Predicting the response to Ondansetron, a 5HT$_3$ receptor antagonist, in irritable bowel syndrome with diarrhoea: the utility of clinical features and objective biomarkers.

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Abstract

Background:

Patients with diarrhoea predominant irritable bowel syndrome (IBS-D) suffer from loose frequent stools with associated urgency and fear of incontinence. Relief from these symptoms is an important unmet need. The 5-HT$_3$ receptor antagonist Alosetron has been shown to increase stool consistency, decrease urgency and reduce abdominal pain leading to a global increase in satisfaction with treatment [1]. Its use is restricted following an increased incidence of ischaemic colitis and this agent is not available in Europe.

The serine proteases family of proteolytic enzymes have been identified as the source of increased faecal proteolytic activity in patients with IBS. These enzymes may be mechanistically important via their action on the Protease activated receptor (PAR) -2, inducing increases in permeability and hypersensitivity.

Aims:

To assess the efficacy of the commonly prescribed 5-HT$_3$ antagonist Ondansetron, in patients with IBS-D and to identify biomarkers that might allow us to predict response defining an Ondansetron responsive endophenotype of IBS.

To structurally characterise faecal serine proteases and define the impact of treatment with Ondansetron.

Methods:

120 patients meeting Rome III criteria for IBS-D entered a randomised, double-blind, placebo controlled, cross-over study of 5 weeks of Ondansetron 4mg versus placebo with dose titration allowed up to two tablets thrice daily in the first 3 weeks. Patients completed daily bowel symptom diaries documenting stool consistency using the Bristol Stool Form Score (BSFS). Gut transit and small bowel water content were measured in the last week of each treatment. The primary endpoint was average stool consistency in the last 2 weeks of treatment.

Faecal samples were obtained from 30 healthy volunteers (HV) and 79 IBS-D patients participating in a trial of Ondansetron versus placebo. Colonic transit was measured using radio-opaque markers. Faecal serine proteases (FSP) were purified from faecal extracts using Benzamidine-Sepharose affinity chromatography. SDS-PAGE profiled components were identified using trypsinolysis and tandem-mass-spectrometry. Functional protease activity in faecal extracts was measured using a colourimetric assay based upon the proteolysis of azo-casein

Results:

Ondansetron significantly improved stool consistency In the intention to treat analysis n= 101, with a 1.39 (95% CI1.20-1.58)point decrease on the Bristol stool form scale whilst taking Ondansetron compared to a 0.51 (95% CI 0.32-

0.72) point reduction whilst taking placebo p=<0.0001. Compared to placebo patients on Ondansetron experienced fewer days with urgency (p=0.01), lower urgency scores (P<0.001), reduced frequency of defecation (p=0.001) and less bloating (p=0. 25) although pain scores did not change significantly.

Protein analysis identified the most abundant FSPs as being of human origin and likely pancreatic juice derived. Functional assays showed increased FSP and faecal amylase in IBS-D compared to HV. Those with higher amylase had significantly higher FSP and greater anxiety. FSP activity correlated negatively with whole gut transit in IBS-D (Spearman r=-0.32, p=0.005) and HV (r=-0.55, p=0.014), but was not affected by treatment with Ondansetron.

Conclusions:

Ondansetron is an effective and well tolerated treatment in patients with IBS-D with a low number of side effects. It slows whole gut transit, but without a demonstrable difference in small bowel water content. Clinical rather than biochemical indicators predicted responsiveness to Ondansetron best. Patients with less severe symptoms are more likely to respond well to Ondansetron which should prove a useful addition to the current rather limited therapies available for this important group of patients.

Previous reports that FSP activity is elevated in some patients with IBS-D has been confirmed. We have increased our understanding of this phenomenon by characterising the proteins responsible for the serine protease activity, showing that most of this activity is likely due to human pancreatic enzymes.

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Abbreviations

AC	Ascending colon
ACWC	Ascending colon water content
AE	Adverse Event
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AUC	Area under curve
BM	Bowel movement
Bmax	Maximum Binding
BSF	Bristol Stool Form
bTFE	balanced Turbo Field Echo
CBT	Cognitive behavioural therapy
CI	Confidence Interval
CNS	Central nervous system
CRF	Case Report Form
CRP	C-reactive protein
CT	Computerised tomography
CTAD	Citrate-dipyridamole-adenosine-theophyline
CTSU	Clinical trials support unit
DNA	Deoxyribonucleic acid
EC	Enterochromaffin
EDTA	Ethylenediaminetetraacetic acid
EMR	Early morning rush
FDA	Food and Drug Administration
GABA	Gamma-amino-butyrate
GCP	Good Clinical Practice
GI	Gastrointestinal
Hb	Haemoglobin
HAD	Hospital anxiety and Depression
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
IBS	Irritable Bowel Syndrome
IBS-C	Constipation predominant IBS
IBS-D	Diarrhoea predominant IBS
IBS-M	IBS with Mixed bowel habit
IBS SSS	IBS symptom severity score
IBS-QOl	IBS quality of life
IEL	Intra epithelial lymphocyte
IMP	Investigational medicinal product
IL	Interleukin
IQR	Inter quartile range
Kd	Dissociation constant
MHRA	Medicines and Healthcare products Regulatory Agency
MMC	Migrating motor complex
MMR	Moderate morning rush

MRCP	Magnetic resonance cholangiopancreatography
mRNA	Messenger Ribonucleic acid
MRI	Magnetic resonance imaging
NHS	National Health Service
NMR	Normal morning rush
NNT	Number needed to treat
OR	Odds Ratio
PAR	Protease activated receptor
PHQ-12	Personal health questionnaire-12
PHQ-15	Personal health questionnaire-15
PIS	Participant Information Sheet
PI-IBS	Post infectious Irritable bowel syndrome
PRO	Patient reported outcome
REC	Research Ethics Committee
RNA	Ribonucleic acid
RR	Relative risk
R&D	Research and Development department
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SBWC	Small bowel water content
SD	Standard deviation
SERT	Serotonin transporter
SMR	Severe morning rush
SOP	Standard operating procedure
SPC	Summary of Product Characteristics
SSC	Study Steering Committee
SSRIs	Selective Serotonin Re-uptake Inhibitors
SUSAR	Suspected Unexpected Serious Adverse Reaction
TCA	Trichloracetic acid
Tds	Three times daily
TNF	Tumour necrosis factor
tTg	Tissue transglutaminase
UC	Ulcerative colitis
5-HT	5-hydroxytyptamine
5-HTTLPR	5-HT transporter length polymorphic region
5-HIAA	5-Hydroxyindoleacetic acid
γGT	Gamma-glutamyltransferase

Candidate statement

All of the work contained in this thesis was written and performed by me unless stated below:

Data collection from the patients recruited at the Manchester site (those with participant numbers beginning 02), 54 in total was done by Maggie Hastings.

I was assisted by Melanie Lingaya and Ravinder Kalley in the preparation and storage of blood and stool samples.

Analysis of liver function tests (LFT), CRP calcium and albumin were performed by the Department of Clinical Chemistry, University Hospital, Nottingham Analysis of the full blood count (FBC) was done by the Deparment of Haematology, Nottingham University Hospitals.Tissue transglutaminase (tTG) was done by the Deparment of Immunology, Nottingham University Hospitals

Assessment of stool faecal protease activity, faecal elastase, faecal amylase, platelet paroxetine binding and plasma 5-Hydroxyindoleacetic acid (5-HIAA) was performed by Dr Gulzar Singh.

Mast cell tryptase analysis was done by the Department of Immunology, University Hospital, Nottingham.

Affinity chromatographic purification of serine proteases, protein electrophoresis and mass spectography were all techniques performed by Dr David Tooth

All MRI scan sequences were written by members of the Sir Peter Mansfield Magnetic Resonance Research Centre. The MRI scanning was done by Caroline Costigan (MR Radiographer).

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1 Introduction

1.1 Irritable bowel syndrome

The irritable bowel syndrome (IBS) is a substantial and difficult clinical problem characterised by abdominal pain, bloating, and disturbed defecation. It is also associated with other intestinal and extra intestinal complaints such as heartburn, dyspepsia headache, backache, chronic fatigue and genitourinary symptoms. Its prevalence is estimated to be between 14 and 24% in women and 5-19% in men in a western population [2] and accounts for up to 40% of consultations in the gastroenterology outpatients. The direct and indirect costs of caring for this group of patients have been estimated to be as high as $30 billion dollars per year in the United States alone[3].

IBS patients show a range of psychosocial and gastroenterological abnormalities and it is this heterogeneity of complaints, which are often variable with time within an individual patient, that presents clinicians and researchers with a challenge, first to define the pathological process (or more likely processes) at work, and ultimately provide safe and effective therapy for this group of patients whose symptoms can lead to a greatly impaired quality of life[4]. Understanding of disease mechanisms and therefore effective treatment discoveries are hampered by a lack of reproducible biomarkers.

Attempts through the years to define the irritable bowel syndrome have been legion. There are to date 5 sets of existing diagnostic criteria and it can be seen in Table 1.1 that the positive predictive value of each set of criteria when applied to unselected patients with lower gastrointestinal symptoms, varies

often with wide confidence intervals. These criteria are evaluated as to their ability to distinguish organic from non organic disease.

There is evidence that the predictive value is altered depending on whether the criteria are applied to men or women[5] and also depending on the ethnic group that is being assessed[6].

Criteria or model	Symptoms, signs, and laboratory investigations included in model	Symptom duration required	Method of defining IBS	Positive likelihood ratio for a diagnosis of IBS
Manning [7]	Abdominal pain relieved by defecation More frequent stools with onset of pain Looser stools with onset of pain Passage of mucus per rectum Feeling of incomplete emptying Patient-reported visible abdominal distension	none	3 positive symptoms	2.9(95% CI 0.93 -1.6)[8]
Kruis[9]	Symptoms: abdominal pain, flatulence , or bowel irregularity Description of pain as "burning, cutting, very strong, terrible,feeling of pressure, dull, boring, or not so bad" Alternating constipation and diarrhoea Signs: abnormal physical findings and/or history pathognomic for any diagnosis other than IBS Erythrocyte sedimentation rate >20mm/2h Leukocytosis >10 000 cells / microlitre Anaemia Impression by the physician that the patient's history suggests blood in the stools	>2 years	Logistic regression model	8.6 (95% CI 2.9-26)[8]
Rome I	Altered stool frequency Altered stool form Altered stool passage Passage of mucus per rectum Bloating or distension	≥ 3 months	Adnominal pain or discomfort relieved with defecation or associated with a change in stool frequency or consistency, plus ≥2 positive symptoms on at least 25% of occasions or days	4.8 (95% CI 3.6-6.5)[8]
Rome II[10]	Pain relieved with defecation Onset of pain associated with a change of frequency of stool Onset of pain associated with a change of form of stool	≥12 weeks(need not be consecutive in the last year)	abdominal discomfort that has ≥2 positive symptoms	
Rome III[11][1]	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	Recurrent abdominal pain or discomfort ≥3 days per month in the last 3months associated with ≥2 positive symptoms	

Table 0-1 Diagnostic criteria and statistical models for the diagnosis of IBS

Our current symptom-based disease definitions are important in providing a diagnosis in a population in whom a positive diagnosis is often key to a meaningful therapeutic relationship with the clinical team. These criteria are also essential in facilitating accurate patient selection into research studies and provide a common language for clinicians and scientists. Despite the trend towards this approach many doctors feel uncertain and treat IBS as a diagnosis of exclusion, based largely on a fear of missing a progressive organic condition. This approach is expensive and often significantly increases patient's anxiety. This concern persists despite studies in both primary and secondary care confirming that patients who meet symptom based criteria for IBS have a low pre-test probability for organic disease, with the exception of celiac disease, and current recommendations do not support the employment a battery of tests in these patients [12, 13].

Symptom based criteria have several weaknesses, inclusion into trials of relatively heterogeneous groups lead to conflicting results from trial to trial, large numbers are needed to generate interpretable data and there is the risk of missing important positive findings in smaller subgroups or endophenotypes who are swamped by the "noise" generated by other subjects whose disease mechanisms are likely different. An area of particular unmet need is the difficulty clinicians have in selecting patients with IBS who will respond to a given treatment. Multiple trials of unsuccessful therapy leaves patients dissatisfied[14], and even with clinically proven preparations numbers needed to treat are high, for example for Alosetron the number needed to treat was still 7[15].

It is clear that the introduction of a test which positively identifies even a small number of these individuals as having IBS via a biologically sound marker would be invaluable to patients, clinicians, scientists and the public purse alike. Furthermore being able to preselect patients most likely to respond to a particular treatment, via such a biomarker, would avoid prolonged trials of ineffective therapy leading to better outcomes and a better satisfied patient group.

1.1.1 Epidemiology

In order to better manage our patient population it is important to know who they are. The classification systems described in the opening paragraphs are the tools by which we define incidence and prevalence. The Manning criteria have traditionally given the highest estimates with values between 9% using a cut off of ≥4 criteria and 20% using ≥ 2 criteria in North America[16]. With Rome II criteria the same group estimate a prevalence of 4.7% although others have found higher rates at 12.1%[17]. In the U.K. and Europe using ≥2 manning criteria estimates a prevalence of 21.6%[18] but again lower values of between 2.9-8.1% are reported using the Rome II criteria[19, 20]. Attempts have been made to define the prevalence of the different IBS subtypes. There is some agreement, with most studies finding the largest subgroup (46.7-63%) to be those complaining of an alternating type [17, 20]. In the US two studies have found the diarrhoea subtype to be most common (53.9-57.6%)[21, 22], and important differences in subtype prevalence may exist in the developing

world, with constipation being reported as occurring in 50.4% of subjects in a Pakistani study[23].

Estimating the incidence of IBS in a given population is challenging. Due to the fluctuating nature of symptoms it is difficult to pick out those cases that are truly incident, from those that represent a flare of this relapsing and remitting condition. The criteria themselves have been shown in short term studies not to be stable with the proportion of subjects in each of the IBS subtypes staying the same, but individuals commonly transitioning between subtypes, particularly between mixed IBS (IBS-M) and constipated IBS (IBS-C)[24]. A UK study including nearly 4000 subjects has however estimated the incidence of new IBS to be between 1.5-2.8% a year, again depending on the criteria used [25].

At least two-thirds of patients with IBS during the aforementioned UK follow up at study entry had persistent symptoms at 10 years[25], and an Icelandic group found that during a decade of follow up a similar number of patients developed IBS as those who lost IBS. Their conclusion was that rather than a true resolution of symptoms that their findings were consistent with a condition where a cluster of symptoms float in time between IBS categories, functional dyspepsia and heartburn. The prognosis of post infectious irritable bowel syndrome (PI-IBS) is somewhat better than unselected IBS but may take years to resolve[26].

1.1.2 Risk factors for the development of IBS

The current view of IBS aetiology is that several factors interact during the life time of the patient to induce IBS. These include gender, early life experience, genetics, infection and personality traits such as anxiety and neuroticism. Of the risk factors studied the role of infection is one of the areas that we have the most data on. Post infectious or PI-IBS can be regarded as a natural experiment in which an insult in the form of an infective illness impacts on an individual with underlying genetic and psychosocial predispositions who then develops IBS. Unlike other IBS patients, there is a clear onset in time and well defined patho-physiological changes. Measuring both psychological as well as pathophysiological features has allowed us to weigh the importance of these different factors in the generation of IBS symptoms.

Around 1 in 10 of patients with IBS [27] believes their IBS began with an infectious illness, and risk factors for developing PI-IBS include in order of importance:

1. The duration of illness and severity, an illness lasting greater than three weeks confers a relative risk of 11.4 (95% confidence interval (CI), 2.2-58) compared to one lasting less than 7 days[28].

2. The toxicity of the infecting bacteria also plays a role illustrated by patients whose cultured *C. jejuni* supernatants demonstrated toxicity to an in vitro cell line. These patients had a relative risk of developing persistently deranged bowel habits of 12.8 (95% CI) 6.1-101) compared with those who had no toxin [29].

3. Patients who smoke are more likely to develop PI-IBS with an OR of 4.8(1.5-15.2)[30]. Whether this is a direct effect of nicotine or more likely smoking is a marker for adverse psychological factors is unclear. However smokers report more anxiety than non-smokers [31], which is also more common in IBS patients.

4. The role of low grade immune activation in IBS has been established in animal and human studies. Increased lymphocytes have been reported throughout the colon in unselected IBS with diarrhoea[32], and in the rectum in PI-IBS[33-35]. This has been associated with increased mRNA for interleukin (IL)-1β [36, 37]. After *Campylobacter jejuni* infection the persistence of inflammatory cells predicts the subsequent development of PI-IBS, a 1-SD increase in enterochromaffin cell (EC) count was a associated with a mean 3.8-fold (95% CI, 1.3–7.5) increase in relative risk[35].

Mast cells are increased in animal models of infection and in the human terminal ileum after Shigella infection [37] and in rectal biopsies of PI-IBS patients [38]. They have also been reported to be increased in the terminal ileum of unselected IBS patients with diarrhoea[39]. PI- IBS may well involve inflammatory changes in the small intestine as well as the colon since small intestinal permeability is increased [29] , a change which could be mediated by increased ileal mast cells.

Other markers of inflammation in IBS patients include higher cortisol and increased peripheral blood mononuclear cells cytokines including IL-6, IL-8[40], tumour necrosis factor (TNF)-alpha, IL-1beta, IL-6, as well as increased

lipopolysaccaride -induced IL-6 levels when compared to healthy controls [41].

5. All subtypes of IBS are more frequent in females than males, with a male: female ratio of between 1.2 and 3.1 in North America and Europe [16, 42]. There is no evidence of any differences between the immune response of men and women to infection and rectal immunocyte numbers are not different[43], yet female gender is frequently reported risk factor for developing PI-IBS[34, 35]. This may in part be due to confounding with anxiety and depression which is commoner in women, since when this is controlled for in multivariate analysis female gender is no longer a risk factor [34, 35]. However female gender did remain a factor in multivariate analysis in one recent study where there was relative risk of 2.36 (1.23 – 3.98) for gender versus 1.82 (1.05 – 1.22) for anxiety [44].

6. Subjects with IBS have higher levels of anxiety neuroticism and depression compared to subjects without IBS. This has also been demonstrated prospectively in patients without IBS where high levels of illness behaviour, anxiety, sleep problems and somatic symptoms predict the subsequent development of IBS [45]. This vulnerability to develop IBS may depend on early learning. Exposure in childhood to parenting styles which reinforce illness behaviour in early life is associated with the later development of functional bowel disorders[46].   The presence of hypochondriasis and neuroticism, which are thought to be enduring rather than acquired traits, increase the risk of PI-IBS, RR=2.0 (1.7-2.5)[47] while each standard deviation

increase in depression, increases the risk of PI-IBS 3.2 fold(1.8 − 8.2)[28].

Adverse life events in the 3 months preceding infection also increase the risk,

RR= 2.0 (1.7 -2.4) [34]. Other psychological factors including perceived stress

RR 1.10 (1.02-1.15) and negative illness beliefs RR 1.14 (1.03-1.27) have also

been shown to increase the risk of developing PI-IBS [48]. Ongoing stress may

initiate an inflammatory response in the human jejunum [49] and also

increase the severity of inflammation in rats with 2,4,6-trinitrobenzene

sulfonic acid (TNBS) colitis[50].

 A genetic predisposition to IBS has been proposed with work looking at

candidate genes within the serotonin and inflammatory pathways amongst

others. To date studies have been small and as yet the effects seen have not

been shown to be reproducible. A single large twin pair study showed

increased concordance for IBS in monozygotic compared to dizygotic twins

(17.2% versus 8.4%) supporting a genetic component to IBS but also

demonstrated that having a mother or father with IBS were independent

predictors of IBS and stronger predictors than having a twin with IBS,

suggesting that social learning has an equal or greater influence than genetic

factors[51].

Age >60 years may protect against the development of PI-IBS[28] while

treatment with antibiotics may be associated with increased risk. Antibiotics

have been shown to cause a transient alteration in the normal microbiota[52]

and one possibility is that increased antibiotic use by patients with IBS results

in an unstable flora. Patients given antibiotics are 4 times more likely to report

bowel symptoms 4 months after treatment initiation than a control population with no antibiotic use[53], and antibiotic use has been reported to be a risk factor for developing IBS per se with an adjusted OR of 3.70 (1.80-7.60)[54].

