-FcεRI complexes on MCs resulting in MC activation with the release of its mediators.[134]

IgE-independent activation can be triggered by inflammatory products such as complement, IgG, cytokines, and chemokines; neuro-hormonal stimuli such as neurotransmitters, neuropeptides, hormones, and growth factors. Activation can also be triggered by exogenous stimuli such as physical factors and drugs.[19,135] MC_T and MC_{TC} have different responses to IgE-independent stimuli. For example, only MC_{TC} respond to complement, substance P and opiates, while only MC_T will respond to platelet activating factor.[19,135] An important receptor in IgE-independent activation is the earlier mentioned MRGXPR2. This receptor can be activated by a variety of triggers including endogenous peptides (e.g., substance P, somatostatin, and oxytocin), venoms, and peptide-based therapeutics (e.g., octreotide).[19,135]

After ligands bind the G-protein-coupled receptor intracellular signalling is commenced via either a G-protein-dependent pathway (through second messengers like Ca^{2+} or cAMP) or a G-protein-independent pathway (through β-arrestin).[136]

Stress, both physical and psychological, activates multiple behavioural and physiological processes aimed at restoring the natural balance. An important physiological process is activation of the hypothalamic–pituitary–adrenal (HPA)-axis. During stress the paraventricular nucleus (PVN) of the hypothalamus releases two neurohormones, arginine vasopressin (AVP) and corticotropin releasing factor or hormone (CRF or CRH). These two hormones induce production and secretion of adrenocorticotropic hormone (ACTH) in the anterior pituitary gland. ACTH in turn activates the glucocorticoid synthesis in the adrenal glands, in humans this is mostly cortisol.[137] Corticotropin releasing hormone activates the HPA-axis, but it will also have an immediate effect on MCs. Activation of MCs by CRH triggers release of pro-inflammatory mediators and CRH as well, further amplifying the effect of central activation.[138]

1.5.2.2 *Mast cells and the nervous system*

MCs and neurons interact continuously with an estimated 70% of MCs in direct contact with nerves, and another 20% within 2μm of nerves in the GI system.[130] On the one hand, MCs operate as sensory cells activated by both immune and non-immune related signals. On the other hand, they are effector cells releasing a variety of biologically active mediators. These mediators have a paracrine function activating a cascade of extrinsic and intrinsic neural networks in the gut.[130]

1.5.2.3 *Mast cells in the gastrointestinal tract*

MCs can be found in the entire gastrointestinal tract. Their density is highest in the lamina propria and submucosa, where they account for 2-3% and 1% of the mononuclear cells respectively. They are only sporadically present in muscle layers and the serosa.[130]

In the gastrointestinal tract, MCs are continuously exposed to a variety of stimuli as they play an important role in the innate and adaptive immune system.[139,140] Activation of MCs and consequent release of mediators has an important impact on gastrointestinal neuromuscular and secretory functions. For example, release of histamine and prostaglandin D2 plays a significant role in chloride and water secretion, as well as control of intestinal motility.[140] MC mediators are able to stimulate epithelial cells, other immune cells, and neurons.[139] Increased luminal secretion, blood flow, and propulsive motor activity are all part of our gastrointestinal defence system aiming at eliminating harmful substances, antigens, toxins, and microbes in our gut.[139] Apart from their role in normal functioning, MCs are shown to sensitise silent nociceptors contributing to visceral hypersensitivity.[140] Research from our group has demonstrated the role of proteases and the potential of protease inhibitors in alleviating visceral hypersensitivity in experimental models.[141,142] Consequently, MCs play a role in several gastrointestinal diseases; for example, food allergy, systemic mastocytosis, inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS).[140]

1.5.2.4 *Mast cells in irritable bowel syndrome*

MC research is increasingly implicated in IBS pathophysiology.[143–146] However, research on the number of MCs in the gut mucosa of IBS patients is contradictory, with some studies

showing an increased number, while others did not detect any differences in numbers. The MCs that are present are more frequently located in the vicinity of afferent nerve terminals in patients with dominant pain symptoms.[145,147–149] MCs can be sensitised or primed for both IgE-dependent and -independent activation by pro-inflammatory cytokines such as IL-6 and IL-33, factors which tend to be elevated in the serum of IBS patients.[150–153] However, when looking at individual subtypes these changes in pro-inflammatory cytokine levels are mainly limited to IBS-D patients, while IBS-C and IBS-M resemble healthy controls.[151,152] Also mast cell mediators like proteases and histamine are reported as contributing factors to visceral pain and barrier dysfunction in IBS patients. Colon tryptase levels are elevated in IBS, while serum tryptase levels are within the normal range, suggesting localised mucosal MC infiltration.[140,147,154] A preliminary clinical trial by Wouters *et al.* from 2016, showed promising results for antihistaminic therapy in IBS patients, presumably by antagonising MC-derived histaminergic effects on afferent nerve endings.[97] Still, little is known about the functional characteristics of these MCs.

1.5.3 Volatile organic compounds

Volatile organic compounds (VOCs) are characterised by a low molecular weight (<300 Da) and a high vapour pressure at room temperature.[155] These compounds are metabolites produced *in vivo* during both physiological and pathophysiological metabolic processes. Additionally, they can originate from the microbial metabolism, and metabolization of exogenous sources like food or drugs.[156] VOCs are excreted in urine, sweat, blood, faeces, and exhaled breath, making them easily accessible to study. Since IBS is associated with

low-grade inflammation and dysbiosis, volatomics may offer a non-invasive tool to reflect these pathophysiological mechanisms, aiding in diagnosis, treatment, and follow-up.[156]

1.5.3.1 *Individual volatile organic compounds in irritable bowel syndrome*

Comparing patients to healthy people allows the identification of VOCs that represent a healthy volatilome. However, although healthy controls (HC) are rarely seen in clinical practice, and, hence, their discrimination has limited clinical utility, information about their baseline volatilome will be of interest to assess whether treatment of patients leads to normalisation of VOCs. More importantly, being able to differentiate between diseases with a similar symptom profile will be key.

To gain a better insight into current knowledge on VOCs in IBS, we performed a systematic literature review.[156] First of all, we reviewed individual VOCs, only the compounds described in multiple studies are described. When comparing HC with IBS patients only one compound, 1-methyl-4-propan-2-ylcyclohexa-1,4-diene, was found to be increased in both faecal and breath samples (figure 1.5).[156–158] When VOCs were used for differential diagnosis, an increase in propan-1-ol in breath and faeces of Crohn disease (CD) patients compared to IBS-D patients was found. When comparing ulcerative colitis (UC) to IBS-D and IBS-D to general inflammatory bowel disease (IBD) patients, no compounds were identified in multiple studies. However, the aforementioned 1-methyl-4-propan-2-ylcyclohexa-1,4-diene was only detected in IBS patients (breath and faeces).[156,158,159]

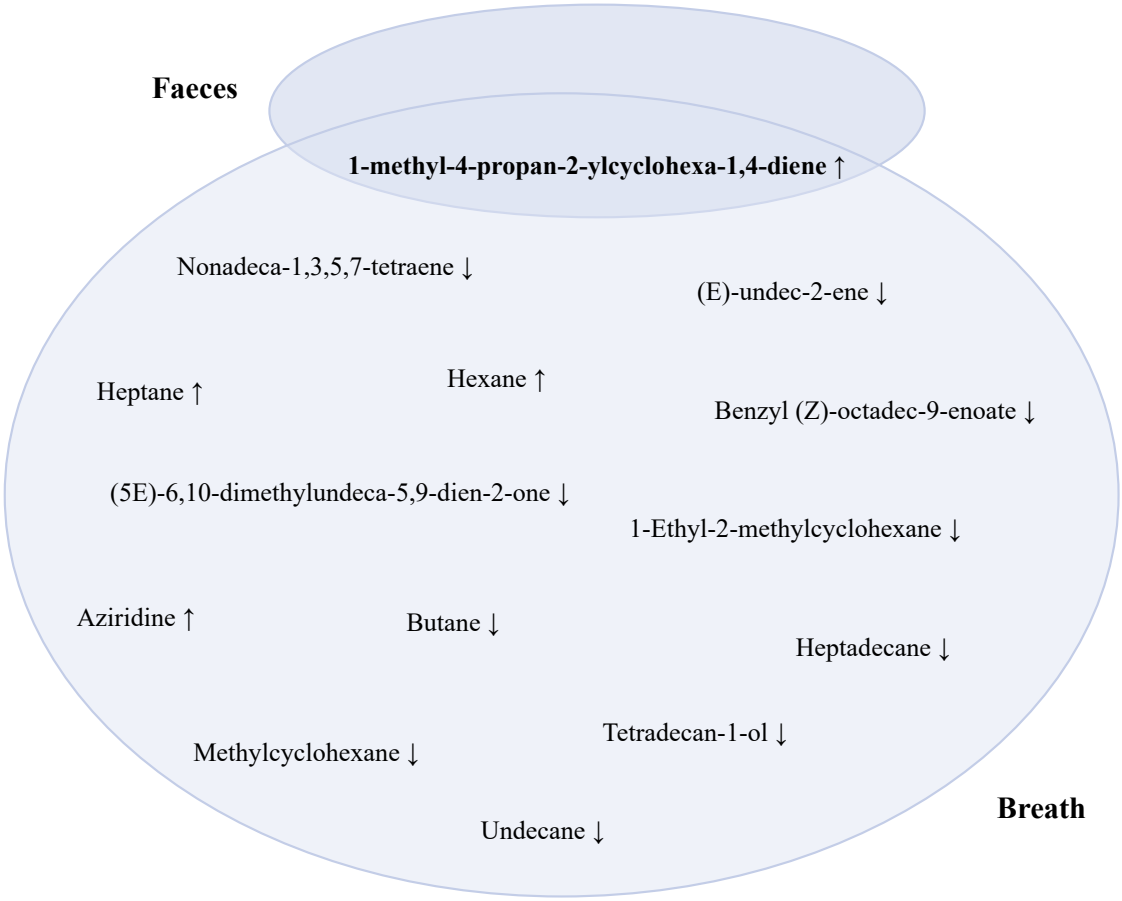


Figure 1.5: Individual VOCs in irritable bowel syndrome. Compounds described in more than one study are in bold. ↑: upregulated. ↓: downregulated

1.5.3.2 *Combining volatile organic compounds in irritable bowel syndrome*

No single VOC has yet been found nor validated by multiple research groups in IBS. Furthermore, individual VOCs are often aspecific, resulting in a limited clinical value to be used as stand-alone biomarker for (differential) diagnosis. Therefore, combining VOCs in biomarker panels has the potential to develop discriminative algorithms with increased sensitivity and specificity to accurately diagnose, differentiate, and monitor patients over time.

When evaluating studies that created VOC models we found that models differentiating IBS patients from healthy controls in breath, faeces, and urine had accuracies, sensitivities, and specificities between respectively 46-68%, 38-89%, and 71-80%.[156–158,160,161] When differentiating IBS patients from IBD patients, accuracies ranged between 70% and 83%, sensitivities between 76% and 90%, and specificities between 62% and 88%.[158,160] When comparing IBS patients with patients with coeliac disease a sensitivity and specificity of 85% were found.[162]

1.5.3.3 *Volatile organic compounds and the gut microbiota*

The gut microbiota play an important role in the pathophysiology of IBS and influence metabolic processes in the body (permeability, digestion). Since the microbiota itself produce VOCs, it can be assumed that the composition has a major influence on VOC analysis.[40,41,163] However, at the moment, it remains unclear whether VOCs are produced by the microbiota, by the patient's intrinsic pathology or both. Smolinska *et al.* focused on this microbial relationship and correlated VOCs to bacterial taxa in CD patients.[163] This study was the first to prove the interplay between VOCs and microbiota and highlights the need to take the intestinal microbial composition into account when studying VOCs. More recently Sagar *et al.* looked at patients with bile acid diarrhoea and IBS-D and found evidence that the metabolic processes of the microbiota are linked to specific VOCs.[164]

1.5.3.4 *Volatile organic compounds and personalised medicine*

For IBS, there is currently no 'one-size-fits-all' treatment, making it a cumbersome process of trial and error, which can negatively impact the patient comfort. In addition, the treatment response needs to be evaluated and adjusted accordingly. Therefore, predictive biomarkers

to preselect the most suitable treatment are of great interest, fulfilling the increasing demand for personalised medicine. Walton *et al*. conducted an interventional study investigating the effect of treatment on VOC composition in faeces in patients with Crohn's disease (CD), ulcerative colitis (UC), and IBS.[159] All patients received two weeks of treatment: CD patients (n = 8) received elemental nutrition wherein proteins are cleaved into individual amino acids, UC patients (n = 12) received oral corticosteroids and 5-aminosalicylic acid derivatives with no specific diet, and IBS patients (n = 4) were treated by an exclusion diet (diet based on Parker *et al.)*.[165] Before treatment, there was a significant increase in the faecal VOC concentrations of ester and alcohol derivates of short chain fatty acids (SCFAs) and indole in CD patients compared to the other groups. In patients with UC and IBS, indole and phenol levels tended to be higher compared to HC. After treatment, faecal VOC concentrations of all groups normalised to those of HC.[159] Another clinical study by Rossi *et al*. randomised IBS patients to a low-FODMAP diet versus sham-diet and a probiotic versus placebo diet for four weeks.[166] Faecal VOCs were analysed at baseline and after treatment by an Odoreader®, which has a gas chromatography (GC) front end and a gas sensor detector and detects patterns. VOC models to predict treatment response at baseline resulted in a high accuracy (low-FODMAP model: 97% and probiotic model: 89%) for the treatment groups. However, when applying the same models in the control groups (sham/placebo), a low accuracy was achieved (low-FODMAP model: 41% and probiotic model: 46%). This implies that these models are specific for the response to low-FODMAP and/or probiotics rather than for response to therapy in general and emphasises the potential use of VOCs as non-invasive predictive markers to optimise personal treatment.[166]

1.5.3.5 *Volatile organic compounds and metabolic pathways*

Most detected individual compounds are found to be endogenous or exogenously present in food or produced by the microbiota. The endogenous compounds play a role in several metabolic pathways such as lipid, butanoate, ethanol, sulphur, propanoate, and ketone metabolism and in the biosynthesis of tropane, piperidine, pyridine alkaloid, and terpenoid backbones. Table 1.6 gives a short overview of relevant compounds found in previous VOC research in IBS and IBD.[156] A substantial amount of the discriminative compounds are SCFAs and part of the butanoate, propanoate, and acetate metabolism. SCFAs are the main metabolic products of anaerobic bacterial fermentation, serving as fuel for intestinal epithelial cells but also modulating electrolyte and water absorption. More importantly, they have anti-inflammatory properties and mediate the effect of the microbiota on the intestinal immune function.[167,168] A second compound appearing in biomarker panels is indole. It is formed by bacterial metabolism of L-tryptophan and has anti-inflammatory properties, again stressing the importance of the effect of the microbiota on VOCs.[169]

It is currently impossible to identify the detected compounds as originating from metabolic pathways and/or from digestion of food or medication. Moreover, all these pathways are in continuous interaction with inflammatory processes. This close synergy might explain the discrepant findings in the different studies and stresses the need to clarify and explore the VOC metabolism and distribution in different body matrices.

Table 1.6: Metabolic pathways

Compound	CAS number†	Source	Concentration	Disease	Pathway	Origin
2-methylbuta-1,3-diene	78-79-5	Breath	↓↑	CD	Biosynthesis of terpenoids, steroids, secondary metabolites; Terpenoid backbone biosynthesis	Endogenous, Plant, Tobacco
3-methylbutanoic acid	503-74-2	Faeces	↑	CD	Protein digestion and absorption; Biosynthesis of alkaloid and secondary metabolites	Endogenous, Plant, Animal
6-methylheptan-2-one	928-68-7	Faeces	↑	CD	Cholesterol oxidation; Synthesis and degradation of ketone bodies	Endogenous, Plant, Animal
Butan-1-ol	71-36-3	Faeces	↑	CD	Degradation of aromatic compounds; Microbial metabolism; Butanoate metabolism	Endogenous, Plant, Animal, Bacteria
Butanoic acid	107-92-6	Faeces	↑	CD	Carbohydrate and protein digestion and absorption; Metabolic pathways; Butanoate metabolism	Endogenous, Plant, Animal, Bacteria
Ethyl butanoate	105-54-4	Faeces	↑	CD	Lipid metabolism	Endogenous, Plant, Animal
Ethyl propanoate	105-37-3	Faeces	↑	CD	Ethanol metabolism	Endogenous, Plant
Heptanal	111-71-7	Faeces	↑	CD	Lipid metabolism	Endogenous, Plant, Animal
Methyl butanoate	623-42-7	Faeces	↑	CD	Lipid metabolism	Endogenous, Plant, Animal
Methylsulfanylmethane	75-18-3	Breath Faeces	↑↓	CD	Sulphur metabolism	Endogenous, Plant, Bacteria

Table 1.6: Metabolic pathways continued

Compound	CAS number†	Source	Concentration	Disease	Pathway	Origin
Pentane	109-66-0	Breath Faeces	↑↓	CD, UC	Lipid metabolism	Endogenous, Plant
Piperidin-2-one	675-20-7	Faeces	↑	CD	Tropane, piperidine and pyridine alkaloid biosynthesis	Endogenous
Propan-1-ol	71-23-8	Breath Faeces	↑	CD	Propanoate metabolism	Endogenous, Plant, Animal, Bacteria, Fungi
Propan-2-one	67-64-1	Breath	↑↓	CD	Metabolic pathways; Propanoate metabolism; Synthesis and degradation of ketone bodies	Endogenous, Plant, Animal, Bacteria, Tobacco
Sulfane	4/06/7783	Breath	↑↓	CD	Carbon metabolism; Microbial metabolism; Sulphur metabolism; Cystine and methionine metabolism	Endogenous, Plant, Animal, Bacteria, Tobacco
1-methyl-4-propan-2-ylcyclohexa-1,4-diene	99-85-4	Breath Faeces	↑	IBS	NA	Endogenous, Plant

† CAS numbers are unique numerical identifiers assigned by the Chemical Abstracts Service; Adapted from Van Malderen *et al.*[156]
CD = Crohn's disease; IBS = irritable bowel syndrome; UC = ulcerative colitis

1.6 Conclusion

Biomarker development is a hot topic in IBS research. However, at the moment there is insufficient scientific data to support a specific biomarker. This is in part due to the heterogeneity of IBS making it difficult to generalise findings and validate them in a large population. Two players that show promise as biomarkers are mast cells (MCs) and volatile organic compounds (VOCs).

Mast cells have been studied for years and it is clear that they play a role in at least a proportion of IBS patients. However, since they are tissue resident cells, they are difficult to study *in vitro* slowing down new discoveries. New techniques are needed to be able to study MCs *in vitro* and functionally characterise these cells.

Volatile organic compounds have been used for years, for example, in breath tests for lactose malabsorption and bacterial overgrowth. Studies in other pathologies and preliminary studies in IBS have shown that there is potential to extend the use of these VOCs as a biomarker. However, previously used methodologies vary greatly making it difficult to repeat and validate results.

Chapter 2 Aims

Irritable bowel syndrome (IBS) is one of the most prevalent chronic gastrointestinal disorders. Despite its prevalence there are still a lot of questions remaining regarding the underlying pathophysiology. Furthermore, there is a lack of biomarkers to diagnose and monitor patients, and targeted therapy is not available. These difficulties lead to high health care costs and frustrated patients and health care professionals.

The **general aim** of this thesis was to further investigate the pathophysiological mechanisms which play a role in IBS and more precisely to evaluate the **potential of cellular and volatile biomarkers**. Before evaluating these novel biomarkers, the epidemiological characteristics of our local IBS population were studied.

A lot of misinformation about IBS is available on the internet making it difficult for patients and health care professionals alike to find reliable sources of information. Therefore, we decided to create a patient-centred informative website about IBS, www.ibsbelgium.org. The primary goal of the website was to provide scientific information in an easily digestible manner. Apart from providing information and breaking taboo the website helped raise awareness among patients about scientific research on IBS. Patients can read more about recently published research studies and can find information to participate in studies from one of the Flemish universities. In **chapter 3** we provide an overview of the epidemiological characteristics of the population visiting our website.

Afterwards, we investigated the potential of cellular biomarkers, more specifically those linked to mast cells (MCs), for diagnosing and monitoring IBS. MCs are tissue resident cells making them difficult to isolate and study *in vitro.* To facilitate future MC research, we optimised and validated a human MC model cultured out of progenitor cells isolated from

peripheral blood. We characterised naïve MCs of both IBS patients and healthy controls to assess if there are any baseline differences. The results of the immunophenotypical and functional characterisation of these MCs are shown in **chapter 4**. The MCs cultured in this model originate from progenitor cells which means they are naïve and have not been exposed to the 'diseased' gut environment. Therefore, we developed an IBS-like environment with the help of supernatant of colonic biopsies of IBS patients. After incubation of the MCs in this IBS-like environment we re-evaluated their immunophenotypical and functional characteristics to assess if any changes in the MC occurred in **chapter 4**.

In a more clinical part of our biomarker quest, we investigated volatile organic compounds (VOCs) as a non-invasive biomarker alternative for diagnosing and monitoring IBS. VOCs are found in bodily excretions like breath and faeces making them easily accessible. However, analysis can be both expensive and difficult with techniques like gas chromatography – mass spectrometry. Therefore, we assessed the feasibility of VOC profiling with the help of ion mobility spectrometry (IMS) in IBS in **chapter 5**. IMS is a cheaper technique which requires no trained professionals. First, we compared VOC profiles of IBS patients with healthy controls in both breath and faecal samples. Second, we further characterised IBS patients with the help of VOC profiling based on clinical characteristics like dominant stool type, psychological comorbidities, and microbiota influencing therapies.

Chapter 3 Epidemiological characteristics

Based on

Van Malderen K, De Man JG, De Winter BY, De Schepper HU. Epidemiological characteristics of a population visiting a patient-centered informative website about irritable bowel syndrome. *Acta Gastro-Enterologica Belgica* (2023) Volume 86:17-25

3.1 Introduction

As described in **chapter 1,** irritable bowel syndrome (IBS) is a prevalent gastrointestinal disorder affecting mostly women and young people.[170] Based on the dominant stool pattern patients can be divided into four subtypes: diarrhoea (IBS-D), constipation (IBS-C), mixed (IBS-M), and unspecified (IBS-U).

The exact aetiology is largely unknown, but patients often report an infectious, traumatic, or stressful event preceding the onset of symptoms.[171] The underlying pathophysiology is multifactorial, and involves increased permeability, dysmotility, dysbiosis, food hypersensitivity, visceral hypersensitivity, inflammation, genetics, and psychological stress.[19,20] There has been a lot of attention for the role of the brain-gut-axis and the interplay between IBS and psychological disorders. A recent meta-analysis noted that psychological comorbidities are prevalent in IBS patients with 44% suffering from anxiety and 36% from depression.[55] There is a complex interplay with stress exacerbating symptoms and excessive symptoms causing further stress. Recent large-scale genome-wide analysis has shown shared genetic pathways between IBS and mood disorders such as anxiety and depression.[172]

Over 60% of patients report a clear link between symptoms and food intake. This makes dietary changes an appealing therapeutic option, however, little is known on the percentage of the population using various dietary options.[31] A group of foods frequently discussed in relation to IBS are the fermentable oligo-, di-, monosaccharides and polyols (FODMAPs). FODMAPs are poorly absorbed in the small intestine causing an osmotic effect with subsequent increased water content in the lumen. When these FODMAPs arrive in the colon they are fermented by the microbiota causing gas production.[31] Apart from these osmotic

and fermentation effects, FODMAPs can also cause immune activation and changes in gut microbiota.[173,174]

IBS has a major impact on quality of life of patients and is associated with high healthcare costs because of difficulties in diagnosis and treatment leading to frequent consultations with health care providers.[17,175] A positive diagnosis of IBS is made with the help of the Rome IV criteria and some limited blood and stool sample testing to exclude other gastrointestinal diseases which can present similarly such as inflammatory bowel disease, celiac disease, and colon cancer as described in Chapter 1. Additional testing can be necessary in the presence of red flag symptoms like blood in the stools, anaemia, weight loss, fever, older age at the start of symptoms, or a family history of colon cancer.[4] Subsequent treatment is focused on the predominant symptom (abdominal pain, constipation, or diarrhoea) and can consist of dietary-, pharmacological-, and/or psychotherapy. Patients are also encouraged to make lifestyle changes with sufficient sleep, relaxation, and physical exercise.[82,88]

Education and a strong physician-patient relationship positively impact symptoms and improve quality of life through illness coherence and acceptance.[83] However, research has shown that 77% of IBS patients feel insufficiently informed and have misconceptions on the cause and appropriate treatment for IBS.[84,85] There is a demand for information coming from patients with an increasing role for internet-based resources.[86,87]

The aim of this study was to evaluate the characteristics of a Dutch speaking population visiting a Belgian patient-centred informative website about IBS. We wanted to gain better insight in the presence of symptoms and red flags, the use of the health care system,

psychological comorbidities, the symptom severity, the quality of life and the lifestyle habits of our local IBS population.

3.2 Methodology

3.2.1 Study population

Participants were recruited through a patient-centred informative website (www.ibsbelgium.org) developed in 2019 by KVM and HDS. Most visitors of the website found it through a Google search. The website was promoted via several social media channels and flyers distributed amongst patients and general practitioners. Apart from promotion on the website and social media no further measures were taken to increase the number of respondents. On the website, visitors had the opportunity to participate in several surveys aimed at evaluating different aspects of IBS. Five surveys will be discussed in this paper, each containing elements of multiple validated questionnaires. Participants were free to decide how many of these surveys they wanted to complete. The 'Symptom assessment' was a short survey on the homepage of the website, it was also the only survey of which patients received a result. The other surveys were solely for the purpose of research with no benefit to the participants and could be found in the 'research section' of the website. Data was collected via QUALTRICS, licensed via the University of Antwerp, except for the 'Symptom assessment' which was a build in feature of the website. Furthermore, apart from the 'Symptom assessment', all surveys were directed at patients who received a diagnosis of IBS while the former was directed towards all visitors of the website who suspected they might have IBS. Before participation, visitors were required to give digital informed consent as approved by the Ethics Committee of the University of Antwerp/Antwerp University

hospital (19/41/449). All data was collected anonymously and cannot be traced back to the individual participant. It is not possible to know if visitors completed more than one survey. Therefore, it was also not possible to correlate the results if patients completed multiple surveys. Patients were excluded if they reported comorbid inflammatory bowel disease, celiac disease, or another gastrointestinal disorder which could influence the reported symptoms.

The Rome IV criteria for IBS were evaluated in each survey, patients were subsequently divided into a Rome positive and Rome negative population for further analysis. In the Rome positive population, patients were further subtyped based on the dominant stool pattern into IBS-diarrhoea, IBS-constipation, IBS-mixed, and IBS-unspecified.

3.2.2 Questionnaires

The questioned red flag symptoms were bloody stools, fever, weight loss (>3kg in the last 3 months), family history of colon cancer, and start of symptoms after 50 years of age. To evaluate symptom severity the IBS symptom severity index (IBS-SSS) was used.[176] The IBS-SSS contains five questions, each scored between 0 and 100, assessing abdominal pain, bloating, stool pattern, and influence of their symptoms on daily life. A total score between 75 and 174 indicated mild IBS, between 175 and 299 moderate IBS, and more than 300 severe IBS. The Visceral sensitivity index (VSI) assesses gastrointestinal (GI) specific anxiety, fear, and hypervigilance.[177] It contains 15 questions each scored between 0 and 5, with a higher score indicating more GI-specific anxiety. To assess comorbid anxiety and depression the Hospital anxiety and depression score (HADS) was used.[178] It consists of two subscales with seven questions each scored between 0 and 3. A score of more than eight

suggests comorbid anxiety or depression.[179] To assess IBS-related quality of life two questionnaires were used. The IBS Quality of life (IBS-QOL) contains 34 questions, and the IBS-36 contains 36 questions.[180,181] They evaluate the influence of IBS on different aspects of daily life. Both questionnaires were normalised to a score between 0 and 100 with a higher score indicating a worse quality of life. Physical activity levels were assessed with the Baecke physical activity questionnaire looking at daily physical activity, sports, and leisure.[182] The Food frequency questionnaire (FFQ) was used to evaluate food intake in the last three months.[183] It also contained some general questions assessing special diets (vegetarian, veganism, FODMAP) and exclusion of certain food groups (dairy, meat, poultry, eggs, fish).

3.2.3 Statistical analysis

Categorical characteristics were expressed as n (%) and analysed with Fisher's exact test (Rome positive versus Rome negative) or Chi square (IBS-D versus IBS-C versus IBS-M versus IBS-U). Continuous variables following a Gaussian distribution were expressed as mean (standard deviation) and analysed using unpaired Student's t-tests (Rome positive versus Rome negative) or one-way ANOVA with Tukeys multiple comparisons test (IBS-D versus IBS-C versus IBS-M versus IBS-U). Non-parametric continuous variables were expressed as median (range) and analysed with Mann-Whitney U (Rome positive versus Rome negative) or Kruskal-Wallis (IBS-D versus IBS-C versus IBS-M versus IBS-U). A significance level of 0.05 was used throughout the analysis.

3.3 Results

3.3.1 Symptom assessment

A total of 2000 participants completed the 'Symptom assessment' evaluating the prevalence of Rome IV criteria for IBS and red flag symptoms (table 3.1, figure 3.1). Of these, 69.2% fulfilled the Rome IV criteria. When patients did not fulfil the Rome IV criteria (more than one reason possible) this was most frequently (49.2%) due to a symptom duration shorter than six months. Other reasons were insufficient relation with defaecation or stool form (45.8%), and insufficient days with abdominal pain (30.7%).

Table 3.1: Symptom assessment

	All	**Rome negative**	**Rome positive**						
			Total	**p-value†**	**IBS-D**	**IBS-C**	**IBS-M**	**IBS-U**	**p-value‡**
N (%)	2000	616 (30.8)	1384 (69.2)		497 (35.9)	244 (17.6)	615 (44.4)	28 (2.0)	
RBPA (%)	232 (11.6)	59 (9.6)	173 (12.5)	0.07	41 (8.2)	38 (15.6)	91 (14.8)	3 (10.7)	**0.004**
Weight loss (%)	232 (11.6)	83 (13.5)	149 (10.8)	0.08	51 (10.3)	26 (10.7)	71 (11.5)	1 (3.6)	0.57
Age >50years (%)	135 (6.8)	54 (8.8)	81 (5.9)	**0.02**	33 (6.6)	17 (7.0)	31 (5.0)	0 (0)	0.31
Fever (%)	69 (3.5)	18 (2.9)	51 (3.7)	0.43	27 (5.4)	6 (2.5)	17 (2.8)	1 (3.6)	0.08
FH CA (%)	284 (14.2)	120 (19.5)	164 (11.9)	0.85	93 (18.7)	51 (20.9)	112 (18.2)	8 (28.6)	0.47
Any red flag symptom (%)	838 (41.9)	261 (42.4)	577 (41.7)	0.81	201 (40.4)	115 (47.1)	251 (40.8)	10 (35.7)	0.27

-C = constipation; -D = diarrhoea; FH CA = family history of colon cancer; IBS = irritable bowel syndrome; -M = mixed; RBPA = red blood loss per anum; -U = unspecified
Significant differences are in bold
†Comparison Rome positive and Rome negative population with Fisher's exact
‡Comparison IBS subtypes with Chi square

When looking at the dominant stool pattern in the Rome IV positive population we found that the predominant subtype was IBS-M (44.4%) followed by IBS-D (35.9%), IBS-C (17.6%), and IBS-U (2.0%).

Approximately 40% of all participants had at least one red flag symptom (figure 3.1). This was not significantly different between Rome positive and negative patients. When patients had at least one red flag symptom they received the advice to consult a health care professional. The most prevalent red flag symptom in all patients was a family history of colon cancer (14.2%). This was followed by weight loss in the Rome negative population (13.5%) and bloody stools in the Rome positive population (12.5%). The prevalence of red flag symptoms was not significantly different between patient subtypes except for bloody stools (p=0.004), which was less prevalent in IBS-D compared to other subtypes.

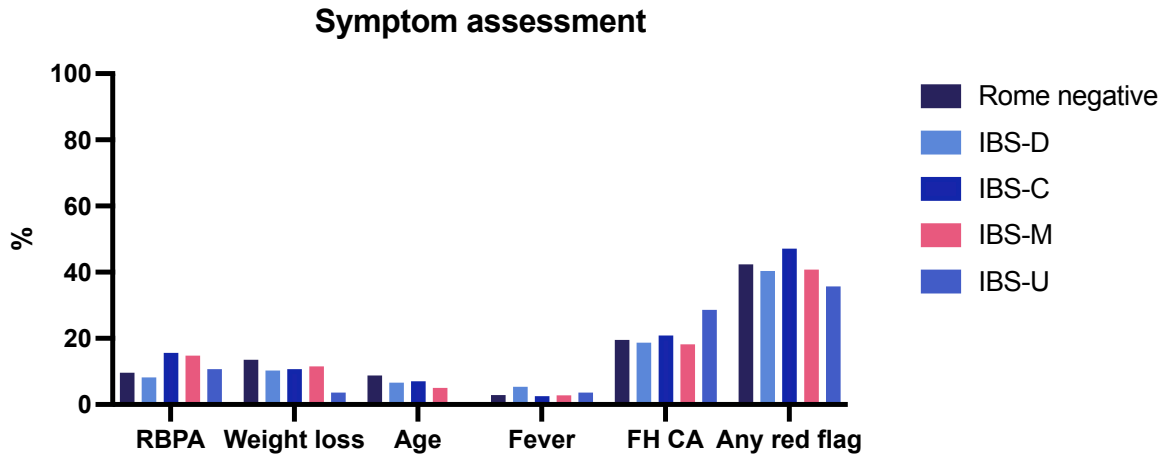


Figure 3.1: Prevalence of red flag symptoms
-C = constipation; -D = diarrhoea; FH CA = family history of colon cancer; IBS = irritable bowel syndrome; -M = mixed; RBPA = red blood loss per anum; -U = unspecified

3.3.2 General assessment

A total of 74 patients completed the 'General assessment' (table 3.2, figure 3.2) of which 68.9% fulfilled the Rome IV criteria. There were no significant differences between patient subtypes when looking at BMI, alcohol use, or smoking. Circa one in five patients reported a post-infectious onset of their symptoms (26.1% Rome negative versus 17.7% Rome positive). Almost all patients (95.9%), had consulted a general practitioner (GP) for their complaints at one point in time, this was however more prevalent in the Rome positive population (87.0% Rome negative versus 100% Rome positive). When we look at the last three months, approximately half of the patients consulted their GP. Seventy-six percent of patients had consulted a gastroenterologist at one point in time and 18% had seen a gastroenterologist in the last three months (figure 3.2).

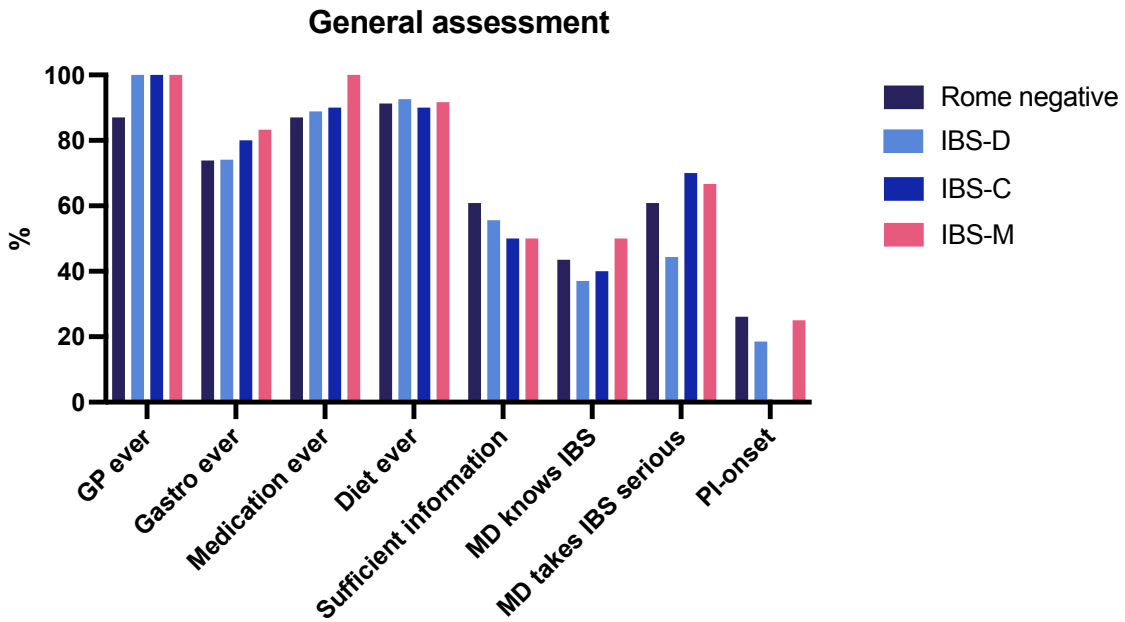


Figure 3.2: Use of the health care system and post-infectious onset
Because of limited sample size IBS-U is not shown.
-C = constipation; -D = diarrhoea; Gastro = gastroenterologist; GP = general practitioner; IBS = irritable bowel syndrome; MD = medical doctor; -M = mixed; PI = post-infectious

Table 3.2: General assessment

	All	Rome negative	Rome positive						
			Total	p-value†	IBS-D	IBS-C	IBS-M	IBS-U	p-value‡
N (%)	74	23 (31.1)	51 (68.9)		27 (36.5)	10 (19.6)	12 (16.2)	2 (2.7)	
Age mean (SD)	41 (14)	44 (15)	41 (13)	0.39	41 (12)	46 (16)	38 (14)	21 (4)	0.11
N females (%)	66 (89.2)	18 (78.3)	48 (94.1)	0.10	26 (96.3)	8 (80.0)	12 (100)	2 (100)	0.19
BMI mean (SD)	24.0 (5.0)	24.1 (5.1)	23.9 (5.0)	0.90	23.8 (5.2)	22.2 (3.2)	26.0 (5.7)	20.6 (3.4)	0.24
Units of alcohol per week median (range)	1 (0 – 25)	1 (0 – 25)	1 (0 – 21)	0.73	1 (0 – 21)	2 (0 – 15)	1 (0 – 6)	0 (0 – 0)	0.24
Smoking (%)	6 (8.1)	2 (8.7)	4 (7.8)	1.00	3 (11.1)	0 (0.0)	1 (8.3)	0 (0.0)	0.70
GP ever (%)	71 (95.9)	20 (87.0)	51 (100)	**0.03**	27 (100)	10 (100)	12 (100)	2 (100)	1.00
GP last 3 months (%)	32 (43.2)	11 (47.8)	21 (41.2)	0.62	12 (44.4)	4 (40.0)	5 (41.7)	0 (0.0)	0.68
Gastro ever (%)	56 (75.7)	17 (73.9)	39 (76.5)	1.00	20 (74.1)	8 (80.0)	10 (83.3)	1 (50.0)	0.74
Gastro last 3 months (%)	13 (17.6)	6 (26.1)	7 (13.7)	0.21	4 (14.8)	2 (20.0)	1 (8.3)	0 (0.0)	0.81
Medication ever (%)	67 (90.5)	20 (87.0)	47 (92.2)	0.67	24 (88.9)	9 (90.0)	12 (100)	2 (100)	0.65
Medication last 3 months (%)	49 (66.2)	16 (69.6)	33 (64.7)	0.79	16 (59.3)	7 (70.0)	9 (75.0)	1 (50.0)	0.86
Diet ever (%)	67 (90.5)	21 (91.3)	46 (90.2)	1.00	25 (92.6)	9 (90.0)	11 (91.7)	1 (50.0)	0.28
Diet last 3 months (%)	55 (74.3)	18 (78.3)	37 (72.5)	0.78	18 (66.7)	9 (90.0)	9 (75.0)	1 (50.0)	0.47
Sufficient information (%)	40 (54.1)	14 (60.9)	26 (51.0)	0.46	15 (55.6)	5 (50.0)	6 (50.0)	0 (0.0)	0.51
MD knows IBS (%)	30 (40.5)	10 (43.5)	20 (39.2)	0.80	10 (37.0)	4 (40.0)	6 (50.0)	0 (0.0)	0.59
MD takes IBS seriously (%)	42 (56.8)	14 (60.9)	28 (54.9)	0.80	12 (44.4)	7 (70.0)	8 (66.7)	1 (50.0)	0.42
Post-infectious onset (%)	15 (20.3)	6 (26.1)	9 (17.7)	0.53	5 (18.5)	0 (0.0)	3 (25.0)	1 (50.0)	0.26

BMI = body mass index; -C = constipation; -D = diarrhoea; Gastro = gastroenterologist; GP = general practitioner; IBS = irritable bowel syndrome; MD = medical doctor; -M = mixed; SD = standard deviation; -U = unspecified; Significant differences are in bold

†Comparison Rome positive and Rome negative population with Fisher's exact for categorical characteristics; Mann-Whitney U for non-parametric continuous variables; Unpaired t-tests for parametric continuous variables

‡Comparison IBS subtypes with Chi square for categorical characteristics; Kruskal-Wallis for non-parametric continuous variables; One-way ANOVA for parametric continuous variables

Most patients (90.5%) had tried any form of medication and/or diet to relieve their symptoms, although we did not ask for the details of the treatment strategy or diet. When comparing the Rome positive and negative population we could see a trend for higher use of the health care system in the last three months for the Rome negative population, however no significance was reached (GP visit p=0.62; gastro visit p=0.21).

