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The Pathophysiology of Irritable Bowel Syndrome and its Treatment

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Thesis submitted to the University Of Nottingham

for the degree of Doctor of Philosophy

December 2020

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Publications and presentations arising from this thesis

Publications

Gunn D, Murthy R, Major G, Wilkinson-Smith V, Hoad C, Marciani L, Remes-Troche J, Gill S, Rossi M, Harris H, Ahn-Jarvis J, Warren F, Whelan K, Spiller R. (2020). **Contrasting effects of viscous and particulate fibers on colonic fermentation in vitro and in vivo, and their impact on intestinal water studied by MRI in a randomized trial**, The American Journal of Clinical Nutrition, *112*(3), 595-602

Gunn D, Garsed K, Lam C, Singh G, Lingaya M, Wahl V, Niesler B, Henry A, Hall IP, Whorwell P, Spiller R. (2019). **Abnormalities of mucosal serotonin metabolism and 5-HT_3 receptor subunit 3C polymorphism in irritable bowel syndrome with diarrhoea predict responsiveness to ondansetron**. *Alimentary Pharmacology and Therapeutics*, *50*(5) 538–546.

Oral Presentations

Gunn, D. **Lactulose increases small bowel but not colonic water content; questioning the osmotic laxative dogma**. British Society of Gastroenterology, Liverpool, UK, February 2021 (delayed due to COVID-19)

Gunn D. **Psyllium reduces colonic hydrogen production following ingestion of inulin in irritable bowel syndrome**. British Society of Gastroenterology, Liverpool, UK, February 2021 (delayed due to COVID-19)

Poster Presentations

Gunn D, Murthy R, Major G, Wilkinson-Smith V, Hoad C, Marciani L, Remes-Troche J, Gill S, Rossi M, Harris H, Ahn-Jarvis J, Warren F, Whelan K, Spiller R. **Varying effects of bran, psyllium and prickly pear cactus fibre (nopal) on small intestinal water content and colonic volume demonstrated using magnetic resonance imaging (MRI).** Federation of Neurogastroenterology and Motility, Amsterdam, Holland, 2018

Gunn, D, Yeldho C, Hoad C, Marciani L, Spiller R. **Lactulose increases small bowel but not colonic water content; features which are not altered by the 5-HT_3 receptor antagonist Ondansetron.** Digestive Diseases Week, Chicago, USA, May 2020 (delayed due to COVID-19)

Gunn D, Abbas Z, Major G, Hoad C, Gowland P, Marciani L, Harris H, Warren F, Gill S, Rossi M, Remes-Troche J, Whelan K, Spiller R. **Psyllium reduces colonic hydrogen production following ingestion of inulin in irritable bowel syndrome.** Digestive Diseases Week, Chicago, USA, May 2020 (delayed due to COVID-19)

Acknowledgements

I'd like to start by thanking my supervisors Prof Robin Spiller and Dr Maura Corsetti for their guidance, feedback and unwavering support, making the whole process both educational and enjoyable. I'm also grateful to the EME programme from the Medical Research Council, who funded the TRITON trial (results still awaited) and my salary through three years in research, as well as the Newton Grant which funded NOPAL and EFIGI studies.

I would like the thank all those from the Nottingham Digestive Diseases Centre, in particular the nurses Helen Handford and Ruth Shephard; Clinical research fellows Tor, PT, Ed, Jane and Mohsen for the laughs, snacks and coffee; and administrative support from Emma, Beth, Natalie, and Lisa.

I wish to acknowledge staff at the Sir Peter Mansfield Imaging Centre for their hard work and tolerance of my stupid questions, particularly Caroline Hoad and Jan Paul.

I must also express my thanks to the co-investigators of NOPAL and EFIGI Fred Warren and Hannah Harris at Quadram Institute for their *in vitro* work; and Kevin Whelan, Megan Rossi and Sammie Gill at King's College London, for their input throughout the studies.

A very large thank you for the love and support goes to my wife Louise, without whom I would be unable to complete this work; and to William, Henry and Florence for keeping a smile on my face.

Lastly I would like to thank all those who have taken part in these studies, without whom there would be nothing to say.

Candidate statement

All work presented here is my own other than the following:

MRI parameter acquisition

Dr Caroline Hoad (CH) devised the MRI sequences and programmes used to measure the agreed physiological variables.

in vitro fermentation

Dr Fred Warren (FW) and Dr Hannah Harris (HH) performed and analysed the *in vitro* fermentation studies at Quadram Institute Biosciences (Norwich, UK) for both NOPAL and EFIGI. HH performed rheology studies for EFIGI. Ms Jennifer Ahn-Jarvis (JAJ) assisted with the *in vitro* fermentation study for NOPAL.

Biopsy analysis

Dr Gulzar Singh (GH) and Ms Melanie Lingaya (ML) analysed rectal biopsies and plasma samples in the original ondansetron trial for IBS-D.

Genetic analysis

Dr Beate Niesler (BN) analysed patient serum for genotypes of interest in the original ondansetron trial for IBS-D.

Study Implementation

Two undergraduate students were each supervised by me in the implementation of a study: Ms Rajani Murthy (RM) was involved in the NOPAL study, and Ms Caroline Yeldho (CY) in the MOLID study. Both contributed to the MRI analysis under the direction of myself and CH.

I am grateful to all for their contribution.

Coronavirus-19 statement

This work was completed during the COVID-19 pandemic, impacting on the analysis and write up which was completed during lockdown.

Abbreviations

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxytryptophan
5HT3RA	Serotonin receptor 3 antagonist
AC	Ascending colon
ANOVA	Analysis of variance
ATI	Amylase-trypsin inhibitors
AUC	Area under the curve
BNF	British National Formulary
BSFS	Bristol stool form score
BT	Breath test
CI	Confidence interval
CRP	C-reactive protein
DF	Dietary fibre
DP	Degree of polymerisation
EC	Enterochromaffin cells
eQTL	Expression quantitative trait loci
FDA	Food and Drug Administration
fMRI	Functional magnetic resonance imaging
FMT	Faecal microbiota transplant
FODMAP	Fermentable oligo-, di-, mono-saccharides and polyols
FSA	Food Standards Agency

GE	Gastric emptying
GFD	Gluten free diet
GI	Gastrointestinal
GPCR	G-protein coupled receptor
HADS	Hospital Anxiety and Depression Scale
HDAC	Histone deacetylase
HPLC	High performance liquid chromatography
HRM	High resolution manometry
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IBS-C	Constipation-predominant irritable bowel syndrome
IBS-D	Diarrhoea-predominant irritable bowel syndrome
IBS-SSS	IBS symptom severity score
IC	Ischaemic colitis
Ig	Immunoglobulin
IQR	Interquartile range
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NDDC	Nottingham Digestive Disease Centre
NHS	National Health Service
NNH	Number needed to harm
NNT	Number needed to treat
PI-IBS	Post-infectious irritable bowel syndrome

PHQ-12	Patient health questionnaire 12 somatic symptom score
PSS	Perceived stress scale
PYY	Peptide YY
RCT	Randomised controlled trial
REC	Research ethics committee
RF	Radiofrequency
RM ANOVA	Repeated measures one-way analysis of variance
RNA	Ribonucleic acid
ROI	Region of interest
ROM	Radio-opaque markers
SBWC	Small bowel water content
SCFA	Short chain fatty acid
SD	Standard deviation
SERT	Serotonin transporter
SIBO	Small intestinal bacterial overgrowth
SNP	Single nucleotide polymorphism
SPMIC	Sir Peter Mansfield Imaging Centre
SSRI	Selective Serotonin Reuptake Inhibitor
T1	Longitudinal relaxation time
T1AC	T1 of the ascending colon
TCA	Tricyclic antidepressant
TI	Inversion time
TPH	Tryptophan hydroxylase

VAS	visual analogue scale
WAPS	weighted average position score
WGTT	whole gut transit time

Abstract

Irritable bowel syndrome (IBS) is a complex disorder of the gut causing abdominal pain, bloating and a change in bowel habit. Despite affecting approximately 10% of the general population, the exact causes are unknown and the treatments inadequate.

This thesis will start by introducing IBS and explore what is known currently about its pathology and treatment options. Chapters Two and Three will look at the role of different fibres in the gut using MRI. Chapter Two will describe the NOPAL study, assessing the effect of fibres of differing physico-chemical composition on intestinal physiology in healthy volunteers. Chapter Three describes the EFIGI study, assessing the effect of fibres alone and co-administered on intestinal physiology of patients with IBS.

The latter part of the thesis will look at the impact of ondansetron, a serotonin receptor-3 antagonist, as a treatment for diarrhoea-predominant IBS (IBS-D). Chapter Four will analyse a previous trial of ondansetron in patients with IBS-D to identify patients who benefit most from this treatment and further subclassify the disease. Chapter Five will describe the MOLID study which uses lactulose in healthy volunteers as a model for IBS-D, assessing the effect of ondansetron on the gut using MRI.

The thesis will conclude in Chapter Six discussing the studies described, and explore further research questions posed.

Aims and objectives

- To review what is currently known about the pathophysiology of irritable bowel syndrome, with particular focus on the treatments serotonin modulation and dietary fibre.

- To outline MRI and breath hydrogen analysis methods used in this thesis.

- To assess the effect of wheat bran, nopal and psyllium on gut physiology in healthy volunteers using MRI and breath tests.

- To assess the effect of co-ingestion of psyllium and inulin on gut physiology in patients with IBS using MRI and breath tests.

- To characterise patients with IBS-D who are most likely to benefit from treatment with ondansetron.

- To assess the effect of ondansetron on gut physiology in a model of IBS-D using MRI.

- To discuss the implications of the findings and further research questions to be answered.

1. Introduction

1.1. Intestinal function and the role of fermentation in the colon

1.1.1. Role of the intestine

To understand the diseases of the intestine, we must first appreciate its mechanisms and function in health. The gastrointestinal (GI) tract, from mouth to anus, is a complex system involving secondary organs such as the liver and pancreas with the main goal of acquiring nutrients necessary for anabolic processes in the body. Macronutrients such as fat, protein and carbohydrate, as well as micronutrients such as vitamins and minerals, must all be transported into the blood stream without compromising the osmotic and electrochemical environment of the epithelium. It must adapt to a large variety of substrates entering the system and at differing time intervals, yet be able to successfully obtain the necessary nutrients in order to survive.

Despite millennia for the GI tract to evolve, the human body alone is unable to digest many complex carbohydrates, which pass unchanged through the small intestine. These fibres are often described as dietary fibre (DF), however the precise definition remains contested.

Although not catabolised in the small intestine, DF exerts physico-chemical effects on the chyme and thus become functionally relevant in transit, mixing and water homeostasis. The fibre enters into a colon colonised by bacteria, which are in a symbiotic relationship with the host. Bacteria are presented with the undigested carbohydrates, which are then metabolised into substances such as short chain fatty acids (SCFA) and essential vitamins such as vitamin K and thiamine, absorbed by the host.

This thesis will focus on the intestine, so will not cover the upper GI tract nor its contribution to digestion in heath and disease. Having briefly given an overview of the role of the intestine, the roles of fibre and serotonin will next be considered.

1.1.2. Dietary Fibre

Since its introduction in the 1953 by Hipsley[1], the term 'dietary fibre' has confused many as regional definitions evolved without international consensus. Put simply, dietary fibre can be defined as plant components that are not broken down by human digestive enzymes. The European Food Safety Authority (EFSA)[2] and US Food and Drug Administration (FDA)[3] more precisely define DF as "carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories (i) edible carbohydrate polymers naturally occurring in the food as consumed, (ii) edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence, or (iii) edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence".

The association between increased dietary fibre and health benefits such as reduction in cardiovascular disease[4], type 2 diabetes[5] and colorectal cancer[6] have been well described. In line with this, the recommended daily intake for adults in the UK is 30g, yet given free choice the average is only 20.1g for men and 17.2g for women[7] stimulating efforts to improve toleration of high fibre diets and fill this "fibre gap".

Fibre has long been categorised by its ability to fully disperse in water, thus the terms "soluble" and "insoluble" fibre have been used. Obvious examples of insoluble fibres include matrix fibres such as lignins, cellulose and wheat bran, whereas natural gel forming fibres such as pectins, gums, and mucilages are soluble. Both share many physical properties including a variable ability to bind water and capacity to bind mineral ions[8]. Soluble fibre, such as psyllium, has been shown to lower serum cholesterol[9] whereas insoluble fibres have demonstrated a greater tendency to lower systolic blood pressure[10]. In the gut, soluble fibre forms a viscous gel which slows transit through the small bowel, slowing absorption of nutrients such as lipids and glucose (thus

lowering the glycaemic index)[11] and are readily accessible to bacteria to facilitate rapid fermentation in the colon. Insoluble fibres more classically act in the colon as a bulking agent, are poorly fermented and increase water absorption[12]. They may also provide ecological niches for bacteria to attach and grow, creating biofilms.

However there is now a greater insight into the structural diversity of fibres at the chemical and physical structural level. Whereas previously fibre was thought only to bulk the stool, we are now aware that fibre components can bind or encapsulate other nutrients and there is spectrum of viscosity, solubility and fermentability. Each of these attributes impact the digestion of the nutrients, rate of delivery, transit through the gut and the availability of substrate for the colonic microbiota[13]. Highly viscous fibres such as psyllium, for example, affect digestion and absorption of co-ingested nutrients in the upper GI tract, and can reduce total glucose and cholesterol absorption by up to 12%[5,14]. Other fibres such as cellulose can slow starch digestion by binding amylase[15] while wheat bran is thought to accelerate transit and increase secretion to increase small bowel water[16,17] by stimulating shear sensitive receptors on enteroendocrine cells[18]. Bran's major impact appears to occur in the colon, where, as we will discuss next, its arabinoxylan component is rapidly fermented.

1.1.2.1. Fermentation and the microbiota

Dietary fibre, as described before, is unable to be broken down by human digestive processes and enters the colon unaltered. Here it is exposed to a complex ecosystem of anaerobic and facultative anaerobes of over 400 bacterial species[19] which catabolise the fibre by partial or total fermentation into SCFAs, carbon dioxide, hydrogen, and energy[20]. SCFAs are actively absorbed and butyrate in particular is preferentially used by the epithelium as an energy source[21]. Hydrogen diffuses into the blood stream, where it is transported to the lungs and exhaled, or passed as flatus. It is also the

substrate for methane producing bacteria, which reduces luminal gas volume by half, and is also exhaled or excreted in flatus. As a result of fermentation, bacteria derive energy to replicate, consequently bacterial mass typically accounts for 30-60% of faecal dry weight[22–25].

The effect of the dietary fibre consumed will also depend on the condition of the microbiome it is mixing with. Long-term diets with little dietary fibre have been shown to result in diminished species richness[26], which is associated with several immune and metabolic disorders such as inflammatory bowel disease and colorectal cancer[27]. Reduced species richness is often associated with a marked distortion of the gut ecosystem, with an increased load of pro-inflammatory Enterobacteria and a reduction in Firmicutes, which are anti-inflammatory and butyrate-producing[26]. This diversity can be modulated by dietary interventions, shown in one trial of 3 months 16g/day inulin and oligofructose to increase *Faecalibacterium prausnitzii*, which exerts anti-inflammatory properties, and *Bifidobacterium,* which correlates with lower serum cholesterol levels[28].

1.1.2.2. Health benefits of fermentation

As well as providing energy for colonocytes, SCFAs exert beneficial immunoregulatory and gut-brain signalling roles. Butyrate and propionate act as histone deacetylase (HDAC) inhibitors, which induce cancer cell cycle arrest, differentiation and cell death. HDAC inhibitors are used extensively as anticancer therapies for diseases such as lymphoma and myloma[29]. They exert anti-inflammatory effects through suppression of pro-inflammatory macrophage signals, as well as regulating T cell cytokine expression[30]. Butyrate further benefits the immune system via G-protein coupled receptor (GPCR) signalling, modulating interleukins to attenuate the inflammatory response and promote gut barrier integrity. Receptors GPR41 and GPR43 have been identified in the colonic mucosa and it has been suggested this is a mechanism by which induce their physiological effects[31].

It is not just the immune system that benefits from colonic fermentation. Products such as acetate and propionate potently activate GPCRs to promote intestinal GLP-1 secretion, reducing blood sugar by stimulation of insulin release. Some enteroendocrine cells have GPR43 receptors, stimulated by propionate to release peptide YY (PYY) which inhibits gastric emptying and has anorectic effects[32]. Acetate also crosses the blood-brain barrier and reduces appetite via a central homeostatic mechanism[33], providing another mechanism to combat metabolic syndrome for conditions such as type 2 diabetes.

Supplementation with the prebiotic galacto-oligosaccharides to modulate the microbiota and its fermentation products have also been shown to reduce stress with a reduction in morning cortisol levels of healthy volunteers[34], consistent with anxiolytic effects of prebiotic microbiota manipulation in animal models[35].

1.1.3. Role of serotonin

Serotonin (5-hydroxytryptamine [5-HT]) has been known to play a key role in gastrointestinal peristalsis, secretion and visceral sensitivity since it was first demonstrated to initiate propulsive motility[36].

90% of serotonin in the gut is produced by enterochromaffin (EC) cells, located in the GI mucosa. Serotonin stimulates intestinal secretion and colonic motility[37], and changes in both EC cell counts and intracellular 5-HT concentrations have been demonstrated in both organic[38–40] and functional disease including IBS[39,41,42].

Tryptophan hydroxylase (TPH), of which there are two forms, is the first and rate limiting step of serotonin synthesis. TPH1 is found in the EC cells, whereas TPH2 is found in nerves; both hydroxylate dietary tryptophan to 5-hydroxytryptophan (5-HTP)[43,44], which is then converted to 5-HT by decarboxylation. Microvilli on EC cells project into the lumen and receptors expressed on their surface sense mechanical and chemical stimuli, including bacterial toxins[45], dietary amines and bile acids [45,46]. They respond by releasing serotonin, mostly basally but some into the lumen, acting predominantly on nerves in the lamina propria.

Studies with TPH1 knockout mice, who lack serotonin in enteroendocrine cells, have demonstrated mucosal serotonin's involvement in motility co-ordination, as in its absence faecal pellets were larger, propulsion was slower and mucosal tone varied[47].

The action of 5-HT depends on its interaction with its receptors; to date seven classes of 5-HT receptors have been described with numerous subtypes[48]. 5-HT_3 and 5-HT_4 receptors are primarily involved in the peristaltic reflex and intestinal secretion, and have thus received the most attention from gastroenterologists. Serotonin acts on adjacent cells[49] and receptors on intrinsic primary afferent neurons in the submucosa to modulate motility and secretion locally. It also communicates with the central nervous system via 5-

HT_3 and 5-HT_4 receptors on the primary afferent neurons of the splanchnic and vagal nerves.

Due to constant mucosal cell exfoliation and absence of synapses, the only mechanism to stop 5-HT's action once secreted is by re-uptake into the epithelial cells of the GI mucosa by the highly selective serotonin transporter (SERT)[50]. It is then metabolised to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase and excreted by the kidneys[51].

1.2. Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterised by abdominal pain, bloating and a change in bowel habit. There is large heterogeneity in symptoms and severity among the patient population, ranging from bothersome to severely incapacitating. The condition is managed predominantly in primary care, and referred to secondary care once initial management strategies have failed or the disease burden is too great for the patient.

Despite its high prevalence the underlying pathophysiology is complex, multifactorial and poorly understood. Subsequently there are many treatments with different therapeutic targets and varying efficacy; psychotherapy is used to target mental health co-morbid conditions that frequently co-exist; dietary modification allows patients to self-manage their condition; and pharmaceutical agents are aimed at managing the symptoms, with no curative intent.

The most debilitating subtype is the diarrhoea predominant form, as patients suffer from urgency and at times faecal incontinence, which severely impacts their quality of life.

1.2.1. Epidemiology

1.2.1.1. Prevalence

Due to population differences and criteria used to evaluate them, estimates of IBS prevalence vary from 5% to 20% of the general population[52–55] with a female predominance of approximately 2:1[56–58]. The Manning criteria (described in the diagnosis section in **Table 1**) tends to give higher estimates than the Rome criteria. Use of the Manning criteria with scores ≥2 in Europe estimates a prevalence of 21.6%[59], however when Rome II criteria are applied estimates fall to 2.9-8.1%[60,61]. Meta-analysis suggested an overall prevalence of 11.2%[52], with geographic variation; a recent study in 33 countries of 73,000 adults found the lowest prevalence in Singapore (1.3%) and highest in Egypt

(7.6%)[62].Using validated postal questionnaires only 30-50% of respondents with symptoms suggestive of IBS sought medical advice[59–61], indicating there is likely to be a large population of IBS sufferers who remain undiagnosed and self-manage.

There are no significant differences in gastrointestinal symptoms between those who see a physician and those who do not, but those who consult report greater levels of anxiety, higher pain scores, a greater impairment on their quality of life[63,64] and are more likely to suffer from IBS-D[65].

Prevalence of IBS subtypes have been variously reported having first being described in the Rome II criteria[66]. Talley *et al*. found distribution was found to be approximately equal in the USA across the 4 subtypes[67], however a survey of 40,000 Europeans found 63% fit the Rome II criteria for alternating IBS[60]. Meta-analysis of studies reporting all 4 subtypes shows a more even distribution (IBS-D 23.4%, IBS-C 22%, IBS-M 24.0% and IBS-U 22.2%)[52].

1.2.1.2. Incidence

Given the fluctuating nature, insidious onset and unpredictable course of symptoms researchers have had difficulty calculating the true incidence. Not all people suffering from symptoms of IBS will see their GP[59–61], so there is a discrepancy between onset of symptoms and date of diagnosis. An American study surveyed a cohort of 690 people on functional GI symptoms 1 year apart. Of the patients without an IBS diagnosis or symptoms in the first survey, 9% had developed symptoms consistent with IBS using the Manning criteria in the second survey; an incidence rate of 67 per 1,000 person-years[68]. Studies using IBS diagnosed by a physician give lower rates of around 2 per 1,000 person years[69–71]. A UK 10-year cohort study of almost 4,000 patients estimated the incidence of new IBS, using Manning criteria, at 1.5% per year[72].

1.2.1.3. Economic impact

Despite no impact on mortality[73], there is significant associated morbidity. Direct care costs have been estimated at £90-£316 per year and patients with IBS are twice as likely to take time off work than the general population[74]. Across 6 European countries mean annual cost to patients and healthcare systems for IBS-C was €1789[75].

Due to the chronic discomfort and unpredictability of bowel habit, optimum performance while present at work is also impaired, leading to costs relating to loss of productivity, roughly estimated at more than $200 million per year in the USA[76]. IBS also places a significant burden on healthcare as patients make twice as many healthcare visits as age-matched controls[77]. A full time GP in the UK will see on average 8 patients with IBS per week and will refer approximately 30% of patients with IBS to secondary care[58].

1.2.2. Diagnosis

There is no specific investigation for IBS, so it is diagnosed on clinical symptoms. The first descriptions of the irritable colon date back to the 19th and 20th centuries[78]. It was a diagnosis of exclusion once infection, inflammation and malignancy had been excluded, and often after several "unsuccessful surgeries"[79].

Manning et al. developed the first set of diagnostic criteria of symptoms in 1978[80] to positively diagnose the condition. This precluded the need for expensive and invasive investigations, easing the anxiety of both the patient and physician. The criteria were developed further by expert consensus to produce the Rome criteria in 1990[81]. Using stricter criteria than Manning *et al.*, it gave a greater likelihood ratio for a diagnosis of IBS[82]. The Rome criteria are now in its fourth iteration, which selects a more severe subgroup of those diagnosed with Rome III criteria[62,83,84]. Details of the different criteria are found in **Table 1**.

Rome IV divides IBS into subtypes by the predominant abnormal stool form; diarrhoea (IBS-D); constipation (IBS-C); mixed if at least a quarter of abnormal stools are diarrhoea and constipation (IBS-M); and unclassified (IBS-U) if the subject never or rarely has abnormal stools[85]. Despite the described criteria, the heterogeneity of patient symptoms and overlap with other disease processes makes diagnosing IBS a challenge for physicians, and some still believe it to be a diagnosis of exclusion[86].

Table 1 - Evolution of diagnostic criteria for irritable bowel syndrome

Criteria	**Method of defining IBS**	**Symptoms**	**Symptom duration required**
Manning[80]	3 positive symptoms	- Pain relieved by defecation - More frequent stools at onset of pain - Looser stools at onset of pain - Visible abdominal distension - Passage of mucus per rectum - Sense of incomplete evacuation	none
Rome I [81]	Abdominal pain or discomfort relieved with defecation or associated with a change in stool frequency or consistency, plus ≥2 associated symptoms on at least 25% of occasions or days	- Altered stool frequency - Altered stool form - Altered stool passage - Passage of mucus per rectum - Bloating or distension	≥ 3 months
Rome II [66]	Abdominal discomfort that has ≥2 associated symptoms	- Pain relieved with defecation - Onset of pain associated with a change of frequency of stool	≥12 weeks (need not be consecutive

		- Onset of pain associated with a change of form of stool	in the last year)
Rome III [87]	Recurrent abdominal pain or discomfort ≥3 days per month in the last 3 months associated with ≥2 associated symptoms	- Improvement with defecation - Onset associated with a change of frequency of stool - Onset associated with a change in form of stool	Symptom onset ≥6 months prior to diagnosis
Rome IV [88]	Recurrent abdominal pain on average at least 1 day/week in the last 3 months, associated with ≥2 of the symptoms	- Related to defecation - Associated with a change in frequency of stool - Associated with a change in form (appearance) of stool	Symptom onset ≥6 months prior to diagnosis

1.2.2.1. Differential diagnosis

Given the lack of specific diagnostic investigation for IBS, the possibility of a misdiagnosis is a cause of anxiety for patient and physician alike. The differential diagnoses for IBS-D include microscopic colitis, coeliac disease, giardiasis, lactose malabsorption, tropical sprue, small bowel bacterial overgrowth, bile acid diarrhoea and colon cancer[89].

1.2.2.2. Classification

IBS is subtyped by the Rome criteria by the predominant stool pattern of abnormal stools; diarrhoea-predominant (IBS-D) when Bristol stool form score (BSFS) is 6 or7, constipation predominant (IBS-C) when Bristol stool form score (BSFS) is 1 or 2 and 'mixed' (IBS-M) when more than a quarter of

abnormal stools are hard (1 or 2) and more than a quarter are loose (BSFS 6 or 7). 'Unclassifiable' describes patients who never or rarely have abnormal bowel movements[85].

An alternative classification in research is if a patient's symptoms develop after an infective episode (post-infection IBS [PI-IBS] and non-post-infective IBS [non-PI-IBS]). An episode of gastroenteritis in the previous year is the strongest predictor of developing IBS[90], with incidence ranging from 7% up to 31% of IBS patients[91–93]. More severe infections, such as acute *Escherichia coli* 0157:H7 and *Campylobacter jejuni,* lead to higher incidence rates of PI-IBS[93]. Microscopically, PI-IBS patients have increased intestinal permeability[94] and deregulation of tight junction formation[95], though these abnormalities are also seen in IBS-D without a history of prior infection. The clinical features of PI-IBS resemble those of IBS-D in 2/3^{rds} of cases[96] with loose, frequent stools and accelerated colonic transit time[97].

1.3. Pathophysiology of IBS

Given the heterogeneity of symptoms and their severity it is not surprising that the pathology is multifactorial, with the burden of each pathological component varying in each patient. In this section the current theories on the pathophysiology of IBS will be discussed, however I will focus on areas relevant to the work in this thesis – namely fibre intake, microbiota and serotonin.

The majority of abdominal IBS symptoms are driven principally by changes in gut motility and an increased perception and upregulation of sensations from the gut - termed visceral hypersensitivity[98]. Many factors, such as genetics, mucosal inflammation, microbiota and host immune responses impact on both these elements.

1.3.1. Altered motility

Although it has been suggested since the 1950s that motility abnormalities exist in patients with IBS[99], early methodological constraints limited investigations to radiographically evaluated transit times and rectal tone via barostat assessment. Colonic motility controls not only the passage of contents aborally, but also the mixing of chyme with the inherent microbiota and then moving it to a mucosal surface for absorption.

Colonic transit time is a gross score to assess time for contents to move through the gut with no ability to assess the functional status of the colon. It has been shown to be prolonged in only approximately 25% of patients with IBS-C[100,101] and accelerated in up to 45% of IBS-D patients[101,102], indicating a more complicated pathophysiology than just time in the lumen. Some authors have argued that it is the gut's exaggerated response to psychological stressors[103] or eating[104] which is altered in IBS. Others have shown an altered response to luminal distension[105] compared to healthy controls, however impaired motility itself may be a cause for luminal distension, by increasing

fermentation time prior to defecation or failure to adequately expel the products of fermentation.

Colonic tone is the state of contraction of the circular smooth muscle in the colon, often measured at rest and after a meal in the rectum via a barostat. Rectal compliance can also be calculated from the slope of the volume versus pressure curve and is defined as the ability to lower tone in order to accommodate increased volumes. It has been postulated compliance and tone are abnormal in IBS, contributing to the painful stimuli and bloating. The sensation of bloating without physical abdominal distension was found to be associated with visceral hypersensitivity, while bloating with distension was not, suggesting the pathogenesis of bloating and abdominal distension are not the same[106].

Another feature of motility which needs to be considered are the patterns of bowel wall contractions found in the colon. Motor patterns are required for mixing of luminal contents to expose the digesta to the microbiota and aid fermentation and also to transport absorbable nutrients to the mucosa. Despite the vital role of the motor patterns for gut function, little is known about them both in health or disease. Early investigations with manometry pressure sensors 10cm apart found no difference between IBS and healthy volunteers[107]. More recent high resolution manometry (HRM) with spatial resolution of 1cm may yet shed more light on this, however to date there are no published results of patients with IBS using HRM. It may be that with increased sensitivity more subtle motor complexes exist or behave differently to healthy controls, and patterns previously labelled as normal have in fact been mislabelled or missed completely[108].

1.3.2. Visceral hypersensitivity

Visceral hypersensitivity is a heightened sensation in response to physiological stimuli, such that normal motility or distension is perceived as pain or discomfort[98]. Colonic distension may occur as a result of increased

stool mass due to bacterial growth or water retention, or due to fermentation producing gas. The hypersensitivity itself is multifactorial, as modulation of sensory information occurs in both the peripheral and central nervous systems (CNS). Following initial studies of pain responses to endoscopically sited balloons[109] and gas inflation[110], it is widely accepted that hypersensitivity to luminal distension is a common finding in patients with IBS[111,112]. Although its prevalence is increased in patients with IBS[113,114], it is not ubiquitous and as such cannot be utilised as a biological marker.

The exact cause and mechanisms of visceral hypersensitivity in IBS are unknown, and detailed analysis is beyond the scope of this thesis. Studies have shown differences suggesting possible mechanisms from mucosa to cortex, including activated mast cells sensitising primary afferent nerves[115], and hypersensitive splanchnic lumber afferents in the periphery[116].

The CNS also plays a role in the modulation of sensations. Induced stress[117] and hypervigilance[118] have been shown to reduce sensory thresholds, while other studies have shown psychological stress can have a distractor effect, raising sensory thresholds[119]. Greater understanding of the sensory dysregulation in IBS has been achieved with the use of functional MRI (fMRI) demonstrating abnormal efferent sensory pathways[120] and increased activation of regions associated with emotional arousal and pain modulation[121].

1.3.3. Factors impacting motility and hypersensitivity

1.3.3.1. Diet

Most patients with IBS can identify certain foods which trigger their IBS symptoms[122,123], and the onset of symptoms can occur as rapidly as 15 minutes after ingestion[124]. There are several possible mechanisms, which include immune and mast cell activation (as will be discussed later in the chapter)[125], chemosensory activation[126,127], and mechanoreceptor activation

due to luminal distention associated with visceral hypersensitivity and altered motility.

Recent work by Fritscher-Ravens *et al.*[128] in IBS patients with atopy and postprandial symptoms used confocal laser endomicroscopy to examine response to intraduodenal infusion of certain foods. They showed microscopic evidence of nonclassical food allergy in 50-60% of this cohort, reacting to wheat, milk, soy or yeast despite no response to classical skin-prick test and normal serum immunoglobulin E. Notably they showed an increase in mucosal permeability and eosinophil density which can lead to immune activation as a mechanism for visceral hypersensitivity. One possible mechanism is thought to be activation of TLR-4 on monocytes, macrophages and dendritic cells by wheat antitrypsin inhibitors, leading to immune activation and cytokine production[129].

The mechanism with the most evidence and one which work in this thesis will focus upon is that of mechanoreceptor activation. In the presence of visceral hypersensitivity and/or abnormal gut motility, ingestion of specific foods can cause gas production and luminal distension felt as bloating, abdominal pain and alterations to bowel habit. These foods are short chain carbohydrates, collectively called FODMAPs (fermentable, oligosaccharides, disaccharides, monosaccharides and polyols), which are poorly absorbed in the small bowel. Fructose and polyols are small osmotically active molecules which increase luminal osmolality thereby increasing water content and distending the small bowel and subsequent ascending colon, causing IBS symptoms[130]. Shepherd *et al.* demonstrated in a randomised placebo controlled 4 –way cross over study that in patients who had previously benefitted from a low FODMAP diet, fructose and fructans challenges induced >75% of patients to report a worsening of their IBS symptoms versus 14% after control glucose challenge[131].

Indigestible oligosaccharides and lactose fail to be absorbed in the small bowel and are delivered to the colon where they are rapidly fermented; this leads to hydrogen and methane production which overwhelms capacity of the

gut to absorb gas into the bloodstream for elimination through the lungs, causing luminal distention. Products of fermentation short chain fatty acids such as acetate, proprionate and butyrate may alter gut motility, although evidence for this is contradictory[132,133]. For each type of FODMAP there is significant mechanistic overlap of increasing luminal water and rapid fermentation, depending on their molecular size and absorption rates[134].

Although dietary exclusion of FODMAPs have been shown to improve symptoms [135] (as will be discussed later), routine dietary advice focusing on a balanced diet and regular exercise has been shown to be equally effective[136] – reinforcing the concept of multifaceted pathogenesis in IBS.

1.3.3.2. Microbiota

The intestinal microbiota includes trillions of bacteria, archaea, fungi, eukaryotes and viruses, outnumbering their host cell numbers ten to one. Despite their abundance, our understanding of the microbiota and its role in gut health remains in its infancy[137]. Alterations to the gut microbiota have been attributed to IBS pathogenesis with a number of potential mechanisms, either directly or by their products of fermentation: activating the local immune system, altering the epithelial permeability and intestinal secretion, modulation of the gut-brain axis, and luminal distention[138]. However the gut microbiome itself is a product of diet, host genetics, environment, and colonic transit[139], factors felt to be relevant to development of IBS symptoms so whether the link is cause or effect remains as yet unclear.

Advances in genetic sequencing using the *16S* rDNA gene[140] have allowed rapid and cheap analysis of stool bacterial composition. Consequently, there is now a wealth of research showing a disrupted biodiversity, richness and composition of gut microbiota - collectively termed dysbiosis – in IBS.

Meta-analysis of this work[141] shows a wide variability in results, however there are consistent findings of an increase in potentially harmful microbiota in patients with IBS, including phylum Proteobacteria, family

Enterobacteriaceae (phylum Proteobacteria), family *Lactobacillaceae* and genus *Bacteroides* (phylum Bacteroidetes). The *Enterobacteriaceae* family contains several pathogenic bacteria including Escherichia, Shigella, Campylobacter and Salmonella. The authors of the meta-analysis postulated the findings could relate to previous intestinal infections or due to a change in the intestinal environment. Potentially, inflammation or increased GI motility could allow for facultative anaerobes such as *Enterobacteriaceae* to establish in the colon where previously strict anaerobes resided. They also found a reduction in potentially protective species in IBS patients such as uncultured Clostridales I and genus *Bifidobacterium.* Although the exact protective mechanism is unclear, a similar species *Faecalibacterium prausnitzii* is considered anti-inflammatory and butyrate-producing, maintaining gut mucosal health.

A recent longitudinal study[142] of 77 patients with either IBS-C or IBS-D analysed data from the gut microbiome and metabolome as well as interplay with host physiologic measurements. As found in earlier work, a higher relative abundance of *Lactobacillaceae* was found in patients with severe IBS-D (with IBS symptom severity score (IBS-SSS) >300) compared to mild-moderate IBS-D. Microbiota diversity and variability over the course of the study was greater in IBS-C than healthy controls and IBS-D patients. When assessing the metabolome, SCFA production in IBS-C was significantly lower than healthy controls, despite no difference in dietary fibre intake.

The inconsistencies found across the different studies are due in part by methodological differences in obtaining and analysing samples, as well as the large heterogeneity in the IBS population. Factors such as age, gender and diet contribute significantly to the differing microbiota. Different diets change the quantities, relative proportions, rate of delivery and hydration of the fermentable substrate to the colon, and will thus alter the colonic microbiota by selecting the bacteria which most readily derives energy from the substrate. One study found a Western diet, high in fat and sugar, led to a decrease in microbial diversity compared to a diet high in fibre, found in rural

African communities[143]. As discussed above, patients with IBS often have a significantly different diet to healthy controls, thus it is not clear if changes to the microbiota are a primary or secondary phenomenon in IBS.