1.1.3 Comorbidities and overlap with other functional GI disorders

Patients with IBS are about twice as likely to be diagnosed with a variety of somatic disorders as other comparison groups[55]. These include fibromyalgia chronic fatigue syndrome, chronic pelvic pain, temporomandibular joint dysfunction and others such as back pain and premenstrual syndrome. The overlap with other functional gastrointestinal diseases is even greater with between 23 and 87% of IBS subjects studied reporting dyspepsia [56, 57]. These disorders share many common aetiological mechanisms, such as visceral hypersensitivity, stress and anxiety and the psychological trait of somatisation, but attempts to define specific disease mechanisms, and thus targeted treatments, to account for the occurrence of these multiple symptoms in patients are as yet inadequate.

The sheer number of these co morbid conditions and symptoms is an important factor in determining quality of life in our patients and impacts significantly on health care costs with IBS patients making twice as many health care visits in a year as age matched controls[58].

1.1.4 Treatment

Because the aetiology of IBS has not been firmly established there is no discrete target for pharmacological therapy. Therapies to date have been focused on symptom relief. Traditional first line therapy has been the use of fibre to regulate defecation, and antispasmodics to improve pain and diarrhoea. The benefits of fibre however have been shown to be small and insoluble fibre can in some make the condition worse[59] . Other first line treatments include laxatives and anti diarrhoeal agents; Loperamide an opiod analogue reduces diarrhoea but has little effect on abdominal pain[60], but is preferable to the use of codeine which has sedating and potentially addictive properties. There is a dearth of controlled evidence in IBS-C regarding the use of traditional laxatives; consensus opinion here is that the use of osmotic laxatives such as polyethylene glycol is preferred, with stimulant laxatives acting erratically with tachyphylaxis and a risk of dependency resulting in recommendations only for occasional use [61]. Encouragingly 2 new agents, Linaclotide, a novel intestinal secretagogue, which works by activating the guanylate cyclase C receptor on the luminal surface of the intestinal epithelium, and Lubiprostone which causes secretion of fluid and electrolytes in the small bowel through the activation of chloride channels, have been demonstrated to be efficacious in patients with both chronic functional constipation and with IBS-C [62-64].

Tricyclic antidepressants are often used in the treatment of chronic pain and their use to modulate the pain experienced by patients with IBS is widely

accepted although evidence for efficacy as been conflicting with society guidelines being cautious in recommending their first line use[61]. A meta-analysis including a total of 575 patients concluded the number need to treat was 4[65]. In practice the use of these drugs is often limited by their side effects which include somnolence and a troublesome dry mouth. Selective serotonin reuptake inhibitors (SSRI's) lead to a significant improvement in health related quality of life but without significant changes in bowel symptoms or pain [61] .

Rifaximin, a poorly absorbed antibiotic that is therefore present in high concentrations in the gastrointestinal lumen has been shown in two large randomised control trials to have benefit in patients with non-constipated IBS. Here the end point, adequate relief from IBS symptoms for at least 2 of the first 4 weeks following treatment was reached in 41% receiving active therapy compared to 32% on placebo, this was sustained for 10 weeks of follow up[66]. However only a minority respond with a number needed to treat of 11. Others have sought to modify the bacterial milieu of the colon by the use of probiotics. These by definition are live microorganisms that confer benefit to the host in this case by quantitative and qualitative changes in the colonic flora. Benefit has been shown in 5 randomised control trials in reducing bloating and flatulence but the variety of species strains and doses of probiotics used means it has thus far been difficult to come to any conclusion about the optimum strategy to use in IBS.

We know that psychological morbidity is important in IBS and an awareness of this should instruct the delivery of all treatments. Specific psychological approaches that have been explored include simple relaxation techniques, cognitive behavioural therapy (CBT), formal psychotherapy and hypnotherapy. Trial design and blinding is challenging in these therapies but evidence exists to suggest at least in expert hands there is benefit in assisting patients in coping with their symptoms and in the case of hypnotherapy delivering lasting improvements in the quality of life and psychological status[61].

It is clear that new strategies are needed and a number of new mechanistic agents targeted at abnormalities thought to be important in the aetiology of IBS have come onto and are coming to market in recent years. Of these by far the largest group is the drugs designed to target the serotonin system, primarily at the 5-hyroxytryptamine (5-HT)$_4$ and 5-HT$_3$ receptors. I will discuss the drugs that have targeted the 5-HT$_3$ receptor in more detail in section 1.2.1.

Prucalopride a 5-HT$_4$ receptor agonist is highly selective and does not display the problems with ischaemic cardiovascular events associated with its predecessor Tegaserod and has been shown to improve symptoms in chronic constipation although not yet in patients with IBS and chronic constipation [67]. Alternative strategies aimed at other sites have met with varied success. Mesalazine has been shown to reduce total colonic immune cell numbers[68] and also to improve pain[69], but targeting the Corticotrophin release

hormone-1 receptor showed early promise without resulting in significant benefit clinically [70].

1.2   5-HT₃ antagonists

The 5-HT$_3$ receptor antagonist's major clinical use has been in the inhibition of chemotherapy induced nausea and vomiting and for the prophylaxis of post operative nausea and vomiting. This class of drugs includes Ondansetron, one of the first to market, Tropisetron, Granisetron, Cilansetron, Alosetron and Ramosetron.

1.2.1 Alosetron

The appearance of Alosetron, which largely replaced Ondansetron in the early 1990's means that most evidence on the role of 5-HT$_3$ antagonists in IBS relates to Alosetron and subsequently Cilansetron and Ramosetron. Alosetron and Cilansetron have been evaluated in several very large randomised clinical trials.   Ramosetron has been shown in 2 randomised control trials to be effective in improving global symptom severity and stool consistency[71, 72] A meta-analysis of Alosetron trials shows a consistent beneficial effect in females with diarrhoea predominant IBS (IBS-D)[73]. Most of the studies lack enough males to make any significant observations but a single study has shown efficacy using the same dose found to be effective in women [74].   The most consistent benefits are an increase in stool consistency, a decrease in urgency and a reduction in abdominal pain leading to a global increase in satisfaction with treatment. In spite of all the clinical evidence of benefit, the mechanism of action in individual patients was never

clearly identified and the number needed to treat was 7. Genetic factors may be important since a previous somewhat underpowered study has shown that the presence of the LL serotonin transporter (SERT) promoter polymorphism was associated with a greater effect of Alosetron in slowing colonic transit when compared to heterozygote patients with the LS polymorphism [75].

A cloud fell over the use of 5-HT$_3$ antagonists with the finding of an increased incidence of ischaemic colitis in patients on treatment. This ischaemic colitis associated with the use of Alosetron is as yet without a clearly demonstrated biological mechanism. 5-HT$_3$ receptors, although not present on the blood vessel wall itself are expressed on sensory endings, and it is hypothesised that blockade of these that potentially inhibits the reflex mechanisms regulating gut blood flow. However a study in anesthetized rats showed that Alosetron did not interfere with splanchnic vascular control mechanisms during occlusion and reactive hyperaemia [76]. This finding has been added with a series of studies that show although there is a small reduction in mesenteric blood flow in rats given intravenous Alosetron, this reduction failed to modify blood flow or intra-luminal pressure in the colon. This appeared to be a class effect, at least in the rat, with similar findings with the injection of Cilansetron. This study went on to show that short term oral administration of Alosetron did not affect the haemodynamics of the superior mesenteric artery and that fasting had no additional effect on the actions of Alosetron on blood flow [77]. An alternative, and more likely, explanation is that it is the increase in incidence of constipation, itself shown to be associated with a reduction in colonic blood flow[78], that predisposes patients to this complication. It

should be noted that most cases of ischaemic colitis were mild and self limiting once treatment was ceased. An important potential contributory factor to the problems experienced with constipation was the use in these trials of a fixed dose in all comers.

1.2.2 Ondansetron

Early studies of 5-HT$_3$ antagonists did use Ondansetron and showed slowing of colonic transit in healthy volunteers. Here mean colonic transit time as measured by radio-opaque markers was 27.8 hrs whilst taking placebo vs. 39.1 hours on Ondansetron, and this effect was seen to be greatest in the left colon and rectosigmoid[79]. Ondansetron was also shown to improve stool consistency in patients with IBS-D in a small pilot study[80], to inhibit the ascending colon response to feeding[81], and increase rectal compliance[82]. In the early 1990's Ondansetron was shown to have a beneficial effect on both functional dyspepsia and IBS-D though the study was probably underpowered. Although it didn't alter abdominal pain it did reduce bowel frequency and improve stool consistency in 50 patients with IBS-D[83]. The effect of Ondansetron on stool consistency is important since it alleviates urgency, one of the most debilitating features of IBS.

1.2.3 Ondansetron pharmacokinetics, safety and rationale for use

Following oral administration, Ondansetron is passively and completely absorbed from the gastrointestinal tract and undergoes first pass metabolism. Peak plasma concentrations of about 30ng/ml are attained approximately 1.5

hours after an 8mg dose. Bioavailability, following oral administration, is slightly enhanced by the presence of food but unaffected by antacids. Studies in healthy elderly volunteers have shown slight, but clinically insignificant, age-related increases in both oral bioavailability (65%) and half-life (five hours) of Ondansetron. Gender differences have been shown in the disposition of Ondansetron, with females having a greater rate and extent of absorption following an oral dose and reduced systemic clearance and volume of distribution (adjusted for weight). Ondansetron has now been used very extensively in sick patients for nearly 20 years and has in contrast to Alosetron never been associated with ischemic colitis. There is early evidence of its efficacy in patients with IBS-D and its safety track record makes it an attractive potential treatment in our patient group.

1.3   Biomarkers in Irritable bowel syndrome

In medicine a biomarker can be used as an indicator of a particular disease state and to predict and monitor response to treatment. An ideal biomarker according to the FDA is one which is specifically associated with a disease state and can differentiate between conditions with similar physiological conditions. It would be desirable if this could be measured in blood urine or stool, removing the need for painful or invasive testing and if the test itself should rapid, accurate, simple, cheap and readily available with a measurable and standard baseline as a reference point.

There already are some candidates in IBS but they are far from ideal. Differences in permeability, motility, visceral hypersensitivity, and evidence of

mucosal inflammation have been proposed and will be discussed in the following sections, but all lack some or many of the characteristics of an ideal marker with many of them being arduous to perform and invasive in nature. Another approach is to use existing markers and software to design panels of tests which can be used to differentiate IBS from organic disease [84]. The hunt is however still on for ideal one stop tests that will allow the definition of disease mechanisms which will in turn lead to drug discovery and a safe and effective treatment for the patient in the clinic or primary care. In the context of this thesis I will be exploring the ability of biomarkers in the serotonin system to predict response to treatment acting at the 5-HT$_3$ receptor, as well as emerging potential markers in the stool as well as the tried and tested measures of transit and the novel field of magnetic resonance imaging (MRI).

1.3.1 Motility and transit

Abnormalities in gut motility have been described in IBS with the use of invasive manometric techniques. Intubation of the small bowel has revealed prolonged periods of small bowel contractions and giant contractions in the distal terminal ileum in patients with IBS. The same study found that cramping abdominal pain was associated with propulsive ileal motility and jejunal bursts were also sometimes associated with abdominal symptoms[85]. Despite this association the impracticality of these measurement techniques precludes their use in routine practice. The advent of the new wireless motility capsule has recently overcome many of these methodological challenges. This capsule measures pressure, temperature and pH and at

present is best validated as a test for transit. Although it does measure pressure, the clinical utility of the pressure data yielded by this technique has not been clearly established[86].

In contrast to manometry the methods available to measure the time it takes for food or other materials to pass through the gastrointestinal (GI) tract are highly patient acceptable. These measures of gut transit include the use of radio-opaque markers and scintigraphy. The most simple of all is the marker method validated by Metcalf et al[87]. Here the subject swallows 20 markers at 9 am on each of three consecutive days with a plain abdominal radiograph on day 4, the transit time is calculated as 1.2 x number of markers present. Validation was performed against multiple daily abdominal films and the faecal output of markers. This simple method provides a good estimate of mean colonic transit in subjects whose transit is <72 hours and is quick and cheap to perform. The technique can be modified in subjects with retention of all sixty markers at day 4 by the addition of a further film on day 7.

There is evidence of accelerated transit in IBS-D patients compared to normal values. In a study of 72 IBS patients and 25 healthy controls total gut transit was accelerated compared to healthy controls and those with IBS-C, no differences between IBS-C patients and controls were seen[88]. A combination of markers and the hydrogen breath test to examine small and large bowel transit in IBS patients demonstrated that small bowel transit times were significantly shorter in patients who complained predominantly of diarrhoea, and significantly longer in patients who complained predominantly

of constipation, compared with controls [60]. However these are not consistent findings with other authors finding no differences in transit times between groups and transit times falling within the normal range which do not appear to correlate well with bowel symptoms in IBS[89].

Colonic transit is significantly associated with changes in stool form accounting for 19-27% of the variance [90], and changes in transit in response to drug treatment appear to correlate consistently with stool form and to a lesser extent stool frequency [91]. It would seem transit is a suitable marker for the assessment of a drug whose aim is to affect bowel habit but there is no evidence to suggest transit is a marker for overall IBS severity.

1.3.2 Visceral hypersensitivity

Visceral hypersensitivity was the first feature of IBS to be seriously considered as a biomarker. This phenomenon of an enhanced perceptual response to a standardised stimulus is measured in a variety of standardised ways; most commonly by the use of a computerised barostat that delivers controlled balloon distension either in the colon or the rectum or by the application of heat or a chemical stimulus. A number of peripheral mechanisms such as mucosal immune activation and mast cell degranulation, as well as changes in central pain processing are potentially responsible for these findings. As a group, patients with IBS show lower average thresholds for pain or discomfort than healthy controls, and 20-60% are hypersensitive at baseline[89]. Pain and bloating scores do correlate with measures of visceral hypersensitivity but there is at best only a moderate correlation with IBS symptoms or response to

treatment[92]. There is considerable variability across patients, between different research groups, and there is an appreciable overlap with the healthy volunteer population[89]. In addition the data obtained are in the laboratory rather than a real life environment where a number of cognitive and emotional variables (both positive and negative) will influence this subjective measure. These factors as well as the considerable time and effort needed to standardise the procedure limits the use of this technique outside expert centres.

1.3.3 Mucosal inflammation

Mucosal immune activation is a cornerstone concept in the aetiology of IBS and shows some promise as a biomarker. Immune cells have been shown to interact with sensory and motor systems indicating a potential role in symptom generation. The use of measures of inflammation as a biomarker is limited by several factors. There is considerable overlap between healthy subjects and those with IBS[35], and the most commonly used method of quantitative histology are time consuming and subject to error, some of these difficulties can be overcome with automated analysis[38], but there is as yet no standard method that can be easily applied to routine clinical samples.

1.3.4 Other emerging biomarkers

A small number of studies suggest that genetic polymorphisms might predict drug responsiveness. Proposed polymorphisms include those found in genes coding drug metabolism enzymes and also the serotonin transporters gene[82]. However these studies are rather small and the results require

replication in larger series before they can be considered potential biomarkers of drug responsiveness. New advances in microbiological techniques are revealing hitherto unknown complexities in the GI tract flora of patients one consistent feature of the many studies in this area being a decrease in overall diversity and the number of aerobic bacteria with an increase in aerobes including Proteobacteria [93]. These may well in the future prove useful biomarkers for studies of treatments likely to alter the flora such as probiotics and antibiotics. The discovery of increased proteolytic activity in the stool of patients with IBS-D [94] has led to the consideration of this activity as a potential biomarker that may be altered by treatment, this will be further discussed in the section 1.5.

1.4 Serotonin and the irritable bowel syndrome

For the purpose of understanding the use of the serotonin system to provide biomarkers in IBS I will first describe the role of serotonin and in particular the 5-HT$_3$ receptor, the target of our therapy, in normal gut physiology. Secondly I will describe the abnormalities of 5-HT signalling found in the periphery (i.e. serum) and thirdly those abnormalities of the serotonin system that are not accessible by simple venepuncture, primarily those seen in the gut mucosa. This is pertinent as it is clear our ideal biomarker would be one easily accessed and venepuncture is easy quick and acceptable to most patients.

1.4.1 Serotonin and its role in the GI tract in health

Serotonin or 5-Hydroxytryptamine is a biogenic amine synthesised from the essential amino acid tryptophan. Tryptophan is hydroxylated by the enzyme

tryptophan hydroxylase to 5-hydroxytryptophan. This is then decarboxylated to 5-hydroxytryptamine by the action of L-amino acid decarboxylase. Complete dietary deficiency of tryptophan will reduce serotonin synthesis within 24 hours but by contrast an increase in intake will not increase synthesis as it is present in normal diet in excess[95]. Serotonin is metabolised 5-HIAA and excreted by the kidneys via glomerular filtration and active secretion into the proximal tubules.

5-HT is present throughout the gastrointestinal tract , in the enterochromaffin cells and the enteric nervous system and makes up 80% of the total body 5-HT[95]. Its primary role is as a mucosal signalling molecule activating receptors on neurons, smooth muscle cells and epithelial cells within the gut regulating peristalsis secretion and sensation[96].

Serotonin signalling is terminated by its uptake via the serotonin transporter (SERT) expressed on the epithelial cells of the gut. SERT is a highly regulated protein that can be influenced by genetic or epigenetic factors. The SERT gene is located on chromosome 17: it spans 37.8 kb and is composed of 14 exons that encode a 630 amino acid protein. It is associated with a 5-HT transporter length polymorphic region (5-HTTLPR) which has a long L and short S variant. This is located 1.2kb upstream of the transcription start site in exon 1 and the length of the polymorphism potentially influences the level of transcription with least SERT expression in the SS type and most in the LL.

The actions of serotonin depend on the interaction of 5-HT with its receptor subclasses which are 7 in number. Receptor subclasses 5-HT$_{1A}$ and P, 5-HT$_2$, 5-HT$_3$, 5HT-$_4$ and 5HT-$_7$ are found in the gut.

1.4.2 5-HT$_3$ receptor

The 5-HT$_3$ receptor is phylogenetically older than the other serotonin receptors and, rather than being a G-protein coupled unit, is a ligand-gated cation channel belonging to the nicotine/gamma-amino-butyrate (GABA) receptor super-family. It is a pentamer consisting of five monomers which form a centrally permeable cylindrical body that is easily penetrated by small cations. There are five monomer subtypes (A-E) and the 5-HT$_3$ receptor exists as a variety of homo or hetero dimers with differing biophysical and pharmacological properties, the functional relevance of these differences is not yet clarified[97].

5-HT$_3$ receptors are located on mononuclear cells, lymphocytes, and intestinal enterochromaffin cells as well as peripheral and central neurons. In the periphery they are found on pre and post ganglionic autonomic neurons and within the myenteric and submucosal plexus. Within the central nervous system (CNS) 5-HT$_3$ receptors are much less prevalent than other subtypes but are found in the areas responsible for the integration of the vomiting reflex, pain processing, the reward system and anxiety control (area postrema, nucleus tractus solitarii, nucleus dorsalis, nervi vagi, nucleus caudatus, nucleus accumbens, amygdale, hippocampus, entorhinal cortex, cingulated cortex and dorsal horn ganglia)[98], and their location at nerve endings suggests a role in

the regulation of neurotransmitter release. CNS effects of 5-HT$_3$ blockade have been found to be anxiolytic [99] and have a role in reducing alcohol intake in early onset alcoholism[100].