Only 61% of the Rome negative population believed there was sufficient information available about IBS, in the Rome positive group this was even lower with 51%. Only 41% of patients thought their physician had sufficient knowledge about IBS and a slightly higher percentage, 57%, felt that their physician took IBS seriously.

3.3.3 Symptom severity and psychological symptoms

Seventy-one patients completed the survey evaluating symptom severity and psychological comorbidities (table 3.3, figure 3.3). 74.6% were Rome positive. Most patients had moderate to severe symptoms with a mean IBS-SSS score of 272 in the Rome negative population and 282 in the Rome positive population. The Rome positive population had a trend towards more patients fulfilling the criteria for comorbid anxiety (67.9% Rome positive versus 55.6% Rome negative) or depression (41.5% Rome positive versus 33.3% Rome negative). Both groups had a VSI score around 45 (45.9 Rome negative versus 47.2 Rome positive). There were no significant differences between the patient subtypes in the Rome positive population for any of the discussed scores.

Table 3.3: Symptom severity and psychological symptoms

	All	Rome negative	Rome positive						
			Total	p-value†	IBS-D	IBS-C	IBS-M	IBS-U	p-value‡
N (%)	71	18 (25.4)	53 (74.6)		20 (28.2)	10 (14.1)	22 (31.0)	1 (1.4)	
N females (%)	59 (83.1)	15 (83.3)	44 (83.0)	1.00	16 (80)	10 (100)	18 (36.4)	0 (0)	0.07
Age mean (SD)	45 (17)	49 (15)	44 (17)	0.27	46 (17)	47 (15)	41 (18)	52 (0)	0.69
IBS-SSS mean (SD)	279 (63)	272 (58)	282 (65)	0.55	278 (68)	272 (53)	290 (69)	300 (0)	0.87
HADS-Anxiety mean (SD)	9.4 (4.2)	8.9 (4.7)	9.6 (4.0)	0.53	9.8 (4.4)	8.8 (2.6)	10.1 (3.9)	2.0 (0)	0.22
Positive anxiety score§ (%)	46 (64.8)	10 (55.6)	36 (67.9)	0.40	15 (75)	7 (70)	14 (63.6)	0 (0)	0.43
HADS-Depression mean (SD)	6.9 (3.8)	6.4 (3.3)	7.0 (4.0)	0.60	7.4 (4.6)	5.4 (4.0)	7.4 (3.5)	6.0 (0)	0.56
Positive depression score§ (%)	28 (39.4)	6 (33.3)	22 (41.5)	0.59	9 (45)	2 (20)	11 (50)	0 (0)	0.34
VSI mean (SD)	46.9 (13.9)	45.9 (14.2)	47.2 (13.8)	0.73	49.3 (15.4)	48.9 (7.2)	45.0 (14.8)	41.0 (0)	0.72

A = anxiety; -C = constipation; -D = diarrhoea; D = depression; HADS = hospital anxiety and depression score; IBS = irritable bowel syndrome; IBS-SSS = symptom severity score; -M = mixed; SD = standard deviation; -U = unspecified

Significant differences are in bold

†Comparison Rome positive and Rome negative population with Fisher's exact for categorical characteristics; Unpaired t-tests for parametric continuous variables

‡Comparison IBS subtypes with Chi square for categorical characteristics; One-way ANOVA for parametric continuous variables

§A positive score is defined as >8

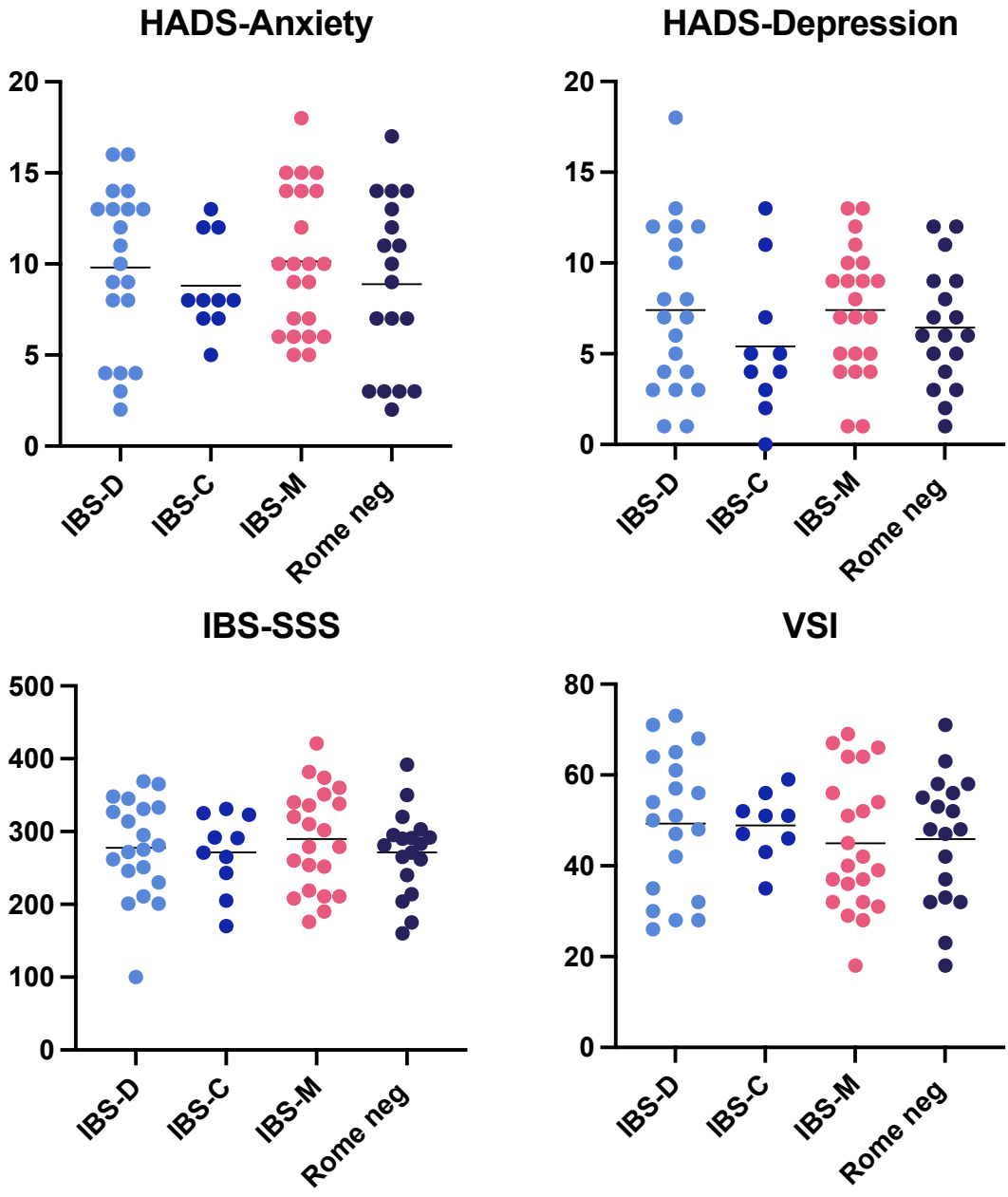


Figure 3: Symptom severity and psychological symptoms
Because of limited sample size IBS-U is not shown. The HADS scales use a score between 0 and 20 with a score of more than eight suggesting comorbid anxiety or depression. The IBS-SSS is presented as a score between 0 and 500 with a higher score indicating more severe IBS. The VSI presents as a score between 0 and 75 with a higher score indicating more gastrointestinal specific anxiety.
A = anxiety; -C = constipation; -D = diarrhoea; D = depression; HADS = hospital anxiety and depression score; IBS = irritable bowel syndrome; IBS-SSS = symptom severity score; -M = mixed; VSI = visceral sensitivity index

3.3.4 Diet and exercise

There were 51 participants who completed the survey about diet and exercise (table 3.4, figure 3.4). Only six participants (11.8%) were Rome negative, the remaining 45 participants (88.2%) were Rome positive. 72.5% of patients had a normal BMI with an average BMI of 24.2 in the Rome negative group and 22.6 in the Rome positive group. The majority of participants (68.6%) spent most of their day at work, while a slightly higher percentage of the Rome positive population, although not significant, (21.7% Rome positive versus 16.7% Rome negative) spent most of their time at home. 73% of participants practiced at least one sport. No Rome negative participants followed the FODMAP diet compared to 24.4% in the Rome positive population. However, the Rome negative population had a trend towards more participants following a lactose-free diet (40.0% Rome negative versus 26.8% Rome positive).

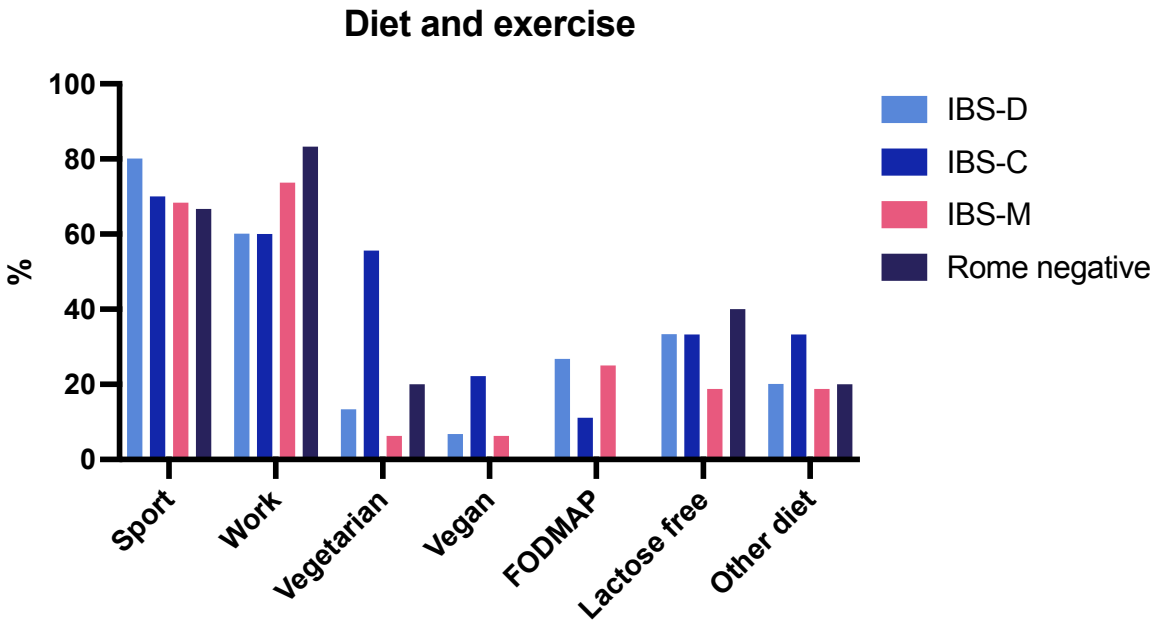


Figure 3.4: Diet and exercise
Because of limited sample size IBS-U is not shown.
-C = constipation; -D = diarrhoea; FODMAP = fermentable oligo-, di-, monosaccharides and polyols; IBS = irritable bowel syndrome; -M = mixed

Table 3.4: Diet and exercise

	All	Rome negative	Rome positive						
			Total	p-value†	IBS-D	IBS-C	IBS-M	IBS-U	p-value‡
N (%)	51	6 (11.5)	45 (88.2)		15 (28.8)	10 (19.2)	19 (37.3)	1 (1.9)	
N females (%)	47 (92.2)	6 (100)	41 (89.1)	1.00	12 (80.0)	9 (90.0)	19 (100)	1 (100)	0.55
Age mean (SD)	41 (14)	45 (12)	41 (15)	0.58	45 (16)	44 (12)	37 (14)	28 (0)	0.28
BMI mean (SD)	22.7 (4.4)	24.2 (6.3)	22.6 (4.2)	0.40	22.5 (4.0)	23.0 (4.1)	22.6 (4.5)	19.6 (0)	0.89
BMI: underweight (%)	6 (11.8)	0 (0.0)	6 (13.0)	1.00	1 (6.7)	1 (10.0)	4 (21.1)	0 (0.0)	0.65
BMI: normal (%)	37 (72.5)	5 (83.3)	32 (69.6)	0.66	12 (80.0)	6 (60.0)	13 (68.4)	1 (100)	0.61
BMI: overweight (%)	4 (7.8)	0 (0.0)	4 (8.7)	1.00	0 (0.0)	3 (30.0)	1 (5.3)	0 (0.0)	0.06
BMI: obese (%)	5 (9.8)	1 (16.7)	4 (8.7)	0.47	2 (13.3)	0 (0.0)	2 (10.5)	0 (0.0)	0.68
Most time: work (%)	35 (68.6)	5 (83.3)	30 (65.2)	0.65	9 (60.0)	6 (60.0)	14 (73.7)	1 (100)	0.79
Most time: house (%)	11 (21.6)	1 (16.7)	10 (21.7)	1.00	4 (26.7)	4 (40.0)	2 (10.5)	0 (0.0)	0.25
Most time: study (%)	5 (9.8)	0 (0.0)	5 (10.9)	1.00	2 (13.3)	0 (0.0)	3 (15.8)	0 (0.0)	0.62
Sport (%)	37 (72.5)	4 (66.7)	33 (73.3)	0.61	12 (80.0)	7 (70.0)	13 (68.4)	1 (100)	0.71
Sport: <1h (%)	1 (2.7)	0 (0.0)	1 (3.0)	1.00	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0.28
Sport: 1-2h (%)	15 (40.5)	0 (0.0)	15 (45.5)	0.13	6 (50.0)	3 (42.9)	6 (46.2)	0 (0.0)	0.81
Sport: 2-3h (%)	8 (21.6)	2 (50.0)	6 (18.2))	0.20	4 (33.3)	1 (14.3)	1 (7.7)	1 (100)	0.09
Sport: 3-4h (%)	5 (13.5)	0 (0.0)	5 (15.2)	1.00	1 (8.3)	1 (14.3)	3 (23.1)	0 (0.0)	0.74
Sport: >4h (%)	8 (21.6)	2 (50.0)	6 (18.2)	0.20	4 (33.3)	1 (14.3)	1 (7.7)	0 (0.0)	0.38
Vegetarian (%)	9 (19.6)[a]	1 (20.0)[b]	8 (19.5)[c]	1.00	2 (13.3)	5 (55.6)[d]	1 (6.3)[e]	0 (0.0)	**0.02**
Vegan (%)	4 (8.7)[a]	0 (0.0)[b]	4 (9.8)[c]	1.00	1 (6.7)	2 (22.2)[d]	1 (6.3)[e]	0 (0.0)	0.56
FODMAP (%)	10 (21.7)[a]	0 (0.0)[b]	10 (24.4)[c]	0.57	4 (26.7)	1 (11.1)[d]	4 (25.0)[e]	1 (100)	0.26
Other diet (%)	10 (21.7)[a]	1 (20.0)[b]	9 (22.0)[c]	1.00	3 (20.0)	3 (33.3)[d]	3 (18.8)[e]	0 (0.0)	0.78
Lactose free (%)	13 (28.3)[a]	2 (40.0)[b]	11 (26.8)[c]	0.61	5 (33.3)	3 (33.3)[d]	3 (18.8)[e]	0 (0.0)	0.70

-C = constipation; -D = diarrhoea; IBS = irritable bowel syndrome; -M = mixed; SD = standard deviation
[a]46 participants; [b]5 participants; [c]41 participants; [d]9 participants; [e]16 participants
Significant differences are in bold

3.3.5 Quality of life

Thirty-four patients completed the survey looking into their quality of life (table 3.5, figure 3.5) of which 73.5% were Rome positive, no participants belonged to the IBS-U subtype. The Rome positive and negative group had similar scores with an average of 63.2 and 55.4 on the IBS-QOL, and 54.9 and 64.0 on the IBS-36. However, when we compared the different IBS subtypes with each other we did see a difference with an average score for the IBS-QOL of 58.5 in IBS-D, 55.8 in IBS-C, and 70.6 in IBS-M (p=0.04). In the IBS-36 these were 51.0, 46.5, and 63.0 respectively (p=0.01).

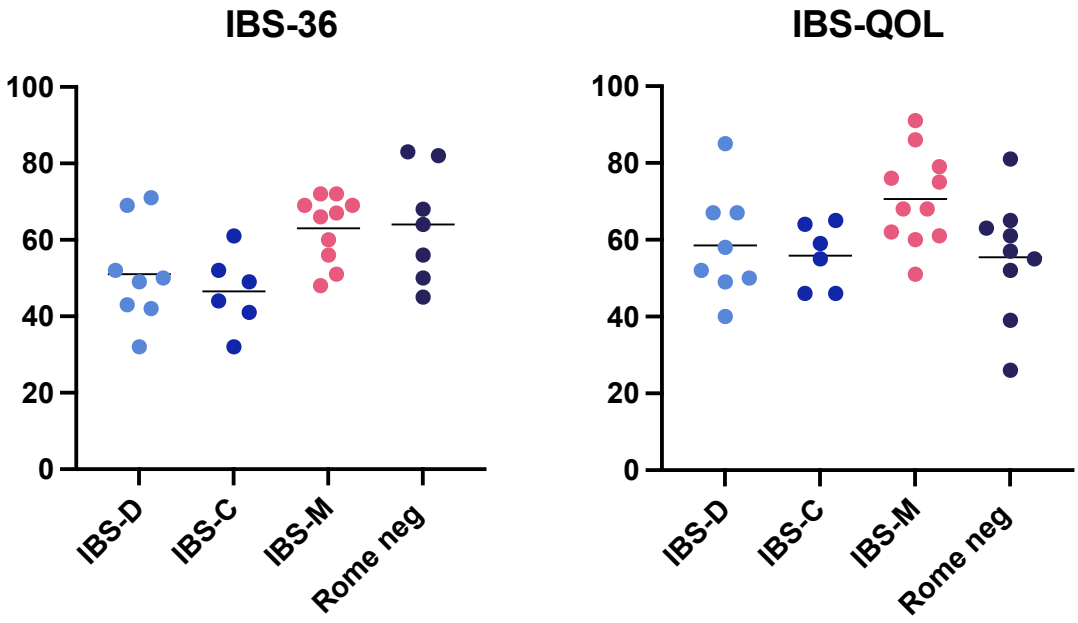


Figure 3.5: Quality of life
-C = constipation; -D = diarrhoea; IBS = irritable bowel syndrome; IBS-QOL = quality of life score; -M = mixed

Table 3.5: Quality of life

	All	Rome negative	Rome positive					
			Total	p-value†	IBS-D	IBS-C	IBS-M	p-value‡
N (%)	34	9 (26.5)	25 (73.5)		8 (32.0)	6 (24.0)	11 (44.0)	
N females (%)	30 (88.2)	8 (88.9)	22 (88.0)	1.00	7 (87.5)	5 (83.3)	10 (90.9)	0.90
Age (mean, SD)	43 (14)	50 (16)	41 (13)	0.12	35 (7)	50 (17)	41 (13)	0.09
IBS-QOL (mean, SD)	61.1 (14.2)	55.4 (15.8)	63.2 (13.4)	0.16	58.5 (14.1)	55.8 (8.4)	70.6 (12.0)	**0.04**
IBS-36 (mean, SD)	56.9 (13.3)	64.0 (14.8)	54.9 (12.5)	0.11	51.0 (13.3)	46.5 (9.9)	63.0 (8.7)	**0.01**

-C = constipation; -D = diarrhoea; IBS = irritable bowel syndrome; IBS-QOL = quality of life score; -M = mixed; SD = standard deviation

Significant differences are in bold

†Comparison Rome positive and Rome negative population with Fisher's exact for categorical characteristics; Unpaired t-tests for parametric continuous variables

‡Comparison IBS subtypes with Chi square for categorical characteristics; One-way ANOVA for parametric continuous variables

3.4 Discussion and conclusion

Irritable bowel syndrome is one of the most prevalent gastrointestinal disorders, but trustworthy information can be difficult to find for patients. After establishing a patient-centred informative website about IBS, we decided to further characterize patients visiting the website. We can assume that people who completed our 'symptom assessment' were suffering from gastrointestinal complaints which they believed could be due to IBS. In the end, 70% of these people fulfilled the Rome IV criteria. However, when participants did not fulfil the criteria, this was in almost half of the cases due to an insufficient duration of their symptoms (less than six months). We can suspect that a number of these participants will continue to experience symptoms and will fulfil the Rome IV criteria when re-evaluating later. Another reason for not fulfilling the Rome IV criteria was insufficient abdominal pain or insufficient changes in the stool pattern. However, this could point towards a milder phenotype or a well-treated patient.

IBS subtypes were further determined with the help of the Bristol stool chart. Most of the patients assessed their IBS as being from the mixed phenotype. This is in contrast with other studies reporting an IBS-D predominant population.[184] Assessing the dominant stool pattern is, however, very subjective and it is unclear if these patients would be classified the same if seen by a health care professional. Furthermore, it has been described that patients often change subtype over time making a single assessment of stool consistency less accurate.[185]

There was a high prevalence of red flag symptoms (42%) in patients completing the 'symptom assessment', mainly a familial history of colon cancer, weight loss, and rectal bleeding were reported. However, since we have no information on pre-existing conditions

or if prior testing has already been performed, we do not know if this statement is applicable to all IBS patients. For example, it is possible that IBS patients experiencing red flag symptoms are more inclined to search the internet for information. It does, however, emphasize the importance of a thorough anamnesis when seeing patients to make an accurate diagnosis and not miss other diseases like colon cancer, inflammatory bowel disease, or celiac disease which might develop over time.

Of the patients completing the 'general assessment', one in five remembered a post-infectious onset of their symptoms which is slightly higher compared to previous studies reporting a prevalence of 6-17%.[186,187] However, this higher prevalence is mainly evident in the Rome negative group with a prevalence of 26% compared to 18% in the Rome positive population. When we take a closer look at these Rome negative PI-IBS patients half of them had a symptom duration shorter than six months. Therefore, it is possible that these patients experience lingering effects from the infection rather than a PI-IBS.

When evaluating the use of our health care system we can see that most patients had consulted a health care professional and tried some form of therapy to relieve their symptoms at one point. The Rome negative population had a higher use of the health care system in the last three months which can be because they are in the process of actively searching for a diagnosis and treatment.

Sadly, less than half of participants believed their treating physician had sufficient knowledge about IBS. However, over half of participants did have the feeling that their physician took IBS seriously. According to participants there is also a major lack in accessible and scientifically correct information for patients which is in accordance with

other studies looking into this topic.[86,87] This emphasizes the urgent need for information and education not only for patients but also for health care professionals.

A large percentage reported severe symptoms, and the presence of comorbid anxiety disorders was also higher than reported in other studies, 65% versus 44% previously.[55] The presence of depression in our population was 40% which is similar to the previously reported 36%.[55] Additionally, most patients reported an important impact of their symptoms on QOL with IBS-D and IBS-M reporting a larger impact on QOL compared to IBS-C. This is in accordance with a previous study comparing QOL between the different subtypes.[188] They found that patients with IBS-D or IBS-M experienced more difficulties with daily activities and avoided food more frequently with the defaecation frequency being an important determinant.[188]

The reported disease severity and impact on QOL could in part be due to an inclusion bias. Patients with severe symptoms might be more inclined to search for information on the internet to better cope with their disease. By searching for information online there is a higher chance of finding our call for participation in digital questionnaires. Furthermore, it is possible that patients participating in scientific research are more actively thinking about their disease throughout the day which can lead to hypervigilance about symptoms.

Most participants had a BMI within the normal range and exercise regularly both of which have a beneficial effect on symptoms. Most participants spent much of their time at work with around one fifth of the Rome positive population spending most of their time on their household. When looking at dietary therapy no Rome negative patients followed the FODMAP diet. There was a larger percentage of Rome negative patients following a lactose-

free diet. This could be because a lactose-free diet is oftentimes a first-line therapy while the FODMAP diet is more elaborate and difficult to follow which is mostly suggested after failing other therapy options. Furthermore, patients can easily initiate a lactose-free diet without any professional guidance making it an easily accessible treatment option early in the disease course.

A limitation of our study is the limited number of participants in some surveys. This might be partly explained by differences in location on the website and the time surveys were online. The ‘Symptom assessment’ was clearly visible on the homepage of the website and while the other surveys were also promoted on the homepage, respondents had to visit the ‘research section’ to participate. Furthermore, the ‘Symptom assessment’ had been available since the start of the website whereas the other surveys were published at a later time.

Furthermore, since inclusion is digital, we are dealing with a poorly defined patient population, and we cannot know for certain if symptoms are related to IBS or another gastrointestinal disease. The Rome IV criteria are relatively strict, and we can assume that part of the Rome negative population was clinically diagnosed and treated as IBS patients. For this reason, we decided to also report data about the Rome negative population separately and compare them to the Rome positive population. Few differences were observed between the Rome negative and positive population. It is likely that, at least part of, the Rome negative population presents a fifth subgroup of IBS patients rather than a distinctly separate population. We repeated statistical analyses with the Rome negative population as a fifth subtype, however, since this did not significantly alter our results and conclusions, we did not report these results.

Lastly, since data was collected anonymously, we were not able to link the answers of the surveys to each other limiting our ability to connect different aspects of IBS. Still, our study suggests areas of interest and reveals several potential research topics for future IBS research. First, it would be interesting to correlate the different aspects we studied with each other. On the one hand, we could achieve this by conducting a large study evaluating all these variables at once. On the other hand, it would be interesting to highlight certain aspects and study these correlations in more detail, for example the relation between dietary measures and QOL. Second, it would be interesting to further study the effect of information and patient – health care professional relationship. For example, do patients reporting insufficient information have different characteristics; what would be the effect of education as an intervention. Third, we demonstrated a high prevalence of red flag symptoms. However, we do not have any additional information about these patients such as prior testing or follow-up. A more in-depth analysis of these patients would be valuable (do they consult a health care professional, which tests are performed, is there another diagnosis in the end).

In conclusion, our study further validates the importance of a thorough characterization of the IBS patients we encounter in our clinical practices. Red flag symptoms are prevalent as well as comorbid psychological disorders. Despite consulting health care professionals and trying different therapies a lot of patients still experience moderate to severe symptoms with an important impact on quality of life. One of the main take-home messages of this study is the obvious need for information of high scientific quality and the need for education of both health care professionals and patients.

Chapter 4 Mast cells in irritable bowel syndrome

Based on

Van Malderen K, Elst J, De Man J, Ebo D, De Winter B, Sabato V, De Schepper HU. Characterisation of human peripheral blood cultured mast cells in the pathophysiology of irritable bowel syndrome. *Research project will be continued*

4.1 Introduction

Irritable bowel syndrome (IBS), as described in **chapter 1,** is a chronic gastrointestinal disorder characterised by abdominal pain and an altered bowel habit. It affects 4-11% of the population making it one of the most prevalent gastrointestinal disorders.[6,189] Four IBS subtypes are described according to the dominant stool pattern: diarrhoea (IBS-D), constipation (IBS-C), mixed (IBS-M), and unspecified (IBS-U).

While the pathogenesis of IBS still needs further research, it is known that multiple factors contribute to the development of symptoms: increased intestinal permeability, dysmotility, intestinal dysbiosis, food hypersensitivity, visceral hypersensitivity, brain-gut axis dysregulation, inflammation, and psychological stress.[19,20] One of the more recent pathophysiological models puts mast cells (MCs) centrally in the interaction between the gut microenvironment and the visceral nerves.[97,143,145] Mature MCs are heterogeneous, tissue-resident, long-lived, granulated cells which are particularly abundant at barrier sites. MCs play an important role in host defence and wound healing through the release of a variety of preformed and newly sensitised mediators, such as vasoactive amines (histamine and serotonin), proteoglycans, proteases, and cytokines. These mediators can affect sensory nerve endings, leading to visceral hypersensitivity and consequently abdominal pain.

MCs can be activated via the classical IgE-mediated pathway, which is crucial in the pathophysiology of allergic disorders. Additionally, there is an equally important IgE-independent activation mechanism, which can be triggered by a variety of substances such as cytokines, hormones, immunoglobulins, neuropeptides, and complement

components.[131,132] Aside from these local effects, MC activation is modulated by central or psychological pathways through the release of corticotropin releasing hormone.[133] MCs are increasingly implicated in the IBS pathophysiology.[143–145] They are more frequently located in the vicinity of afferent nerve terminals in patients with dominant pain symptoms.[145,147–149] Colon tryptase levels are elevated in IBS, while serum tryptase levels are within the normal range, suggesting localised mucosal MC infiltration.[147,190] Still, little is known about the functional characteristics of these MCs.

The aim of this research project was to develop a MC culture model to be able to study these cells *in vitro* in IBS conditions. First, immunophenotypic and functional characteristics of naïve MCs of IBS patients and healthy controls were studied and compared. Second, we developed a gut/IBS-like environment and evaluated its effect on the characteristics and functionality of the MCs.

4.2 Methodology

4.2.1 Study population

Healthy controls (HC) and IBS patients were recruited via the tertiary referral motility clinic of the department of Gastroenterology and Hepatology of the Antwerp University Hospital (UZA), the University of Antwerp, and a patient-centred informative website (www.ibsbelgium.org). HC who donated colonic biopsies for the supernatant development were recruited from the colonic cancer screening program, all had a negative colonoscopy. Patients were only included if they fulfilled the Rome IV criteria for IBS. Exclusion criteria for both patients and HC were the presence of inflammatory bowel disease (IBD), celiac

disease, any history of malignancy in the gastrointestinal tract, pregnancy, breastfeeding, a history of anaphylactic reactions, and the use of immunosuppressive medication. HC were also excluded if they experienced chronic gastrointestinal complaints. A general profile with medical history and medication list was compiled. All participants gave written informed consent approved by the Ethics Committee of the University of Antwerp/Antwerp University Hospital (16/44/466). Samples were registered and stored in the "Biobank Antwerpen", Antwerp, Belgium (ID: BE 71030031000).

4.2.2 Mast cell culture

Participants were asked to donate 50 mL peripheral blood out of which $CD34^+$ progenitor cells were isolated according to the protocol developed by the laboratory of Immunology.[191,192] Mononuclear cells were isolated using a density gradient separation (Histopaque, Merck, Saint Louis, Missouri, USA). $CD34^+$ progenitor cells were subsequently magnetically separated using the EasySep Human CD34 selection Kit according to the manufacturer's instructions (Stemcell technologies, Vancouver, Canada). These progenitor cells were cultured in a serum-free methylcellulose-based medium (MethoCult SFH4236, Stemcell technologies) supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL) (GIBCO Thermofisher, Waltham, Massachusetts, USA), low-density lipoprotein (LDL, 10 µg/mL, Stemcell technologies), 2-mercaptoethanol (55 µmol/L, GIBCO Thermofisher), stem cell factor (SCF, 100 ng/mL, Miltenyi Biotec, Bergisch Gladbach, Germany), and interleukin-3 (IL-3, 100 ng/mL, Peprotech, Cranbury, New Jersey, USA) for two weeks. Afterwards, the cells were cultured for another three

weeks in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO Thermofisher) supplemented with SCF (10 ng/mL).

4.2.3 Mast cell immunophenotyping

After five weeks the mast cells were immunophenotyped by staining for surface markers with anti-human CD117-APC (clone 104D2, Biolegend, San Diego, California, USA), anti-human FcεRI-PE (clone AER37, Biolegend), anti-human CD203c-PeCy7 (clone NP4D6, Biolegend), anti-human CD63-FITC (clone H5C6, BD Biosciences, Franklin Lakes, New Jersey, USA), anti-human MRGPRX2-PE (clone K125H4, Biolegend), anti-human CD300a-PE (clone E59.126, Beckman Coulter, Brea, California, USA), anti-human CD32-PE (clone FLI8.26, BD Biosciences). Mature human MCs were defined as $CD117^+$ and $CD203c^+$. Viability was measured using 7AAD (BD Biosciences). Cells were stained for 20 min in the dark at 4°C after which they were washed and resuspended in PBS with 0.1% sodium azide (PBS-NaN_3).

Early-stage apoptosis was measured using Annexin V-PeCy7 (Biolegend). Cells were stained with CD45-FITC and CD117-APC for 20 min in the dark at 4°C after which they were washed and resuspended in a $CaCl_2$ buffer. Next, cells were stained with the Annexin V and incubated for 15 min in the dark at room temperature before measurement.

4.2.4 Mast cell functionality

The functionality was measured via upregulation of CD63 (lysosomal degranulation marker) after adding the neurokinin substance P (natural ligand for MRGPRX2, thus IgE-independent activation; Sigma Aldrich, Sant Louis, Missouri, USA) and anti-FcεRI (IgE-

dependent mechanism with cross-linking of the FcεRI with anti-IgE receptor; Thermo Fisher). First, MC were suspended in Tyrode buffer (37°C) at a concentration of $5x10^5$ cells/mL after which 100 µL of cells was stimulated with 100 µL Tyrode buffer as a negative control, 100 µL substance P (final concentration of 74 µmol/L), or 100 µL anti-FcεRI (final concentration of 2.5 µg/mL) during 3 and 20 min at 37°C. Activations were stopped by placing the cells on ice. Subsequently, the supernatant was removed after centrifugation (500g, 4°C, 5 min). The cells were resuspended in PBS with 0.1% bovine serum albumin (BSA) and stained with anti-human CD117-APC, anti-human CD203c-PeCy7, anti-human CD63-FITC, and anti-human MRGPRX2-PE for 20 min at 4°C in the dark. After staining, MCs were fixated with Lyse/Fix buffer (BD Biosciences) and lastly, cells were washed and resuspended in PBS-NaN_3.

4.2.5 Colonic supernatant

Since colonic supernatant had never been used on MC cultures, we first optimised the development of a supernatant suitable for these cultures. After an enema, colonic biopsies were collected in the sigmoid, to eliminate confounding based on location in the gut, using a standard biopsy forceps and a flexible gastroscope. Biopsies were immediately submerged in a saline solution and further processed within an hour.[193] Before adding the medium the biopsies were weighed and for every 1 mg of tissue 30 µL of medium was added. Biopsies were incubated for 12 h at 37 °C under 95% O_2 and 5% CO_2 since previous research has shown cytokine release was maximal between 12-18 h.[194] After incubation supernatant was collected and stored at – 80 °C until time of experiments.

4.2.6 Flow cytometric analysis

Flow cytometric analyses were performed on a calibrated FACSCanto II flow cytometer (BD immunocytometry systems) equipped with three lasers (450 nm, 488 nm, and 633 nM). Compensation settings were performed using the BD CompBeads (BD Biosciences) and data were analysed using Kaluza analysis 2.1 software (Beckman Coulter). To set a marker between positive and negative cells according to the 99th percentile, a fluorescence minus one (FMO) sample was used. Density was measured with the use of standardised fluorospheres (SPHERO Ultra Rainbow Calibration particles, Spherotech). Figure 4.1 shows an example of flowcytometric output and gating of CD117+CD203c+ cells.