1.3.3.3. Serotonin dysregulation

Serotonin is a key signalling chemical in the brain, and altered levels contribute to disorders such as depression, anxiety and obsessive-compulsive disorders. Many of these conditions are successfully treated with 5-HT modulators such as selective serotonin receptor inhibitors (SSRIs). As discussed earlier, serotonin plays a significant role in the co-ordination of gut motility and secretion, and in IBS, disorders such as depression and anxiety are common co-morbidities. This suggests serotonin dysregulation, in both the brain and the gut, is a factor in the pathophysiology of IBS too.

Polymorphisms of the rate limiting step in serotonin production, TPH, have been extensively studied in psychiatric illnesses in which 5-HT has been implicated in its pathophysiology[144–146]. The first study to assess if there was an association between TPH polymorphism and IBS found a non-significant trend of association between single nucleotide polymorphisms (SNPs) and daily reporting of GI symptoms in IBS Caucasian women[147]. Others demonstrated a higher, although still non-significant, TPH1 expression in IBS-D patients compared with controls[148]. In mice without TPH1, no mucosal reflexes were present with rare propagation of motor complexes, and peristalsis relied on stretch reflexes alone[47]. This study highlights that although 5-HT is important for normal colonic motility, other compensatory mechanisms are in place to ensure the bowel's function isn't solely reliant on this one neurotransmitter.

In IBS-C, where colonic transit is often reduced, plasma 5-HT concentrations are reduced[41,149] and 5-HT_4 agonists are successfully used to loosen stool consistency[150,151]. Conversely studies have shown greater 5-HT in IBS-D[152,153] and used 5-HT_3 receptor antagonists to firm stool consistency[154].

Activity of serotonin is controlled not only by synthesis but also by reuptake via SERT. The 5-HT transporter gene-linked polymorphic region (5-HTTLPR) has long (*l*) and short (*s*) allelic variants which may impact on the function of SERT. The *s* allele is thought to reduce the activity of the promoter region, reducing uptake of 5-HT and thereby increasing exposure of the mucosa to 5-HT[155]. 5-HTTLPR *l/s* or *s/s* genotypes have been reported more commonly in IBS-D patients than controls[156], however no association has also been shown[157–159]. Reports of altered responsiveness to the 5-HT_3 receptor antagonist, Alosetron[158] have not been confirmed in a larger study with ondansetron[154].

Polymorphisms in the TPH1 genes have been inconsistently associated with IBS patients. Jun *et al.* reported associations between single nuclear polymorphisms (SNPs) rs4537731 and rs211105 and diarrhoea and bloating symptoms in IBS patients, but no association with the diagnosis of IBS[160].

1.3.3.4. Genetics

Several studies have shown a propensity for IBS in families[161–163], indeed one study found the relative risk of IBS is doubled if a subject has a relative with IBS[161]. Similarly, patients with IBS are more likely (33.9%) than controls (12.6%) to have a family history of IBS[162]. Twin studies have sought to further assess the genetic component of IBS[164–167], and confirmed a significant genetic component in IBS. In an American study of over 6000 twin pairs, concordance for IBS was significantly greater in monozygotic (17.2%) than dizygotic (8.4%) twins. However a British study of 1870 twin pairs found no difference between monozygotic and dizygotic twins[168].

Single genetic mutations with powerful effects have been identified in a small subgroup of patients which appears to be driving their symptoms. A loss of function mutation of the *SCN5A* voltage gated sodium channel leading to increased abdominal pain was seen in a pilot study[169] then found in up to 2% of IBS patients in a genome-wide association study of 584 IBS patients and

1380 healthy controls[170]. This was treated with the anti-arrhythmic mexiletine, normalising bowel habit and shows promise for curative treatment for a small proportion of IBS patients.

The gene encoding tumour necrosis factor superfamily 15 (TNFSF15) was found to be associated with IBS[171] and confirmed in UK cohorts[172], however in a genome-wide association study no significant association was found[173]. More than 60 genes have been proposed to predispose to IBS, however they are yet to be validated[174] as most genetic association studies in IBS are significantly underpowered. The largest genome-wide study contained only 5500 individuals with IBS, compared with over 30,000 in inflammatory bowel disease studies[175,176]. Given experience in IBD where most SNPs give relative risks of 1.2-1.5 it is likely that adequately powered studies in IBS will need orders of magnitude larger numbers (>10,000) than most previous studies which were very underpowered and hence inconsistent.

More recently, variants in the sucrase-isomaltase gene have been shown to lead to defective or reduced enzyme activity and predispose to IBS[177]. As will be discussed later, this has implications in treatment strategies for this subgroup of IBS patients.

1.3.3.5. Low grade inflammation

A complex organisation of immune cells are closely associated with the intestinal mucosa to protect from pathogens, and it has been hypothesised that derangements in this system lead to the symptoms of IBS. Several studies have shown low grade inflammation in the colonic mucosa of IBS patients, suggesting it plays a role in the pathophysiology[178,179], however some results are contradictory[180].

In patients whose IBS isn't triggered by an infection (non-PI-IBS), findings of inflammation have been inconsistent. Piche *et al.* found significantly greater mast cells in the colonic mucosa which correlated with severity of fatigue[181], however El-Salhy *et al.* found no significant differences in colonic mucosa mast cell and leucocyte density between non-PI-IBS and healthy controls[182]. Meta-analysis of colonic immune cells in IBS found an increase in mast cells and $CD3^+$ T cells in biopsies of IBS patients[183]. Mast cells release mediators including serine proteases which could evoke neuronal hyperexcitability. This idea is supported by the correlation noted between the number of mast cells < 5 µm from enteric nerves with severity of abdominal pain in patients with IBS[115]. In a cross-section of IBS subtypes, increased intestinal permeability[184], increase in pro-inflammatory cytokines[185] and reduction in anti-inflammatory cytokines[186] have been found and postulated as potential mechanisms.

Evidence is more compelling for the role of low grade inflammation in PI-IBS[91,187]. Increased leukocytes have been found repeatedly throughout the colon in PI-IBS[96,97,188]; greater numbers of the pro-inflammatory cytokine interleukin 1β was demonstrated in those with IBS symptoms versus asymptomatic patients after gastroenteritis[187]; and raised rectal mucosal $CD3^+$ and $CD8^+$ lymphocytes have been shown following *Campylobacter jejuni* infection[189] and in those who develop PI-IBS when compared with infected individuals who recover completely[96]. The inconsistency of results may result differing study methodologies, limited patient numbers and the subtle differences in subject populations.

1.3.3.6. Immune response

The role of the immune system reacting to food allergens in food intolerances remains debated. Immunoglobulin (Ig) E-mediated antibodies to food allergens are yet to be proven in the pathogenesis of IBS, however there is a small increase in incidence of atopy in the IBS population[190]. IgG-mediated immune reactions have also been proposed[191] but have not yet been replicated[192].

As discussed earlier, a study in 108 IBS patients with IgE-negative food allergies showed microscopic mucosal reactions to endoscopically delivered food challenges[128]. Confocal laser endomicroscopy was used to identify increases in intraepithelial lymphocytes and increased mucosal permeability, which then leads to local inflammation causing symptoms as described above.

1.3.4. Altered gut-brain interactions

Psychosocial factors are important in IBS and often co-exist. One study demonstrated 17% of patients with IBS also suffer from major depression and 32% from generalised anxiety disorder[193]. Abuse in childhood or adulthood is associated with IBS[194] and for some it is considered that IBS forms a somatisation disorder[195]. However in the patients without co-morbid psychological disturbances this theory is unlikely[196].

As well as modulating afferent stimuli such as in visceral hypersensitivity, the efferents from the CNS communicate with the gut, affecting secretion, motility and blood flow. Central mood states such as anxiety and stress have been shown to accelerate intestinal transit[197] and response to visceral stimuli.

Functional magnetic resonance imaging (fMRI) studies have allowed for a greater understanding of this pathway, and have demonstrated IBS patients have increased regional blood flow in response to rectal balloon distention compared to controls and IBD patients[198]. Amitriptyline, an anti-depressant,

has been shown to reduce rectal pain and was correlated with reduced cortical blood flow on fMRI[199].

1.3.5. Summary

Motility and hypersensitivity drive abdominal symptoms in IBS, with many different factors underlying these changes. As more pathophysiological specifics are discovered, further subtyping of IBS can occur and potential treatment targets identified and current treatments, as will be discussed next, can be better utilised.

1.4. Treatments for IBS

As discussed above, there are a large number of interconnecting factors in the pathophysiology of IBS, and consequently there are multiple treatments of differing mechanisms. This section will discuss potential treatments but will focus on those relevant to work in this thesis, namely diet manipulation, especially fibre supplementation, and serotonin receptor antagonists.

1.4.1. Diet

Many patients with IBS believe that diet plays an important role in their IBS[200], which leads to food avoidance behaviour, and is associated with a reduced quality of life[122]. Consequently significant food groups are excluded without advice from dieticians, which can lead to nutritional deficiencies[201].

In a cohort of patients who expressed symptoms after eating certain meals, Fritscher-Ravens *et al.* identified patients who reacted to endoscopically delivered food antigens. Removal of that trigger resulted in total relief of IBS symptoms 1 year after the intervention[202]. However this trial was open label and as the recruited patients believed their symptoms were food related, highly susceptible to a placebo effect.

While some foods trigger symptoms, others have been shown to provide relief. Supplementing the diet is one way to introduce foods beneficial to those with IBS. Supplements such as *Aloe vera* show only a modest efficacy[203], whereas peppermint oil has demonstrated effective relief of IBS symptoms, notably by the active ingredient's intestinal smooth muscle relaxation, leading to reduction in abdominal discomfort[204].

1.4.1.1. Probiotics

The gut microbiota can be supplemented directly by use of probiotics. Probiotics are "live microorganisms that, when administered in adequate amounts, confer health benefits to the host"[205]. Probiotics induce changes in the gut microbiota and their metabolites which may interact with the immune system as well as the nervous system. Thus they can infer benefits by competing with pathogen binding[206]; enhancing the mucosal barrier function[207]; reducing gas production[208]; improving mucosal response to stress[209]; inhibiting inflammatory responses such as stimulation of regulatory T cells[210]; and reducing visceral hypersensitivity by increased expression of μ-opioid receptors[211]. The relative importance of these mechanisms, which have often been demonstrated only in cell culture or laboratory models, is still debated.

Systematic review and meta-analysis of trials investigating probiotics demonstrated some specific probiotic combinations reduced the risk of persistent IBS symptoms (RR 0.79, 95% CI 0.68-0.91,*p*=0.001) and improved flatulence but not bloating or urgency[212]. There was however evidence of publication bias, potentially skewing results as negative studies remain unpublished. It remains unclear which strains, alone or in combination, at which dose or duration will benefit which subtype of IBS.

1.4.1.2. Dietary fibre

Supplementation of fibre is a traditional first line treatment for patients with constipation and thus is commonly used in IBS-C. The goal for constipation is to increase stool mass, soften stools and accelerate transit time. However in IBS-C these goals are conflicted by also trying to reduce abdominal pain and bloating, which can be exacerbated as a consequence of fibre fermentation, gas production and luminal distention. The use of fibre supplementation is further complicated by the wide variety in physicochemical properties of

different fibres impacting the gut differently – thus the advice to simply increase dietary fibre may not be specific enough for patients with IBS.

Psyllium, a soluble fibre, has been shown to increase stool water content and stool mass[213] by forming a viscous gel which is also relatively resistant to fermentation resulting in little gas production. Contrastingly, wheat bran, an insoluble fibre, accelerates colonic transit[214] and is rapidly fermented to increase bacterial biomass and subsequent stool mass[215].

In a direct study of psyllium or bran versus placebo in patients with undifferentiated IBS, a greater proportion of responders were found in the psyllium group than placebo for two of the three months, whereas wheat bran was more effective than placebo only in the third month. Notably, early withdrawal from the trial was most common in the wheat bran group due to worsening of IBS symptoms[216].

In a meta-analysis of fifteen studies and 1048 patients, fibre supplements (of all types) were statistically better than placebo in reducing global IBS symptoms (RR: 1.27). The relative risk of improving symptoms was marginally higher for soluble than insoluble fibres, 1.49 *versus* 1.27[217]. Bijkerk *et al.* reviewed eight studies of fibre supplementation specifically in IBS-C and demonstrated an improvement in symptoms for both soluble (RR 1.60) and insoluble fibres (RR 1.54)[218].

1.4.1.3. Low FODMAP diet

FODMAP intake, especially fructose, has increased over the past 30 years in Western diets as availability of fruits and concentrated fruit juices increases, coupled with the extensive use of partially hydrolysed fructose and glucose containing corn syrup in processed foods[219]. FODMAPs are short chain carbohydrates which are poorly digested in the small intestine, and consequently are rapidly fermented in the colon causing gas production and increasing intestinal osmolality. The low FODMAP diet was introduced to try and improve symptoms for patients with IBS, and works in three stages; firstly

to remove all FODMAPs in the diet to reduce abdominal symptoms; then re-introduce individual foods containing FODMAPs to identify specific triggers and individual FODMAP threshold levels; and finally develop a personalised diet which is less restrictive, excluding only the triggers[220]. Such a complex intervention requires close supervision with a dietician to be successful.

In recent years the low FODMAP diet has been demonstrated to be beneficial to IBS patients, and meta-analysis showed the restricted diet improved quality of life and IBS symptom severity[221]. However two high quality studies have identified no differences in improvement in symptoms with a modified NICE guidelines, in a mixed IBS population[136] and in IBS-D[222], with success rates of 40-50% for both diets. The modified NICE diet is simpler to follow and does not require dietician input.

In patients who do not respond to low FODMAP diets, it may be that other pathologies are driving their abdominal symptoms. Genetic mutations in disaccharidase enzymes such as sucrase-isomaltase lead to reduced catabolic activity and increased delivery of substrate to the colon, resulting in bloating, abdominal pain and diarrhoea[177]. They can be misdiagnosed as IBS-D[223] and do not respond to low FODMAP diets but require a different dietary strategy.

1.4.2. Serotonin modulators

As discussed earlier, serotonin is a key neurotransmitter in the gut and is a therapeutic target to modulate gut motility. 5-HT receptor 3 antagonists have been found to improve symptoms of IBS-D, and 5-HT receptor 4 agonists have improved symptoms in IBS-C.

1.4.2.1. 5-HT_3 receptor antagonists

5-HT_3 receptor antagonists (5HT3RAs) were developed as anti-emetics for treatment of chemotherapy induced nausea and vomiting[224] and were soon found to be constipating[225], particularly slowing left colonic transit[226].

Alosetron is a potent selective antagonist for 5-HT_3 receptors and was the first 5HT3RA to be introduced to the market. It has been shown in randomised control trials (RCTs) to be beneficial in the treatment of women[227–230],and men[231] with IBS-D. Reports of ischaemic colitis and serious complications of constipation after its introduction in 2000 lead to its voluntary suspension from the market. It was re-introduced in the USA in 2002 at a lower dose and a more restrictive indication of women with severe IBS-D who had failed to respond to other therapies[232].

Ramosetron is another 5HT3RA which has been used for prevention of nausea and vomiting in chemotherapy and postoperatively for many years. Meta-analysis of RCTs of ramosetron versus placebo use in IBS-D showed a relative risk of relief of overall IBS symptoms of 1.7 (95%CI 1.48-1.95)[233] for both males and females. Although constipation was more common than placebo, there were no reported incidences of serious side effects such as ischaemic colitis. It is currently licenced for use in Japan, Korea and Thailand.

In the UK, ondansetron is the most common 5HT3RA used for nausea and vomiting. It has been shown to harden stool consistency in both healthy volunteers and IBS-D patients[234], as well as slow colonic transit[235]. A trial involving 125 IBS-D patients compared ondansetron to placebo and found fewer days with urgency, lower urgency scores, and reduced frequency of defecation. However it had no impact on pain scores[154].

Meta-analysis of safety and efficacy of 5HT3RAs for the treatment of IBS-D reviewed 21 RCTs, demonstrating a pooled RR of global IBS symptom improvement versus placebo or mebeverine of 1.56 (95% CI 1.43, 1.71), improvement of abdominal pain versus placebo of 1.33 (95% CI 1.26, 1.39) and improvement of stool consistency of 1.63 (95% CI 1.33, 1.99). The RR of an adverse event with a 5HT3RA was 1.15 (95% CI 1.08, 1.22), which was predominantly constipation although there were nine cases of ischaemic colitis with alosetron.

1.4.2.2. 5-HT_4 receptor agonists

5-HT_4 receptor agonists stimulate motility and secretion through increasing acetylcholine release for excitatory motor neurones[236]. Tegaserod was initially shown to improve symptoms in IBS-C and chronic constipation[150], but was withdrawn after being associated with an increase in cardiovascular adverse events[237].

Prucalopride, a more selective 5-HT_4 receptor agonist with a safe pharmacological profile[237], is used predominantly in functional constipation with little evidence currently for IBS-C[238], however symptoms such as abdominal pain and bloating responded well with prucalopride in constipation trials[239].

1.4.3. Other treatments

Treatments important in the management of IBS but of less relevance to the work in this thesis, will now be mentioned for completeness.

1.4.3.1. Peppermint oil

Mint plants have been used to aid GI symptoms since ancient Egyptian times[240]. Peppermint oil decreases small bowel contractility[241] and when applied to the colonic mucosa inhibited colonic motor activity measured by manometry[242]. In IBS it has been shown to reduce overall GI symptoms, with a number needed to treat (NNT) with peppermint oil to improve global IBS symptoms by 12 weeks of 3[243], however long term efficacy is unknown.

1.4.3.2. Antispasmodics

Antispasmodics target abdominal pain, the main symptom of IBS, and are proven to be safe and effective (NNT = 5, number needed to harm (NNH) = 20). Hyoscine butylbromide has shown to significantly reduce the risk of

persistent symptoms after treatment[244], and a multicentre study in 427 IBS-D patients showed pinaverium reduced IBS symptoms after a 4 week period versus placebo[245]. Phloroglucinol, a non-specific antispasmodic works by directly inhibiting smooth muscle calcium channels and has the advantage of no anticholinergic side effects. In a recent 3-week trial of 72 patients with IBS-D, a significantly higher proportion of patients had at least moderate improvement in symptoms in the treatment arm than placebo (62% vs 30%)[246]. Although just failing to show differences in the primary endpoints, trends shown warrants further investigation with larger cohorts and more robust, FDA recommended endpoints.

1.4.3.3. Laxatives and motility accelerants

For patients with constipation, simple laxatives such as senna and docusate are often used to control symptoms and can be bought over the counter. Lactulose, an osmotic laxative, can cause bloating and so is poorly tolerated in IBS-C.

Linaclotide is a secretagogue which increases luminal secretion of chloride and bicarbonate ions, increasing intraluminal osmolality and resulting in softer stools. Meta-analysis of 3 clinical trials involving 2024 patients demonstrated an increase in bowel frequency and reduction in abdominal pain and bloating[247]. In mice, linaclotide stimulated cGMP release, which inhibited nociceptors, and in humans it reduced abdominal pain more than placebo[248].

Lubiprostone stimulates luminal fluid secretion by activating chloride channels, and has been shown to improve global IBS symptoms in IBS-C[249].

1.4.3.4. Antidiarrhoeals

Loperamide is a common over the counter medication used for symptomatic relief of diarrhoea; however evidence for its use in IBS is limited. In a double-

blind RCT of unselected IBS patients, loperamide improved stool consistency versus placebo (p<0.01) but failed to improve abdominal pain and worsened nocturnal abdominal pain[250].

Eluxadoline is a mixed μ- and κ-opioid receptor agonist and δ-opioid receptor antagonist which was approved by the National Institute for Clinical Excellence (NICE) for use as a second line agent in IBS-D patients in 2017[251,252]. Its exact mechanism of action is as yet unclear, however its low bioavailability suggests it is locally acting in the GI tract. Clinical studies have demonstrated an improvement in both abdominal pain and stool consistency compared to placebo (responder rate 29% vs 19%, p<0.001)[253], however side effects of pancreatitis and Sphincter of Oddi dysfunction exclude patients with a history of biliary tree pathology and alcohol abuse.

1.4.3.5. Antidepressants

Visceral hypersensitivity is thought to contribute to the chronic pain experienced in IBS. Modulation of neuropathic pain perception by antidepressants has shown to be effective[254]. Although tricyclic antidepressants (TCAs) and SSRIs have both been shown effective in relieving the pain and global symptoms of IBS[255] the consensus from multiple systematic reviews on TCAs are conflicting[256–259]. Consequently, guidance from the British Society of Gastroenterologists (BSG) advises cautious TCA use in IBS as a second line agent.

1.4.3.6. Rifaxamin

Rifaxamin, a non-systemic broad-spectrum antibiotic, has been studied in patients with non-constipating IBS to target gut microbiota dysbiosis. In a study of >1000 patients, a greater number of patients in the rifaximin group had adequate relief of global IBS symptoms (41% vs 32%, p<0.001) and adequate relief of bloating (40% vs 30%, p<0.001)[260]. In the IBS-D population,

rifaximin has been given and treatments repeated in those whose symptoms relapse[261]. Concerns persist however on the unknown side effects to long term use, specifically antibiotic resistance, antibiotic-induced diarrhoea and *Clostridium difficile* colitis[262]. Given the enormity and heterogeneity of the IBS-D population, rifaximin is an unlikely therapy suitable for all and may be further suited to an as yet undefined subgroup.

1.4.3.7. Faecal Microbiota Transplant

This new treatment aims to target the dysbiosis responsible for IBS symptoms. In the largest faecal microbiota transplant (FMT) trial to date 165 non-PI-IBS patients received placebo (own stool), 30g or 60g stool from a single donor. 3 months after treatment, a 50 point reduction in IBS-SSS was seen in 24% vs 77% ($p<0.0001$) vs 89% ($p<0.0001$) of patients[263]. Success depends on the quality of the donor stool, however long term data for such a treatment is still awaited, meta-analysis from RCTs does not suggest FMT benefits global IBS symptoms[264] and the use of a single donor is likely to be ineffective for a global condition.

1.4.3.8. Psychological treatment

The role of psychological therapies for functional bowel disorders remains controversial and their therapy goals are broad. Some aim to impact the enteric nervous system or maladaptive coping mechanisms, whereas other treatments focus on co-morbid anxiety, depression or somatisation disorders.

Meta-analysis of efficacy in IBS versus physician's "usual management" showed a relative risk of symptoms persisting in cognitive behavioural therapy of 0.6, relaxation therapy of 0.82, hypnotherapy of 0.48, and dynamic psychotherapy of 0.6 while self-administered CBT showed little effect on symptoms[265]. There was however significant heterogeneity between studies,

small sample numbers and evidence of publication bias, calling into question by the meta-analysis authors the validity of the findings.

1.4.4. Summary of treatments

The pathology of IBS is multifaceted and the burden for each mechanism of pathology varies between patients. It is therefore unwise to advise a set treatment that is suitable for all, and treatment should focus on the most troubling symptom for that patient. Basnayake *et al*. demonstrated the benefit of multidisciplinary care (including dieticians, psychiatrists, behavioural therapists and a gut focused hypnotherapist) in such patients, reducing the IBS-SSS by ≥50 points in 66% *versus* 38% if seen by a gastroenterologist alone[266].

1.5. Magnetic Resonance Imaging

Chapters two, three and five are based upon studies using magnetic resonance imaging (MRI) to evaluate responses in the gut to different challenges. The principals of MRI will be discussed briefly here, so that its application in this thesis can be understood. The different MRI parameters used in the thesis will next be explained, and studies validating their use described.

Magnetic resonance imaging (MRI) was first developed in 1976, with images of a human finger, and progressed to the first cross-sectional abdominal image of the co-creator Sir Peter Mansfield produced in Nottingham in 1978. It is now the imaging modality of choice for many clinical indications with MRI scanners operational in almost all hospitals in the UK.

The use of MRI for specialities such as neurology have been rapidly and broadly adopted, but for gastroenterology the application of MRI to assess GI function is still in its infancy. The lack of ionising radiation or radioactive agents further increases MRI's appeal, as repeated and prolonged scans are safe. MRI offers imaging in three dimensions with excellent resolution, especially in soft tissues, including the ability to image water and fat based liquids.

The scientific basis upon which MRI depends is the presence of hydrogen atoms in the human body, each containing a single proton and single electron. The protons are constantly "spinning" in random orientations, and possess a magnetic moment such that on application of a magnetic field they will line up either in the direction of the field or in the opposite direction. To align in the opposite direction of the field requires slightly more energy. Within the body the majority of atoms cancel each other out and are called paired atoms. Due to the mis-match of energy states there will be more protons aligning with the field (requiring less energy) and these are termed unpaired.

In a 1.5T magnetic field, there are approximately 9 unpaired protons per 2 million, which equates to 6 x 10^{15} unpaired protons per voxel (2 x 2 x 5mm) of water. These unpaired protons are 'seen' by the MRI.

When a radiofrequency (RF) pulse is applied to protons within a magnetic field, lower energy state protons gain energy and move to the higher energy state aligning in the opposite direction to the field. When the RF pulse stops, the protons return to their original orientation, and as they do so they release energy in the form of electromagnetic waves which can be detected by receiving coils placed next to the subject. It is the time taken to return to the original orientation which is used to infer properties of the tissues.

A second effect of the RF pulse is to bring into phase the spins of protons affected. Spin of the proton is an interaction between the dipole, the magnetic field and gravity. Proton spin will decay back out of congruity when the RF pulse is turned off, and interactions between adjacent protons determines the speed of this decay, providing another physico-chemical property of the material.

The two time constants, the time to recover the magnetic vector and time for phase alignment to decay, are referred to respectively as the longitudinal relaxation time (T1) and the transverse relaxation time (T2) respectively. They represent a material's composition, so if the T1 and T2 values of tissues of interest are known, a suitable MRI sequence can be devised to highlight that tissue in contrast to the surrounding tissues, as illustrated in **Figure 1**. Here the image on the right is heavily T2 weighted, so highlighting areas of free water while the image on the left is T1 weighted providing better anatomical detail.

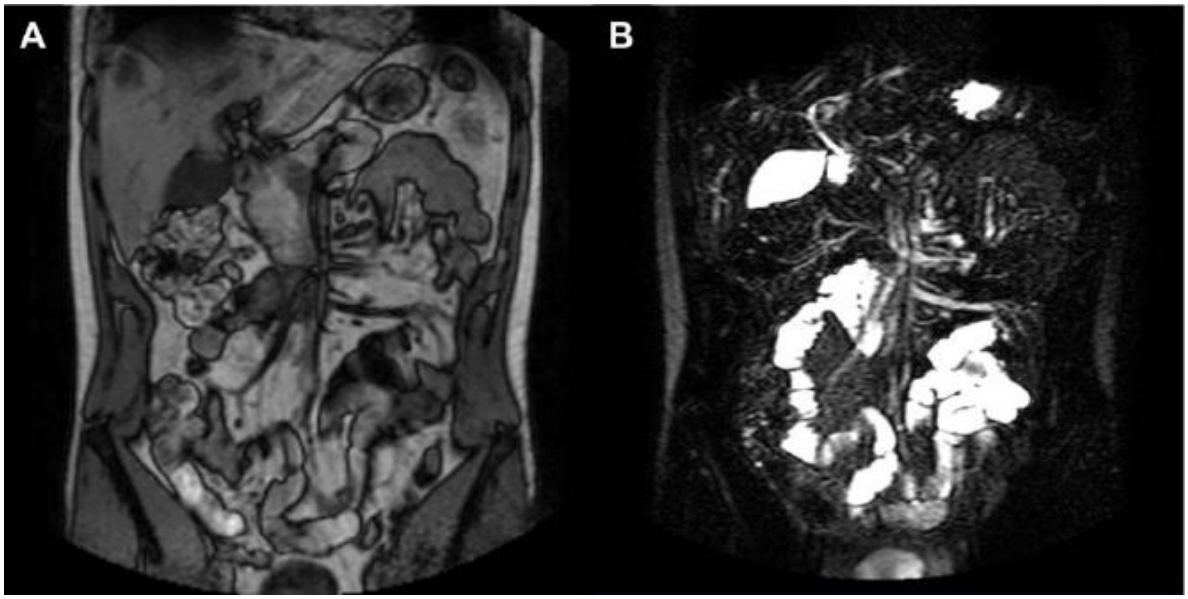


Figure 1 - MRI images of the same patient illustrating the different tissue enhancement of (A) T_1 weighted and (B) strongly T_2 weighted scan sequences

The key attraction in MRI's use clinically is the wide variation in T1 and T2 between water, soft tissue and hard tissues in the body. Other parameters such as the power of the RF pulse, degree to which the magnetic field is altered or the time between consecutive images can be varied to further highlight the desired are to be imaged.

Further discussion of MRI theory is beyond the scope of this thesis, however knowledge of the basic principles are advantageous when evaluating the experimental work described. The parameters measured were small bowel water content, colonic volume, colonic gas, T1 of the ascending colon and colonic transit. The parameters and how they are measured will be discussed next.

1.5.1. Small bowel water content

Water content of the small bowel was previously only able to be assessed using intubation studies, infusing a non-absorbable marker solution then subsequently aspirating intestinal fluid via a nasojejunal tube to assess dilution/ conentration of the marker[267]. These had significant limitations in

their ability to only assess non-viscous fluids, and so likely underestimated water content bound in more viscous digesta.

Work to measure intestinal water by MRI was first published by Hykin *et al.* in 1994[268], using a combination of operator-dependent image analysis to remove unwanted organs such as the gallbladder while setting an objective threshold to estimate water content in the remaining areas of interest.

This technique has been developed further and in 2007 Hoad *et al.* published a validation study measuring small bowel water non-invasively by MRI while instilling via a nasojejunal tube, boluses of a mannitol saline solution previously shown in perfusion studies to have no net water absorption[269]. The instilled volumes showed a mean difference from MRI volumes of just 2% with a standard deviation of 10%[270]. Using MRI the necessary image can be acquired in a single breath hold and the bowel doesn't require modification to "prepare" it such as cleansing or gas insufflation; consequently the results are much closer to the true physiological state and more tolerable than intubated studies.

The MRI sequence in this thesis uses a magnetic resonance cholangiopancreatography sequence. Originally designed to study the biliary tract, it is turbo spin-echo (TSE) sequence which is strongly T2 weighted which is sensitive to mobile water. The method assumes any voxel in the region of interest (ROI) with a signal above a given threshold contains free water. The threshold chosen must normalise for inter- and intra- subject variations due to patient and receiver coil positioning as well as scanner instabilities. Signal intensity from cerebral spinal fluid is used at each time point to set this threshold. Located centrally in the magnetic field, away from variable receiver characteristics at the edge of the field of view, and kept under tight physiological homeostasis and thus constant composition, it is the ideal standard.

Image analysis uses in-house software written in IDL® (Research Systems Inc, Boulder, Colorado, USA). The data set for the subject and time point is loaded,

and the operator selects the centre of the CSF visible in each slice. The programme uses the signal from the CSF to calculate an intensity threshold whereby every pixel with signal over that threshold is assumed to be free water. This technique has been previously validated using a process of optimisation to find the conversion from the CSF signal in each of the subject's MRI images, to the threshold level that, when applied to MR images to remove unwanted signal, gave the most accurate estimate of small bowel water against a known volume of infused fluid[270]. The operator manually draws around areas of small bowel to create an ROI for each slice (see **Figure 2**). Care is taken to avoid anatomy such as the gall bladder, stomach, large blood vessels and the large bowel, and an alternate contemporaneous MRI image is used to identify the anatomy if required. Once the ROI has been drawn for all slices the volume with signal intensity greater than the threshold is calculated. This volume is the small bowel water content, expressed in mm^3.

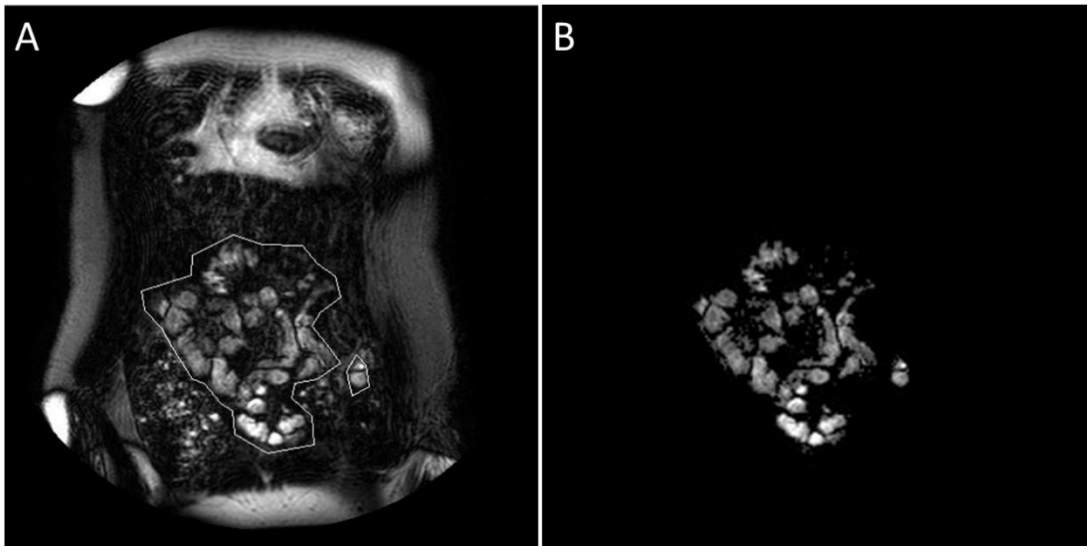


Figure 2 - Analysis of MRI images for small bowel water content. A) A region of interest (ROI) is drawn around the small bowel. B) Voxels not in the ROIs and under the water threshold are excluded, leaving the volume to be calculated

1.5.2. Colonic volume and gas

Assessment of colonic volume has previously been limited to cadaveric, intra-operative or CT and barium contrast studies – however each have limitations

due to bowel preparations and preservation techniques. Pritchard *et al.* were able to measure the volumes of the undisturbed colon from caecum to sigmoid flexure in both healthy volunteers and patients with IBS-D using MRI[271]. They found a normal range for fasted colonic volume from 75 healthy volunteers of (mean ± SD) 203 ± 75ml in the ascending colon, 198 ± 79ml in the transverse colon and 160 ± 86ml in the descending colon, which was not significantly different from 25 patients with IBS-D.

Using MRI, the sequences used generate a sharp contrast as the tissue changes from intraluminal to colonic wall, visible on the image as a sharp line around the colon. Image analysis software (Analyze9™ Mayo Foundation, Rochester, MN USA) was used in this thesis to allow the operator to draw around visible colon on each coronal slice. The slices are then combined to create a 3-dimensional object map from which volume can be derived.

Colonic gas is measured from the same colonic volume data using in-house software written in IDL. For each set of images for each time point the MRI analyst selects multiple index regions of interest of clearly defined free air. This is outside the subject's body just under the arms. These regions are used to provide a mean image intensity of gas which is used as a threshold. Areas within the colon regions of interest used to calculate volume are defined as gas when 5 or more adjoining voxels, equating to a volume of 0.1ml, has an intensity below the calculated threshold. This technique has been used successfully in several studies[130,272–275]. Murray *et al*. demonstrated in healthy volunteers mean fasting colonic gas is approximately 40mls, however 5 hours after consuming 40g inulin this increased by 100mls[276].

1.5.3. Whole gut transit time

Whole gut transit time (WGTT) is the time taken for food ingested to travel the entire length of the gastrointestinal tract. For the past 40 years the gold standard has been to use radio-opaque markers (ROM) ingested at set times prior to an abdominal radiograph[277,278]. MRI offers the advantage of no

ionising radiation, is non-invasive and images can often be achieved in a single breath hold. 5 capsules filled with gadolinium are ingested 24 hours prior to an MRI scan, and the WGTT is then calculated using the method described by Chaddock *et al.*[279] called weighted average position score (WAPS). Capsule positions are identified in the images then scored using **Figure 3.** Frequently, some capsules can be separated by several segments from the rest of the capsules in the colon. Owing to the low number of capsules, a weighing score was calculated to reduce the impact of the outliers, depending on the difference of a capsule to the median capsule score. For a difference of 0 and 1 the weighting factor was 1, for all differences larger than 1 the weighting factor was the inverse of the difference. A weighted mean position score of the MRI marker capsules was thus determined for each participant. A non-weighted least square fit was applied to the MRI marker capsule scores and their corresponding ROM transit scores to calculate a transit time in hours for the MRI marker capsules. This is based on the equation*:* $y = mx + c$, Where x is the average MRI marker position, m and c are unique coefficients determined from the validation study in which MRI transit was compared with simultaneously measured transit using the gold standard radio-opaque marker technique described by Metcalf *et al.*[280] (m = 0.03, c = -0.12), and y is the transit time in hours[279].