Activation of 5-HT$_3$ receptors can be immunomodulatory, for example via the induction of T cell proliferation via the protein kinase C-dependent phospholipase D pathway [101]. These immunoregulatory pathways may have a net anti-inflammatory effect with Tropisetron being shown to have an inhibitory effect on the secretion of TNF alpha and interleukin 1beta in rheumatological patients. Topical Tropisetron showed clinical macroscopic and microscopic improvements in acetic acid induced experimental colitis in rats similar to that seen with dexamethasone [102]. Other studies have shown Tropisetron to exert its effects through non 5-HT$_3$ pathways, inhibiting T cell proliferation via a calcineurin inhibiting function and inhibiting the signalling pathway leading to NF kappa b activation [103]. Ondansetron may have less potent effects with partial inhibition of T cell proliferation and may therefore not have these other non 5-HT$_3$ mediated immune effects [103]. Important in our patient group is the effect 5-HT$_3$ antagonism has on neurogenic inflammation and noiciception in the periphery where it can inhibit the stimulated release of substance P, neurokinin A and calcitonin gene related peptide from primary afferents[104]. it is this substance P related pain modulation that is thought to be the mechanism by which 5-HT$_3$ receptor antagonists are effective in fibromyalgia in other pain syndromes such as migraine[105] and chronic neuropathic pain[106]. Benefit in fibromyalgia is also gained from a reduction in fatigue which has been demonstrated in other

non painful conditions such as the fatigue associated with chronic hepatitis C virus infection [107]. Taken together the evidence points to usefulness of a 5-HT_3 blockade in conditions linked to inflammatory stimuli and altered pain perception in the context of chronic pain, making this an attractive target for IBS therapy.

1.4.3 Biomarkers within the serotonin system

1.4.3.1 Platelet free plasma 5HT

Free platelet poor plasma serotonin represents the serotonin that is newly released by gut enterochromaffin (EC) cells not yet taken up into platelets via the SERT transporter. Conversely platelet serotonin content represents changes in serotonin over time. Studying plasma serotonin requires the careful collection of blood via a large bore needle without the use of a tourniquet in order to minimise platelet activation and serotonin release. In this way increases in postprandial serotonin in IBS patients have been shown. It is hypothesised that serotonin release (greatest postprandially) leads to the increase in symptoms that is often observed in patients after eating. A small pilot study found no differences in fasting 5-HT in IBS patients, but higher post-prandial 5-HT concentrations with a longer duration of 5-HT peak than healthy volunteers[108]. These findings have been confirmed in larger study which also showed IBS-D patients to have higher fasting 5-HT than healthy control and patients with IBS-C, with increased area under the curve (AUC) postprandially in IBS-D compared to healthy volunteers but no increase compared to fasting in the IBS-C group [109]. This increase in postprandial 5-

HT has also been shown in PI-IBS[110] and demonstrated to weakly correlate with postprandial symptoms[111].

1.4.3.2 Plasma 5-Hydroxyindoleacetic acid (5-HIAA)

Using platelet poor plasma to measure 5-HIAA has been less frequently studied. In patients with IBS-C a decrease in fasting 5-HIAA was observed but there was a preserved 5-HIAA:5-HT ratio suggesting normal breakdown of a reduced amount of serotonin in these patients. In the same study IBS-D subjects were found to have normal 5-HIAA but a decreased 5-HIAA: 5-HT ratio after feeding [109]. This points to the capacity for serotonin breakdown failing to match the amount of released serotonin indicating a disorder of metabolism and/or reuptake rather than synthesis and/or release of 5-HT. Importantly plasma 5-HIAA represents the relative activity of the enzyme monoamine oxidase not only in the platelets but also in other tissues such as the liver and lungs and as such should only be regarded as a surrogate marker of 5-HT turnover.

1.4.3.3 Plasma tryptophan

A potentially understudied component of the serotonin system is the contribution another metabolic pathway of dietary tryptophan, the kyneurine pathway might have in IBS patients. A study in 10 male patients looking at metabolites of tryptophan via this pathway found increased levels of L-kyneurine and an increased ratio of L-kyneurine to tryptophan. Coupled with an increase in neoptrin whose production also relies on the enzyme indolamine 2,3-dioxygenase authors conclude that there may be an induction

of this enzyme potentially via an inflammatory mechanism   [112]. More recently other have found higher free serum tryptophan in patients but an inhibition of the kynurenine pathway[113], these conflicting results and the small numbers of subjects point to the need for further studies before the role of this pathway can be evaluated as a biomarker.

1.4.3.4 SERT polymorphisms

DNA extraction and analysis is relatively easy and cheap and it is feasible to look for polymorphisms in large populations to better understand disease mechanisms and increasingly importantly to predict response to treatment. In IBS SERT polymorphisms have been proposed as possible candidates underlying a genetic predisposition to develop the condition. Evidence that SERT function is important includes the finding that blockade of SERT with a selective serotonin reuptake inhibitor leads to an increase in gut contractility[114] and the intriguing observation that SERT knockout mice have alternating diarrhoea and constipation[115]. The hypothesis being that deficient reuptake of serotonin leads to a supra-physiological level of serotonin and so the generation of symptoms. SERT polymorphisms have also have been linked to response to Alosetron. Here in patients who were genotyped for 5-HTTLPR those with an LL polymorphism had greater response to alosetron treatment as assessed by increase in colonic transit time was better in those with LL polymorphisms. The proposed mechanism being enhanced efficacy of alosetron in an environment with less available serotonin [75].

Many studies have looked at the incidence of the 5-HTTLPR polymorphisms in groups of patients with IBS and the results are conflicting. In a meta-analysis of 1034 patients in comparison with 1377 controls it was concluded that 5-HTTLPR genotypes are not risk factors for IBS[116] . This is despite some studies finding evidence of association when examining patients by their subtype. Consistent with the hypothesis that a reduction in SERT function leads to a greater availability of serotonin and enhanced bowel activity, 2 studies have found an association between those with IBS-D and the SS genotype [117, 118] and in keeping with idea that IBS is a disorder of the gut brain axis patients with an SS genotype and IBS are reported to have a higher lifetime history of depression [119]. More promisingly the correlation between SERT polymorphisms and a therapeutic response to drugs acting on the serotonin system has been demonstrated [75]. I will discuss the regulation of gut mucosal SERT expression and its role in IBS in the section looking at changes detectable using biopsy sampling.

1.4.3.5 SERT binding kinetics

Serotonin transport across the human blood platelet membrane through SERT has been widely used as a cellular model of neuronal 5-HT reuptake, however attempts to link platelet SERT function to different psychological states have had mixed success despite common molecular and physiological features. In the case of bowel SERT function the circulating platelets pass directly through the mucosal environment and therefore platelet SERT function may be more closely linked to the function of the mucosal SERT by being exposed to the

same environment. In a Study of 12 female patients taking 1mg bd of Alosetron the activity of platelet SERT receptors was assessed. Here the maximal binding capacity of the ligand and its receptor (Bmax), represents an estimate of the total number of binding sites expressed on the cell membrane and the dissociation constant Kd represents the affinity the drug has for its receptor. The patients with IBS had a lower Bmax than healthy volunteers coupled with a higher Kd representing a lower expression of SERT and a lower affinity for the ligand at its binding site. This did not change after Alosetron treatment. This combination of findings would create in the patient a situation where decreased uptake of serotonin leads to the presence of an over physiological concentration of serotonin in the gut and so symptoms, this is supported by the correlation of low density SERT on platelet membranes with more severe symptoms [120]. A second study using IBS-D patients found an increase in Paroxetine binding compared to controls. This was inversely correlated with platelet 5-HT uptake and associated with decreased mucosal SERT mRNA. The authors hypothesise that in the presence of reduced SERT the number of binding sites for Paroxetine increase as part of a potential compensatory mechanism[121]. Despite this discrepancy the ability to perform this assay on stored samples and the intriguing nature of results so far suggest platelet SERT could be a convenient biomarker for increased serotonin availability and warrants further investigation.

1.4.3.6 SERT mRNA expression

Reduced SERT expression has been demonstrated in the rectal biopsies of patients with ulcerative colitis IBS-D and IBS-C. This finding was accompanied by reduced tryptophan hydroxylase levels and reduced 5-HT content [122]. The finding of reduced SERT in conditions with mucosal damage and pronounced inflammation has been replicated in patients with celiac disease, but more importantly has also been seen in patients with IBS-D and subtle low grade inflammation[121] . In this study the reduction in SERT correlated with intraepithelial lymphocyte (IEL) numbers even in the range considered normal by conventional histopathology.

These findings have not been demonstrated in all studies [123] and may simply represent the patient heterogeneity which is so difficult to overcome. In addition interpreting the functional significance of altered mRNA levels is complex. SERT function relies on its apical position on the cell membrane, phosphorylation by Protein Kinase C leads to internalisation and reduction in 5-HT uptake in SERT expressing cell lines. Therefore measures of phosphorylated SERT may give a better clue to the levels of functional transporter in the mucosa.

1.4.3.7 Enterochromaffin cells

EC cells are serotonin-containing enteroendocrine cells that are distributed through the length of the gut, greatest in number in the duodenum and rectum[124]. These cells are orientated with their base in contact with the basement membrane and their apex, covered by microvilli, extending out into

the lumen where they can sense and transduce luminal stimuli. Activation leads to the release of pre-stored serotonin from dense apical and basal granules and can be via mechanical, neural or chemical means. These cells are increased in number in inflammation and infection and it is hypothesised that their prolonged presence after the resolution of an infectious stimulus leads to PI-IBS. Increased numbers of these cells have been found in PI-IBS [29, 35, 125] and in a mouse model of post infectious bowel dysfunction infection with *Trichinella spiralis* where these changes were accompanied by reduced SERT expression via a T cell dependent mechanism [126]. EC cell hyperplasia is seen in patients with inflammatory bowel disease (IBD) and may be a contributing mechanism of visceral hypersensitivity and symptoms in IBS[127].

More recently the EC hyperplasia has been shown to be controlled by T lymphocytes, which activate IL-13 receptors found on EC cells [128]. *T. spiralis* infection leads to long lasting motor and sensory dysfunction [129] associated with increased EC numbers and reduced SERT expression [126]. The same model shows long term increases mucosal 5-HT content and spontaneous release and also increased afferent nerve response to distension which can be inhibited by the 5-HT$_3$ receptor antagonist, Ondansetron [130].

1.4.3.8 Mucosal 5HT content

Changes in mucosal 5-HT content of biopsies have been demonstrated in patients with C-IBS with elevated 5-HT concentrations when compared with IBS-D and controls[131]. A tendency towards increased 5-HT content in IBS-C patients has also been found by members of our own department [110]. This

is in contrast to the finding by others that mucosal 5-HT content is reduced in IBS-C, IBS-D and ulcerative colitis (UC) [122]. Opiate induced constipation has not been shown to alter mucosal 5-HT content or mucosal SERT suggesting these findings are primary as opposed to being secondary to altered motility [132].

1.4.3.9 Mucosal 5HT release

A better understanding of the role of serotonin might therefore be gained by looking at mucosal release and turnover as the vast majority of serotonin in the gut is stored away in the EC cell and is only active during the time between release and reuptake. Mucosal 5-HT turnover as assessed by mucosal 5-HIAA/5-HT ratio has been shown to be decreased in both IBS-C and PI-IBS patients. IBS-C patients who release less 5-HT and hence generate less of the 5-HIAA metabolite might be predicted to have a reduced 5-HIAA/5-HT. hover in PI-IBS there is increased 5-HT release so in this case reduced 5-HIAA/5-HT ratio suggests a defect in SERT or monoamine oxidase as previously discussed [110]. In a small study that looked at basal and stimulated 5-HT release from biopsy of patients with IBS-D, IBS-C and UC controls no group differences were seen[131] but the trauma of biopsy may make this a poor model for studying 5-HT release.

1.4.3.10   Summary of abnormalities in the serotonin system

The overriding picture is of an altered serotonin system but with multiple potential mechanisms, including increased release, reduced uptake, differences in receptor number and sensitivity at peripheral, mucosal and

central sites. In addition none of the parameters discussed have thus far displayed characteristics of a good biomarker, with to date no data on reproducibility reliability, sensitivity and specificity.

1.5 Serine proteases

The serine proteases family of proteolytic enzymes have been identified as the source of increased faecal proteolytic activity in patients. These enzymes may be mechanistically important via their action on the Protease activated receptor (PAR) -2, inducing increases in permeability and hypersensitivity.

Proteolytic enzymes make up 2% of the human genome and almost a third of these proteases can be classed as serine proteases, so called because the active site contains a nucleophillic serine residue. Classification is into 12 clans according to catalytic mechanism and 40 families on the basis of common ancestry. They are widely distributed in nature and are found in all cellular life including viruses. The chymotrypsin like clan is the largest and best studied of these, and members play a critical role in digestion (chymotrypsin, trypsin and pancreatic elastase), haemostasis, apoptosis, reproduction and the immune response (tryptase, neutrophil elastase, complement factor B,C and D, cathepsin G)[133].

The largest pool of serine proteases in the intestinal tract of an animal model is derived from the pancreas under physiological conditions[134]. Trypsin and elastase enter the colon in much greater concentration than that found in stool, with the proteolytic activity of human ileal effluent being in the order of 20 times that found in the faeces[135]. Protease inhibition studies with

washed faecal bacteria have shown that they produce serine-, cysteine-, and metallo-proteinases, and that these bacteria have low levels of trypsin and chymotrypsin like activity when whole. This activity greatly increases when the bacteria are lysed. It is clear that the proteolytic activity of the colonic flora [136] and the colonic transit time regulate the delivery of these to the rectum.

Mast cells are another potential sources of serine proteases in the gut. Tryptase makes up to 25% of the total cellular proteins and is expressed by almost all subsets of human mast cells. There is some controversy as to the true in vivo potency of tryptase as a PAR activator with the consensus being that the activation is considerably less than that produced by trypsin and that the action of tryptase is likely to important when it is present in high concentrations during inflammation and mast cell activation[133].

1.5.1 Protease activated receptors (PARs)

PARs are G-protein coupled membrane bound receptors that are activated in a unique way. The same general mechanism activates all types and consists of an extracellular free amino acid terminus which is cleaved by a protease; this exposes a new terminus which then binds to the second of remaining extracellular loops of the receptor resulting in the initiation of the signal. There is no known function of the amino-terminal fragment that is cleaved during this process.
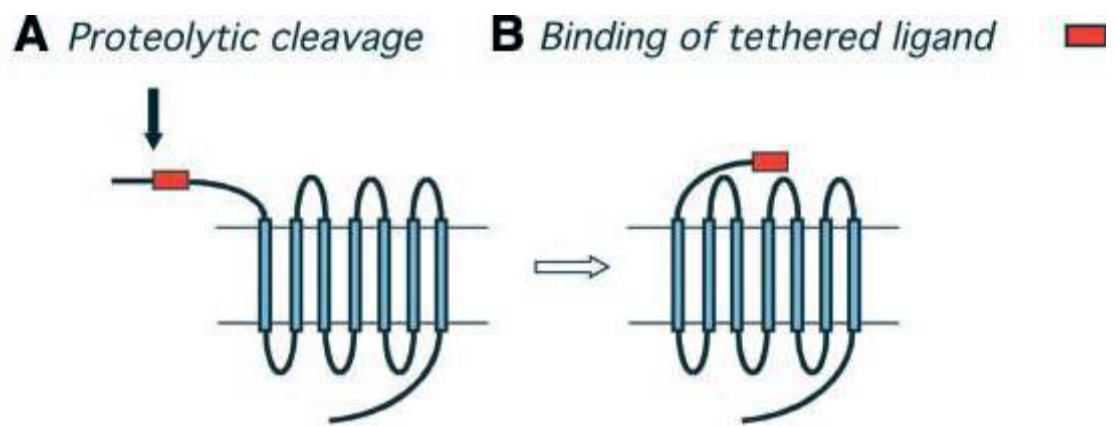
Figure 1-1 Showing A, proteolytic cleavage of the extracellular free amino acid terminus, and B, binding of the new terminus to the remaining extracellular loops of the receptor. [137]

PARs exist in 4 forms, and are distributed throughout the body with many important roles processes as diverse as coagulation and pain signalling; I will focus here however on their role and distribution in the GI tract. PAR-1, the first of this family to be cloned and identified, is present in the endothelial cells of the lamina propria of the small intestine, intestinal epithelial cells, smooth muscle cells and the neurones of the enteric nervous system. PAR-2 is present throughout the GI tract but is found in lesser concentrations in the stomach. Its presence has been localised to enterocytes, smooth muscle cells, mesenteric afferent nerves and the neurons of the myenteric and submucosal plexuses, as well as vascular smooth muscle, endothelial cells and also neutrophils mast cells and lymphocytes. PAR-3 is expressed in the stomach and small intestine and PAR-4 is highly expressed in the pancreas, small bowel and colon[137].

Activation of PAR-1 and PAR-2 stimulates chloride ion secretion from the intestinal mucosa, an important physiological response to inflammation and infection allowing diarrhoea to assist in clearing the noxious agent. In addition the presence of PARs in the myenteric plexus suggests a role in the control of motility, and the net effect of their activation in vivo appears to stimulate intestinal transit. PAR-2 also has a role in regulating pancreatic, gastric, and salivary secretion, with tissue specific pro and antisecretory effects. Under conditions of inflammation, during which proteases are generated and released, it appears that intraluminal administration of the selective PAR2 activating peptide (PAR-2-AP) SLIGRL-NH2 provokes inflammation in a wild type mouse but not its PAR-2 deficient counterpart [138] . The induction of this inflammation is in part via a neurogenic mechanism involving release of peptides from sensory nerves since this is suppressed by sensory nerve ablation [139]. Activation of PAR-2 by mast cell tryptase [140], trypsin or the agonist SLIGRL increases colonic paracellular permeability [141].

Multiple proteases can activate a PAR and the ability of a protease to activate a PAR depends on its secreted concentration, the presence or absence of co factors, and the relative abundance of specific inhibitory proteins. This is illustrated by the pancreatic trypsins whose activity depends on the release of zymogen trypsinogen, the presence of enteropeptidase which activates trypsinogen and the existence of a large array of endogenous trypsin inhibitors. Much less is known about the regulation of extra pancreatic

trypsins which are known to be present in endothelial and epithelial cells as well as the nervous system[137].

1.5.2 Serine proteases and IBS

Evidence for the role and action of these serine proteases in IBS has grown rapidly with initial animal studies showing that trypsin and tryptase are capable of activating colonic PAR-2 receptors in mice and that this activation results in inflammation and increased paracellular permeability [138]. Activation of PAR-2 receptors by injection of a sub inflammatory dose of agonist into the paw of both rats and mice results in visceral hyperalgesia, an important feature of IBS in patients [142]. Supernatants from IBS patient biopsies have increased proteolytic activity compared to controls and these supernatants when injected into the colons of mice causes hypersensitivity again via a PAR-2 dependent mechanism [143]. Bueno and colleagues have found increased levels of serine protease activity in the stool supernatant of patients with IBS-D and hypothesised that the source of these enzymes may be bacterial or from mucosal mast cells [94]. In this study there was no increase in pancreatic elastase in the IBS-D patients and coupled with the finding of no increase in activity in infectious diarrhoea they conclude that fast transit and delivery of pancreatic enzymes to the colon is not an important source of this activity. However this argument may be erroneous. Elastase may be atypical in not being rapidly degraded by colonic bacteria and so its levels may not change with accelerated transit. Furthermore the absence of increase in infectious diarrhoea may reflect the fact that subjects suffering

acute gastroenteritis often do not eat and hence may not secrete as much pancreatic enzymes. The source of increased faecal serine protease (FSP) activity is an important next question in establishing its role as a biomarker and a target for new treatments.

1.6 *Gastrointestinal Imaging in irritable bowel syndrome*

IBS patients have a disorder of gut function and not structure; conventional GI imaging is directed at the diagnosis of structural and mucosal alterations and employs a range of endoscopic and radiological techniques. Most if not all patients presenting with symptoms consistent with IBS will undergo at least one of these investigations whilst on their diagnostic pathway, most usually with the aim of excluding organic disease. This has the effect of exposing the patient to radiation and the small but important risk of perforation and bleeding at endoscopy as well as resulting in anxiety and uncertainty. These tests have been demonstrated to have a very low diagnostic yield, for example in 2 large multinational studies colonic evaluations, including barium enema, computerised tomography (CT) or endoscopy were normal in 98% of screened patients[144], and perhaps should not be performed with such regularity.

Less risky evaluation such as ultrasound may be helpful in selected cases but has not been shown to add valuable information in the majority of subjects. A study of 125 IBS patients diagnosed via symptom-based criteria concluded

that abdominal ultrasound was not necessary and may actually be counterproductive because the identification of irrelevant anatomic abnormalities could conceivably lead to unnecessary patient concern and additional, more invasive tests or procedures such as cholecystectomy[145].