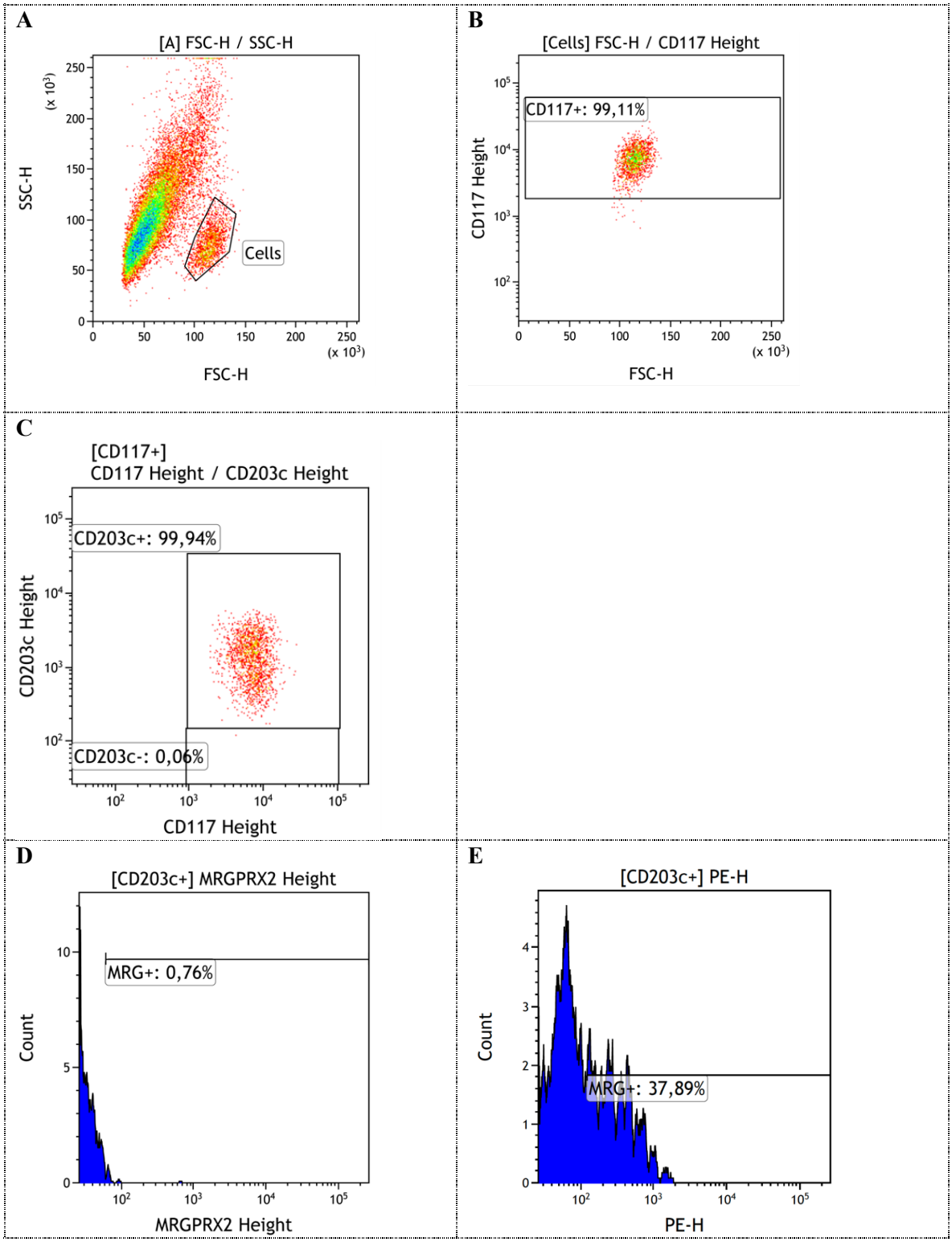


Figure 4.1: Example of flowcytometric output and gating. (A) First, cells are gated based on their size (forward scatter (FSC)) and granularity (side scatter (SSC)); (B) Second, cells expressing CD117 are selected; (C) Third, cells expressing CD203c are selected and used for further analysis; (D) Last, fluorescence minus one (FMO) samples are used to set a marker between positive and negative cells; (E) Example of expression MRGPRX2 after setting FMO marker.

4.2.7 Statistical analysis

For statistical analysis Graphpad Prism version 9 was used. Continuous variables following a Gaussian distribution were expressed as mean (standard deviation) and analysed using unpaired Student's t-tests, one-way ANOVA, or two-way ANOVA as appropriate. Nonparametric continuous variables were expressed as median (range) and analysed with Mann-Whitney U, Friedman test, or Wilcoxon matched-pairs signed rank test as appropriate. A p-value of < 0.05 was considered significant.

4.3 Results

4.3.1 Study population

The IBS patients and HC samples were collected from a total pool of 14 HC and 10 IBS (Table 4.1) of which some samples were used in one or both assays (immunophenotypic and/or functional) resulting in 7 IBS patients being compared to 12 HC for the immunophenotypic characterisation and 7 IBS patients and 12 HC for the functional characterisation. The populations were predominantly female and had a similar age distribution (table 4.1).

Table 4.1: Study population

	Healthy control (n=14)	**IBS (n=10)**
Subtype	NA	IBS-D: 6 IBS-C: 2 IBS-M: 2
Age[#]	30 (25 – 57)	36 (21-68)*
Gender M:F	4:12	0:10

[#]Median (range) *No significant difference with healthy control (Mann-Whitney U); p-value 0.48

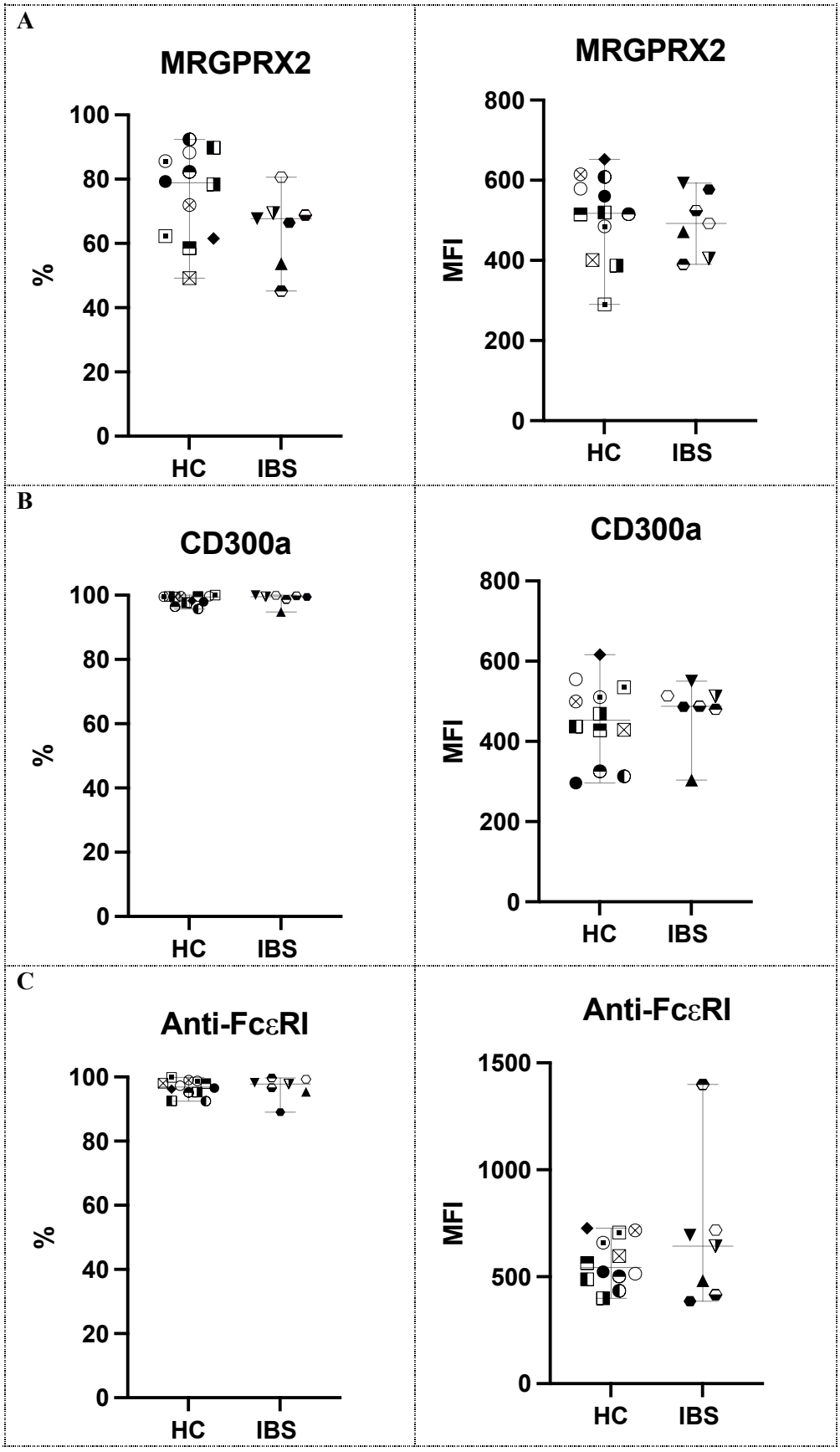
4.3.2 Immunophenotypic characterisation naïve mast cells

Results of the immunophenotypic characterisation are shown in table 4.2 and visually presented in figure 4.2. Both IBS patients and HC had a similar percentage and density expression of CD300a, FcεRI, and CD32 with all of them nearing 100% expression. There was a trend towards a slightly lower expression of MRGPRX2 in IBS patients (67.7% versus 78.8%), this was however not significant (figure 4.2, A; p = 0.14). Viability of the MCs was at least 94% and similar in IBS patients and HC (figure 4.2, E).

Table 4.2: Expression and density of immunophenotypic characteristics

	Healthy control (n=12)	**IBS (n=7)**	**p-value***
	Expression % (range)[#]		
MRGPRX2$^+$	78.8 (49.2 – 92.4)	67.7 (45.2 – 80.7)	0.14
CD300a$^+$	99.5 (95.7 – 100)	99.6 (94.8 – 100)	0.45
FcεRI$^+$	96.9 (92.4 – 99.9)	97.8 (89.0 – 99.7)	0.65
CD32$^+$	99.1 (98.5 – 99.9)	99.0 (98.4 – 100)	0.97
7AAD	2.43 (0.13 – 5.73)	2.05 (1.24 – 2.55)	0.34
	Density MFI (range)[#]		
MRGPRX2$^+$	518 (290 – 653)	493 (391 – 593)	0.65
CD300a$^+$	453 (296 – 617)	487 (303 – 551)	0.54
FcεRI$^+$	544 (400 – 727)	643 (386 – 1400)	0.97
CD32$^+$	424 (145 – 660)	410 (128 – 744)	1.00

MFI = median fluorescence intensity; IBS = irritable bowel syndrome
[#]Median (range) *Mann-Whitney U


A
MRGPRX2
%
100
80
60
40
20
0
HC
IBS
MRGPRX2
MFI
800
600
400
200
0
HC
IBS
B
CD300a
%
100
80
60
40
20
0
HC
IBS
CD300a
MFI
800
600
400
200
0
HC
IBS
C
Anti-FcεRI
%
100
80
60
40
20
0
HC
IBS
Anti-FcεRI
MFI
1500
1000
500
0
HC
IBS

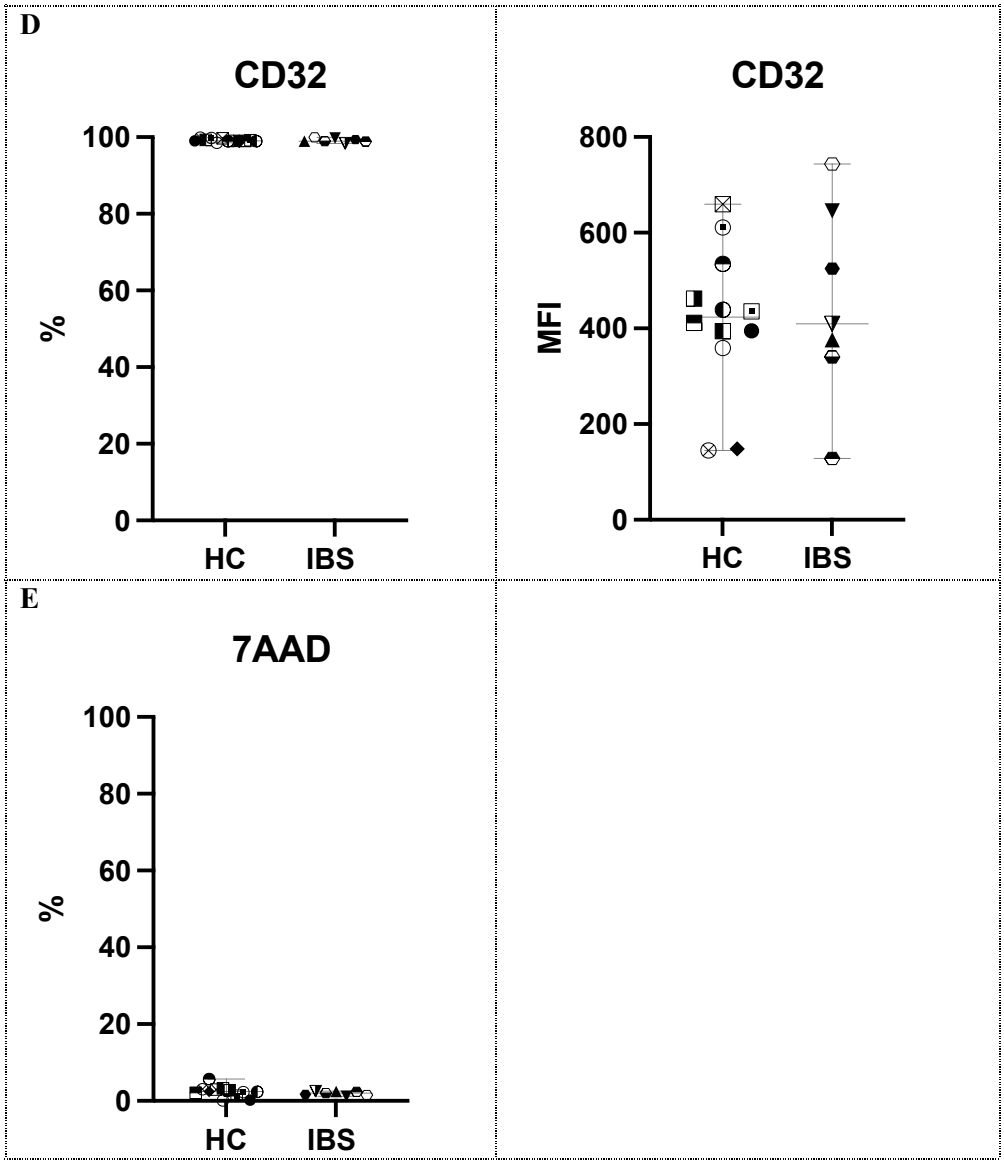


Figure 4.2: Expression and density of immunophenotypic characteristics. Expression of (A) MRGPRX2; (B) CD300a; (C) anti-FcεRI; (D) CD32; (E) 7AAD.
N=19 HC = healthy control; IBS = irritable bowel syndrome; Results are expressed as median with range. Each symbol represents an individual HC or IBS patient.

4.3.3 Functionality naïve mast cells

Results of the functionality of the MCs are shown in table 4.3 and visually represented in figure 4.3. HC and IBS patients had a similar upregulation of CD63 after stimulation with anti-FcεRI for 20 min (4.2% and 5.7%; figure 4.3, C) and 3 min (6.4% and 7.1%; figure 4.3, D). After stimulation with substance P there was a trend towards a higher upregulation of CD63 in HC (73.7% versus 49.4% at 20 min), which did not reach significance (figure 4.3, A; p = 0.20).

Table 4.3: Expression of functionality MCs

	Healthy control (n=12)	**IBS (n=7)**	**p-value***
	Expression % (range)[#]		
Substance P 20min	73.7 (28.4 – 89.2)	49.4 (30.8 – 86.4)	0.20
Substance P 3min	72.0 (24.7 – 91.0)	47.0 (23.9 – 88.3)	0.14
anti-FcεRI 20min	4.2 (0.8 – 25.1)	5.7 (1.3 – 16.1)	0.95
anti-FcεRI 3min	6.4 (1.6 – 40.4)	7.1 (1.9 – 21.36)	0.84

Expression of activation marker (CD63) after activation with substance P and anti-FcεRI.
IBS = irritable bowel syndrome
[#]Median (range) *Mann-Whitney U

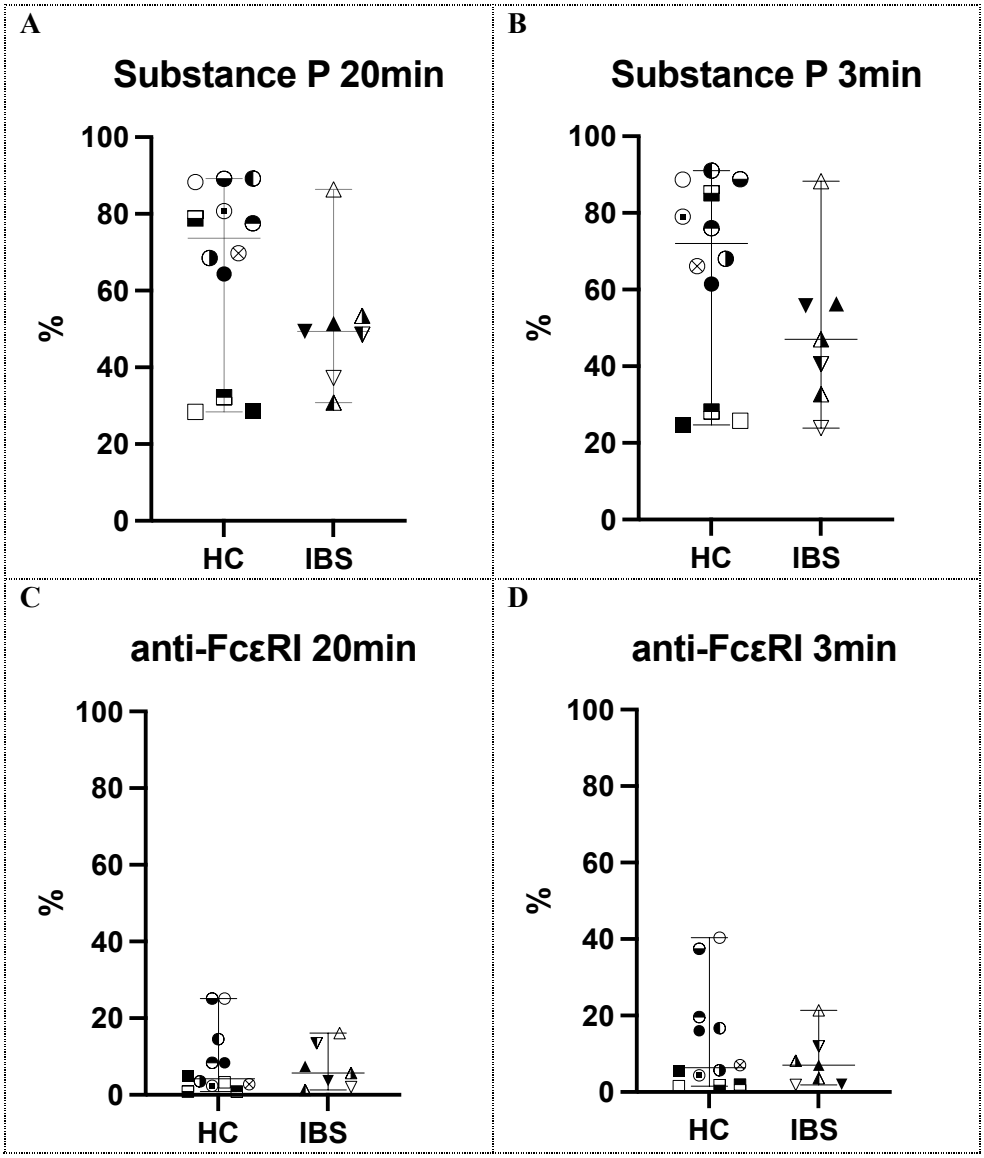


Figure 4.3: Expression of functionality. Expression of activation marker (CD63) after activation with (A) Substance P for 20 min; (B) Substance P for 3 min; (C) anti-FcεRI for 20 min; (D) anti-FcεRI for 3 min. N=19 HC = healthy control; IBS = irritable bowel syndrome; Results are expressed as median with range. Each symbol represents an individual HC or IBS patient.

4.3.4 Development of an IBS-like environment with colonic supernatant

4.3.4.1 *Optimalisation of the medium used in supernatant development*

For the optimalisation procedure, sigmoid biopsies of healthy controls were used. Three different media were evaluated to produce the supernatant. HBSS (Hank's buffer saline) and

RPMI (Roswell Park Memorial Institute; supplemented with FBS, penicillin/streptomycin, and gentamicin/amphotericin B) were selected based on literature developing colonic supernatant for other applications.[194,195] These two media were compared to IMDM (Iscove's Modified Dulbecco's Medium) which we use to culture the MC. Adding HBSS to the MC culture had a tendency to increase cell death as measured by 7AAD (median 3.4% in HBSS versus 1.5% in IMDM and 2.1% in RPMI; figure 4.4, A). RPMI and IMDM medium mostly resulted in a similar expression of surface markers and an upregulation of CD63 after activation. However, in some cultures there was a tendency for a lower expression of MRGPRX2 (median 28% versus 62%; figure 4.4, B; $p = 0.63$) and an associated lower upregulation of CD63 after activation with substance P (median 56% versus 69%; figure 4.4, C; $p = 0.13$). Since our MC were already cultured in IMDM, we decided to use IMDM for the development of our colonic supernatant.

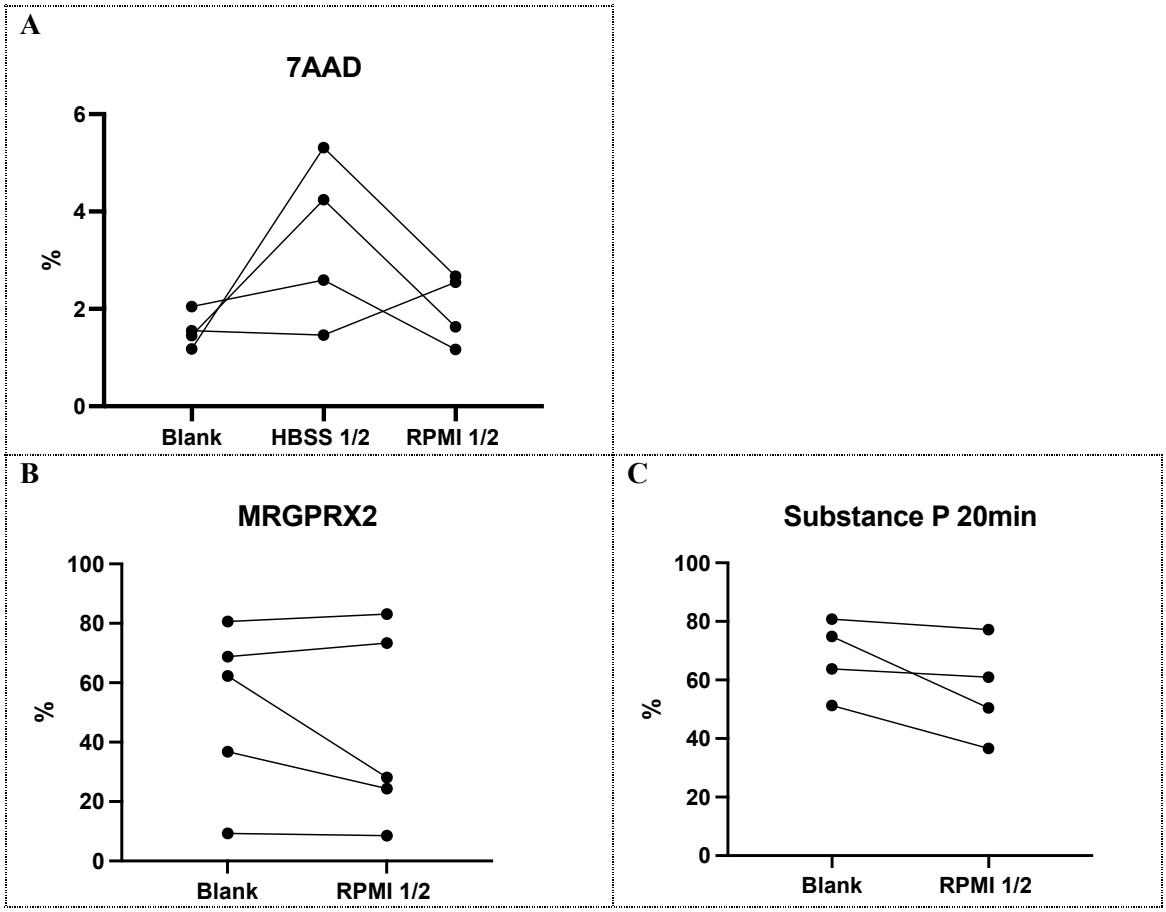


Figure 4.4: Optimalisation of medium supernatant. (A) Expression of 7AAD in IMDM, HBSS, and RPMI medium N=4; (B) Expression of MRGPRX2 in IMDM versus RPMI N=5; (C) Expression of CD63 after activation with substance P for 20 min in IMDM versus RPMI N=4
(A) Friedman test; (B) and (C) Wilcoxon matched pairs signed rank test

4.3.4.2 *Troubleshooting contamination*

After incubation of the MC cultures with supernatant, a fungal or bacterial infection was noted in some of the cultures. To prevent this in the future we decided to add gentamicin/amphotericin B (GA) to the supernatant. Furthermore, every supernatant was cultured before adding to the MC culture to assess for any resistant pathogens.

Before adding GA to all cultures, we assessed if there was any influence on the functionality of the MC as measured by upregulation of CD63 after stimulation. No significant differences could be observed after stimulation with substance P (median 65% with GA versus 69%

without GA; p = 0.63) or anti-FcεRI (median 14% with GA versus 9% without GA; p = 1.00) as shown in figure 4.5.

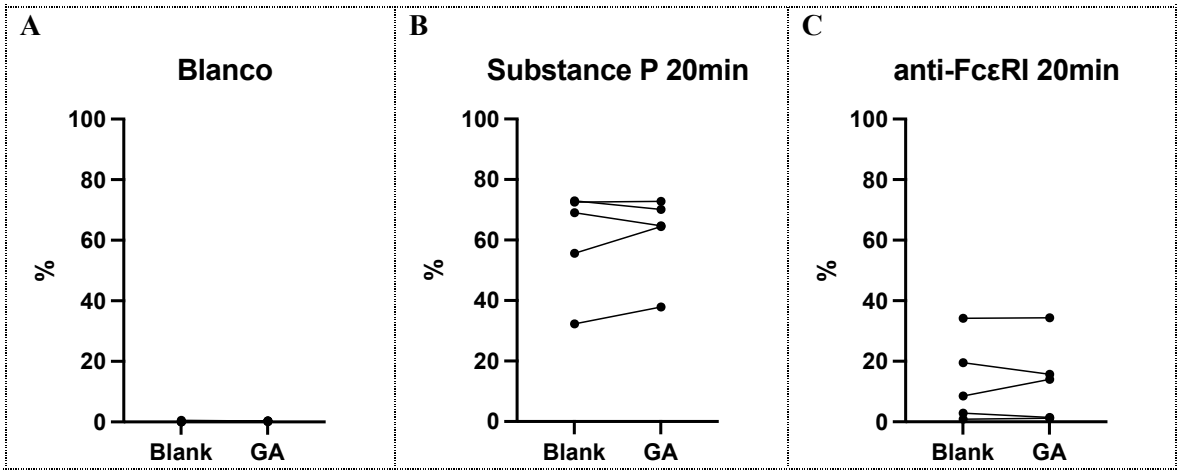


Figure 4.5: Expression of functionality with and without gentamicin/amphotericin B (GA). Expression of activation marker (CD63) (A) Before activation (B) After activation with substance P for 20 min; (C) After activation with anti-FcεRI for 20 min. N=5 Wilcoxon matched-pairs signed rank test

4.3.4.3 *Optimalisation of incubation time and concentration*

Before starting characterisation, we assessed the viability of the MCs after incubation with the supernatant at different time points. A blank condition was used as comparison, in which only IMDM medium was added to correct for dilution of the culture.

No significant differences between the different time points were found (p = 0.92) as shown in figure 4.6, therefore we decided to incubate the MC cultures with the supernatant for 48 h to allow enough time for the MC to adapt.

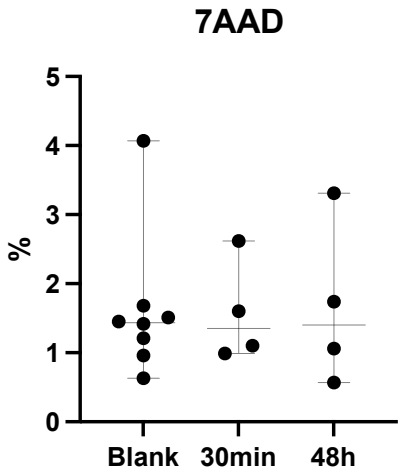


Figure 4.6: Expression of 7AAD after incubation with colonic supernatant at two different time points (30 min and 48 h) compared to a blank condition. Results are expressed as median with range. Mixed effects analysis.

After deciding to incubate the MC cultures for 48 h the viability was tested after incubation with different concentrations of supernatants. When incubating the MC cultures with a 1/10 or 1/20 dilution of the supernatant we did not find any significant differences in viability (median blank condition 1.24%, 1/10 2.06%, and 1/20 1.40%; $p = 0.27$). However, when the MC cultures were incubated with a 1/2 supernatant there was a clear migration of the cell population on flow cytometry towards debris (figure 4.7). Therefore, we decided to only use the 1/10 and 1/20 supernatant dilutions for the following experiments.

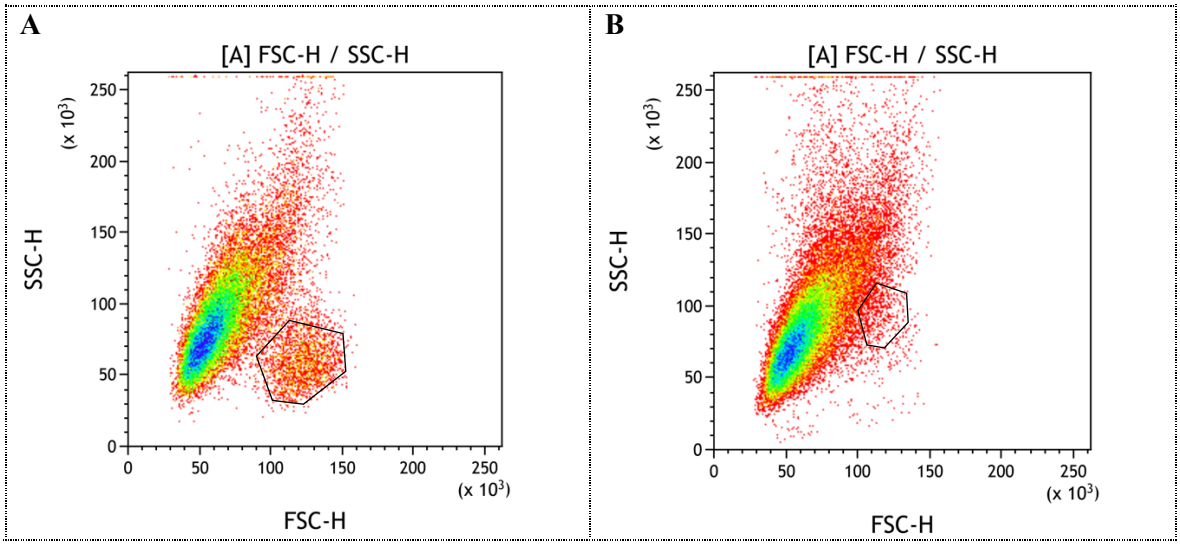


Figure 4.7: Migration of cell population on flow cytometry after adding a 1/2 supernatant. MCs are selected in black. (A) Normal situation; (B) After adding 1/2 supernatant

4.3.5 Characterisation of mast cells in an IBS-like environment

4.3.5.1 *Immunophenotypic characterisation of mast cells in an IBS-like environment*

Patients MCs were incubated with autologous matched colonic supernatant (1/10 and 1/20 concentration) for 48 h. Immunophenotypic characteristics of the MC were measured and compared to a blank condition to which no supernatant was added (figure 4.8). When we looked at the expression of MRGPRX2 and anti-FcεRI we found no significant differences between the different conditions. However, when we looked at the density of these receptors, we found a significant decrease after adding 1/10 supernatant of both MRGPRX2 and anti-FcεRI ($p = 0.03$). This effect was less pronounced after adding 1/20 supernatant ($p = 0.15$). Viability of the cells, measured by expression of 7AAD, remained stable (figure 4.8, C).

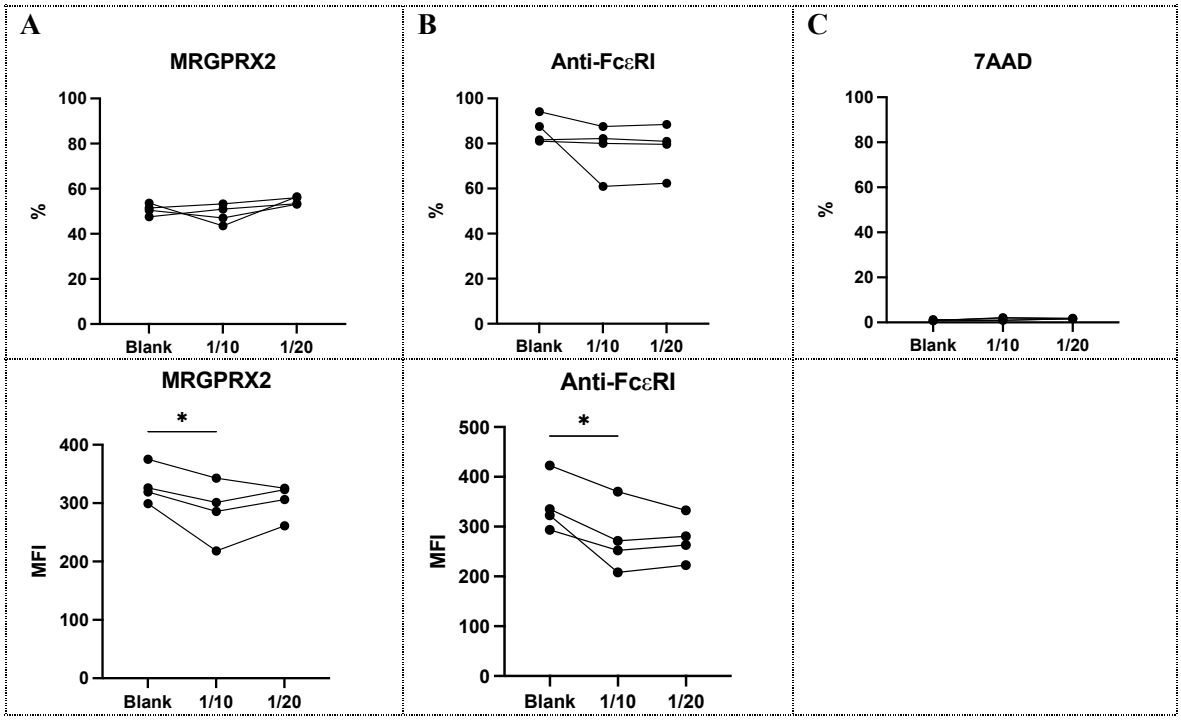


Figure 4.8: Expression (top row) and density (bottom row) of immunophenotypic characteristics in patients after incubation with matched supernatant. Expression of (A) MRGPRX2; (B) anti-FcεRI; (C) 7AAD. N=4
* p-value < 0.05 (Friedman test, Dunn's multiple comparisons test)

Subsequently, these supernatants were added to an unmatched cell culture of a healthy control to evaluate if the observed effects remained (figure 4.9). We did not find an effect on MRGPRX2 or viability. We did, however, find a significant effect on both the expression ($p = 0.02$ after adding 1/10) and density ($p= 0.02$ after adding 1/20) of anti-FcεRI in the control MCs.

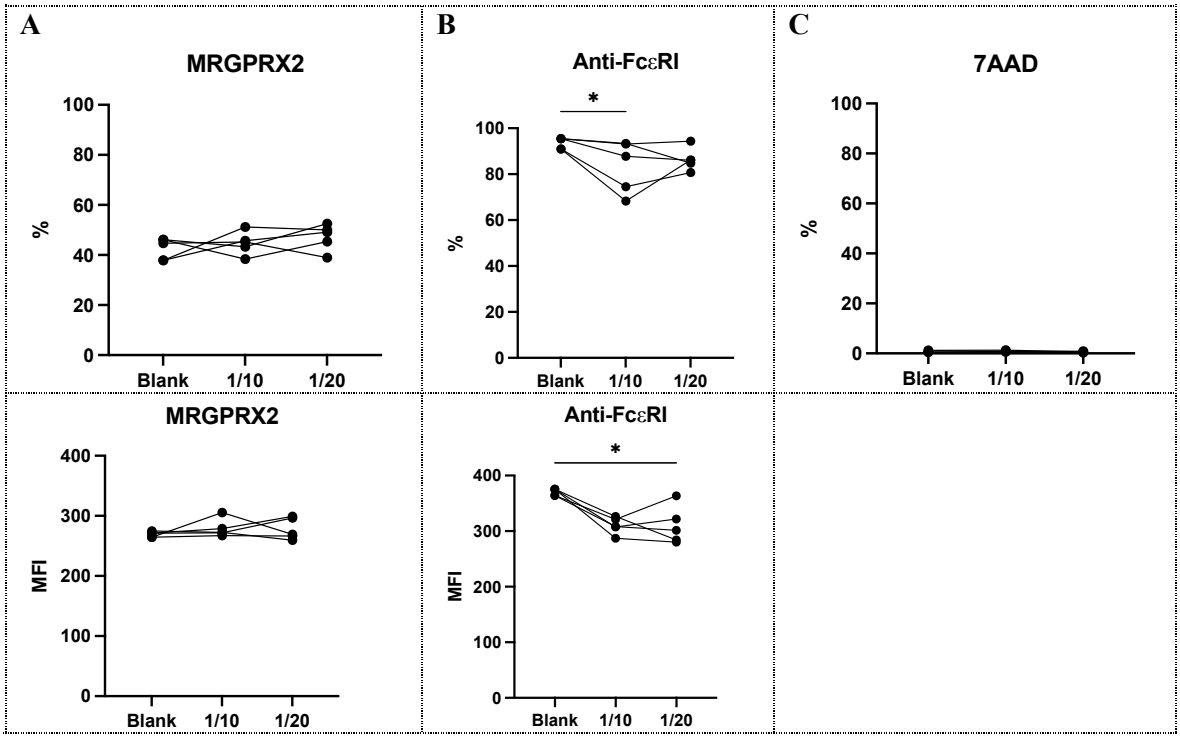


Figure 4.9: Expression (top row) and density (bottom row) of immunophenotypic characteristics in control after incubation with patient supernatant. Expression of (A) MRGPRX2; (B) anti-FcεRI; (C) 7AAD. N=5
* p-value < 0.05 (Friedman test, Dunn's multiple comparisons test)

4.3.5.2 *Functionality of mast cells in an IBS-like environment*

After determining the immunophenotypic characteristics of the MCs incubated with the supernatant, we evaluated the functionality of these cells after activation with substance P and anti-FcεRI. A decrease in upregulation of CD63 was observed for both IgE-dependent and -independent activation after adding the 1/10 diluted supernatant (figure 4.10).