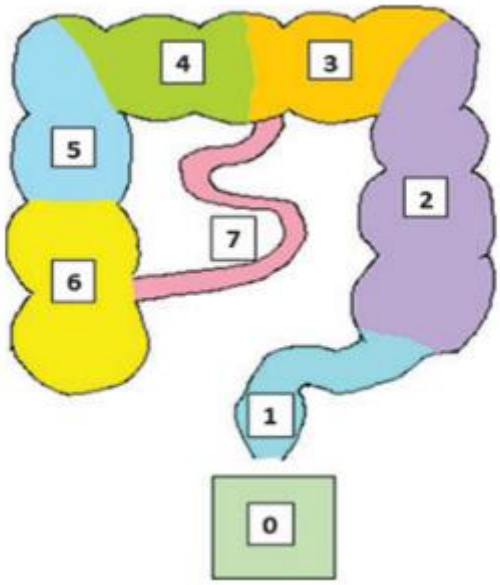


Figure 3 - Segmented colon used to score MRI markers for a weighted average position score (WAPS), reproduced from Chaddock et al[279].

In the MOLID study, whole gut transit was being assessed after therapeutic doses of lactulose in healthy individuals. In order to prevent total loss of marker capsules due to fast transit, the WAPS technique was modified such that the capsules were given 12 hours prior to imaging rather than 24 hours. As this modified technique has not been validated, the results are given as a rate in arbitrary units (a.u.).

1.5.4. T1 of the ascending colon

As described previously, the longitudinal relaxation time (T1) is a time constant for protons to return back to baseline alignment after excitation by a RF pulse. The T1 varies depending on the physical and chemical environment the protons are in via interactions with surrounding molecules, and in colonic chyme T1 will fall with a reduction in water, associated with a more solid chyme.

A single sagittal slice through the ascending colon is used with eight different inversion times (TI) ranging from 100 to 5000ms. Inversion times reflect water

proton mobility with cerebrospinal fluid having (at 1.5 T) a TI of the order of 2400 ms and liver tissue a TI of the order of 500 ms. For each time point, an ROI is drawn in the intraluminal ascending colon image. Using in house software created in IDL (see **Figure 4**) then processed using Matlab (Math-Works, Natick, MA, USA), data from the 8 corresponding images of varying inversion times are analysed to give a T1 in milliseconds. This process is repeated for two other ROIs drawn on the same image so that ROIs are at the top, middle and bottom of the ascending colon, and the average of the three T1s used. This process has used in several published works[213,281–284] and been validated by comparing T1 of descending colon content with the percent water assessed by freeze drying the next stool passed after the MRI scan. T1 correlated with percent water (r = 0.65, p<0 .001) so T1 can be used to non-invasively assess the water content of the chyme[213].

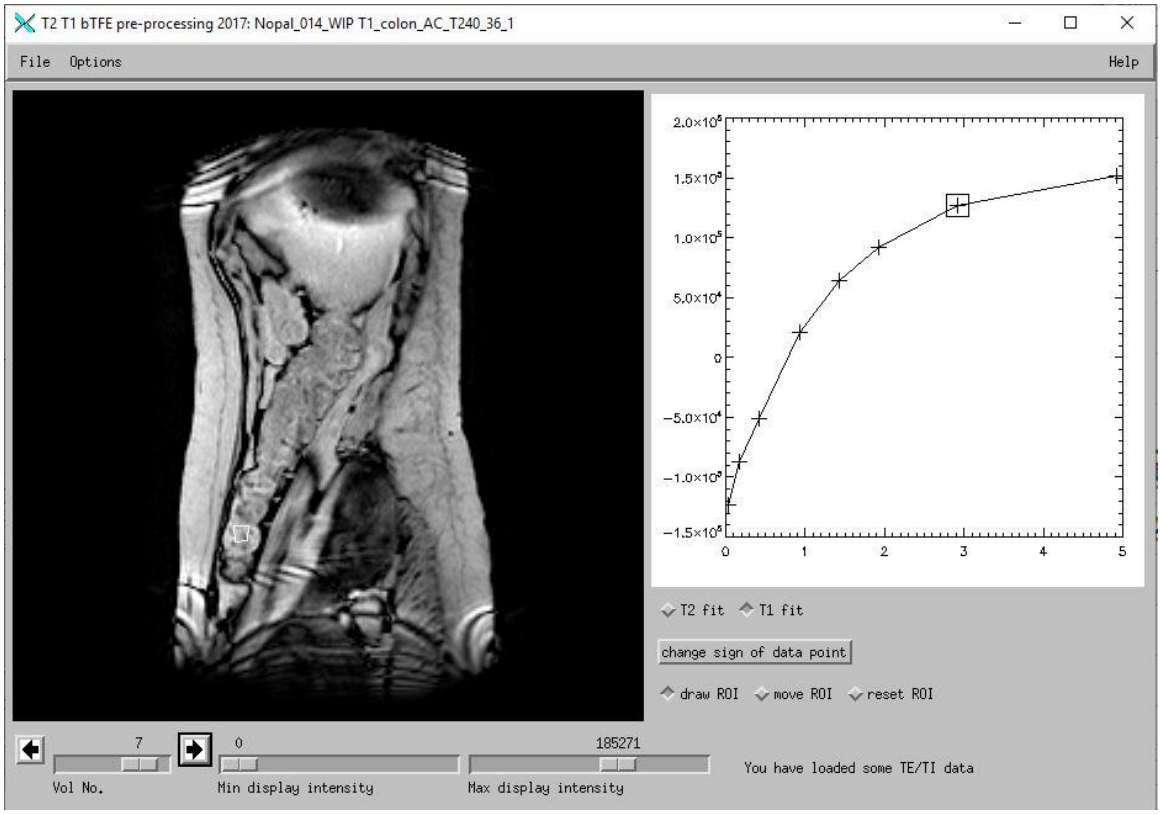


Figure 4 - Screenshot of T1 of the ascending colon analysis using IDL software.

1.5.5. Small bowel motility

Altered small bowel motility has been implicated in the pathology of many gastrointestinal disorders, and found using invasive techniques in many diseases[285,286] including IBS[287]. Due to the limitations accessing the small bowel, measurement is severely restricted, and the physiology is significantly altered by presence of the intraluminal equipment[288].

MR enterography techniques have been developed to assess small bowel pathologies such as Crohn's disease, however this requires dilated small bowel loops to clearly visualise the bowel wall, and often uses mannitol solution[289]. While a useful tool to assess bowel wall lesions and propulsive movements, the motility in a grossly dilated small bowel is clearly not physiological and so a new technique has been developed to measure an unprepared, fasted bowel. This technique is used in chapter four of this thesis, and shall now be described.

Participants in the MRI machine were instructed to remain still for a period of 5 minutes, only breathing shallowly. Dynamic volume cine loops were used to depict small bowel motility. The data was then processed using GIQuant™ (Motilent, Ford, UK), using an algorithm to correct for respiratory motion[290]. It then applied non-linear optic flow registration[291] to the image to correct for local deformation caused by bowel wall motion and luminal flow. It is this degree to which the image is deformed relative to the previous image (chronologically) which gives us the metric as an index for motility.

The data output from image registration is further analysed using in-house Matlab-based software (Math-Works, Natick, MA, USA). An ROI was drawn over the visible small bowel of each coronal slice of each time point. Solid organs, the large bowel, stomach, major blood vessels and mesentery were excluded. For each pixel in the ROI, a power spectrum of the intensity changes across the time series (smoothed using a running average of 5 pixels to reduce noise) was calculated and then summed across all frequencies. This metric is termed the total power, and reflects bowel motility, both in terms of

segmental oscillations and bolus movement of contents. A larger total power motility index represents higher small bowel motility.

This process relies on the analyst drawing an ROI around areas of small bowel, avoiding other intra-abdominal viscera and vessels. Inter-observer variability between an experienced and inexperienced MRI data analyst, measured using the Intra-class correlation coefficient, was 0.979, $p<0.0001$. Intra-observer variability was >0.96, $p<0.0001$[292].

Evaluation of this technique has been achieved by assessing expected response to stimuli. Khalaf *et al.* demonstrated an increase from subjects' fasting to initial postprandial AUC_{power} motility of 122% ± 98.7% (n=20)[292]. Using pharmacological provocation, Menys *et al.* demonstrated the analysis is sensitive to changes in motility stimulated by 0.5mg IV neostigmine ($p<0.001$) and suppressed by 20mg IV butylscopolamine ($p<0.001$; n=30)[293].

1.6. Breath hydrogen analysis

Studies in chapters two and three use the measurement of breath hydrogen to indirectly quantify colonic fermentation. Gas concentrations were measured by chromatography on a GastroCH4ECK (Bedfont, UK). Study participants were asked to take a deep inspiration, hold their breath for 15 seconds, and then exhale fully into the mouthpiece attached to the machine to achieve an end expiratory breath sample. Their use in clinical practice and rationale of such a technique will now be described.

Breath tests (BT) are commonly used in clinical practice to aid diagnosis for a wide range of GI conditions, including small intestinal bacterial overgrowth (SIBO) and carbohydrate maldigestion and dysfunction. They measure gases produced in the small and large intestine, which diffuse into the circulation and are expired through the lungs. Intestinal gas principally comprises of hydrogen, carbon dioxide and methane, with lower concentrations of oxygen, nitrogen, hydrogen sulphide and ammonia[294]. Intestinal gas is either swallowed air or air mixed in food, due to chemical reactions in the gut, diffusion of gases from the blood stream or a product of microbial metabolism[295]. Significantly to the work in this thesis, hydrogen and methane are exclusively produced by the microbiota due to fermentation in the gut.

Carbohydrate malabsorption or intolerance syndromes which may result from a deficiency in the relevant catabolic enzyme can be diagnosed with a breath test. Lactose and fructose are commonly tested, and if undigested or absorbed are delivered to the colonic microbiota and fermented, leading to an increase in breath hydrogen as well as symptoms of gas, bloating and abdominal pain.

Despite its increased use in research, a systematic review of 13 BT studies to diagnose SIBO found no consistency in methodologies to either conduct the studies or interpret the results[296], and in 2017 a North American consensus was published to establish necessary standards and reduce heterogeneity between studies. They instituted guidance for patients prior to BTs (including

avoidance of antibiotics and motility agents, complex carbohydrates, smoking and exercise), clinical indications for the test (diagnosis of SIBO, methane assessment in constipation, carbohydrate malabsorption), adequate dosing and duration of the tests, and their interpretation[297].

The consensus advocated for incorporated measurement of methane and hydrogen in breath tests. The majority of methanogenic species utilise 4 mols of hydrogen and 1 mol of carbon dioxide for every 1 mol of methane[298], therefore potentially impacting hydrogen measurement[299]. Unfortunately due to equipment failure, methane results were unavailable for work in this thesis.

1.7. Summary and application to this thesis

Irritable bowel syndrome is a prevalent condition which is frequently self-managed, yet when physician assistance is required, treatments available often fail to satisfactorily reduce symptom burden. Due to the numerous pathophysiological mechanisms which manifest as IBS, treatments which work well for one may be ineffective for another.

In primary care, dietary modification is often the first step. For those with IBS-C and IBS-M, an increase in fibre intake is often advised. This is often unhelpfully vague, as patients with IBS commonly suffer from bloating and flatulence with subsequent pain as a consequence of colonic fermentation, and an obvious fibre such as wheat bran is poorly tolerated[216]. Knowledge of different fibre types is not mainstream, thus without specialist input self-managing in this way is likely to be futile. The work in chapter 2 aims to further increase the knowledge on mechanism of action of three different fibres in order to better tailor advice to patients. The work also seeks to gain insight into the mechanism of action of nopal, a readily available fibre indigenous to countries with warmer climates which would otherwise have to import fibre supplements.

For patients with IBS, severely limiting diets can lead to deficiencies in both micronutrients and probiotics for the colonic microbiota, with consequent potential harm. The work in chapter three aims to combine two different types of fibre so that probiotics of inulin, a common food additive, can still be delivered to the microbiome, but by co-ingesting with the viscous psyllium there may be a reduction in rate of fermentation and thus moderation of symptoms.

Patients suffering from diarrhoea predominant IBS tend to be referred to secondary care for further investigation to exclude differential diagnoses, and are more likely to take pharmacological treatments to improve symptoms. Serotonin plays a significant role in gut motility, demonstrated by receptor agonism and antagonism benefitting constipation and diarrhoeal subtypes,

respectively. The 5HT3RA ondansetron is used to treat IBS-D, however there is a wide range in its efficacy. Chapter four further analyses a clinical trial of patients with IBS-D being treated with ondansetron, to try and identify clinical features or biomarkers of those most likely to benefit from the drug. Despite our knowledge of ondansetron's action on the $5HT_3$ receptor, we do not know where in the gut it acts or its impact on small bowel and colonic water content. Chapter five uses MRI to assess the impact of ondansetron in lactulose-induced diarrhoea in healthy individuals as a model of IBS-D.

2. A pilot study of the effect of wheat bran, psyllium and nopal on intestinal water content using Magnetic Resonance Imaging (NOPAL)

This chapter describes the NOPAL study designed to assess the impact of three fibres with differing physico-chemical properties on colonic fermentation, assessed *in vivo* using MRI and *in vitro* in a model of colonic fermentation. It will discuss the fibres chosen, methods and results of the study, and the implications of the findings. The work was published in the American Journal of Clinical Nutrition in July 2020[282].

2.1. Background

The underlying physico-chemical and functional properties of dietary fibres vary widely. Gel forming fibres such as psyllium have evolved as mucilage plant polymers with extremely high water-holding capacity which despite their large molecular weight (in excess of 1MDa) are easily able to hydrate. Such fibres form highly viscous solutions and gels when dissolved in water. In contrast, some fibres such as wheat bran, which has a large particle size (>100 μM), have very limited solubility and do not form a gel nor contribute significantly to viscosity in the bowel[13].

Clinical evidence shows that some viscous, gel-forming fibres (e.g. psyllium) benefit patients with irritable bowel syndrome whereas fibre from different sources (e.g. bran) may worsen symptoms[218], suggesting that the differing physico-chemical properties impact on mode of action in the gut, although this has to date not been studied in detail.

Psyllium fibre is a hemicellulose rich mucilage comprising highly branched arabinoxylan, composed of a xylan polymer densely decorated with arabinose and xylose sidechains[300]. Although this is poorly fermented it facilitates water-holding in the small bowel causing an increase in both small bowel and colonic water content as well as colonic volume assessed by magnetic resonance imaging (MRI)[213]. Constipated patients have lower colonic water content as measured using T1, MR the relaxation time constant, whose value correlates with stool water. This can be normalised by therapeutic doses of psyllium (7g three times daily)[213], which are widely used to treat constipation.

The main fibre component of wheat bran is also an arabinoxylan, which comprises the majority of the cell wall in wheat but, unlike the arabinoxylan in psyllium, is highly fermentable[301]. Wheat bran acts as a substrate for the colonic microbiota and is fermented to produce significant amounts of short-chain fatty acids (SCFA)[302,303]. Several studies have shown that wheat bran also accelerates oro-caecal transit[17] and increases small bowel water content (SBWC)[16].

Nopal fibre is an extract from the nopal cactus *Opuntia ficus-indica* (see **Figure 5**). In contrast with psyllium, nopal fibre is primarily a pectic mucilage comprising a complex mixture of galacturonan, rhamnogalactans and rhamnogalacturonans as well as arabinoxylans, which are gel forming[304–306] but readily fermentable. Nopal has been traditionally used as a laxative in North Africa and Mexico[307] but its effects on human gut microbiota and function have yet to be examined.



Figure 5 – Nopal cactus (Opuntia ficus-indica) grown in Mexico

The three fibres used in this study, with the contrasting physico-chemical properties described above, can be expected to be associated with different physiological behaviour in the gastrointestinal tract but these have not previously been directly compared in humans.

The aims of the study were to compare equal doses of wheat bran, nopal and psyllium fibres on gas production by microbial fermentation *in vitro*; and their dynamic effects on SBWC, colonic volume and water content of the chyme in the ascending colon *in vivo* using MRI in healthy human volunteers.

2.2. Methods

Two studies were performed; the first examined the effect of the three fibres in a laboratory model of colonic fermentation (*in vitro* fermentation study) and the second examined the effect of ingesting the three fibres for two days on healthy subjects' SBWC, colonic volume and colonic water content using MRI and breath hydrogen (human MRI study).

The fibres used for both the *in vitro* fermentation study and the human MRI study were; coarse wheat bran (Holland and Barrett, Hinckley, UK), nopal provided as dehydrated cactus leaf (OroVerde Nopal Cactus Green Leaf Powder, Cuernavaca, Mexico) and psyllium husk (98%, Supernutrients, Bath, UK). Their composition is shown in

Table 2**,** analysed by Medallion Labs (Minneapolis, MN, USA) using standard AOAC methods and by Quadram Institute Biosciences (Norwich, UK) using Megazyme Fructan HK enzymatic assay kit (Megazyme, Bray, IE) according to manufacture recommendations[308].

Table 2 - Composition of the three test fibres. [1]Quantified using high-performance anion exchange chromatography with pulsed amperometric detection. ND: not detectable

Test	Wheat Bran	Nopal	Psyllium
Resistant Starch	<2%	<2%	<2%
Total Dietary Fibre	41.3%	50.8%	88.9%
- Soluble Fibre	6.2%	13.2%	23.5%
Total Fructans[1]	1.2%	0.1%	-
Total Sugars	4.4%	4.9%	-
Mannitol	trace	0.1%	-
Glucose	2.0%	1.4%	-
Fructose	0.7%	2.2%	-
Sucrose	ND	1.2%	-

Raffinose	0.1%	trace	-
1-Kestose	0.1%	trace	-
Maltose	1.42%	0.1%	-
Nystose	ND	trace	-
Kestopentose	ND	ND	-

2.2.1. Ethical Approval

The human MRI study was approved by the University of Nottingham Faculty of Medicine & Health Sciences Research Ethics Committee (51-1707) and prospectively registered on clinicaltrials.gov (NCT03263065). The study opened in September 2017 and closed in May 2018. Ethical approval for collection of stool samples from healthy people for the *in vitro* study was obtained from London - Westminster Research Ethics Committee (REC) (15/LO/2169).

2.2.2. *in vitro* fermentation study

In vitro fermentations for the three test fibres were carried out using a well-established model of the human colon seeded with microbiota obtained from healthy human volunteers[309–312]. Prior to the fermentation, wheat bran and nopal underwent *in vitro* digestion using the INFOGEST, a validated international consensus method[313] that mimics small intestinal digestion and absorption of non-fibre carbohydrates that would otherwise be fermented in the *in vitro* fermentation model. Digestions were performed using the INFOGEST digestion method[313] with the addition of amyloglucosidase (final concentration 3 U/ml) at the intestinal phase. On completion, pre-digested fibre samples were frozen and lyophilized for 6 days. Once dry, samples were washed with absolute ethanol to release unbound sugars. Ethanol was added at the concentration of 20ml ethanol / g dried substrate, the sample mixed and incubated at room temperature for approximately 90 minutes. Samples were centrifuged to allow excess ethanol to be removed, and the remainder

evaporated through for three days. Once complete the final mass of substrate was recorded. Psyllium did not undergo digestion as it is 98% dietary fibre.

Gas production from the three fibres was measured using single stage anaerobic colon models[314]. In brief, per 125ml vessel; 0.5g of pre-digested wheat bran, pre-digested nopal or psyllium were mixed with 76ml of media, as described by Williams *et al.*[314], kept anaerobic under a constant stream of CO_2. Once sealed, bottles were injected with 5ml of a vitamin and buffer solution, 1ml of the reducing solution[314] and pre-warmed overnight at 37°C.

Faecal samples were obtained from five healthy individuals who had no history of gastrointestinal disease nor antibiotic use in the previous three months, and who were non-smokers. Individual faecal samples were diluted in pre-reduced phosphate buffered saline (10% w/v) and strained to remove particulate. Each fibre substrate was fermented in triplicate per volunteer faecal sample. Each vessel was inoculated with 3ml of slurry by injection and incubated at 37°C for 10 days. Gas production was measured at regular intervals using a pressure transducer (Omega USB-H transducer, Omega Engineering, Manchester, UK) and syringe. At each time point, the pressure in the bottle was recorded with the transducer and the volume measured by removing gas with a syringe to bring the pressure in the bottle to atmospheric pressure. Data are reported as cumulative gas volume produced during fermentation, averaged from five volunteers measured in triplicate per fibre, a total of 15 fermentation studies per fibre.

2.2.3. Human MRI study

This was a single centre, randomized, three-treatment crossover study of wheat bran, nopal and psyllium's effects on SBWC, colonic volume and water content of the chyme in the ascending colon assessed by MRI, and on exhaled breath hydrogen breath concentration. This study did not use a negative control such as glucose as an extensive data set, which defines the normal range, already exists and would have significantly increased the cost for little

gain. The study followed the principles of Good Clinical Practice in accordance with the Declaration of Helsinki.

2.2.3.1. Participants

Healthy volunteers were recruited by poster advertisement on University of Nottingham campuses and gave written informed consent. Participants were eligible for inclusion if they were over 18 years old and were able to give informed consent. The exclusion criteria were pregnancy, history of pre-existing gastrointestinal disorder including irritable bowel syndrome, previous intestinal resection, any serious medical condition, contraindications to MRI scanning, and inability to stop medications known to alter intestinal motility. All subjects assessed completed the study protocol.

2.2.3.2. Test fibres and controlled diet

The wheat bran, nopal and psyllium consumed in the study were identical to those used in the *in vitro* fermentation except that wheat bran and nopal did not undergo pre-digestion. All fibres were stored in a sealed container in a cool, dry and dark environment. Doses were standardized to provide approximately 7.5 g of total fibre per dose so participants received 20.6 g wheat bran, 14.8 g nopal and 8.4 g psyllium per dose (see **Figure 6**). The pre-weighed test fibre was mixed with 300ml of water and taken with breakfast (see **Figure 7**) and lunch (see **Figure 8**) the day before the study visit and then again at the research centre on the day of the study visit (therefore three doses in total over 24-hour period). Participants consumed the three fibres in random order with study days separated by at least 6 days to ensure adequate washout.

The order of fibre consumption was determined by random sequence generated using the online program www.randomization.com. The researchers were not blinded to the order of fibre allocation as they prepared

the supplement and water mix on the day. Although participants were not informed about the order of fibre allocation, the differing appearance, taste and texture of the fibre supplement meant that they could not be formally blinded to the fibre consumed that day. However, all study MRI and other data were link-anonymized via a study ID and MRI analysis was done blind to the intervention.

Whilst consuming the fibre supplements (i.e. the day before and the day of the study visit), participants were instructed to avoid caffeine, alcohol and strenuous exercise and provided with a standardized controlled diet (see **Table 3**), that was low in fermentable carbohydrates (low FODMAP diet, known to reduce the symptoms of bloating[220]) and otherwise low in fibre. This aimed to reduce the intra- and inter-individual variability in colonic fermentation due to background diet and effects on gastrointestinal motility.



Figure 6 - 20.6g Wheat bran, 8.4g psyllium and 14.8g nopal used for the study

Table 3 - Nutritional composition of low fibre, low FODMAP meals provided to participants the day before the study day

Breakfast	**Energy (kcal)**	**Carbohydrate (g)**	**Protein (g)**	**Fat (g)**	**Fibre (g)**
30g rice pops cereal (Sainsbury's) with 100ml lactose free milk	158	29.6	5.5	1.9	0.4
coffee/ tea 125ml with lactose free milk (dash)	9.6	0.6	0.8	0.3	0.6
Lunch	**Energy (kcal)**	**Carbohydrate (g)**	**Protein (g)**	**Fat (g)**	**Fibre (g)**
220 g Sainsbury's creamed rice pudding	211	37	6.8	3.7	<0.5
34g Sainsbury's seedless raspberry jam	85	21	<0.5	<0.5	0.9
100 ml Sainsbury's pure orange juice from concentrate	42	8.6	0.6	<0.5	<0.5
Dinner	**Energy (kcal)**	**Carbohydrate (g)**	**Protein (g)**	**Fat (g)**	**Fibre (g)**

Lean corned beef (Princes) 200g	194	1.0	25	10	-
Half of 300g tinned whole carrots in water (Sainsbury's)	25	4.3	0.5	0.3	1.9
Steamed basmati plain rice (Tilda) 250g	358	70.4	7.2	4.8	1.8
2 Highland All Butter Shortbread finger biscuits (Sainsbury's)	208	23.8	2	11.6	1



Figure 7 - Supervised study breakfast. 30g rice pops cereal (Sainsbury's) with 100ml lactose free milk, and 1 serving of study fibre (wheat bran pictured)



Figure 8 - Study lunch: 220g rice pudding, 34g seedless raspberry jam, 100ml orange juice

2.2.3.3. Magnetic Resonance Imaging

Subjects were scanned on a research-dedicated 3.0 T Philips Achieva scanner (Best, The Netherlands) using a parallel imaging SENSE 16-element torso coil. A range of MRI sequences were used to image the abdomen including:

1) A balanced gradient echo (called balanced turbo field echo, bTFE or trueFISP) sequence (TR = 3.0 ms, TE = 1.5 ms, FA 80^{o}, SENSE factor 2.0) to acquire 50 transverse images each with an in-plane resolution of 2.00 mm x 1.77 mm and slice thickness of 5 mm, with no gap between slices. This was used to measure gastric volumes.

2) A single shot, fast spin echo sequence (rapid acquisition with relaxation enhancement, RARE) to acquire in a single breath-hold 24 coronal images with in-plane resolution interpolated to 0.78 mm x 0.78 mm and a slice thickness of 7 mm, with no gap between slices (TR = 8000 ms, TE_{eff} = 320 ms, AQR = 1.56 mm x 2.90 mm). This sequence yields high intensity signals from areas with fluid and little signal from body tissues and was used to measure small bowel water content.

3) A high resolution bTFE sequence to acquire images of the contents of the ascending colon (TR = 3.1 ms, TE = 1.56 ms, acquired resolution = 1.50 mm x 1.50 mm, reconstructed resolution= 0.86 x 0.86, 8 slices 5 mm thick, up to 5.0 mm gap to cover whole ascending colon, flip angle 45^{o}). This was used to measure T1 in the ascending colon.

4) Segmental colonic volume were assessed using a coronal dual echo fast field echo sequence (24 contiguous slices with TE = 2.3/4.6 ms TR = 158 ms, in-plane resolution 1.76 × 1.76 mm, slice thickness of 7 mm, SENSE factor = 2) during an expiration breath hold of 13 s and then a transverse dual echo FFE sequence (45 contiguous slices with TE = 2.3/4.6 ms TR = 296 ms with in-plane resolution 1.76 × 1.76 mm, slice thickness 7 mm, SENSE factor = 2) under a 20-s expiration breath hold, as previously published [271].

Images were acquired with an expiration breath-hold between 13 and 24 seconds. Participants spent approximately 15 minutes inside the magnet at

any one time. The methods to analyse MRI images obtained are discussed in section 1.5.

2.2.3.4. Sample size and statistical analysis

Our previous studies of psyllium showed a mean (SD) increase in T1AC of 0.35 (0.42) s (unpublished data on file) after therapeutic doses of psyllium which is a mild laxative and this increase represents a minimal clinically significant difference. Using the PS Power and Sample Size Calculations program, version 3.0.43 with a false discovery rate of 0.05 and power of 80% we calculated that we would need 13 subjects in order to demonstrate such a difference.

Parametric data are presented as mean (SD) and non-parametric data as median (interquartile range). All statistical analysis was performed using Graphpad Prism version 8.2.1 for Windows (GraphPad Software, La Jolla California USA). Repeated measures one-way ANOVA followed by Tukey's multiple comparisons test was performed for area under the curve (AUC) volume versus time for *in vitro* gas production, comparison of time to onset, T1AC, SBWC and total colonic volumes. Equal variance was not assumed, the Geisser-Greenhouse correction was used, and normality of the distributions was assessed by the D'Agostino & Pearson test. Friedman's test followed by Dunn's multiple comparisons test was used to assess non-parametric breath hydrogen data at 4 hours. Onset of fermentation was assessed from the inflection point.

2.2.3.5. Protocol

On the day prior to the study day, the allocated test fibre was provided at two meals (breakfast and lunch). All food was provided as low fibre, low FODMAP meals (see

Table 3), including a supervised and standardized breakfast and lunch, and a standardized dinner was provided for participants to consume in the evening

at home. Participants arrived the following morning at the Sir Peter Mansfield Imaging Centre at the University of Nottingham after an overnight fast and verbally confirmed compliance with dietary restrictions. MRI safety questionnaires were completed with the radiographer. Participants underwent a fasted MRI scan and measurement of breath hydrogen by exhaling into a gas analyser (GastroCH4ECK, Bedfont, UK). Participants then consumed the same meal and fibre supplement as was taken for lunch the previous day. MRI scans were performed immediately after the meal and then hourly for four hours with hydrogen breath tests every half hour (see **Figure 9**). MRI parameters included SBWC, colonic volume and T1 of the chyme in the ascending colon (T1AC). T1 is the time constant for the water hydrogen protons to return to their equilibrium state following radiofrequency excitation. More watery chyme has a longer T1 relaxation time, and the T1 of the descending colon has been recently shown to correlate with stool water content[213]. MR images were analysed as detailed in Chapter 1.

2.2.3.6. Endpoints

The primary endpoint was the T1AC 4 hours post-meal ingestion, measured by MRI. Secondary endpoints included the fasting T1AC and change in small bowel water content, colonic volume, and breath hydrogen over the same time period (0-4 hours). We also compared fasting values to our normal range for T1AC and colonic volumes. There were no changes to the pre-specified endpoints during the course of the study.

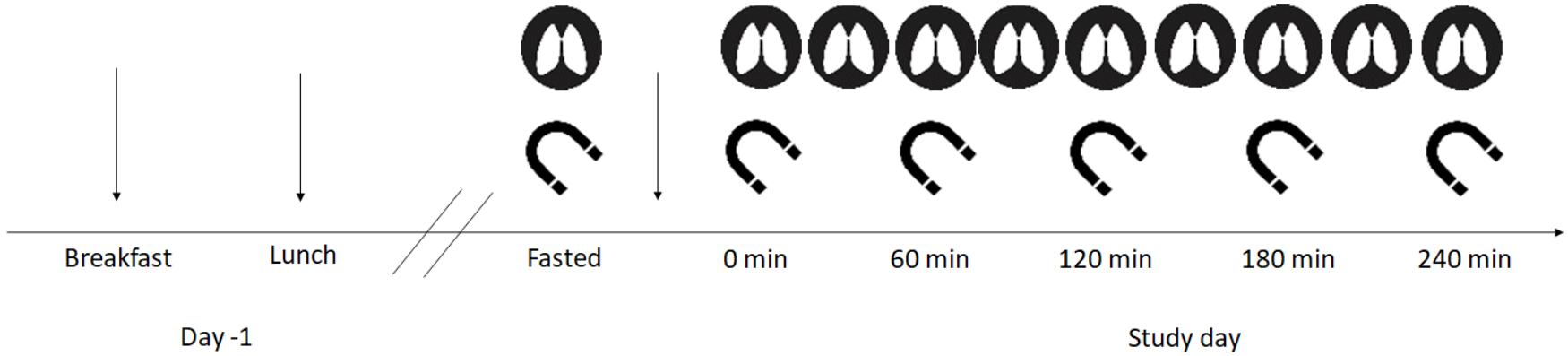


Figure 9 - Schematic of events during the human MRI study. MRI scans are represented by , hydrogen breath tests by and test meal ingestion by ↓. Test meals comprised 7.5g total fibre with a low fibre, low FODMAP meal. Each scan day was separated by a washout period of at least 6 day

2.3. Results

2.3.1. *in vitro* fermentation study

AUC total gas production over 48 hours was significantly different between fibres (one-way ANOVA F=9.07, *p*=0.01), with a significantly greater AUC for wheat bran compared with psyllium, mean (95% CI) difference 370.4 (76.8, 664.0) ml.hr, *p*=0.02, but not nopal, mean (95% CI) difference 164 (-117.6, 446.4) ml.hr, *p*=0.2 (see **Figure 10** and **Figure 11**). Onset to gas production was significantly longer for psyllium than wheat bran, mean (95% CI) difference 8.4 (2.9, 13.9) hours, *p*=0.004 and compared to nopal 10.1 (4.6, 15.6) hours, *p*=0.001.

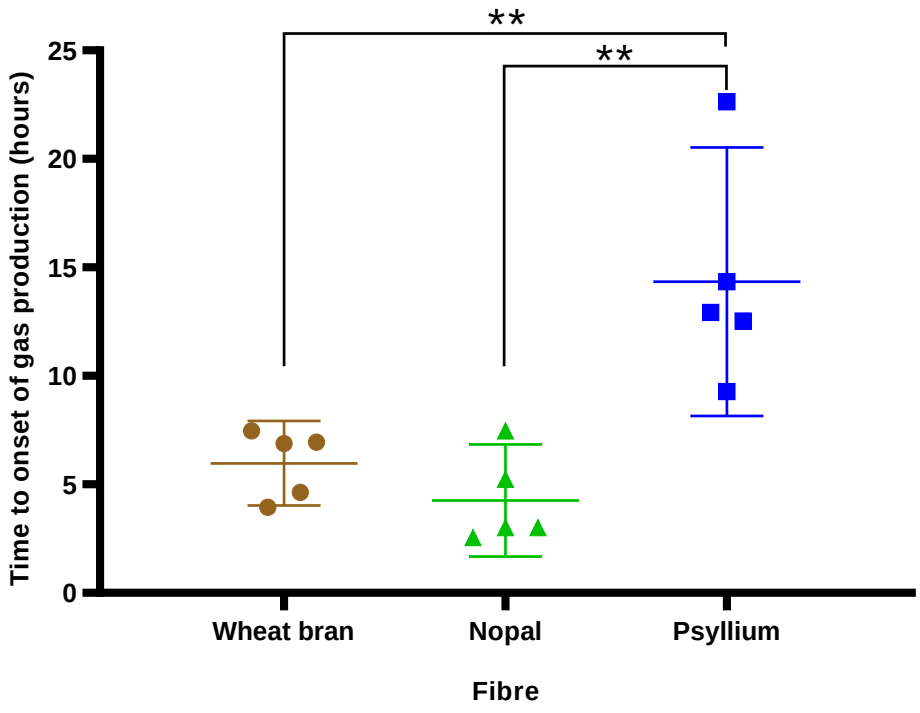


Figure 10 - In vitro onset of gas production (in hours) when combined with the study fibres, demonstrating significantly longer onset time (defined by the inflection point in the time versus volume curve) for psyllium than wheat bran (14 (5) hours versus 6 (2) hours. Data are presented as mean and 95% confidence intervals, n=5 (in triplicate).

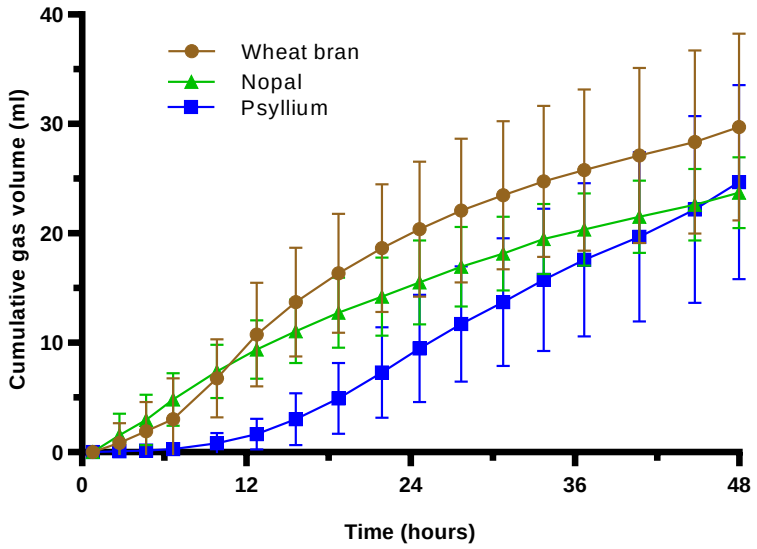


Figure 11 - In vitro stool sample gas production when combined with the pre-digested fibres over 48 hours. AUCs were significantly different between fibres (F=9.07, p=0.0109), with a significantly greater AUC for wheat bran compared with psyllium, mean (95% CI) difference 370.4 (76.8, 664.0) ml.hr, p=0.02, but not nopal, mean (95% CI) difference 164 (-117.6, 446.4) ml.hr, p=0.2. Data are presented as mean and 95% confidence intervals, n=5 (in triplicate).

2.3.2. Human MRI study

Fourteen participants completed the human MRI study (64% female, aged median [interquartile range, IQR] 20 [20-22] years with BMI median [IQR] 22.8 [21.1-25.8]). All participants consumed the allocated fibres with no adverse effects. Due to equipment failure 11 complete data sets were available for analysis for SBWC and for breath hydrogen (see **Figure 12** for CONSORT diagram).