The use of transit measurement via transit markers, scintigraphy, and wireless capsules has already been discussed and these techniques have a clear role in assessing gut function. Other more conventional and commonly available imaging modalities have demonstrated potential new disease mechanisms. Accarino et al have produced elegant work looking at abdominal gas volumes and distribution using CT scanning. They found that in contrast to patients with dysmotility total abdominal volume did not increase during bloating episodes in patients with IBS, rather that abdominal protrusion was mediated via alteration of posture and diaphragmatic descent [146] . However CT gives a large radiation dose and this research technique should be avoided in IBS patients in routine clinical practice.

1.6.1 MRI imaging in functional GI disorders

In the early days of MRI it was difficult to image the GI tract. With long acquisition times, resolution was poor, and artefact common. New advances in MRI imaging methods have now made it possible to detect mural and transmural disease and these techniques are also ideally placed to assess abnormalities of gut function. A good example of the use of functional

imaging is MRI defecography, used clinically in the understanding of evacuatory disorders.

The images are acquired via signal arising from the endogenous hydrogen protons, this means that the image contrast depends on the physicochemical environment of the organ of interest, fine tuning of the MRI sequences allows highlight of areas of interest for example the distribution of free water within the bowel itself. This has the potential to remove the need for unpleasant purging or the use of additional contrast medias making the test on the whole very patient acceptable. The avoidance of radiation facilitates multiple examinations during interventions such as eating a test meal or receiving a treatment in an otherwise undisturbed system. There are of course some limitations to this emerging technique. The software to assess GI motility and other functional parameters has yet to be fully automated and is time consuming, MRI scanning time remains costly and some patients will not find the enclosed environment acceptable.

To date several novel insights using MRI have been made. Measuring distribution of freely mobile small bowel water using a validated technique[147], in patients with IBS-D compared to controls has resulted into several new insights. Patients have a reduction in total fasting small bowel water content (SBWC), median 42 ml compared to 100-150 ml which was associated with faster transit and a hypertonic "spaghetti" bowel. This would be predicted to deliver an increased amount of water to the colon and soften the stool. The scores of the first day's stool showed a significant negative

correlation with the AUC for SBWC, a low value of which indirectly confirmed the suggested increased delivery of water to the ascending colon [148].

As well as giving insights into gut function in a disease state this technique has been successfully employed to investigate the effects of drugs on gut function. Measurement of intraluminal cross-sectional diameter changes in selected loops of bowel distended with oral contrast in healthy volunteers has allowed the inhibitory properties of hyoscine butlybromide[149] and glucagon[150] to be demonstrated. In another example using the same technique a reduction in net gut water, either by stimulation of absorption or inhibition of secretion with loperamide was demonstrated where previous invasive intubation methods had concluded Lopermide acts solely via change in intestinal transit [151]. In a double blind randomised placebo control trial of Ondansetron in healthy volunteers fasting small bowel water was significantly increased in the Ondansetron arm when compared to placebo. Ondansetron is known to inhibit colonic motility[79] and the migrating motor complex (MMC) in rodents[152], and a second arm of this study was able, again using MRI, to demonstrate a reduction in antroduodenal motility[153].

1.7 Clinical trial design in IBS

There is no structural abnormality that defines IBS, and given the heterogeneity of symptoms experienced by patients and the absence of a reliable biomarker, optimal endpoints for clinical trials have been difficult to

establish. Therefore most usually response to treatment is assessed by improvement in symptoms. This is a subjective outcome and there are several factors including the large placebo response seen in this group[154] that need to be considered in the design of a trial to ensure the most meaningful result is obtained. The use of patient reported outcomes (PRO) has been until now the most common method of collecting endpoint data. These endpoints should be validated and identify relief from important symptoms based on their pathophysiological mechanisms in patients with IBS.   Several recommendations have been made and an understanding of these has informed the study design that will be outlined in full in chapter 3 [155]. With this in mind we have designed a trial that uses PROs enhanced by a mechanistically appropriate set of proven (transit) and potential biomarkers in the serotonin system. In order to make this as clinically applicable as possible and patient acceptable we will also explore the new field of MRI Imaging as a possible biomarker of responsiveness to treatment.

1.8 Aims of the thesis

This thesis aims to identify biomarkers that allow us to predict response to our therapeutic intervention and to define an Ondansetron responsive endophenotype of IBS.

I will test the following hypotheses:

1. The 5-HT_3 receptor antagonist Ondansetron is a well tolerated and effective treatment in patients with IBS-D.

2. Ondansetron acts to increase small bowel water and slow colonic transit.

3. Response to Ondansetron will be more effective in those with abnormally increased mucosal serotonin availability at baseline.

4. Faecal serine protease activity will be reduced by treatment with Ondansetron as a result of increased colonic transit.

2 Ondansetron in patients with diarrhoea predominant Irritable bowel syndrome "identifying the responder": Study design

2.1 Study configuration

This was a 2 centre (Nottingham Digestive Disease Centre and Biomedical Research unit in conjunction with the department of Neurogastroenterology at the Wythenshawe Hospital, Manchester), randomised placebo controlled cross-over study of Ondansetron 4-8mg TDS with dose titration in IBS-D patients. The trial was conducted according to the principles of good clinical practice (GCP), ethical approval was granted by the Nottingham Research Ethics Committee 2 (REC). The trial was registered on clinicaltrials.gov and received the necessary approval from the Medicines and Healthcare Regulatory authority (MHRA). Funding was provided by the National Institute for Health Research. A summary of the study design can be seen in figure 2.1
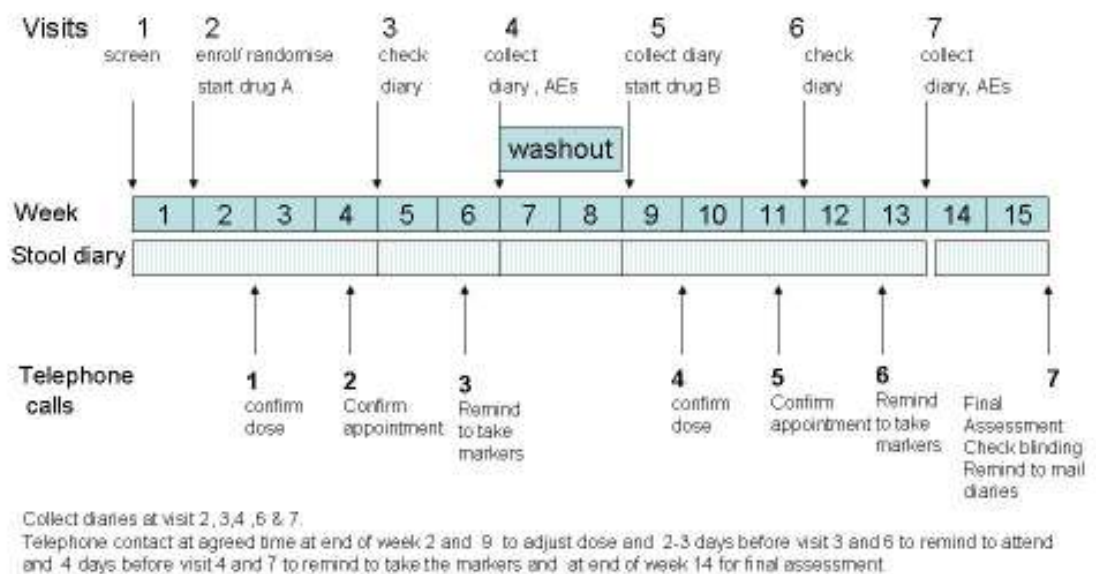
Figure 2-1 Summary of the study design for the trial Ondansetron in patients with diarrhoea predominant Irritable bowel syndrome "identifying the responder".

2.1.1 Power calculation

Change in stool consistency from baseline in the final 2 weeks of treatment was the primary end point of the study. Previous studies in Nottingham show a mean (SD) stool consistency of 3.8 (1.1), n=28 with a normal distribution. Using the sampsi command in Stata, 111 subjects were required for a one-sample t-test with 90% power to detect a shift of 0.4 points on the stool consistency scale at a 1% two-sided significance. 0.4 was chosen as the difference obtained by Camilleri using alosetron in IBS-D[156]. Since the standard deviation is estimated from a small sample (itself prone to sampling variation) the sample size was multiplied by a correction factor of 1.111[157] to give a final sample size estimate of 123 evaluable subjects. An 18% drop out was assumed giving a final number of recruits of 150. This was a deliberately conservative calculation since the magnitude of effect was believed to likely be larger as this study used the Bristol Stool Form Score which runs from 1-7 while Camilleri used a scale running from 1-4.

2.1.2 Patient recruitment, inclusion and exclusion criteria

Participants were recruited from IBS clinics at the Queens Medical Centre Nottingham and the Wythenshawe Hospital Manchester, and from Professor Spiller's list of patients who have previously taken part in research studies. Adverts were placed in the clinical areas of the Queens's Medical centre. Patients were also recruited from general practice via collaboration with the Trent Primary Care Research Network. All patients who expressed an interest

in taking part were sent a detailed information sheet, this contained the contact information of the study team and every effort was made to answer any questions regarding the trial before and during the first screening visit. Inclusion and exclusion criteria are listed in Table 2.2 and 2.3. Since many patients were on SSRIs or tricyclic antidepressants these were not excluded provided they had been on medication at least 3 months and that the dose remained unaltered throughout the study.

Inclusion criteria
IBS-D patients meeting the Rome III criteria.
Male or female aged 18-75 years
Women of child bearing potential (who have a negative pregnancy test) must agree to use methods of medically acceptable forms of contraception during the study, (e.g. implants, injectables, combined oral contraceptives, sexual abstinence or vasectomised partners)
Patients who were able to give informed consent.

Table 2-1 Ondansetron in patients with diarrhoea predominant Irritable bowel syndrome "identifying the responder" inclusion criteria.

Exclusion criteria
Women who were pregnant or breastfeeding
Patients that, in the opinion of the investigator, were considered unsuitable.
Patients who have had abdominal surgery which may cause bowel symptoms similar to IBS (appendicectomy and cholecystectomy were not an exclusion)
Patient unable to stop anti-diarrhoeal drugs
Patients who were currently participating in another clinical trial or who had been in a trial in the previous three months

Table 2-2 Ondansetron in patients with diarrhoea predominant Irritable bowel syndrome "identifying the responder" exclusion criteria.

2.1.3 Randomisation and blinding

The randomisation was based on a computer generated pseudo-random code using random permuted blocks of randomly varying size, created by the Nottingham Clinical Trials Support Unit (CTSU) in accordance with their standard operating procedure (SOP) and held on a secure server. The research team obtained a randomisation reference number for each participant by means of a remote, internet-based randomisation system developed and maintained by the Nottingham CTSU. The sequence and decode of treatment allocations was concealed until all interventions had been assigned and recruitment, data collected, and all other trial-related assessments was complete.

2.2 Drug preparation and dosing

Each participant received five weeks of placebo and five weeks of Ondansetron. The investigational medicinal product (IMP) was produced by

over encapsulation in a gelatin capsule of either a 4mg Ondansetron tablet (Pliva, Zagreb, Croatia) or placebo. The placebo formulation matched that of the Ondansetron in appearance and composition, except for the active drug. This was done by Bilcare (Crickhowel ,Powys, UK). The pharmacy at NUH then packaged, labelled and QP released (carried out by the designated person) blinded treatment packs. These packs were labelled with a single panel label according to clinical trial regulations. Subject name, randomisation number, storage conditions and date of dispensing were added to the label at the time of dispensing.

The Ondansetron dose was titrated, and as such varied from 4mgs alternate days to 8mgs tds. Patients were instructed to commence treatment with 1 capsule once a day. Depending on the response, patients were asked to increase the dose to a maximum of 8mg tds. If stool consistency increased to hard (stool form 1 or 2), or if bowel frequency dropped below 1 per day the dose was reduced to a minimum of one tablet taken every 2 days. Patients were encouraged to ring in to discuss dose adjustment. Dose adjustment was completed within the first 3 weeks. During the final 2 weeks patients completed the stool diary on a steady dose of drug. Following each treatment period patients underwent   a washout period of between 2 and 3 weeks.

Patients were allowed to take Loperamide 2mgs BD as rescue medication in the event of uncontrolled diarrhoea. They were asked to discontinue this for the final two weeks since stool consistency in this period was the primary end point of the study.

2.3 Visit schedule

2.3.1 Visit 1 -Screening

At this visit the inclusion and exclusion criteria were checked and if the patient was eligible to enter informed was consent taken, followed by:

1) A physical examination, including a pregnancy test for women of child-bearing potential.

2) Taking of blood samples for:

  i) Routine screening test (Haemoglobin (Hb), tissue transglutaminase (tTG) antibodies, Calcium, Gamma-glutamyltransferase (γGT), alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Albumin and C-reactive protein (CRP)) if these were not done within the last 3 months.

  ii) Collection of DNA for analysis of genetic polymorphisms of SERT.

  iii) Fasting whole blood assessment of biomarkers of serotonin metabolism including the maximum binding (Bmax) of H^3-labelled paroxetine binding to platelet membranes and H^3-5-HT uptake together with plasma to be assayed for 5-HIAA

3) Flexible sigmoidoscopy with mucosal biopsies to exclude microscopic colitis (if not done in preceding 2 years).

4) Nottingham only: Flexible sigmoidoscopy and 8 rectal biopsies. 2 for routine histology and immunostaining for 5-HT containing cells, 2 for assessment of 5-HT release and 2 for preservation in RNA Later for RNA extraction and

assessment for mRNA of inflammatory cytokines, 2 for snap freezing for subsequent protein assays

We also performed a psychometric assessment at this visit using the following questionnaires: Hospital Anxiety and Depression Scales (HAD)[158], Personal Health Questionnaire (PHQ15) [159], Perceived Stress Questionnaire [160] and IBS quality of life (IBS QOL) [161] score. Patients were then asked to complete a one week stool diary using the Bristol Stool Form Score. The IBS symptom severity score (IBS SSS) which provides and overall assessment of symptoms and has been shown to be sensitive and responsive to treatment effects was also performed at this baseline visit (all study questionnaires are contained in appendix 1).

2.3.2 Visit 2 - Enrolment and Randomisation – (end of Week 1):

All sites: The stool diaries were examined to confirm loose stools >25% and hard stools <25% allowing classification of Rome III IBS. The results of the blood tests were reviewed to ensure no other diseases were present. The patients were then randomised using the previously described web based randomisation and allocated treatment. At this visit a stool sample collected that day was stored at -80°C for later serine protease analysis as well as future microbiological analysis using the DNA based HITChip. Patients were then given the first 1x 100 capsules consisting of 4mg tablets of Ondansetron or placebo.

2.3.3 Visit 3 (end of Week 4):

All sites: Patients returned to the hospital after completing 3 weeks of stool diary recording on treatment for a diary check. Patients were reminded to make no further dose adjustments and to no longer use rescue Loperamide in the final 2 weeks of the treatment period. They were also given 3 containers each containing 24 radio-opaque pellets one to be taken on each of the last three days of week 6.

2.3.4 Visit 4 (end of Week 6):

All Sites: On the day of the visit the colonic transit of the radio-opaque pellets were assessed by plain abdominal X-ray. A second IBS-SSS was completed, blood samples were taken for safety assessment (FBC & Liver function tests), and stool sample for serine protease activity and future microbiological assessment. Patients then stopped all study medication for the wash out period. Any un-used study medication was collected. The wash-out period was usually 2 weeks during which the patient continued to complete a stool diary. It some patients the washout was extended usually to allow them to meet work or family commitments during study period two.

Nottingham only: A fasting MRI scan was carried out to assess small bowel and colonic water content.

2.3.5 Visit 5 (end of Week 8):

All Sites: After confirming that the patient's bowel habit had returned to baseline a new supply of 1x 100 capsules of study medication and new stool diaries were issued.

2.3.6 Visit 6 (end of Week 11):

All sites: Patients returned to the hospital after completing 3 weeks of stool diary recording for a second diary check. Patients were again reminded to make no further dose adjustments and to no longer use rescue Loperamide in the final 2 weeks of the treatment period. During this visit the patients were as previously given 3 capsules each containing 20 radio-opaque pellets to be taken on each of the last 3 days of week 6.

2.3.7 Visit 7 Study end (end of Week 13):

All Sites: as visit 4, in addition subjects were asked to express their preference for the first or second treatment and state whether they would like to continue to receive it. They were also reminded to mail in their final stool diaries in order to demonstrate the wearing off of any drug effect.

Nottingham only: A fasting MRI scan was carried out to assess small and large bowel water content.

2.4 Healthy volunteers

21 age and sex matched healthy volunteers were recruited to provide normal values for comparison with our patient group.

2.4.1 Recruitment

Participants were recruited via ethically approved adverts placed in the non-clinical areas of the Queens's Medical centre. All participants who expressed an interest in taking part were sent a detailed information sheet, this contained the contact information of the study team and every effort was made to answer any questions regarding the trial before and during the first screening visit. Inclusion and exclusion criteria are listed in Table 2.3 and 2.4.

Inclusion criteria
Healthy Volunteer
Male or female aged 18-75 years
Volunteers who are able to give informed consent

Table 2-3 Inclusion criteria for healthy volunteers.

Exclusion criteria
IBS
Women who are pregnant or breastfeeding
Volunteers that, in the opinion of the investigator, are considered unsuitable
Volunteers who have had abdominal surgery (Please note, appendicectomy and cholecystectomy is not an exclusion)
Concomitant use of drugs that affect stool consistency (e.g. opiate analgesia)
Volunteers currently participating in another clinical trial or who have been in a trial in the previous three months

Table 2-4 Exclusion criteria for healthy volunteers.

2.4.2 Visit schedule

Visit 1: After signing a consent form healthy volunteers had:

1) A physical examination, including a pregnancy test for women of child-bearing potential.

2) Taking of blood samples for:

i) Routine screening test (Hb, tTG antibodies, Calcium, γGT, ALT, ALP, Albumin and CRP) if these were not done within the last 3 months.

ii) Collection of DNA for analysis of genetic polymorphisms of SERT.

iii) Fasting whole blood assessment of biomarkers of serotonin metabolism including the maximum binding (Bmax) of H^3-labelled paroxetine binding to platelet membranes and H^3-5HT uptake together with plasma to be assayed for 5-HIAA

3) Flexible sigmoidoscopy and 8 rectal biopsies. 2 for routine histology and immunostaining for 5HT containing cells, 2 for assessment of 5HT release and 2 for preservation in RNA Later for RNA extraction and assessment for mRNA of inflammatory cytokines, 2 for snap freezing for subsequent protein assays

We also performed a psychometric assessment at this visit using the following questionnaires: HAD, PHQ15, Perceived Stress Questionnaire. Volunteers were then asked to complete a one week stool diary using the Bristol Stool Form Score.

Volunteers were also given capsules each containing 24 radio-opaque pellets one to be taken on each of the last three days before visit 2.

Visit 2: The colonic transit of the radio-opaque pellets were assessed by plain abdominal X-ray and a fasting MRI scan was carried out to assess small and large bowel water.

2.5 Case Report Forms (CRFs)

Each participant was assigned a study identity code number at randomisation, for use on CRFs other study documents and the electronic database. The documents and database also used patient initials and date of birth (dd/mm/yy). CRFs were held securely in accordance with regulations. The investigator made a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Study Number (the Study Recruitment Log), to permit identification of all participants enrolled in the study, in case additional follow-up was required. Computer held data including the study database was held securely and password protected. All data was stored on a secure dedicated web server.

2.6 Stopping rules and discontinuation

Participants were able to withdraw from the study at any point. Patients reporting rectal bleeding were asked to stop their treatment and attend for a flexible sigmoidoscopy to diagnose the cause. If the bleeding was due to minor haemorrhoidal bleeding they were allowed to continue treatment if they wished to. Other adverse events were assessed by the supervising doctor

who decided whether they should be allowed to continue in the trial. Participants were made aware (via the information sheet and consent form) that should they withdraw the data collected up to their withdrawal could not be erased and would still be used in the final analysis.

2.7 Adverse events

All adverse events were assessed for seriousness, expectedness and causality, definitions of an Adverse Event (AE), Serious Adverse Event (SAE) and Suspected Unexpected Serious Adverse Reactions (SUSARs) are contained in appendix 2.