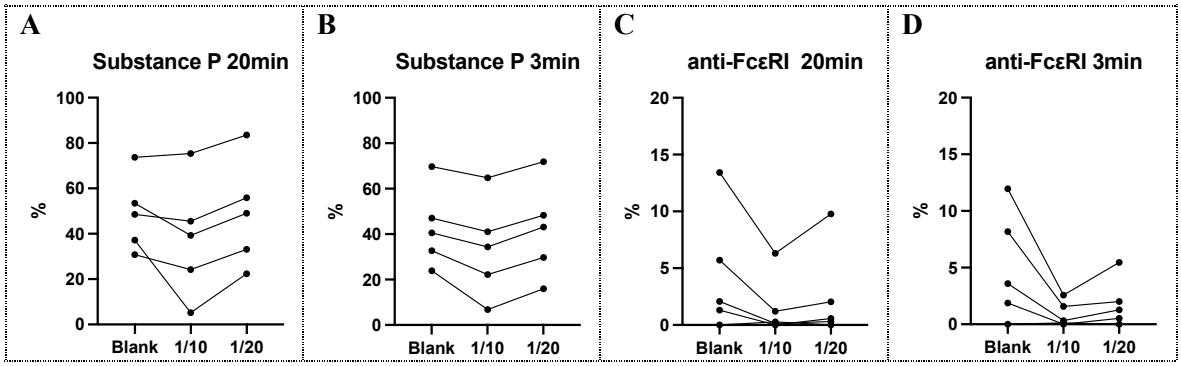


Figure 4.10: Expression of functionality in patients after incubation with matched supernatant. Expression of activation marker (CD63) after activation with (A) Substance P for 20 min; (B) Substance P for 3 min; (C) anti-FcεRI for 20 min; (D) anti-FcεRI for 3 min. N=5 Friedman test, Dunn's multiple comparisons test

As with the immunophenotypic characteristics the effect of the patient supernatant on the functionality of the MCs of a healthy control was evaluated (figure 4.11). Again, a decrease in upregulation of CD63 could be observed. The effect was more pronounced after activation with anti-FcεRI (p=<0.01 after 3min and 20 min) compared to substance P (p=<0.01 after 3 min).

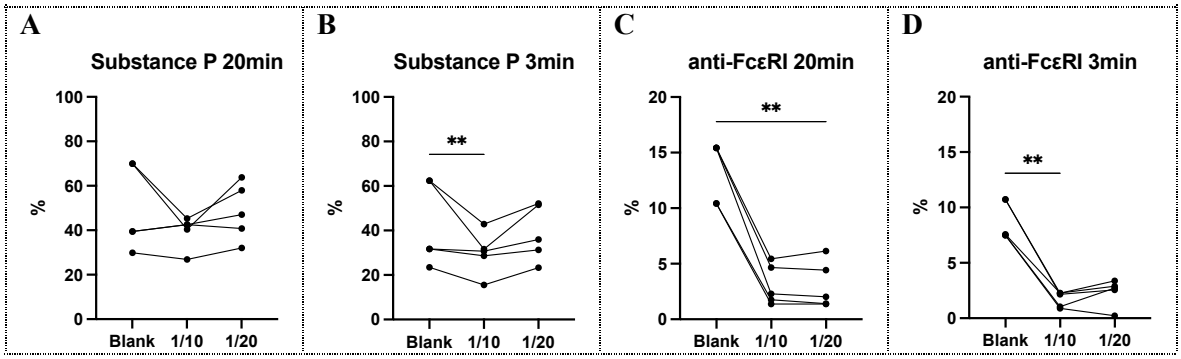


Figure 4.11: Expression of functionality in control after incubation with patient supernatant. Expression of activation marker (CD63) after activation with (A) Substance P for 20 min; (B) Substance P for 3 min; (C) anti-FcεRI for 20 min; (D) anti-FcεRI for 3 min. N=5
** p-value < 0.01 (Friedman test, Dunn's multiple comparisons test)

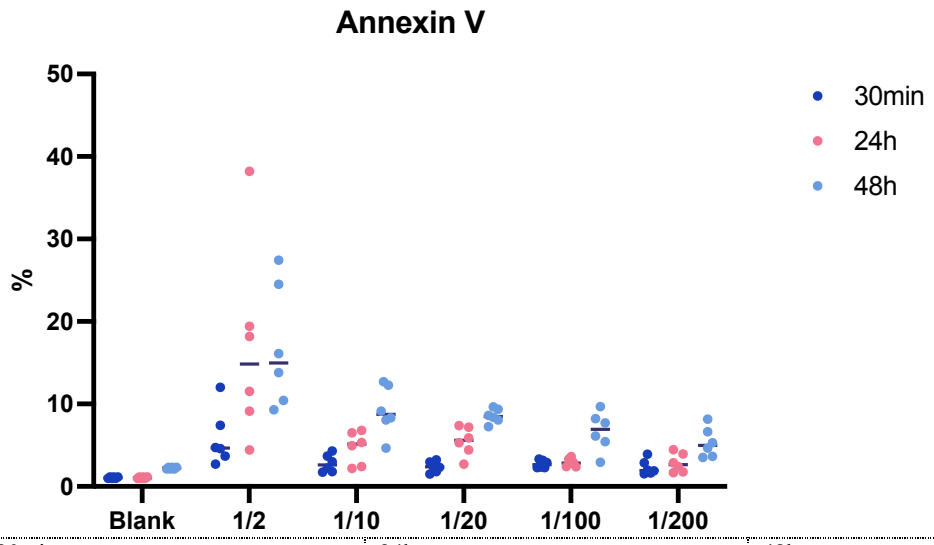
Considering previous research into the role of MCs in IBS we hypothesised to find an increase in activation after incubation with the supernatant, the opposite of what we found. A decrease in reactivity could be explained by toxicity of the supernatant resulting in cell damage and cell death. However, when assessing viability with 7AAD no differences could

be observed indicating no signs of cell damage and death by the supernatant itself. 7AAD does have its limitations since it will only stain cells in late apoptosis or necrosis. Therefore, we decided to re-examine a potential toxicity effect by using annexin V which detects cells in early apoptosis.

4.3.5.3 *Measurement toxicity colonic supernatant*

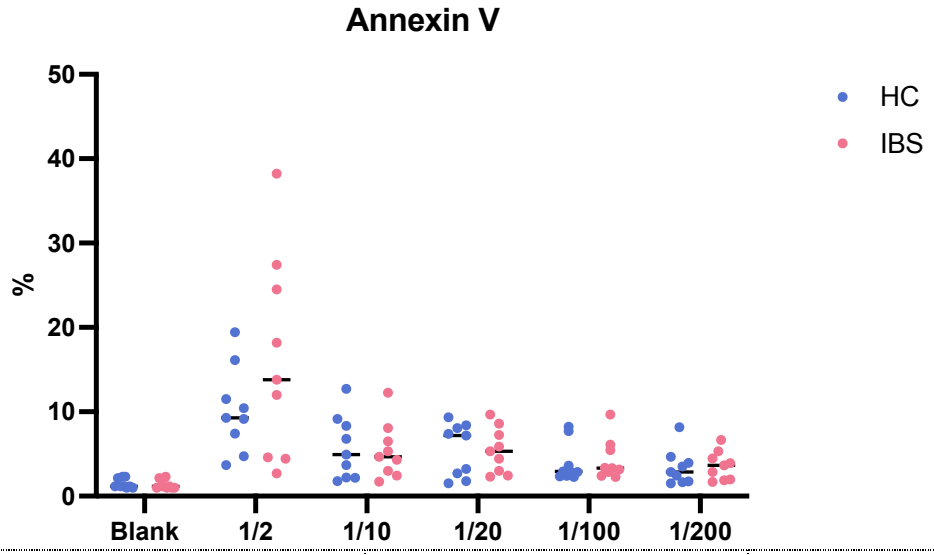
To evaluate toxicity, we tested the colonic supernatant of three IBS patients and three healthy controls on MCs originating from the same cell culture (from a HC). Different incubation times (30 min, 24 h, and 48h) and concentrations (1/2, 1/10, 1/20, 1/100, 1/200) were compared with a blank condition. A significant effect of both time ($p=<0.001$) and concentration ($p=<0.001$) could be observed (figure 4.12, A). The toxicity at all incubation times and concentrations is similar after adding supernatant of IBS patients or healthy controls, indicating a toxicity of colonic supernatant in general rather than a disease specific effect (figure 4.12, B).

A



30min	24h	48h
Blank vs. 1/2	Blank vs. 1/2	Blank vs. 1/2*
Blank vs. 1/10*	Blank vs. 1/10*	Blank vs. 1/10**
Blank vs. 1/20*	Blank vs. 1/20**	Blank vs. 1/20****
Blank vs. 1/100***	Blank vs. 1/100***	Blank vs. 1/100*
Blank vs. 1/200	Blank vs. 1/200*	Blank vs. 1/200*

B



HC	IBS
Blank vs. ½**	Blank vs. 1/2*
Blank vs. 1/10*	Blank vs. 1/10*
Blank vs. 1/20**	Blank vs. 1/20**
Blank vs. 1/100*	Blank vs. 1/100**
Blank vs. 1/200*	Blank vs. 1/200**

Figure 4.12: Expression of Annexin V after incubation with colonic supernatant. A) Influence of different incubation times and concentrations; B) Colonic supernatant from healthy controls versus patient
HC = healthy control; IBS = irritable bowel syndrome
Repeated measures two-way ANOVA with Geisser-Greenhouse correction
Dunnet's multiple comparisons test; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001

4.4 Discussion and conclusion

In recent years there has been increasing evidence of the important role of MCs in the pathophysiology of IBS. Several studies have shown an increased number of MCs in the gut mucosa of IBS patients, while this could not be confirmed by others.[145,147,148,196] Furthermore, these MCs are more frequently located in the vicinity of afferent nerve fibres. Besides colonic tryptase levels are increased in IBS, while serum tryptase levels are within the normal range, suggesting a localised mucosal MCs pathophysiology[145,154]. MCs are tissue resident cells which differentiate based on their environment making them hard to study *in vitro*. To be able to study patient specific MCs we would need a novel technique to isolate and culture MCs from patient samples. In this research project we validated the culture of human MCs derived from peripheral blood progenitor cells in healthy controls and IBS patients based on earlier validated protocols to culture human MCs in the domain of immunology and mastocytosis.[191,192]

Since these MCs have not been exposed to the 'diseased' gut environment we call them 'naïve MCs'. We compared these naïve MCs of IBS patients with the naïve MCs of healthy controls. When looking at the immunophenotypic characteristics of the MCs we did not find any significant differences between the MCs of IBS patients and HC. We did, however, find a trend for lower expression of the MRGPRX2 receptor in IBS patients, which was accompanied by a lower upregulation of CD63 after stimulation with substance P. Nonetheless, these results should be further validated.

The absence of significant differences between the naïve MCs of IBS patients and healthy controls suggests a disturbance in the peripheral rather than central regulation of MC which

is in accordance with previous research on MCs in IBS.[140,147,154] To be able to further investigate our findings and unfold the full potential of MC cultures it would be interesting to incubate the MCs in an IBS or gut-like environment. This gut-like environment should stimulate the MCs to further differentiate as they would in the gut mucosa *in vivo*. A gut-like environment can be created with the help of an inflammatory IBS cocktail based on nerve activating components found in colonic biopsy supernatant and serum of IBS patients.[197] However, all the components of this cocktail (serotonin, tryptase, histamine, and TNFα) are mediators which are released by the MCs themselves upon activation.[150,198] Furthermore, it is likely that other components (e.g. allergens, microbial products like LPS, hormones, cytokines, complement,…), or a combination of components, in the gut environment are of importance.

For this reason, we decided to develop a colonic biopsy supernatant based on literature and guidelines[195,196,199,200] allowing the patient's MCs to be incubated in their own gut-environment. While colonic supernatant has previously been used in research, this was mainly to study its effect on, for example, dorsal root ganglions, and not cell cultures similar to our MC cultures.[196,199,200] Extensive validation and optimalisation was therefore required before being able to regularly use the supernatant on our MC cultures. Two different media (HBSS and RPMI) were selected based on literature and compared to IMDM (Iscove's Modified Dulbecco's Medium) which is the medium used to culture the MCs. HBSS (Hank's buffer saline) is a balanced saline solution only suited for the short-term maintenance of cells. When we incubated the MCs with supernatant made with HBSS there was indeed an increase in cell death, measured by 7AAD. Both RPMI (Roswell Park Memorial Institute) and IMDM are more complex media suitable for long-term maintenance of cells. Both gave

similar results when looking at the expression of surface markers and upregulation of CD63 after activation. However, in some cultures with RPMI there was a tendency for a lower expression of MRGPRX2 and an associated lower upregulation of CD63 after activation with substance P, compared to cultures where RPMI was not added. Taking this into account in combination with the fact that our MCs were already cultured in IMDM, we decided to use IMDM for the further development of the colonic supernatant.

Processing steps of the supernatant were limited, however, to achieve some degree of standardisation biopsies were weighed after which a corresponding amount of medium was added. With this first version of the supernatant, we had some cultures in which yeasts started growing. Therefore, we decided to add gentamicin/amphotericin B (GA) to the supernatant since this had no effect on the characteristics or functionality of the MCs.

Different incubation times and concentrations of the supernatant were tested to assess the optimal conditions to immerse our MCs in the IBS/gut-like environment. When comparing three different incubation times (30 min, 24 h, 48 h) we did not find any significant differences when looking at viability based on 7AAD. Therefore, we decided to incubate our MCs for 48 h allowing them enough time to adapt to the changing environment. Next, three different concentrations of the supernatant were tested (1/2, 1/10, 1/20). While the 1/10 and 1/20 diluted supernatants did not have a significant effect on viability, the 1/2 concentration proved to be toxic with a migration of cells towards debris on flow cytometry.

After this initial optimisation we incubated patients MCs with their matched colonic supernatant to evaluate the effect on both immunophenotypic characteristics and functionality of the MCs. Surprisingly, we found a decrease in the density of membrane

receptors and a decrease in the upregulation of CD63 after activation with both substance P and anti-FcεRI. We expected the opposite effect since previous research looking into the role of MCs in IBS found an increased activity of the MCs of IBS patients. [145,147–149] Since our findings were more pronounced when adding a higher concentration of supernatant, we wanted to make sure there was no influence of toxicity on our results. An increase in damaged or apoptotic cells could explain a decrease in overall functionality. In the initial optimisation we assessed viability with 7AAD which was always acceptable. However, 7AAD will only increase in late-stage apoptosis and necrosis limiting its use since it will not detect early-stage apoptosis which can already have an influence on the characteristics and functionality of MCs. Therefore, we decided to retest the potential toxicity of the supernatant with the help of annexin V which is able to detect early-stage apoptosis. Supernatants of both healthy controls and IBS patients were tested to make sure the toxicity was not limited to the "diseased" samples. After staining with annexin V we found a clear influence of concentration and time with a marked increase of toxicity when incubating for longer than 24 h or with a supernatant more concentrated than 1/20. No difference was found after incubation with supernatant from healthy controls or IBS patients, indicating a toxicity of colonic supernatant in general. Therefore, we can hypothesise it being highly likely that the observed downregulation can be attributed to toxicity rather than as a consequence of IBS. However, at the moment it is not possible to formulate a definite conclusion on the effect of supernatant on the characteristics and functionality of MCs. While we made progress in the validation and optimisation of this new technique, there are still questions to be answered and work to be done. First, the toxicity of the supernatant should be further explored. On the one hand, we need to determine the factors contributing to the toxic effect, for example

endotoxins or the microbiota and its metabolites. On the other hand, we need to investigate interventions to reduce this toxicity. Efforts should be made to determine some of the main components of the supernatant and assess their influence on the MCs. Deactivating certain elements or reducing their concentration could be considered, weighing the pros and cons, since less toxicity could be associated with a loss of crucial compounds in the regulation of MCs thereby losing some of its potential in IBS research. Another future possibility to standardise the supernatants is using the EC50 values of the endotoxins in the supernatants. Rather than decreasing toxicity, another option would be to use annexin V to select only cells without signs of apoptosis to use in further analysis.

In conclusion, in this research project we validated the use of peripheral blood MC cultures in IBS. When comparing the immunophenotypic and functional characteristics of naïve MCs of IBS patients with healthy controls we could not find any significant differences. A next step in the validation and use of MC cultures in IBS research was to incubate these MCs in an IBS/gut-like environment, based on colonic supernatant and assess its influence on the MCs. While incubation with the supernatant did alter characteristics and functionality of the MCs, it was the opposite of what was expected. Furthermore, in its current form the supernatant negatively impacts viability of the MCs. While the use of colonic supernatant shows promise, further optimisation and validation is needed before the technique can be used to study IBS.

Chapter 5 Volatile organic compounds in irritable bowel syndrome

Based on

Van Malderen K, Hanning N, Lambrechts H, Haverhals T, Van Marcke S, Ceuleers H, De Man JG, De Winter BY, Lamote K, De Schepper HU. Volatile organic compound profiling as a potential biomarker in irritable bowel syndrome: a feasibility study. *Frontiers in Medicine Section Gastroenterology* (2022) Volume 9

5.1 Introduction

As mentioned in **chapter 1** there is a lack of biomarkers to aid in the identification and follow-up of patients with irritable bowel syndrome (IBS). The study of volatomics, focusing on the analysis of volatile organic compounds (VOCs), is a rapidly growing developmental area in the biomarker universe. VOCs are metabolites produced *in vivo* during physiological processes and pathophysiological metabolic activities.[155] Additionally, they can originate from the microbial metabolism, and by metabolization of exogenous products like food or drugs.[156] VOCs are excreted in urine, sweat, blood, faeces, and exhaled breath, making them easily accessible to study. Since IBS is associated with low-grade inflammation and dysbiosis, volatomics may offer a non-invasive tool to reflect these pathophysiological mechanisms, aiding in the diagnosis, treatment, and follow-up of this disease.[156]

In the past, researchers have tried to differentiate IBS patients from healthy controls (HC) by volatile biomarkers. These studies were mainly based upon VOCs analysed by gas chromatography–mass spectrometry (GC-MS) and two studies used high field asymmetric waveform ion mobility spectrometry (FAIMS).[156–158,160–162,164,166,201,202] Almost all these studies investigated VOCs in faecal samples with only two looking at VOCs in breath, and two looking at VOCs in urine. Results of these studies were not consistently positive and resulted in AUCs ranging between 0.44 and 0.95. [156–158,160–162,164,166,201–203] Sagar *et al.* looked at patients with bile acid diarrhoea and IBS-D and found evidence that metabolic processes of the microbiota are linked to specific VOCs.[164147]

At the moment, GC-MS is considered as the golden standard in VOC detection and analysis. It is a highly sensitive technique that allows explicit identification of individual compounds.

However, it is also a labour-intensive technique needing trained technicians and it is associated with high analytical costs. In this research project we examined the possibilities of multicapillary column/ion mobility spectrometry (MCC/IMS). It is an easy to use and less costly alternative compared to GC-MS.

Since studies in breath of IBS patients are still sparse, the aim of this study is to analyse and compare VOC profiles in both breath and faecal samples of IBS patients and healthy controls using the MCC/IMS methodology.

5.2 Methodology

5.2.1 Study population

Patients with IBS and healthy controls (HC) were recruited via the tertiary referral motility clinic of the department of Gastroenterology and Hepatology of the Antwerp University Hospital (UZA), via the University of Antwerp, and a patient-centred informative website (ibsbelgium.org). IBS patients were only included if they fulfilled the Rome IV criteria for IBS. Exclusion criteria for both patients and HC were the presence of inflammatory bowel disease (IBD), celiac disease, any history of malignancy in the gastrointestinal tract, pregnancy, and breastfeeding. HC were also excluded if they experienced any gastrointestinal complaints. Patients were further categorized according to their dominant stool pattern and in compliance with the Rome IV diagnostic criteria: diarrhoea (IBS-D), constipation (IBS-C), or mixed (IBS-M).[204] No patients with the unspecified subtype (IBS-U) were included. A general profile including the patients biometrics, medical history, and medication list was compiled. Additional information was collected through digital

questionnaires at the time of inclusion (IBS-symptom severity system (IBS-SSS), Hospital Anxiety and Depression Scale (HADS), Visceral Sensitivity Index (VSI), Irritable Bowel Syndrome Quality of Life Questionnaire (IBS-QOL), food diary, and exercise habits). After inclusion, breath and faecal samples were collected within the same week. Samples were collected between August 2019 and April 2021.

All participants gave written informed consent approved by the Ethics Committee of the Antwerp University Hospital (19/38/419). Samples were registered and stored until analysis in the "Biobank Antwerpen", Antwerp, Belgium (ID: BE 71030031000). The study was performed according to the Helsinki declaration.[205]

5.2.2 Analytical methods

There are numerous analytical methods available to detect VOCs (table 5.1). At the moment, gas chromatography - mass spectrometry (GC-MS) is considered the golden standard in VOC detection as stated above. It is a labour-intensive and complex technique allowing explicit identification of individual compounds. It requires *offline* sampling including different preconcentration steps which offers the possibility to store samples for batch analysis but also increases the risk of introducing contamination and bias. Furthermore, analytical costs are high and trained technicians are needed. In contrast, multicapillary column/ion mobility spectrometry (MCC/IMS) and Field asymmetric ion mobility spectrometry (FAIMS) offer an easy to use and less costly alternative which provide real-time (*online*) analysis and a (pseudo)identification of compounds, which makes it a more interesting technique to use in the clinical practice. Another alternative is selective ion flow tube-mass spectrometry (SIFT-MS) which also provides real-time (*online*) analysis.

However, it is associated with a higher cost and provides an absolute quantification of detected VOC. The electronic nose (E-nose) is another *offline* sampling technique. It is a less costly alternative to GC-MS but has limited detection abilities since it relies on an array of sensors which recognise patterns in VOC composition.[156]

Table 5.1: Comparison of analytical methods

Method	Description	Real-time analysis	Cost	Risk of contamination	Sample preparation	Online/ Offline	Storage time
Gas chromatography-mass spectrometry (GC-MS)	Separation of chemical components based on their relative affinity with a capillary column. Components elute from the GC-column with different retention times after which they are captured, ionised, accelerated, deflected, and detected by the MC.	-	+	+	+	Offline	+
Ion mobility spectrometry (IMS)	Separation of chemical components based on differences in ion mobilities within an electric field	+	-	-	-	Online	-
Selective ion flow tube-mass spectrometry (SIFT-MS)	Absolute quantification of trace VOC by ionisation with precursor/reagent ions	+	+	-	-	Online	-
Electronic nose (E-nose)	Array of sensors creating a smell "fingerprint" with pattern recognition modules resembling the olfactory system	-	-	+	+	Offline	+
Field asymmetric ion mobility spectrometry (FAIMS)	Separation of chemical components based on differences in ion mobilities within an electric field	+	-	-	-	Online	-

+ = applicable/high/long; - = not applicable/low/short; Online = immediate analysis of the sample; Offline = preconcentration of samples and possibility of storing samples for later analysis

In this research project it was decided to use IMS to sample VOCs because it is affordable, easy to use, and it offers a real-time analysis.

5.2.3 Sampling of breath

A BioScout breath analysing device (B&S Analytik, Dortmund, Germany) operating on VOCan v2.7 software was used for breath sampling. Details regarding the set-up are shown in table 5.2. This device consists of a BreathDiscovery ion mobility spectrometer coupled to

a multicapillary column, which is connected to a SpiroScout ultrasound-controlled breath sampler (Ganshorn Medizin Electronic) by a sample loop. Participants were asked to refrain from eating, drinking, brushing their teeth, taking medication, and smoking at least two hours before breath sampling. Patients were then asked to rinse their mouth with distilled water, put on a nose-clip and breathe tidally for 3 min through the SpiroScout sampler connected to a bacteria filter. Subsequently, 10 mL of alveolar air was collected and immediately analysed. After breath sampling, a patient-related background sample was collected to correct for potential environmental contamination.

Table 5.2: MCC-IMS characteristics

Parameter	
Ionisation source	^{63}Ni (95 MBq)
Electrical field strength	320 V/cm
Length of the drift region	12 cm
Diameter of the drift region	15 mm
Length of the ionisation chamber	15 mm
Shutter opening time	300 µs
Shutter impulse time	100 ms
Drift gas	α_2-nitrogen gas
Drift gas flow	100 ml/min
Carrier gas flow	100 ml/min
Working temperature	Ambient temperature
Pressure	Ambient pressure (101 kPa)
Pre-separation	Multi-capillary column OV-5, polar, 1000 packed columns, 3 mm diameter (Multichrom Ltd., Novosibirsk, Russia)
Column temperature	40°C, isothermal, adjusted
Tubing	PTFE

5.2.4 Sampling of faeces

Faecal samples were collected in a plastic container in the same week as the breath samples, preferably on the same day. Participants were asked to hand in the faecal sample within 4 h after defaecation after which the sample was aliquoted and stored at -80°C without the

addition of any buffers. Samples were left to defrost overnight at 4°C before analysis. For the faecal analysis, a BioScout breath analysing device operating on VOCan v4.1 software was used (B&S Analytik, Dortmund, Germany). The sample loop of the MCC/IMS was connected to a custom-made stainless-steel IMS-box.[206] In this closed box, 0.5 grams of faeces was heated at 37°C for one hour. Subsequently, the IMS box was flushed with nitrogen gas (α_1-nitrogen gas; 99.999% pure; Air Liquide Medical, Schelle, Belgium), sampling 10 mL of headspace air followed by immediate analysis by MCC/IMS. Background samples of the empty set-up were collected to correct for potential environmental and instrumental contamination.

5.2.5 Data handling

All MCC/IMS data were analysed using VisualNow Software v3.9 (B&S Analytik, Dortmund, Germany) as previously described.[207,208] In short, the raw IMS chromatograms were denoised through baseline correction using a low pass filter and aligned. Next, data were normalised to the reactant ion peak (RIP) and RIP-tailing was compensated by subtracting a median spectrum from each chromatogram within the data set. Further, the data were smoothed, and the chromatograms were visually inspected for the presence of VOCs. An example of a chromatogram can be found in figure 5.1. If a VOC was present in either breath/faecal or background sample, they were manually selected and analysed, resulting in a list of VOC peak intensities (maximum peak height in the selected peak area). For every VOC, the alveolar gradient was calculated by subtracting the intensity of the VOC in the background sample from the intensity of the VOC in the corresponding breath/faecal sample.

These gradients were used as independent variables for further statistical analysis. Faecal VOC gradients are shown as ‘PF’ and breath VOC gradients as ‘PB’.

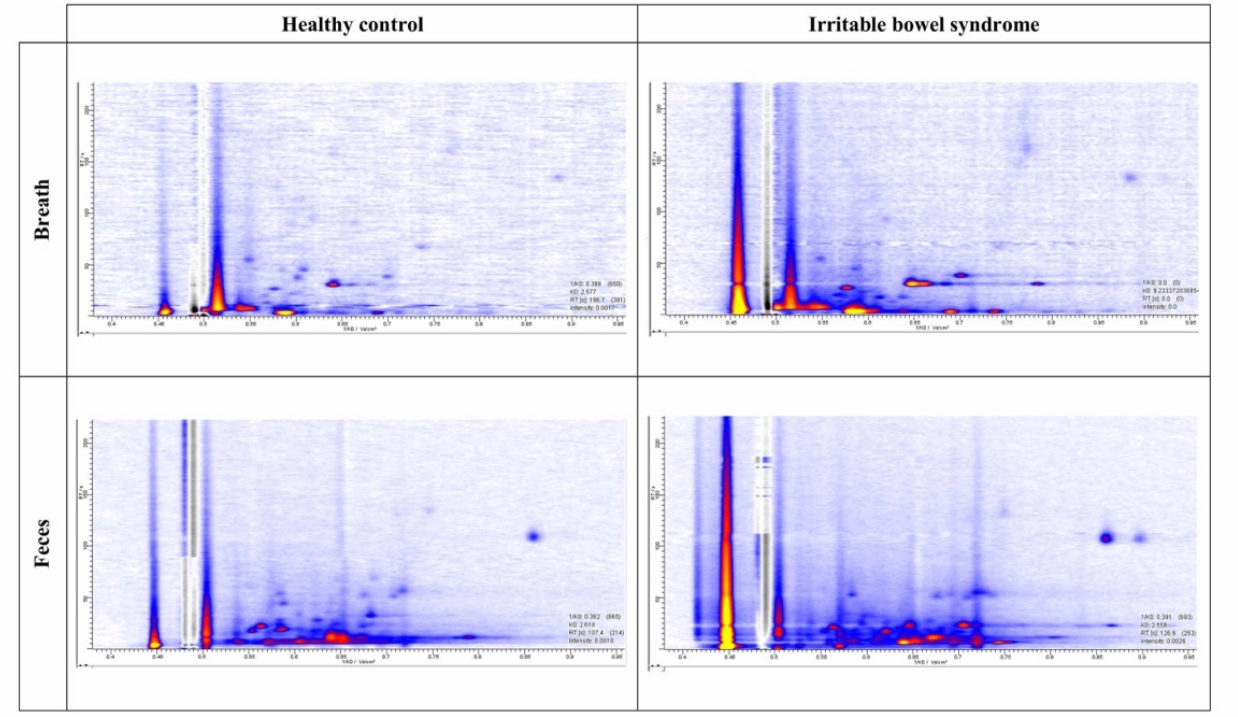


Figure 5.1: Example of chromatograms. Intensities are presented with the help of a colour code from low intensity (blue) to high intensity (yellow). A larger variation and higher intensity of VOCs can be observed in the faecal samples.

5.2.6 Statistical analysis

Categorical patient characteristics are expressed as *n* (%) and compared using Pearson’s χ^2 test, followed by pairwise comparisons between the subpopulations with a Bonferroni correction for multiple comparisons. Continuous variables are expressed as median (range) and comparisons between groups were made using the Kruskal-Wallis test, followed by Dunn’s post hoc test with a Bonferroni adjustment for multiple comparisons. All analyses were performed using R (version 4.1.1) in RStudio (version 1.4.1717). A significance level of α=0.05 was used throughout the analysis.

Logistic least absolute shrinkage and selection operator (lasso) regression with leave-one-out cross-validation (LOOCV) was used to select the VOCs that best differentiated IBS patients from HC, or IBS subtypes from other subtypes. Besides VOCs, the clinical characteristics age and gender were included as potential predictors to prevent confounding. Models were fitted using the R package *glmnet* (version 4.1-2). The alveolar gradient of these VOCs selected by the lasso regression in breath, faeces, or in models containing VOCs from both matrices were then used as independent variables in a logistic regression model which was internally validated by LOOCV. To prevent overfitting of the data, a maximum of two predictors was considered in each logistic model. Using the predicted outcome of all subjects, receiver operating characteristic (ROC) curves were generated. Relevant cut-off values were chosen to optimize accuracy and the model's sensitivity and specificity were estimated based upon these cut-off values. The accuracy of classification models based on VOCs derived from faeces, breath, or a combination of both was compared by testing for a difference in the area under the curve (AUC) of the respective ROC curves using a bootstrapping approach, as implemented by the pROC package (version 1.18.0). The AUC is a different performance characteristic compared to accuracy and does not depend on the earlier mentioned cut-off values but can be considered as a general indicator of the discriminatory capacity of the model.

To explore the impact of differences in the IBS phenotype on the VOC profiles, the IBS population was stratified based on the presence of depression (HADS score >8 on depression subscale), anxiety (HADS score >8 on anxiety subscale), use of antibiotics, use of probiotics, symptom severity (IBS-SSS), quality of life (IBS-QOL), and visceral sensitivity (VSI).[176–178,209] For continuous variables, the stratification was based on the median value in the

population. Logistic lasso regression models were then used to generate VOC profiles that differentiated the two IBS subpopulations.

5.3 Results

5.3.1 Study population

In total, 101 subjects were included (figure 5.2). Five patients did not meet the Rome IV criteria and were excluded, leaving 96 participants for breath sampling (24 HC; 27 IBS-D; 21 IBS-C; 24 IBS-M). Of these, 81 participants had a matched faecal sample (19 HC; 22 IBS-D; 19 IBS-C; 21 IBS-M). Baseline characteristics of the study population are shown in table 5.3. There were no statistically significant differences between the groups for age, gender, or BMI. There was, however, a trend towards a more female predominant population in the IBS groups compared to controls. IBS patients had significantly higher scores on IBS-SSS, VSI, and IBS-QOL compared to controls, with no differences between the subtypes. In addition, IBS-M patients had a significantly higher HADS score for anxiety compared to controls, while IBS-D and IBS-C were not significantly different from HC for the HADS score.

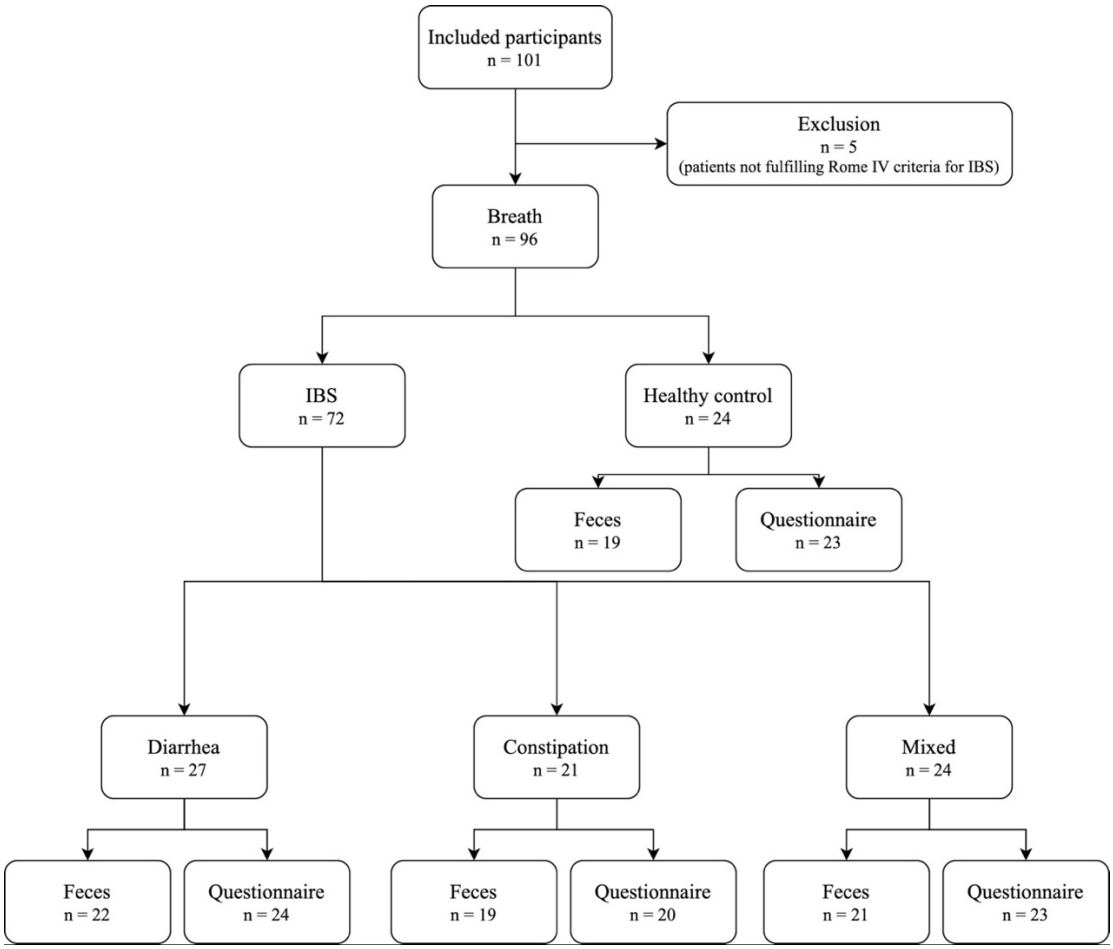


Figure 5.2: Flow of study participants. IBS = irritable bowel syndrome

Table 5.3: Baseline characteristics

	HC	IBS-D	IBS-C	IBS-M	p-value
Number	24	27	21	24	
Gender Males:Females	11:13	3:24	5:16	4:20	p = 0.0567[§]
Age (years; range)[a]	27 (18 – 70)	37 (18 – 78)	38 (20 – 77)	32 (23 – 64)	p = 0.2161[§]
BMI (kg/m²; range)[a]	22.7 (18.4 – 29.7)	22.9 (18.7 – 37.5)	22.6 (16.8 – 33.5)	25.0 (19.5 – 39.1)	p = 0.5032[§]
IBS-SSS (range)[a]	4 (0 – 92)	220 (68 – 400)	251 (135 – 398)	264 (61 – 383)	p < 0.0001[§&]
VSI (range)[a]	9 (0 – 40)	24.5 (5 – 62)	27 (0 – 70)	28 (1 – 55)	p = 0.0005[§&]
Median IBS-QOL (range)[a]	20 (19 – 33)	48 (22 – 78)	50 (24 – 78)	52 (22 – 97)	p < 0.0001[§&]
Positive HADS-An (%)	4/23 (17%)	13/24 (54%)	10/19 (53%)	16/23 (70%)	p = 0.0038[¶$]
Positive HADS-Dep (%)	1/23 (4%)	2/24 (8%)	4/19 (21%)	6/23 (26%)	p = 0.1238[¶]
Antibiotic use (%)	1/24 (4%)	5/27 (19%)	6/21 (29%)	2/24 (8%)	p = 0.0906[¶]
Probiotic use (%)	1/24 (4%)	8/27 (30%)	6/21 (29%)	6/24 (25%)	p = 0.1082[¶]

An = anxiety; C = constipation; D = diarrhoea; Dep = depression; GI = gastrointestinal; HADS = hospital anxiety and depression score; HC = healthy control; IBS = irritable bowel syndrome; IBS-SSS = symptom severity score; IBS-QOL = quality of life; M = mixed; SD = standard deviation; VSI = visceral sensitivity index.