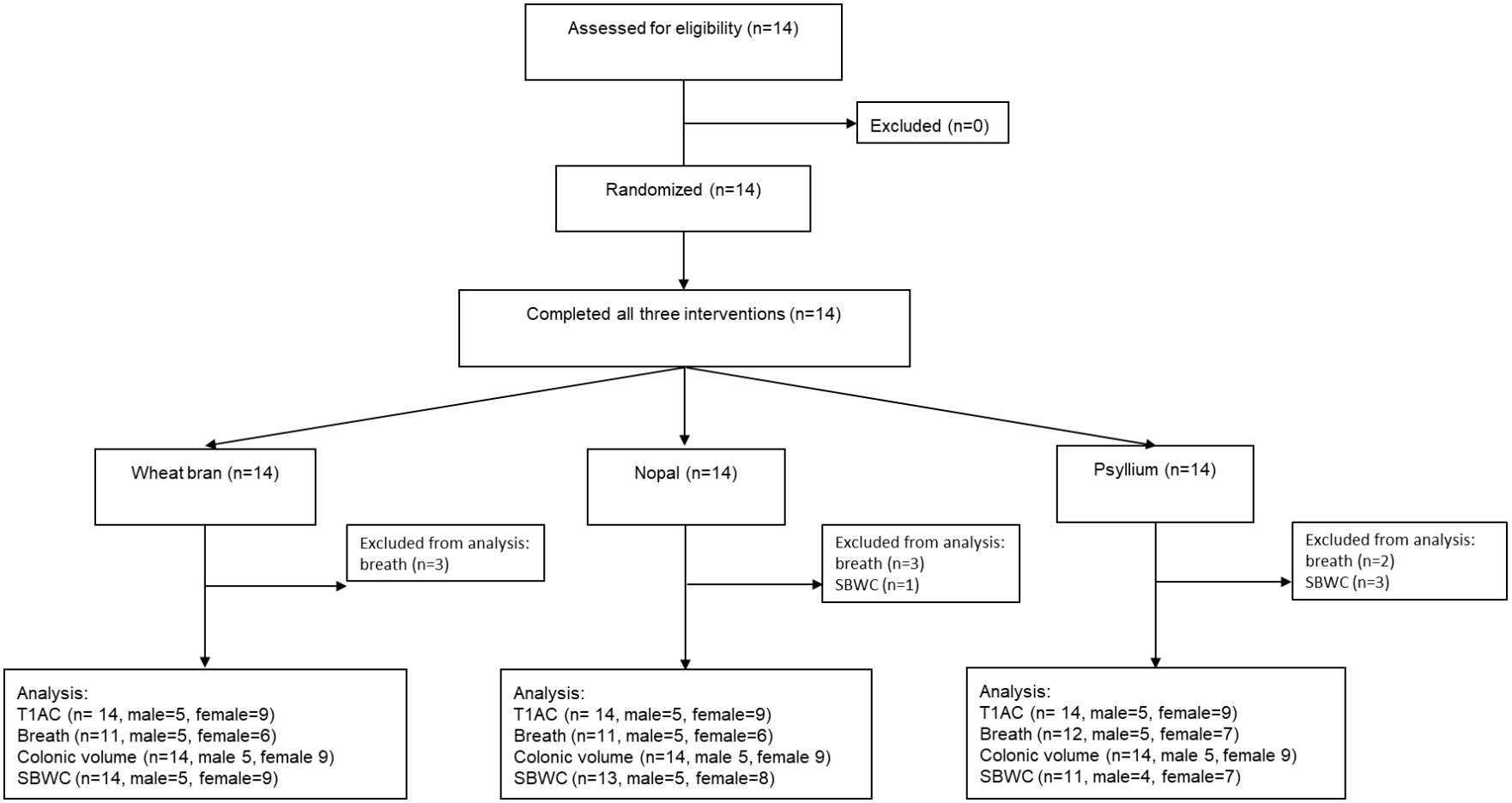


Figure 12 – CONSORT diagram of the study

2.3.2.1. Primary endpoint

As

Table 4 and **Figure 13** show, fasting values of T1AC were similar for the three fibres despite receiving 2 doses the previous day. However, over the study day T1AC rose significantly with psyllium but not wheat bran or nopal so that the differences were greatest at the end of the study day. T1AC at 4 hours showed a significant difference between the fibres (one-way ANOVA F=23.2, $p<0.0001$) and Tukey's multiple comparisons showed a significant T1 increase for psyllium compared to both wheat bran and nopal, mean (95% CI) difference 0.439 (0.207, 0.672)s, $p=0.0007$, and 0.338 (0.17, 0.505)s, $p=0.0004$, respectively.

Table 4 - T1 (in seconds) in the ascending colon of participants, fasted and 4 hours postprandially, indicating a more watery colonic chyme for psyllium than nopal and wheat bran after ingestion

	Ascending colon T1, mean (SD)	
Fibre	**Fasting**	**4 hours after meal and fibre**
Wheat bran, mean (SD)	0.98 (0.19)	0.82 (0.18)
Nopal, mean (SD)	0.97 (0.16)	0.92 (0.16)
Psyllium, mean (SD)	0.99 (0.17)	1.26 (0.29)[1]

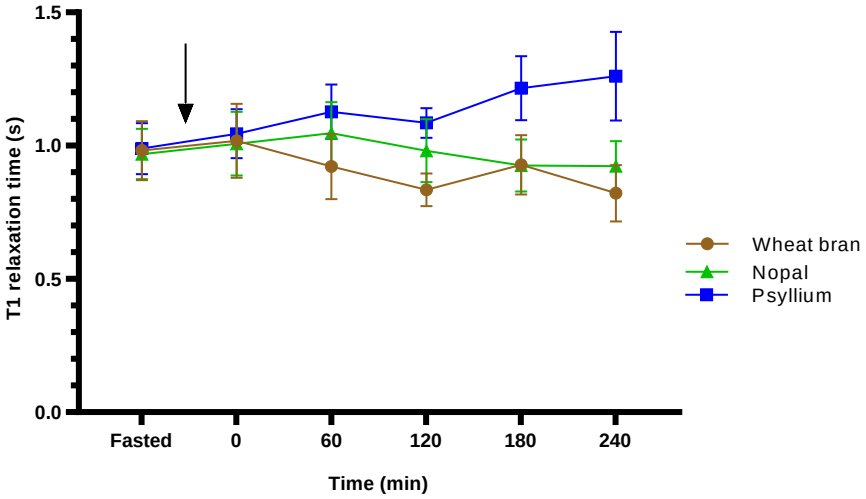


Figure 13 - Time course of T1 relaxation in the ascending colon (T1AC) (mean, 95%CI) following fibre ingestion on the MR imaging day (n=14).

2.3.2.2. Secondary endpoints

2.3.2.2.1. Fasting T1AC

24 hours of fibre pre-feeding resulted in at least 75% of fasting T1AC values lying above the 90th centile of the normal range with no significant differences between the three fibres (repeated measures one-way ANOVA F=0.05, *p*=0.93) (see **Figure 14**). Normal values for T1AC after an 8-hour fasting period have previously been obtained from 29 healthy volunteers from previous studies, published[281] and unpublished, on the same 3.0 T Philips Achieva MRI scanner, and are shown as the median and 10th- 90th centiles).

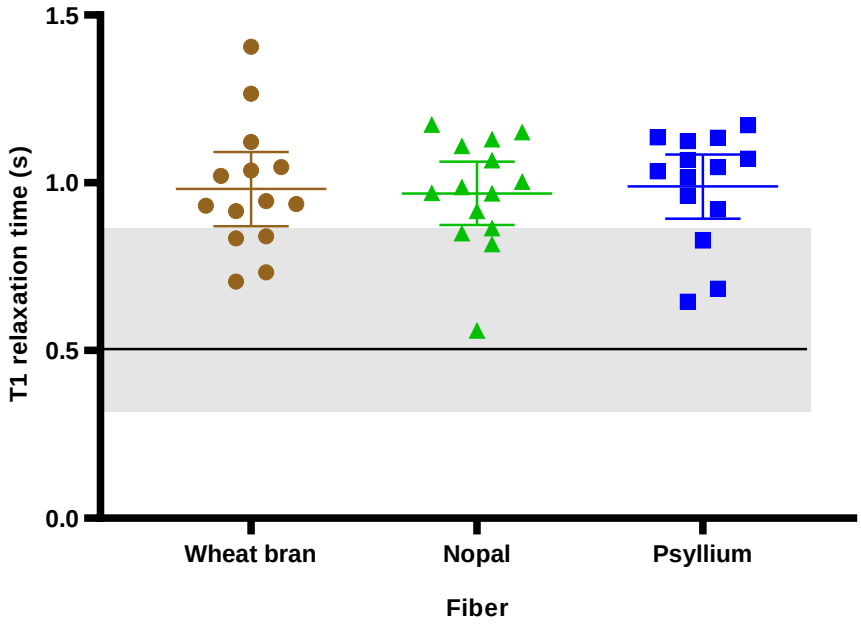


Figure 14 - Fasting T1 of the ascending colon (T1AC) (mean, 95%CI) after 24 hours of fibre pre-feeding (n=14). Fasting T1AC in 29 healthy volunteers on the same 3.0 T MRI scanner represent normal values, and are denoted as a solid black line (median) and 10th – 90th centiles (grey).

2.3.2.2.2. Small bowel water content

There was a significant increase in SBWC for all fibres from fasting to 4 hours (see **Figure 15**). AUC analysis demonstrated a significant difference between fibres (repeated measures one-way ANOVA F=4.8, *p*=0.02); nopal stimulating significantly more small bowel water than wheat bran (mean (95% CI) difference 7.1 (0.6, 13.8) L.min, *p*=0.03).

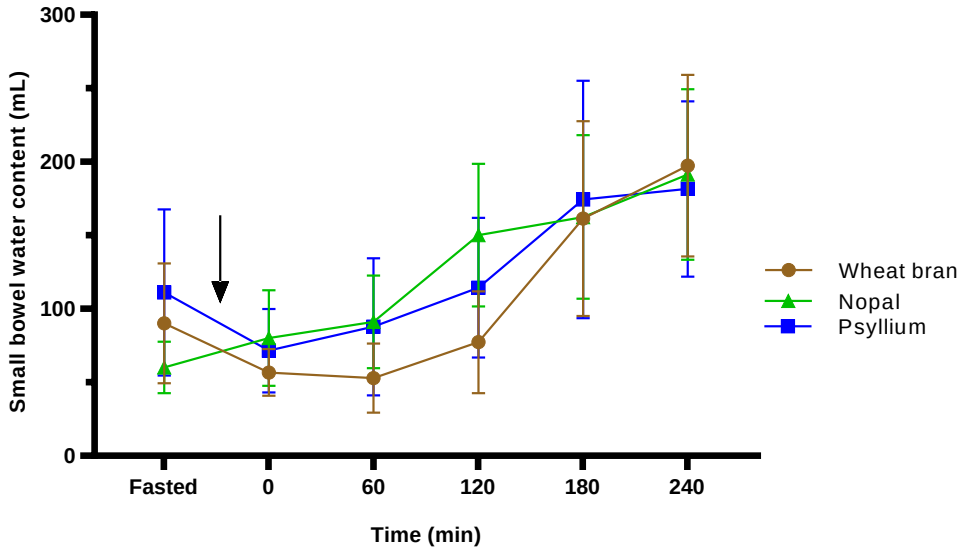


Figure 15 - Time course of Small Bowel Water Content (SBWC) (mean, 95%CI) following fibre ingestion on the MR imaging day (n=11). Ingestion of the test meal is designated by ↓ .

2.3.2.2.3. Colonic Volume

There were significant differences in the fasting colonic volume between fibres after 24 hours of pre-feeding (repeated measures one-way ANOVA F=20.5, *p*<0.0001); participants pre-fed with psyllium for 24 hours had larger fasting total colonic volumes than both nopal (mean (95% CI) difference 128 (71, 185) ml, *p*=0.0001) and wheat bran (mean (95% CI) difference 129 (53, 205) ml, *p*=0.002) with no significant difference between nopal and wheat bran. AUC for the study duration was significantly different (F=40, *p*<0.0001); psyllium was greater than nopal (mean (95% CI) difference 36.0 (24.1, 47.8) L.min, *p*<0.0001) and wheat bran (mean (95% CI) difference 45.8 (31.1, 60.4) L.min, *p*<0.0001), with no difference between nopal and wheat bran (see **Figure 16**). Normal colonic volumes after an 8-hour fasting period (mean, SD) have been obtained from 34 healthy volunteers from a previous study[272] on a 1.5T Philips Achieva MRI scanner and are demonstrated in grey).

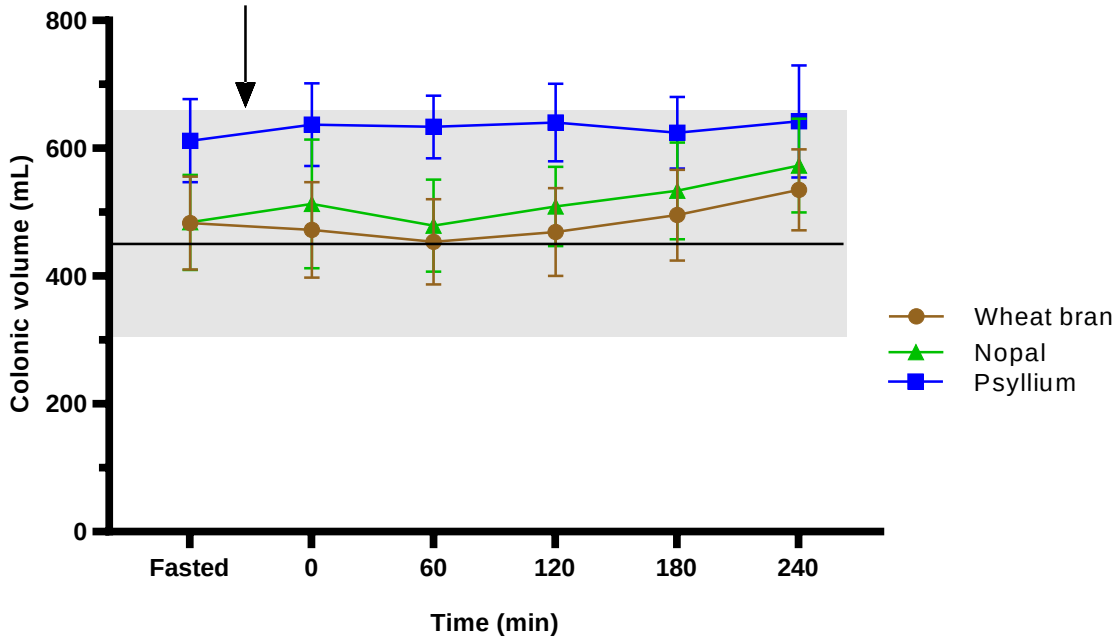


Figure 16 - Time course of total colonic volumes (mean, 95%CI) following fibre ingestion on the MR imaging day (n=14). Ingestion of the test meal is designated by ↓ . Total colonic volumes in 34 healthy volunteers represent normal values, and are denoted as a solid black line (mean) ± standard deviation (grey).

2.3.2.2.4. Breath Hydrogen

Fasting breath hydrogen concentrations were not different, however after 4 hours there was a significant difference between the fibres (Friedman's test, $p<0.0001$). Breath hydrogen concentration was significantly higher for both wheat bran and nopal *versus* psyllium (mean difference (SD) 56.1 (42.8) ppm, $p=0.0003$ and 32.3 (32.4) ppm, $p=0.04$, respectively), with no difference between wheat bran and nopal (see **Figure 17**).

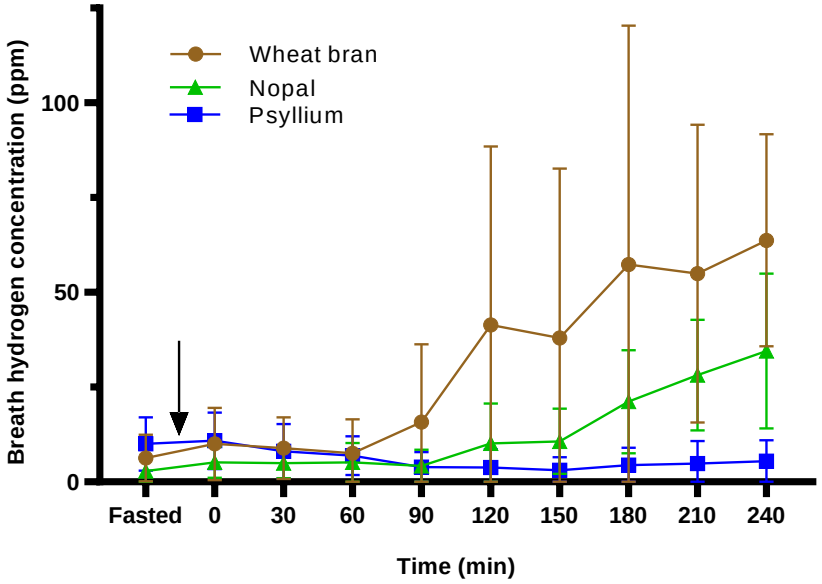


Figure 17 - Time course of breath hydrogen concentration (mean, 95% CI) following fibre ingestion on the MR imaging day (n=10). Ingestion of the test meal is designated by ↓ .

2.4. Discussion

The laxative effect of the many and various dietary fibres is well recognized but the individual underlying mechanisms have until recently been unclear. Our study utilizes three very different fibres and shows that all three increase colonic water but by different mechanisms. We confirmed the results from our previous study[213] by showing that psyllium is highly effective in acutely trapping water in the small bowel, which rose steadily in the hours after meal ingestion. It should be noted that without fibre supplementation postprandial SBWC between 180-240 minutes has been shown to average under 100mls[16] whereas in our study it was 178mls. The *in vitro* fermentation studies, showing more rapid fermentation of wheat bran and nopal fibre compared to psyllium, match the earlier and more substantial rise in breath hydrogen seen *in vivo*. Psyllium is only very slowly fermented which will ensure a prolonged "trapping" of water in the colon. The larger colonic volume after psyllium may also reflect a lack of stimulation of colonic motility compared to the other more fermentable fibres. Psyllium would be predicted to produce fewer short chain fatty acids, which are known to stimulate 5-HT release from colonic enteroendocrine cells[315] which is known to have a prokinetic effect.

Previous publications show that postprandial SBWC is strongly influenced by nutrient absorption and osmotic factors. Glucose[16], bread or rice meals[316] lead to rapid falls in SBWC over the next 1-2 hours as glucose and sodium are actively absorbed by small intestinal transporters with accompanying passive water absorption. Psyllium slows nutrient absorption[317], possibly by increasing viscosity and reducing the mixing which is essential to allow access of luminal contents to the mucosa. Psyllium potently retains water within its complex network making it unavailable for absorption. We have shown in the current study that repeated doses of psyllium lead to an increase in colonic volumes and water content as assessed by the MRI parameter, T1. The rise in colonic volume may be due to the fact that, unlike wheat bran[318], psyllium does not significantly accelerate whole colonic transit[213,319], a feature which would reduce colonic volumes by increasing the frequency of defecation.

Wheat bran by contrast, being less viscous, cannot trap water like psyllium but does however produce a similar increase in SBWC. Previous studies[17] had showed that 15g of both wheat bran and plastic particles caused similar acceleration of meal transit suggesting that this is driven by mechanical rather than chemical stimulation of the mucosa. Earlier studies have shown that stroking intestinal mucosa activates neurogenic secretion[320] which could accelerate transit. More recently, it has been shown that a subpopulation of enterochromaffin cells express mechanosensitive piezo-2 ion channels[18]. Enterochromaffin cells are stimulated by shear forces to release serotonin[321] which stimulates crypt secretions. This may be an important mechanism to dilute luminal contents if they become too viscous and threaten to cause intestinal obstruction[16,322]. Another potential mechanism through which particulate fibre can increase postprandial water is inhibiting amylase digestion of starch in the rice meal through adsorption of amylase to the particle surface[15]. Wheat bran is also known to increase faecal bacterial mass, a factor that accounts for a substantial proportion of stool mass[22] and may thus exert a laxative effect. Given that both viscous and particulate fibre increase small bowel water content but by different mechanisms, it is perhaps not unexpected that nopal, which contains both mucilage and particulate fibre, had a greater effect on small bowel water than either psyllium or wheat bran alone.

Towards the second half of the 4-hour study, small bowel contents would be expected to start entering the ascending colon and hence increase T1AC. At this point psyllium seemed to be most effective. This may reflect the slow breakdown and fermentation rate of psyllium's highly branched structure, demonstrated by the delayed onset *in vitro* of gas production in our fermentation study and the virtual absence of a rise in breath hydrogen in the MRI study. The undegraded psyllium will continue to trap water, making it unavailable for absorption, hence increasing colonic volumes. Wheat bran, with a particulate structure, a less branched arabinoxylan and a small amount of fructans, is more rapidly fermented *in vitro* and shows a clear rise in breath

hydrogen *in vivo*. This rapid fermentation would increase bacterial mass and produce SCFA that stimulate sodium and water absorption[323]. Fermentation products may also stimulate motility and accelerate transit thereby reducing colonic volumes though direct evidence of the impact of SCFA on motility is contradictory, with some studies suggesting stimulation[132] and others not[133]. More recently it has been shown that SCFA stimulate colonic motility in rats via the release of 5-hydroxytryptamine (5-HT)[324] that stimulates colonic peristalsis. Using germ free and mice colonized with human microbiota it has been shown that the presence of colonic microbiota increases serotonin synthesis and release by enteroendocrine cells[325], providing a mechanism whereby dietary fibre modulation of colonic microbiota could accelerate transit.

We assessed fasting values of T1AC after two doses of fibre the day before to understand the longer-term effects. Despite the greater rise in T1AC soon (2-4 hours) after acute ingestion of psyllium compared to the other fibres, by 24 hours their effects were similar; all three fibres increasing T1AC to the upper limit of our normal range. While both wheat bran and nopal increase small bowel water, this does not appear to increase colon volumes in the short term. This may be because, as shown by the greater breath hydrogen response, the more readily fermentable components of both wheat bran and nopal are rapidly fermented and absorbed thus limiting any increase in colonic volume. Alternatively, it may reflect greater stimulation of motility by wheat bran and nopal which would also reduce colonic volumes but demonstrating this would require further studies. Previous studies have shown a link between increased colonic volumes and the sense of distension and bloating[326] that may limit the use of psyllium in constipated patients.

2.4.1. Study Limitations

This pilot study was able to illustrate differences in gut physiology when ingesting the test fibres, however it was powered for the primary endpoint T1AC. We have assessed multiple MRI endpoints but did not correct the *p*

values for this. While we can be confident that our primary endpoint result is not due to chance, secondary endpoints need confirming in further studies.

Greater insight into the mechanisms of action would have been obtained with stool analysis, both with a baseline and post treatment stool diary, and stool dry weight, water content and consistency. Microbiota analysis, although underpowered, might have shown trends consistent with previous literature in acute changes in response to the different prebiotics and give greater weight to conclusions drawn. Although MRI was able to measure T1AC, SBWC and colonic volume, the study was not designed to also measure transit. This is thought to be of particular relevance for psyllium, slowing colonic fermentation due to steric hindrance and thus reducing products of fermentation which have shown to be prokinetic. Further, more complete studies should include these analyses for a comprehensive understanding of the dietary fibres investigated.

2.5. Conclusion

In summary, both viscous and particulate fibre stimulate an increase in postprandial small bowel water content and an increase in colonic T1. Possible mechanisms include inhibiting absorption of both water and nutrients or stimulating intestinal secretion. Psyllium appears more effective in trapping small bowel water and its slow metabolism means that colon volumes remain increased over at least 24 hours. Nopal and wheat bran, despite not being viscous, also increase small bowel water but are rapidly fermented in the colon and do not lead to colonic distension. Short studies of nopal in IBS have shown encouraging results[327], warranting larger trials powered to assess symptomatic, physiological and microbiological endpoints.

3. Effects of differing fibres on colonic fermentation *in vitro* and in patients with Irritable Bowel Syndrome (EFIGI)

This chapter describes the EFIGI study designed to assess the impact of co-administration of two fibres with very different physico-chemical properties, assessed in patients with IBS using MRI and *in vitro* in a model of colonic fermentation. It will discuss the fibres chosen, methods and results of the study, and the implications of the findings.

3.1. Background

Irritable bowel syndrome patients often report an unpleasant awareness of intestinal gas, associated with a sensation of abdominal distension often described as bloating. The connection between gas and symptoms is complex as signalling from gut to brain is influenced by many factors including past experience, mood and psychological factors such as somatisation[98]. Although cross-sectional studies of unselected IBS patients have not shown excessive intestinal gas compared with healthy controls[328], increases in gas have been related to symptoms[130], and manoeuvres which reduce gas can alleviate symptoms. The origins of intestinal gas and its transit down the gastrointestinal tract have been extensively studied[329,330] and in the upper gut include swallowed air and carbon dioxide generated when gastric acid meets duodenal bicarbonate, though this is rapidly absorbed. Colonic gas consists predominantly of carbon dioxide, hydrogen and methane[331] derived from bacterial anaerobic fermentation of poorly absorbed dietary FODMAPs (fermentable, oligo-di-and mono-saccharides and polyols) along with mucus and shed epithelial cells. High fibre diets increase colonic volumes, both gaseous and nongaseous with the most marked increase in gas occurring in the pelvic colon[332]. How this relates to symptoms is complicated by variable sensitivity to distension, but reduction of intake of foods high in FODMAPs reduces symptoms in unselected IBS patients in clinical trials in 40-50% of patients[136,222]. However only a few trials have adequately controlled for placebo effect[135,333] and there is a substantial risk of bias in all these trials[334].

Nevertheless such diets are now widely used in clinical practice. The popularity of such diets and their widespread use without adequate dietician support has given rise to concerns because of the associated reduction in colonic microbiota thought to provide important health benefits including reduced risk of cardiovascular disease, diabetes, hypertension, obesity and diverticulitis[335]. Alternative approaches to control of colonic fermentation are therefore being actively pursued.

Viscous fibres such as psyllium, an arabinoxylan polymer that resists digestion in the human upper gastrointestinal tract[25], improve symptoms in unselected IBS[336] though until now the mechanism was unclear. We have recently used magnetic resonance imaging (MRI) to show that psyllium's open network of polymers traps water in the small bowel and increases ascending and descending colon water content. We believe that this is the basis of its laxative effect, softening stool so that it is easier to pass[213]. However the small bowel effects may also be important since psyllium forms a highly viscous solution in the upper small intestine which interferes with absorption[337]. Psyllium is only slowly fermented so its viscosity effects are likely to persist for some hours after it reaches the colon. We have previously used radio-isotopic imaging to demonstrate that psyllium 3.5g three times daily substantially reduced by lactulose-associated acceleration of proximal colonic transit in healthy volunteers but limitations of the method used, means the mechanisms of action were unclear[338].

Inulin is an important example of poorly absorbed dietary polysaccharide commonly found in wheat, onion, banana, garlic, and leeks[339], food types often associated with symptoms in IBS patients[122]. Food manufacturers seeking health benefit claims have sought to use inulin, a storage fructan polymer easily obtained from chicory root, to increase the fibre content of many processed foods. Its simple linear structure means that compared to other sources of fibre it is rapidly fermented and this limits tolerance to it. This is particularly true for IBS patients in whom distension of the colon by fermentation of inulin generates symptoms not seen in healthy volunteers[130].

The current study uses MRI to test the hypothesis that psyllium can reduce the increase in colonic gas induced in IBS by inulin, which reaches the colon largely intact where it is rapidly fermented. This produces large amounts of gas[273] which correlates with symptoms of flatulence, bloating, discomfort and pain in IBS patients[130]. We further investigated the potential mechanisms of action by assessing viscosity of test substances and conducting *in vitro* fermentation experiments with stool donated from the same participants.

The aim of the study was to investigate how co-administration of dietary fibres affects gut physiology, particularly colonic gas production. Comparisons were made between a single fermentable fibre (inulin), a non-fermentable fibre (psyllium) and a combination of the two. *In vitro* assessment allowed further insight into the mechanisms of fibre co-administration in the colon. The study also explored differences in response between constipation and diarrhoea-predominant IBS subtypes.

3.2. Methods

Two studies were performed both investigating the effect of psyllium and inulin, alone and when co-administered, on gas production in IBS. An *in vivo* study investigated this using MRI and breath hydrogen in patients with IBS and *in vitro* we measured the viscosity of test solutions and investigated fermentation in a laboratory model of colonic fermentation using stool samples from participants in the *in vivo* study.

The carbohydrates used were inulin (Orafti® HP, BENEO, Mannheim, Germany) which is 99.9% pure with average degree of polymerisation >23 and psyllium husk (Supernutrients, Bath, UK) which is 98% pure, and the placebo used was food grade dextrose (Thornton & Ross Ltd, Huddersfield, UK).

3.2.1. Ethical Approval

The trial was approved by East Midlands Nottingham 1 Research Ethics Committee, reference 17/EM/0277 on 10/10/17, prospectively registered on clinicaltrials.gov (NCT03265002) and performed between July 2018 and October 2019.

3.2.2. Human MRI study

This was a single-centre, 4-period, 4-treatment, placebo-controlled, crossover trial. Each treatment was taken once by each participant, with randomisation of treatment order. The treatments were inulin, psyllium, inulin and psyllium, and dextrose (control).

3.2.3. Participants

Participants were recruited through Digestive Diseases services at Nottingham University Hospitals NHS Trust and the Circle Treatment Centre, through primary care via the NIHR Clinical Research Network in England and

through general advertisement. Eligible participants were aged over 16, fulfilling Rome IV criteria[85] for either diarrhoea- or constipation-predominant IBS. Exclusion criteria included: pregnancy, contraindications to MRI scanning, inability to cease use of supplementary fibres or laxatives, inability to stop drugs affecting GI motility, history of other pre-existing gastrointestinal disorders or resection, intention to change smoking habit during the study, excess alcohol intake, or anyone who in the opinion of the investigator would be unable to comply with the study protocol.

3.2.4. Test drinks and controlled diet

To standardise dietary sources of colonic gas production, participants were provided with a low fibre, low FODMAP meal to eat at home the evening prior to and during the study day (see **Table 5** and **Table 6** for nutritional information).

The test drinks contained a) 20 g inulin powder (20 g inulin fibre), b) 20 g psyllium powder (20 g psyllium fibre), c) 20 g inulin powder and 20 g psyllium powder, and d) 20 g dextrose powder as a placebo control. Inulin and dextrose were dissolved in boiling water which was subsequently cooled in a refrigerator overnight. In order to increase tolerability, test drinks containing psyllium were divided into four 125 ml drinks with a quarter of the psyllium added to each just prior to ingestion. In total, each test drink included 500 mls water, consumed over 5 minutes. All test substances were stored in a sealed container in a cool, dry and dark environment prior to consumption.

Table 5 - Nutritional composition of standardised meal consumed the evening prior to MRI scans

	Energy (kcal)	Carbohydrate (g)	Protein (g)	Fat (g)	Fibre (g)
Lean corned beef (Princes) 200g	194	1.0	25	10	-
Half of 300g tinned whole carrots in water (Sainsbury's)	25	4.3	0.5	0.3	1.9
Steamed basmati plain rice (Tilda) 250g	358	70.4	7.2	4.8	1.8
2 Highland All Butter Shortbread finger biscuits (Sainsbury's)	208	23.8	2	11.6	1

Table 6 - Nutritional composition of meals consumed during the study day

	Energy (kcal)	Carbohydrate (g)	Protein (g)	Fat (g)	Fibre (g)
220 g Sainsbury's creamed rice pudding	211	37	6.8	3.7	<0.5
34g Sainsbury's seedless raspberry jam	85	21	<0.5	<0.5	0.9
100 mL Sainsbury's pure orange juice from concentrate	42	8.6	0.6	<0.5	<0.5

3.2.5. Magnetic Resonance Imaging

A range of MRI sequences were used to image the abdomen to obtain the various endpoints including:

1) Colonic gas was assessed as previously published[274] using a dual echo gradient echo sequence (TR 175 ms, TE1= 2ms, TE2 = 4.3 ms, FA 80^{o}, ASSET 2) to acquire 24 coronal images with a slice thickness 7mm (no gap) and reconstructed in plane resolution of 1.76 x 1.76 mm^2. This was used to measure colonic volumes and was acquired during a breathhold. An additional identical sequence was also acquired with the R.F. power set to zero to acquire the noise distribution across the images for gas measurements.
2) Small bowel water content was measured as previously reported[270] using a single shot fast spin echo (SSFSE) sequence with fat saturation (TE_{eff} = 325 ms, Echo spacing 5ms) to acquire 32 coronal images with a 7mm slice thickness (no gap) and reconstructed in plane resolution of 0.78 x 0.78 mm^2.

Participants spent approximately 15 minutes inside the magnet at any one time. The methods to analyse MRI images obtained are discussed in section 1.5.

3.2.6. Protocol

Participants were provided with written information about the study at least 24 hours prior to informed consent. Eligibility was confirmed and information including past medical history, current medications, height, weight, and smoking history were recorded. Their IBS subtype was confirmed according to Rome IV criteria using a one week stool diary documenting stool frequency and consistency on the Bristol Stool Form Scale (BSFS)[85]. Participants completed the Hospital Anxiety and Depression Scale (HADS)[340] and the Patient Health Questionnaire 12 Somatic Symptom scale (PHQ-12)[341] questionnaires. Eligible participants were randomised to the order of test

drink intake and given a dietary advice sheet for the day preceding each study day. A standardised meal was provided for the evening prior to MRI scans (**Table 5**).

Participants fasted from 20:00 the day prior to the study day and were allowed only sips of water for essential tablets as necessary. On the morning of the study they attended the University of Nottingham Sir Peter Mansfield Imaging Centre and underwent a fasted MRI scan on a 1.5T GE MRI scanner using a parallel imaging 12-element torso coil. They also had breath hydrogen measured by exhaling into a gas analyser (GastroCH4ECK, Bedfont, UK), and recorded symptoms of flatulence, bloating and abdominal pain using a modified Gastrointestinal Symptom Rating Scale[342]. Participants then consumed the allocated test drink. MRI scans were performed immediately after the drink and then hourly for 6 h while hydrogen breath tests and symptom scores occurred every half hour (see **Figure 18** for study day details). After 210 minutes participants received a 338kcal meal of rice pudding, jam and orange juice (see **Table 6** for nutritional information) and completed a hydrogen breath test and symptom score. Participants spent approximately 15 minutes inside the MRI at any one time. A washout period of at least six days was used between study days.

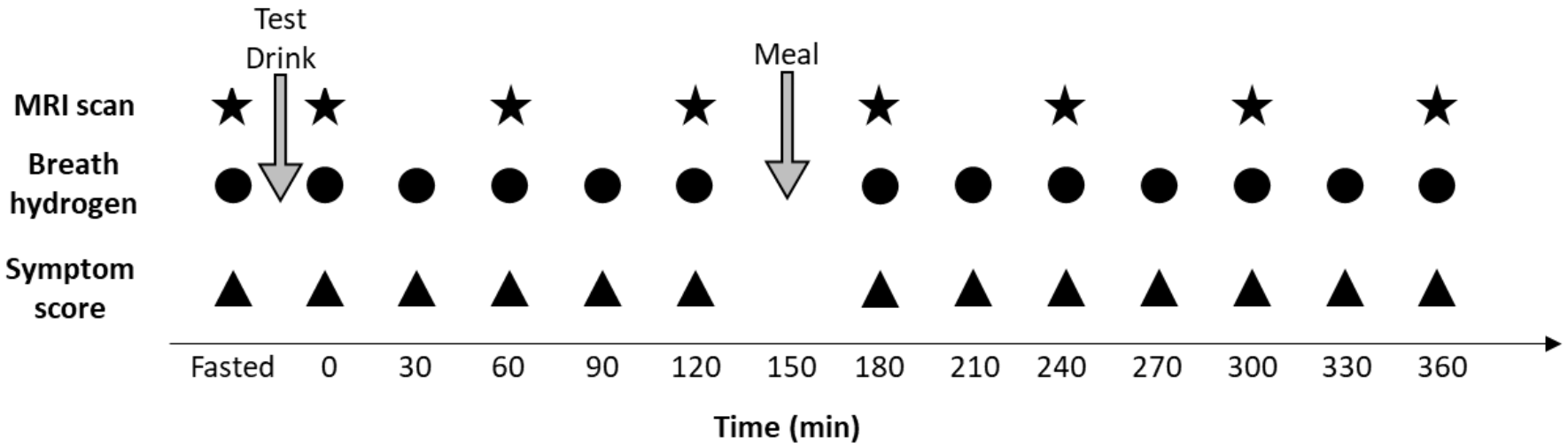


Figure 18 - Schematic of the study day. MRI scans were performed fasted, immediately after the test drink and at hourly intervals while breath hydrogen and symptom scores were obtained at 30 minute intervals. Test drinks were consumed before time 0 scan and the standardised meal given at 210 minutes.