Reporting of adverse events

Participants were asked to contact the study site immediately in the event of any serious adverse event. All adverse events were recorded and closely monitored until resolution, stabilisation, or until it had been shown that the study medication or treatment was not the cause.

In concordance with Good Clinical Practice all SAEs would have been recorded and reported to the MHRA and REC as part of the annual reports. SUSARs would be reported within the statutory timeframes to the MHRA and REC. The Chief investigator was responsible for all adverse event reporting. There were no SAEs or SUSARs during this study.

2.8 Compliance

Patients kept a daily diary of drug ingestion, which was checked at each visit. Non-compliant participants were noted and encouraged to be compliant.

Unused pills were returned for counting. Ingestion of more than 75% of prescribed doses was considered compliant as assessed from the diary and count of returned tablets at each visit. Analysis per protocol excluded non-compliant individuals.

2.9 Primary and secondary endpoints Statistics including handling of missing data

Analysis was performed by myself with the assistance of the statistician once the treatment codes had been broken. Data was entered into an Excel spread sheet and then imported into SPSS and Graph Pad Prism which were used for analysis.

The distribution of each parameter analysed was assessed for normality using the Shapiro-Wilks test.

Every effort was made to avoid missing data. However, data omitted from diary cards will be imputed by taking an average of the day before and the day after.

2.9.1 Definition of populations analysed

Full Analysis set: All randomised participants, who take at least one dose of study medication and for whom at least one post-baseline assessment of the primary endpoint is available.

Per protocol set: All participants in the Full Analysis set who are deemed to have no major protocol violations that could interfere with the objectives of the study.

<u>Primary Outcome Measure</u> =Difference in average stool consistency in the last 2 weeks of treatment of Ondansetron versus placebo.

<u>Secondary Outcome Measures</u> (all in last 2 weeks)

1) Change from baseline in stool frequency

2) Change from baseline in number of days with pain urgency and bloating

3) Change from baseline in IBSSSS

4) Proportion of patients preferring ondansetron versus placebo

5) Proportion wanting to continue with ondansetron versus placebo

6) Percentage satisfactory relief

These results are described in detail in chapter 4, Patient reported outcomes.

Chapter 5 describes the secondary outcome measures of difference between ondansetron and placebo periods with respect to:

1) Colon transit

2) Fasting SBWC

3) Fasting ACWC

Chapter 6 contains the secondary outcome measures pertaining to laboratory outcomes.

Chapter 7 contains the results of experiments to define the origin of faecal serine proteases and the effect of Ondansetron on faecal serine protease activity,

2.9.2 Statistical methods

Values were assessed for normality using the Shapiro-Wilks statistic and results are expressed as mean (95%, confidence interval, CI) for normally distributed data or median (interquartile range, IQR) if not normally distributed.

The paired or unpaired students t test was used for paired or unpaired parametric data, and the Wilcoxon matched pairs or Mann-Whitney U for non-parametric data.

Difference in responder status between those with *ss , sl* and *ll* version of the SERT DNA polymorphism was assessed using Chi Squared test for trend.

Correlation of outcome variables was perfomed using either Pearson correlation coefficient or the nonparametric Spearman Rank Order correlation coefficient. Logistic regression was used to assess the relative value of these different measures in predicting response to Ondansetron.

3 Materials and Methods

Here all the imaging and laboratory methods utilised in this thesis will be outlined in order starting with imaging, followed by analysis of blood samples, methods for analysing biopsies and finally stool analysis.

3.1 Magnetic resonance imaging

MRI was previously acronymed NMR or nuclear magnetic resonance reminding us that the physics behind the images is concerned with the nucleus. In the context of clinical MRI this is related to the nucleus of the hydrogen atom (also called the water proton) that is so abundant in the body. A detailed understanding of the physics behind the formation of a MRI image is beyond the scope of this thesis. This section summarises briefly the theory[162] with the only aim of introducing and describing the main MRI parameters measured during this work.

The intrinsic nature of the NMR phenomenon requires a complex quantum mechanical description. In such a framework, the water protons have a magnetic moment arising from their spin angular momentum. This is a physical property meaning, in simple words, that each water hydrogen nucleus can be thought of as a little magnetic compass. Normally these magnetic moments in a sample will be randomly orientated. When the water protons in a sample are exposed to a main static magnetic field then their energy levels split into two separate values, one corresponding to the magnetic moments (compasses) aligning along the direction of the external

magnetic field (parallel) and one corresponding to aligning opposite to the direction of the external magnetic field (anti parallel).

The parallel alignment requires less energy and is therefore slightly favoured. This creates a net sample magnetisation parallel to the applied field. It can be shown that the time evolution of this magnetisation in the main external magnetic field can be written as a clockwise rotation about the direction of the external magnetic field with a given angular frequency. This rotational frequency is called the Larmor frequency. In this equilibrium state no signal arising from this magnetisation is detected using a receiver coil.

In order to get information from the proton spins they need to be excited. This is done with a short burst of radiofrequency, termed a radiofrequency (or RF) pulse. This excitation is only possible if the pulse is delivered at the given Larmor frequency.

Starting again with the information that the water protons have a magnetic moment which can be thought of as a little magnetic compass, when this is placed in a large external static magnetic field the magnetic moment will experience a torque causing it to spin about the direction of the main magnetic field in a gyroscopic fashion (see Figure 2.1) with a given precession frequency called the Larmor frequency. The Larmor frequency depends on the properties of the nucleus involved and the strength of the magnetic field.
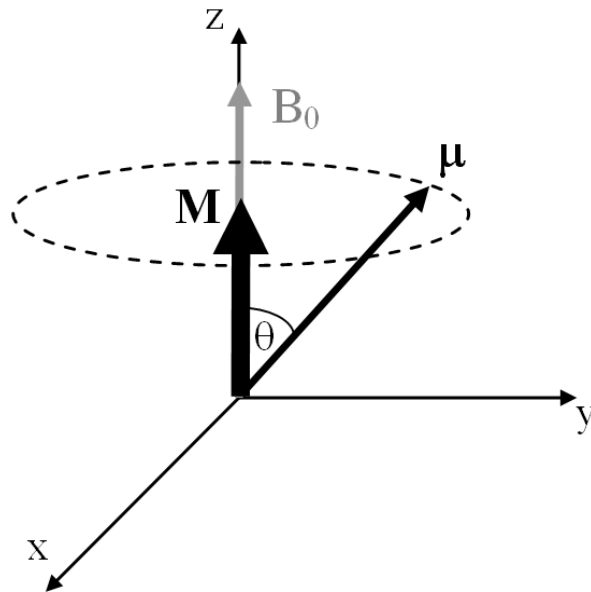
Figure 3-1 Precession of a magnetic moment μ about the static field B_0 at a constant angle θ. In this reference frame the resulting magnetisation M is all along the main magnetic field B_0 (longitudinal) whilst the component of M in the perpendicular (transverse) xy plane is zero.

In analogy of what was described before, in this equilibrium state no signal arising from this magnetisation **M** is detected using a receiver coil. The application of a RF pulse delivered at the given Larmor frequency will tip the magnetisation away from the z axis creating two components, one along the z axis (longitudinal magentisation) and one perpendicular to it in the xy plane (transverse magnetisation).After excitation these two separate components will then return (relax) to equilibrium with two intrinsically different mechanisms called relaxation times T1 and T2 as described below.

3.1.1 T_1 longitudinal (spin-lattice) relaxation

The longitudinal component of the magnetisation will return to thermal equilibrium by interacting with the surrounding lattice which functions as a

thermal reservoir. This is a first-order process characterised by a time constant T1 which is called the spin-lattice relaxation time or longitudinal relaxation time. Therefore T1 is the constant which describes the time required for the longitudinal component of the magnetisation to return to 63% of its original value. The rate at which energy can be transferred depends amongst other mechanisms on the size and rotation and translational motion characteristics of the molecules and therefore how easily they can move within this lattice. So large molecules usually move too slowly to transfer energy quickly and the best transfer of energy occurs at medium correlation times. T_1 is therefore generally shorter in solutions than in solids. However in pure liquids small molecules move fast and their motion is too rapid to permit efficient energy transfer so a pure liquid will also have a relatively long T_1. In addition T_1 will also be affected by macromolecules such as proteins with hydrophilic bonding sites, as well as bound water the hydrophilic sites also slow the motion of free water in the near vicinity allowing it to transfer energy more efficiently and so shortening the T_1.

3.1.2 T_2 transverse (spin-spin) relaxation

The component of the magnetisation that is in the transverse, xy plane decays away after excitation but in this case the decay is due to random and irreversible loss of coherence between the ensemble of spins. This does not require transfer of energy to the lattice as it is just a dephasing process until each proton is spinning randomly and they are completely out of phase. In the liquid phase and with some assumptions this can be regarded again as a first-

order process characterised by a time constant T2 which is called the spin-spin relaxation time or transverse relaxation time. Therefore T_2 is the constant which describes the time for the transverse magnetisation to fall 63% of its original value. This spin-spin relaxation is also affected by physical state and molecular size. Solids and large molecules have a short T_2; by contrast T_2 is long in free water. Like T_1, T_2 is also affected by the presence of macromolecules, here they increase the efficiency of spin-spin interactions and shorten T2. Some T_1 and T_2 values for human tissue are given in table 2.1[162] to illustrate how this can be exploited.

Tissue	T_1 at 1.5 Tesla in milliseconds	T_2 at 1.5 Tesla in milliseconds
Skeletal muscle	870	47
Liver	490	43
Kidney	650	58
Spleen	780	62
Fat	260	84
Gray matter	920	101
White mater	790	92
Cerebrospinal fluid	>4000	>2000
Lung	830	79

Table 3-1 Table showing the T_1 value in ms of Tesla.

3.1.3   MRI imaging

The theory summarised so far shows that one can excite the water protons in a sample and that the decay characteristics of the signal contain rich

information about the physicochemical and motional environment of the protons. The use of specially designed RF pulses that selectively excite only a portion of the sample in conjunction with linear magnetic gradients that superimpose to the protons a predictable spatial variation in the Larmor frequency allow sampling and reconstruction of two-dimensional planar images.

For an image to have diagnostic utility there must be contrast between the MR signal of different tissue types. The intensity of the signal is a function of T1 and T2 and also proton density, chemical shift and motion. The relative contribution of each parameter is controlled by adjusting the RF pulses, applied gradients and timing of the data acquisition. These parameters are set in the imaging sequence and the sequences used in this study will be described in the following methods.

3.1.4 Equipment

MRI scanning was performed on the state-of-the-art, research dedicated 1.5T Philips Achieva Scanner sited at the University of Nottingham

3.1.5 Image sequences

Each subject was positioned supine in the scanner with a body coil wrapped around the abdomen. Firstly, a coarse scout scan was taken to locate the position of the abdominal organs and plan the position of the image planes followed by a calibration scan allowing automatic setup of the scanner specific

to the subject at that time point. The subjects spent approximately 20 minutes inside the magnet at each visit.

Small bowel water content (SBWC) was assessed with a single shot, fast spin echo sequence (similar to that used for magnetic resonance cholangiopancreatography (MRCP), (effective echo time(TE)=320 milliseconds)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 (acquired voxel size = 1.56 X 2.83 X 7mm^3).This sequence yields high-intensity signals from areas with liquid fluid and little signal from body tissues.

Secondly, a dual-gradient echo (dual-echo fast field echo [FFE], TE1 = 2.3 milliseconds, TE2 = 4.6, repetition time =158 milliseconds) imaging sequence was used to visualize anatomy acquiring 24 coronal plane and 45 transverse images with in-plane resolution of 1.76mm X 1.76 mm and a slice thickness of 7 mm, with no gap between slices. Each image set was acquired on an expiration breath hold, the duration of which varied between 13 and 24 seconds.

T1 in the ascending colon (AC) was measured using a single slice balanced Turbo Field Echo (bTFE) with a preparatory 180 deg inversion pulse applied before acquiring the imaging data. Data were acquired from 8 different inversion times (TI) (time between inversion pulse and imaging pulses) ranging from 25 - 4925 ms. T2 in the AC was measured using a single slice bTFE with a preparatory spin echo pulse (90deg-TE/2-180deg-TE/2--90deg) applied before

acquiring the imaging data. Data were acquired from 10 different echo times (TE) ranging from 20-637 ms. For both sequences there was a 15 second gap between each acquisition to allow the system to return to equilibrium.

3.1.6 Image processing

3.1.6.1   Small bowel water content (SBWC)

Image analysis was performed using the software Intestine Analyse 6 written in IDL by Dr Caroline Hoad at the Sir Peter Mansfield Magnetic Resonance Research Centre (SPMMRC) (Research Systems Inc., Boulder,Colorado, USA). This method of estimating SBWC assumes that in the MRCP images any pixel in the peritoneal cavity with signal intensity above a given threshold is filled with 'free' water. This threshold is used in order to normalize for intra- and inter-subject variations in signal due to scanner instabilities, subject repositioning and coil loading. The signal from cerebral spinal fluid is used to calculate the threshold as it covers multiple slices, is near the centre of the field of view and it is known to have a very accurately regulated and hence constant composition. The use of this threshold has been validated against the infusion of known volumes of water[147].

The volume of free mobile water in the small bowel was then calculated by integrating the volume of all image pixels with signal greater than the threshold, after manually excluding regions containing the colon, kidneys, gallbladder, bladder and visible blood vessels. An example of the images used for this calculation can be seen in figure 3.2.
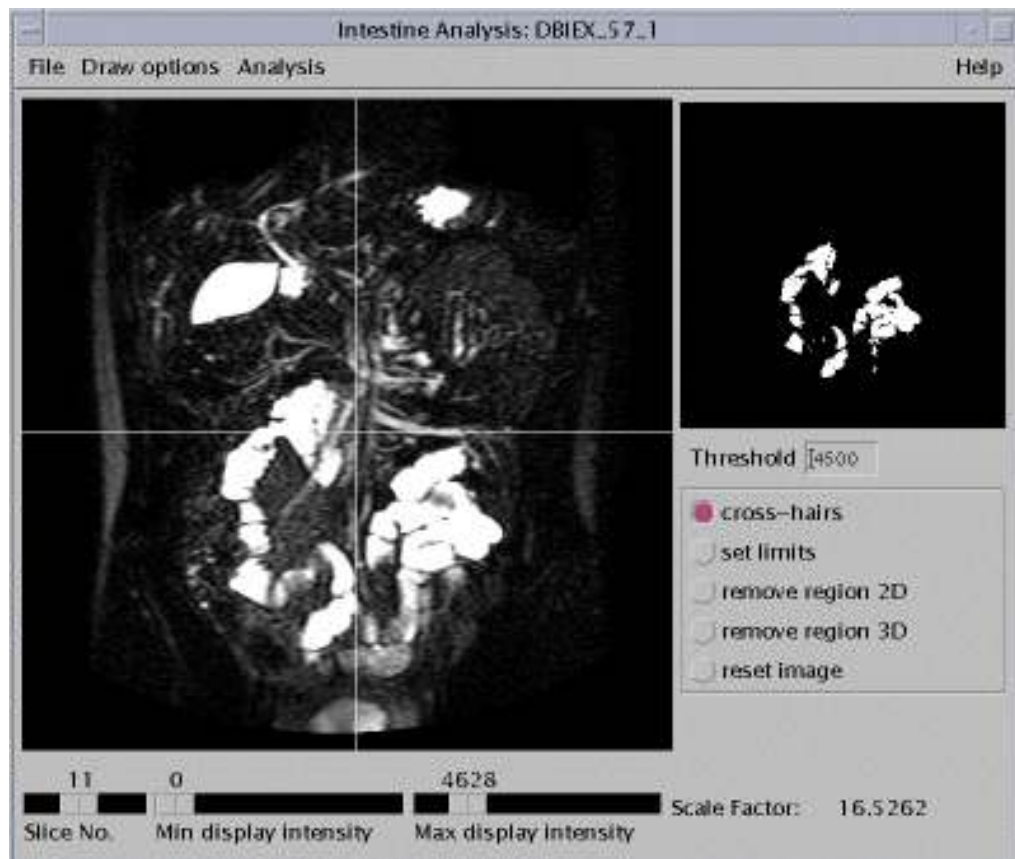
Figure 3-2 showing a slice of the MRCP series loaded into the Intestine Analyse 6 programme; here free water has been defined as all areas above the threshold 4500. The gall bladder, biliary tree and transverse colon have been excluded and the total SBWC of this slice can be seen in the top left window.

3.1.6.2 Ascending colon water content

Free ascending colon water was measured as for small bowel water, by manually excluding the signal from the small bowel, kidneys, gallbladder and visible blood vessels as before.

3.2 Whole gut transit

The method of assessing whole gut transit was adapted from that published by Metcalf et al[87]. Subjects took 20 silicon markers impregnated with 13.5% barium (Altimex, Nottingham, UK) at 9am each morning for three consecutive days. A plain abdominal film was then taken on the morning of day 4. In the absence of clear outlines of the bowel, markers located to the right of the

vertebral spinous processes above the pelvic brim on the right were allocated as right colon. Markers to the left of the vertebral spinous processes and above an imaginary line from the fifth lumbar vertebrae to the anterior superior iliac spine were assigned to the left colon. Markers in between these two lines were assigned to the rectosigmoid and rectum. However, if bowel outlines clearly showed a pelvic caecum, a transverse colon, or a large sigmoid loop above the fifth lumbar vertebrae, markers were judged to be' in the anatomic segment based on the gaseous outlines.
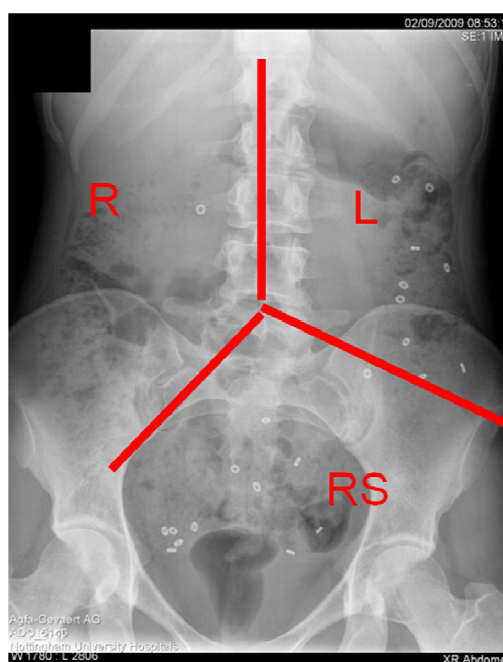
Figure 3-3 Showing a plain abdominal film dived into 3 sections, R= right colon, L =left colon and RS= rectosigmoid.

3.3 Bloods for routine clinical testing

Blood was colected into 1x 4.0ml ethylenediaminetetraacetic acid (EDTA) K2E Vacutainer tube (Becton Dickinson Ltd, Oxford, UK) and 1x 6.0ml SSTII vacutainer tube (Becton Dickinson Ltd, Oxford, UK) Analysis of liver function tests (LFT), CRP calcium and albumin were performed by the Department of

Clinical Chemistry, University Hospital, Nottingham using a Vitros 5.1 analyser (Johnson and Johnson Ltd, UK). Analysis of the full blood count (FBC) was done by the Deparment of Haematology, Nottingham University Hospitals. tTG was done by the Deparment of Immunology, Nottingham University Hospitals by automated ELISA on a DS2 analyser (Dynex Ltd Worthing, UK).

3.4 Platelet paroxetine binding

3.4.1 Blood sampling

All samples were taken via a 17g butterfly needle without the use of a torniquet with the patient in a seated positon. This method minimises platlet activation and thus the release of serotonin enabling a single venepuncture episode to provide blood suitable for all of the assays. Blood for platelet paroxetine binding was collected into 5x 6.0 ml EDTA K2E Vacutainer tubes (Becton Dickinson Ltd) and platelt plugs prepared within 2 hours of collection

3.4.2 Preparation of platelet rich plasma

Platelets were isolated by differential centrifugation. Briefly, anti-coagulated blood was centrifuged at 200g at 20 °C for 15 minutes to remove residual red and white blood cells. The supernatant platelet rich plasma (PRP) was centrifuged at 4500g at 20°C for 20 minutes and subsequently platelets were pelleted. The platelet pellet was frozen at -80oC until analysis.