[a]Median (range); [§]Median (range) with Kruskal-Wallis test, followed by Dunn's post hoc test with a Bonferroni adjustment for multiple comparisons; [¶]n (%) with Pearson's χ^2 test, followed by pairwise comparisons between the subpopulations with a Bonferroni correction for multiple comparisons; [&]Significant differences between HC and each of the patient subtypes, not amongst patient subtypes; [$]Significant difference between HC and IBS-M

5.3.2 Optimisation of faecal sampling

Since no protocols were available for faecal analysis with MCC/IMS the first step consisted of optimisation of the faecal sampling. Based on literature on faecal analysis with GC-MS three different weight categories (0.5 g, 1 g, 2 g) and three different temperatures (37°C, 50°C, 60°C) were tried.[156] All conditions were tested in threefold (table 5.4). Samples were heated in a custom-made stainless-steel IMS box and an empty box was used as a negative control.[206]

Table 5.4: Conditions faecal sampling optimalisation

Temperature Weight	37°C	50°C	60°C
0.5 g	3x	3x	3x
1 g	3x	3x	3x
2 g	3x	3x	3x

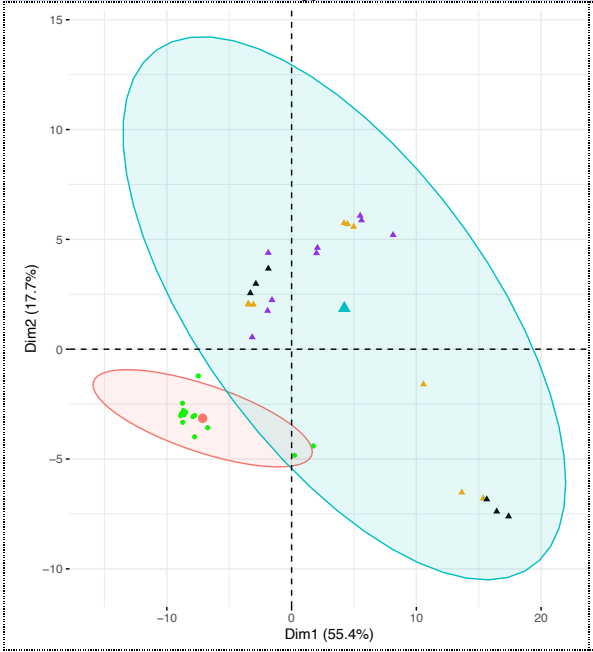
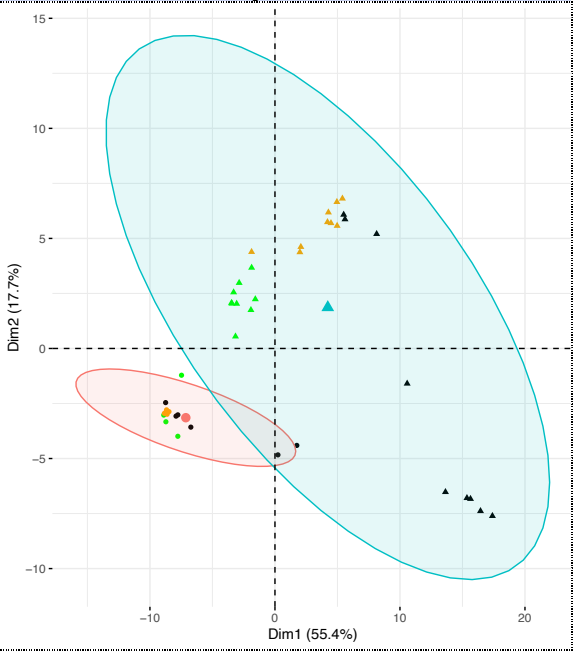
Weight	Temperature
	
Purple = 0.5g; Orange = 1g; Black = 2g; Circle = Negative control; Triangle = Faeces	Green = 37°C; Orange = 50°C; Black = 60°C; Circle = Negative control; Triangle = Faeces

Figure 5.3: PCA plot different weight and temperature conditions. PCA = principal component analysis; Negative control = empty box

Results of the different conditions are visually represented with the help of principal component analysis (PCA) plots (figure 5.3). In the different temperature and weight conditions we tested, a clear clustering of the negative control measurements can be seen. When looking at the different weight categories the measurements of 0.5 g and 1 g cluster together while the 2 g measurements are separate. A similar image can be seen in the temperature measurements with a clustering of the 37°C and 50°C while the 60°C

measurements are separate. Both the higher weight categories and the higher temperature categories gave a hyperintense chromatogram making interpretation of the data more difficult.

Considering the difficulties in interpretation with higher weight or temperature in combination with a clustering at lower weight and temperature we decided to go further with 0.5 g faeces at 37°C for future analyses.

5.3.3 Volatile analysis

In total, 92 and 211 VOCs were identified in respectively breath (PB) and faecal (PF) samples (supplementary tables 5.5.1 and 5.5.2). Supplementary table 5.5.3 summarises the VOCs selected by the lasso regression analysis followed by LOOCV. Figure 5.4 shows the ROC curves in the different matrices. We first determined whether it was feasible to differentiate IBS patients from healthy controls based upon VOCs in breath and faecal samples using MCC/IMS.

5.3.3.1 *Pooled IBS patients versus healthy controls*

The fit and internal validation of the logistic regression models and the characteristics of the VOCs used in these models are shown in tables 5.5 and 5.6 respectively. In breath, IBS patients were differentiated from HC with an AUC of 0.62 (0.47 – 0.76), 97.2% (91.2% - 99.5%) sensitivity, and 20.8% (8.1% - 40.3%) specificity. Based upon faecal VOCs, a higher AUC was obtained (0.80 (0.69 – 0.91)) with a specificity of 21.1% (7.1% – 43.3%), and a sensitivity of 100% (95.3% – 100%). Lastly, combining the breath and faecal VOC matrices

into one model averaged the performance of the individual matrices, resulting in an AUC of 0.69 (table 5.5, figure 5.4).

The differences in performance (in terms of AUC) were not significantly different. However, there was a trend when comparing breath and faecal models with a higher performance in the faeces-based classifier (breath versus faeces: $p = 0.054$; breath versus combination: $p = 0.541$; faeces versus combination: $p = 0.284$).

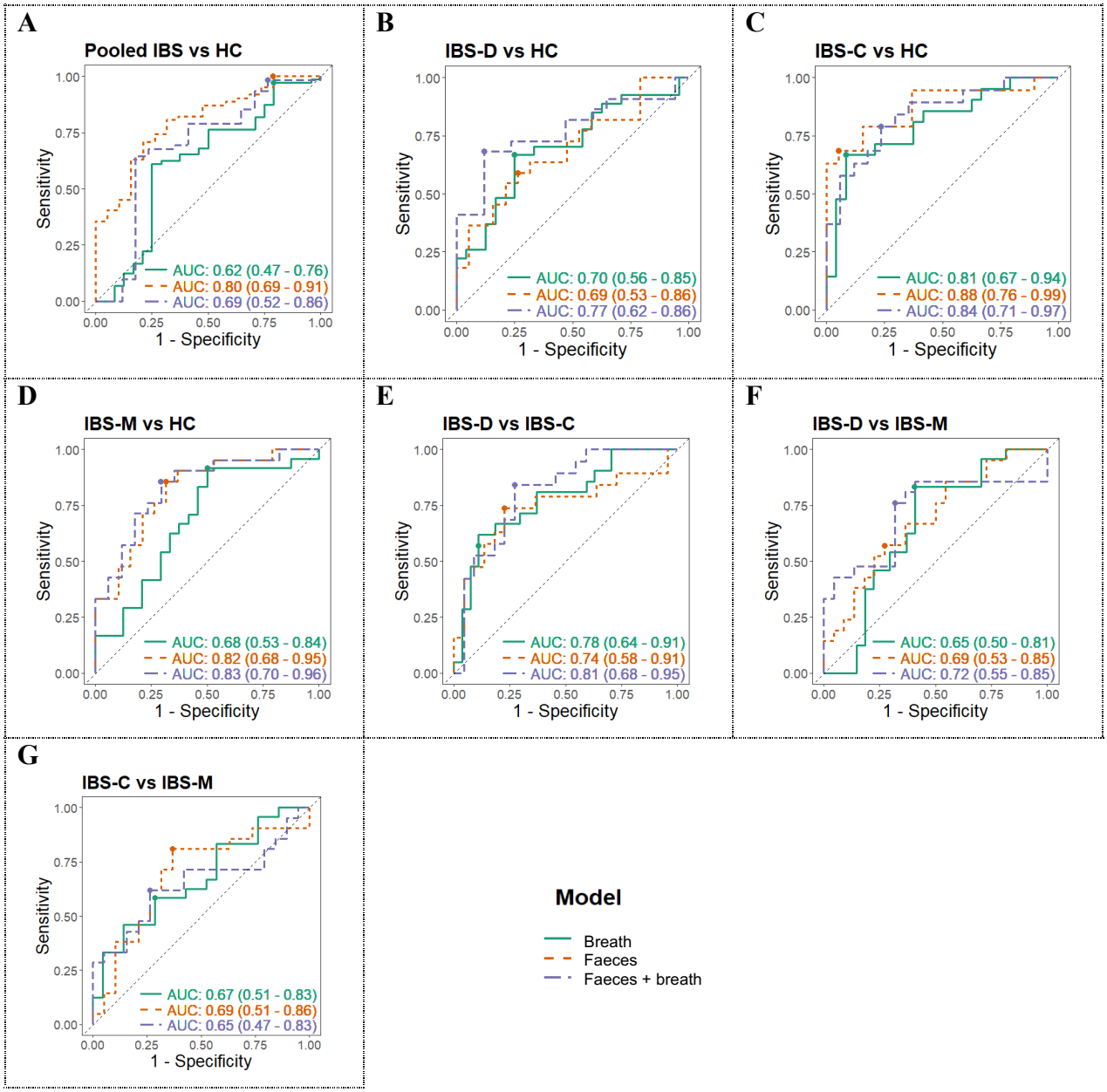


Figure 5.4: Receiver operating characteristic (ROC) curves in different matrices. Each panel shows the ROC curve of the breath, faecal, and combined model; (A) Pooled IBS patients versus HC (B) IBS-D versus HC (C) IBS-C versus HC (D) IBS-M versus HC (E) IBS-D versus IBS-C (F) IBS-D versus IBS-M (G) IBS-C versus IBS-M. C = constipation; D = diarrhoea; HC = healthy control; IBS = irritable bowel syndrome; M = mixed

Table 5.5: Differentiating models based on VOC pattern

	Pooled IBS vs HC	IBS-D vs HC	IBS-C vs HC	IBS-M vs HC	IBS-D vs IBS-M	IBS-D vs IBS-C	IBS-C vs IBS-M
Breath							
Sens % (95%CI)	97.2 (91.2 – 99.5)	66.7 (47.6 – 82.4)	66.7 (44.9 – 84.1)	91.7 (75.1 – 98.6)	83.3 (64.6 – 94.5)	57.1 (35.8 – 76.7)	58.3 (38.3 – 76.5)
Spec % (95%CI)	20.8 (8.1 – 40.3)	75.0 (55.1 – 89.2)	91.7 (75.1 – 98.6)	50.0 (30.6 – 69.4)	59.3 (40.3 – 76.4)	88.9 (72.7 – 97.1)	71.4 (49.8 – 87.5)
Acc % (95%CI)	78.1 (69.1 – 85.5)	70.6 (57.1 – 81.8)	80.0 (66.5 – 89.8)	70.8 (56.9 – 82.3)	70.6 (57.1 – 81.8)	75.0 (61.4 – 85.7)	64.4 (49.8 – 77.3)
AUC (95%CI)	0.62 (0.47 – 0.76)	0.70 (0.56 – 0.85)	0.81 (0.67 – 0.94)	0.68 (0.53 – 0.84)	0.65 (0.50 – 0.81)	0.78 (0.64 – 0.91)	0.67 (0.51 – 0.83)
Faeces							
Sens % (95%CI)	100 (95.3 – 100)	59.1 (38.1 – 77.9)	68.4 (45.5 – 86.1)	85.7 (65.9 – 96.2)	57.1 (35.8 – 76.7)	73.7 (51.0 – 89.6)	81.0 (60.2 – 93.6)
Spec % (95%CI)	21.1 (7.1 – 43.3)	73.7 (51.0 – 89.7)	94.7 (76.7 – 99.7)	68.4 (45.5 – 86.1)	72.7 (51.7 – 88.1)	77.3 (56.6 – 91.2)	63.2 (40.4 – 82.2)
Acc % (95%CI)	81.5 (72.0 – 88.8)	65.9 (50.5 – 79.1)	81.6 (67.0 – 91.6)	77.5 (62.7 – 88.4)	65.1 (50.1 – 78.2)	75.6 (60.9 – 86.9)	72.5 (57.3 – 84.6)
AUC (95%CI)	0.80 (0.69 – 0.91)	0.69 (0.53 – 0.86)	0.88 (0.76 – 0.99)	0.82 (0.68 – 0.95)	0.69 (0.53 – 0.85)	0.74 (0.58 – 0.91)	0.69 (0.51 – 0.86)
Breath and faeces							
Sens % (95%CI)	98.4 (92.3 – 99.9)	68.2 (47.0 – 84.9)	78.9 (56.7 – 92.9)	85.7 (65.9 – 96.2)	76.2 (54.9 – 90.7)	84.2 (62.8 – 95.8)	61.9 (40.3 – 80.5)
Spec % (95%CI)	23.5 (8.0 – 47.5)	88.2 (66.3 – 98.0)	76.5 (52.5 – 92.0)	70.6 (46.4 – 88.3)	68.2 (47.0 – 84.9)	72.7 (51.7 – 88.1)	73.7 (51.0 – 89.6)
Acc % (95%CI)	82.3 (72.7 – 89.5)	76.9 (61.9 – 88.1)	77.8 (62.2 – 89.1)	78.9 (63.9 – 89.7)	72.1 (57.4 – 83.9)	78.0 (63.6 – 88.7)	67.5 (52.0 – 80.6)
AUC (95%CI)	0.69 (0.52 – 0.86)	0.77 (0.62 – 0.92)	0.84 (0.71 – 0.97)	0.83 (0.70 – 0.96)	0.72 (0.55 – 0.88)	0.81 (0.68 – 0.95)	0.65 (0.47 – 0.83)

Acc = accuracy; AUC = area under the curve; C = constipation; CI = confidence interval; D = diarrhoea; HC = healthy control; IBS = irritable bowel syndrome; M = mixed; Sens = sensitivity; Spec = specificity; VOC = volatile organic compound

Table 5.6: Characteristics VOCs used in logistic regression models

VOC	Model	1/K0	RT	1/K0 radius	RT radius
Breath					
PB0	IBS-C vs HC*; IBS-C vs IBS-M*	0.770	161.5	0.006	5.5
PB1	Quality of life; Quality of life*	0.739	67.4	0.006	2.3
PB2	IBS-D vs IBS-M; Probiotics	0.757	30.2	0.005	1.7
PB11	IBS-D vs HC; IBS-D vs IBS-M; IBS-D vs HC*	0.684	30.2	0.005	2.1
PB12	IBS-D vs IBS-C*	0.704	30.0	0.007	1.7
PB14	Quality of life; Quality of life*	0.619	93.1	0.006	3.2
PB23	Visceral sensitivity	0.502	6.9	0.006	2.0
PB28	Anxiety; Anxiety*	0.609	8.5	0.006	1.8
PB29	Antibiotics*	0.530	11.5	0.005	1.6
PB45	IBS-C vs HC; IBS-C vs IBS-M; IBS-C vs IBS-M*	0.666	13.6	0.005	2.1
PB57	IBS-M vs HC	0.707	43.7	0.005	1.6
PB61	IBS-C vs HC; IBS-D vs IBS-C; IBS-C vs IBS-M; IBS-C vs HC*	0.558	18.6	0.003	1.9
PB63	Antibiotics	0.550	7.5	0.004	1.5
PB66	Visceral sensitivity	0.603	37.8	0.004	1.6
PB74	Depression	0.597	64.8	0.009	2.3
PB77	Anxiety; Anxiety*; Visceral sensitivity*	0.884	37.3	0.008	1.0
PB78	Depression; Symptom severity; Depression*	0.640	157.7	0.007	4.8
PB81	Pooled IBS vs HC; Pooled IBS vs HC*; Symptom severity	0.810	3.0	0.004	2.0
PB89	IBS-D vs IBS-C; IBS-D vs IBS-C*	0.625	30.6	0.005	1.4
Faeces					
PF0	Depression	0.859	108.2	0.005	4.6
PF3	Antibiotics	0.548	5.0	0.007	1.1
PF9	IBS pooled vs HC; IBS-M vs HC; IBS-M vs HC*	0.528	0.5	0.006	0.7
PF10	Anxiety; Visceral sensitivity; Visceral sensitivity*	0.571	4.1	0.004	1.6
PF32	IBS-D vs IBS-M	0.588	19.2	0.004	1.5
PF37	IBS-D vs IBS-M	0.451	9.1	0.003	2.6
PF43	IBS-D vs IBS-C	0.662	9.1	0.005	1.1
PF56	IBS pooled vs HC; IBS-D vs HC; IBS-C vs HC; IBS-M vs HC; IBS-M vs HC*	0.684	31.6	0.008	2.0
PF63	IBS-D vs IBS-M*	0.583	14.1	0.005	1.6
PF64	IBS-D vs IBS-M*	0.650	2.5	0.007	1.5
PF65	Quality of life	0.670	28.2	0.003	3.8
PF74	Probiotics	0.528	9.1	0.005	0.8
PF80	Anxiety	0.554	17.5	0.004	1.2
PF84	Quality of life	0.572	9.0	0.006	1.1
PF87	Symptom severity; Symptom severity*	0.573	0.0	0.004	1.0
PF92	IBS-D vs HC	0.640	13.5	0.003	1.3
PF116	Depression; Depression*	0.539	11.2	0.003	2.7
PF170	IBS-C vs HC	0.573	64.0	0.004	1.8
PF172	IBS-D vs IBS-C	0.583	24.8	0.004	1.7
PF207	Symptom severity; Symptom severity*	0.708	21.5	0.003	0.9
PF208	IBS-C vs IBS-M; Probiotics*	0.578	18.5	0.003	3.1

C = constipation; CI = confidence interval; D = diarrhoea; HC = healthy control; IBS = irritable bowel syndrome; M = mixed; PB = breath volatile; PF = faecal volatile; VOC = volatile organic compound.
*Combination model

5.3.3.2 *IBS subtypes versus healthy controls*

Most of the models differentiating the individual IBS subtypes with HC appear less optimal than the pooled IBS patient models, as shown in table 5.5. In breath, the best classification was found when differentiating IBS-C patients from HC with a specificity of 91.7% (75.1% – 98.6%), sensitivity of 66.7% (44.9% – 84.1%), and AUC of 0.81 (0.67 – 0.94). In faeces, this differentiation between IBS-C and HC was possible with 94.7% (76.7% – 99.7%) specificity, 68.4% (45.5% – 86.1%) sensitivity, and an AUC of 0.88 (0.76 – 0.99). This faecal model was based upon the VOCs PF56 and PF170, the former one was also included in the pooled IBS model. Pooling VOCs of both matrices resulted in a similar outcome (table 5.5, figure 5.4).

IBS-D patients were better differentiated by breath VOCs 0.70 (0.56 – 0.85) AUC, 66.7% (47.6% – 82.4%) sensitivity, and 75.0% (55.1% – 89.2%) specificity. In faeces, differentiation resulted in an AUC of 0.69 (0.53 – 0.86) and in the model combining both VOCs the resulting AUC was the highest at 0.77 (0.62 – 0.92).

Differentiating IBS-M patients and HC in breath resulted in an AUC of 0.68 (0.53 – 0.84). Again, faecal VOCs performed better, based upon the VOCs PF9 and PF56 from the pooled IBS model with an AUC of 0.82 (0.68 – 0.95), similar as the combined model (AUC 0.83 (0.70 – 0.96)). Given that IBS is a heterogenous disorder with a multifactorial aetiology, we hypothesized that VOC profiles might also differ between different IBS subtypes. Therefore, the role of volatomics in differentiating IBS subtypes from each other was investigated (table 5.5, figure 5.4).

5.3.3.3 *Differentiating IBS patient subtypes*

In breath, the best classification was obtained by comparing IBS-D and IBS-C patients, resulting in an AUC of 0.78 (0.64 – 0.91) (specificity of 88.9% (72.7% – 97.1%), sensitivity of 57.1% (35.8% – 76.7%)). This was amongst other things based upon PB61 which was also found to differentiate between IBS-C and IBS-M patients, and IBS-C versus HC. Differentiating IBS-M patients from IBS-D and IBS-C patients resulted in lower AUCs (respectively 0.65 (0.50 – 0.81) and 0.67 (0.51 – 0.83)). When focusing on faecal volatiles AUCs ranged between 0.69 and 0.74 (table 5.5, figure 5.4). There was no overlap in faecal VOCs when subtyping IBS-patients or when different IBS subtypes were compared to HC.

When volatiles from both breath and faeces were combined, the classifier's performance for IBS-C versus IBS-M was 0.65 (0.47 – 0.83), specificity of 73.7% (51.0% – 89.6%), and sensitivity of 61.9% (40.3% – 80.5%). An acceptable differentiation was found when comparing IBS-D with IBS-C patients, characterized by an AUC of 0.81 (0.68 – 0.95), 72.7% (51.7% – 88.1%) specificity, and 84.2% (62.8% – 95.8%) sensitivity, and when comparing IBS-D to IBS-M patients, resulting in an AUC of 0.72 (0.55 – 0.88), 76.2% (54.9% – 90.7%) sensitivity, and 68.2% (47.0% – 84.9%) specificity.

In general, an acceptable differentiation of IBS patients from HC was observed, as well as between subtypes based upon different VOCs. Subsequently, we wanted to evaluate the influence of other clinical characteristics on VOC profiles to further elucidate the between-patient variability. Therefore, patients were reclassified based on clinical characteristics independent of Rome subtype (table 5.7). For these analyses HC were not included.

5.3.1 Analysis based on clinical characteristics other than stool pattern

5.3.1.1 *Psychological comorbidities*

Thirty-nine out of the 66 IBS patients that completed the HADS questionnaire (59.1%) scored positive for anxiety (HADS anxiety >8) and were compared with patients scoring negative for anxiety. Both breath and faecal analysis allowed a modest differentiation between both groups with an AUC of 0.66 (table 5.7). None of the contributing VOCs used in the logistic regression were found to overlap with the differentiation between subtypes (table 5.6). Combining the VOCs of both matrices did not improve the differentiating capacity (AUC 0.66 (0.52 – 0.80)).

Twelve out of 66 IBS patients (18.2%) scored positive for depression (HADS depression >8), and all patients scoring positive for depression also scored positive for anxiety, making this a subpopulation of the previous analysis. Based upon breath volatiles, a differentiation between depressed and non-depressed patients was found with an AUC of 0.64 (0.39 – 0.90) (specificity of 93.9% (84.3% – 98.4%), sensitivity of 44.4% (16.1% – 75.9%)). In faeces, the AUC was higher at 0.78 (0.61 – 0.95) (33.3% (9.3% – 66.7%) sensitivity, 98.0% (90.4% – 99.9%) specificity) and, as with anxiety, none of the contributing VOCs were found to overlap with the differentiation between subtypes. The combined model performed similar with a specificity of 95.9% (87.2% – 99.3%), sensitivity of 55.6% (24.1% – 83.9%), and an AUC of 0.76 (0.54 – 0.98).

Table 5.7: Differentiating models based on clinical characteristics in patients

	Psychological comorbidities		Microbiota influencing therapies		Symptom scores		
	Depression	**Anxiety**	**Antibiotics**	**Probiotics**	**Quality of life**	**Visceral sensitivity**	**Symptom severity**
Breath							
Sens % (95%CI)	44.4 (16.1 – 75.9)	84.8 (69.6 – 94.2)	53.8 (27.5 – 78.7)	52.9 (29.8 – 75.2)	86.2 (70.0 – 95.4)	62.1 (43.7 – 78.2)	89.4 (78.0 – 96.0)
Spec % (95%CI)	93.9 (84.3 – 98.4)	40.0 (22.4 – 59.8)	95.9 (87.2 – 99.3)	86.7 (74.4 – 94.4)	55.2 (37.1 – 72.3)	72.4 (54.3 – 86.3)	36.4 (12.8 – 66.3)
Acc % (95%CI)	86.2 (75.5 – 93.4)	65.5 (52.7 – 76.9)	87.1 (77.0 – 93.8)	77.4 (65.8 – 86.5)	70.7 (58.1 – 81.3)	67.2 (54.5 – 78.4)	79.3 (67.5 – 88.3)
AUC (95%CI)	0.64 (0.39 – 0.90)	0.66 (0.52 – 0.80)	0.79 (0.63 – 0.95)	0.70 (0.54 – 0.86)	0.69 (0.55 – 0.83)	0.66 (0.51 – 0.80)	0.56 (0.32 – 0.81)
Faeces							
Sens % (95%CI)	33.3 (9.3 – 66.7)	75.8 (59.1 – 88.0)	23.1 (6.3 – 50.8)	47.1 (24.8 – 70.3)	69.0 (50.7 – 83.7)	58.6 (40.3 - 75.3)	95.7 (86.7 – 99.3)
Spec % (95%CI)	98.0 (90.4 – 99.9)	56.0 (36.5 – 74.2)	95.9 (87.2 – 99.3)	88.9 (77.1 – 95.8)	58.6 (40.3 – 75.3)	62.1 (43.7 – 78.2)	18.2 (3.2 – 48.3)
Acc % (95%CI)	87.9 (77.6 – 94.6)	67.2 (54.5 – 78.4)	80.6 (69.5 – 89.1)	77.4 (65.8 – 86.5)	63.8 (50.9 – 75.3)	60.3 (47.4 – 72.3)	81.0 (69.5 – 89.6)
AUC (95%CI)	0.78 (0.61 – 0.95)	0.66 (0.52 – 0.81)	0.75 (0.58 – 0.91)	0.72 (0.57 – 0.87)	0.61 (0.46 – 0.76)	0.57 (0.42 – 0.72)	0.66 (0.48 – 0.85)
Breath and faeces							
Sens % (95%CI)	55.6 (24.1 – 83.9)	60.6 (43.4 – 76.0)	46.2 (21.3 – 72.6)	52.9 (29.8 – 75.2)	86.2 (70.0 – 95.4)	69.0 (50.7 – 83.7)	95.7 (86.7 – 99.3)
Spec % (95%CI)	95.9 (87.2 – 99.3)	68.0 (48.2 – 83.9)	83.7 (71.4 – 92.1)	95.6 (86.1 – 99.2)	55.2 (37.1 – 72.3)	62.1 (43.7 – 78.2)	18.2 (3.2 – 48.3)
Acc % (95%CI)	89.7 (79.8 – 95.7)	63.8 (50.9 – 75.3)	75.8 (64.1 – 85.2)	83.9 (73.2 – 91.5)	70.7 (58.1 – 81.3)	65.5 (52.7 – 76.9)	81.0 (69.5 – 89.6)
AUC (95%CI)	0.76 (0.54 – 0.98)	0.66 (0.52 – 0.80)	0.73 (0.57 – 0.89)	0.75 (0.59 – 0.91)	0.69 (0.55 – 0.83)	0.64 (0.49 – 0.78)	0.66 (0.48 – 0.85)

Acc = accuracy; AUC= area under the curve; CI = confidence interval; Sens = sensitivity; Spec = specificity.

5.3.1.2 *Microbiota influencing therapies*

Thirty IBS patients (41.7%) used antibiotics and/or probiotics in the three months prior to sample collection. Patients using antibiotics (13/72, 18.1%) were differentiated by breath volatiles from those not using antibiotics with an AUC of 0.79 (0.63 – 0.95), 95.9% (87.2% – 99.3%) specificity, and 53.8% (27.5% – 78.7%) sensitivity). Based upon faecal volatiles, the AUC was 0.75 (0.58 – 0.91) (23.1% sensitivity (6.3% – 50.8%) and 95.9% (87.2% – 99.3%) specificity). In the combined model, the classifier's performance was lower (AUC 0.73 (0.57 – 0.89)).

Patients on probiotics (20/72, 27.8%) were accurately differentiated from those not using probiotics based upon VOCs in breath (AUC 0.70 (0.54 – 0.86)), faeces (AUC 0.72 (0.57 – 0.87)), or both combined (AUC 0.75 (0.59 – 0.91)).

5.3.1.3 *Symptom scores*

IBS patients were also differentiated based on the scores of the IBS-QOL questionnaire, IBS-SSS, and VSI (table 5.7). Based upon volatiles in breath, patients with a high score on IBS-QOL could be differentiated from those with a low score with an AUC of 0.69 (0.55 – 0.83). Faecal volatiles on the other hand, were not as performant (AUC of 0.61 (0.46 – 0.76)). Combining both breath and faecal VOCs, groups resulted in the same differentiating values as breath models (AUC 0.69 (0.55 – 0.83)). Also, no VOCs were found to be overlapping with those which were able to differentiate IBS subtypes.

Furthermore, patients with a high VSI could be differentiated from patients with a low VSI in breath, faeces, and combined models (respectively AUCs of 0.66 (0.51 – 0.80), 0.57 (0.42 – 0.72), 0.64 (0.49 – 0.78)).

Differentiating patients based on symptom severity (IBS-SSS) performed similar with AUCs ranging between 0.56 and 0.66, albeit at the cost of low specificities.

5.4 Discussion and conclusion

Research and clinical practice are still eagerly awaiting the discovery of biomarkers to diagnose and characterize patients with IBS. A recent development in this area is the field of volatomics studying volatile organic compounds (VOCs).[156]

A recent systematic review from our group stressed that current volatomics research in IBS is heterogenous and limited to mostly faecal analysis, but still suggested promising clinical applications.[156] Our study is the first to demonstrate the use of VOCs in diagnosing and subtyping patients with IBS in breath and faecal samples using the more clinically applicable MCC/IMS. The results of our models are in line with previous studies using GC-MS.[157,158,160–162,166,201,202] When differentiating IBS patients from HC, faecal volatiles performed better compared to breath. However, as there are limited differences in differentiating values, breath sampling could be preferred given the ease of providing and analysing a sample. This is further strengthened by the fact that both breath and faecal volatiles comparably differentiated each IBS subtype from HC. However, combining volatiles of both biological samples showed no added value in this feasibility study. The highest classification characteristics across all matrices were observed when pooling IBS subtypes and comparing them to HC (AUC of 0.80 versus 0.62). This showed the feasibility of the method and the presence of different VOCs between HC and symptomatic patients with IBS and suggests that VOC measurement and identification could possibly evolve into a clinically useful biomarker. However, when comparing individual IBS subtypes (IBS-D, IBS-C, and IBS-M) with HC, the differentiating potential was found to be slightly lower. This is somewhat unexpected as we hypothesised that subtyping patients based on their dominant stool pattern

(confer Rome IV criteria) would increase the ability of VOCs to differentiate patients from HC, because of underlying differences in pathophysiology. Still, when we differentiated these patient subtypes from each other, results were also acceptable. One VOC in breath, PB61 (RT 18.6), was found as an important classifier when differentiating IBS-C patients from IBS-D, IBS-M, and HC, possibly linking its presence to the IBS-C subtype. There was little to no overlap in VOCs when subtyping IBS patients or when subtypes were compared to HC, which could suggest the existence of subtype-specific volatiles that could relate to the dominant stool pattern.

IBS patients were also differentiated based on clinical characteristics other than stool pattern. VOCs that contributed to these differentiations were different from those allowing subtyping of IBS patients, suggesting that other parameters could play a role in the subtyping of IBS patients.

Since the microbiota are omnipresent in the human colon and faeces, and since they produce specific VOCs[163], it is expected that the microbial composition would be better reflected in faecal VOC profiles compared to breath profiles. However, while the classification models differentiating IBS patients having used probiotics in the last three months versus IBS patients not using probiotics had a similar performance in faeces and breath (AUC of 0.72 and 0.70), the models utilising antibiotic use in the last three months had a slightly better performance when using breath (AUC of 0.75 versus 0.79). Hence, the microbiota have an influence on VOC composition in general and these VOCs could reflect a change in microbial composition. This has also been demonstrated by Smolinska *et al.* in patients with Crohn's disease and Sagar *et al.* in patients with bile acid diarrhoea and IBS-D.[163,164]

However, more research is needed to further elucidate the origin and relationship between the microbiota on the one hand and its manipulation using medication and VOC profiles on the other hand.

Psychological characteristics like the presence of depression or anxiety had a higher differentiating ability in faeces compared to breath models. This could be explained by differences in underlying pathophysiology and metabolism in IBS patients with comorbid anxiety or depression and the role of the gut-brain-microbiome axis.[210] This is in accordance with the recent publications of Black *et al.* demonstrating that classifying patients based on psychological burden had a higher stability over time compared to a classification based on dominant stool pattern.[211,212]

Breath and faecal volatiles were also able to differentiate patients based on questionnaires assessing symptom severity, quality of life, and visceral sensitivity. Considering that those clinical characteristics, other than dominant stool pattern, are able to accurately differentiate patients demonstrates the importance of questioning these characteristics and maybe consider alternative classifications of IBS patients rather than purely using stool pattern as per the Rome criteria. The heterogeneity of IBS, both in clinical presentation and underlying pathophysiology, further demonstrates the need to thoroughly characterize patients when looking for novel biomarkers.

Despite these positive findings, our study did have limitations which should be considered for future research. First, this feasibility study had a moderate sample size that needs to be increased in future validation trials. As a result, despite the use of lasso regression in order

to avoid overfitting of the data, the analysis based on clinical characteristics oftentimes involved a small sample size which could still lead to overfitting and overoptimistic results.

Secondly, only HC and IBS patients were included as the intent was to explore the feasibility of sampling and analysing VOCs by MCC/IMS in these populations. When further investigating the role of volatomics in clinical practice, large-scale studies should be initiated enclosing other common gastrointestinal disorders, such as celiac disease and inflammatory bowel disease, since these are important differential diagnoses of IBS. If VOCs can differentiate IBS from these organic disorders, they could be further developed into a diagnostic biomarker test, which is one of the major unmet needs in IBS management.

Thirdly, we only collected samples at a single time point. Little is known about the natural evolution of VOC profiles over time. Hence, long-term follow-up of a patient population to evaluate spontaneous fluctuation and the impact of specific therapies on VOC profiles will help understand and optimize the current classification models.

We also did not record the consistency of the faecal samples and it is currently unknown if stool consistency *per se* has an influence on VOC output during measurement. Nevertheless, further optimization of faecal VOC analysis, taking stool consistency into account, is advised.

In conclusion, we demonstrated the potential of VOCs in the characterization of patients with IBS. VOCs accurately differentiated IBS patients from HC. In addition, independent VOCs were found to differentiate IBS patients when classified into the classical subtypes based on their dominant stool pattern (Rome IV criteria) compared to controls. Furthermore,

volatiles were able to distinguish patients based on clinical characteristics, other than their dominant stool pattern, such as psychological states, symptom scores, and microbiota-influencing treatment, suggesting the possibility of alternative subtyping of IBS patients. Over the years the validity of the Rome criteria has been questioned partly based on its subjectivity and strictness.[213,214] However, with our increasing knowledge about IBS it has also become clear that the underlying mechanisms of the different subtypes are not as clear cut and distinct as once believed. Dividing patients based on dominant stool pattern might no longer be the best option. New subtypes based on other clinical characteristics and underlying pathophysiological mechanisms would be more interesting depending on the purpose of subtyping patients.

We therefore plead for the inclusion of other clinical characteristics besides the dominant stool pattern when developing biomarkers for IBS in general and using volatomics in particular. The results of this study should be validated in a larger population including an extensive clinical characterisation of patients and their microbiota analysis.