3.2.7. Endpoints

The primary endpoint was change in colonic gas area under the curve from 0-360 minutes (AUC) in ml.min with secondary endpoints being colonic volume AUC (L.min) and small bowel water content AUC (L.min), all measured by MRI. Other secondary endpoints included breath hydrogen in parts per million and severity of flatulence, bloating and abdominal pain, each scored 0-3 with half integers.

3.2.8. *In vitro* fermentation study

Gas production from the fermentation of the test substrates was measured using the ANKOM RF gas production system (ANKOM Technology, Macedon, NY, USA). Briefly, per 125ml bottle, 0.5g of psyllium, inulin, or dextrose were added to each fermentation bottle. Additionally, 0.5g of psyllium and 0.5g of inulin were also added to bottles to test the impact of the substrates combined on gas production (inulin + psyllium). To remove any discrepancies in gas produced from sources other than the test substrates, a non-substrate blank was used. To each fermentation bottle, 76ml of media, 5ml of a vitamin and phosphate buffer solution, and 1ml of the reducing solution[314] were added under a constant stream of CO_2. Once sealed, the substrates were allowed to hydrate, and bottles pre-warmed overnight at 37°C.

Bottles were seeded with faecal samples from eight of the IBS individuals from the human MRI study (four IBS-C, four IBS-D). Faecal samples were frozen at -80°C, therefore prior to testing, each faecal sample was defrosted at room temperature. Once defrosted they were diluted in pre-reduced PBS (10% wt/vol), homogenised in a stomacher and strained to remove particulates. Each substrate was fermented in triplicate per volunteer faecal sample. Each bottle was inoculated with 3ml of slurry, sealed, and incubated at 37°C in a shaking water bath (80 rpm) for five days.

Using the ANKOM RF system, the gas pressure was automatically measured every 15 minutes. Gas production from fibre was calculated using previous

methods [343]. Data are reported as cumulative gas volume produced during fermentation, averaged from eight IBS individuals and measured in triplicate per individual/substrate type, thus a total of 24 individual fermentations were performed per substrate.

3.2.8.1. Viscosity assessment

Substrate viscosity was assessed using an AR-G2 rheometer (TA instruments, Delaware, USA) using a cup and vane geometry. Analysis was performed at the same concentration as predicted to occur *in vivo* (4% solution assuming 20 g is distributed in a colonic volume of around 500ml) and *in vitro* (0.5% solution). Inulin was solubilised in boiling water and stored at 4^{o}C overnight. Psyllium was mixed with water or the inulin solution immediately prior to analysis at a frequency of 6.28 rad/s (1Hz).

3.2.9. Sample size and statistical analysis

The sample size was calculated using data from a previous study of the effect of inulin on colonic gas in IBS[130]. To detect an increase of 40 ml of gas at 80% power and alpha of 1.66%, we calculated 20 participants would be required to complete the study[344]. We planned to enrol up to 25 participants to allow for 20% dropout. We considered 40 ml to be the minimally clinically important difference, representing approximately a 20% increase in ascending colon volume.

Profiles of values over time are summarised as AUC from 0-360 minutes for the MRI study and from 0-48 hours for the *in vitro* fermentation studies. Differences between the AUC of the change from fasting values for MRI colonic gas, AUC for breath hydrogen and for *in vitro* gas production were compared for the 4 test carbohydrates using Friedman test with Dunn's multiple comparisons as a *post hoc* test. The differences in colonic volume AUC, small bowel water content (SBWC) AUC and patient symptoms at 6

hours were analysed using repeated measures one-way analysis of variance (RM ANOVA) with Tukey's multiple comparisons as the *post hoc* test. Comparisons of data between the two IBS subtypes were performed using Mann Whitney test.

In the *in vitro* fermentation studies, gas production values from inulin and psyllium co-administration were measured and theoretical values were calculated by summation of measured values for inulin alone and psyllium alone. Calculated inulin and psyllium was treated as an additional variable for all analyses. Comparisons of *in vitro* data between IBS subtype per substrate were performed using Mann Whitney test. Correlations between *in vivo* AUC MRI colonic gas and *in vitro* AUC gas production were performed using simple linear regression.

All statistical analysis was performed using Graphpad Prism version 8.2.1 or later for Windows (GraphPad Software, La Jolla California USA). Normally distributed data are presented as mean ± SD and non-normally distributed data as median (interquartile range).

3.3. Results

3.3.1. Human MRI study

3.3.1.1. Participants

26 patients attended the initial screening visit, of which 19 completed the study (for CONSORT diagram see **Figure 19**). 15 were female and 4 male, with a median age of 39 (range 19-65) years. Nine participants suffered from constipation-predominant IBS and ten with diarrhoea-predominant IBS. There were no significant differences in age, gender, anxiety or depression scores between IBS-C and IBS-D (

Table 7). IBS-D patients had a higher BMI (35.0 ± 7.1 kg/m^2 *versus* 26.2 ± 5.7 kg/m^2, p=0.009), and as expected had a greater frequency of bowel movements (p=0.02) and higher BSFS score (p=0.02) than IBS-C patients. All tolerated the study procedures well.

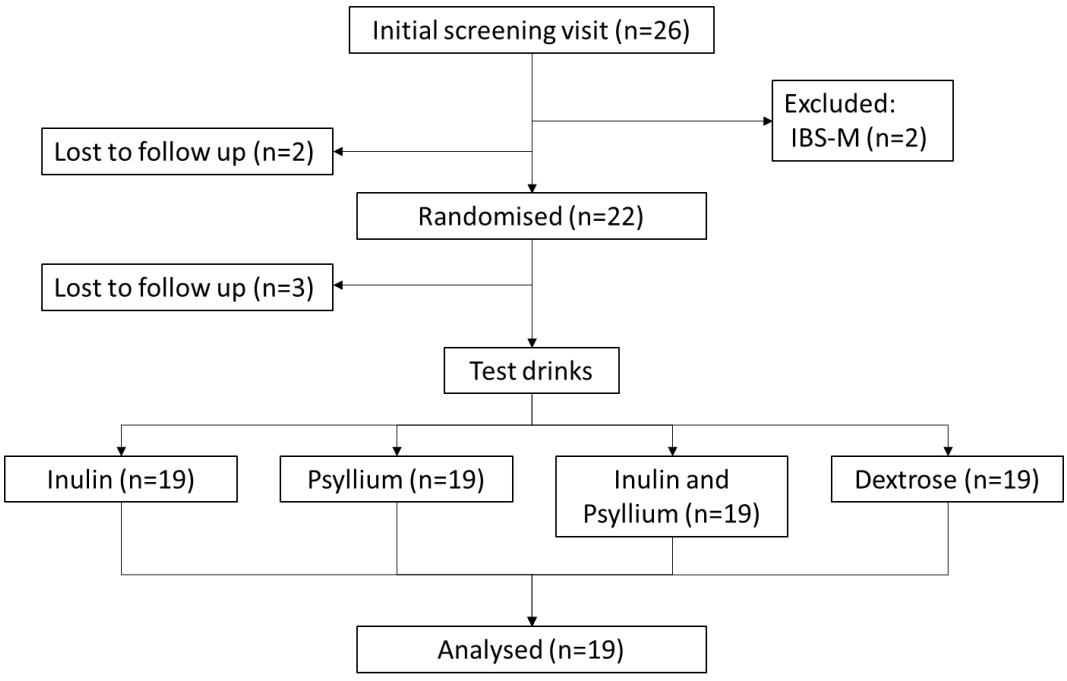


Figure 19 - CONSORT diagram of the study. IBS-M, Mixed-type irritable bowel syndrome

Table 7 - Characteristics of study participants by irritable bowel syndrome subtype. HADS-A, hospital anxiety and depression scale anxiety subscale; HADS-D, hospital anxiety and depression scale depression subscale; IBS, irritable bowel syndrome; PHQ-12, Patient Health Questionnaire 12 Somatic Symptom scale.

Characteristic	**Constipation-predominant IBS (n=9)**	**Diarrhoea-predominant IBS (n=10)**	***P* value**
Age, median (min-max)	39 (19-65)	43 (19-65)	0.92
Gender, M/F	2/7	2/8	>0.99
BMI (kg/m^2), mean ± SD	26.2 ± 5.7	35.0 ± 7.1	0.009
HADS-A, mean ± SD	6.8 ± 6.3	5.6 ± 3.7	0.62
HADS-D, mean ± SD	2.8 ± 3.1	3.6 ± 2.4	0.34
PHQ-12, mean ± SD	4.6 ± 4.4	7.7 ± 3.4	0.10
Weekly bowel frequency, mean ± SD	7.7 ± 3.0	12.4 ± 5.0	0.02
Weekly Bristol stool form scale score, mean ± SD	2.1 ± 0.8	5.3 ± 0.8	0.02

3.3.1.2. Colonic gas and colonic volume

Colonic gas, as measured by MRI, rose steadily during the course of the study period, reaching a maximum at 360 minutes (**Figure 20A**). The AUC of the change in colonic gas from fasting to 360 minutes [AUC gas, the primary endpoint) was significantly affected by the fibre given (**Figure 20B**) (Friedman test, *p*=0.005). Inulin caused the greatest rise in colonic gas (AUC gas 3145 (848-6502) ml.min) which was significantly reduced by the co-administration of inulin and psyllium (618 (62-2345) ml.min, *p*=0.02), the latter not being significantly different than dextrose (*p*>0.99).

Total colonic volume remained unaltered by dextrose, rose steadily for psyllium and inulin alone but the greatest rise occurred with inulin and psyllium co-administration (**Figure 21** and

Table 8). RM ANOVA showed a significant difference between test drinks (p=0.01); post hoc analyses showed greater AUCs for inulin (353.8 ± 85.0 L.min) and inulin and psyllium co-administration (362.5 ± 109.0 L.min) than dextrose (300.2 ± 73.0 L.min, p=0.002 and p=0.005 respectively). Since fermentation would be predicted to be maximal in the ascending colon at 360 minutes we also measured just the ascending colon which clearly showed the differences between the test drinks (p=0.0016). Co-administration of inulin and psyllium significantly increased colonic volume at 360 minutes compared to inulin alone or psyllium alone (p=0.0026 and p=0.018 respectively, **Figure 21B**).

*Table 8 - Colonic volumes (mean ± SD of area under the curve, in L.min) of all participants after each test drink, demonstrating a larger volume for inulin and inulin and psyllium co-administration than dextrose (indicated by *) but no differences between subtypes. p value corresponds to the difference between constipation and diarrhoea-predominant IBS subtypes.*

Test substrate	**Colonic volume (AUC, L.min)**			***P* value**
	All participants	**IBS-C**	**IBS-D**	
Inulin	353.8 ± 85.0*	319.1 ± 88.9	375.8 ± 76.2	0.153
Psyllium	336.4 ± 97.1	296.5 ± 105.7	376.4 ± 72.5	0.0802
Inulin and psyllium	362.5 ± 109*	330.6 ± 108.3	384.2 ± 105.1	0.278
Dextrose	300.2 ± 73	275.5 ± 80.3	322.6 ± 56.4	0.153

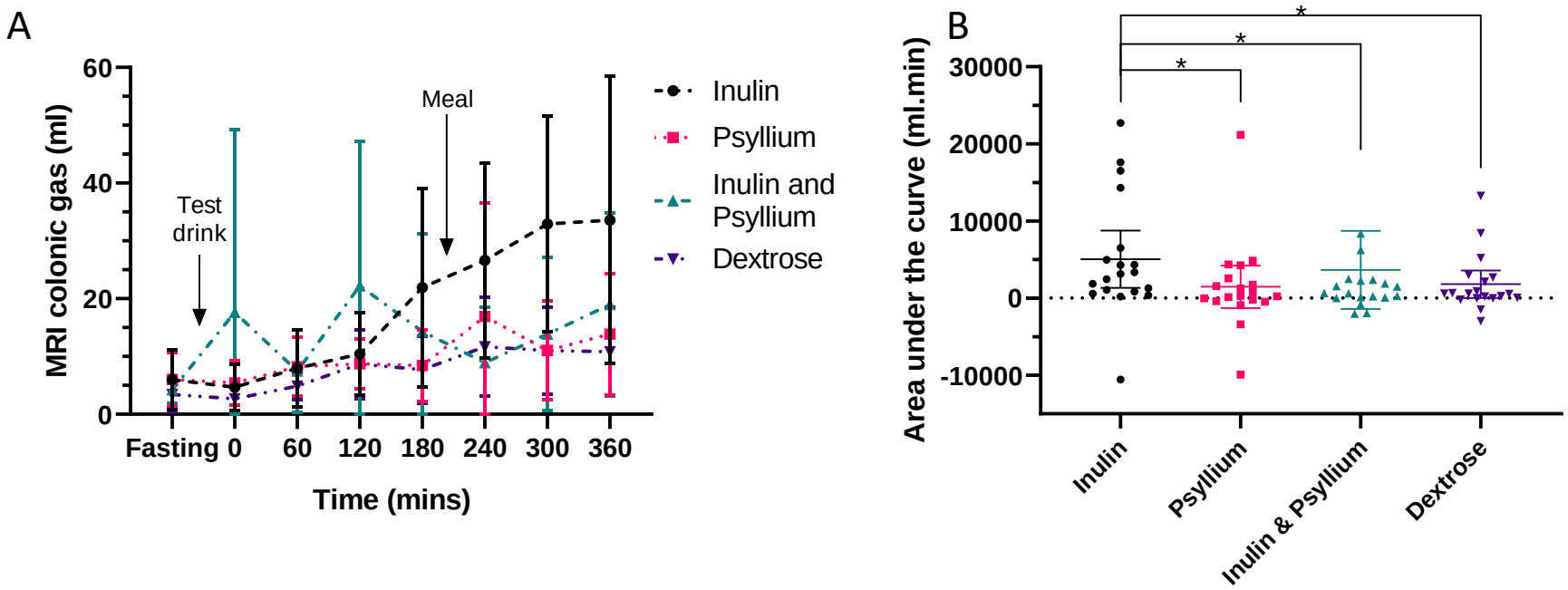


*Figure 20 - Change in MRI colonic gas from fasting values for each test drink (n=19). (A) Time course over the duration of the study, showing significantly greater gas production for inulin compared to psyllium, dextrose and inulin and psyllium co-administration at 360 minutes (p=0.0097). (B) Area under the curve (AUC) for individual participants after each test drink (on the x axis). Inulin produced a significantly larger AUC than the other 3 test drinks. Data shown are mean ± 95% CI. *p<0.05*

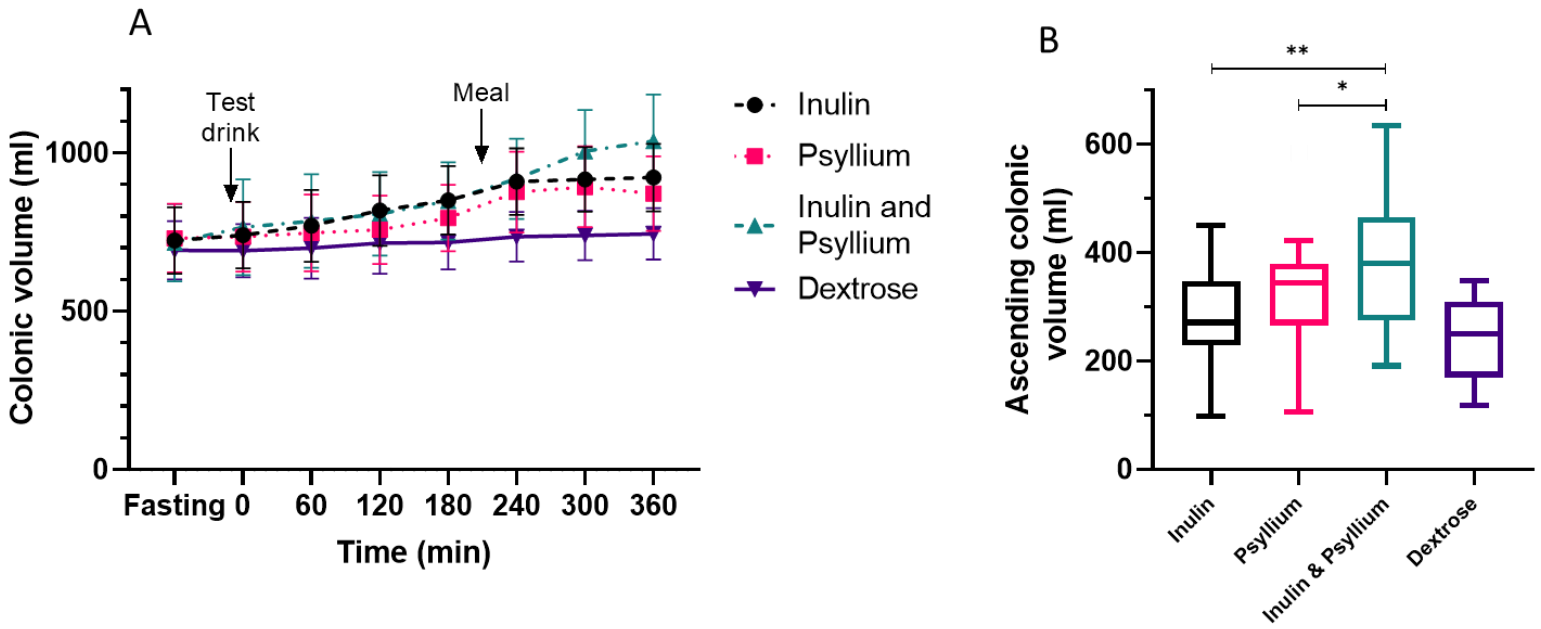


Figure 21 - Colonic volumes after the test drink (n=19). (A) Total colonic volumes after dextrose remained stable but rose significantly after the other 3 test drinks. AUC for both inulin and inulin and psyllium co-administration were significantly greater than dextrose, p=0.002 and p=0.005, respectively. Data shown are mean ± 95% CI. (B) Ascending colonic volume 360 minutes after each test drink (on the x axis), represented by Tukey box and whiskers plot. Inulin and psyllium co-administration significantly increased the volume compared to inulin or psyllium alone (p=0.003 and p=0.02 respectively).

3.3.1.3. Breath hydrogen

Psyllium produced no discernible increase in breath hydrogen, whereas breath hydrogen began to rise within 60 minutes of ingestion of inulin (**Figure 22**). Co-administration of inulin and psyllium both slowed and reduced the observed rise in breath hydrogen during the study period.

Significant differences were found in AUC breath hydrogen between the test drinks (p<0.0001). Post hoc analysis showed AUC for breath hydrogen after inulin, 7230 (3255-17910) ppm.hr, was significantly greater than after both psyllium (555 (180-915) ppm.hr, p<0.0001) and dextrose (750 (180-1140) ppm.hr, p<0.0001). Co-administration of inulin and psyllium reduced the AUC to 1035 (360-4320) ppm.hr, which was significantly lower than inulin alone, p=0.0065.

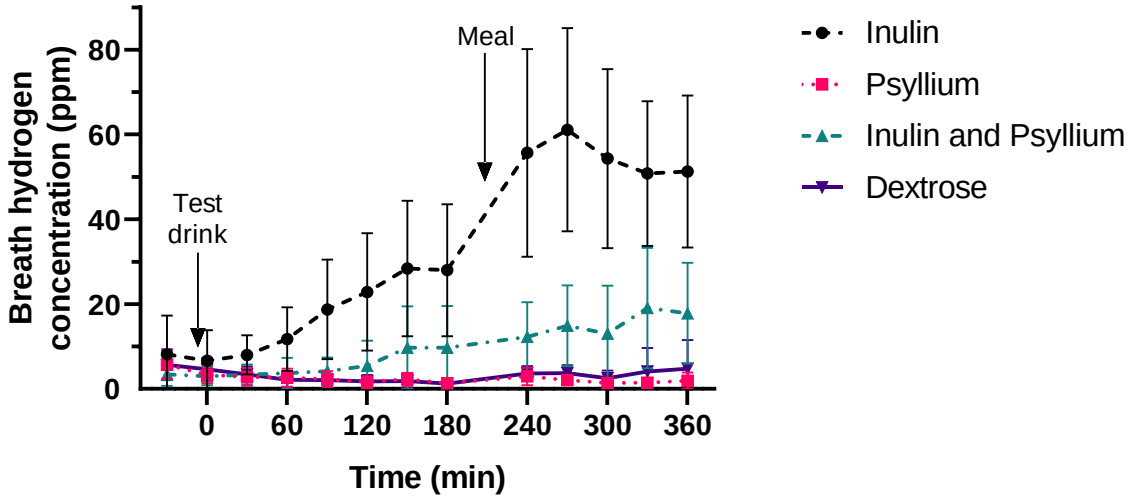


Figure 22 - Breath hydrogen at fasting and every 30 minutes after the test drink (n=19). Breath hydrogen rose steadily after 30 minutes with the inulin test drink but area under the curve analysis demonstrated a significantly reduced rise when inulin was co-administered with psyllium, p=0.0065. Both psyllium and dextrose produced significantly less breath hydrogen than inulin alone, both p<0.0001. Data shown are mean ± 95% CI

Using the time for breath hydrogen to rise more than 10ppm from baseline we can estimate that inulin, when given alone passed rapidly through the small bowel reaching the caecum within 143 ± 86mins (n=19). This was

apparently slower at 176 ± 86mins (n=8) for co-administered inulin and psyllium. However it should be noted that in 11 subjects, breath hydrogen did not rise by 10ppm and so transit time could not be calculated nor could a transit time for psyllium nor dextrose alone.

3.3.1.4. Small bowel water content

Test drinks containing psyllium resulted in greater SBWC than the dextrose control. Significant differences were found in SBWC AUC between test drinks (p<0.0001); inulin alone (47.2 ± 22.9 L.min) did not significantly differ from dextrose ((42.4 ± 23.4 L.min, p=0.63) but co-administration of inulin and psyllium caused a significant increase (86.9 ± 42.8 L.min, p=0.0007). Psyllium alone induced the greatest SBWC (102.7 ± 49.1 L.min), significantly greater than inulin and psyllium co-administration (p=0.03). (**Figure 23**).

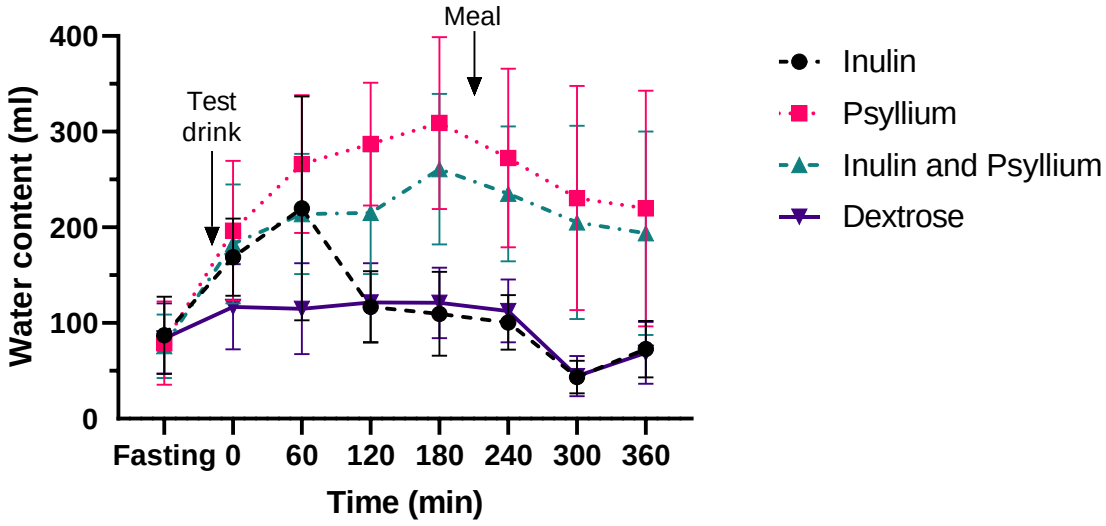


Figure 23 - Small bowel water content (SBWC) rose after test drinks containing psyllium but hardly at all after dextrose. Comparison of areas under the curve (AUCs) showed psyllium was associated with the highest values, significantly greater than inulin plus psyllium, p=*0.03. Adding psyllium to inulin produced a significant rise in SBWC,* p=*0.0007. Inulin and dextrose were not significantly different. Data shown are mean ± 95% CI.*

3.3.1.5. IBS symptoms

Symptoms were typically mild to moderate, the main symptom being flatulence which rose steadily during the study period for all test drinks. Six hours post ingestion there were significant differences between test drinks for flatulence (p=0.01); inulin leading to more severe symptoms than psyllium (1.2 ± 0.8 *versus* 0.5 ± 0.5, p=0.04). Co-administration of inulin and psyllium resulted in intermediate values which were not significantly different from inulin alone (see **Figure 24**).

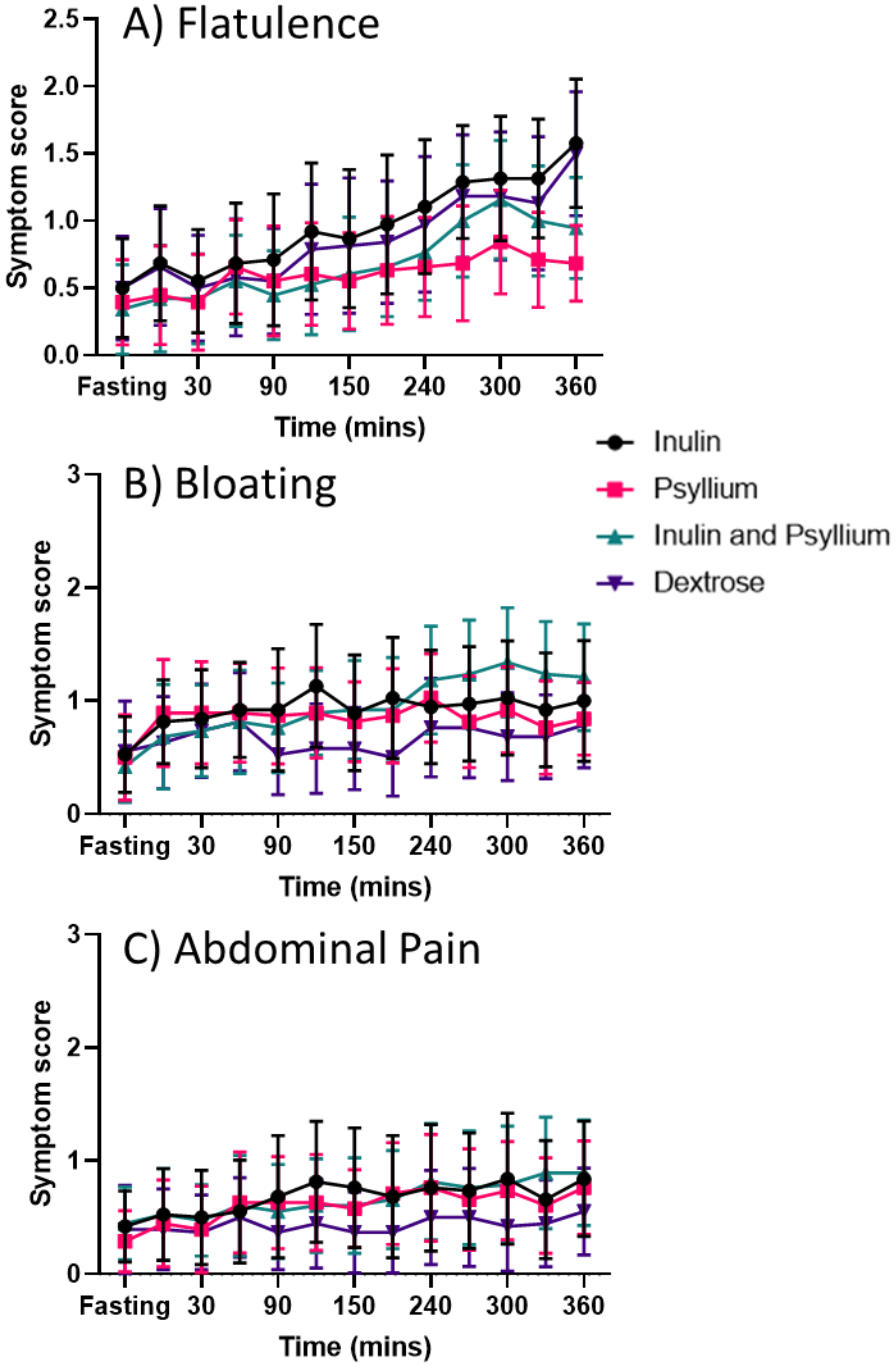


Figure 24 – Reported gastrointestinal symptoms, fasted and every 30 minutes after the test drink for A) flatulence, B) bloating and C) abdominal pain. Six hours post ingestion there were significant differences between test drinks for flatulence (p=0.01); inulin leading to more severe symptoms than psyllium (1.2 ± 0.8 versus 0.5 ± 0.5, p=0.04). Data are mean ± 95% CI.

3.3.1.6. IBS subtype

IBS subtype influenced the AUC gas which was larger in IBS-D compared with those with IBS-C for both inulin (Mann Whitney test, *p*=0.01) and inulin with psyllium co-administration (*p*=0.03), but not for dextrose or psyllium alone (see **Figure 25** and

Table 9). No differences between IBS subtypes were found for colonic volumes, breath hydrogen, SBWC or reported symptoms (see

Table 9).

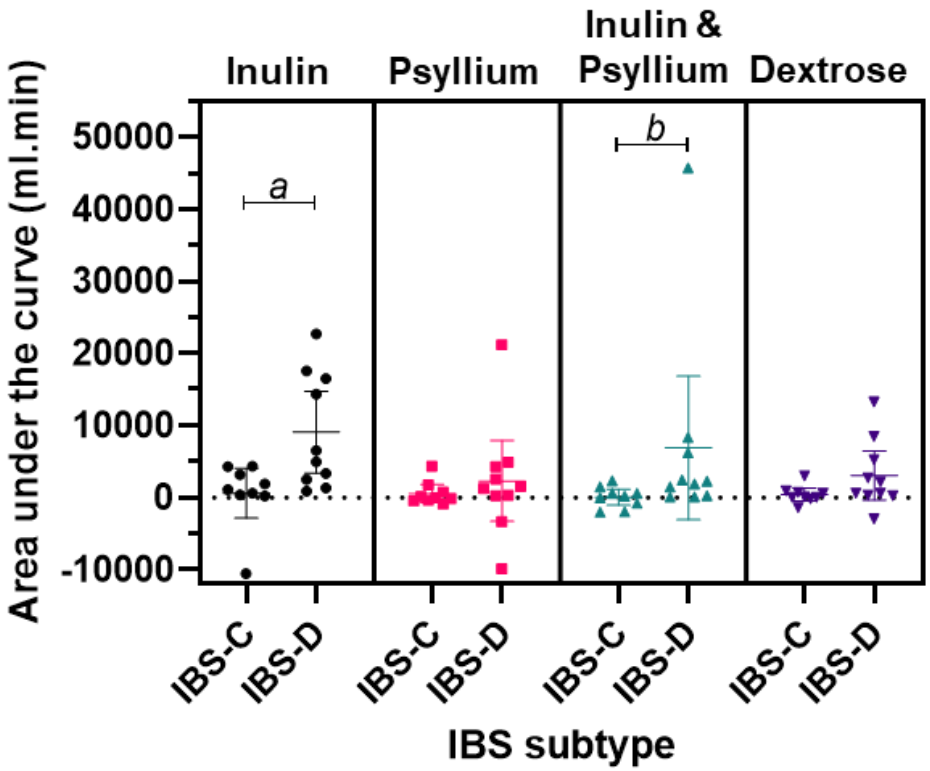


Figure 25 - Area under the curve (AUC) change in MRI colonic gas from fasting values for IBS-C versus IBS-D (n=19), demonstrating significantly greater AUCs for IBS-D after both inulin and inulin and psyllium co-administration test drinks. Data shown are mean ± 95% CI. a, p=0.01; b, p=0.03.

Table 9 - Exploratory analysis of endpoints divided by IBS subtype. P values relate to testing for differences between the subtypes using Mann-Whitney tests or unpaired t-tests as appropriate. AUC, area under the curve; IBS-C, constipation-predominant irritable bowel syndrome; IBS-D, diarrhoea-predominant irritable bowel syndrome.

	Test drink	**IBS-C**	**IBS-D**	***p* value**
MRI colonic gas change from fasting AUC (ml.min)	Inulin, median (IQR)	1080 (282-3717)	5728 (2172-16772)	0.01
	Psyllium, median (IQR)	-12 (-429-1255)	1402 (-660-4418)	0.24
	Inulin and Psyllium, median (IQR)	212 (-1400-1063)	2057 (198-6742)	0.03
	Dextrose, mean ± SD	416 ± 1186	3063 ± 4750	0.12
Colonic volume AUC (L.min)	Inulin, mean ± SD	319.1 ± 88.9	375.8 ± 76.2	0.15
	Psyllium, mean ± SD	296.5 ± 105.7	376.4 ± 72.5	0.08
	Inulin and psyllium, mean ± SD	330.6 ± 108.3	384.2 ± 105.1	0.28
	Dextrose, mean ± SD	275.5 ± 80.3	322.6 ± 56.4	0.15
Small bowel water content AUC (l.min)	Inulin, mean ± SD	46.7 ± 28.2	47.6 ± 18.6	0.45
	Psyllium, mean ± SD	112.3 ± 55.9	94.0 ± 43.2	0.43
	Inulin and Psyllium, mean ± SD	94.2 ± 50.0	80.2 ± 36.5	0.32

	Dextrose, mean ± SD	41.9 ± 32.7	42.8 ± 12.3	0.94
Breath hydrogen AUC (ppm.hr)	Inulin, mean ± SD	12432 ± 9354	12623 ± 12331	0.97
	Psyllium, median (IQR)	900 (390-1080)	615 (300-949)	0.33
Breath hydrogen AUC (ppm.hr)	Inulin and Psyllium, median (IQR)	1290 (510-6330)	1133 (338-4913)	0.62
	Dextrose, median (IQR)	1065 (653-2055)	608 (41-1958)	0.32
Flatulence score AUC (arbitrary unit.min)	Inulin, median (IQR)	150 (79-443)	281 (203-773)	0.16
	Psyllium, median (IQR)	135 (64-315)	206 (62-319)	0.92
	Inulin and Psyllium, median (IQR)	105 (41-341)	270 (113-465)	0.29
	Dextrose, mean ± SD	253 ± 278	406 ± 364	0.32
Bloating score AUC (arbitrary unit.min)	Inulin, median (IQR)	15 (8-420)	379 (219-958)	0.04
	Psyllium, mean ± SD	262 ± 268	407 ± 321	0.30
	Inulin and Psyllium, median (IQR)	173 (49-338)	450 (191-840)	0.08
	Dextrose, median (IQR)	98 (0-236)	278 (81-435)	0.11

Abdominal pain score AUC (arbitrary unit.min)	Inulin, median (IQR)	30 (0-64)	184 (98-939)	0.01
	Psyllium, median (IQR)	38 (8-184)	218 (43-572)	0.09
	Inulin and Psyllium, median (IQR)	0 (0-143)	289 (73-600)	0.03
	Dextrose, median (IQR)	0 (0-53)	120 (6-373)	0.14

3.3.2. *in vitro* fermentation study

Bacterial fermentation of all test fibres and the placebo dextrose resulted in the production of gas which was greatest for dextrose (AUC 4500 ± 1144 ml.hr), significantly higher than for inulin or psyllium alone (p<0.001 for both (**Figure 26**)).

Of the substrates likely to reach the colon intact, co-administration of inulin and psyllium to the fermentation model generated the most gas (AUC of 2991 ± 1169 ml.hr) whereas inulin alone produced less gas (1122 ± 758 ml.hr), although the difference was not significant, p=0.056. Psyllium resulted in the lowest amount of gas production (324 ± 146 ml.hr), which was significantly lower than co-administration of inulin and psyllium (p=0.02). The calculated values for inulin and psyllium fermentation (the sum of inulin alone plus psyllium alone) gave a lower mean AUC (1446 ± 704 ml.hr) than that measured during co-administration, although this difference was not significant (p=0.1).

There were no differences in *in vitro* gas production for any fibre between IBS subtypes. Gas production *in vitro* and *in vivo* were both highly variable but correlated for inulin (r^2=0.58, p=0.03) but not for psyllium (r^2=0.14, p =0.35) or co-administration of inulin and psyllium (r^2=0.003, p=0.89 (**Figure 27**)).