3.4.3 Radio-immune assay

This assay was performed by Gulzar Singh (GS).

Preparation of reagents

1. **50mM Tris Buffer pH7.4**

   1 day before beginning the assay 6.10g of Tris, 7.02g NaCl and 380mg KCL were weighed out and added to 1l of double deionised water . The Ph was adjusted to 7.4 with hydrochloric acid. This was stored at 4˚C.

2. **[3H]Paroxetine**

   A 10nM solution was prepared using a commercial stock solution (Perkin-Elmer, Waltham, Massachussetts, US) specific activity 24.4 Ci/mmol, 902.8 GBq/mmol in Tris buffer. Immediately before use this was diluted again with Tris buffer to give a 1nM working solution.

3. **Fluoxetine hydrochloride**

   Fluoxetine hydrochoride (Sigma-Aldrich) 10nM/l was diluted using double deionised water to give concentrations of 200, 100, 50, 20, 10 and 1 um/l when added to the total reaction volume.

The platelet pellets were disolved in 500ul of ice cold Tris and pippetted out into an eppendorph, the original container of the pelllet was washed with a further 400ul of Tris to ensure no platelets remained adhered to the eppendorph. The eppendorph was then placed on ice. The platelets were homogenised with a sonicator (soniprep 150,MSE: Wolf Laboritories, Pocklington, York , England) at an amplitude of 10μm (2 strokes of 10 seconds) and then placed on ice for 30 minutes. This was done to ensure the platelet mebranes came out of solution before the sample was centriuged at

3000 G for 10 minutes at 4°C. The supernatant was then discarded and 2 further cycles of washing were performed with the pellet being dissolved by sonication in a further 1ml of cold Tris for 20 seconds, rested on ice and centrifuged as previously. This was done to remove any endogenous 5-HT.

The assay was performed in triplicate in the prescence (non-specific binding) and absence (total binding) of fluoxetine. Reagents were added to LP4 tubes (Sarstedt, Numbrecht, Germany) as detailed in the following table.

	Total binding	Non-specific binding
3H paroxetine –1nM	100µl	100µl
Tris buffer	1700µl	1600µl
fluoxetine	-	100µl
Platelet membrane suspension	50µl	50µl

Table 3-2 Paroxetine binding assay reagents.

The reaction was incubated at room temperature for 1 hour. During the incubation a Brandel harvester (Brandel, Gaithersburg, US.) was prepared by washing 3 times with ice cold Tris. The reaction was then halted by the addtion of ice cold Tris, followed by immediate filtration under vaccum using the Brandel Harvester and ice cold Tris buffer as a washing solution.The circles of filter paper that had absorbed the 3H paroxetine were pricked out and placed in 7mL plastic Scintillation vials, with a PV3push in cap (Meridian, Epson Surrey, UK), containing 3mls of Emulsifier Scintillation Plus(Perkin-Elmer, Waltham, Massachussetts, US).These were incubated overnight and

counted on a Wallac B counter (Perkin-Elmer, Waltham, Massachussetts, US) to determine radioactivity.

3.4.4 Calculation SERT binding kinetics

Radioactive counts were averaged for total and nonspecific binding. Specific binding was then determined by subtracting nonspecific binding from total binding averages. Values were calculated using Prism 5.0 (Graph Pad Software) and the Scatchard transformation method. The technique is based on the assumption that 1) the drug interacts reversibly with a single molecular site on its receptor and that 2) the formation of the drug receptor complex obeys the law of mass action. Under these circumstances the relationship between the drug concentration and receptor occupancy at the equilibrium is described by the equation: $B = Bmax \_ F / (F \_ Kd)$, where B is the amount of drug bound to receptors, Bmax the amount of drug required to saturate a population of receptors and a measure of the number of receptors present in the sample, F is the free drug concentration and Kd is the disassociation constant, a measure of the strength of ligand binding. In this plot, the X-axis is specific binding and the Y-axis is specific binding divided by free radioligand concentration. Here the Bmax is the X intercept and Kd is the negative reciprocal of the slope. An example plot is seen in figure 3.4.
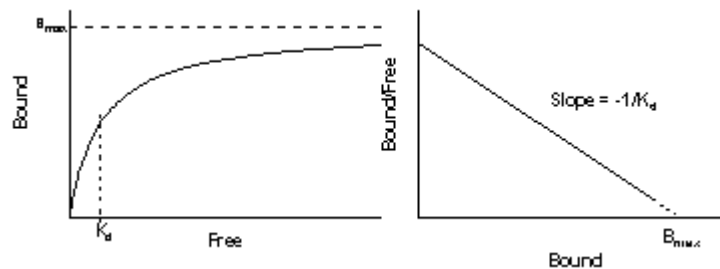
Figure 3-4 An example Scatchard plot here the X axis is specific binding (usually labeled "bound") and the Y axis is the ratio of specific binding to concentration of free radioligand (usually labeled "bound/free"). Bmax is the X intercept; Kd is the negative reciprocal of the slope

3.5   Serum plasma 5-HIAA analysis

3.5.1   Blood sampling and preparation

10 mls of blood was collected from subjects as in section 3.4.1. Samples were collected directly into prechilled 5ml plastic syringes containing 0.5ml of citrate-dipyridamole-adenosine-theophyline (CTAD) platelet stabalising solution that had been aspirated from 4.5ml Diatube H vacutainer tubes (Becton-Dickinson Ltd). Blood was drawn into the syringe to give a total volume of 4.5ml. Care was takent to ensure rapid smooth blood flow while avoiding turbulence again to minimise platelet activation. The blood-anticoagulent mixture was placed immediately into the chilled empty vacutainer the lid replaced and the sample placed on ice.

Platlet poor plasma for the analysis of 5-HIAA was produced in a single step by centrifuging the vacutainer tubes at 3500xG for 25 minutes at 4$^\text{o}$C within 5 minutes of being taken. Plasma was removed using a plastic pipette and stored until analysis at -80$^\text{o}$C.

3.5.2 High performance liquid chromatography (HPLC)

HPLC is a technique that separates small molecules on the basis of size, polarity, solubility or adsorption characteristics and is considered the method of choice for analysing biogenic amines. The analysis of serotonin in this study was performed using reversed phase ion-pair HPLC with electrochemical detection. In reversed phase HPLC a non-polar solvent chemically bonded to a pourous support matrix packed into a chromatographic coloumn forms a "stationary phase" while a polar solvent pumped through the coloumn at high pressure forms a "mobile phase". Molecules passing through the column interact with the stationary phase through non-polar interactions so that hydrophobic molecules elute more slowly from the coloumn. By adding an ion-pairing agent such as sodium octyl sulphate (a negatively charged molecule with a hydrophobic side chain) to the mobile phase the elution of serotonin which is positively charged at experimental pH can be selectively retarded. Electrochemical detectors are highly sensitive and specific and measure the change in current or potential as sample molecules pass between 2 electrodes within a flow cell. One electrode works as a reference cell while the other is held at a voltage high enough to cause oxidation so the current generated by electron transfer is directly proportional to the concentration of the electrocactive substance.

Quantification of plasma 5-HIAA was carried out as follows by GS. Plasma samples were sonicated in 0.2M perchloric acid for 30 seconds containing 0.1% sodium metabisulfite, and centrifuged at 15 000 g for 15 min at 4°C.

Detection and subsequent quantification of 5-HT in the supernatant involved the use of reverse-phase, ion-pair high-performance liquid chromatography (HPLC) coupled with electrochemical detection. Briefly, the method employed a TARGA (75×2.1 mm internal diameter; Higgins Analytical CA).A solvent delivery pump (L-7110, Merck Hitachi,Poole,U.K) was used to circulate mobile phase (0.15 M sodium dihydrogen orthophosphate, 1 mM EDTA, 1.0 mM 1-octane sulphonic acid sodium salt,14% methanol, (adjusted to pH 4.7 with *o*-phosphoric acid, filtered and degassed). Samples were injected onto the column via a Perkin Elmer autosampler series 200(Bucks, U.K) with a cooling tray set at 4°C. An electrochemical detector (Antec Leyden, Netherlands).The flow rate was 0.15 mL/min and the glassy carbon working electrode potential was set + 0.70 V with reference to a saturated KCl-filled Ag/AgCl reference electrode. The current produced was monitored by 'System Gold' software on an IBM PC, with automated data collection (Analogue Interface Module 406).

3.6 *Genotyping for SERT promoter polymorphisms*

3.6.1 Blood sampling

Blood was collected as in section 3.4.1 into a 4.0ml EDTA K2E Vacutainer tube (Becton Dickinson Ltd) and stored at $- 80^{o}$C until analysis.

3.6.2 Genotyping

DNA was extracted from 200 ml of each blood sample using the QIAamp DNA Blood Mini Kit (Qiagen Cat. No. 51106) and then genotyped by KBiosciences

(Hoddesdon, UK) using Taqman methodology for allelic discrimination. All genotyping was performed blinded to clinical status by Caroline Swan.

3.7   Serine protease activity

3.7.1   Stool sample collection

Stool samples were collected by the patient at home into 3 separate pots (Sterilin, Newport, UK) on the morning of the visit, no patient travelled for >30 miles to reach the hospital and the expectation was that samples were usually no >2 hours old on recipt in the department. The samples were immediately frozen at -80C˚.

3.7.2   Stool supernatant preparation

Tris buffer was prepared and frozen in 50ml aliquots at -80oC. 500mls of 20nM Tris required the addition of 3.02g of trizma base , 0.1855g of potassium chloride and 3.505g of sodium chloride to double deionised water. The PH was adjusted to 8.2 with the use of hydrochloric acid.

1g of stool was homogenized in 5ml of Tris buffer pH8.2. The resulting supernatant was centrifuged at 3500G at 4˚c for 20 minutes to remove any insoluble fibrous material. The remaining supenatant was aspirated with a plastic pipette and frozen at -80˚C in 0.5ml aliquots.

3.7.3   Serine protease assay

The assay was performed by Gulzar Singh.

Preparation of reagents

1. **Trypsin standard**

   1mg of trypsin 10,000u/mg (Sigma Aldrich, UK) was dissolved in 10mls of double deionised water and frozen at -80˚C

2. **2% azocaesin**

   1g of azocaesin (Sigma Aldrich) was dissolved completely in 50mls of double deionised water. The solution was stored at 4^oC and used within 24 hours of preparation.

3. **Tris buffer**

   Tris buffer was prepared as for stool supernatant extraction, and used once defrosted within 24 hours.

4. **10% Trichloracetic acid (TCA)**

   10g of TCA were dissolved in 100ml of double deionised water and stored in a locked chest at room temperature.

5. **Stool supernatant**

   0.5ml of stool supernant was defrosted and filtered using a 0.2micron filter (Nalgene, Rochester, US) immediately before use in the assay.

All assays were performed in triplicate in a 96 well plate (Sigma Aldrich, UK). First 0.1ml of Tris was added to each microplate well. 0.1mls of 1% trypsin standard was added to wells 1A and 1H, this was diluted 2-fold serially using a

multichannel pipette to column 12 and the residual 0.1ml discarded to waste. 0.1ml of a positive control stool supernatant with a known value for serine protease activity was added to wells B1 B5 and B9. 0.1ml of Tris was added to wells G1-12 as a blank control. Finally 0.1ml of test stool supernatant were added to rows C1, 5 and 9, D1, 5 and 9, E1, 5 and 9, and F1, 5 and 9. This allowed 4 patient samples to be tested in triplicate per plate. 2-fold serial dilution of rows B to H 1-4, 5-8 and 9-12 respectively was performed and the residual 0.1ml discarded to waste. 0.1mL of 2% azo-casein was added to each well, and mixed briefly by tapping by hand. Wells were sealed using adhesive film-seals and transferred to a 37°C incubator for 30 minutes. The film seal was then removed and 0.1mL 10% TCA was added to each well. Microplates were resealed and centrifuged for 10 minutes at >2k RCF. Finally the seal was again removed and using the multi-channel pipette 0.2 ml of supernatant was transferred to the corresponding wells of a second analytical microplate. The absorbance was read at 440nm on a plate reader and the plate reader software used to determine the unknown relative protease levels against Trypsin calibration values. The values for stool supernatant serine protease activity were expressed as units of trypsin/mg of protein. The protein content of the stool supernatant was assayed using the Bradford method[163].

3.8 Faecal elastase

Faecal elastase was assayed by GS using a commercial ELISA kit (ScheBo Biotech AG, Giessen Germany). Briefly the method comprises of: an initial

binding step where pancreatic elastase in the sample binds to a specific monoclonal antibody on a pre-coated plate. A further incubation with a complex of monoclonal anti-elastase 1-Biotin and Peroxidase –streptavidin allows binding to the bound pancreatic elastase. The peroxidise oxidises 2, 2'-Azino-bis-(3-ethylybenzothiazolin-6-sulfonic acid) diammonium salt (ABTS) which turns dark green. The concentration of oxidised ABTS is determined photometrically.

Preparation of reagents

1. 100mls of wash buffer was diluted in 400ms of double deionised water and stored at 4-8°C until required.

Specimen preparation

Stool samples were prepared using the E1 Quick-Prep^{TM} dosing device. These devices contain ready to use extraction buffer and a comb which captures a fixed dose of sample, it can be seen in figure 3.5.

Figure 3-5 The E1 Quick-Prep^{TM} dosing device

1. The yellow dosing tip was removed and inserted into the stool sample to a depth of 1cm ensuring all notches of the comb like tip were filled with stool.

2. The dosing tip was reinserted into the tube through the blue cone and turned clockwise to close.

3. The tube was vortexed to mix and incubated at room temperature for 10 minutes.

4. A further vortex was performed after incubation to ensure no stool was left on the dosing tip.

5. After the particles had settled the blue cone and dosing tip were removed and 10µl of stool sample extract were diluted to a concentration of 1:70 using 700µl of wash buffer.

Test procedure

1. All tests were carried out in duplicate.

2. 50µl of diluted stool extract or provided standard (15, 50, 150 and 500µg/g) were added to individual wells of the pre-coated plate.

3. The plate was incubated at room temperature for 30 minutes.

4. The contents of the plate were decanted and each of the wells of the plate washed 3 times with 250µl of wash buffer. Any remaining fluid

was removed by inverting the plate and tapping onto a clean paper towel.

5. 50μl of anti-E1-biotin and peroxidise-streptoviridin complex was added to each well.

6. The plate was incubated in the dark at room temperature for 15 minutes.

7. The contents of the plate were decanted and the wells washed as in step 4.

8. 100μl of substrate solution was added to each well.

9. The plate was incubated as in step 6.

10. The substrate reaction was stopped by adding 100μl of stop solution to each well.

11. The absorption at 405nm was read on a plate reader, between 5 and 30 minutes after adding the stop solution, using 492nm as a reference, and the pancreatic elastase concentration calculated from a calibration curve constructed using the provided standards.

3.9 Faecal amylase

Faecal amylase was assayed by GS using a commercial ELISA kit (Immunodiagnostik AG,Bensheim, Germany). Briefly the method comprises of: an initial step where there was binding of pancreatic amylase in stool

supernatant in to a specific mouse monoclonal antibody on a coated plate. This is followed by a washing step to remove any excess before incubation with a monoclonal anti pancreatic amylase antibody. A further wash removes all unbound substances before the chromogenic substrate tetramethylbenzidine is added. An acidic stop solution halts the reaction and there is a colour change from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of pancreatic amylase in the sample. The concentration in the patient samples is calculated from a standard curve that is obtained using standards supplied by the manufacturer.

Preparation of regents

1. The ELISA wash buffer was diluted to a 1:10 concentration with deionised water

2. Both the standards and the control were reconstituted with 250µl of deionised water. The vials were allowed to stand for 10minutes before being gently inverted to mix and ensure complete reconstitution.

3. 10µl of the anti-pancreatic amylase monoclonal antibody was diluted with 10mls of wash buffer and stored at 2-4°C until required.

Specimen preparation

1. 100mg of stool was weighed and diluted in 5ml of wash buffer.

2. The sample was then centrifuged at 3000G for 10 minutes.

3. 1ml of sample supernatant was then placed in an eppendorf and centrifuged at 13,000G for a further 5 minutes.

4. 25µl of the final supernatant was then diluted to a concentration of 1:40 with a further 975µl of the wash buffer.

Test procedure

1. All tests were carried out in duplicate

2. Each well of the pre-coated microtiter plate was washed 5 times with 250µl of wash buffer

3. 100µl of each of the standard solutions (0; 440; 1750; 7000; 28000 mU/l) and the prepared patient stool supernatants were added to individual wells of the pre-coated plate.

4. The plate was incubated for 1 hour at room temperature whilst shaking on a horizontal mixer.

5. The contents of the plate were then decanted and the wells washed 5 times with 250µl of wash buffer.

6. 100µl of tetramethylbenzidine was then added to each well of the pre-coated plate

7. The plate was incubated at room temperature for a further 10 minutes.

8. The reaction was stopped by the addition of 50 µl of a sulphuric acid containing stop solution to each well.

9. The absorption at 450nm was read on a plate reader, using 650nm as a reference, and the pancreatic amylase concentration calculated from a calibration curve constructed using the provided standards.

10. The concentration of pancreatic amylase per sample was calculated as in the follow example:

Example sample weight: 80 mg (1ml Stool = 1g) = 0,08 ml

Dilution step 1: 5ml / 0,08ml = 62,5

Dilution step 2: 40

Dilution factor: 62,5 x 40 = 2500x the value obtained using the calibration curve.

3.10 Mast cell tryptase

A fluorescence reporter assay system (PHADIA-CAP, Phadia Ltd, Uppsala, Sweden) was used to specifically quantitate mast cell tryptase. The assay was demonstrated to give undetectable readings when samples were spiked with pancreatic Trypsin. This assay was performed by the Department of Immunology, University Hospital, Nottingham.

3.11 Serine protease identification

All of the following analyses were performed by Dr David Tooth.

3.11.1　Affinity chromatographic purification of
serine　proteases

Faecal serine proteases were purified by passing faecal extracts at 1mL/min through a Benzamidine-sepharose column (1mL, HiTrap, GE Healthcare, Amersham, UK) which specifically retains serine proteases. Samples were loaded and washed (10 column-volumes) in 50mM Tris, pH 7.4, 0.5M sodium chloride buffer and retained components were eluted by the step-wise adjustment to 50mM Glycine pH 3.0 buffer. Stool extracts were buffer-exchanged to wash-buffer, prior to loading using gel-permeation (PD-10, GE-Healthcare, Amersham, UK), according to the vendor protocol. Chromatography was profiled by monitoring protein absorbance at 280nm and components in flow- through and eluate fractions were collected for subsequent characterisation.

3.11.2　Protein electrophoresis

SDS-Polyacrylamide gels (SDS-PAGE, Bis-Tris using MES buffer, Invitrogen, Paisley, UK,) were electrophoresed under thiol-reducing conditions, according to vendor guidelines. Protein components were visualised by chemical staining using colloidal Coomassie Brilliant Blue G-250 according to vendor guidelines.

3.11.3　Identification of Protein components

Protein components in gel bands were excised, reduced using dithiothreitol, alkylated using iodoacetamide and proteolysed using Porcine Trypsin in 0.2M ammonium bicarbonate pH8.0. All protocols were standard procedures

essentially according to Hellman et al [164].Proteolytic peptides were extracted captured and desalted to a chromatographic trap (Dionex C18, 0.3 x 5mm) and subjected to micro-capillary-high-pressure-liquid-chromatography with Tandem-mass-spectrometry (nano-LC-MS/MS) using a hybrid quadrupole-time-of-flight (Waters Q-TOF2) instrument equipped with a nano-electrospray ion-source in positive-ion mode and calibrated against synthetic peptides and product ions. Data-dependent product-ion spectra were acquired 'on-the-fly' during test-experiments. The MASCOT server (www.matrixscience.com) was used to interrogate genome databases using precursor- and product-ion data and reports were returned together with (MOWSE) probability scores.