5.5 Supplementary tables

Table 5.5.1: Detected volatile organic compounds in breath samples

VOC	1/K0	RT	1/K0 radius	RT radius
PB0	0.770	161.5	0.006	5.5
PB1	0.739	67.4	0.006	2.3
PB2	0.757	30.2	0.005	1.7
PB3	0.737	3.7	0.008	1.9
PB4	0.713	4.5	0.006	2.6
PB5	0.689	3.5	0.008	2.3
PB6	0.639	4.2	0.005	1.7
PB7	0.624	3.5	0.004	1.7
PB8	0.700	38.5	0.005	2.0
PB9	0.661	30.2	0.007	2.2
PB10	0.644	30.6	0.005	2.2
PB11	0.684	30.2	0.005	2.1
PB12	0.704	30.0	0.007	1.7
PB13	0.611	17.1	0.005	2.2
PB14	0.619	93.1	0.006	3.2
PB15	0.586	45.1	0.005	1.8
PB16	0.547	54.5	0.006	2.4
PB17	0.587	3.9	0.006	1.5
PB18	0.577	26.1	0.005	1.4
PB19	0.569	19.3	0.006	1.6
PB20	0.540	7.6	0.006	2.0
PB21	0.527	6.5	0.006	1.6
PB22	0.515	7.5	0.005	2.3
PB23	0.502	6.9	0.006	2.0
PB24	0.515	1.5	0.009	0.9
PB25	0.490	0.5	0.006	1.2
PB26	0.458	4.5	0.007	4.0
PB27	0.782	30.7	0.008	2.1
PB28	0.609	8.5	0.006	1.8
PB29	0.530	11.5	0.005	1.6
PB30	0.637	17.6	0.008	2.6
PB31	0.796	2.3	0.009	2.0
PB32	0.653	4.2	0.006	2.1
PB33	0.600	22.7	0.005	1.8
PB34	0.586	10.6	0.005	2.1
PB35	0.664	91.5	0.007	2.5
PB36	0.568	13.6	0.005	1.6
PB37	0.596	34.1	0.006	2.1
PB38	0.609	44.2	0.005	2.2
PB39	0.607	4.0	0.006	1.7
PB40	0.682	75.0	0.009	2.8
PB41	0.695	17.1	0.008	2.1

VOC	1/K0	RT	1/K0 radius	RT radius
PB46	0.643	34.5	0.005	1.3
PB47	0.886	134.5	0.008	3.4
PB48	0.887	74.0	0.009	3.4
PB49	0.824	17.1	0.009	2.3
PB50	0.687	38.8	0.007	2.2
PB51	0.538	2.0	0.007	1.5
PB52	0.765	13.1	0.009	1.3
PB53	0.654	44.3	0.005	2.5
PB54	0.606	114.4	0.012	4.3
PB55	0.741	114.1	0.006	2.6
PB56	0.825	32.7	0.007	1.8
PB57	0.707	43.7	0.005	1.6
PB58	0.557	1.5	0.006	1.0
PB59	0.515	34.2	0.003	4.1
PB60	0.458	34.7	0.003	4.0
PB61	0.558	18.6	0.003	1.9
PB62	0.558	13.6	0.003	2.5
PB63	0.550	7.5	0.004	1.5
PB64	0.515	15.1	0.002	2.0
PB65	0.515	11.1	0.002	1.1
PB66	0.603	37.8	0.004	1.6
PB67	0.578	5.5	0.003	0.9
PB68	0.634	30.3	0.003	1.7
PB69	0.653	17.1	0.006	1.6
PB70	0.515	25.3	0.003	2.1
PB71	0.884	152.4	0.010	4.5
PB72	0.780	7.9	0.007	2.8
PB73	0.456	19.6	0.003	3.4
PB74	0.597	64.8	0.009	2.3
PB75	0.596	54.9	0.008	2.9
PB76	0.887	127.1	0.005	3.6
PB77	0.884	37.3	0.008	1.0
PB78	0.640	157.7	0.007	4.8
PB79	0.609	97.5	0.004	2.7
PB80	0.729	67.4	0.003	2.2
PB81	0.810	3.0	0.004	2.0
PB82	0.762	192.0	0.007	5.2
PB83	0.579	2.6	0.004	1.0
PB84	0.572	7.0	0.004	1.1
PB85	0.780	13.0	0.005	1.6
PB86	0.611	12.1	0.006	1.4
PB87	0.652	12.6	0.006	1.7

PB42	0.740	38.8	0.008	1.4
PB43	0.678	55.4	0.006	3.3
PB44	0.726	8.6	0.008	1.6
PB45	0.666	13.6	0.005	2.1

PB88	0.663	20.0	0.004	1.3
PB89	0.625	30.6	0.005	1.4
PB90	0.681	7.6	0.004	1.3
PB91	0.693	28.8	0.003	2.2

RT = retention time; VOC = volatile organic compound. The order of the VOCs does not have a reason but the order in which they were selected.

Table 5.5.2: Detected volatile organic compounds in faecal samples

VOC	1/K0	RT	1/K0 radius	RT radius
PF0	0.859	108.2	0.005	4.6
PF1	0.652	5.5	0.006	1.3
PF2	0.650	0.5	0.008	0.6
PF3	0.548	5.0	0.007	1.1
PF4	0.547	0.7	0.004	0.8
PF5	0.450	4.0	0.003	2.1
PF6	0.506	0.5	0.003	2.2
PF7	0.502	4.1	0.006	1.6
PF8	0.529	5.0	0.006	0.9
PF9	0.528	0.5	0.006	0.7
PF10	0.571	4.1	0.004	1.6
PF11	0.893	109.2	0.007	5.8
PF12	0.853	24.7	0.005	1.7
PF13	0.828	23.4	0.005	1.4
PF14	0.759	55.3	0.009	2.3
PF15	0.726	55.5	0.005	2.5
PF16	0.682	54.8	0.010	2.0
PF17	0.716	32.2	0.006	1.2
PF18	0.700	31.7	0.005	1.3
PF19	0.700	26.8	0.009	1.4
PF20	0.798	11.7	0.008	1.4
PF21	0.785	8.6	0.006	1.2
PF22	0.764	8.2	0.008	1.6
PF23	0.744	7.0	0.007	1.3
PF24	0.720	6.6	0.008	1.7
PF25	0.450	19.2	0.003	3.0
PF26	0.450	29.7	0.003	3.7
PF27	0.669	13.7	0.008	1.5
PF28	0.652	14.6	0.008	1.8
PF29	0.649	23.8	0.006	1.2
PF30	0.563	21.7	0.006	1.5
PF31	0.547	29.2	0.003	3.6
PF32	0.588	19.2	0.004	1.5
PF33	0.594	2.0	0.005	1.5
PF34	0.602	33.4	0.003	3.9
PF35	0.691	7.7	0.007	1.7
PF36	0.586	5.8	0.009	1.0
PF37	0.451	9.1	0.003	2.6

VOC	1/K0	RT	1/K0 radius	RT radius
PF46	0.450	63.3	0.003	5.8
PF47	0.547	20.1	0.005	1.8
PF48	0.507	9.1	0.009	1.1
PF49	0.583	28.7	0.006	1.6
PF50	0.771	10.6	0.004	1.2
PF51	0.749	10.4	0.007	1.6
PF52	0.628	27.7	0.006	1.7
PF53	0.583	35.6	0.007	1.6
PF54	0.583	44.7	0.007	1.5
PF55	0.778	18.2	0.008	1.6
PF56	0.684	31.6	0.008	2.0
PF57	0.677	21.6	0.006	1.7
PF58	0.505	14.6	0.003	2.7
PF59	0.505	20.6	0.003	3.1
PF60	0.505	28.2	0.003	3.5
PF61	0.624	17.1	0.005	1.8
PF62	0.610	17.6	0.005	1.7
PF63	0.583	14.1	0.005	1.6
PF64	0.650	2.5	0.007	1.5
PF65	0.670	28.2	0.003	3.8
PF66	0.909	55.4	0.007	3.0
PF67	0.914	24.2	0.006	2.0
PF68	0.649	65.4	0.005	2.0
PF69	0.585	53.5	0.005	2.1
PF70	0.527	18.2	0.003	3.0
PF71	0.848	28.2	0.005	1.3
PF72	0.786	11.1	0.004	1.1
PF73	0.671	10.1	0.005	1.3
PF74	0.528	9.1	0.005	0.8
PF75	0.584	8.1	0.004	1.0
PF76	0.547	15.1	0.005	1.5
PF77	0.645	27.7	0.006	1.3
PF78	0.623	5.5	0.005	1.1
PF79	0.622	0.5	0.005	1.5
PF80	0.554	17.5	0.004	1.2
PF81	0.598	10.5	0.005	1.2
PF82	0.863	32.1	0.006	1.8
PF83	0.769	32.1	0.006	1.3

PF38	0.551	9.4	0.007	1.3
PF39	0.617	13.7	0.007	1.4
PF40	0.608	9.1	0.005	1.2
PF41	0.651	9.1	0.006	1.0
PF42	0.640	6.6	0.006	1.1
PF43	0.662	9.1	0.005	1.1
PF44	0.611	6.1	0.005	1.1
PF45	0.717	13.2	0.007	1.6
PF92	0.640	13.5	0.003	1.3
PF93	0.698	22.5	0.006	1.7
PF94	0.692	11.0	0.006	1.3
PF95	0.720	26.0	0.005	1.3
PF96	0.721	22.5	0.005	1.4
PF97	0.645	19.5	0.004	1.3
PF98	0.637	21.0	0.004	1.1
PF99	0.679	43.1	0.004	1.1
PF100	0.547	61.9	0.003	6.0
PF101	0.836	22.0	0.004	1.5
PF102	0.858	19.6	0.005	1.8
PF103	0.877	13.5	0.004	2.0
PF104	0.681	14.0	0.004	1.6
PF105	0.698	16.5	0.004	1.5
PF106	0.617	10.1	0.003	1.2
PF107	0.744	20.1	0.004	2.0
PF108	0.865	12.0	0.007	2.4
PF109	0.845	11.5	0.007	1.3
PF110	0.823	12.0	0.005	1.3
PF111	0.679	48.1	0.005	1.6
PF112	0.721	41.1	0.003	4.5
PF113	0.744	25.0	0.004	2.1
PF114	0.760	24.0	0.005	1.9
PF115	0.548	50.1	0.003	2.4
PF116	0.539	11.2	0.003	2.7
PF117	0.562	9.6	0.003	2.6
PF118	0.694	2.0	0.003	2.1
PF119	0.513	5.1	0.004	0.9
PF120	0.647	58.3	0.006	2.0
PF121	0.681	17.7	0.005	1.9
PF122	0.668	19.2	0.004	1.3
PF123	0.595	25.3	0.004	1.3
PF124	0.571	15.0	0.003	2.8
PF125	0.559	5.5	0.004	1.4
PF126	0.763	0.5	0.006	1.3
PF127	0.554	250.4	0.007	5.9
PF128	0.722	92.8	0.006	4.3
PF129	0.711	51.0	0.004	2.5
PF130	0.866	23.5	0.004	1.7
PF131	0.749	133.3	0.005	3.4
PF84	0.572	9.0	0.006	1.1
PF85	0.650	45.1	0.006	3.6
PF86	0.639	36.6	0.007	1.6
PF87	0.573	0.0	0.004	1.0
PF88	0.722	0.0	0.005	1.4
PF89	0.720	10.2	0.007	1.0
PF90	0.481	0.4	0.004	0.8
PF91	0.642	9.0	0.004	1.0
PF140	0.672	6.5	0.005	1.0
PF141	0.962	55.1	0.007	2.8
PF142	0.841	18.5	0.005	2.2
PF143	0.825	18.5	0.007	1.8
PF144	0.801	21.0	0.004	3.3
PF145	0.882	54.6	0.004	3.2
PF146	0.959	131.1	0.005	4.4
PF147	0.772	14.0	0.004	1.1
PF148	0.849	18.5	0.003	1.5
PF149	0.819	28.5	0.005	1.7
PF150	0.933	56.0	0.005	2.7
PF151	0.878	75.2	0.007	2.8
PF152	0.941	75.0	0.006	2.6
PF153	0.896	93.7	0.006	3.4
PF154	0.711	131.0	0.006	6.8
PF155	0.642	156.8	0.006	7.6
PF156	0.665	253.3	0.005	9.4
PF157	0.594	80.0	0.009	2.8
PF158	0.583	91.5	0.005	4.1
PF159	0.582	227.1	0.004	7.4
PF160	0.620	49.1	0.006	1.3
PF161	0.621	228.7	0.006	7.5
PF162	0.604	152.2	0.006	6.7
PF163	0.627	106.5	0.004	4.4
PF164	0.580	76.0	0.005	5.5
PF165	0.583	177.2	0.005	5.2
PF166	0.653	109.6	0.004	5.2
PF167	0.546	39.3	0.003	4.6
PF168	0.538	27.4	0.003	3.7
PF169	0.567	38.0	0.003	2.6
PF170	0.573	64.0	0.004	1.8
PF171	0.554	54.0	0.004	2.5
PF172	0.583	24.8	0.004	1.7
PF173	0.529	6.5	0.003	0.9
PF174	0.597	40.8	0.003	2.8
PF175	0.546	82.5	0.003	6.8
PF176	0.505	46.2	0.003	4.5
PF177	0.504	74.2	0.003	6.1
PF178	0.559	32.7	0.003	3.9
PF179	0.534	46.0	0.003	2.1

PF132	0.578	106.9	0.005	4.3
PF133	0.713	66.7	0.005	2.8
PF134	0.684	37.1	0.005	1.3
PF135	0.653	53.6	0.005	1.6
PF136	0.816	7.6	0.005	1.3
PF137	0.720	3.3	0.005	1.3
PF138	0.594	13.1	0.005	0.9
PF139	0.631	3.4	0.005	1.0
PF188	0.686	69.0	0.004	3.5
PF189	0.610	28.5	0.005	1.5
PF190	0.680	3.5	0.003	2.3
PF191	0.649	37.0	0.003	4.1
PF192	0.678	65.2	0.003	6.1
PF193	0.699	41.5	0.003	4.7
PF194	0.619	38.2	0.003	4.1
PF195	0.617	20.2	0.003	3.1
PF196	0.654	19.7	0.003	2.0
PF197	0.725	68.7	0.003	6.1
PF198	0.680	51.5	0.006	0.6
PF199	0.565	7.2	0.001	1.0
PF180	0.524	41.0	0.003	4.2
PF181	0.538	18.3	0.003	2.4
PF182	0.525	14.0	0.002	1.7
PF183	0.525	25.5	0.003	3.7
PF184	0.584	49.1	0.004	1.7
PF185	0.504	7.0	0.004	1.0
PF186	0.572	51.5	0.003	1.4
PF187	0.571	41.5	0.002	1.8
PF200	0.706	24.3	0.003	1.2
PF201	0.649	74.7	0.003	2.5
PF202	0.711	75.5	0.003	3.4
PF203	0.719	86.3	0.003	2.6
PF204	0.562	16.2	0.003	2.8
PF205	0.686	24.5	0.003	2.0
PF206	0.717	56.5	0.003	1.8
PF207	0.708	21.5	0.003	0.9
PF208	0.578	18.5	0.003	3.1
PF209	0.708	10.5	0.003	2.6
PF210	0.654	29.0	0.003	2.3

RT = retention time; VOC = volatile organic compound. The order of the VOCs does not have a reason but the order in which they were selected.

Table 5.5.3: Selected volatile organic compounds by lasso regression

	Pooled IBS vs HC	IBS-D vs HC	IBS-C vs HC	IBS-M vs HC	IBS-D vs IBS-C	IBS-C vs IBS-M	IBS-D vs IBS-M
Breath							
VOCs[#]	*PB11, PB31, PB37, PB57, PB58, PB66, PB70, PB81*	*PB7,* ***PB11****, PB31,* ***PB35****,* ***PB37****,* ***PB41****, PB45,* ***PB57****,* ***PB66****, PB70, PB75, PB78, PB80,* ***PB81****, PB89, PB90*	*PB45,* ***PB61***	***PB2****,* ***PB14****,* ***PB21****, PB24, PB29,* ***PB31****, PB32,* ***PB36****,* ***PB37****,* ***PB38****, PB44, PB51, PB54, PB55,* ***PB57****, PB58,* ***PB59****, PB60, PB63,* ***PB66****,* ***PB71****, PB73, PB74,* ***PB75****,* ***PB76****,* ***PB77****, PB78, PB80,* ***PB81****,* ***PB83****, PB88*	*PB0,* ***PB7****,* ***PB12****, PB35, PB56,* ***PB61****, PB63, PB71, PB77,* ***PB89***	***PB45***	***PB2****, PB5,* ***PB11****, PB12,* ***PB14****,* ***PB15****, PB32,* ***PB33****,* ***PB35****, PB42, PB45, PB55,* ***PB58****, PB63, PB71, PB74,* ***PB82****, PB84, PB88, PB89, PB90*
Faeces							
VOCs[#]	*PF0, PF51,* ***PF9****, PF42, PF49,* ***PF56****, PF94, PF125,* ***PF170****, PF184*	*PF9, PF51,* ***PF56****,* ***PF71****,* ***PF84****,* ***PF90****,* ***PF92****,* ***PF94****, PF105, PF124, PF127, PF138, PF143,* ***PF152****, PF154,* ***PF170****,* ***PF172****, PF180, PF184*	*PF0,* ***PF1****, PF2, PF9, PF10, PF34, PF37,* ***PF49****,* ***PF56****, PF75, PF107, PF119, PF126, PF134,* ***PF170****, PF172*	*PF8,* ***PF9,*** *PF49,* ***PF56****,* ***PF78****, PF125, PF206*	*PF0, PF1, PF11, PF21, PF34,* ***PF37****, PF41,* ***PF43****, PF49, PF51, PF52, PF55, PF80, PF116,* ***PF119****, PF127, PF129, PF140, PF146,* ***PF152****, PF156, PF159,* ***PF172****, PF199, PF208*	*PF32, PF36,* ***PF40****, PF41, PF61, PF63, PF65, PF81, PF87, PF105, PF117,* ***PF125****, PF126, PF131, PF137, PF143, PF183,* ***PF208***	***PF3****,* ***PF32****, PF35,* ***PF36****,* ***PF37****,* ***PF40****, PF44,* ***PF51****,* ***PF53****, PF63,* ***PF64****, PF74,* ***PF77****,* ***PF80****, PF90,* ***PF105****, PF111,* ***PF117****,* ***PF123****, PF125, PF131, PF138, PF166, PF172, PF185, PF206,* ***PF208***
Breath and faeces							
VOCs[#]	***PF0****, PF1,* ***PB2****,* ***PF9****,* ***PB11****,* ***PF30****,* ***PB32****,* ***PF42****,* ***PB37****, PB58,* ***PB81****, PB53,* ***PF56****,* ***PB57****,* ***PB58****, PB59, PB62, PB71,* ***PB73****, PF75, PF79,* ***PF94****,* ***PF124****,* ***PF125****, PF127,* ***PF170***	***PB11****, PB15, PB32, PB37,* ***PB57****,* ***PB81****,* ***PF56****,* ***PB62****,* ***PB67****, PB84, PF84,* ***PF90****,* ***PF92****,* ***PF94****, PF127,* ***PF146****, PF152,* ***PF170****,* ***PF172****, PF205*	***PB0****, PF3, PF9, PF22, PB24, PB53,* ***PF56****,* ***PB61****, PF75, PB79, PB81,* ***PB89****, PF119, PF170*	***PF9****,* ***PF56****, PF78*	*PB0, PF3,* ***PB12****, PB33, PF41, PF43,* ***PF52****, PF53, PB61, PB77,* ***PB89****,* ***PF119****, PF146, PF152, PF163,* ***PF172***	***PB0****, PB12, PF32,* ***PB45****, PB61, PF63, PF125, PF143, PF208*	*PB2, PF3, PB7, PB12, PB15, PB30, PB35, PF36, PF37, PB42, PB46, PF49, PF51, PB55,* ***PF63****,* ***PF64****, PB83, PB87,* ***PB89****, PF111, PF119, PF123, PF125, PF144, PF172*

C = constipation; CI = confidence interval; D = diarrhoea; HC = healthy control; IBS = irritable bowel syndrome; M = mixed; VOC = volatile organic compounds [#]*VOCs selected in >30% of the cross-validation models (VOCs in bold are selected in >80% of models).*

Chapter 6 General discussion

6.1 Characteristics of irritable bowel syndrome

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder which has been receiving increasing attention over the years. Research output has been steadily growing with a marked increase in IBS publications from 270 in 2016 to 424 in 2021.[215] But also amongst the general public awareness about this disease has increased substantially. Patients are becoming more vocal, and progress has been made in breaking the taboo surrounding gut disorders. In 2019 we created a website (www.ibsbelgium.org) to help break the stigma and provide scientifically based information about IBS in an easy-to-understand manner. With over 1000 visitors a month it is clear people are actively searching for information. But is it enough? When we asked patients visiting our website if they believe sufficient information is available only 54% answered positively. However, less than 1% of visitors answered this question which raises the question if we can generalise these results to the average IBS patient visiting the site. All in all, while progress has been made, there is still a long way to go.

IBS is characterised by abdominal pain and changes in the stool pattern which can range from constipation to diarrhoea to a combination of both. Stool consistency is evaluated with the help of the visual Bristol stool scale making it a rather subjective scoring method. However, applications are being developed to evaluate stool consistency via photographs analysed by artificial intelligence, providing a more objective and accurate assessment.[216] In our research studies patients most frequently characterised themselves as a diarrhoea or mixed phenotype which is in accordance with the literature. Apart from these classical symptoms, patients often experience other symptoms as well, both intra- and extraintestinal.

Frequently occurring extraintestinal symptoms are fatigue, symptoms related to autonomic dysfunction, pain, and psychological symptoms such as anxiety and depression. Psychological comorbidities are often considered important in the pathophysiology of IBS. In our epidemiological study we also found a high prevalence of both anxiety (65%) and depression (39%). However, the link between psychological disorders and IBS is a classic case of the chicken-or-egg story. On the one hand, psychological disorders can trigger and exacerbate symptoms. On the other hand, excessive symptoms and its impact on daily life can lead to the development of anxiety or depression. A study from 2021 found genetic susceptibility loci shared by IBS and psychological disorders indicating that shared pathophysiological pathways may provide a clue for the association of both disease entities.[65]

IBS has an important impact on quality of life (QOL), rivalling diseases like diabetes and end-stage renal disease.[15–17] Our epidemiological study confirmed this with QOL-scores averaging around 60 out of 100 with a higher score indicating a worse QOL. In comparison, the HC in our VOC study had a score around 20 out of 100. Patients with IBS-D and IBS-M generally reported a larger impact on QOL than IBS-C. This is also in accordance with previous studies which found that patients with IBS-D or IBS-M experienced more difficulties with daily activities and more frequently avoided food, with defaecation frequency being an important determinant.[188]

6.2 Diagnosis of irritable bowel syndrome

At the moment, no diagnostic tests for IBS are available and a positive diagnosis is made with the help of the Rome IV criteria, the absence of red flag symptoms, and some limited

testing. Two thousand visitors of our website completed the ‘“self-test” which assessed the Rome IV criteria and the prevalence of red flag symptoms (red blood loss per anum or RBPA, weight loss, older age, family history of colon cancer, fever). After completing this test, patients received a result with the likelihood of them suffering from IBS, therefore, we can assume that visitors completing the questionnaire were experiencing gastrointestinal symptoms which they believed could be caused by IBS. Of the 2000 visitors completing the questionnaire, 70% fulfilled the Rome IV criteria. When patients did not fulfil the Rome IV criteria this was frequently due to insufficient symptom duration (less than six months). It is probable that a proportion of these patients will continue to experience symptoms and will fulfil the Rome IV criteria when re-evaluating at a later timepoint. Another reason for not fulfilling the Rome IV criteria was insufficient abdominal pain or insufficient changes in the stool pattern. However, as with the symptom duration, in a proportion of patients this could be explained by either a milder phenotype or a well-treated patient. These reasons for not fulfilling the Rome IV criteria demonstrate the limitations of using symptom-based scores to diagnose IBS. In clinical practice, therefore, the Rome IV criteria are often interpreted more loosely, and diagnosis is partly based on physician experience. Of the participants completing the self-test, 42% had at least one red flag symptom with a family history of colon cancer, weight loss, and RBPA being the most prevalent ones. This is a staggering amount which demonstrates the importance of a thorough evaluation of new patients to exclude diseases like colon cancer or inflammatory bowel disease.

A diagnostic test for IBS would be of interest for both patients and health care professionals. The diagnosis of IBS is often surrounded by a lot of questions and uncertainty which can cause scepticism in patients and lead to a prolonged diagnostic process with excessive tests

and high costs. Apart from diagnosing patients there is also a demand for biomarkers to aid in personalised medicine, in the prediction of the treatment response, and the follow-up. However, biomarker development in IBS is a slow and extensive process, hindered by the heterogeneity of the patient population.

In this thesis we studied two biomarker groups: cellular and volatile biomarkers. When looking at cellular biomarkers we decided to focus on mast cells (MCs) since there is increasing evidence of the important role these immune cells play in certain groups of patients. Next, we assessed the use of volatomic profiles as biomarkers in IBS.

6.2.1 Mast cells in irritable bowel syndrome

MCs are inherently difficult to study *in vitro* since they are tissue resident cells, making them challenging to isolate and sustain *ex vivo*. A large part of this research project was therefore focused on the development and validation of a MC culture model based on the research in allergies and mastocytosis.[192] CD34+ progenitor cells were isolated from peripheral blood samples and incubated with IL-3 and stem cell factor for 5 weeks causing the differentiation of a proportion of the cells. The advantages of this cell culture model are the need for only one blood sample and the fact that the technique has been used in other pathologies for years.[150,191,192] However, there are also some disadvantages, it is labour-intensive, time-consuming, and has a variable and often limited yield.

MCs cultured from peripheral blood progenitors are naïve MCs as they have not yet been exposed to a tissue environment. Even in physiological circumstances MCs will differentiate based upon the tissue type where they settle. We characterised the naïve MCs of both IBS

patients and healthy controls (HC) to evaluate if there are any baseline differences in the expression or functionality of these cells. When looking at the immunophenotypic characteristics, we found no significant differences in expression of markers related to activation (FcεRI, MRGPRX2) or inhibition (CD300a, CD32).[217] To evaluate the functionality of the MCs, upregulation of CD63 after activation with substance P and anti-FcεRI was measured. Again, no significant differences in the functionality of the naïve MCs were found when comparing IBS patients to HC. We did notice a slight decrease in the expression of MRGPRX2 and an upregulation of CD63 after activation with substance P in IBS patients.

However, as mentioned, these naïve MCs were not yet exposed to the gut environment. We hypothesised that, since IBS is a gut disorder, the MCs would differentiate and change expression after being submerged in an IBS or gut-like environment. We decided to create a lifelike environment with the help of a supernatant distilled from matched colonic biopsies. This technique has been used extensively in the past, but mainly to study its effect on dorsal root ganglion (DRG) neurons.[199,200] Therefore, some optimisation and validation was needed to be able to use the supernatant of the colonic biopsies on the MC cultures. We limited the processing steps in the development of the supernatant to keep it as 'natural' as possible. Biopsies were incubated in IMDM medium for 12 to 18 hours, to optimise cytokine release. Gentamicin/amphotericin B and penicillin/streptomycin were added to prevent contamination of the supernatant-incubated MC cultures.

The MC cultures were incubated with the supernatant for 48 hours to allow the MCs to adapt like they would *in vivo*. In a first series of experiments incubation with the supernatant

resulted in a decreased functionality with less upregulation of CD63 upon activation. This is the exact opposite of what we hypothesised since previous research on MCs has revealed an increased activity in IBS. To assess for a potential toxic effect of the supernatant, which would explain the decreased functionality, the viability of the cells was tested with 7AAD, which showed a normal viability. However, the 7AAD measurement will only be positive in a late-stage apoptosis or necrosis. Therefore, we decided to also use annexin V to determine the early-stage apoptosis.[218] After staining with annexin V we noticed a clear correlation between the degree of apoptosis and the incubation time and the concentration of the colonic supernatant. There was a distinct increase in toxicity after incubation with a concentration of 1/20 or higher and after incubation for more than 24 h. No significant differences could be detected after incubation with supernatant from healthy controls versus IBS patients indicating a toxicity of the supernatant in general rather than a specific effect of IBS supernatant. Further research is needed to evaluate whether a decrease in toxicity is associated with an altered effect on characteristics and functionality of the MCs. In the 'Future perspectives' paragraph we will further elaborate on the potential problem solving.

In conclusion, we succeeded in optimising and validating the use of a MC culture model for IBS research. With the MC culture we are able to study MCs *in vitro* which provides opportunities not only in pathophysiological research, but also in the development and assessment of therapeutics targeting the MCs. Furthermore, we developed an IBS/gut-like environment with the help of colonic biopsies. However, while this novel technique is promising and substantial progress has been made in optimising its use, some further optimisation is needed.

6.2.2 Volatile organic compounds in irritable bowel syndrome

The second biomarker group that was studied in this thesis are the volatile biomarkers or volatile organic compounds (VOCs). VOCs are omnipresent and are excreted in all bodily fluids and vapours making them an easily accessible and non-invasive biomarker option. They are produced during both physiological and pathophysiological metabolic processes, by our microbiota, and through the metabolization of food and drugs. As such, they can provide an insight into the functioning of different aspects of the human body. However, there are also some limitations to the use of VOCs. Single VOCs are often aspecific making it necessary to combine VOCs into models to increase their specificity in detecting diseases. Furthermore, it is not possible to directly detect the origin of a VOC which can hinder further investigations. For example, some VOCs can be produced by metabolic processes but also by our microbiota or through the metabolization of food making the unravelling of the pathophysiological background more difficult.

Since VOC research in IBS was scarce when we started this research project, we conducted a systematic literature review to gain a better insight into the current knowledge on VOCs in IBS. We found a large variety in methodological approaches making it difficult to compare studies. Most studies used faecal samples with only a few using breath or urine samples. All matrices have their own advantages and disadvantages. On the one hand, faecal samples are easy to collect and store and might provide a more integral view on gastrointestinal diseases. However, patients are often reluctant to collect and hand in a faecal sample. On the other hand, while breath is easy to collect and use for real-time analysis, there are difficulties in storing samples for later analysis.[219]

The most frequently used analytical method is gas chromatography – mass spectrometry (GC-MS) which is considered the golden standard in VOC research. It is a highly sensitive technique that allows detailed identification of individual compounds. However, it is also a labour-intensive technique needing trained technicians, and it is associated with high analytical costs. It requires offline sampling including different pre-concentration steps which offers the possibility to store samples for batch analysis but also increases the risk of introducing contamination and bias. All these factors limit the use of GC-MS in clinical practice. For this thesis, we decided to use multicapillary column/ion mobility spectrometry (MCC/IMS) and assess its possibilities in IBS research. MCC/IMS is an easy to use and less costly alternative compared to GC-MS. It provides real-time (online) analysis, improving its use in a clinical setting. A potential limitation of MCC/IMS is that it only allows for a pseudo-identification (retention time and ion mobility but not a specific compound), making it impossible to identify specific individual compounds. For this reason, VOCs detected with MCC/IMS are usually combined into differentiating models (statistically generated models consisting of a combination of individual compounds) without a clear identification of each compound. Nevertheless, as most VOCs are aspecific, the use of differentiating models could be the preferred way to go in biomarker research.

For this research project we collected both breath and faecal samples from IBS patients and HC. Since MCC/IMS had not been used in IBS research prior to our study we first wanted to assess the feasibility of the technique by differentiating patients from HC. We were able to accurately differentiate IBS patients from HC with an AUC of 0.80 in faecal samples, 0.62 in breath samples, and 0.69 when breath and faecal volatiles were combined. While the faecal models tended to perform better than the breath models, there were no significant

differences between both modalities. Considering these limited differences, breath samples could be preferred in clinical practice given the ease of sample collection and analysis. Furthermore, breath tests are already frequently used in clinical practice to diagnose, for example, carbohydrate malabsorption. Combining both breath and faecal volatiles did not have a significant added value compared to breath or faeces individually.

Surprisingly, when we compared HC to the individual subtypes, we found lower, although still acceptable, differentiating values. Considering the differences in the underlying pathophysiological mechanisms we expected the differentiating values to be higher. Furthermore, when differentiating the individual subtypes based on the Rome IV criteria from each other the differentiating values were often even lower. This suggests that the classic subtyping of IBS patients based on the dominant stool pattern might not be the best option in VOC research or even IBS research in general.

In a next phase we evaluated the power of other clinical characteristics like psychological comorbidities, symptom scores, and microbiota influencing therapies (probiotics and antibiotics) in differentiating IBS patients from each other. Differentiation based on psychological comorbidities and microbiota influencing therapies resulted in models with either a high specificity or sensitivity while the respective sensitivity or specificity was rather low. Symptom scores like QOL, symptom severity, and the visceral sensitivity index had the lowest differentiating values of all the variables tested. There is a substantial heterogeneity in pathophysiological mechanisms and clinical presentations in IBS. Combined with the accuracy with which the models based on clinical characteristics were able to differentiate IBS patients from each other, this demonstrates the importance of a thorough

characterisation of patients in both clinical practice and research, surpassing pain, and stool pattern.

In conclusion, we demonstrated the potential of VOCs, measured with MCC/IMS, in the characterisation of patients with IBS. With the help of VOC profiles, we could accurately differentiate IBS patients from HC. Furthermore, volatiles were able to distinguish patients based on clinical characteristics, other than their dominant stool pattern, such as psychological states, symptom scores, and microbiota-influencing treatments, showing the possibility of an alternative way of subtyping IBS patients.

6.3 Treatment of irritable bowel syndrome

In this thesis we have proven the potential of both mast cells and volatile organic compounds in biomarker research. As mentioned, biomarkers are not only useful to diagnose patients, but can also play a role in personalised medicine, for example, in the selection of the best treatment option for a patient or in the follow-up of a treatment strategy. IBS cannot be cured, and the treatment is currently symptom-based. Ideally there should be a multidisciplinary approach with lifestyle changes, diet, psychotherapy, and pharmacotherapy depending on the individual patient. Finding the best treatment option can be a long process of trial-and-error leading to frustration and high health care costs. In our epidemiological study, we demonstrated that 96% of patients had consulted their general practitioner concerning their symptoms and 76% had consulted a gastroenterologist. Almost all patients tried some sort of pharmacotherapy (91%) and/or dietary changes (91%) at one point in time, while only 66% and 74% respectively had done so in the last three months.

The first step in IBS treatment remains education and a good patient-physician relationship. As mentioned earlier, a substantial proportion of patients do not believe sufficient information about IBS is available. And even less patients (41%) believe their physician has sufficient knowledge about IBS. However, 57% does feel like their physician takes IBS seriously. Although education and a good patient-physician relationship are the cheapest and easiest treatment options, they seem to be severely lacking from a patient's perspective. While development of biomarkers and novel treatment options is crucial, a serious effort should be made to improve education of both patients and health care professionals.

6.4 Conclusion

In conclusion, while IBS research has come a long way in the past decades, there is still more to discover, ranging from basic biomarker research to increased educational efforts. In this thesis we started with an epidemiological characterisation of a Dutch speaking IBS population. The results emphasised the importance of a thorough characterisation and the unmet needs of patients. Subsequently, we took a deep dive into cellular and volatile biomarkers. We developed and validated an IBS mast cell culture model which opens up opportunities for *in vitro* MC studies and made progress in the optimalisation of an IBS/gut-like environment based on colonic supernatant. Furthermore, we demonstrated the feasibility of MCC/IMS in IBS research providing a cheaper and easily accessible alternative to the classic GC-MS.

6.5 Future plans and perspectives

We started this thesis with an exploration of disorders of the gut brain interaction (DGBI), more specifically IBS, and the continuous interaction between the gut and central processes. We know, from literature, that MCs play an important role in the gut-brain axis. The mast cells can be activated by both peripheral and central pathways and in turn, release their mediators which can not only change the MCs direct environment but also influence the peripheral and central nervous system. In this thesis we further investigated the role of MCs through a human mast cell culture model. Another topic we studied were VOCs, which are volatile organic compounds resulting from different metabolic processes, reflecting homeostasis and disturbances not only in the gut but in the entire body.

We believe MCs and VOCs are promising research topics to study DGBI's. In this last part of the general discussion, we will take a further look at the research potential of MCs and VOCs in IBS research."

6.5.1 Mast cells as cellular biomarkers in irritable bowel syndrome

In this thesis we validated the use of a MC culture model originating from peripheral blood progenitor cells. Furthermore, we developed a gut/IBS-like environment for MCs, based on colonic supernatant. However, while substantial progress has been made, some further optimisation and validation of the technique is needed. The colonic supernatant, at the moment, is a black box with little understanding on its composition. Some form of characterisation seems opportune. On the one hand, a broad characterisation with mass-spectrometry could give a general idea on the composition. Afterwards, elements of interest

could be isolated and studied in more detail. On the other hand, we could directly study elements which we believe could play a role in the effects of the supernatant on MCs like endotoxins or proteases. However, this narrows the view and could cause us to miss other important elements we might not be aware of at the moment.

Another element which might play an important role in the supernatant are the gut microbiota. The microbiota are a crucial element in the gut environment, are in continuous interaction with the host, produce metabolites, and influence MCs. However, since our cell cultures are a sterile environment the presence of living microbes could cause contamination. For this reason, antibiotics and anti-fungal drugs are added to the supernatant to eliminate or subdue the present microbiota. However, this gives rise to two main concerns. First, the degradation of the microbiota produces a variety of waste products which can cause an increase of certain components in the supernatant like endotoxins. Second, by eliminating the microbiota we also eliminate an important factor of the gut environment and its natural interplay with the MCs. To gain a better insight in the role of the microbiota it might be interesting to perform a basic microbial culture or more elaborate sequencing on the supernatant before using it in further experiments.

Apart from characterising the colonic supernatant, the experimental conditions in which the supernatant is added to the MC cultures should also be explored further. We demonstrated the importance of both the concentration and incubation time. However, one could also consider eliminating toxic components from the supernatant, for example by using a filter. Furthermore, annexin V could be used to select a healthy cell population, excluding the apoptotic cells from analysis thereby eliminating their influence on the results.

Currently, we studied the effect of the colonic supernatant on MCs by assessing its immunophenotypic characteristics and functionality with the help of flow cytometry. However, assessing if an effect is due to a change in the MCs versus as a result from an unwanted side effect like toxicity, can be challenging. Therefore, it could be interesting to study the MCs in more depth and look into the RNA expression of receptors of interest.

Up to now, we incubated the patient's supernatant on a matched MC culture and a MC culture from a healthy control to evaluate if there is a different effect. However, it would also be interesting to evaluate if matching is necessary or if it would be possible to use a standardised MC culture with the supernatant of different patients.

When the MC culture and its IBS/gut-like environment are fully validated a wealth of new research opportunities becomes available. On the one hand, the technique can be used in pathophysiological research to further elucidate the underlying mechanisms of IBS and the detailed role of the MC. For example, the MCs could be sensitised with food antigens to evaluate their influence on the functionality. On the other hand, the technique could also be used in diagnostic research or in the development of novel therapeutics in IBS. For example, new drugs could first be tested on human MCs before administering it to patients to evaluate potential negative effects.