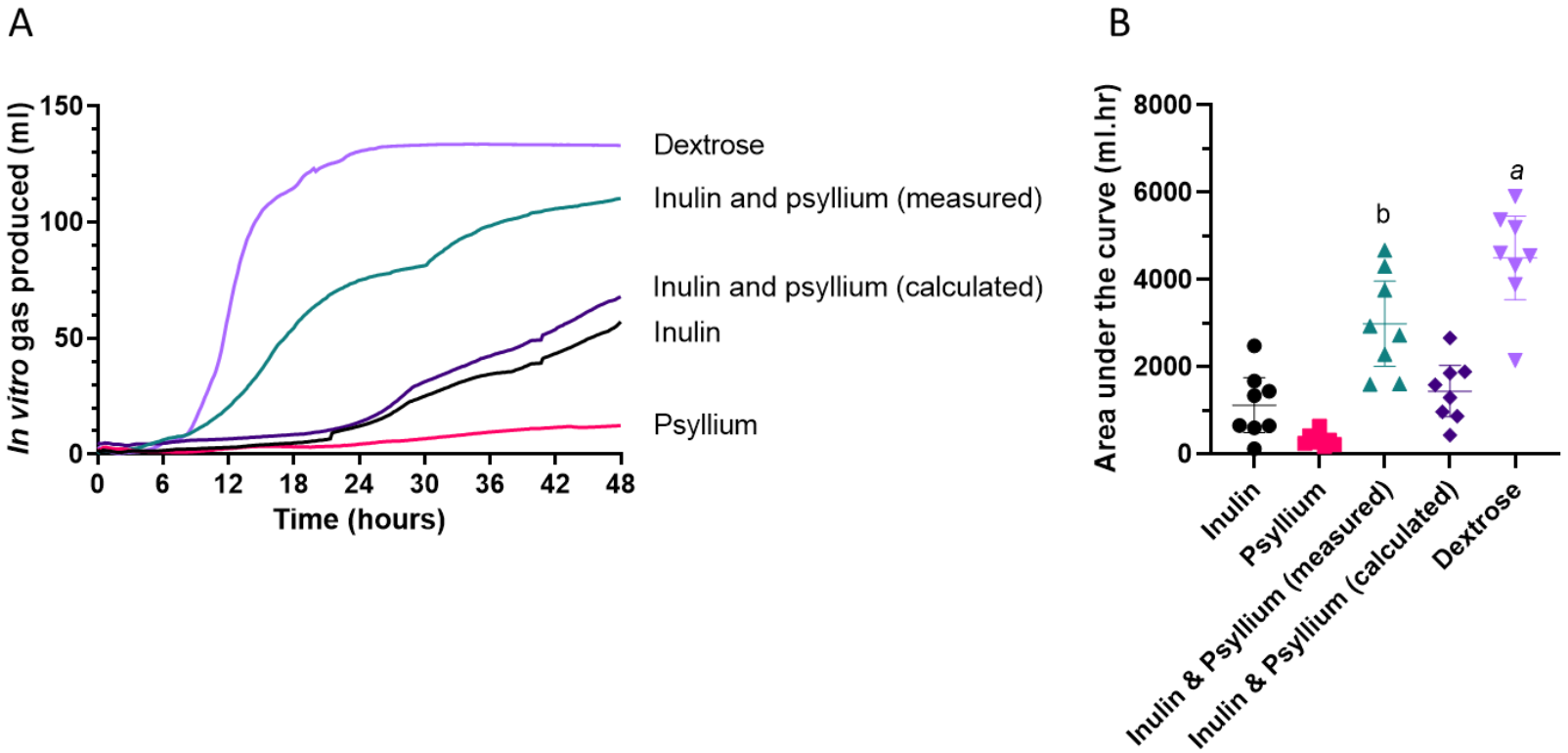


Figure 26 - 48 hours of in vitro gas production of substrates inoculated using stool from patients who participated in the human MRI study (n=8). (A) Median cumulative gas production showing an early rapid fermentation of dextrose followed by the inulin and psyllium combination. (B) Area under the curve (AUC) of in vitro gas production for each test drink (on the x axis). Data shown are mean ± 95% CI. a, Dextrose AUC is significantly greater than inulin (p=0.0008), psyllium (p=0.0001), and calculated inulin and psyllium (p=0.001). b, Inulin and psyllium combination AUC is significantly greater than psyllium (p=0.002).

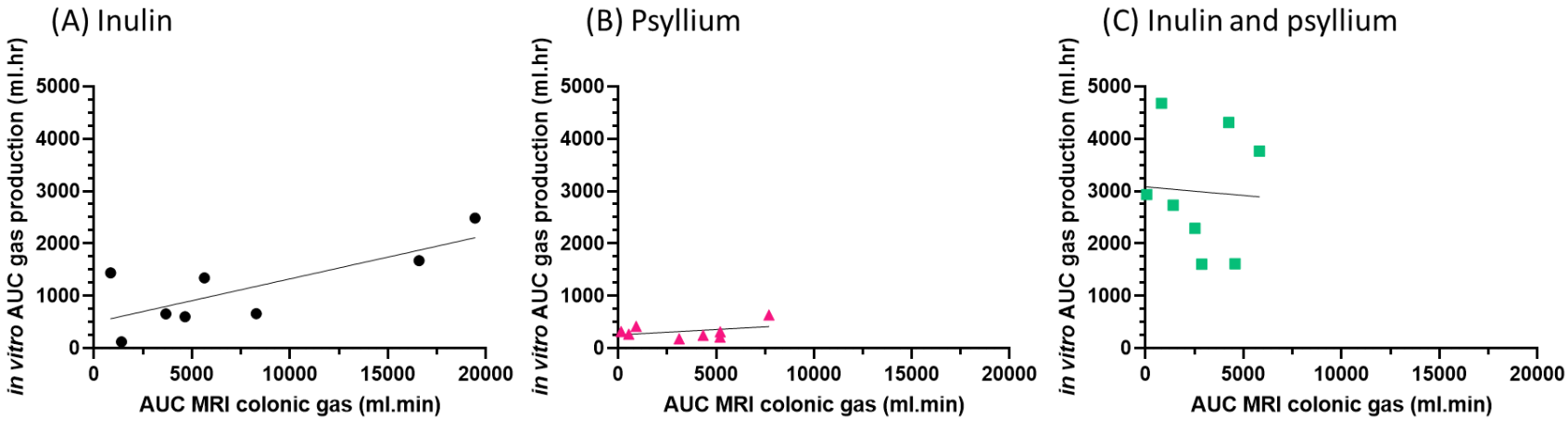


Figure 27 – Correlations between in vitro *area under the curve (AUC) gas production (ml.hr) and* in vivo *AUC colonic gas (ml.min) as assessed by MRI for A) inulin* r^2*=0.58,* p*=0.03, B) psyllium* r^2*=0.14,* p*=0.35 and C) inulin and psyllium in combination* r^2*=0.003,* p*=0.89.*

3.3.2.1. Viscosity

Data shows that the addition of inulin to the psyllium did not alter the viscosity compared to psyllium alone (see **Figure 28A**). Varying the temperature from room to body temperature did not affect results (see **Figure 28B**). The 4% solution was more viscous than the 0.5% solution, but both solutions yielded gel structures as demonstrated by an increase in G' (storage modulus) compared to the G" (loss modulus). These gels also behaved the same whereby they lost their structure, becoming a liquid at the same oscillation strain (see **Figure 28C**).

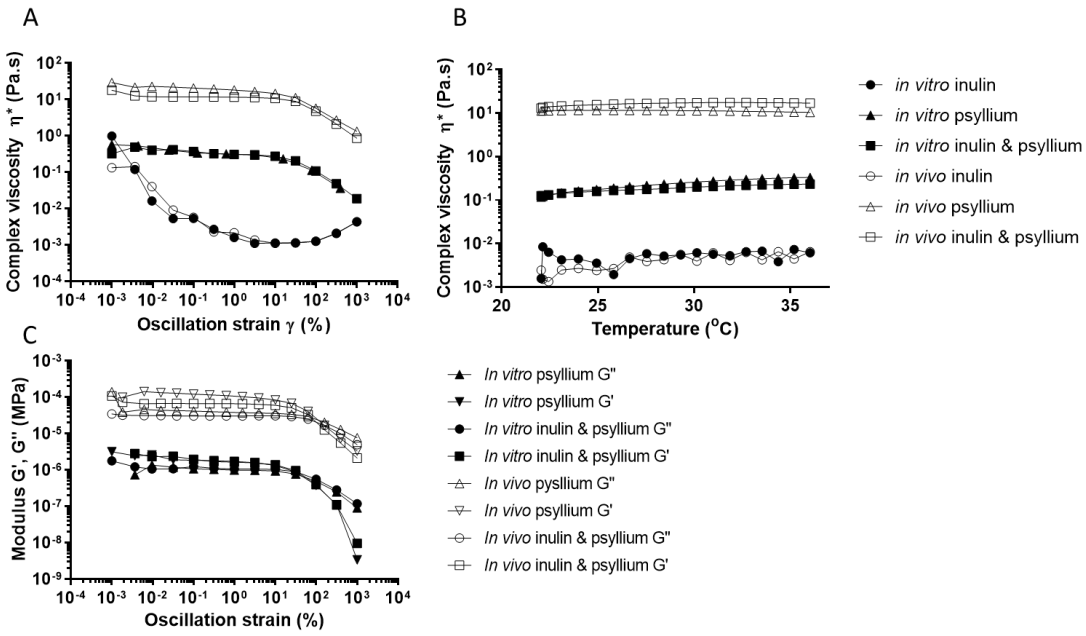


Figure 28 - Rheometric analysis of test fibres. (A) Viscosity at varying oscillation strains, (B) viscosity at varying temperatures from room temperature to body temperature, and (C) shear modulus at varying oscillation strains. in vitro solution concentration was 0.5% while in vivo was 4% solution. Parameters for data within oscillation strain sweeps ranging from 10^{-3} to 10^{3} at 37°C and a frequency of 6.28 rad/s A) viscosity of test fibres at different oscillations.

3.4. Discussion

Our study confirms previous data[130,273] from healthy volunteers and IBS patients that inulin has little effect in the small bowel but is rapidly fermented in the colon causing a rise in colonic gas and breath hydrogen. We additionally confirmed our original hypothesis that both these effects can be largely inhibited by concurrent ingestion of psyllium.

Whether fermentation produces gas depends on complex cross-feeding networks in which some bacteria degrade inulin while others such as Bifidobacteria utilise the products to generate short chain fatty acids (SCFAs) including butyrate[345]. The production of H_2, CH_4 and CO_2 after ingestion of large amounts of inulin reflects its rapid fermentation in an anaerobic environment. It is possible that slowing fermentation would allow more efficient metabolism with transfer of reducing equivalents to acetate and other SCFAs rather than gas. We have previously shown both *in vitro* and *in vivo* that psyllium is poorly fermented and produces a much smaller rise in breath hydrogen than equal doses of more readily fermentable fibres including wheat bran and the prickly pear cactus fibre, nopal[282]. It follows therefore that adding psyllium to inulin would not be expected to increase the gas burden but why it reduces colonic gas requires further explanation.

Our *in vitro* studies were designed to explore the possibility that combining inulin and psyllium would inhibit fermentation directly. However this actually enhanced gas production in the model where all substrate was added at the beginning and well mixed with the stool microbiota. Thus the effect we observed *in vivo* was not due to any direct inhibition of colonic fermentation by psyllium. Furthermore, we showed that the inulin and psyllium combination had the same high viscosity seen with psyllium alone which did not per se inhibit fermentation. We hypothesise therefore that the *in vivo* effect is due to a combination of the reduction in the rate of delivery of inulin to the colon together with restriction of mixing of microbiota with the inulin bolus as it enters the colon.

This hypothesis is supported by our observation in subjects who produced hydrogen, that oro-caecal transit was delayed by around 23% by psyllium. When we simulated *in vitro* the concentrations of psyllium likely to occur in the colon we found a high viscosity, 10Pa.s, which is likely to impede mixing in the colon and thereby delay access of colonic bacteria to the inulin. Previous MRI studies show that the arriving insoluble material, which coming from the small bowel has a low bacterial count, is distributed largely in the middle of the colon[213]. Tagging studies show that within the haustra, close to the colonic wall, there is much less movement and mixing which may favour anaerobic fermentation[346]. Psyllium might impair movement from the main stream into the haustral pockets and hence delay fermentation.

While the dose of inulin we used (20g) substantially exceeds the UK average daily intake which is around 4g, the dose is less than the 25-30g recommended as daily intake of total dietary fibre so for a mechanistic study it is not unphysiological. Ileostomy[347] and intubation studies[25] show that >85% of ingested inulin enters the colon intact where it is largely completely metabolised since very little is excreted in stool[348]. Inulin supplements selectively increase Bifidobacterium and Anaerostipes[349,350], genera which comprise a quarter of the colonic bacteria and in concert with other bacteria are capable of rapidly fermenting inulin and producing large amounts of short chain fatty acids, especially butyrate which is thought to underlie its potential health benefits.

The dose of psyllium we used, 20g would be expected to transit through the small intestine largely intact where it would be distributed within approximately 400-600 ml of the ascending and transverse colon[271]. The unusually high side-chain density of psyllium limits bacterial access so that it is poorly fermented in the colon compared with other viscous fibres[351,352], producing little gas as the current study confirmed. Although time constraints on scanning prevented study of events later in the day, from other studies we can expect the fermentation to continue slowly during transit to the descending colon. Further benefits from delaying fermentation would include

shifting SCFA production to more distal colon where the highest cancer risk lies. This has been seen in pigs with another laxative fibre, wheat bran[353], a feature confirmed in humans using wheat bran and resistant starch[354]. The water trapping softens stool leading to increased stool frequency in both healthy and constipated subjects[213], without changing transit despite an increase in colonic volume. This is unexplained since distension of the colon by balloon[355] or osmotic laxatives like macrogols[326] stimulate propulsive colonic contractions. We can speculate that psyllium reduces access to colonic sensors of luminal stimulants such as bile acids and faecal proteases thus avoiding the cramps and diarrhoea which can occur with other laxatives. A recent study using xyloglucan supports the idea that enhancing the barrier between lumen and mucosa by dietary means has promise in the treatment of IBS[356]. Further studies are plainly warranted of this simple and safe method of alleviating symptoms in IBS patients.

3.4.1. Study Limitations

Potential mechanisms for the reduction in colonic gas and breath hydrogen seen with co-administration have been discussed above, however a significant limitation is the measurement of transit in the study participants. A rise in breath hydrogen more than 10ppm was only demonstrated in 8 subjects when inulin and psyllium were co-administered, with a high degree of variability. Analysis of the MRI images failed to reliably demonstrate a visible head of meal in the caecum as the fibre was not ingested with a meal. Thus a more robust assessment of transit, such as with MRI transit markers[279], would have given greater power to assess if changes to transit times were present.

Participants had generously given 7 hours of their time on four occasions for this study. Owing to the large time commitments, unfortunately there was a higher degree of drop outs and subsequently we were one participant short of the target from power calculations.

Analysis of the data showed IBS-D participants had larger AUCs for colonic gas than patients with IBS-C. This may be a reflection of the slower transit often seen in IBS-C, so a study looking at MRI parameters for a longer duration than 6 hours may have seen a delayed rise in gas as a result. Another, more pragmatic alternative would be for participants to continue their symptom recording at home for a day after the study to see if subtype differences occurred.

We expected inulin fermentation to be reduced when co-administered with psyllium, and have reasoned that this is demonstrated by changes in breath hydrogen and MRI colonic gas. Another, more direct measure of this would have been to quantify the amount of inulin remaining in the stool and might have helped the interpretation of the data.

MRI gas measurements are based on selecting adjacent voxels with signal intensity below a threshold (defined from an area outside the patient) which occupy volumes greater than 0.1ml, so will miss smaller, more homogenously dispersed gas. The values therefore are the minimum amount of gas present. The large inter-individual variation in gas volumes can in part be explained by our observation that large gas pockets can rapidly move through the colonic regions and be expelled, causing large differences in gas volumes seen on MRI from one scan to the next.

3.5. Conclusion

Our small mechanistic study has demonstrated that adding psyllium to inulin reduces gas production in IBS patients and suggests that by choosing diets with adequate amounts of viscous fibre, patients may be able to obtain the health benefits of high fibre diets without exacerbating their IBS symptoms, particularly flatulence. Larger clinical trials are now indicated to confirm the clinical value of such mechanistic insights.

4. Predicting responders to ondansetron for the treatment of diarrhoea-predominant irritable bowel syndrome

This chapter aims to find features of patients suffering from IBS-D who show a beneficial response to the 5-hydroxytryptamine receptor 3 antagonist, ondansetron. This is achieved by further analysis of a previously completed clinical trial[154] of 125 IBS-D patients in a double-blind trial of ondansetron versus placebo. The work in this chapter was published in Alimentary Pharmacology & Therapeutics in June 2019[357].

4.1. Background

When patients with irritable bowel syndrome with diarrhoea (IBS-D) are asked to report their most bothersome symptoms, erratic bowel habit and urgency are the most common[358]. Urgency and the associated occasional faecal incontinence causes significant distress and anxiety, which substantially impairs their quality of life. Serotonin (5-hydroxytryptamine (5-HT)), a neurotransmitter in the gut has many effects which are relevant to this aspect of IBS, stimulating intestinal secretion and colonic motility[37]. 5-HT receptor 3 antagonists (5HT3RAs) including alosetron, cilansetron and ramosetron which block these effects and slow whole gut transit have been shown in meta-analysis to be effective treatments for IBS-D[359]. While alosetron has been withdrawn because of ischaemic colitis (IC)[232], ramosetron, a potent highly specific 5HT3RA has been shown to be similarly effective and recently another 5HT3RA, ondansetron, has also been shown to be effective without causing IC.

Previous studies which focused on post-infectious IBS (PI-IBS), 2/3^{rds} of whom have IBS-D, had shown an increased number of 5-HT containing enteroendocrine cells[96]. In animal models of PI-IBS[360] colonic mucosal 5-HT was elevated immediately after infection and 5-HIAA/5-HT ratios were increased. In contrast, serotonin transporter gene (SERT gene *SLC6A4*) mRNA expression was depressed up to 56 days post infection suggesting long lasting accelerated mucosal 5-HT turnover. This was associated with enhanced firing of afferent neurons during colonic distension, a feature which could be blocked by ondansetron. This led us to hypothesise that increased 5-HT availability in the intestinal mucosa could be the driver of symptoms and the explanation of the benefit of 5HT3RAs in IBS-D.

There are marked individual differences in responsiveness to 5HT3RAs which have been correlated with common polymorphisms in key genes governing the synthesis and reuptake of 5-HT, as well as the structure of the 5-HT receptors. Responsiveness to alosetron was shown in one trial to be greater with the homozygous l/l variant of the 5-HTTLPR (serotonin-transporter-

linked polymorphic region) of *SLC6A4*[158]. Furthermore, the gene *TPH1* encoding tryptophan hydroxylase 1, the rate limiting enzyme for serotonin synthesis in enterochromaffin cells of the gut, contains several single nucleotide polymorphisms (SNPs), including rs4537731 and rs211105, which have been reported to predict responsiveness to ramosetron[361]. Finally, the SNP of *HTR3C* rs6766410 predicts chemotherapy induced vomiting[362], which is known to be driven largely by serotonin. This led us to hypothesize that the sensitivity to ondansetron might in part be dependent on genetic variability due to polymorphisms in these genes.

We now present the biomarkers of mucosal 5-HT metabolism along with the genetic markers obtained from patients participating in our double blind crossover trial whose clinical results have been previously reported[154], aiming to further characterise IBS-D patients who respond to ondansetron therapy.

4.2. Methods

4.2.1. Design

This work further analyses results from a randomised, double-blind, placebo-controlled crossover study of ondansetron 4 mg versus placebo[154]. 125 patients with IBS-D meeting Rome III criteria[363] were recruited from gastroenterology clinics in Nottingham and Manchester and randomised to receive either 5 weeks of ondansetron then 5 weeks of placebo or placebo followed by ondansetron, with at least 2 weeks washout between. Doses were titrated for the first 3 weeks of each treatment period, maintaining a constant dose for the final two weeks. 21 Healthy volunteers (HV) were recruited for comparison. They completed the same questionnaires as IBS-D patients, underwent rectal mucosal biopsy and provided normal values for colonic transit studies.

4.2.2. Data Collection

The following personal baseline data were collected: age, sex, Hospital Anxiety and Depression Scale (HADS)[340] score, Patient Health Questionnaire 12 Somatic Symptom (PHQ-12)[341] score, and Perceived Stress Scale (PSS)[364] score. IBS Quality of Life Questionnaire[365] and IBS Severity Scoring System[366] scores were collected at baseline and at the end of each treatment period. A previously described daily stool diary[16] was used to collect stool form information (using the Bristol Stool Form Score (BSFS)[367], abdominal pain perception, urgency to defecate and abdominal bloating (all three scored on a 0-3 scale). Frequency of defecation and number of days with bloating, pain or urgency were recorded. Whole gut transit time (WGTT) was measured after 5 weeks of each treatment using the Metcalf's radio-opaque marker technique[280].

4.2.3. Biopsy analysis

All patients recruited from Nottingham were invited to undergo unprepared flexible sigmoidoscopy to obtain high rectal/sigmoid biopsies and 57 consented to this additional procedure. Two of the samples were immediately frozen in liquid nitrogen for analysis of serotonin and 5-HIAA content by HPLC, 2 in RNALater for gene expression studies, 1 for incubation at 25°C in 95% O_2 for assay of 5-HT release from a 0-30 minute period as previously described[368]. Finally, one biopsy was embedded in paraffin for routine histology to exclude microscopic colitis. Real time PCR was used to assess the relative expression of *TPH1* with respect to the house keeping gene hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). RNA was extracted and analysed as we have previously reported[369].

4.2.4. Plasma 5-HIAA

We assessed fasting plasma 5-HIAA using HPLC as previously described[41].

4.2.5. Genotyping

Blood samples were taken at visit 1. DNA was extracted from 200 µl of citrated blood using the QIAamp DNA Blood Mini Kit (Qiagen Cat. No. 51106) and subsequently genotyped for 62 patients who provided samples for the following polymorphisms: 5-HTTLPR (serotonin-transporter-linked polymorphic region) residing within the promoter region of the serotonin transporter gene *SLC6A4* as described previously[370]. Genotyping of the SNPs within the tryptophan hydroxylase gene *TPH1* rs211105 (non-coding SNP), rs4537731 (non-coding SNP), and in 5-HT_3 receptor genes including *HTR3A* c.-42C>T (rs1062613), *HTR3B* p.Y129S (rs1176744), *HTR3C* p.N163K (rs6766410) and *HTR3E* c.*76G>A (rs56109847) was carried out by the KASPar® assay (KBiosciences, Ltd, Hoddesdon, United Kingdom) using KASP by design primer mixes as recommended by the manufacturer. Thermal cycling was performed in Mastercycler vapo.protect thermal cyclers (Eppendorf). An initial 15 minute

incubation at 95°C was followed by 20 cycles consisting of 10 s at 94°C, 5 s at 57°C, and 10 s at 72°C, followed by 23 cycles consisting of 10 s at 94°C, 5 s at 57°C, and 10 s at 72°C. After thermal cycling, results were analysed using the fluorescence plate reader of the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California). 10% of the samples were repeated to ensure genotyping accuracy.

4.2.6. Responder Definition

Our original study was a pilot study, so we used stool form rather than pain as our primary endpoint since it was known to have had a much lower placebo response rate. Therefore in this paper we used the US Food and Drug Administration (FDA) definition of a 'stool consistency responder' as a 'patient who experiences a 50 percent or greater reduction in the number of days per week with at least one stool that has a consistency of Type 6 or 7 compared with baseline'[371]. Our pain assessments on a 0-3 scale did not allow us to calculate a pain responder rate according to FDA guidelines which were published after our study was initiated.

4.2.7. Data analysis

Analysis was performed using Graphpad Prism version 7.0c (GraphPad Software, La Jolla California, USA). Efficacy parameters were calculated for each patient as the difference in the endpoints measured in the last 2 weeks of the ondansetron and placebo periods.

Baseline and rectal biopsy data were analysed for differences between patients and healthy volunteers using unpaired t-tests and Mann-Whitney tests on parametric and non-parametric data, respectively. Subsequent analysis based on their stool form responder status and mean ondansetron dose was done as below.

Genotype data was correlated with stool form responder status, baseline clinical features, biopsy results, final ondansetron dose, WGTT and *TPH1* mRNA expression. Chi-squared tests were performed for stool form responder status, and one-way ANOVA and Kruskal-Wallis tests were performed on parametric and non-parametric data respectively.

4.3. Results

4.3.1. Participants

Healthy volunteers were age and sex matched to patients, mean patient and volunteer ages were 41 and 43 years and percentage female were 71% and 76% respectively. As shown in **Table 10** the 125 patients taking part in the trial were as expected, more anxious, more stressed and showed greater somatic symptoms with significantly higher HAD, PSS and PHQ-12 scores than HV. There were no differences between patients consented for biopsy and those who did not. As required for entry into the trial[154] IBS-D patients reported significantly higher bowel frequency.

Table 10 - Demographics of study participants. Data are mean (SD), unless stated. P values, where significant, demonstrate differences between the patient population and healthy volunteers with no difference between the two patient groups. P *values are obtained from one way ANOVA and Kruskal-Wallis tests for parametric and non-parametric data, respectively*

Variable	**Patients without biopsy (n=68)**	**Patients with biopsy (n=57)**	**Heathy volunteers (n=21)**	***P* value**
Age	40 (12)	42 (12)	43 (18)	0.59
Sex (women), n (%)	53 (78)	36 (63)	16 (76)	0.17
Patient Health Questionnaire 12	8 (3.5)	7.3 (3.7)	2.2 (1.8)	<0.0001
Anxiety	9.8 (4.3)	9.5 (4.8)	5.2 (2.7)	0.0001
Depression, median (IQR)	5.5 (3-10)	4 (2-8.8)	1 (1-2)	<0.0001
Hospital Anxiety and Depression Scale	16.3 (7.4)	14.8 (8.2)	6.8 (3.3)	<0.0001
Perceived Stress Scale	19 (7.5)	17.5 (7.9)	11.2 (6.0)	0.0003
bowel frequency, median (IQR)	2.6 (1.9-4)	2.8 (2-4)	1.1 (1-1.4)	<0.0001
Stool form	5.4 (0.8)	5.4 (0.6)	3.5 (0.7)	<0.0001

4.3.2. Rectal Biopsies

Rectal biopsies were obtained from 57 of the 125 patients completing the trial and 21 HVs. Biopsy 5-HIAA levels were significantly higher in IBS-D patients as were the 5-HIAA /5-HT ratios (see **Figure 29**). There were however no significant differences between patients and controls for biopsy 5-HT nor 5-HT release in the first 30 minutes (see

Table 11).

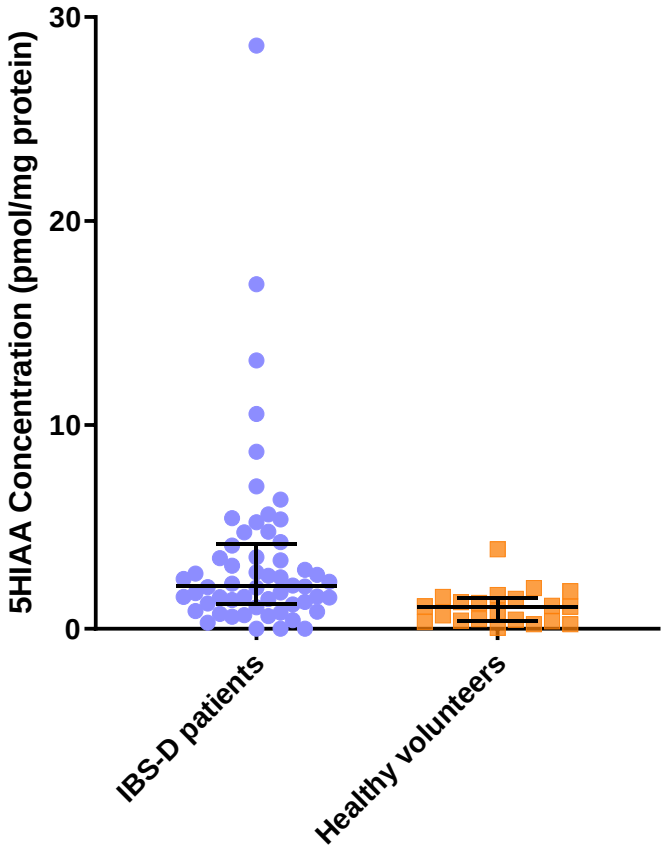


Figure 29 - Colonic biopsy 5-HIAA concentrations in IBS-D patients and healthy volunteers. Median and IQR shown. 5-HIAA: 5-Hydroxyindoleacetic acid

Table 11 - Colonic biopsy results for IBS-D patients and healthy volunteers. Data are median (IQR). P values obtained from Mann-Whitney tests. 5-HT: serotonin, 5-HIAA: 5-Hydroxyindoleacetic acid

Variable	**Patients (n=57)**	**Healthy volunteers (n=21)**	***P* value**
Biopsy 5-HIAA (pmol/mg protein)	2.1 (1.2-4.2)	1.1 (0.4-1.5)	0.0001
Biopsy 5-HIAA/5-HT	0.06 (0.03-0.16)	0.02 (0.02-0.05)	0.0038
Biopsy 5-HT (pmol/mg protein)	31.2 (20.1-46.0)	39.1 (16.5-46.7)	0.9288
Biopsy 5-HT release in 30mins (pmol/mg wet weight)	17.2 (13.5-23.1)	23.8 (13.8-44.2)	0.078
Biopsy 5-HT release in 30mins/ Biopsy 5-HT content	0.8 (0.3-1.1)	0.8 (0.2-2.2)	0.851

4.3.3. Plasma 5-HIAA

Despite higher biopsy levels, median plasma 5-HIAA (nmol/l) was significantly lower in patients versus healthy volunteers (16.2 *versus* 20.8, $p<0.0001$**;** see **Figure 30**).

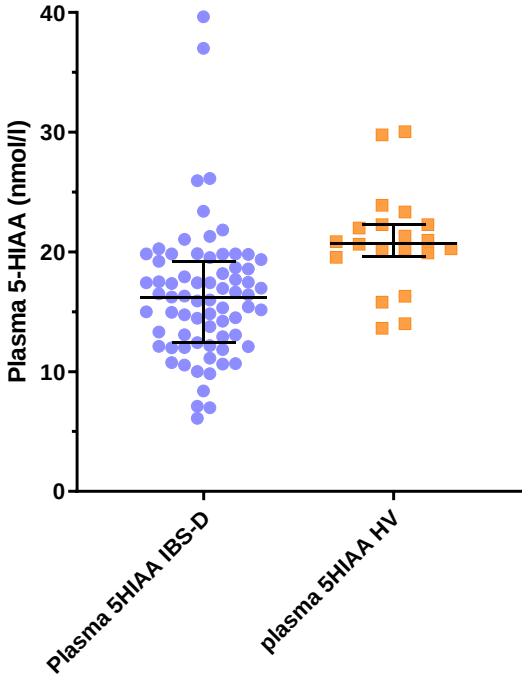


Figure 30 - Plasma 5-HIAA concentrations in IBS-D patients and healthy volunteers. Median and IQR shown. 5-HIAA: 5-Hydroxyindoleacetic acid

4.3.4. Stool form responders

107/125 IBS-D patients completed daily stool diaries to allow assessment of response, of which 82 patients met FDA criteria for stool form responders. As shown in **Table 12** they had significantly lower baseline abdominal pain scores which occurred on fewer days per week and reported lower urgency scores on fewer days per week. Responders tended to be younger, with lower HAD scores and have a slower colonic transit; however these differences failed to reach conventional statistical significance. There were no significant differences in either baseline stool form or frequency.

Table 12 - Demographics, stool diary results and markers of serotonin turnover of stool form responder and non-responder patients. Data are mean (SD) unless stated. P values obtained from unpaired t tests and Mann-Whitney tests for parametric and non-parametric data respectively. 5-HT: serotonin, 5-HIAA: 5-Hydroxyindoleacetic acid. †Numbers in each group: n=40 stool form responder; n=9 stool form non-responder. ‡Numbers in each group: n=53 stool form responder; n=10 stool form non-responder

Variable	Stool form responder (n=82)	Stool form non-responder (n=25)	*P* value
Age	40 (12)	45 (11)	0.054
Sex (female), n (%)	61 (74)	16 (64)	0.45
Hospital Anxiety and Depression Scale	14.7 (7.0)	17.8 (8.3)	0.077
Patient Health Questionnaire 12	7.4 (3.4)	8.2 (3.9)	0.32
Placebo whole gut transit (hours), median (IQR)	18 (9-31)	10 (6.3-24.8)	0.063
Baseline days with abdominal pain, median (IQR)	5 (3-7)	7 (5-7)	0.018
Baseline average abdominal pain score	1.2 (0.7)	1.8 (0.8)	0.0023
Baseline days with urgency, median (IQR)	6 (4.8-7)	7 (6.5-7)	0.0014
Baseline average urgency score, median (IQR)	1.4 (1-2)	2.4 (1.6-2.6)	<0.0001
Baseline days with bloating, median (IQR)	6 (3-7)	6 (3.5-7)	0.50
Baseline average bloating score	1.3 (0.8)	1.4 (0.9)	0.44
Baseline average stool form	5.3 (0.7)	5.5 (0.8)	0.25

Baseline average stool frequency, median (IQR)	2.6 (1.9-3.9)	2.9 (1.9-3.9)	0.73
Biopsy 5-HT (pmol/mg protein), median (IQR) †	28.6 (18.3-42)	44.3 (25.5-65.7)	0.064
Biopsy 5-HIAA (pmol/mg protein), median (IQR) †	2.1 (1-4.1)	1.6 (1-4)	0.66
Biopsy 5-HIAA /5-HT, median (IQR) †	0.06 (0.03-0.20)	0.03 (0.02-0.08)	0.27
Plasma 5-HIAA (nmol/l), median (IQR) ‡	16.5 (13.1-19.8)	16.3 (12.7-18)	0.70

4.3.5. Features of patients showing increased responsiveness to ondansetron (super-responders)

39 IBS-D patients adjusted their daily dose to <4mg ondansetron per day, compared to 55 requiring a dose of ≥4mg Ondansetron. Patients on <4mg per day (super-responders) were more commonly female (92% *versus* 58%, *p*=0.003) and had firmer baseline stools (5.1 *versus* 5.4, *p*=0.0159). Other baseline clinical features including pain, urgency and bloating as assessed from days with each symptom and average severity scores; HADS, anxiety, depression and PHQ-12 scores were not significantly different (see **Table 14**).

There were however significant differences in 5-HT concentration in rectal biopsies which were significantly lower in super-responders (21.3 (17.0-31.8) *versus* 37.7, (21.4-61.4), *p*=0.036) (see **Figure 31**). There were no differences between the groups in biopsy 5-HIAA, 5-HIAA /5-HT, plasma 5-HIAA, and biopsy *TPH1* mRNA expression (see **Table 13**). However super-responders, despite the lower dose, showed a 4-fold greater increase in whole gut transit time when on ondansetron compared to placebo (15.6 *versus* 3.9 hours, *p*=0.040) (see **Figure 32**).

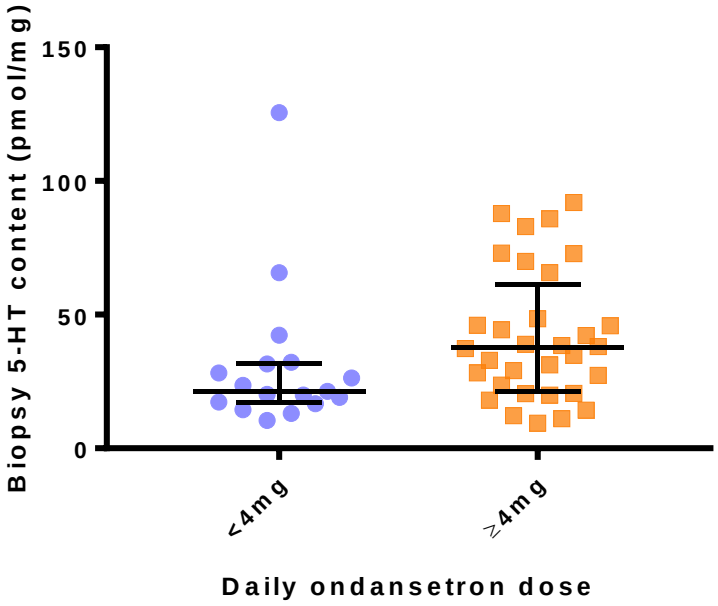


Figure 31 - Biopsy serotonin content in those taking <4mg and ≥4mg ondansetron daily. Median and IQR shown

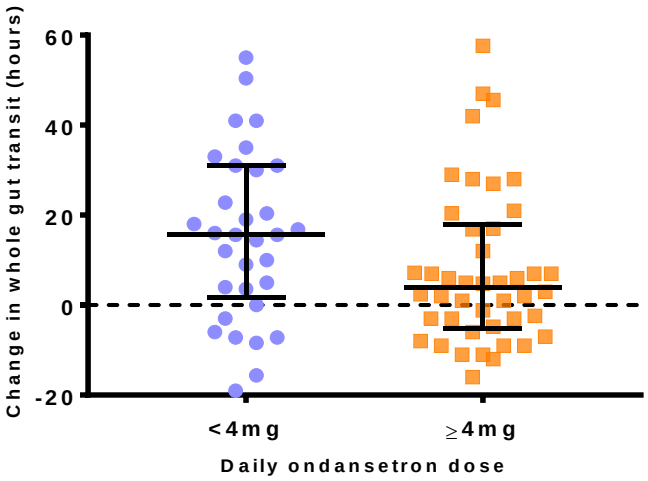


Figure 32 - Change in whole gut transit (in hours) between ondansetron and placebo regimens, separated by those taking ≥4mg and <4mg ondansetron daily. Median and IQR shown.