4 Results: Patient reported outcomes.

4.1 Study populations

Of the 125 patients that were recruited from both sites, 71 were enrolled in Nottingham and 54 in Manchester, along with 20 age and sex matched controls from the Nottingham site only. Patients recruited in Manchester were significantly more depressed (4.5 (2-8) vs. 5.5 (3-10) p= 0.04) and had an increased IBS-SSS (281.8 ± 10.06 vs. 342.2 ± 9.75 p=<0.001) with the mean value in Nottingham being classified as moderate and in Manchester severe. This was coupled with a lower quality of life (488.0 ± 18.41 vs. 446.0 ± 14.23 p=0.0006) than those recruited in Nottingham. Although the Manchester recruits had significantly higher pain, bloating and urgency scores they did not experience symptoms on more days per week, these findings are summarised in table 4.1. The mean age of patients recruited was not significantly different between sites and the number of men in the study was 37 or 29% of the total study population.

	IBS-D Nottingham	IBS-D Manchester	P=	IBS-D total
Number	71	54		125
Age	39.75 ± 1.50	42.7 ± 1.57	0.18	41.02 ± 1.10
Sex (Male/Female)	23/48	13/41		36/89
Anxiety (<7)	9.37 ± 0.59	10.7 ± 0.53	0.39	9.7 ± 0.41
Depression (<7)	4.5 (2-8)	5.5 (3-10)	0.04	5 (2.75-9)
PHQ-15 (0-30)	11.79 ± 0.52	12.8 ± 0.52	0.18	12.22 ± 0.38
PSSS (0-40)	17.44 ± 0.95	19.43 ± 0.10	0.16	18.31 ± 0.69
IBS-SSS (0-500)	281.8 ± 10.06	342.2 ± 9.75	<0.001*	308.1 ± 7.56
IBS-QOL	488.0 ± 18.41	391.5 ± 20.19	0.0006	446.0 ±14.23
Stool Form (1-7)	5.29 ± 0.08	5.46 ± 0.11	0.18	5.24 ± 0.06
Stool Frequency	2.93 (2.1-4.18)	2.57 (1.85-3.64)	0.22	2.7 (1.86-4)
Pain (0-3)	1.21 ± 0.09	1.61 ± 0.11	0.005*	1.37 ± 0.07
Days with pain/week	5.5 (3-7)	6.5 (4.75-7)	0.06	6 (4-7)
Urgency (0-3)	1.54 ± 0.08	1.82 ± 0.09	0.03*	1.66 ± 0.06
Days with urgency/week	6 (5-7)	7 (5-7)	0.67	7(5-7)
Bloating (0-3)	1.2 ± 0.10	1.56 ± 0.12	0.02*	1.35 ± 0.08
Days with bloating/week	6 (3-7)	6 (5-7)	0.22	6 (3-7)

Table 4-1 Characteristics of patients: Data shown as mean (SEM) or median (IQR). Where stool form and frequency as well as pain, urgency and bloating are all weekly averages.

4.2 Study drop outs and adverse events

4.2.1 Drop outs

Of the 125 patients recruited, 5 did not complete the screening phase. 1 patient received a diagnosis of microscopic colitis at screening biopsy and was therefore ineligible. 3 patients did not wish to take part after their initial screening visit, all expressed concerns about time needed to attend study visits. 1 patient developed swine flu during the screening period and subsequently was excluded.

During the study there were 6 dropouts whilst taking Ondansetron, 5 in the first treatment period. 2 patients were lost to follow up, all were contacted by phone and post but failed to attend subsequent study visits. The GP was informed of their withdrawal. 2 patients discontinued secondary to constipation; both responded to dose reduction but did not wish to continue. 1 patient discontinued secondary to a previously diagnosed back problem that prevented them from easily attending study visits.

The same number of patients dropped out whilst taking placebo. In the first treatment arm, 1 patient had difficulty travelling, 1 patient simply wanted to discontinue and a third withdrew due to back pain. In the second treatment period 2 patients withdrew due to lack of efficacy and diarrhoea and 1 secondary to worsening abdominal pain.

This is summarised in figure 4.1.

```
┌─────────────────────────────────┐  ┌──────────────────┐
│        125 recruited            │  │ 5 excluded       │
└─────────────────────────────────┘  │ 1 screen fail    │
┌─────────────────────────────────┐  │ 3 declined       │
│        120 randomised           │  │ 1 swine flu      │
└─────────────────────────────────┘  └──────────────────┘

┌─────────────────────────────┐   ┌─────────────────────────────────┐
│ Drug 1 Ondansetron = 61     │   │ Drug 1 placebo = 59             │
│ 3 lost to follow up         │   │ 1discontinued back pain         │
│ 2 discontinued, constipation│   │ 2 discontinued, patient preference│
└─────────────────────────────┘   └─────────────────────────────────┘

┌─────────────────────────────┐   ┌─────────────────────────────────┐
│ Drug 2 Placebo = 56         │   │ Drug 2 Ondansetron = 56         │
│ 1 discontinued, abdominal pain│ │ 1 discontinued due to back pain │
│ 2 discontinued, lack of efficacy│ └─────────────────────────────────┘
└─────────────────────────────┘
```
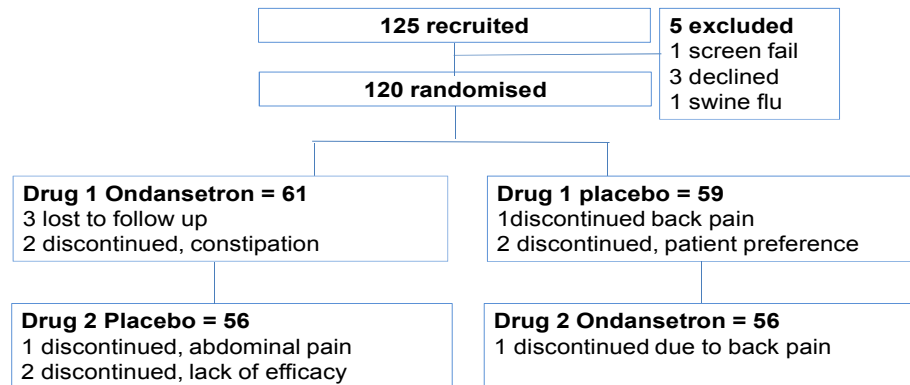
Figure 4-1 Consort diagram: showing exclusions and drop outs from the study Ondansetron in patients with diarrhoea predominant Irritable bowel syndrome "identifying the responder".

4.2.2 Adverse events

There were 28 adverse events, 1 in the screening period, 10 whilst taking placebo and 17 whilst taking Ondansetron. 3 patients withdrew from the trial secondary to adverse events in the Ondansetron arm and 3 withdrew in the placebo arm. There were no serious adverse events. The adverse events are summarised in table 4.2.

Treatment	Adverse event	Severity	Action
Ondansetron	Abdominal pain	Mild	None
Ondansetron	Abdominal pain	Severe	None
Ondansetron	Abnormal liver function tests	Mild	Other
Ondansetron	Back pain	Moderate	Withdrawn
Ondansetron	Constipation	Mild	Dose reduction
Ondansetron	Constipation	Mild	Dose reduction
Ondansetron	Constipation	Mild	Dose reduction
Ondansetron	Constipation	Moderate	Withdrawn
Ondansetron	Constipation	Moderate	Dose reduction
Ondansetron	Constipation	Moderate	Dose reduction
Ondansetron	Constipation	Moderate	Withdrawn
Ondansetron	Constipation	Severe	Dose reduction and over the counter laxatives
Ondansetron	Headache	Mild	None
Ondansetron	Headache	Mild	None
Ondansetron	Rectal bleeding	Mild	None
Ondansetron	Rectal bleeding	Mild	Flexible sigmoidoscopy
Ondansetron	Rectal pain	Moderate	Simple analgesia
Placebo	Chest pain	Moderate	Cardiology review
Placebo	Elbow pain	Moderate	Simple analgesia
Placebo	Back pain	Severe	Withdrawn
Placebo	Headache	Mild	None
Placebo	Headache	Severe	Simple analgesia
Placebo	Diarrhoea	Severe	Withdrawn
Placebo	Abdominal pain	Moderate	Withdrawn
Placebo	Rectal bleeding	Mild	Flexible sigmoidoscopy
Placebo	Rectal bleeding	Mild	Flexible sigmoidoscopy
Placebo	Testicular lump	Mild	Urology review

Table 4-2 Adverse events during the study Ondansetron in patients with diarrhoea predominant Irritable bowel syndrome "identifying the responder"

There were 8 instances of constipation in the Ondansetron arm and none in the placebo arm of the study. The incidence of constipation is likely to be directly attributable to Ondansetron, all but 1 of the patients responded to dose reduction alone. 2 patients however did not wish to continue taking part in the trial. The single patient who took laxatives purchased an over the counter remedy before contacting the study team. 2 patients experienced exacerbations of their abdominal pain whilst taking Ondansetron and 1 whilst taking placebo, these symptoms are likely due to exacerbation of the patient's irritable bowel syndrome and no specific treatment was required. The patient in the placebo arm did not wish to continue and was withdrawn. I patient had abnormal liver function tests at the end of the Ondansetron arm of treatment, after referral to hepatology and appropriate investigation a diagnosis of Epstein Barr virus infection was made, during follow up liver function returned to normal. 4 patients reported rectal bleeding during the trial, 2 whilst taking Ondansetron and 2 whilst taking placebo. All but one had a flexible sigmoidoscopy the next available working day, the 4th patient refused. All bleeding was diagnosed as local blood loss from the anal canal. 2 patients in each arm reported headache, 1 whilst on placebo was classed as severe and required the prescription of simple analgesia. The chest pain, back pain and elbow pain reported were all exacerbations of previously diagnosed disorders and the testicular lump was found to be not clinically significant.

Despite the significant difference in number of patients experiencing constipation with Ondansetron compared to placebo there was no significant

difference in overall number of adverse events between the placebo and Ondansetron arms, Fisher's exact test p=0.2.

13 patients were excluded from the per protocol analysis. During the 2 year course of the study 3 patients under follow up by the medical team in Nottingham received another diagnosis: 1 chronic pancreatitis, 1 bile salt malabsorption, 1 diarrhoeal illness responding to steroids, it is interesting to note none of these patients responded to Ondansetron.

3 patients provided insufficient diary data to be included in the analysis.

There were 8 protocol violations, 5 patients took loperamide during endpoints, 3 during both placebo and Ondansetron and 2 during the Ondansetron arm. 2 patients were included at the Manchester site with a high CRP at entry. 1 patient had documented poor compliance. Compliance was monitored by asking to patient at study visits and by a final pill count of all returned medicines.

4.3   Baseline diaries

A wealth of information is contained in the baseline diaries of 119 patients with IBS-D (1 patient did not adequately complete the baseline diary) and 20 healthy controls. The study is powered on change in mean daily stool form but the diaries provide an opportunity to investigate other abnormalities in stool form and pattern as well as pain, bloating, and urgency to better understand the symptoms our patients experience on a weekly basis.

4.3.1 Stool form.

The average weekly Bristol stool form (BSF) of patients and healthy volunteers was normally distributed (Shapiro-wilks) using a t test as expected patients at baseline had a significantly greater mean weekly BSF than the healthy volunteers 3.5 (±0.16) vs. 5.4 (±0.06), p=0.0001. This is represented in Fig 4.2.

Baseline weekly average stool form.
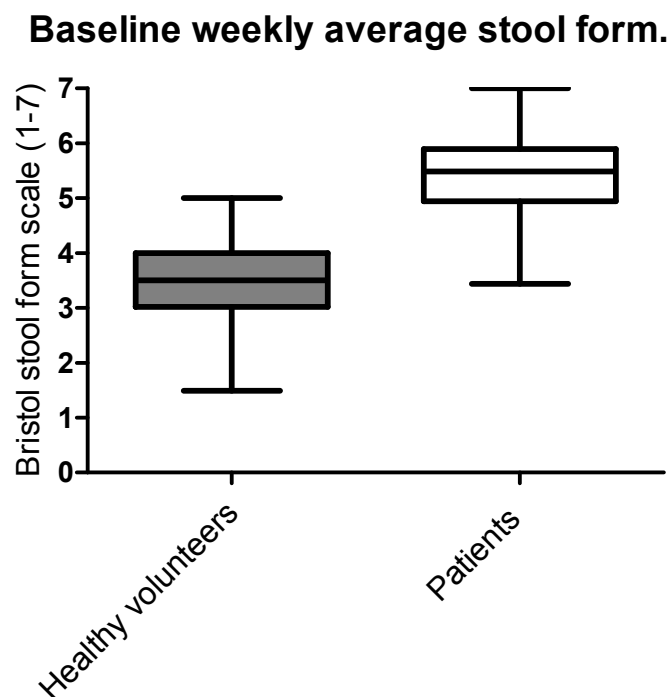
Figure 4-2 Baseline weekly average stool form in patients and healthy volunteers, with the mean weekly stool form being 3.5 (±0.16) in healthy volunteers vs. 5.4 (±0.06) in patients, p=0.0001.

4.3.2 Stool form variability.

The unpredictability of symptoms in IBS-D has been suggested to be a major cause of distress in patients, and this intra-individual variability is not captured by a weekly mean BSF score. Indeed Basseri et al have used a visual analogue measure of variability between constipation and diarrhoea to

predict the incidence and severity of IBS-C[165]. In order to understand the unpredictability and variability reported I have examined the stool diaries of patients and healthy volunteers to determine the minimum and maximum weekly BSF, the difference between these two values is termed the stool form variability.

Somewhat surprisingly there was no difference in weekly stool form variability between patients and healthy volunteers, median variability being 3 (2-3.8) points on the BSF scale vs. 3(2-5) p= 0.3. Similarly there is no association between stool form variability and quality of life r^2=<0.0004, IBSSS r^2= 0.004, pain r^2 =0.007, urgency r^2 =0.01, or bloating r^2 =0.02.

Stool form variability at best weakly correlates with mean stool form , with some with the greatest mean stool form score on the Bristol Stool Form Scale, i.e. loosest stool on average having the least variability in form over 7 days (r^2 =0.05 p=0.005). There is also a similarly weak correlation between stool frequency and stool form variability (r^2 0.04 p=0.02) with some of those with very high frequency having higher stool form variability.

In order to determine whether stool form variability was related to any of the psychological and somatic measurements collected I divided patients and volunteers into those with low stool form variability (0-3 points on the BSF scale) and high stool form variability (4-6 points on the BSF scale). There were no differences in psychological distress, 13.88 (±0.98) vs. 14.77 (±0.91) points p=0.5, or somatisation as measured by the PHQ12, 7.66 (±0.48) vs. 6.0 (±0.43) points p=0.1 between these groups.

4.3.3 Number of days with stool form >5.

It is the incidence of very loose stool and subsequent urgency and threat of incontinence that is the driver to many patients consultation[166], this is intended to be captured in the measure of number of days per week with stool form 6 and 7 proposed as a measure of stool form response to a treatment by the FDA.

At baseline patients had many more days with stool form >5 than healthy volunteers 5.5 (4-7), compared to 0 (0-0.75) p=<0.0001. The number of days with stool form >5 in patients correlated positively with psychological distress, the sum of the anxiety and depression components of the HADS score, with an r^2 of 0.05 p= <0.01. There was also a correlation with, IBSQOL r^2 = 0.1 p=0.0009 and IBSSS r^2= 0.04 p=0.02, making this a simple single measure of severity. Those with more days a week with stool form >5 had more urgency r^2 0.01 p=<0.001 but not pain, bloating or a higher PHQ12.

4.3.4 Stool frequency.

Baseline stool frequency was non-normally distributed (Shapiro-Wilks). As expected patients had a greater average daily stool frequency than healthy volunteers 2.7 (1.9-4) vs. 1.1 (1-1.4) p=<0.00001, Mann Whitney-U. This difference was surprisingly small reflecting the fact that episodes of diarrhoea do not occur every day

Baseline weekly average stool frequency
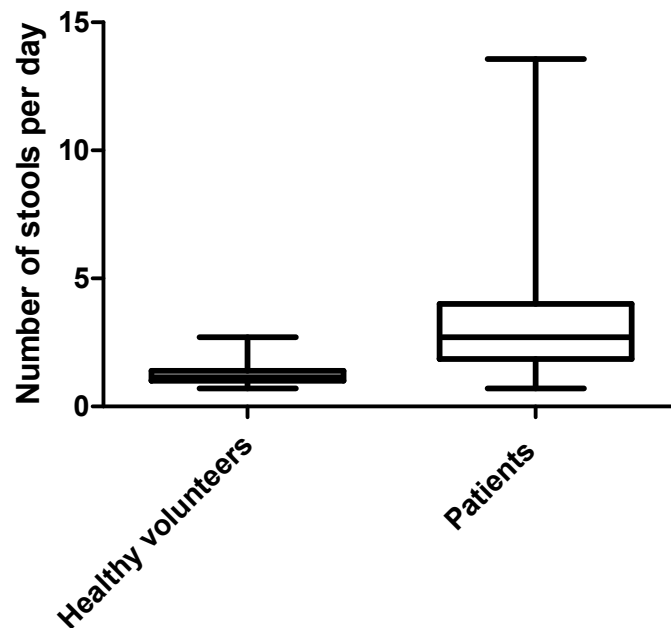
Figure 4-3 Baseline weekly average stool frequency in patients and healthy volunteers, with the mean weekly stool in patients 2.7 (1.9-4) vs. 1.1 (1-1.4) in healthy volunteers p=<0.00001.

4.3.5 Early morning rush (EMR)

A commonly recognised clinical phenotype and a major source of unpredictable symptoms and high stool frequency is that of the "early morning rush". Here the patient complains of multiple and frequent bowel motions (BM) soon after waking. To investigate the effect of Ondansetron on this commonly reported symptom I have defined a morning rush as 2 or more BM in less than 1 hour occurring after midnight and before 12 midday. Using this definition 60% of patients had at least one day with a morning rush in the baseline week compared to 10% of healthy volunteers. Patients and volunteers were further stratified into 3 groups. Those with 1-3 episodes of EMR a week were classified as normal morning rush (NMR), this group included all of the healthy volunteers. Those with 3-4 days a week with EMR

were termed moderate morning rush (MMR) and those with 5-7 days a week were classified as severe morning rush (SMR).

16 % of patients had MMR and 15 % of patients had SMR. Those with SMR had greater psychological distress (anxiety + depression component of the HADS score) than those with NMR, 19.11 (± 1.8) vs. 13.48 (±0.8) p=0.021, this can be seen in figure 4.4. This group also have more somatic symptoms as measured by the PHQ-12, 9.2 (± 0.9) vs. 6.5 (± 0.4) p=0.02.

EMR and psychological distress
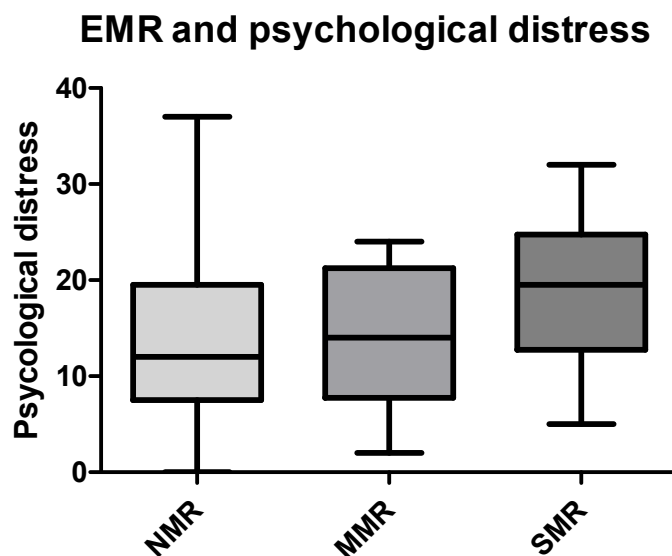
Figure 4-4EMR and psychological distress with SMR had greater psychological distress (anxiety + depression component of the HADS score) than those with NMR, 19.11 (± 1.8) vs. 13.48 (±0.8) p=0.021.