Furthermore, while the MC culture model and gut-like environment have a lot of potential applications in IBS research, the technique could also be adapted and used in other gastrointestinal diseases. For example, in functional dyspepsia, which has similar pathophysiological mechanisms, an alternative supernatant based on gastric or duodenal biopsies could be developed.

6.5.2 Volatile biomarkers in irritable bowel syndrome

With our feasibility study we demonstrated the potential of MCC/IMS in IBS research, however some further optimisation is needed. First, inclusion of a larger population is needed to further validate these results. Furthermore, since some of the subgroup's analysis contained a limited number of participants some overfitting of the data is to be expected. While we tried to correct this by using a lasso regression analysis, sampling a larger population would be opportune.

Second, as GC-MS is still considered the golden standard in VOC research, a next step would be to validate the results by analysing samples in parallel with both techniques MCC/IMS and GC-MS.

Third, in this study only IBS patients and HC were included. However, in clinical practice there is a need to diagnose patients presenting with similar symptoms. For example, a patient presenting with diarrhoea and abdominal pain could suffer from IBS but other diseases like inflammatory bowel disease, coeliac disease, or microscopic colitis can all present with similar symptoms. Therefore, future studies should include and compare other gastrointestinal diseases with a symptom profile similar to IBS, like the aforementioned inflammatory bowel disease and coeliac disease.

This preliminary study also provides a lot of future research possibilities. First, it is known that the microbiota produce VOCs and Sagar *et al.* also demonstrated the link between VOCs and microbiota metabolites.[164] The fact that the use of microbiota influencing therapies can accurately differentiate IBS patients further validates the importance of a patient's

microbiota in VOC research. More research looking into the interaction between the microbiota and the VOC profiles, via extensive microbiome analysis using techniques such as 16S RNA or shotgun sequencing, is needed. Especially considering the important role the microbiota play in IBS pathophysiology.

Second, while MCC/IMS only allow pseudo-identification of VOCs, GC-MS does allow identification of individual compounds of interest, which could form the basis for further pathophysiological research.

Third, while we only collected breath and faecal samples, other human excretions could also be used for VOC analysis such as urine or biopsy material. Furthermore, VOC analysis could be of interest in animal models and even cell cultures.

It is clear that VOC analysis could play a role in both clinical and preclinical research. It would be interesting to assess the influence of different therapeutics on VOC profiles and perhaps even predict treatment response or determine the most suitable treatment option for an individual patient. Even without active treatment, VOC profiles could be used to follow patients and gain better insights into the natural fluctuation of symptoms over time.

Finally, while the research opportunities are endless, one of the objectives should also be to develop VOC tests (breath, faecal, urine, …) for use in clinical practice, thereby advancing IBS care. Since there are currently no tests available establishing the diagnosis can be an expensive and tiring process. Furthermore, symptom control is often difficult resulting in frequent consultations and the use of a variety of different therapeutic options. VOC tests

could improve IBS care by providing a quick diagnosis and aid in the selection of the most suitable treatment options.

Chapter 7 Summary

Irritable bowel syndrome (IBS) is a chronic gastrointestinal disorder affecting 4-11% of the population. While we know its pathophysiology to be multifactorial, a lot of questions on the exact mechanisms remain unanswered. In this thesis we investigated some of these pathophysiological mechanisms, more specifically we looked into the potential of cellular and volatile biomarkers. At the moment, no biomarkers for IBS are readily available in clinical practice hindering efficient and accurate diagnosis and follow-up of patients.

We started the thesis by evaluating the epidemiological characteristics of a Dutch speaking population visiting a patient-centred informative website. Red flag symptoms and comorbid psychological disorders were prevalent validating the importance of a thorough characterisation of IBS patients both in clinical practice and research. Despite treatment patients frequently experienced moderate to severe symptoms with an important impact on their quality of life indicating the need of more targeted treatment options. Perhaps one of the most important messages of this study is the urgent need for high-quality, scientifically substantiated information and education for both patients and health care professionals.

In the second research project we validated the use of mast cells (MCs) cultured from peripheral blood progenitors from patients and healthy controls to investigate the potential of these cell cultures as cellular biomarkers. We compared the immunophenotypic characteristics and functionality of naïve MCs from IBS patients and healthy controls (HC) and did not find any basal significant differences. Next, we developed an IBS/gut-like environment based on colonic supernatant in which the MCs can be incubated. We optimised the used medium and need for anti-microbial drugs. Furthermore, we evaluated optimal incubation times and concentration and assessed toxicity of the supernatant. While the use

of the IBS/gut-like environment has a lot of research potential, further optimisation and validation is needed. At the moment, the colonic supernatant is still a black box containing a variety of compounds with an unknown effect on MCs. A characterisation of the supernatant and identification of compounds of interest is needed. Next, the composition of the supernatant should be optimised to reduce cell toxicity.

In the third research project we validated the potential of volatile organic compounds (VOCs) in IBS. We demonstrated the feasibility of ion mobility spectrometry as a cheaper and easily accessible alternative to GC-MS. With the help of VOC models, we could accurately differentiate IBS patients from healthy controls. Furthermore, we could differentiate IBS patients from each other, and from healthy controls, with the help of VOC models based on the dominant stool pattern. We were also able to differentiate IBS patients from each other with VOC models based on alternative clinical characteristics like psychological comorbidities, microbiota-influencing therapies, and symptom scores. This further validates the possibility of alternative subtyping based on clinical characteristics or pathophysiological mechanisms in IBS research. We, therefore, advocate to include these characteristics in the development of biomarkers, especially when studying volatomics.

In conclusion, in this thesis we started with an epidemiological study demonstrating the importance of a thorough characterisation and the need for information and education for both patients and health care professionals. Subsequently, we took a look at cellular and volatile biomarkers. We validated an IBS mast cell culture models which can be used for *in vitro* research on the role of MCs in IBS. Next, we started developing an IBS/gut-like environment based on colonic supernatant. While promising, this technique requires further

optimisation and validation to reduce toxic side effects. Furthermore, we demonstrated the feasibility of MCC/IMS to study volatile biomarkers in IBS, thereby providing a more accessible alternative to classic methods like GC-MS.

Chapter 8 Samenvatting

Het prikkelbare darmsyndroom (PDS) is een chronische gastro-intestinale aandoening die 4-11% van de bevolking treft. De onderliggende pathofysiologie is multifactorieel, maar de exacte mechanismen zijn nog steeds niet volledig uitgeklaard. In deze thesis hebben we enkele pathofysiologische mechanismen verder bestudeerd met nadruk op het potentieel van cellulaire en volatiele biomerkers. Momenteel zijn er geen biomerkers voor PDS beschikbaar in de klinische praktijk wat de efficiënte en accurate diagnose en follow-up van patiënten verhindert.

We zijn deze thesis gestart met een evaluatie van de epidemiologische karakteristieken van een Nederlandstalige populatie die een patiëntgerichte, informatieve website over PDS bezochten. Zowel alarmsymptomen als comorbide psychologische aandoeningen waren frequent aanwezig. Dit benadrukt het belang van een grondige karakterisatie van PDS patiënten zowel in de klinische praktijk als in wetenschappelijk onderzoek. Patiënten hadden vaak nog matige tot ernstige symptomen met een belangrijke impact op de levenskwaliteit ondanks behandeling. Dit toont de noodzaak van meer gerichte behandelingsopties in PDS. Eén van de belangrijkste conclusies van deze studie was de urgente nood voor wetenschappelijk onderbouwde informatie en educatie van hoge kwaliteit voor zowel patiënten als zorgverleners.

In een tweede onderzoeksproject hebben we het gebruik van mestcellen, gekweekt uit perifeer bloed voorlopercellen van patiënten en gezonde controles, gevalideerd. We onderzochten het potentieel van deze celculturen als cellulaire biomerkers. Eerst hebben we de immunofenotypische karakteristieken en functionaliteit van de naïeve mestcellen van patiënten en gezonde controles met elkaar vergeleken, wat geen significante verschillen

tussen beide groepen toonde. Vervolgens hebben we een PDS-darmomgeving gecreëerd, op basis van colon supernatant, waarin deze cellen geïncubeerd konden worden. We hebben het gebruikte medium en de nood voor antimicrobiële medicatie geëvalueerd waarna we verschillende incubatietijden en concentraties hebben getest. Om af te sluiten hebben we de toxiciteit van het supernatant bepaald. Het gebruik van deze PDS-darmomgeving heeft duidelijk potentieel in het wetenschappelijk onderzoek, maar verdere optimalisatie en validatie is nog nodig. Het colon supernatant is momenteel nog een zwarte doos die een variëteit aan componenten bevat die een effect kunnen hebben op de mestcellen, verdere karakterisatie en optimalisatie is dan ook nodig.

In het derde onderzoeksproject hebben we het potentieel van volatiele organische componenten (VOC) onderzocht. We hebben aangetoond dat ionen mobiliteit spectrometrie (IMS) een goedkoper en eenvoudiger alternatief is voor de klassieke gas chromatografie massa spectrometrie. Met behulp van VOC-modellen konden we PDS patiënten accuraat onderscheiden van gezonde controles. Het was eveneens mogelijk om met behulp van VOCs individuele subtypes van elkaar en gezonde controles te onderscheiden. Ook andere klinische karakteristieken zoals psychologische co-morbiditeiten, microbioom beïnvloedende therapieën en symptoom scores konden gebruikt worden om patiënten van elkaar te onderscheiden. Dit bewijst het potentieel om alternatieve subtypes gebaseerd op klinische karakteristieken of pathofysiologische mechanismen in PDS onderzoek te gebruiken. We bevelen dan ook aan om deze karakteristieken mee te nemen in de ontwikkeling van nieuwe biomerkers, zeker wanneer volatomics worden gebruikt.

Om te concluderen, in deze thesis zijn we gestart met een epidemiologische studie die het belang van een grondige patiënten karakterisatie aantoonde. Anderzijds demonstreerde het de nood voor informatie en educatie van zowel patiënten als zorgverleners. Vervolgens hebben we het potentieel van enkele cellulaire en volatiele biomerkers beoordeeld. We valideerden het gebruik van een PDS mestcel cultuurmodel voor *in vitro* research naar de rol van mestcellen in PDS. We zijn eveneens gestart met de ontwikkeling van een PDS-darmomgeving gebaseerd op colon supernatant. Hoewel deze techniek duidelijk potentieel heeft is verdere optimalisatie en validatie nodig om toxische effecten te reduceren. Om te eindigen demonstreerden we de bruikbaarheid van ionen mobiliteit spectrometrie om volatiele biomerkers in PDS te bestuderen, wat deze techniek een interessant alternatief maakt voor klassieke methodes zoals gas chromatografie massa spectrometrie.

Chapter 9 References

1 Drossman DA. Functional Gastrointestinal Disorders: History, Pathophysiology, Clinical Features, and Rome IV. *Gastroenterology* 2016; **150**: 1262-1279.e2.

2 Corazziari E. Definition and epidemiology of functional gastrointestinal disorders. *Best Pract Res Clin Gastroenterol* 2004; **18**: 613–31.

3 Stanghellini V, Chan FKL, Hasler WL, *et al.* Gastroduodenal Disorders. *Gastroenterology* 2016; **150**: 1380–92.

4 Camilleri M. Diagnosis and Treatment of Irritable Bowel Syndrome: A Review. *JAMA* 2021; **325**: 865–77.

5 Lacy BE, Mearin F, Chang L, *et al.* Bowel Disorders. *Gastroenterology* 2016; **150**: 1393-1407.e5.

6 Sperber AD, Bangdiwala SI, Drossman DA, *et al.* Worldwide Prevalence and Burden of Functional Gastrointestinal Disorders, Results of Rome Foundation Global Study. *Gastroenterology* 2021; **160**: 99-114.e3.

7 Oka P, Parr H, Barberio B, Black CJ, Savarino E v., Ford AC. Global prevalence of irritable bowel syndrome according to Rome III or IV criteria: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* 2020; **5**: 908–17.

8 Shorey S, Demutska A, Chan V, Siah KTH. Adults living with irritable bowel syndrome (IBS): A qualitative systematic review. *J Psychosom Res* 2021; **140**: 110289.

9 Canavan C, West J, Card T. The epidemiology of irritable bowel syndrome. *Clin Epidemiol* 2014; **6**: 71–80.

10 Houghton LA, Heitkemper M, Crowell MD, *et al.* Age, Gender, and Women's Health and the Patient. *Gastroenterology* 2016; **150**: 1332-1343.e4.

11 Camilleri M. Sex as a biological variable in irritable bowel syndrome. *Neurogastroenterology & Motility* 2020; **32**: e13802.

12 Gupta A, Mayer EA, Acosta JR, *et al.* Early adverse life events are associated with altered brain network architecture in a sex- dependent manner. *Neurobiol Stress* 2017; **7**: 16–26.

13 Lacy BE, Mearin F, Chang L, *et al.* Bowel Disorders. *Gastroenterology* 2016; **150**: 1393-1407.e5.

14 Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. *Scand J Gastroenterol* 1997; **32**: 920–4.

15 Mönnikes H. Quality of life in patients with irritable bowel syndrome. *J Clin Gastroenterol* 2011; **45**. DOI:10.1097/MCG.0B013E31821FBF44.

16 Lea R, Whorwell PJ. Quality of Life in Irritable Bowel Syndrome. *PharmacoEconomics 2001 19:6* 2012; **19**: 643–53.

17 Cassar GE, Youssef GJ, Knowles S, Moulding R, Austin DW. Health-Related Quality of Life in Irritable Bowel Syndrome: A Systematic Review and Meta-analysis. *Gastroenterology Nursing* 2020; **43**: E102–22.

18 Gralnek IM, Hays RD, Kilbourne AA, Naliboff B, Mayer EA. The impact of irritable bowel syndrome on health-related quality of life. *Gastroenterology* 2000; **119**: 654–60.

19 Zhang L, Song J, Hou X. Mast cells and irritable bowel syndrome: From the bench to the bedside. *J Neurogastroenterol Motil* 2016; **22**: 181–92.

20 Defrees DN, Bailey J. Irritable Bowel Syndrome: Epidemiology, Pathophysiology, Diagnosis, and Treatment. *Primary Care - Clinics in Office Practice* 2017; **44**: 655–71.

21 Videlock EJ, Chang L. Latest Insights on the Pathogenesis of Irritable Bowel Syndrome. *Gastroenterology Clinics* 2021; **50**: 505–22.

22 Holtmann GJ, Ford AC, Talley NJ. Pathophysiology of irritable bowel syndrome. *Lancet Gastroenterol Hepatol* 2016; **1**: 133–46.

23 Chey WY, Jin HO, Lee MH, Sun SW, Lee KY. Colonic motility abnormality in patients with irritable bowel syndrome exhibiting abdominal pain and diarrhea. *Am J Gastroenterol* 2001; **96**: 1499–506.

24 Gunnarsson J, Simrén M. Peripheral factors in the pathophysiology of irritable bowel syndrome. *Digestive and Liver Disease* 2009; **41**: 788–93.

25 Clemens C. Association between pain episodes and high amplitude propagated pressure waves in patients with irritable bowel syndrome. *Am J Gastroenterol* 2003; **98**: 1838–43.

26 Vassallo M, Camilleri M, Phillips SF, Brown ML, Chapman NJ, Thomforde GM. Transit through the proximal colon influences stool weight in the irritable bowel syndrome. *Gastroenterology* 1992; **102**: 102–8.

27 Kellow JE, Eckersley GM, Jones M. Enteric and central contributions to intestinal dysmotility in irritable bowel syndrome. *Digestive Diseases and Sciences 1992 37:2* 1992; **37**: 168–74.

28 Kellow JE, Gill RC, Wingate DL. Prolonged Ambulant Recordings of Small Bowel Motility Demonstrate Abnormalities in the Irritable Bowel Syndrome. *Gastroenterology* 1990; **98**: 1208–18.

29 Hanning N, Edwinson AL, Ceuleers H, *et al.* Intestinal barrier dysfunction in irritable bowel syndrome: a systematic review. *Therap Adv Gastroenterol* 2021; **14**: 1756284821993586.

30 Ghosh SS, Wang J, Yannie PJ, Ghosh S. Intestinal Barrier Dysfunction, LPS Translocation, and Disease Development. 2020; **4**. DOI:10.1210/jendso/bvz039.

31 Tuck CJ, Vanner SJ. Dietary therapies for functional bowel symptoms: Recent advances, challenges, and future directions. *Neurogastroenterology & Motility* 2018; **30**: e13238.

32 Son Choung R, Talley NJ, of Medicine P, Fiterman S. Food Allergy and Intolerance in IBS. *Gastroenterol Hepatol (N Y)* 2006; **2**: 756.

33 Werlang ME, Palmer WC, Lacy BE. Irritable Bowel Syndrome and Dietary Interventions. *Gastroenterol Hepatol (N Y)* 2019; **15**: 16.

34 Barbaro MR, Cremon C, Stanghellini V, Barbara G. Recent advances in understanding non-celiac gluten sensitivity. *F1000Res* 2018; **7**. DOI:10.12688/F1000RESEARCH.15849.1.

35 Liebregts T, Adam B, Bredack C, *et al.* Immune Activation in Patients With Irritable Bowel Syndrome. *Gastroenterology* 2007; **132**: 913–20.

36 Card T, Enck P, Barbara G, *et al.* Post-infectious IBS: Defining its clinical features and prognosis using an internet-based survey: *https://doi.org/101177/2050640618779923* 2018; **6**: 1245–53.

37 Klem F, Wadhwa A, Prokop LJ, *et al.* Prevalence, Risk Factors, and Outcomes of Irritable Bowel Syndrome After Infectious Enteritis: a Systematic Review and Meta-analysis. *Gastroenterology* 2017; **152**: 1042.

38 Lee YY, Annamalai C, Rao SSC. Post-Infectious Irritable Bowel Syndrome. *Curr Gastroenterol Rep* 2017; **19**: 1–10.

39 Thursby E, Juge N. Introduction to the human gut microbiota. *Biochemical Journal* 2017; **474**: 1823.

40 Rajilić-Stojanović M, Jonkers DM, Salonen A, *et al.* Intestinal microbiota and diet in IBS: causes, consequences, or epiphenomena? *Am J Gastroenterol* 2015; **110**: 278–87.

41 Sundin J, Öhman L, Simrén M. Understanding the Gut Microbiota in Inflammatory and Functional Gastrointestinal Diseases. *Psychosom Med* 2017; **79**: 857–67.

42 Sundin J, Öhman L, Simrén M. Understanding the Gut Microbiota in Inflammatory and Functional Gastrointestinal Diseases. *Psychosom Med* 2017; **79**: 857–67.

43 Pecora F, Persico F, Gismondi P, *et al.* Gut Microbiota in Celiac Disease: Is There Any Role for Probiotics? *Front Immunol* 2020; **11**: 957.

44 Liu HN, Wu H, Chen YZ, Chen YJ, Shen XZ, Liu TT. Altered molecular signature of intestinal microbiota in irritable bowel syndrome patients compared with healthy controls: A systematic review and meta-analysis. *Dig Liver Dis* 2017; **49**: 331–7.

45 Fan WT, Ding C, Xu NN, Zong S, Ma P, Gu B. Close association between intestinal microbiota and irritable bowel syndrome. *Eur J Clin Microbiol Infect Dis* 2017; **36**: 2303–17.

46 Wu GD, Chen J, Hoffmann C, *et al.* Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011; **334**: 105–8.

47 David LA, Maurice CF, Carmody RN, *et al.* Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014; **505**: 559–63.

48 Tana C, Umesaki Y, Imaoka A, Handa T, Kanazawa M, Fukudo S. Altered profiles of intestinal microbiota and organic acids may be the origin of symptoms in irritable bowel syndrome. DOI:10.1111/j.1365-2982.2009.01427.x.

49 Sahakian AB, Jee SR, Pimentel M. Methane and the gastrointestinal tract. *Dig Dis Sci* 2010; **55**: 2135–43.

50 Carabotti M, Scirocco A, Maselli MA, Severi C. The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Annals of Gastroenterology : Quarterly Publication of the Hellenic Society of Gastroenterology* 2015; **28**: 203.

51 Mayer EA, Labus JS, Tillisch K, Cole SW, Baldi P. Towards a systems view of IBS. *Nature Reviews Gastroenterology & Hepatology 2015 12:10* 2015; **12**: 592–605.

52 Park SH, Videlock EJ, Shih W, Presson AP, Mayer EA, Chang L. Adverse childhood experiences are associated with irritable bowel syndrome and gastrointestinal symptom severity. *Neurogastroenterology & Motility* 2016; **28**: 1252–60.

53 Rahal H, Videlock EJ, Icenhour A, *et al.* Importance of trauma-related fear in patients with irritable bowel syndrome and early adverse life events. *Neurogastroenterology & Motility* 2020; **32**: e13896.

54 Ju T, Naliboff BD, Shih W, *et al.* Risk and Protective Factors Related to Early Adverse Life Events in Irritable Bowel Syndrome. *J Clin Gastroenterol* 2020; **54**: 63.

55 Hu Z, Li M, Yao L, *et al.* The level and prevalence of depression and anxiety among patients with different subtypes of irritable bowel syndrome: a network meta-analysis. *BMC Gastroenterol* 2021; **21**: 1–18.

56 Sykes MA, Blanchard EB, Lackner J, Keefer L, Krasner S. Psychopathology in Irritable Bowel Syndrome: Support for a Psychophysiological Model. *Journal of Behavioral Medicine 2003 26:4* 2003; **26**: 361–72.

57 Zhou Q, Verne GN. New insights into visceral hypersensitivity—clinical implications in IBS. *Nature Reviews Gastroenterology & Hepatology 2011 8:6* 2011; **8**: 349–55.

58 Farzaei MH, Bahramsoltani R, Abdollahi M, Rahimi R. The Role of Visceral Hypersensitivity in Irritable Bowel Syndrome: Pharmacological Targets and Novel Treatments. *J Neurogastroenterol Motil* 2016; **22**: 558.

59 van Thiel IAM, de Jonge WJ, Chiu IM, van den Wijngaard RM. Microbiota-neuroimmune cross talk in stress-induced visceral hypersensitivity of the bowel. *Am J Physiol Gastrointest Liver Physiol* 2020; **318**: G1034–41.

60 Deiteren A, de Man JG, Ruyssers NE, Moreels TG, Pelckmans PA, de Winter BY. Histamine H4 and H1 receptors contribute to postinflammatory visceral hypersensitivity. *Gut* 2014; **63**: 1873–82.

61 Yang S, Chang MC. Chronic Pain: Structural and Functional Changes in Brain Structures and Associated Negative Affective States. *Int J Mol Sci* 2019; **20**. DOI:10.3390/IJMS20133130.

62 Yu Z, Liu LY, Lai YY, *et al.* Altered Resting Brain Functions in Patients With Irritable Bowel Syndrome: A Systematic Review. *Front Hum Neurosci* 2022; **16**: 208.

63 Henström M, D'Amato M. Genetics of irritable bowel syndrome. *Mol Cell Pediatr* 2016; **3**: 1–5.

64 Camilleri M, Zhernakova A, Bozzarelli I, D'Amato M. Genetics of irritable bowel syndrome: shifting gear via biobank-scale studies. *Nature Reviews Gastroenterology & Hepatology 2022* 2022; : 1–14.

65 Eijsbouts C, Zheng T, Kennedy NA, *et al.* Genome-wide analysis of 53,400 people with irritable bowel syndrome highlights shared genetic pathways with mood and anxiety disorders. *Nature Genetics 2021 53:11* 2021; **53**: 1543–52.

66 Mahurkar-Joshi S, Chang L. Epigenetic Mechanisms in Irritable Bowel Syndrome. *Front Psychiatry* 2020; **11**: 805.

67 Chang L, Lembo A, Sultan S. Spotlight: IBS Treatment. *Gastroenterology* 2022; **163**: 153.

68 Hatcher S, Arroll B. Assessment and management of medically unexplained symptoms. *BMJ : British Medical Journal* 2008; **336**: 1124.

69 Begtrup LM, Engsbro AL, Kjeldsen J, *et al.* A positive diagnostic strategy is noninferior to a strategy of exclusion for patients with irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2013; **11**. DOI:10.1016/J.CGH.2012.12.038.

70 Engsbro AL, Begtrup LM, Haastrup P, *et al.* A positive diagnostic strategy is safe and saves endoscopies in patients with irritable bowel syndrome: A five-year follow-up of a randomized controlled trial. *Neurogastroenterol Motil* 2021; **33**. DOI:10.1111/NMO.14004.

71 Grad S, Dumitrascu DL. Irritable Bowel Syndrome Subtypes: New Names for Old Medical Conditions. *Dig Dis* 2020; **38**: 122–7.

72 Hadjivasilis A, Tsioutis C, Michalinos A, Ntourakis D, Christodoulou DK, Agouridis AP. New insights into irritable bowel syndrome: from pathophysiology to treatment. *Ann Gastroenterol* 2019; **32**: 554.

73 Hammer J, Eslick GD, Howell SC, Altiparmak E, Talley NJ. Diagnostic yield of alarm features in irritable bowel syndrome and functional dyspepsia. *Gut* 2004; **53**: 666–72.

74 Sood R, Camilleri M, Gracie DJ, *et al.* Enhancing Diagnostic Performance of Symptom-Based Criteria for Irritable Bowel Syndrome by Additional History and Limited Diagnostic Evaluation. *American Journal of Gastroenterology* 2016; **111**: 1446–54.

75 Lacy BE, Pimentel M, Brenner DM, *et al.* ACG Clinical Guideline: Management of Irritable Bowel Syndrome. *Am J Gastroenterol* 2021; **116**: 17–44.

76 Moayyedi P, Andrews CN, MacQueen G, *et al.* Canadian Association of Gastroenterology Clinical Practice Guideline for the Management of Irritable Bowel Syndrome (IBS). *J Can Assoc Gastroenterol* 2019; **2**: 6–29.

77 Camilleri M. Diagnosis and Treatment of Irritable Bowel Syndrome: A Review. *JAMA* 2021; **325**: 865–77.

78 Chang L, Sultan S, Lembo A, Verne GN, Smalley W, Heidelbaugh JJ. AGA Clinical Practice Guideline on the Pharmacological Management of Irritable Bowel Syndrome With Constipation. *Gastroenterology* 2022; **163**: 118–36.

79 Lembo A, Sultan S, Chang L, Heidelbaugh JJ, Smalley W, Verne GN. AGA Clinical Practice Guideline on the Pharmacological Management of Irritable Bowel Syndrome With Diarrhea. *Gastroenterology* 2022; **163**: 137–51.

80 Chey WD, Hashash JG, Manning L, Chang L. AGA Clinical Practice Update on the Role of Diet in Irritable Bowel Syndrome: Expert Review. *Gastroenterology* 2022; **162**: 1737-1745.e5.

81 Drossman DA, Tack J, Ford AC, Szigethy E, Törnblom H, van Oudenhove L. Neuromodulators for Functional Gastrointestinal Disorders (Disorders of Gut−Brain

Interaction): A Rome Foundation Working Team Report. *Gastroenterology* 2018; **154**: 1140-1171.e1.

82 Johannesson E, Ringström G, Abrahamsson H, Sadik R. Intervention to increase physical activity in irritable bowel syndrome shows long-term positive effects. *World Journal of Gastroenterology : WJG* 2015; **21**: 600.

83 Hulme K, Chilcot J, Smith MA. Doctor-patient relationship and quality of life in Irritable Bowel Syndrome: an exploratory study of the potential mediating role of illness perceptions and acceptance. *Psychol Health Med* 2018; **23**: 674–84.

84 O'Sullivan MA, Mahmud N, Kelleher DP, Lovett E, O'Morain CA. Patient knowledge and educational needs in irritable bowel syndrome. *Eur J Gastroenterol Hepatol* 2000; **12**: 39–43.

85 Halpert A, Dalton CB, Palsson O, *et al.* What Patients Know About Irritable Bowel Syndrome (IBS) and What They Would Like to Know. National Survey on Patient Educational Needs in IBS and Development and Validation of the Patient Educational Needs Questionnaire (PEQ). *Official journal of the American College of Gastroenterology | ACG* 2007; **102**. https://journals.lww.com/ajg/Fulltext/2007/09000/What_Patients_Know_About_Irritable_Bowel_Syndrome.21.aspx.

86 Halpert A, Dalton CB, Palsson O, *et al.* Patient educational media preferences for information about irritable bowel syndrome (IBS). *Dig Dis Sci* 2008; **53**: 3184–90.

87 Drossman DA, Morris CB, Schneck S, *et al.* International survey of patients with IBS: symptom features and their severity, health status, treatments, and risk taking to achieve clinical benefit. *J Clin Gastroenterol* 2009; **43**: 541.

88 Kindt S, Louis H, de Schepper H, *et al.* Belgian consensus on irritable bowel syndrome. *Acta Gastroenterol Belg* 2022; **85**: 360–82.

89 Bijkerk CJ, Muris JWM, Knottnerus JA, Hoes AW, de Wit NJ. Systematic review: the role of different types of fibre in the treatment of irritable bowel syndrome. *Aliment Pharmacol Ther* 2004; **19**: 245–51.

90 McKenzie YA, Alder A, Anderson W, *et al.* British Dietetic Association evidence-based guidelines for the dietary management of irritable bowel syndrome in adults. *J Hum Nutr Diet* 2012; **25**: 260–74.

91 van Lanen AS, de Bree A, Greyling A. Efficacy of a low-FODMAP diet in adult irritable bowel syndrome: a systematic review and meta-analysis. *Eur J Nutr* 2021; **60**: 3505–22.

92 Vandeputte D, Joossens M. microorganisms Effects of Low and High FODMAP Diets on Human Gastrointestinal Microbiota Composition in Adults with Intestinal Diseases: A Systematic Review. DOI:10.3390/microorganisms8111638.

93 Harvie RM, Chisholm AW, Bisanz JE, *et al.* Long-term irritable bowel syndrome symptom control with reintroduction of selected FODMAPs. *World J Gastroenterol* 2017; **23**: 4632.

94 Lembo A, Sultan S, Chang L, Heidelbaugh JJ, Smalley W, Verne GN. AGA Clinical Practice Guideline on the Pharmacological Management of Irritable Bowel Syndrome With Diarrhea. *Gastroenterology* 2022; **163**: 137–51.

95 Ford AC, Harris LA, Lacy BE, Quigley EMM, Moayyedi P. Systematic review with meta-analysis: the efficacy of prebiotics, probiotics, synbiotics and antibiotics in irritable bowel syndrome. *Aliment Pharmacol Ther* 2018; **48**: 1044–60.

96 Dale HF, Rasmussen SH, Asiller ÖÖ, Lied GA. Probiotics in Irritable Bowel Syndrome: An Up-to-Date Systematic Review. *Nutrients* 2019; **11**. DOI:10.3390/NU11092048.

97 Wouters MM, Balemans D, van Wanrooy S, *et al.* Histamine Receptor H1-Mediated Sensitization of TRPV1 Mediates Visceral Hypersensitivity and Symptoms in Patients with Irritable Bowel Syndrome. *Gastroenterology* 2016; **150**: 875-887.e9.

98 Camilleri M, Boeckxstaens G. Dietary and pharmacological treatment of abdominal pain in IBS. DOI:10.1136/gutjnl-2016-313425.

99 Aguilera-Lizarraga J, Florens M v., Viola MF, *et al.* Local immune response to food antigens drives meal-induced abdominal pain. *Nature 2021 590:7844* 2021; **590**: 151–6.

100 Trifan A, Burta O, Tiuca N, Petrisor DC, Lenghel A, Santos J. Efficacy and safety of Gelsectan for diarrhoea-predominant irritable bowel syndrome: A randomised, crossover clinical trial. *United European Gastroenterol J* 2019; **7**: 1093–101.

101 Alexea O, Bacarea V, Piqué N. The combination of oligo- and polysaccharides and reticulated protein for the control of symptoms in patients with irritable bowel

syndrome: Results of a randomised, placebo-controlled, double-blind, parallel group, multicentre clinical trial. *United European Gastroenterol J* 2016; **4**: 455.

102 Noergaard M, Traerup Andersen J, Jimenez-Solem E, Bring Christensen M. Long term treatment with stimulant laxatives – clinical evidence for effectiveness and safety? *https://doi.org/101080/0036552120181563806* 2019; **54**: 27–34.

103 Lawrensia S, Raja A. Bisacodyl. *xPharm: The Comprehensive Pharmacology Reference* 2021; : 1–4.

104 Chang L, Sultan S, Lembo A, Verne GN, Smalley W, Heidelbaugh JJ. AGA Clinical Practice Guideline on the Pharmacological Management of Irritable Bowel Syndrome With Constipation. *Gastroenterology* 2022; **163**: 118–36.

105 Kessing BF, Smout AJPM, Bennink RJ, Kraaijpoel N, Oors JM, Bredenoord AJ. Prucalopride decreases esophageal acid exposure and accelerates gastric emptying in healthy subjects. *Neurogastroenterology & Motility* 2014; **26**: 1079–86.

106 Omer A, Quigley EMM. An update on prucalopride in the treatment of chronic constipation. *Therap Adv Gastroenterol* 2017; **10**: 877.

107 Layer P, Stanghellini V. Review article: linaclotide for the management of irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2014; **39**: 371.

108 Sajid MS, Hebbar M, Baig MK, Li A, Philipose Z. Use of Prucalopride for Chronic Constipation: A Systematic Review and Meta-analysis of Published Randomized, Controlled Trials. *J Neurogastroenterol Motil* 2016; **22**: 412.

109 Black CJ, Yuan Y, Selinger CP, *et al.* Efficacy of soluble fibre, antispasmodic drugs, and gut-brain neuromodulators in irritable bowel syndrome: a systematic review and network meta-analysis. *Lancet Gastroenterol Hepatol* 2020; **5**: 117–31.

110 Montero AM, Jones S. Roles and Impact of Psychologists in Interdisciplinary Gastroenterology Care. *Clin Gastroenterol Hepatol* 2020; **18**: 290–3.

111 Black CJ, Thakur ER, Houghton LA, Quigley EMM, Moayyedi P, Ford AC. Efficacy of psychological therapies for irritable bowel syndrome: systematic review and network meta-analysis. *Gut* 2020; **69**: 1441–51.

112 Basnayake C, Kamm MA, Stanley A, *et al.* Standard gastroenterologist versus multidisciplinary treatment for functional gastrointestinal disorders (MANTRA): an

open-label, single-centre, randomised controlled trial. *Lancet Gastroenterol Hepatol* 2020; **5**: 890–9.

113 Lackner JM, Jaccard J, Firth R, *et al.* Factors Associated With Efficacy of Cognitive Behavior Therapy vs Education for Patients With Irritable Bowel Syndrome. *Clinical Gastroenterology and Hepatology* 2019; **17**: 1500-1508.e3.

114 Shah K, Ramos-Garcia M, Bhavsar J, Lehrer P. Mind-body treatments of irritable bowel syndrome symptoms: An updated meta-analysis. *Behaviour research and therapy* 2020; **128**. DOI:10.1016/J.BRAT.2019.103462.

115 Ford AC, Lacy BE, Harris LA, Quigley EMM, Moayyedi P. Effect of Antidepressants and Psychological Therapies in Irritable Bowel Syndrome: An Updated Systematic Review and Meta-Analysis. *American Journal of Gastroenterology* 2019; **114**: 21–39.

116 van Oudenhove L, Levy RL, Crowell MD, *et al.* Biopsychosocial Aspects of Functional Gastrointestinal Disorders. *Gastroenterology* 2016; **150**: 1355-1367.e2.

117 Lackner JM. Skills over pills? A clinical gastroenterologist's primer in cognitive behavioral therapy for irritable bowel syndrome. *https://doi.org/101080/1747412420201780118* 2020; **14**: 601–18.

118 Lackner JM, Jaccard J, Keefer L, *et al.* Improvement in Gastrointestinal Symptoms After Cognitive Behavior Therapy for Refractory Irritable Bowel Syndrome. *Gastroenterology* 2018; **155**: 47–57.

119 Everitt HA, Landau S, O'Reilly G, *et al.* Cognitive behavioural therapy for irritable bowel syndrome: 24-month follow-up of participants in the ACTIB randomised trial. *Lancet Gastroenterol Hepatol* 2019; **4**: 863–72.

120 Lee HH, Choi YY, Choi MG. The Efficacy of Hypnotherapy in the Treatment of Irritable Bowel Syndrome: A Systematic Review and Meta-analysis. *J Neurogastroenterol Motil* 2014; **20**: 152–62.

121 Lindfors P, Ljótsson B, Bjornsson E, Abrahamsson H, Simrén M. Patient satisfaction after gut-directed hypnotherapy in irritable bowel syndrome. *Neurogastroenterol Motil* 2013; **25**. DOI:10.1111/NMO.12022.

122 Atkinson AJ, Colburn WA, DeGruttola VG, *et al.* Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001; **69**: 89–95.

123 Camilleri M, Halawi H, Oduyebo I. Biomarkers as a diagnostic tool for irritable bowel syndrome: where are we? *Expert Rev Gastroenterol Hepatol* 2017; **11**: 303–16.

124 Strimbu K, Tavel JA. What are Biomarkers? *Curr Opin HIV AIDS* 2010; **5**: 463.

125 Hungin APS, Whorwell PJ, Tack J, Mearin F. The prevalence, patterns and impact of irritable bowel syndrome: an international survey of 40,000 subjects. *Aliment Pharmacol Ther* 2003; **17**: 643–50.

126 Valent P, Cerny-Reiterer S, Herrmann H, *et al.* Phenotypic heterogeneity, novel diagnostic markers, and target expression profiles in normal and neoplastic human mast cells. *Best Pract Res Clin Haematol* 2010; **23**: 369–78.

127 Moon TC, St Laurent CD, Morris KE, *et al.* Advances in mast cell biology: new understanding of heterogeneity and function. *Mucosal Immunol* 2010; **3**: 111–28.

128 Serhan N, Cenac N, Basso L, Gaudenzio N. Mas-related G protein-coupled receptors (Mrgprs) – Key regulators of neuroimmune interactions. *Neurosci Lett* 2021; **749**: 135724.

129 Gupta K, Harvima IT. Mast cell-neural interactions contribute to pain and itch. *Immunol Rev* 2018; **282**: 168–87.

130 Buhner S, Schemann M. Mast cell-nerve axis with a focus on the human gut. *Biochim Biophys Acta* 2012; **1822**: 85–92.