Table 13 - Colonic transit and markers of colonic mucosal serotonin turnover in IBS-D patients, grouped by those requiring less than and greater than 4mg per day of ondansetron. Data are median (IQR). P values obtained from Mann-Whitney tests. 5-HT: serotonin, 5-HIAA: 5-Hydroxyindoleacetic acid. TPH1: Tryptophan hydroxylase 1.

Variable	<4mg Ondansetron (n=17)	≥4mg Ondansetron (n=32)	*P* value
Biopsy 5-HT (pmol/mg protein)	21.3 (17.0-31.8)	37.7 (21.4-61.4)	0.0357
Biopsy 5-HT release in 30mins (pmol/mg wet weight)	23.8 (10.9-26.4)	17.1 (14-21.8)	0.23
Biopsy 5-HT release in 30mins/ Biopsy 5-HT content	1.1 (0.6-2.1)	0.4 (0.3-0.8)	0.0818
Plasma 5-HIAA (nmol/l) †	17 (14.2-20.0)	16.0 (12.3-18.1)	0.1733
Biopsy *TPH1* mRNA relative expression ‡	0.61 (0.54-0.80)	0.61 (0.42-0.95)	0.6554
Placebo whole gut transit time (hours) §	18 (7-30)	14 (7-29)	0.7545
Difference in whole gut transit time on ondansetron versus placebo(hours) ¶	15.6 (1.8-31)	3.9 (-5.1-17.9)	0.0398

†numbers in each group: n=22 <4mg ondansetron; n=41 ≥4mg Ondansetron.

‡numbers in each group: n=17 <4mg ondansetron; n=37 ≥4mg Ondansetron.

§numbers in each group: n=35 <4mg ondansetron; n=51 ≥4mg Ondansetron.

¶numbers in each group: n=33 <4mg ondansetron; n=46 ≥4mg Ondansetron.

Table 14 - Comparison of baseline variables between patients taking <4mg and ≥4mg ondansetron daily. Data are mean (SD) unless stated. P values obtained from unpaired t-tests and Mann-Whitney tests for parametric and non-parametric data, respectively.

Variable	<4mg ondansetron (n=39)	≥4mg ondansetron (n=55)	*P* value
Age	39 (12)	42 (12)	0.20
Sex (women), n (%)	36 (92%)	32 (58%)	0.0003
Patient Health Questionnaire 12	7.7 (3.5)	7.3 (3.7)	0.56
Perceived Stress Scale	18 (7)	18 (9)	0.59
Hospital Anxiety and Depression Scale	15 (7)	15 (8)	0.92
IBS-Symptom severity scale	299 (81)	297 (90)	0.91
Days with abdominal pain, median (IQR)	5 (3-7)	6 (4-7)	0.31
Average abdominal pain score	1.2 (0.7)	1.4 (0.8)	0.39
Days with urgency, median (IQR)	6 (4-7)	7 (5-7)	0.12
Average urgency score	1.4 (0.6)	1.7 (0.8)	0.079
Days with bloating, median (IQR)	6 (3-7)	6 (3-7)	0.58
Average bloating score, median (IQR)	1.3 (0.7-1.9)	1.4 (0.4-2)	0.84
Average stool form (BSFS)	5.1 (0.7)	5.4 (0.7)	0.016
Average stool frequency, median (IQR)	2.6 (2-4)	2.9 (2.1-3.9)	0.80

4.3.6. Effect of genotype on treatment response

Stool form responders were more likely to carry the homozygous CC genotype rs6766410 of the *HTR3C* gene which was found in 33% of responders compared to 14% of non-responders, (*p*=0.0066, see

Table 15). The polymorphism in *HTR3E* rs561098476 showed a trend towards association with more responders carrying the GG genotype compared to non-responders, although this was non-significant (*p*=0.09). Patients carrying the genotype TT of the *HTR3A* rs1062613 reported significant improvement of both days with bloating (1.7 (2.6) *versus* 5.0 (2.5), *p*=0.035) and average bloating score (0.38 (0.7) *versus* 1.4 (1.0), *p*=0.018). The significances were only found comparing the genotypes TT vs CT, and not with CT *versus* CC or TT *versus* CC, probably due to the small number of TT individuals. No other significant associations with any of the genotypes tested were found for the secondary endpoints such as 5-HT or 5-HIAA levels, or 5-HIAA/5-HT ratio were detected. Surprisingly, no significant effect was seen of the two *TPH1* SNPs and *TPH1* mRNA levels (see **Table 16** and **Table 17).**

Table 15 - Effect of the polymorphism HTR3C rs6766410 on IBS-D patients' FDA stool responder status, expressed as a percentage of responder status patient population; and clinical features while on ondansetron. Data are median (IQR) unless stated. P *values for clinical features while on ondansetron are obtained from one-way ANOVA and Kruskal-Wallis tests for parametric and non-parametric data, respectively. For FDA stool responder and polymorphisms the* P *value is obtained from a Chi-squared test.*

Variable	**AA genotype (n=15)**	**CA genotype (n=54)**	**CC genotype (n=28)**	***P* value**
Stool responder (n=22), %	14	53	33	0.0066
Stool non-responder (n=73), %	18	68	14	
Average stool form, mean (SD)	3.6 (1.5)	4.1 (1.3)	3.9 (1)	0.51
Average stool frequency	2 (1.1-3.1)	1.8 (1.4-2.9)	1.6 (1.2-2.4)	0.59
Days with abdominal pain	4 (1.4-6.5)	6 (2.5-7)	5 (1.5-6.5)	0.092
Average abdominal pain score	0.6 (0.2-1.4)	1.4 (0.6-2.1)	0.9 (0.3-1.3)	0.024
Days with urgency	3.8 (2.3-6.6)	5 (2.4-6.5)	3 (1-6.3)	0.29
Average urgency score, mean (SD)	1.2 (1)	1.2 (0.8)	0.8 (0.7)	0.13
Days with bloating	5 (2-6.5)	5.8 (1.9-7)	3 (0.5-6.3)	0.21
Average bloating score	0.8 (0.1-1.4)	1.4 (0.3-2.1)	0.6 (0.1-1.5)	0.16

Table 16 - Effect of TPH1 rs211105 genotypes on TPH1 mRNA levels. Data are mean (SD). TPH1 - Tryptophan hydroxylase 1. P value obtained from one way ANOVA

	TT genotype	**GT genotype**	**GG genotype**	***P* value**
TPH1 mRNA level	0.66 (0.33)	0.70 (0.30)	0.59 (0.22)	0.78

Table 17 - Effect of TPH1 rs4537731 genotypes on TPH1 mRNA levels. Data are mean (SD). TPH1 - Tryptophan hydroxylase 1. P value obtained from one way ANOVA

	TT genotype	**CT genotype**	**CC genotype**	***P* value**
TPH1 mRNA level	0.60 (0.21)	0.71 (0.37)	0.67 (0.28)	0.49

4.3.7. TPH1 mRNA expression

78 patient rectal biopsies were analysed for *TPH1* mRNA expression levels. There were no significant correlations with baseline clinical features, biopsy 5-HT or biopsy 5-HIAA concentrations (see **Table 18**).

Table 18 - Correlation of TPH1 mRNA levels with baseline symptoms and biopsy serotonin. P values obtained from Pearson correlation coefficients for parametric data and Spearman correlation for non-parametric data. 5-H: serotonin, 5-HIAA: 5Hydroxyindoleacetic acid, TPH1: Tryptophan hydroxylase 1

Biopsy *TPH1* mRNA Comparator	**r value**	**Correlation significance (*p*)**
Average stool form	-0.12	0.35
Average stool frequency	0.035	0.79
Days with abdominal pain	-0.025	0.85
Average abdominal pain	-0.070	0.60
Days with urgency	0.0085	0.95
Average urgency	-0.072	0.58
Biopsy 5-HT	0.17	0.23
Biopsy 5-HIAA	0.15	0.27
Biopsy 5-HIAA /5-HT	0.0052	0.97

4.4. Discussion

Serotonin metabolism has been previously shown to be altered in IBS-D patients with greater postprandial 5-HT in platelet poor plasma[41,149] and reduced SERT mRNA levels in both duodenal[368] and colonic biopsies in some[39] but not all studies[372]. The current study found significantly increased concentrations of the 5-HT metabolite 5-HIAA together with an increased 5-HIAA/ 5-HT ratio suggesting greater turnover of 5-HT in IBS-D patients. The biopsies also released more 5-HT when expressed as a proportion of the basal content thus supporting the idea that IBS-D is characterised by increased mucosal 5-HT availability. This provides a rationale for the use of 5HT3RAs as treatment for IBS-D.

Paradoxically we also found our patients had significantly lower fasting plasma 5-HIAA levels. Plasma 5-HIAA is known to be increased by dietary intake of foods containing tryptophan[373,374] and as IBS patients often limit their diet in an attempt to reduce symptoms[201,375] it is possible they were avoiding such foods. However, since we did not assess diet this must remain speculative. Previous studies have found no difference in fasting 5-HIAA in IBS-D patients compared to controls[149] suggesting there are unknown sources of variation.

Previous studies and meta-analyses have demonstrated that 5HT3RAs are effective treatments in IBS-D[359], particularly benefiting urgency and loose stools[376]. However, the number of patients needed to treat (NNT) to get one responder more than with placebo is 7.7, indicating that there are subgroups of individual patients who respond better, as is true of all IBS treatments. During our initial trial, which uniquely allowed the patient to titrate to their optimum dose, we observed striking differences in the doses patients chose, with over half using 4mg or less per day while some used the maximum allowed of 24mg per day. These differences suggest that there existed a subgroup with greater sensitivity to ondansetron which we have termed “super-responders”. We therefore looked to see what biomarkers predicted “super-responder status”.

We found that rectal biopsies of the super-responders had a significantly lower 5-HT concentration than those requiring larger doses. When the 5-HT released from the biopsies over 30 minutes was expressed as a percentage of the biopsy 5-HT concentration, there was a trend for this to be elevated but this was not statistically significant owing to wide variability in this measure. This may be because it depends on many factors including the trauma associated with the biopsy which is difficult to control.

The super-responders showed a 4-fold greater change in transit even though they were taking a dose which was a quarter of the non-super-responders, again objectively supporting the idea that super-responders are very much more sensitive to the drug. Having less serotonin in the biopsy could come about either because of reduced synthesis or accelerated turnover. The increased 5-HT release when expressed as a percentage of biopsy serotonin content would tend to support this interpretation and might explain why they were so much more sensitive to ondansetron if excess 5-HT was driving their symptoms. Future studies might use *in vivo* assessment of 5-HT release as has recently been done in animals to address this issue[377].

IBS-D patients have fast colonic transit which may reduce the degradation of bile acids and endogenous proteases, both of which can sensitise the rectum. We have previously reported from this same patient group that the faecal protease levels correlated negatively with transit time and positively with average urgency in IBS-D patients[378]. Urgency is strongly related to fast transit in IBS-D[379] but just exactly why slowing transit helps is uncertain. It is possible that allowing more time for the colonic microbiota to deconjugate bile acids and degrade endogenous proteases[378] may be important. However further intervention trials using other agents to slow transit will be needed to decide what is unique about the 5HT3RA's action.

Our finding that stool form responders were more likely to carry the CC genotype rs6766410 of *HTR3C* fits with the data of Fasching *et al*.[362] which suggested CC was more sensitive to chemotherapy induced vomiting, known to be driven largely by serotonin. This substitution has a predicted possible

functional effect on the receptor (polyphen2[380] score 0.798). If those with CC genotype are more sensitive to the effects of 5-HT this might help explain the benefit of a drug which blocks its action. Although we chose to study variants with previous evidence for potential contributions these findings needs to be interpreted with caution as analyses were not corrected for multiple testing and subject numbers are small. Further studies are indicated to address this issue fully.

4.4.1. Study limitations

Like many mechanistic studies which make substantial demands on patients we were probably underpowered for many of our secondary endpoints. We only requested biopsies for patients recruited in Nottingham and only 57 of the 70 Nottingham patients had colonic biopsies. Wide variability in some of our measures meant that the study was underpowered for some of the analyses. Furthermore, all the analysis is post hoc and not corrected for multiple testing and so could be due to chance, however trends were observed which give direction to further, larger and more definitive studies.

We were unable to link known SNPs in the genes encoding the SERT gene *SLC6A4* or *TPH1* with a clinically significant pattern of symptom features nor responsiveness to ondansetron, as has been suggested by others for the related drug ramosetron[381], but our numbers were probably too low for such an analysis. Also relevant is the fact that we did not find a correlation between *TPH1* SNPs examined and mRNA levels for *TPH1*. These data are compatible with data in Gtex[382] showing that rs21105 has no significant Expression quantitative trait loci (eQTL) signatures. rs4537731 has significant eQTL signatures in skin and thyroid but not in nerve or gut tissue.

Our pilot study asked subjects for pain scores on a 0-3 scale, which is insufficient to define a decrease in pain by at least 30% as recommended by the FDA. Consequently, we defined response by the change in stool consistency which is suboptimal in an IBS study. Future studies should take

this into account by using an 11-point pain scale allowing an assessment using both pain and stool consistency response according to FDA guidance.

4.5. Conclusion

The fact that super-responders show evidence of increased 5-HT turnover fits with previous data and is supported by the finding that *TPH1* inhibitors improve symptoms in IBS-D[383]. However, rectal biopsy assessment of 5-HT turnover is never going to be a feasible clinical test, so the value of our findings is more that it encourages us to look to further subdivide IBS-D according to specific 5-HT system related mechanisms. Some plainly have serotonin excess, but others appear to have quite different mechanisms. Examining the response to 4mg daily of ondansetron could be a simple test to identify at least one subgroup.

5. Mechanism of action of ondansetron in lactulose induced diarrhoea (MOLID)

This chapter describes the MOLID study designed to assess the mechanism of action of ondansetron, a 5-HT_3 receptor antagonist, in a model of lactulose induced diarrhoea in healthy volunteers, using MRI. It will discuss what is already known about ondansetron and lactulose, describe the methods and results of the study, and the implications of the findings.

5.1. Background

The British National Formulary describes Lactulose as an "osmotic laxative", a class of laxatives that are thought to soften stool by increasing the amount of water in the large bowel[384]. This conclusion is based on studies which used very large doses (up to 290 mosmoles) and showed that at such high doses, the watery stools contained substantial amounts of lactulose[385]. However at the normal clinical doses of 30-60 mosmoles it induces laxation with a very low incidence of liquid stools[386], something which has never been adequately explained.

Lactulose is a synthetic disaccharide, 4-O-β-D-galactopyranosyl-D-fructofuranose resistant to human digestive enzymes, confirmed by ileostomy studies showing that it is 100% excreted[385]. It has a molecular weight of 342, meaning that the usual clinical dose of 10-20g (29-58 mosmols) exerts an osmotic effect in the permeable small intestine, predicted to require 100-200 ml to create a solution isosmotic to interstitial fluid (290 mosomol/l). In addition the very low Na^+ content will create a steep electrochemical gradient causing Na^+ and water influx from interstitial fluid into the small bowel increasing the water content still further[387]. However the colon is able to absorb up to 5 litres of saline over 24 hours[388], so such a small increase in fluid input into the colon would not be expected to cause laxation unless there was some other effect.

Intubation studies show that orally ingested lactulose appears in the caecum within 1 hour of oral ingestion where it is rapidly fermented. Its caecal concentration peaks at 2 hours associated with the appearance of lactic acid and a fall in caecal pH, with maximal effect at 4 hours[389].

Until recently there has been no study of the effect of clinically relevant doses on either small bowel or colonic water. Recent studies using magnetic resonance imaging (MRI) have made it possible to demonstrate that it does indeed increase small bowel water content[390] but its impact on large bowel water content is unclear.

We wanted to further understand the mode of action of lactulose induced loose stools as a possible model of irritable bowel syndrome with diarrhoea (IBS-D). We were also interested to see whether the 5-hydroxytryptamine receptor 3 antagonist (5HT3RA) ondansetron would attenuate lactulose's effect, since it is known to benefit IBS-D reducing urgency and loose stools associated with a slowing of left sided colonic transit[154].

Preclinical rodent studies indicate that serotonin (5-HT) mediates intestinal secretion in response to several diarrhoeic infections including cholera and rotavirus which can be blocked by 5HT3RAs[391]. It may also play a role in the meal evoked stimulation of pancreatic secretions[391,392].

The aims of this study were to determine the mechanism of lactulose's effect on stool consistency by assessing the physiology of the small bowel and ascending colon, and if ondansetron could inhibit postprandial secretions and/or reduce the laxative effect of lactulose.

5.2. Methods

This was a double-blind, two-period, two-treatment crossover trial of ondansetron (8mg/tablet) and lactulose versus placebo and lactulose.

5.2.1. Ethical Approval

The trial was approved by the University of Nottingham Faculty of Medicine & Health Sciences Research Ethics Committee (85-1807) on 7th August 2018, prospectively registered on clinicaltrials.gov (NCT03833999), and conducted between September 2018 and July 2019.

5.2.2. Healthy Volunteers

Healthy volunteers were recruited by general advertisement on social media and University of Nottingham campuses. Eligible participants were aged 18 or older and able to give informed consent. Exclusion criteria were pregnancy or breast feeding; pre-existing gastrointestinal disorder; prior abdominal surgery other than appendicectomy or cholecystectomy; congenital long QT syndrome or prolonged QTc on screening ECG; contraindication to MRI scanning; inability to lie flat or exceed scanner limits of weight (120kg); inability to stop drugs known to alter GI motility; participation in night shift work in the week prior to the study; being in another trial or being in the opinion of the investigator otherwise unsuitable.

5.2.3. Randomisation

All participants participated in both study arms, the order of study being randomised using the online program www.randomization.com. Each study day was separated by at least 6 days in order to minimize carryover effects.

5.2.4. Interventions

The investigational medicinal product (IMP) was either 8 mg ondansetron (Milpharm, UK) or placebo, both identically over-encapsulated by the Pharmacy Production Unit at Nottingham University Hospitals NHS Trust so that they were identical in appearance. Lactulose (Teva, UK) was provided as lactulose syrup 13.6g/20ml. Participants drank 300ml of Fortisip (Nutricia, UK) and 150ml water as a meal substitute. This is a nutritionally complete milkshake style supplement, 300ml containing 450 kcal, 18g protein, 55.2g carbohydrate and 17.4g fat.

5.2.5. Magnetic Resonance Imaging

Subjects were scanned on a research dedicated 3T Philips Achieva MRI scanner, using a parallel imaging SENSE 16-element torso coil. A range of MRI sequences were used to image the abdomen including:

1. A balanced gradient echo (called balanced turbo field echo, bTFE or trueFISP) sequence (TR = 3.0 ms, TE = 1.5 ms, FA 80^{o}, SENSE factor 2.0) to acquire 50 transverse images each with an in-plane resolution of 2.00 mm x 1.77 mm and slice thickness of 5 mm, with no gap between slices. This was used to measure gastric volumes and thus assess gastric emptying.

2. A single shot, fast spin echo sequence (rapid acquisition with relaxation enhancement, RARE; effective echo time TE = 400 ms) to acquire in a single breath-hold, 20 coronal images with in-plane resolution interpolated to 0.78 mm × 0.78 mm and a slice thickness of 7 mm, with no gap between slices (acquired voxel size = 1.4 × 1.76 × 7 mm^3). This sequence yields high-intensity signals from areas with freely mobile fluid and little signal from body tissues and was used to measure small bowel water content.

3. A high resolution bTFE sequence to acquire images of the contents of the ascending colon (TR = 3.1 ms, TE = 1.56 ms, acquired resolution = 1.50 mm x 1.50 mm, reconstructed resolution= 0.86 x 0.86, 8 slices 5 mm thick, up to 5.0 mm gap to cover whole ascending colon, flip angle 45^{o}). This was used to measure the T1 of chyme in the ascending colon.

4. MRI marker pill positions were determined from images acquired at 2 stations using a coronal 3D dual-gradient echo sequence (TE1 = 1.07 ms, TE2 = 1.9 ms, repetition time =3.0 ms) and mDIXON readout[393]. The field of view at each station was 250 mm (HF) x 375 mm (LR) by 200 mm (AP) with acquired slice thickness 3.6 mm, interpolated to 1.8 mm with a reconstruction matrix of 384. These 2 sets of images were combined into a single image of matrix size 481 (HF) x 384 (LR) and 37 slices of thickness 5.4 mm and were used to visualize the pills within the colonic anatomy.

5. Small bowel motility was assessed from a cine bTFE coronal acquisition across 8 slices of the small bowel. The motility of each slice was assessed from data acquired during 1 minute of gentle free breathing.

5.2.6. Protocol

The study comprised of two three-day periods, taking either ondansetron or placebo in random order with a washout period of at least 6 days (see **Figure 33**). Participants attended visit 1 where informed written consent was obtained and they were screened against the inclusion and exclusion criteria. This included a 12-lead ECG, MRI safety screening questionnaire, height, weight, smoking history, past medical history and current medications. For 24 hours prior to their attendance at visit 2 they were instructed to eat their usual diet but avoid alcohol and beans or pulses, not to engage in strenuous exercise, nor to change their usual smoking habit.

On the morning of visit 2 fasted participants attended the Sir Peter Mansfield Imaging Centre (SPMIC) at the University of Nottingham. Once consent and MRI safety had been re-confirmed they underwent a fasted scan. Participants were then given one IMP (either placebo or 8mg ondansetron) with 50mls water and a meal comprised of 300mls Fortisip and 150mls water, then had a second MRI scan (T=0). Further scans occurred 120, 240 and 360 minutes after the meal. At the end of the day participants consumed 20mls lactulose and one IMP, and were asked to take a further IMP that evening at home.

The following day, the study was completed in participants' homes. While following the same dietary and lifestyle rules as prior to visit 2, they consumed 20mls lactulose twice daily, IMP three times daily and 5 MRI marker pills at 8pm.

On the third day, they returned to SPMIC for visit 3. Compliance and MRI safety was re-confirmed, then participants had a fasted scan where marker position was assessed. A dose of IMP with 50mls water, 20ml lactulose and the same meal as visit 2 was consumed, followed by MRI scans at 0, 120 and 240 minutes.

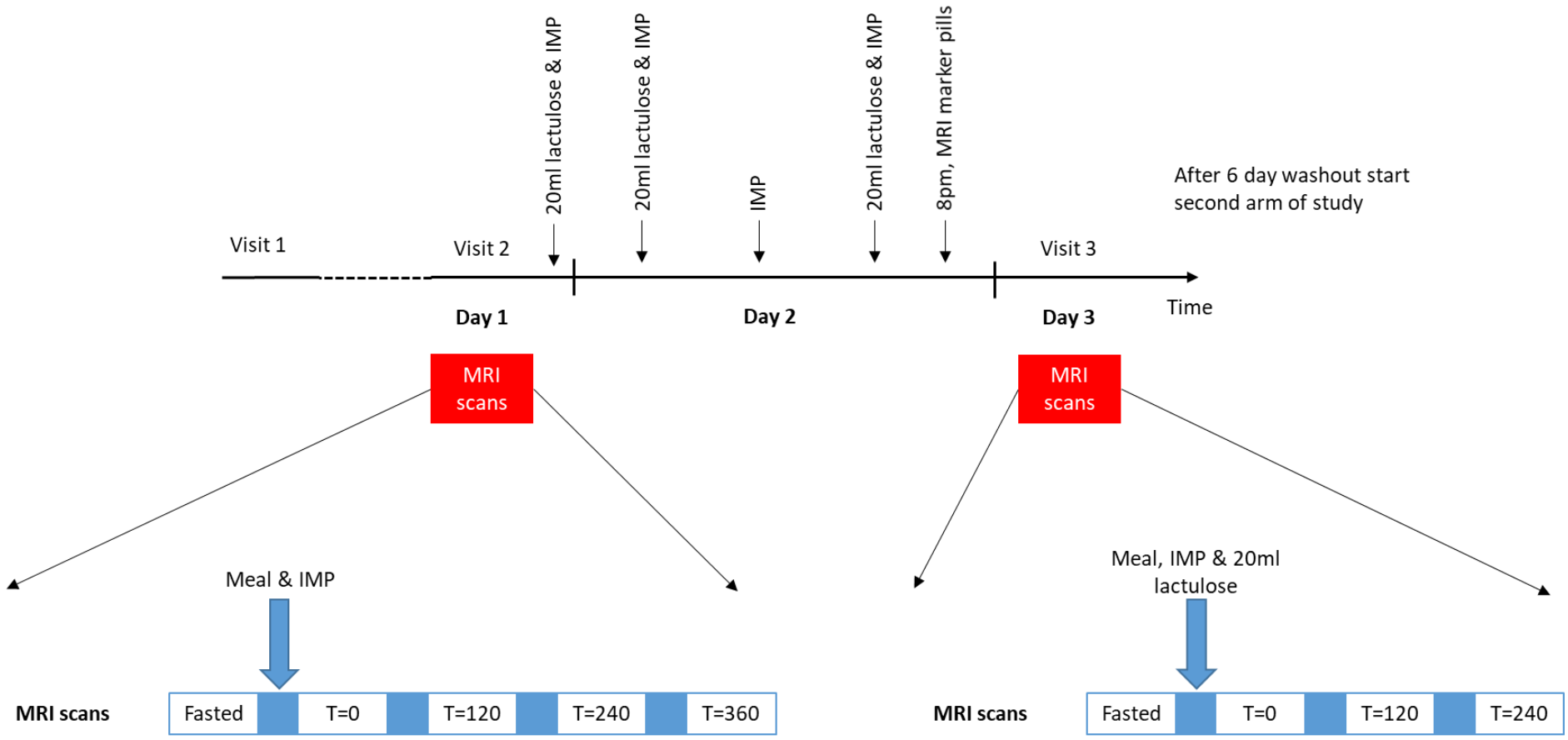


Figure 33 - Schedule of MOLID study events. IMP - investigational medicinal product

5.2.7. Endpoints

The primary endpoint was area under the curve from time 0-240 minutes (AUC_{0-240}) of small bowel water content (SBWC, ml.min) on visit 3. Secondary endpoints were AUC_{0-360} of SBWC on visit 2; T1 relaxation time in the ascending colon (T1AC, s) fasted and at the end of study days; small bowel motility index (arbitrary units, a.u.) 120 and 240 minutes postprandially; whole gut transit rate (a.u.) on visit 3, assessed by weighted average position score (WAPS)[279]; and gastric volume at 120 minutes to confirm passage of meals into the small bowel.

5.2.8. Data analysis

5.2.8.1. Small bowel water content

SBWC was measured as previously validated[270], using in house software written in IDL (Research Systems Inc. Boulder, Colorado, USA). For each image slice at each time point a region of interest (ROI) was drawn around the small bowel, and structures such as blood vessels, bladder and gall bladder were excluded. Any pixel with signal intensity above a calculated threshold (set by the subject's cerebral spinal fluid) in the heavily T2 weighted images were assumed to be filled with free water.

5.2.8.2. Small bowel Motility

All motility data were processed as previously described[292]. Briefly, free breathing MRI data were processed to correct for respiratory motion[290] before applying the nonlinear optic flow registration[291] to correct local deformation caused by bowel wall motion and luminal flow. An ROI was then demarcated around any visible small bowel on each coronal image on MATLAB-based software (MathWorks, Natick, MA). For each pixel in the registered dataset, a power spectrum of the intensity changes across the time series (smoothed using a running average of 5 pixels to reduce noise) was

calculated and then summed across all frequencies. This metric is termed the total power, and reflected bowel motility, both in terms of segmental oscillations and bolus movement of contents. A larger total power motility index represents higher small bowel motility.

5.2.8.3. Whole gut transit rate

Whole gut transit was assessed using the Weighed Average Position Score (WAPS) of the MRI marker capsules[279]. We have previously validated the use of such marker pills to measure transit and found they correlate well with values obtained using the standard radio-opaque marker technique[279]. We modified this method by having the subjects take the marker 12 rather than the conventional 24 hours before the MRI scan because, if transit is rapid as we expected with lactulose, the marker technique can fail if all the markers have left the body by the time the scan takes place. Dosing 12 hours before assessing markers has been validated with the radio-opaque marker technique and shown to work well for those with fast transit[394]. From the fasted set of MRI images on visit 3 a transit score was calculated by subdividing the bowel into eight sections and each capsule was scored according to its position in the colon. A weighting factor was calculated for each capsule depending on the difference of the capsule score from the median capsule score.

5.2.9. Sample size and statistical analysis

Previous studies using the same scanning technique have shown the AUC SBWC 0-4 hours to be mean (SD) 252(105) L.min after 7.5g psyllium. We expect lactulose 20ml to at least double SBWC as shown by others[390]. Using n=12 would give us >99% power to detect the effect of lactulose. The magnitude of the ondansetron effect on SBWC is unknown but it does produce a >50% change in transit time[154]. Using n=16 would give us 80% power to detect a change of 79 L.min in AUC SBWC comparing ondansetron

to placebo which represents a 31% change, usually taken to be the minimally important difference in many motility parameters.

Parametric data are represented by mean (SD) and non-parametric data by median (IQR). All statistical analysis was performed using Graphpad Prism version 8.2.1 or later for Windows (Graphpad Software, La Jolla California USA). Data were tested for normality using the D'Agostino & Pearson normality test. Assessing the effect of lactulose, paired t-tests were performed for SBWC AUC, T1AC AUC and small bowel motility at 120 and 240 minutes. Wilcoxon matched pairs signed rank tests were performed for fasting SBWC and fasting T1AC.

Assessing ondansetron versus placebo, paired t-tests were performed for T1AC at 360 minutes on day one, fasted on day 3 and at 240 minutes on day 3; small bowel motility at 240 minutes; and gastric volumes at 120 and 240 minutes on day 1 and day 3. Wilcoxon matched pairs signed rank tests were performed for SBWC AUC on day 1 and 3; small bowel motility at 120 and 240 minutes on day 1 and 120 minutes on day 3; and whole gut transit on the fasted scan on day 3.

5.3. Results

16 participants completed the study, 11 female, mean age 22 (range 20-33), BMI 23kg/m^2 (standard deviation 3.3). All participants tolerated the lactulose without the need to modify their dose. A comprehensive list of the results can be found in **Table 19**.

5.3.1. Effect of lactulose

Comparing participants while taking placebo, comparison of study day 1 (without lactulose) and day 3 (with lactulose) allows analysis of the lactulose's effect on the gut.

5.3.1.1. Small bowel water

36 hours of lactulose (20ml twice daily) numerically increased fasting small bowel water, the mean difference between day 1 *versus* day 3 was 27ml (95% CI -4, 57), however this failed statistical significance, *p*=0.057. Over the course of the study day SBWC was significantly greater on day 3 than day 1, $AUC_{0\text{-}240}$ mean difference 13.7 L.min (95% CI 4.2, 23.1), *p*=0.0078 (see **Figure 34**).

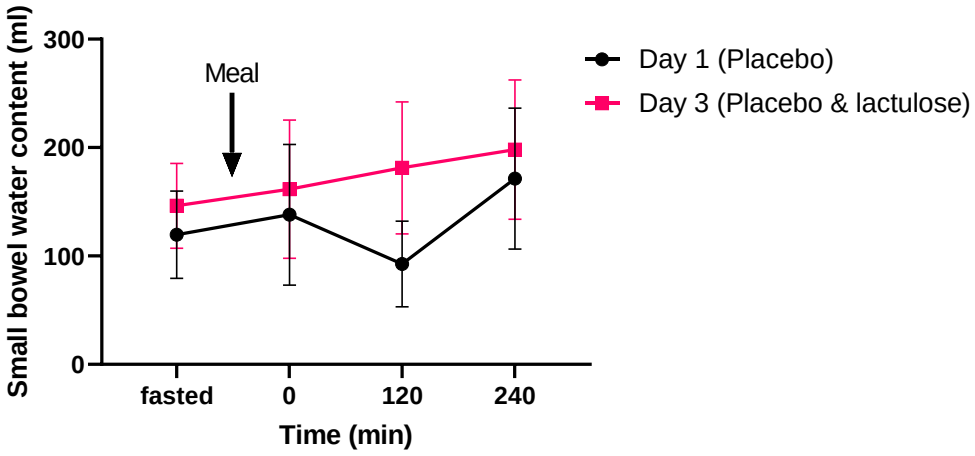


Figure 34 - Effect of lactulose on postprandial small bowel water content (SBWC). Area under the curve analysis demonstrates a significantly greater SBWC for placebo with lactulose than placebo alone (43.3 [25.0] L.min versus 29.7 [16.9] L.min, p=0.0078). Data are mean ± 95% CI

5.3.1.2. Small bowel motility

Small bowel motility significantly increased when taking lactulose (see **Figure 35**). Total power was greater after placebo & lactulose than placebo alone at both 120 minutes, mean difference 289 a.u. (95% CI 63, 514), *p*=0.016, and 240 minutes, mean difference 390 a.u. (95% CI 154, 626), *p*=0.004. An example of motility analysis can be seen in **Figure 36.**

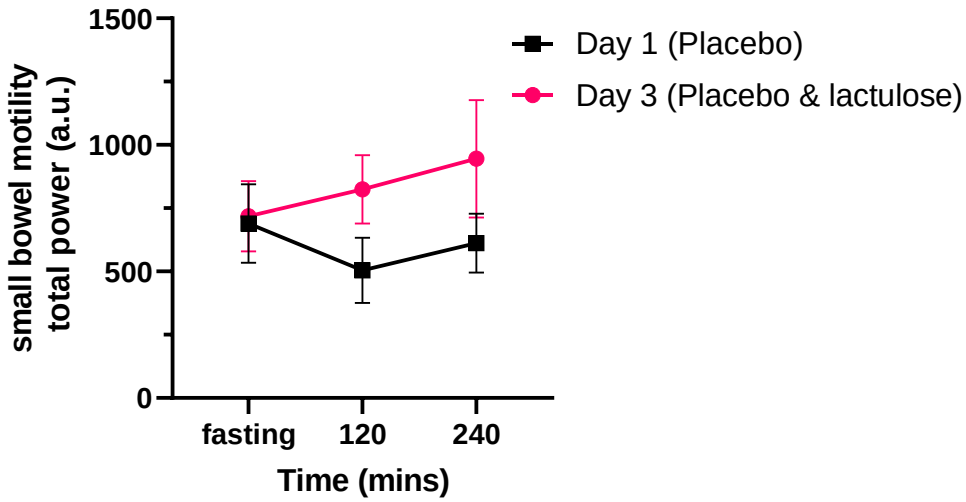


Figure 35 - Small bowel motility on day 1 when taking placebo versus day 3 when taking placebo and lactulose, demonstrating a significantly greater small bowel motility when taking lactulose at T=120 (p=0.016) and 240 minutes (p=0.004). Data are mean ± 95% CI

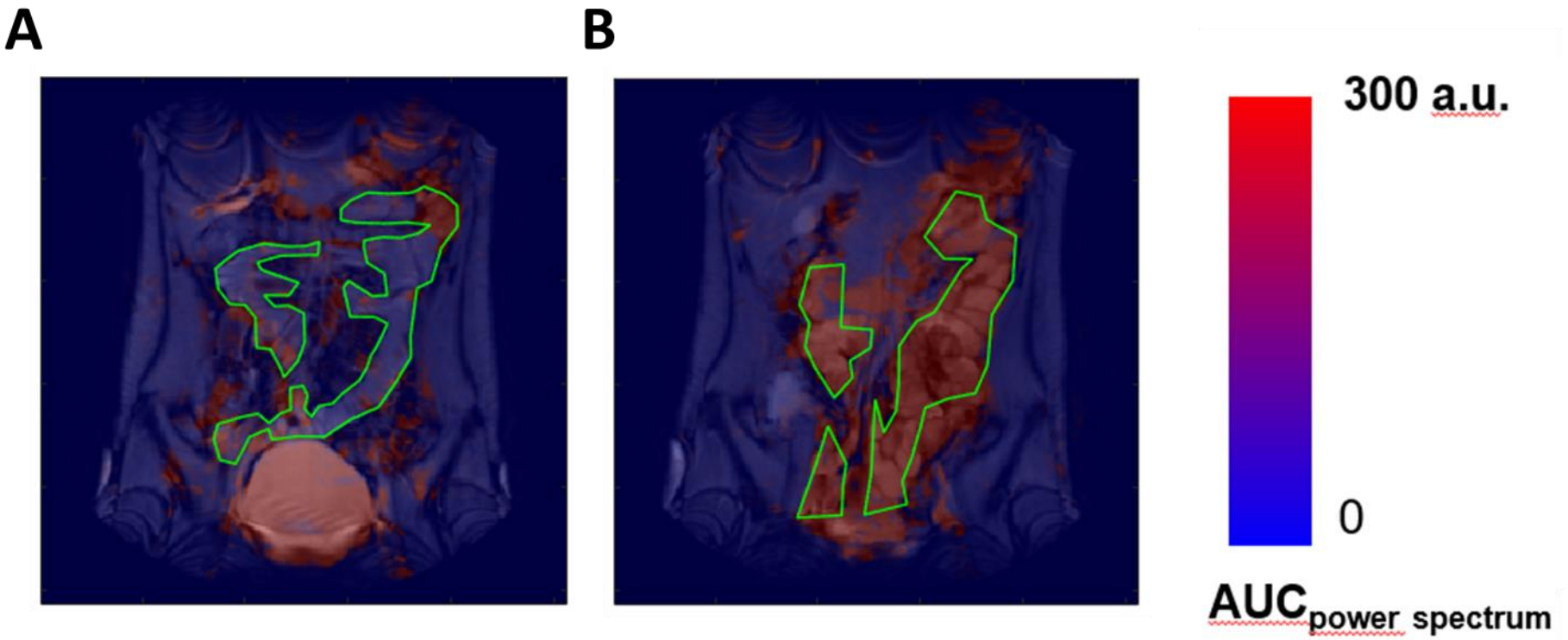


Figure 36 - Comparison of small bowel motility 240 minutes after a meal, having taken (A) placebo only, and (B) placebo and 20ml lactulose three times daily for 36 hours. In A, areas of small bowel remain grey, yet in B small bowel is shaded in red, indicating an area of motion. Areas of motility are coloured in red

5.3.1.1. T1 of the Ascending Colon

Despite an increase in SBWC and motility, there was no effect on T1AC, either fasted, mean difference 0.07s (95% CI -0.16, 0.31), p=0.72, or over the whole study day, $AUC_{0\text{-}240}$ mean difference 11min.s (95% CI -23, 47), p=0.48 (see **Figure 37**).

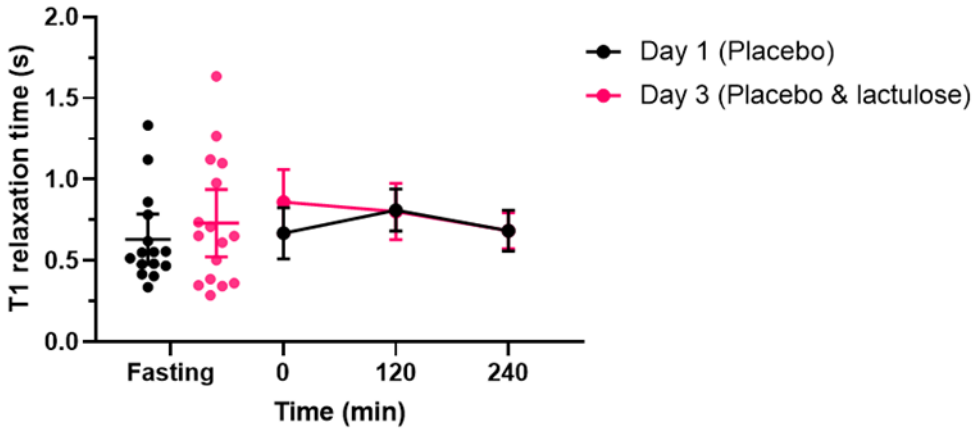


Figure 37 - T1 relaxation time in the ascending colon from fasting to 240 minutes after the meal, taking placebo versus placebo and lactulose. Individual values for each participant are plotted for the fasted scan. Error bars are mean ± 95% CI

5.3.2. Ondansetron versus placebo

5.3.2.1. Small bowel water

No differences were found in SBWC AUC between participants taking ondansetron or placebo, either after one dose, mean difference 1.7 L.min (95% CI -15.5, 18.8), p>0.99, or after 36 hours of treatment and lactulose, mean difference 3.2 L.min (95% CI -12.0, 18.4), p=0.71.

5.3.2.2. Small bowel motility

After 1 dose of ondansetron, small bowel motility at 120 and 240 minutes was unchanged compared to placebo, mean differences -6 a.u. (95% CI -208, 196), *p*=0.9, and -108 a.u. (95% CI -367, 151), *p*=0.34, respectively.

Similarly, after 36 hours of ondansetron and lactulose, there were no differences at 120 and 240 minutes after the meal, mean difference 66 a.u. (95% CI -136, 268), p=0.91, and 87 a.u. (95% CI -304, 478), p=0.62, respectively (see **Figure 38**).

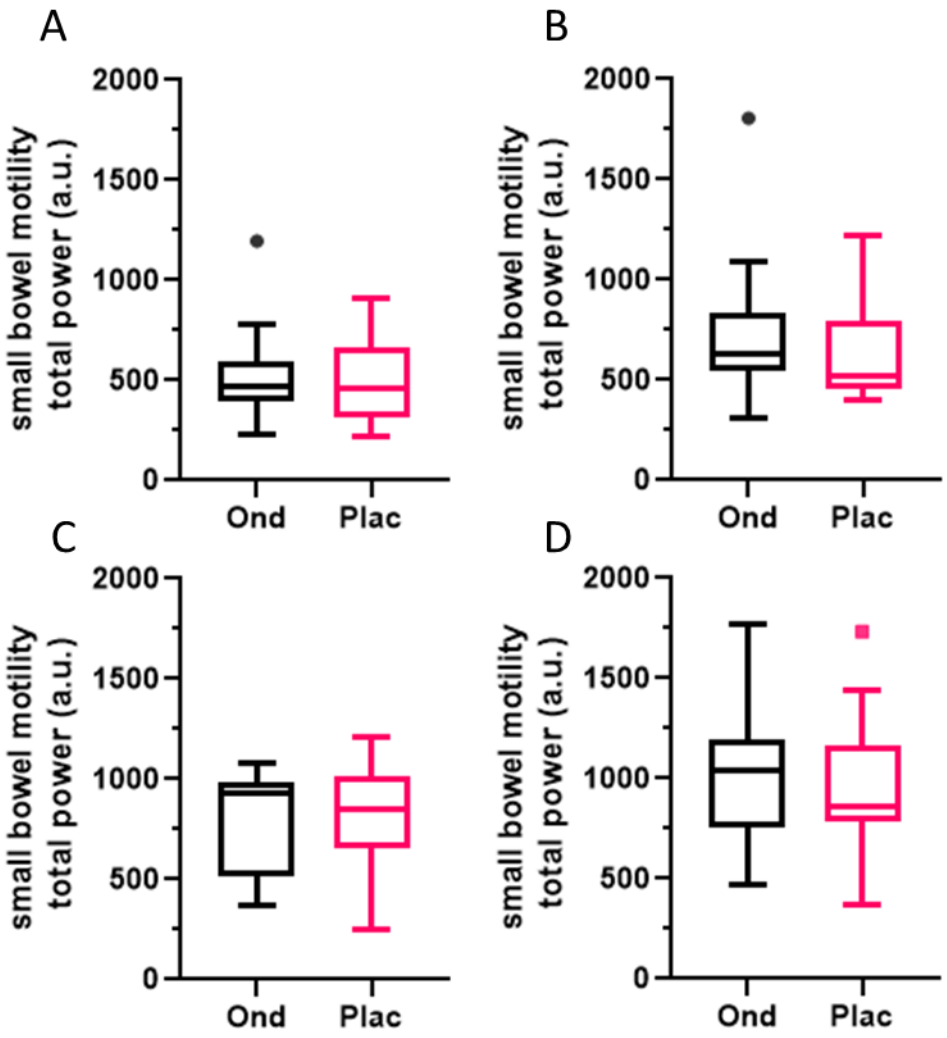


Figure 38 - Small bowel motility when taking ondansetron or placebo, showing no significant differences. A) Day 1, T=120min. p=0.90. B) Day 1, T-240min. p=0.34. C) Day 3, after 36 hours lactulose (20ml twice daily), T=120min. p=0.91. D) Day 3, after 36 hours lactulose (20ml twice daily), T=240min. p=0.62. Data are presented as Tukey box plots.

5.3.2.3. T1 of the Ascending Colon

360 minutes after taking one dose of ondansetron, T1AC was not significantly altered versus placebo, mean difference 0.08s (95% CI -0.11, 0.27), *p*=0.37. After 36 hours of therapeutic lactulose, there was no significant difference in fasting T1AC between ondansetron and placebo, mean difference 0.03s (95% CI -0.26, 0.31), *p*=0.84. Similarly, 240 minutes after the meal, T1AC was not altered by ondansetron, mean difference 0.03s (95% CI -0.15, 0.20), *p*=0.76.

5.3.2.4. Transit

Whole gut transit after 36 hours of lactulose showed no significant difference between ondansetron and placebo, mean difference 0.2 a.u. (95% CI -1.6, 1.9), *p*=0.63.

5.3.2.5. Gastric volume

No differences were found between ondansetron and placebo in gastric volumes 120 minutes after ingestion, either acutely, mean difference 13ml (95% CI -17, 43), *p*=0.37, or after 36 hours of IMP and lactulose, mean difference 16ml (95% CI -45.7, 13.8), *p*=0.26.

Table 19 - MR parameters assessed during the study. On Visit 2 ondansetron or placebo is given immediately prior to the study. On visit 3 participants have received ondansetron or placebo thrise daily and lactulose 20mls twice daily for 36 hours prior to the study day. a.u., arbitrary units; AUC, area under the curve; SBWC, small bowel water content; T1AC, T1 of the ascending colon; WAPS, weighted average position score. Data are represented as mean ± SD.

	Parameter	**Ondansetron**	**Placebo**	**Mean difference**	***p* value**
Day 1	Gastric volume (ml) at 120 minutes	94 ± 32	107 ± 64	13 ± 50	0.37
	SBWC (L.min) AUC 0-360	46 ± 30	47 ± 25	2 ± 32	0.99
	Motility (a.u.) at 120 minutes	511 ± 241	505 ± 232	-6 ± 349	0.90
	Motility (a.u.) at 240 minutes	738 ± 380	630 ± 226	-108 ± 429	0.34
	T1AC (s) at 360 minutes	0.51 ± 0.14	0.59 ± 0.27	0.08 ± 0.34	0.37
Day 3	Gastric volume (ml) at 120 minutes	100 ± 44	84 ± 38	-16 ± 44	0.26
	SBWC (L.min) AUC 0-240	40 ± 28	43 ± 25	3 ± 43	0.63
	Motility (a.u.) at 120 minutes	788 ± 256	854 ± 251	66 ± 318	0.91
	Motility (a.u.) at 240 minutes	910 ± 306	997 ± 466	87 ± 467	0.62
	T1AC (s) fasting	0.71 ± 0.30	0.74 ± 0.40	0.03 ± 0.53	0.84
	T1AC (s) at 240 minutes	0.66 ± 0.21	0.68 ± 0.21	0.03 ± 0.33	0.76
	WAPS (a.u.)	2.5 ± 2.4	2.7 ± 2.7	0.2 ± 3	0.63
	Ascending colonic volume (ml) fasted	191 ± 84	226 ± 97	35 ± 112	0.27
	Ascending colonic volume (ml) at 240 minutes	157 ± 63	186 ± 92	29 ± 89	0.34
	Total colonic volume (ml) fasted	507 ± 301	644 ± 299	137 ± 313	0.11
	Total colonic volume (ml) at 240 minutes	502 ± 173	616 ± 340	114 ± 323	0.29

5.4. Discussion

Despite its widespread use there are very few studies looking at lactulose's mechanism of action, and only one using MRI[390]. Our study differs from previous studies examining the role of osmotic forces in lactulose induced diarrhoea in using clinically relevant doses which produce stool softening but not profuse watery stools. As others have reported using 10g of lactulose in 200ml of water[390], small bowel water content increased. The osmotic load we gave of 35 mosmoles would be predicted to require 120ml to generate a solution isotonic to the interstitial fluid in the gut mucosa. Additionally we would expect the low Na^+ concentration to cause intestinal secretion down the electrochemical gradient. We actually observed that 13.6g lactulose increases SBWC by 89ml, considerably less than the 300ml reported by Undseth *et al.*[390]. This difference is most likely due to the fact that Undseth gave lactulose alone while we wanted to study the effect of lactulose when given as it is in clinical practice, i.e. combined with normal food intake. The Fortisip we used is a simple mixed nutrient meal which includes simple sugars and proteins whose rapid absorption would stimulate water absorption and thus reduce SBWC. Another factor reducing the observed increase would be rapid passage of small bowel fluid into the colon as has been observed in ileostomy patients after ingesting lactulose[395]. This idea is supported by the increase in motility we demonstrated in this study. The mechanism underlying the stimulation of small bowel motility is uncertain but could be a response to bowel distension which is known to produce propulsive motility in both humans[396] and animals[397].

Given that lactulose is neither hydrolysed nor absorbed in the small bowel[398] the osmotically "trapped" small bowel water and lactulose passes unchanged into the ascending colon. Here however it is rapidly anaerobically fermented with evolution of hydrogen within 10 minutes[399] and short chain fatty acids within 60 minutes[389]. The novelty of our observation is that we have showed that the speed of fermentation means that lactulose did not significantly alter colonic water content, neither acutely nor after repeated dosing. Short chain

fatty acids are known to be rapidly absorbed, co-transported with Na^+ which would tend to reduce colonic water content[323]. This suggests that its undoubted laxative effect may be due to the stimulatory effects of products of fermentation on the colon.

The key products of lactulose fermentation in humans are acetate and lactic acid whose production leads to marked acidification of caecal contents with a 10 fold rise in hydrogen ion concentration[389]. This fermentation will start in the mouth with its abundant microbiota and although then inhibited by gastric acid, it may continue further down the small intestine towards the terminal ileum where bacterial numbers often exceed 10^6 organisms/ml. Several studies have indicated that SCFAs stimulate propulsive motility in the terminal ileum[132] while in the colon they may both inhibit and stimulate propulsive motility depending on the type and concentration[400]. SCFAs act by activating the G protein coupled receptors (GPRs), GPR41 and GPR43 which are expressed on Peptide YY containing enteroendocrine cells in rats[31] and humans[401]. Human studies infusing solutions of SCFAs showed no obvious change in colonic motility patterns[133]. However several animal studies indicate that SCFAs stimulate colonic motility via mechanisms involving serotonin release[324,402]. Animal studies also show the microbiota stimulate tryptophan hydroxylase-1 (TPH1), the rate limiting enzyme in serotonin production[325] though the precise mechanism is unclear. More recently it has been shown that the GLP-1 containing enteroendocrine cells appear to be the most responsive to microbial metabolites and that the resulting GLP-1 release activates serotonin secretion from neighbouring enterochromaffin cells[315].

Despite this possible link between acidification of the ascending colon by fermentation of lactulose and serotonin release we were unable to show that this could be altered by 5-HT3RAs though this might be because other receptors, 5-HT1, 4 or 7 are involved. There may be species differences in these effects since despite animal studies suggesting 5-HT3RAs could block postprandial pancreatic secretion we could not see any change in

postprandial small bowel water content which is stimulated powerfully by high fat meals[403], presumed to be via pancreatic secretions.

Earlier studies in healthy volunteers suggested that ondansetron inhibited the normal increase in left sided colonic tone in response to feeding[404]. Ondansetron also slows colonic transit in both healthy volunteers and IBS-D patients in whom the main effect was on the descending and sigmoid colon[154]. Unlike the ascending and transverse colon these are regions where the rate of absorption is usually less, suggesting that ondansetron's anti-diarrhoeal effect may be primarily due to altered colonic motility rather than enhanced absorption or inhibition of secretion. We were however unable to show any effect using our lactulose diarrhoea model. This may be because the model does not adequately mimic all aspects of IBS-D. Future studies should examine in detail the impact of ondansetron on left sided colonic motor patterns, particularly examining its impact on retrograde motor patterns.

However our study has thrown new light on the mode of action of lactulose when used as a laxative. At normal therapeutic doses the osmotic effect seems less important and our study suggests lactulose acts as a prokinetic, mostly likely via SCFAs or other products of fermentation. Defining these more precisely may allow the production of more potent prokinetic agents while avoiding the undesirable effects of fermentation such as intestinal gas.

5.4.1. Study limitations

This study used MRI techniques to assess the effect of ondansetron, which has been shown to harden stool consistency in patients with IBS-D[154], in a model using lactulose in healthy volunteers. 20mls lactulose was given twice daily in the model, increasing small bowel water and small bowel motility, however did not alter water content of chyme in the ascending colon measured by the MR parameter T1. This has lead us to question the mechanism of action of lactulose, as products of its fermentation may act as a prokinetic. Correlating these findings with participant stool diaries would have

given greater clarity to the data, as it may also be that 20mls twice daily in young healthy volunteers is not enough to loosen stools to the degree seen in IBS-D.

Replication of this study in patients with IBS-D would be of value for three main reasons; it may give further insights into site of action of ondansetron in IBS-D, if there are differences it may help better understand the differences in GI physiology between IBS-D and healthy volunteers, and if results are similar it gives validation to this model used.

Previous work has shown ondansetron significantly slows transit in the descending colon[154], which may be where it mainly acts. We looked at water content in the ascending colon, the main site of fermentation to see if ondansetron acted in this area and found no difference to placebo. Further MRI studies should assess the T1 in the descending colon to see if the slowed transit also correlates with a reduction in water content of the chyme.

6. Discussion

6.1. Role of diet in IBS pathophysiology

Most IBS patients can identify foods which trigger their symptoms – some within 15 minutes of starting a meal and in others the symptoms are more delayed. Logically these appear to be two different pathologies, and recent work has presented evidence of nonclassical food allergies acting at a mucosal level as a possible mechanism[128]. The study however has several limitations and should be interpreted with caution: provocation tests use uncooked wheat, which has higher proportions of potentially irritant proteins including amylase-trypsin inhibitors (ATIs) than normally ingested; the patient population was selected to include only those believing their symptoms were triggered by foods prior to the study and the trial was open label, thus at very high risk of bias; wheat was found to be the most common antigen causing symptoms, and patients counselled to avoid this were essentially following a simplified low FODMAP diet. Wheat is commonly described as exacerbating IBS symptoms however it remains unclear which component is primarily responsible[405]; it is a complex of several potential triggers including proteins gluten, wheat germ agglutinin and ATIs; as well as carbohydrates arabinoxylan, which is rapidly fermented and makes up 32% of wheat[406], and fructan, a FODMAP[125].

More delayed reactions can be attributed to processes more distally in the gut, such as the result of colonic fermentation. In patients with IBS-D, a faster transit through the small bowel delivers FODMAPs into the ascending colon at an increased rate. In this nutrient rich environment, bacteria are limited in their ability to ferment by the need to maintain redox balance, so different SCFAs are produced and reducing equivalents are removed by production of hydrogen[407]. The sudden increase in large volumes of gas are unable to be absorbed by the colonic mucosa to be exhaled via the lungs, and in IBS-C, gas expulsion is also impaired[408]. This leads to luminal distention, which in the viscerally hypersensitive gut, is felt as pain.

Diet has a role in the pathophysiology, and thus by modifying the diet it can be manipulated into the management of the condition. The low FODMAP diet has seen a significant improvement in symptoms for many IBS patients. As a proof of concept, a study compared a 3 week intervention of low FODMAP diet versus a high FODMAP diet and demonstrated a reduction in IBS-SSS with a low but not a high FODMAP diet[409]. Meta-analysis of 7 RCTs comparing low FODMAP diet to various control interventions in 397 participants showed a significant relative risk of reduction in global symptoms of 0.69 compared with controls[410].

Such a dietary manipulation doesn't come without its own risks however. The low FODMAP diet is incredibly restrictive and potentially damaging to the microbiota, as it restricts prebiotics to the microbiota leading to reductions in species such as *Bifidobacterium*[411] which have potential health benefits[412]. Long term outcomes and safety of the dietary intervention is also lacking.

This is further complicated in cases where patients haven't been positively diagnosed using specific criteria such as Rome IV, but as a diagnosis of exclusion after malignancy, inflammatory bowel disease, coeliac disease and lactose intolerance. As discussed earlier, the differential diagnosis includes many other pathologies, and a meta-analysis suggests 10% of patients with IBS-D have severe bile acid malabsorption[413]. These patients will become more symptomatic after eating foods high in fats, which stimulate bile acid secretion which irritates the colon.

6.2. Fibre supplementation in healthy volunteers

The NOPAL study adds to what is known about mechanisms of action of fibres in the gut. The particulate fibre wheat bran, viscous fibre psyllium and nopal, which has properties of both, despite being different physico-chemically, all increased small bowel water content (SBWC) and ascending colonic water, assessed by the MR parameter T1 (T1AC) in healthy volunteers.

There are several potential mechanisms for this. In the small bowel, we postulate that the particulate wheat bran acts via Piezo2 mechanosensitive ion channels on enterochromaffin cells. This stimulates serotonin release[18] which increases fluid secretion in a mechanism thought to prevent luminal obstruction. Wheat bran also adsorbs amylase[15], reducing starch break down and increasing the intraluminal oncotic pressure.

Psyllium's highly branched arabinoxylan structure is unable to be broken down by host enzymes, so throughout its journey in the small bowel it holds water in a complex matrix, increasing viscosity, impeding mixing and access to the mucosa. This slows breakdown of nutrients and subsequent absorption[414], increasing small bowel water.

Nopal, comprising of both viscous and particulate elements, could act via both mechanisms however it is unknown if the particulate elements would be large enough to exhibit the same effect as wheat bran. The combination of the two mechanisms might explain why nopal was found to have a significantly greater area under the curve small bowel water content than wheat bran.

In the colon, the difference in the physico-chemical properties between the fibres led to significantly different measurements. Wheat bran, which contains substantial amounts of arabinoxylans, was rapidly fermented by the colonic microbiota producing hydrogen gas as a by-product, evidenced by high breath hydrogen concentrations. As a result of the nutrient rich environment, bacteria proliferate and increase stool bacterial mass and may

exert a laxative effect, although this was not measured in this study so can only be speculated.

Psyllium's complex structure is not readily fermented, taking 14 hours *in vitro*, and consequently subjects showed no rise in breath hydrogen during the 4 hour study period. Prefeeding the fibres for 24 hours increased all T1 values to the upper limit of normal and by the time the healthy volunteers attended for an overnight fasted scan there was no distinction between the fibres. Postprandial T1 values however were significantly higher for psyllium during the study day, showing not only the high water holding capacity of psyllium, but in the absence of fermentation, this water remained held in the psyllium matrix for the duration of the study. As nopal and wheat bran were fermented, it is possible the water was absorbed into the mucosa along with SCFAs, and consequently had lower T1AC values.

The lesser known fibre in this study, nopal, is derived from the cactus *Opuntia ficus-indica* native to Mexico, which despite only recent attention from the scientific community, has been used for health-promoting properties for thousands of years in rural communities[415]. IBS affects patients from all corners of the earth, and in Mexico prevalence is estimated at 4.4% using Rome III criteria and up to 35% using Rome II[416]. In contrast to the UK population, where constipation and diarrhoea subtypes are approximately equal, in Mexico IBS-D is significantly rarer[417]. There is *in vitro* evidence that nopal acts as a prebiotic similar to inulin, increasing *Bifidobacterium* species and decreasing *Enterobacteriacia*[418]; and in a 1 week RCT of nopal supplementation in IBS, more patients reported adequate relief from IBS symptoms taking 20g and 30g per day than placebo[327]. In countries where nopal is indigenous, this cheap and readily available fibre supplement can be used to help those with IBS-C. We have shown nopal to be as effective at increasing water content in the small bowel and ascending colon as two fibres which are popular in Western healthcare systems, and may have a place in the management of IBS if evidence of future trials remains favourable.

6.3. Fibre supplementation in patients with IBS

For patients with IBS, the limiting factor for fibre intake is often resultant gas production causing bloating and abdominal pain[130] due to visceral hypersensitivity which is not present in healthy individuals[112]. Consequently, IBS patients avoid foods which can cause bloating, and severely limit their dietary intake[122]. As well as the potential for micronutrient deficiencies on such a restricted diet, their microbiota is starved of prebiotics and there is an increase in potentially pathogenic species such as *Enterobacteriacae* and *Proteobacteria* with a decrease in potentially protective species such as *Bifidobacterium*[141].

The NOPAL study demonstrated the differences in physico-chemical properties of different fibres, measured by MRI and breath hydrogen analysis in healthy individuals. EFIGI aimed to utilise these properties in patients with IBS, such that by combining a viscous and readily fermentable fibre, fermentation and the associated benefits of prebiotics still occurred but at a reduced rate thus reducing gas production and hopefully diminished symptoms.

Inulin made no difference to water in the small bowel, confirming previous work[348] that it exerts little osmotic force and passes through largely undigested. However once in the colon, fermentation rapidly occurred and pockets of gas, visible on MRI, developed and correlated with a rise in breath hydrogen concentrations. 6 hours after the test drink, ingesting inulin lead to higher flatulence symptom scores than psyllium.

Co-administration of the viscous fibre psyllium with inulin may seem initially an unattractive prospect for the viscerally hypersensitive gut. However *in vivo* the combination significantly slowed, but crucially didn't stop, fermentation in the colon; demonstrated by a reduction from inulin values in MRI gas and breath hydrogen. Our initial hypothesis was that fermentation may have been impeded by the increased viscosity of psyllium reducing the mixing of inulin with the colonic microbiota. Our *in vitro* fermentation studies on stool

samples from the same subjects found gas production was increased with inulin and psyllium co-administration compared to inulin alone, suggesting the differences seen *in vivo* were not due to a direct inhibition of colonic fermentation. The differences between *in vivo* and *in vitro* in this study were that in the *in vitro* model, all the fibre is added into the chamber at the beginning and the stool is well mixed with the fibre. Thus we hypothesised two potential mechanisms; firstly, *in vivo* psyllium is slowing rate of inulin delivery to the colon.

This is supported by the observation that in participants who produced hydrogen after co-administration (n=8), oro-caecal transit was 23% slower than inulin alone. However this needs further study using a method of measuring oro-caecal transit time which does not depend on fermentation, using the breath hydrogen technique it was only possible to measure this in 40% of subjects co-administered psyllium. Secondly, psyllium is restricting the mixing of chyme in the colon, limiting access of the microbiota to inulin. MRI studies have shown chyme from the small bowel is distributed largely in the middle of the colon[213], and MRI tagging has shown a marked decrease in movement close to the mucosa in the haustral pockets, which would favour anaerobic fermentation[346]. If psyllium delays movement of inulin from the centre to the periphery of the lumen, then fermentation would also be delayed.

Although time constraints on scanning prevented study of events later in the day, from other studies we can expect the fermentation to continue slowly during transit through the distal colon. Further benefits of co-administration include delaying fermentation in the proximal colon, shifting SCFA production and particularly butyrate production more distally, where the highest cancer risk lies. Studies have shown that adding wheat bran to the diet reduced carcinogenesis in rats fed resistant starch[419], thought to be mediated by changes in transit and fermentation though the precise mechanism was unclear. Subsequent studies showed that fermentation of resistant starch in pigs was delayed and shifted distally by co-administration of wheat bran[353] a

phenomenon confirmed in humans[354]. The steric impedance of psyllium may lend itself as a useful tool when co-administered with a prebiotic to increase butyrate production in the descending colon and dilute the concentration of potential deleterious compounds ammonia and phenols[420].

Such changes in fermentation throw new light on the epidemiological associations between colorectal cancer and fibre intake and suggest future studies will need to take account the type of fibre, alone or in combination, and not just its amount.

6.4. Treatment of IBS-D with ondansetron

For those unable to manage their symptoms with dietary modification, medications such as ondansetron, a 5-HT_3 receptor antagonist (5HT3RA), have shown to benefit those with IBS-D[359]. Serotonin (5-HT) is found predominantly in the gut and stimulates intestinal secretion and motility; 5HT3RAs block these effects with a resultant slower gut transit. Although the mechanism of action on the receptor is known, the site of action and its precise effect on the physiology is unclear. Li *et al.* demonstrated serotonin stimulated pancreatic secretions which reduced with 5-HT_3 receptor antagonism in a rat model[421].

In the MOLID study, using therapeutic doses of the FODMAP lactulose as a model of IBS-D in healthy volunteers, we were unable to show a change in SBWC with the 5HT3RA ondansetron. The Fortisip meal given to our volunteers contained 17.4g fat, thus the serotonin-mediated mechanism to stimulate pancreatic secretions seen in rodents may not be present in humans.

The negative findings of the MOLID study suggest ondansetron's constipating effect occurs more distally than the region assessed, in the descending and sigmoid colon. Retrograde motor patterns have been identified in healthy individuals as the predominant motor pattern in the descending colon[422], hypothesised to act as a recto-sigmoid brake[423]. Earlier work using manometry catheters with lower spatial resolution described alosetron, a 5HT3RA, increasing colonic contractions in the descending colon[424] which may reflect the retrograde motility seen with HRM, however as yet this has not been studied so remains speculative. Colonic transit in the descending colon of patients with IBS-D is prolonged when taking ondansetron[154], further implicating this region as ondansetron's site of action.

Previous work has shown an increase in small bowel water and luminal distention after lactulose in patients with IBS compared to healthy volunteers[390], concluding it is small bowel motility rather than secretion and absorption that is altered in IBS. Undseth *et al.* used 10g lactulose in 200ml

water which produced a 300ml increase in small bowel water after 1 hour, whereas in MOLID in healthy individuals we used 13.6g lactulose with 200ml water and 300ml Fortisip, yet we found a small bowel water increase of only 89ml after 2 hours. Fortisip is a balanced meal supplement, containing many readily absorbed proteins and carbohydrates. Rapid absorption of substrates and water is likely to have reduced the impact of lactulose, and the degree to which IBS also affected small bowel water remains unclear.

The MOLID study also found evidence to reclassify the mechanism of action of lactulose, described in the British National Formulary (BNF) as an osmotic laxative[384]. Lactulose is rapidly fermented in the ascending colon, producing hydrogen within 10 minutes[399] and SCFAs within 60 minutes[389]. Despite an increase in small bowel water and motility, the rapid fermentation of lactulose and subsequent absorption of SCFAs, co-transported Na^+ and water, meant water content of the chyme in the ascending colon was unchanged, questioning its categorisation as an osmotic laxative such as polyethylene glycol. SCFAs including acetate have been shown to increase colonic motility and transit in animal models by increasing 5-HT[324], however other studies contradict these findings[425]. SCFAs act on mucosal receptors to influence colonic motility[31], and their effects may be both location and dose-dependent[132,400].

Response to treatment with ondansetron is not predictable with a NNT of 7.7, and clinical experience shows there is a wide variety in dosages prior to reduction in frequency or hardening of stool. This may be due in part to the heterogeneity of patient symptoms and variety of diagnostic rigour in the work up for IBS-D. Patients recruited to the trial further analysed in chapter four fit the Rome III criteria for IBS-D and had all undergone colonoscopy, nuclear medicine scans to assess for bile acid malabsorption and serology to exclude coeliac disease. In this group, responders to ondansetron had less severe abdominal pain and urgency and tended to be younger and less anxious. Biopsy analysis of 57 patients compared to 21 controls demonstrated increased concentrations of the 5-HT metabolite 5-HIAA together with an

increased 5-HIAA/5-HT ratio, suggesting an increase in 5-HT turnover. This fits with previous literature that serotonin metabolism is altered in IBS-D, having previously been shown to have greater postprandial plasma 5-HT[41] and reduced 5-HT reuptake via lower concentrations of SERT mRNA[39].

Trial patients were able to titrate their dose to control their symptoms, and a subgroup of them required <4mg ondansetron per day. These 'super responders' showed a four times greater slowing of their whole gut transit time than others taking a greater dose, suggesting they were more sensitive to the drug. Biopsy 5-HT concentrations were lower, which paired with the increased 5-HT release expressed as a percentage of biopsy 5-HT content, supports the conclusion that the low 5-HT is due to an accelerated turnover. These patients were more commonly female and had firmer baseline stools, however we were unable to describe a biochemical marker to alert the clinician they would be suitable to ondansetron therapy.

6.5. Future directions

The studies in this thesis, like many studies before it, have offered up as many questions as they have answered, as listed below:

1. **Do naturally occurring mixed fibre types behave similarly to two co-ingested but differing fibre types?**

In the NOPAL study, the fibre nopal (with both components of both viscous and particulate fibre) gave intermediate values for water content and breath hydrogen compared to the particulate wheat bran and viscous psyllium. EFIGI demonstrated a reduction in colonic gas production when co-administering inulin, a rapidly fermentable FODMAP, with psyllium, a viscous fibre. Nopal and the inulin-psyllium combination now require head-to-head analysis to see if they are comparable in their gas production profiles, symptom induction and fermentation products.

2. **Does nopal benefit patients with IBS?**

The work has already begun to answer this question; in a 1 week study of 60 patients with IBS (of all subtypes), 20g and 30g per day led to more patients reporting adequate relief of symptoms[327]. This pilot study shows nopal has a potential to help relieve symptoms in IBS, and warrants further larger trials of longer duration to assess the impact of nopal in each subtype.

3. **Can the knowledge of different fibre types be applied into real world dietary habits?**

In NOPAL and EFIGI, the fibres were given with water and study meals were uncomplicated. In the real world, complex foods are co-ingested in meals, making it difficult to extrapolate findings from the study. Whole foods such as kiwifruit, which has both fructose and gelling components, increase small bowel and colonic water, is well tolerated without causing symptoms of excessive gas and produces more frequent stools in healthy volunteers[281] and IBS-C[426]. It is possible that the gelling agents in kiwifruit prevent gas

production from the FODMAPs it contains. This suggests that it is not just the FODMAP content but the physical environment within which it lies which is key and the diets should reflect this. Thus fructose in apple juice might be avoided but whole apples tolerated due to the presence of pectin, even though they contain the same amount of fructose.

Studies assessing whole dietary components, perhaps by giving set meal plans, while complicated to devise and orchestrate, would give more meaningful and applicable results to consumers, and could lead to a range of ready-prepared meals suited to IBS patients with a balance of fibre types to reduce symptoms and provide necessary prebiotics.

4. **What is the microbial impact of foods with mixed fibre types and fibre co-administration?**

Inulin supplementation has been shown to act as a prebiotic, increasing colonic Bifidobacteria. The effect of co-administration of inulin and psyllium, while hoping to mediate abdominal symptoms, has as yet an unknown impact on the microbiota. A study designed to assess both the effect on symptoms and the microbiota profile would give a greater understanding to the dietary manipulation of the microbiota, both in the short and long term. Nopal too, being relatively new to the scientific community, has an unknown effect on microbiota which needs further assessment prior to its advocation in IBS.

5. **How does lactulose alter stool consistency?**

Our data from the MOLID study suggests lactulose does not alter water content of chyme in the ascending colon, contrary to the widely held belief that it is an osmotic laxative[427]. Lactulose metabolites lactic acid and acetate may act directly as a prokinetic in the colon, or indirectly on the microbiota due to its reduction in pH. Studies examining the effect of these metabolites infused into the colon, via MRI using T1 relaxation of the ascending and

descending colon, and correlating results to stool water content, would give further insight into lactulose's mechanism.

6. Where does ondansetron alter motility to slow transit?

Our data suggest ondansetron does not alter upper GI secretions implying that the effect on transit is due to altered motility. This raises the question of where this effect is exerted. As discussed earlier, 5HT3RAs slow transit[154] and increase colonic contractions[424] in the descending colon, which is where retrograde motor patterns are most active[423]. Further work should use HRM to establish in a placebo-controlled trial if ondansetron alters motility in the descending colon.

7. Does ondansetron provide adequate relief of symptoms in IBS-D?

Two RCTs assessing the use of ondansetron in IBS-D have been published[154,428] and a third is ongoing[429]. Using ondansetron at a titrated dose to a median 4mg per day, Garsed *et al.* demonstrated an improvement in stool consistency, faecal urgency and bloating[154]. Although pain scores were not significantly different, a greater proportion of patients reported "adequate relief" from their IBS symptoms and IBS severity score was significantly lower than placebo, suggesting, as previously reported[358], that urgency and stool consistency are the more bothersome symptoms.

Similarly, Plasse *et al.* showed an improvement in stool consistency, their primary endpoint, and a non-significant reduction in pain with ondansetron[428]. The medication in this study was 12mg ondansetron split into 3mg immediate release and 9mg in a controlled-release matrix, giving a bimodal release in a once daily tablet.

Once results from the third trial are known, meta-analysis is required to build the evidence for ondansetron's use in IBS-D. If the results are favourable, evaluation by NICE would be required in order to bring ondansetron into use in primary care, where most of IBS-D is managed.

7. References

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