131 Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol* 2006; **6**: 218–30.

132 Noto CN, Hoft SG, DiPaolo RJ. Mast Cells as Important Regulators in Autoimmunity and Cancer Development. *Front Cell Dev Biol* 2021; **9**: 2814.

133 Vanuytsel T, van Wanrooy S, Vanheel H, *et al.* Psychological stress and corticotropin-releasing hormone increase intestinal permeability in humans by a mast cell-dependent mechanism. *Gut* 2014; **63**: 1293–9.

134 He SH, Zhang HY, Zeng XN, Chen D, Yang PC. Mast cells and basophils are essential for allergies: mechanisms of allergic inflammation and a proposed procedure for diagnosis. *Acta Pharmacol Sin* 2013; **34**: 1270–83.

135 Lieberman P, Garvey LH. Mast Cells and Anaphylaxis. *Curr Allergy Asthma Rep* 2016; **16**: 1–7.

136 Lafleur MA, Werner J, Fort M, Lobenhofer EK, Balazs M, Goyos A. MRGPRX2 activation as a rapid, high-throughput mechanistic-based approach for detecting peptide-mediated human mast cell degranulation liabilities. *https://doi.org/101080/1547691X20201757793* 2020; **17**: 110–21.

137 Stephens MAC, Wand G. Stress and the HPA Axis: Role of Glucocorticoids in Alcohol Dependence. *Alcohol Res* 2012; **34**: 468.

138 Theoharides TC, Donelan JM, Papadopoulou N, Cao J, Kempuraj D, Conti P. Mast cells as targets of corticotropin-releasing factor and related peptides. *Trends Pharmacol Sci* 2004; **25**: 563–8.

139 de Winter BY, van den Wijngaard RM, de Jonge WJ. Intestinal mast cells in gut inflammation and motility disturbances. *Biochim Biophys Acta* 2012; **1822**: 66–73.

140 Ramsay DB, Stephen S, Borum M, Voltaggio L, Doman DB. Mast Cells in Gastrointestinal Disease. *Gastroenterol Hepatol (N Y)* 2010; **6**: 772.

141 Hanning N, de bruyn M, Ceuleers H, *et al.* Local Colonic Administration of a Serine Protease Inhibitor Improves Post-Inflammatory Visceral Hypersensitivity in Rats. *Pharmaceutics 2021, Vol 13, Page 811* 2021; **13**: 811.

142 Ceuleers H, Hanning N, Heirbaut J, *et al.* Newly developed serine protease inhibitors decrease visceral hypersensitivity in a post-inflammatory rat model for irritable bowel syndrome. *Br J Pharmacol* 2018; **175**: 3516.

143 Wouters MM, Vicario M, Santos J. The role of mast cells in functional GI disorders. *Gut* 2016; **65**: 155–68.

144 Boeckxstaens GE, Wouters MM. Neuroimmune factors in functional gastrointestinal disorders: A focus on irritable bowel syndrome. *Neurogastroenterol Motil* 2017; **29**. DOI:10.1111/NMO.13007.

145 Cheng L, Luo QQ, Chen SL. The role of intestinal mast cell infiltration in irritable bowel syndrome. *J Dig Dis* 2021; **22**: 143–51.

146 Hasler WL, Grabauskas G, Singh P, Owyang C. Mast cell mediation of visceral sensation and permeability in irritable bowel syndrome. *Neurogastroenterology & Motility* 2022; **34**: e14339.

147 Guilarte M, Santos J, de Torres I, *et al.* Diarrhoea-predominant IBS patients show mast cell activation and hyperplasia in the jejunum. *Gut* 2007; **56**: 203.

148 Barbara G, Stanghellini V, de Giorgio R, *et al.* Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 2004; **126**: 693–702.

149 Deiteren A, de Man JG, Ruyssers NE, Moreels TG, Pelckmans PA, de Winter BY. Histamine H4 and H1 receptors contribute to postinflammatory visceral hypersensitivity. *Gut* 2014; **63**: 1873–82.

150 Cop N, Ebo DG, Bridts CH, *et al.* Influence of IL-6, IL-33, and TNF-α on human mast cell activation: Lessons from single cell analysis by flow cytometry. *Cytometry B Clin Cytom* 2018; **94**: 405–11.

151 Bennet SMP, Polster A, Törnblom H, *et al.* Global Cytokine Profiles and Association With Clinical Characteristics in Patients With Irritable Bowel Syndrome. *Am J Gastroenterol* 2016; **111**: 1165–76.

152 Bashashati M, Moradi M, Sarosiek I. Interleukin-6 in irritable bowel syndrome: A systematic review and meta-analysis of IL-6 (-G174C) and circulating IL-6 levels. *Cytokine* 2017; **99**: 132–8.

153 Joulia R, L'Faqihi FE, Valitutti S, Espinosa E. IL-33 fine tunes mast cell degranulation and chemokine production at the single-cell level. *Journal of Allergy and Clinical Immunology* 2017; **140**: 497-509.e10.

154 Liang WJ, Zhang G, Luo HS, Liang LX, Huang D, Zhang FC. Tryptase and Protease-Activated Receptor 2 Expression Levels in Irritable Bowel Syndrome. *Gut Liver* 2016; **10**: 382–90.

155 Romano A, Capozzi V, Spano G, Biasioli F. Proton transfer reaction–mass spectrometry: online and rapid determination of volatile organic compounds of microbial origin. Appl Microbiol Biotechnol. 2015; **99**. DOI:10.1007/s00253-015-6528-y.

156 van Malderen K, de Winter BY, de Man JG, de Schepper HU, Lamote K. Volatomics in inflammatory bowel disease and irritable bowel syndrome. *EBioMedicine* 2020; **54**. DOI:10.1016/j.ebiom.2020.102725.

157 Baranska A, Mujagic Z, Smolinska A, *et al.* Volatile organic compounds in breath as markers for irritable bowel syndrome: a metabolomic approach. *Aliment Pharmacol Ther* 2016; **44**: 45–56.

158 Ahmed I, Greenwood R, de Costello BL, Ratcliffe NM, Probert CS. An Investigation of Fecal Volatile Organic Metabolites in Irritable Bowel Syndrome. *PLoS One* 2013; **8**: 1–13.

159 Walton C, Fowler DP, Turner C, *et al.* Analysis of Volatile Organic Compounds of Bacterial Origin in Chronic Gastrointestinal Diseases. *Inflamm Bowel Dis* 2013; **19**: 2069–78.

160 Shepherd SF, McGuire ND, de Lacy Costello BPJ, *et al.* The use of a gas chromatograph coupled to a metal oxide sensor for rapid assessment of stool samples from irritable bowel syndrome and inflammatory bowel disease patients. *J Breath Res* 2014; **8**. DOI:10.1088/1752-7155/8/2/026001.

161 Cauchi M, Fowler DP, Walton C, *et al.* Application of gas chromatography mass spectrometry (GC–MS) in conjunction with multivariate classification for the diagnosis of gastrointestinal diseases. *Metabolomics* 2014; **10**: 1113–20.

162 Arasaradnam RP, Westenbrink E, McFarlane MJ, *et al.* Differentiating coeliac disease from irritable bowel syndrome by urinary volatile organic compound analysis - A pilot study. *PLoS One* 2014; **9**: 1–9.

163 Smolinska A, Tedjo DI, Blanchet L, *et al.* Volatile metabolites in breath strongly correlate with gut microbiome in CD patients. *Anal Chim Acta* 2018; **1025**: 1–11.

164 Sagar NM, Duboc H, Kay GL, *et al.* The pathophysiology of bile acid diarrhoea: differences in the colonic microbiome, metabolome and bile acids. *Scientific Reports 2020 10:1* 2020; **10**: 1–12.

165 Parker TJ, Naylor SJ, Riordan AM, Hunter JO. Management of patients with food intolerance in irritable bowel syndrome: the development and use of an exclusion diet. *Journal of Human Nutrition and Dietetics* 1995; **8**: 159–66.

166 Rossi M, Aggio R, Staudacher HM, *et al.* Volatile Organic Compounds in Feces Associate With Response to Dietary Intervention in Patients With Irritable Bowel Syndrome. *Clinical Gastroenterology and Hepatology* 2018; **16**: 385-391.e1.

167 Vinolo MAR, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. *Nutrients* 2011; **3**: 858–76.

168 Tedelind S, Westberg F, Kjerrulf M, Vidal A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: A study with relevance to inflammatory bowel disease. *World Journal of Gastroenterology : WJG* 2007; **13**: 2826.

169 Whitfield-Cargile CM, Cohen ND, Chapkin RS, *et al.* The microbiota-derived metabolite indole decreases mucosal inflammation and injury in a murine model of NSAID enteropathy. *Gut Microbes* 2016; **7**: 246–61.

170 Canavan C, West J, Card T. The epidemiology of irritable bowel syndrome. *Clin Epidemiol* 2014; **6**: 71–80.

171 Zia JK, Lenhart A, Yang PL, *et al.* Risk Factors for Abdominal Pain–Related Disorders of Gut–Brain Interaction in Adults and Children: A Systematic Review. *Gastroenterology* 2022; **163**: 995-1023.e3.

172 Eijsbouts C, Zheng T, Kennedy NA, *et al.* Genome-wide analysis of 53,400 people with irritable bowel syndrome highlights shared genetic pathways with mood and anxiety disorders. *Nature Genetics 2021 53:11* 2021; **53**: 1543–52.

173 Mcintosh K, Reed DE, Schneider T, *et al.* FODMAPs alter symptoms and the metabolome of patients with IBS: a randomised controlled trial. DOI:10.1136/gutjnl-2015-311339.

174 Liu J, Chey WD, Haller E, Eswaran S. Low-FODMAP Diet for Irritable Bowel Syndrome: What We Know and What We Have Yet to Learn. *Annu Rev Med* 2020; **71**: 303–14.

175 Poulsen CH, Eplov LF, Hjorthøj C, *et al.* Irritable bowel symptoms, use of healthcare, costs, sickness and disability pension benefits: A long-term population-based study. *Scand J Public Health* 2019; **47**: 867–75.

176 Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system : a simple method of monitoring irritable bowel syndrome and its progress. 1997.

177 Labus JS, Bolus R, Chang L, *et al.* The Visceral Sensitivity Index: development and validation of a gastrointestinal symptom-specific anxiety scale. DOI:10.1111/j.1365-2036.2004.02007.x.

178 Zigmond AS, Snaith RP. The Hospital Anxiety and Depression Scale. *Acta Psychiatr Scand* 1983; **67**: 361–70.

179 Stern AF. The Hospital Anxiety and Depression Scale. *Occup Med (Chic Ill)* 2014; **64**: 393–4.

180 Groll D, Vanner SJ, Depew WT, *et al.* The IBS-36: a new quality of life measure for irritable bowel syndrome. *Am J Gastroenterol* 2002; **97**: 962–71.

181 Patrick DL, Drossman DA, Frederick IO, Dicesare J, Puder KL. Quality of life in persons with irritable bowel syndrome: development and validation of a new measure. *Dig Dis Sci* 1998; **43**: 400–11.

182 Baecke JAH, Burema J, Frijters JER. A short questionnaire for the measurement of habitual physical activity in epidemiological studies. *Am J Clin Nutr* 1982; **36**: 936–42.

183 Stefanik PA, Trulson MF. Determining the frequency intakes of foods in large group studies. *Am J Clin Nutr* 1962; **11**: 335–43.

184 Lovell RM, Ford AC. Global Prevalence of and Risk Factors for Irritable Bowel Syndrome: A Meta-analysis. *Clinical Gastroenterology and Hepatology* 2012; **10**: 712-721.e4.

185 Engsbro AL, Simren M, Bytzer P. Short-term stability of subtypes in the irritable bowel syndrome: prospective evaluation using the Rome III classification. *Aliment Pharmacol Ther* 2012; **35**: 350–9.

186 Beatty JK, Bhargava A, Buret AG. Post-infectious irritable bowel syndrome: Mechanistic insights into chronic disturbances following enteric infection. *World Journal of Gastroenterology : WJG* 2014; **20**: 3976.

187 Longstreth GF, Hawkey CJ, Mayer EA, *et al.* Characteristics of patients with irritable bowel syndrome recruited from three sources: implications for clinical trials. *Aliment Pharmacol Ther* 2001; **15**: 959–64.

188 Singh P, Staller K, Barshop K, *et al.* Patients with irritable bowel syndrome-diarrhea have lower disease-specific quality of life than irritable bowel syndrome-constipation. *World J Gastroenterol* 2015; **21**: 8103–9.

189 Canavan C, West J, Card T. The epidemiology of irritable bowel syndrome. *Clin Epidemiol* 2014; **6**: 71–80.

190 Santos J, Guilarte M, Alonso C, Malagelada JR. Pathogenesis of irritable bowel syndrome: The mast cell connection. *Scand J Gastroenterol* 2005; **40**: 129–40.

191 Cop N, Decuyper II, Faber MA, *et al.* Phenotypic and functional characterization of in vitro cultured human mast cells. *Cytometry B Clin Cytom* 2017; **92**: 348–54.

192 Elst J, Sabato V, van der Poorten MLM, *et al.* Peripheral blood cultured mast cells: Phenotypic and functional outcomes of different culture protocols. *J Immunol Methods* 2021; **492**: 113003.

193 Nasser Y, Boeckxstaens GE, Wouters MM, Schemann M, Vanner S. Using human intestinal biopsies to study the pathogenesis of irritable bowel syndrome. *Neurogastroenterology & Motility* 2014; **26**: 455–69.

194 Valdez-Morales EE, Overington J, Guerrero-Alba R, *et al.* Sensitization of peripheral sensory nerves by mediators from colonic biopsies of diarrhea-predominant irritable bowel syndrome patients: a role for PAR2. *Am J Gastroenterol* 2013; **108**: 1634–43.

195 Piche T, Barbara G, Aubert P, *et al.* Impaired Intestinal barrier integrity in the colon of patients with irritable bowel syndrome: Involvement of soluble mediators. *Gut* 2009; **58**: 196–201.

196 Nasser Y, Boeckxstaens GE, Wouters MM, Schemann M, Vanner S. Using human intestinal biopsies to study the pathogenesis of irritable bowel syndrome. *Neurogastroenterology and Motility* 2014; **26**: 455–69.

197 Ostertag D, Buhner S, Michel K, *et al.* Reduced responses of submucous neurons from irritable bowel syndrome patients to a cocktail containing histamine, serotonin, TNFα, and tryptase (IBS-cocktail). *Front Neurosci* 2015; **9**: 465.

198 Herr N, Bode C, Duerschmied D. The Effects of Serotonin in Immune Cells. *Front Cardiovasc Med* 2017; **4**: 48.

199 Ibeakanma C, Vanner S. TNFalpha is a key mediator of the pronociceptive effects of mucosal supernatant from human ulcerative colitis on colonic DRG neurons. *Gut* 2010; **59**: 612–21.

200 Valdez-Morales EE, Overington J, Guerrero-Alba R, *et al.* Sensitization of peripheral sensory nerves by mediators from colonic biopsies of diarrhea-predominant irritable bowel syndrome patients: A role for PAR2. *American Journal of Gastroenterology* 2013; **108**: 1634–43.

201 Aggio RBM, White P, Jayasena H, de Lacy Costello B, Ratcliffe NM, Probert CSJ. Irritable bowel syndrome and active inflammatory bowel disease diagnosed by faecal gas analysis. *Aliment Pharmacol Ther* 2017; **45**: 82–90.

202 Walton C, Fowler DP, Turner C, *et al.* Analysis of volatile organic compounds of bacterial origin in chronic gastrointestinal diseases. *Inflamm Bowel Dis* 2013; **19**: 2069–78.

203 Chan DK, Leggett CL, Wang KK. Diagnosing gastrointestinal illnesses using fecal headspace volatile organic compounds. *World J Gastroenterol* 2016; **22**: 1639.

204 Lacy BE, Mearin F, Chang L, *et al.* Bowel Disorders. *Gastroenterology* 2016; **150**: 1393-1407.e5.

205 WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects – WMA – The World Medical Association. https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/ (accessed Jan 4, 2022).

206 Kreuer S, Hellbrück R, Fink T, *et al.* Development of a device to measure MCC-IMS peaks of pure analytes (IMS-BOX). *International Journal for Ion Mobility Spectrometry* 2013; **16**: 281–6.

207 Lamote K, Vynck M, van Cleemput J, Thas O, Nackaerts K, van Meerbeeck JP. Detection of malignant pleural mesothelioma in exhaled breath by multicapillary column/ion mobility spectrometry (MCC/IMS). *J Breath Res* 2016; **10**. DOI:10.1088/1752-7155/10/4/046001.

208 Lamote K, Vynck M, Thas O, van Cleemput J, Nackaerts K, van Meerbeeck JP. Exhaled breath to screen for malignant pleural mesothelioma: a validation study. *European Respiratory Journal* 2017; **50**: 1700919.

209 Patrick DL, Drossman DA, Frederick IO, DiCesare J, Puder KL. Quality of life in persons with irritable bowel syndrome: development and validation of a new measure. *Dig Dis Sci* 1998; **43**: 400–11.

210 Holtmann GJ, Ford AC, Talley NJ. Pathophysiology of irritable bowel syndrome. *Lancet Gastroenterol Hepatol* 2016; **1**: 133–46.

211 Black CJ, Yiannakou Y, Guthrie E, West R, Houghton LA, Ford AC. Longitudinal follow-up of a novel classification system for irritable bowel syndrome: natural history and prognostic value. *Aliment Pharmacol Ther* 2021; **53**: 1126–37.

212 Black CJ, Yiannakou Y, Guthrie EA, West R, Houghton LA, Ford AC. A Novel Method to Classify and Subgroup Patients With IBS Based on Gastrointestinal Symptoms and Psychological Profiles. *Am J Gastroenterol* 2021; **116**: 372–81.

213 Camilleri M. Irritable Bowel Syndrome: Straightening the road from the Rome criteria. *Neurogastroenterology & Motility* 2020; **32**: e13957.

214 El-Salhy M. Recent advances in the diagnosis of irritable bowel syndrome. *Expert Rev Gastroenterol Hepatol* 2015; **9**: 1161–74.

215 Zhang T, Ma X, Tian W, *et al.* Global Research Trends in Irritable Bowel Syndrome: A Bibliometric and Visualized Study. *Front Med (Lausanne)* 2022; **0**: 1931.

216 Pimentel M, Mathur R, Wang J, *et al.* Assessment of stool images using artificial intelligence via a smartphone application is more accurate than patient reported outcomes. *Gastroenterology* 2022; **162**: S-63.

217 MacGlashan D, Hamilton RG. Parameters Determining the Efficacy of CD32 to Inhibit Activation of FcεRI in Human Basophils. *J Allergy Clin Immunol* 2016; **137**: 1256.

218 Zimmermann M, Meyer N. Annexin V/7-AAD staining in keratinocytes. *Methods Mol Biol* 2011; **740**: 57–63.

219 el Hassani S el M, Berkhout DJC, Bosch S, Benninga MA, de Boer NKH, de Meij TGJ. Application of Fecal Volatile Organic Compound Analysis in Clinical Practice: Current State and Future Perspectives. *Chemosensors 2018, Vol 6, Page 29* 2018; **6**: 29.

Chapter 10 Curriculum vitae

10.1 Education

2005 - 2007	Latin and science (Koninklijk Atheneum Dendermonde)
2007 - 2011	Science and mathematics (Koninklijk Atheneum Dendermonde)
2011 - 2014	Bachelor of Medicine (University of Antwerp, *Great distinction*)
2014 - 2018	Master of Medicine (University of Antwerp, *Great distinction*)
2018 - 2023	PhD in Medicine (University of Antwerp)
2018 - 2026	Advanced Master Specialistic Medicine, internal medicine (University of Antwerp)

10.2 Training

2013	BLS/AED (University of Antwerp)
2014	Laboratory animal sciences (University of Antwerp)
2015	Electrocardiography (University hospital Antwerp)
	Trauma X conference (Imperial college London)
2016	Language course English (Altissia)
2017	Basic emergency ultrasound course (University of Antwerp)
	E-course faecal microbiota (United European Gastroenterology)
	E-course functional constipation (United European Gastroenterology)
	E-course capsule endoscopy (United European Gastroenterology)
	Research internship (Mayo Clinic Rochester)
2018	Personal effectiveness (University of Antwerp)
	Biomedical e-sources (University of Antwerp)
	Communicating effectively (University of Antwerp)
	Language course French (Altissia)
	E-course statistics in medicine (Stanford University)
2019	QUALTRICS (University of Antwerp)
	Writing Academic papers (University of Antwerp)
	Optimizing cooperation in international research groups (University of Antwerp)
	Postgraduate writing course (Biocodex)
2020	E-course basic principles of online marketing (Google digital atelier)
	E-course data science: R Basics (Harvard University)
2021	Basic training medical hypnotherapy (Vlaamse Wetenschappelijke Hypnosevereniging)
	Leadership and teamworking (University of Antwerp)
	Summerschool neurogastroenterology (Queen Mary University London)
2022	Motility course (Vlaamse vereniging voor gastro-enterologie)

10.3 Professional experience

2009	European Youth parliament
2008 - 2011	TRUST (student guidance counselor)
2015	WHO meeting
2011 - 2016	Volunteer Red Cross
2012 - 2016	Secretary at a general practitioner's office (De Kouter)
2014 - 2016	Open day University of Antwerp, Medical department
2014 - 2016	Project SOS, sexual education (EMSA)
2016	Teddy bear hospital (EMSA)
2014 - 2017	European medical student's association
2019 - present	Founder and moderator patient website IBS Belgium

10.4 Scientific output

10.4.1 Publications related to the thesis

Van Malderen K, De Man J, De Winter B, De Schepper H, Lamote K. Volatomics in inflammatory bowel disease and irritable bowel syndrome. *EBioMedicine* 2020; **54** https://doi.org/10.1016/j.ebiom.2020.102725.

Kindt S, Louis H, De Schepper H, Arts J, Caenepeel P, De Looze D, Gerkens A, Holvoet T, Latour P, Mahler T, Mokaddem F, Nullens S, Piessevaux H, Poortmans P, Rasschaert G, Surmont M, Vafa H, **Van Malderen K**, Vanuytsel T, Wuestenberghs F, Tack J. Belgian consensus on irritable bowel syndrome. *Acta Gastro-Enterologica Belgica* 2022; **85** doi:10.51821/85.2.10100

Van Malderen K, Hanning N, Lambrechts H, Haverhals T, Van Marcke S, Ceuleers H, De Man J, De Winter B, Lamote K, De Schepper H. Volatile organic compound profiling as a potential biomarker in irritable bowel syndrome: a feasibility study. *Frontiers in Medicine Section Gastroenterology* 2022; https://doi.org/10.3389/fmed.2022.960000

Van Malderen K, De Man J, De Winter B, De Schepper H. Epidemiological characteristics of a population visiting a patient-centered informative website about irritable bowel syndrome. *Acta Gastro-Enterologica Belgica* 2023; **86** doi:10.51821/86.1.10885

Van Malderen K, Elst J, De Man J, Ebo D, De Winter B, Sabato V, De Schepper HU. Characterisation of human peripheral blood cultured mast cells in the pathophysiology of irritable bowel syndrome. *Research project will be continued*

10.4.2 Abstracts related to the thesis

Van Malderen K, De Man J, De Winter B, De Schepper H, Lamote K. Volatomics in inflammatory bowel disease and irritable bowel syndrome: a systematic review *Poster BWGE* 2020

Van Malderen K, Janssens E, De Man J, De Winter B, De Schepper H, Lamote K.Volatile organic compound profiling of breath samples as a biomarker to discriminate between patients with irritable bowel syndrome and healthy controls: a feasibility study *Oral presentation BWGE* 2020

Van Malderen K, De Man J, De Winter B, De Schepper H, Lamote K. Volatomics in inflammatory bowel disease and irritable bowel syndrome: present and future *Poster DDW* 2020

Van Malderen K, Janssens E, De Man J, De Winter B, De Schepper H, Lamote K. Discriminating between patients with irritable bowel syndrome and healthy controls with the help of volatile organic compounds profiling in breath samples: a feasibility study *Poster DDW* 2020

Van Malderen K, De Man J, De Winter B, De Schepper H. Prevalence of Rome IV criteria for irritable bowel syndrome and red flag symptoms in visitors of a patient centered informative website *Poster UEG week* 2020

Van Malderen K, De Man J, De Winter B, De Schepper H. Characteristics of a population visiting a patient centred informative website: prevalence of Rome IV criteria for irritable bowel syndrome and red flag symptoms *Oral presentation BWGE* 2021

Van Malderen K, Lambrechts H, Haverhals T, Van Marcke S, De Man J, De Winter B, Lamote K, De Schepper H. Exhaled breath analysis for diagnosis and subtyping of patients with irritable bowel syndrome *Poster Neurogastro* 2021

Van Malderen K, Lambrechts H, Haverhals T, Van Marcke S, De Man J, De Winter B, Lamote K, De Schepper H. Comparing volatile organic compound profiling in breath and faecal samples to discriminate between patients with irritable bowel syndrome and healthy controls: a feasibility study *Poster Neurogastro* 2021

Van Malderen K, Hanning N, Lambrechts H, Haverhals T, Van Marcke S, De Man J, De Winter B, Lamote K, De Schepper H. Volatile organic compound (VOC) profiling in breath and faecal samples discriminates patients with irritable bowel syndrome from healthy controls *Oral presentation BWGE* 2022

Van Malderen K, Hanning N, Lambrechts H, Haverhals T, Van Marcke S, De Man J, De Winter B, Lamote K, De Schepper H. Discriminating patients with irritable bowel syndrome from healthy controls with volatile organic compound (VOC) profiling in breath and faecal samples *E-poster DDW* 2022

10.4.3 Publications not related to the thesis

Van Malderen K, Halawi H, Camilleri M. Insights on efficacious doses of PAMORAs for patients on chronic opioid therapy or opioid-naïve patients. *Neurogastroenterology and Motility* 2018; **30**: e13250. https://doi.org/10.1111/nmo.13250

Van Malderen K, Camilleri M. Large Meckel's diverticulum and dilated adjacent small intestine presenting with intestinal obstruction. *Clinical Gastroenterology and Hepatology* 2017; **16**: A33

Somers M, Peleman C, **Van Malderen K**, Verlinden W, Francque S, De Schepper H. Manometric and ultrasonographic characteristics of patients with coexisting fecal incontinence and constipation. *Acta Gastro-enterologica Belgica* 2017; **80**(4): 463-469

Van Malderen K, Vijayvargiya P, Camilleri M, Larson DW, Cima R. Malignancy and Meckel's diverticulum: A systematic literature review and 14-year experience at a tertiary referral center. *United European Gastroenterology Journal.* 2018; **6**(5):739-747. doi:10.1177/2050640617752771

Chedid, V, Vijayvargiya, P, Carlson, P, **Van Malderen K**, Acosta A, Zinsmeister A, Camilleri M. Allelic variant in the glucagon-like peptide 1 receptor gene associated with greater effect of liraglutide and exenatide on gastric emptying: A pilot pharmacogenetics study. *Neurogastroenterology and motility* 2018; **30**: e13313. https://doi.org/10.1111/nmo.13313

Van Malderen K, Hanning N, De Man J, De Winter B, De Schepper H. Development of irritable bowel syndrome and functional dyspepsia after COVID-19 infection. *In preparation*

Van Malderen K, De Man J, De Winter B, De Schepper H. Prevalence of symptoms outside of the intestinal tract in irritable bowel syndrome. *In preparation*

10.4.4 Abstracts not related to the thesis

Peleman C, **Van Malderen K**, Verlinden W, De Schepper H. Phasic and continuous protocols in anorectal manometrical assessment of rectal sensitivity: agreement and correlation with clinical presentation. *Poster UEG week* 2016

Van Malderen K, Peleman C, Spinhoven M, De Schepper H. Agreement between dynamic transrectal ultrasound and MR defecography in patients with constipation. *Poster DDW* 2017

Van Malderen K, Somers M, Spinhoven M, De Schepper H. Agreement between dynamic transrectal ultrasound and MR defecography in patients with constipation. *Oral presentation BWGE* 2018

Van Malderen K, De Man J, De Winter B, De Schepper H. The prevalence of gastrointestinal symptoms during acute COVID-19 infection assessed using a digital questionnaire *Pitch BWGE* 2022

Van Malderen K, De Man J, De Winter B, De Schepper H. The prevalence of gastrointestinal symptoms in acute COVID-19 infection evaluated with a digital questionnaire *E-poster DDW* 2022

10.4.5 Science communication

Mindfulness vermindert klachten van prikkelbare darmsyndroom (2020) *Nina, Goed Gevoel*

Waarom beland ik voor het examen op het toilet? (2021) *Technopolis*

Als het rommelt in je buik (2021) *Libelle*

Er was eens… het prikkelbare darmsyndroom (2022) *Kennismakersplatform Fonds wetenschappelijk onderzoek Vlaanderen*

Over het muurtje: prof. Dr. Kristof De Witte en Dr. Kathleen Van Malderen (2022) *Kennismakersplatform Fonds wetenschappelijk onderzoek Vlaanderen*

Geen baas in eigen buik (2023) *Libelle*

10.4.5.1 *Articles IBS Belgium*

Hoe werken onze darmbacteriën (2019)
Stellingen prikkelbare darmsyndroom (2019)
De rol van lactose in het prikkelbare darmsyndroom (2019)
Hoe werken laxeermiddelen (2019)
De rol van gluten in het prikkelbare darmsyndroom (2019)
Stoelgangstransplantatie bij het prikkelbare darmsyndroom (2020)
Nieuwe studie: Stoelgangstransplantatie bij het prikkelbare darmsyndroom (2020)
Maag- en darmklachten bij het coronavirus (2020)
Wat onze neus ons kan leren over het prikkelbare darmsyndroom (2020)
Functionele maag- en darmaandoeningen (2020)
Functionele diarree (2020)
Hoe werkt het prikkelbare darmsyndroom (2020)
Functionele constipatie (2020)
Functionele dyspepsie (2020)
Functionele buikpijn (2020)
Functionele bloating (2020)
Wat is het belang van lotgenotencontact (2020)
Functionele nausea en braken (2020)
Speciale editie Nutrients (2020)
Functioneel boeren (2020)
Nutrients: Gefermenteerde voeding (2020)
Dyschezie of bekkenbodemdysfunctie (2020)
Update maag- en darmklachten bij het coronavirus (2020)
Functionele reflux (2020)
Fecale incontinentie (2020)
Globus gevoel (2020)
Wat zijn de oorzaken van chronische diarree (2020)
Functionele anorectale pijn (2020)
Hoogtepunten UEG congres (2020)
Functionele dysfagie (2020)
Functioneel galblaaslijden (2020)
Reflux hypersensitiviteit (2020)
Functionele pijn op de borst (2021)
Lokale immuunrespons tegen voeding als oorzaak van het prikkelbare darmsyndroom (2021)
Resultaten zelftest IBS Belgium (2021)
Ruminatie syndroom (2021)
Wat is de rol van genetica (2021)
PDS symptomen bij inflammatoire darmziekten (2021)
Minds PDS klachten tijdens COVID-19 lockdown (2021)
Wat is de rol van hypnose (2021)

Symptomen bij PDS buiten de darmen (2021)
Wat zijn de oorzaken van functionele buikpijn bij kinderen (2021)
Wat is de behandeling van functionele buikpijn bij kinderen (2021)
Studies Universiteit Antwerpen (2021)
Het effect van voeding en darmbacteriën op PDS symptomen (2021)
Wat is urgency (2021)
Evolutie van diagnostische criteria voor het prikkelbare darmsyndroom (2022)
Voorkomen van maag- en darmklachten bij COVID-19 (2022)
De geur van adem en stoelgang bij het prikkelbare darmsyndroom (2022)
Wat is de rol van pepermunt (2022)
Wat is de rol van ademtesten in het prikkelbare darmsyndroom (2022)
Wat weten we over ebastine (2022)
Wat zijn lekkende darmen (2022)
Wat is de link tussen onze darmen en depressieve gevoelens (2022)
Wat is de rol van biomerkers in het bloed (2022)
Wat is de link tussen de bekkenbodem en een opgeblazen gevoel (2022)
Wat is de rol van antibiotica (2022)
Risicofactoren voor het ontstaan van het prikkelbare darmsyndroom (2023)

Chapter 11 Dankwoord

Een doctoraat is een team effort en geen solo, een marathon en geen sprint. Ik wil dan ook iedereen bedanken die er mee voor heeft gezorgd dat ik mijn onderzoek succesvol heb kunnen afronden.

Eerst en vooral mijn promotor prof. Dr. Heiko De Schepper. Bedankt om mij de kans te geven dit avontuur te starten. Voor het vertrouwen dat me toeliet om zelfstandig te werken en zo met vallen en opstaan ontzettend veel te leren. Voor alle kansen om nieuwe dingen te ontdekken, maar ook om me af te remmen wanneer ik te veel wou doen. Bedankt om mij de mogelijkheid te geven in te zetten op wetenschapscommunicatie en deze passie met mij te delen. Ik hoop dat we de komende jaren nog veel mensen kunnen bereiken en het taboe rond het prikkelbare darmsyndroom kunnen doorbreken. U heeft me niet enkel de kans gegeven een betere onderzoeker te worden, maar ook een betere arts.

Mijn co-promotoren prof. Dr. Vito Sabato en prof. Dr. Benedicte De Winter. Bedankt voor jullie steun en waardevolle advies. Jammer genoeg liep niet alles even vlot, maar door jullie suggesties tijdens onze meetings was er steeds een plan voor de volgende stappen. Het is mede dankzij jullie dat ik steeds de moed had om verder te gaan. Prof. Dr. Didier Ebo, prof. Annemieke Smet, dr. Kevin Lamote, bedankt voor jullie adviezen en het delen van kennis en expertise uit jullie respectievelijke onderzoeksdomeinen.

Joris, bedankt voor je praktische ondersteuning bij al mijn vragen en luisterend oor wanneer er problemen waren. Jessy, Christel, Michel dank u om mij in te leiden in de wondere wereld van de mestcel culturen. Er waren tegenslagen, maar ik kon steeds op jullie rekenen voor advies en eindeloos geduld bij het vinden van oplossingen. Zonder jullie zou het nooit gelukt zijn.

Prof. Camilleri, thank you for the amazing two months I was able to spend in your research lab before I even started with my PhD project. You gave me an introduction into clinical research and thought me a lot about writing research papers.

Nikita, vier jaar geleden starten we samen aan dit avontuur. We konden steeds op elkaar rekenen om frustraties te delen, maar ook voor een peptalk en advies kon ik bij jou terecht. Mijn doctoraat zou niet geweest zijn wat het nu is zonder jouw statistische kennis en eindeloze geduld om mij dingen uit te leggen.

Baptiste, de laatste maanden van mijn doctoraat hebben we elkaar veel gezien. Wat een eenvoudige opdracht had moeten zijn bleek uiteindelijk een parcours vol hindernissen. Bedankt om steeds tijd voor mij vrij te maken ook al kwam je hierdoor soms in de problemen met je eigen planning. Wout, ook jij was er deze laatste maanden vaak bij toen we zochten naar oplossingen. Samen konden we onze frustraties delen over het gebruik van de celcultuur. Niet enkel tijdens de werkuren, maar ook hierbuiten kon ik steeds op jullie beiden rekenen.

Eline, Kathleen, Eline en Amber, de andere collega's van mijn onofficiële bureau. Ook op jullie kon ik steeds rekenen voor een goed gesprek. Er zijn weinig zaken die niet aan bod zijn gekomen tijdens de vele gesprekken. Denise, Philip, Hanne mijn bureaugenoten van de eerste jaren. Jullie maakten mij wegwijs in het onderzoek en leerden me de werking van het labo kennen. Ook bedankt aan alle andere doctoraatsstudenten die ik doorheen de jaren hebben mogen kennen ook jullie waren steeds beschikbaar voor een goede babbel, Stijn, Wilco, Sam, Jonas, Mikhaïl, Kristien, Eline, Tom, Shivani, Axelle, Arno, Cédric, Veerle, Eline.

Sara, bedankt om mij al die jaren geleden te laten kennis maken met wetenschappelijk onderzoek. Jouw enthousiasme en passie voor onderzoek zijn een doorslaggevende factor geweest in mijn beslissing dit avontuur aan te gaan. Ook nu is het steeds een plezier ervaringen en advies te delen.

Ook wil ik graag alle laboranten bedanken voor hun hulp en dan zeker Marleen, Petra en Lieve. Ik kon steeds op jullie rekenen en ook wanneer er weer materiaal vermist was zochten jullie graag mee.

Frauke, Sophie en Eveline, ik kon steeds op jullie rekenen voor het contacteren en inplannen van patiënten. Ook voor een gezellige babbel tussen de consultaties door kon ik altijd bij jullie terecht. Ook alle andere artsen, ASO's en het secretariaat van de gastro-enterologie in het UZA zou ik willen bedanken. Het was steeds fijn om met jullie te kunnen samen werken.

En dan aan alle vrienden waar ik al vele jaren op kan rekenen. Magali, Hanne, Ann, Caroline, Vincent, Gregory, Steven en Kimberly. Bedankt voor alle steun, voor het luisterend oor en de fijne herinneringen die we de voorbije jaren hebben gemaakt.

Mama, papa, Joris, Eline bedankt voor jullie jarenlange steun. Jullie hebben me gemaakt tot de persoon die ik vandaag ben en stimuleerden me steeds om het maximale te bereiken.

Om af te sluiten wil ik graag één van de belangrijkste personen bedanken, Bjorn. Je was mijn onvoorwaardelijke steun en toeverlaat. Je bood niet alleen een luisterend oor, maar ook afleiding op moeilijke momenten. De laatste jaren waren een avontuur en niet enkel op

professioneel vlak, voor ons allebei trouwens. We besloten samen een huis te bouwen, overleefden de coronacrisis en kwamen er sterker uit. Het was niet altijd even makkelijk, maar ik kon steeds op je rekenen. En toen kwam de website. Ik kan je niet genoeg bedanken voor alle uren die je hier, gratis, aan werkte. Weekenden, avonden en soms zelfs nachten. We kunnen trots zijn op wat we samen hebben bereikt, maar één ding is zeker, zonder jou was dit nooit mogelijk geweest.