The SMR group had greater mean daily stool frequency than those with NMR and MMR 5.6 (3.5-8.2) vs. 2.14 (1.7-3.0) and 3.7 (2.7-4.4) p=<0.0001. The SMR group was also found to have a higher IBSSS 366 (± 18.4) vs. 296.6 (± 9.4) and 295.9 (±17.2) p= 0.005, and this was accompanied by a lower IBSQOL 334.3 (±21.1) vs. 467.3 (± 17.9) and 467.3 (± 33.4) p=0.004. Whole gut transit (in

hours) whilst taking placebo, or at baseline in the case of the healthy volunteers, was significantly faster in the SMR group with transit times of 7h (5.5-15) vs. 19h (10-39) in the NMR and 16h (8.5-24.5) in the MMR groups, p=0.03, See Fig 4.5.
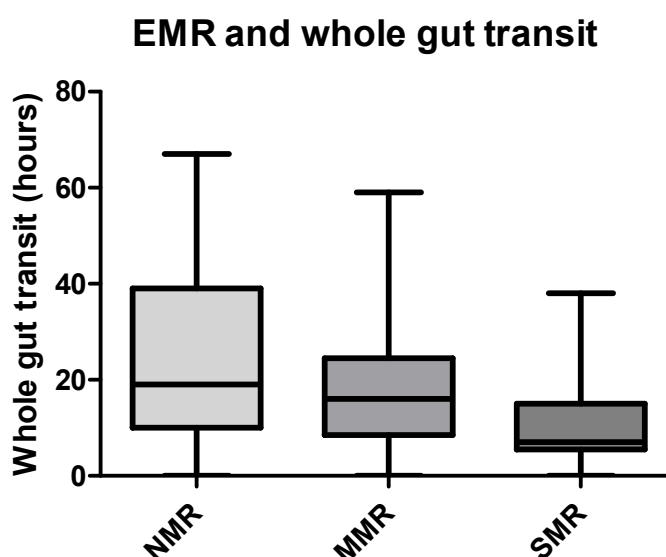
Figure 4-5 EMR and whole gut transit (in hours). Placebo, or baseline transit in the case of the healthy volunteers, was significantly faster in the SMR group with transit times of 7h (5.5-15) vs. 19h (10-39) in the NMR and 16h (8.5-24.5) in the MMR groups, p=0.03.

4.3.6 Days with pain, urgency and bloating

As expected in accordance with the entry criteria patients had significantly more days with pain, bloating and urgency per week than healthy volunteers, see table 4.3.

	Healthy volunteers	Patients	P value
Pain score (0-3)	0 (0-0)	6 (4-7)	<0.0001
Urgency score (0-3)	0.5(0-1)	7(5-7)	<0.0001
Bloating score (0-3)	0(0-2)	6 (3-7)	<0.0001

Table 4-3 Days with pain, urgency and bloating inpatients and healthy volunteers.

4.3.7 Anxiety depression and somatisation

Values for anxiety depression and somatisation at baseline were significantly higher in the patient population than in healthy volunteers this is summarised in table 4.4

	Healthy volunteers	Patients	P value
Anxiety normal <7	5.2 (± 0.6)	9.7 (± 0.4)	<0.0001
Depression normal <7	1.5 (1-2.3)	5 (2.5-9)	<0.0001
PHQ12 (0-24)	2.3 (±0.4)	7.7 (±0.3)	<0.0001

Table 4-4 Baseline anxiety depression and somatisation in patients and healthy volunteers.

4.4 Primary endpoint

The primary endpoint was the change in stool form from baseline. Change in stool form from baseline was not normally distributed. In the intention to treat analysis n= 101, there was a 1.39 (95% CI1.20-1.58)point decrease on the Bristol stool form scale whilst taking Ondansetron compared to a 0.51 (95% CI 0.32-0.72) point reduction whilst taking placebo p=<0.0001, Wilcoxon matched pairs. In the per protocol analysis n=95 there was a 1.47 (95% CI 1.27-1.26) point decrease on the Bristol stool form scale whilst taking Ondansetron, compared to 0.51 (95% CI 0.30-0.71) points whilst taking placebo p=<0.0001, Wilcoxon matched pairs signed rank test. The intention to treat data is represented graphically in Fig 4.6.

Change in stool form from baseline (intention to treat)
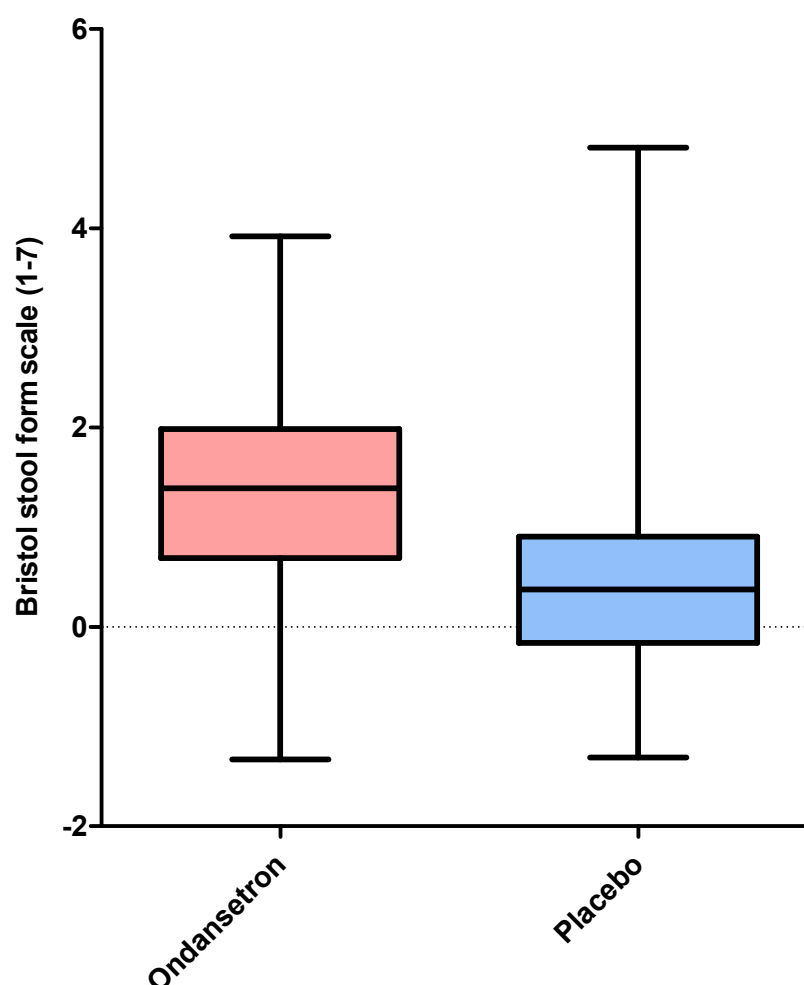
Figure 4-6 Primary endpoint, the change in stool form from baseline with Ondansetron compared to placebo (intention to treat) n= 101, Showing a 1.39 (95% CI1.20-1.58)point decrease on the Bristol stool form scale whilst taking Ondansetron compared to a 0.51 (95% CI 0.32-0.72) point reduction whilst taking placebo p=<0.0001, Wilcoxon matched pairs signed rank test.

4.5   Secondary endpoints

Secondary endpoints are presented as intention to treat only, and are summarised in table 4.5.

4.5.1 Stool frequency

Change in stool frequency from baseline was non-normally distributed (Shapiro-Wilks). There was a significant reduction in mean stool frequency

compared to baseline of 0.86 (IQ 0.24-1.7) stools per day whilst taking Ondansetron compared to 0.44 (IQ 0-1.13) whilst taking placebo p=0.001, Wilcoxon matched pairs signed rank test.

4.5.2 Days with pain urgency and bloating

There was a reduction in the number of days per week patients report urgency of any severity with Ondansetron of 1.5 (IQ 0-4) days, compared to 0.5 (IQ 0-2) days with placebo p= 0.01. Average weekly scores for the severity of urgency (0-3), where 0 is no urgency, 1 is mild urgency, 2 is moderate urgency and 3 is severe urgency) were also reduced when taking Ondansetron by 0.62 (95% CI 0.48-0.77) compared to 0.34 (95% CI 0.21-0.47) whilst taking placebo p =<0.0001.

There was no significant change in the number of days per week patients reported pain of any severity with Ondansetron with a median reduction from baseline of 0.25 (IQ 0.0-1.5) days, compared to 0.0 (IQ 0.0- 1.5) with placebo p=0.37. Average weekly scores for the severity of pain (0-3, where 0 is no pain, 1 is mild pain, 2 is moderate pain and 3 is severe pain) were also not significantly improved from baseline when taking Ondansetron,   0.25 (95% CI 0.12-0.37) compared to 0.17 (95% CI 0.06-0.29) points whilst taking placebo, p = 0.12.

Additionally there was no significant change in the number of days per week patients report bloating of any severity with a median reduction whilst taking Ondansetron of 0.25 (IQ 0-1.5) days, compared to 0.0 (IQ -0.37-1) days with placebo, p= 0.14. Average weekly scores for the severity of bloating (0-3,

where 0 is no bloating, 1 is mild bloating, 2 is moderate bloating and 3 is severe bloating) were again unchanged from baseline when taking Ondansetron by 0.12 (IQ -0.14- 0.5)points, compared to 0.07 (IQ -0.29-0.43) points whilst taking placebo, p =0.25.

	Ondansetron	Placebo	p
Decrease in stool frequency	0.86 (IQ 0.24-1.7)	0.44 (IQ 0-1.13)	0.001
Decrease in urgency score (0-3)	0.62 (95% CI 0.48-0.77)	0.34 (95% CI 0.21-0.47)	<0.0001
Decrease in days/week with urgency	1.5 (IQ 0-4)	0.5 (IQ 0-2)	<0.0001
Decrease in abdominal pain score (0-3)	0.25 (95% CI 0.12-0.37)	0.17 (95% CI 0.06-0.29)	0.12
Decrease in days/week with pain	0.25 (IQ 0.0-1.5)	0.0 (IQ 0.0- 1.5)	0.37
Decrease in bloating score (0-3)	0.25 (IQ 0-1.5)	0.0 (IQ -0.37-1)	0.14
Decrease days/week with bloating	0.12 (IQ -0.14- 0.5)	0.07 (IQ -0.29-0.43)	0.25

Table 4-5 Secondary endpoints (intention to treat).

4.5.3 IBS symptom severity score (IBS SSS)

There is a significant reduction in the IBS SSS compared to baseline when taking Ondansetron of 83 ± 9.8 points compared to 37 ± 9.7 points whilst taking placebo, p=0.001. The maximum achievable score is 500. Mild, moderate and severe cases are indicated by scores of 75 to 175, 175 to 300 and > 300 respectively. Healthy controls score below 75 and patients scoring in this range can be considered to be in remission[167]. At baseline only 6% of patients had mild disease, 36% had moderate disease and 58% had severe disease. After taking Ondansetron (n=106) 9% had entered remission with 26% having mild, 40% having moderate and 25% having severe disease. This is compared to with Placebo (n=108) where 4% had entered remission, 15 % had mild, 43 % had moderate and 39 % had severe disease, p=0.04 Chi squared.

4.5.4 Proportion of patients preferring ondansetron versus placebo

When asked at the final study visit which treatment they preferred significantly more patients, 74%, preferred Ondansetron with only 17% preferring placebo, 9% patients preferred neither treatment with Ondansetron or placebo, chi squared p=<0.0001. This is shown in fig 4.7

Proportion of patients preffering Ondansetron versus Placebo
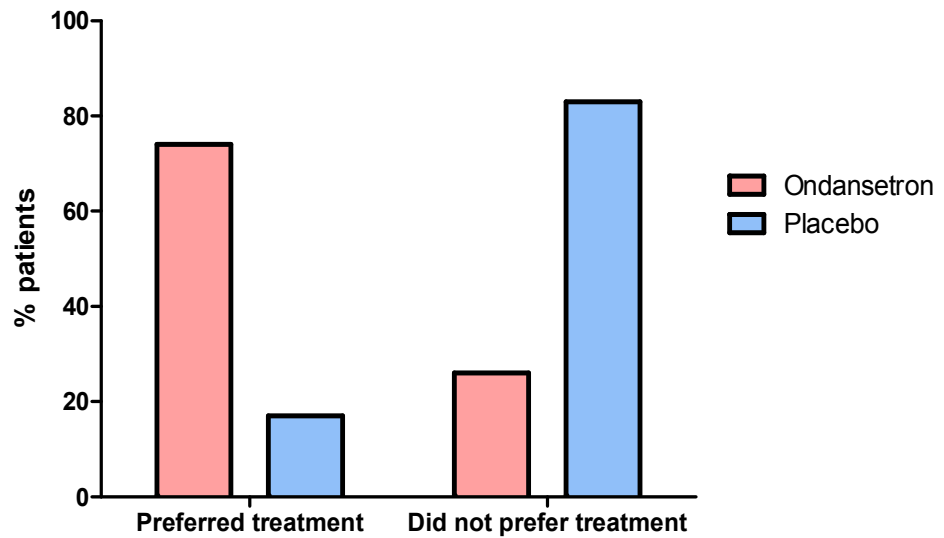
Figure 4-7 Proportion of patients preferring Ondansetron vs. Placebo, 74%, preferred Ondansetron with only 17% preferring placebo, 9% patients preferred neither treatment with Ondansetron or placebo, chi squared p=<0.0001.

4.5.5 Proportion wanting to continue with ondansetron versus placebo

After completing the 15 week trial 73% of patients if given the choice would have chosen to continue taking Ondansetron compared to 18% who would have preferred to continue placebo, chi squared p=<0.001. 9% of patients did not wish to continue either Ondansetron or placebo, 1% would have continued either Ondansetron or placebo, this is shown in fig 4.8.

Proportion wanting to continue Ondansetron versus Placebo
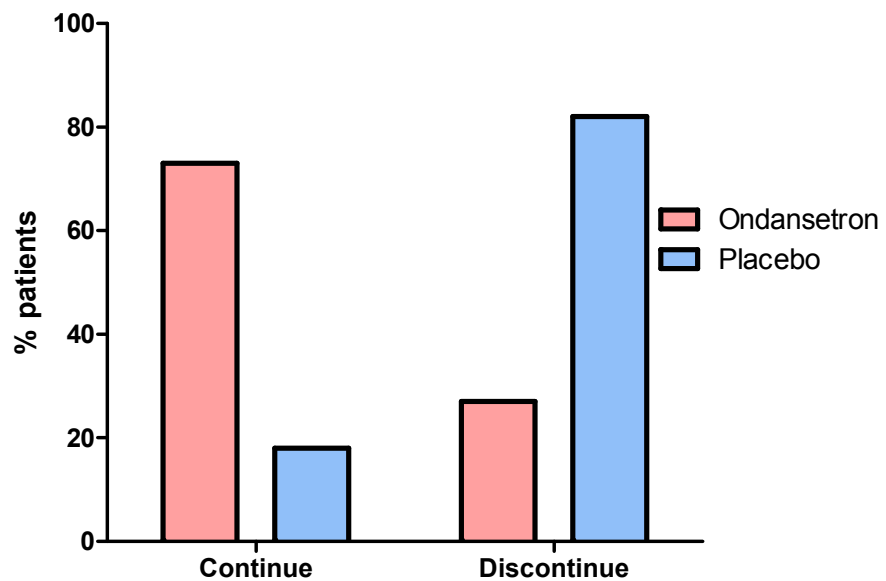
Figure 4-8 Proportion wanting to continue Ondansetron vs. Placebo where 73% of would have chosen to continue taking Ondansetron compared to 18% who would have preferred to continue placebo, chi squared p=<0.001.

4.5.6 Percentage satisfactory relief

67 % of patients when asked at the end of the study if they had recieved satisfactory relief from their symptoms said yes whilst taking Ondansetron, compared to 18% who described satisfactory releif after taking placebo, chi squared p=<0.0001. 18% of patients did not get satisfactory relief from their symptoms whilst taking either placebo or Ondansetron. 3% of patients got satisfactory from their symptoms with both the ondansetron and placebo treatment arms, fig 4.9.

Percentage satisfactory relief
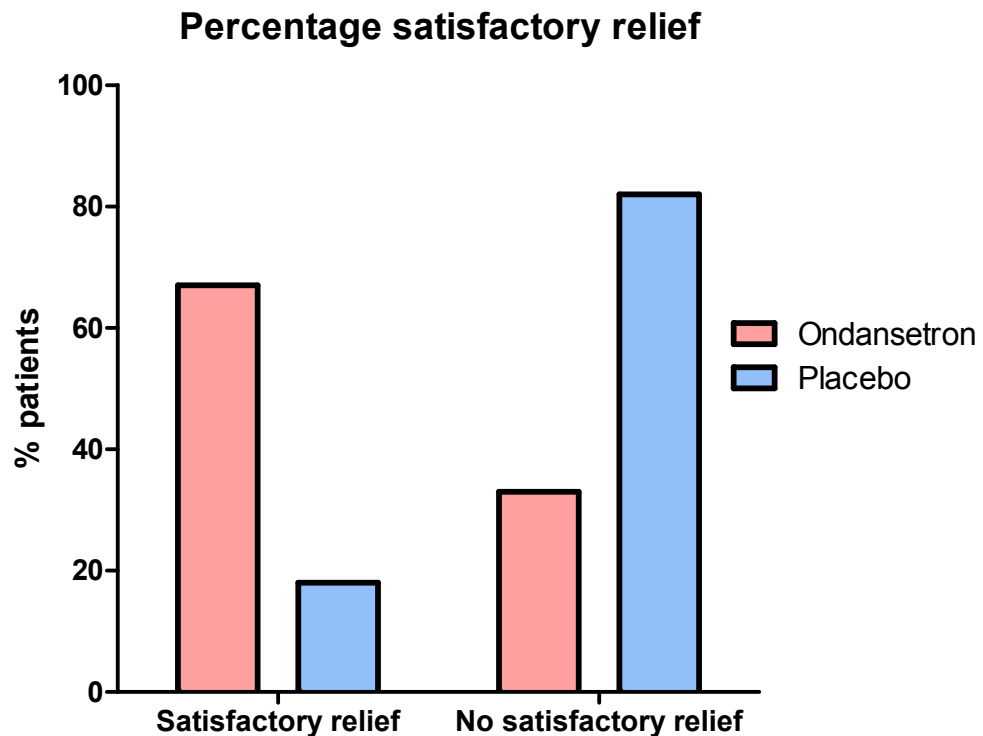
Figure 4-9 Percentage satisfactory relief at the end of treatment with Ondansetron was 67 % compared to 18% with placebo, chi squared p=<0.0001

4.6 Number needed to treat (NNT)

The number needed to treat is a measure of the effectiveness of an intervention; it is an expression of the average number of patients needed to treat for one patient to benefit, the ideal number to treat is 1, where everyone improves with treatment and nobody improves with control. In order to calculate this for Ondansetron it is necessary to define what a response to treatment is.

There are several potential methods of describing what constitutes a response to treatment. Adequate relief, patient preference, and improvement in stool form can all be used. the definition of a responder to treatment has been contentious and I am helped by recent guidelines for clinical trials in IBS from

the US Food and Drug Administration (FDA) [168] which propose that for a patient to be a stool form responder to a medication they should have a reduction of 50% or greater in the number of days a week they have a stool with a BSF >5 this is termed an FDA response. We have seen using the baseline diaries that days with stool form >5 correlate with symptom severity, quality of life and psychological distress adding weight to this being a clinically meaningful measure of improvement with treatment. I will outline the number needed to treat in this trial using these 4 different measures of response.

4.6.1 Patient preference

17% of patients preferred placebo and 83% did not, whilst 74% patients preferred Ondansetron and 26% did not giving a NNT of 2 (95% CI 1.5-2.2).

4.6.2 Adequate relief

18% of patients had an adequate relief whilst taking placebo, whilst 82% did not. Whilst taking Ondansetron 67% patients had adequate relief whilst 33% did not giving a NNT of 3 (95% CI 1.7-2.7)

4.6.3 Improvement in mean stool form

A clinically significant reduction in mean BSF would be a reduction of 1 whole point on the scale. Using this definition of response 31% patients taking Ondansetron did not respond to treatment and 69% did, 81% patients did not respond to the placebo whereas 19% did giving a number needed to treat of 3 (95% CI 1.6-2.6).

4.6.4 FDA response

33% of patients had a response according to the FDA criteria to placebo with 67% not responding, whilst taking Ondansetron 70% responded and 30% did not giving a NNT of 3 (95% CI 2-4.2), fig 4.10.

Percent response according to FDA criteria
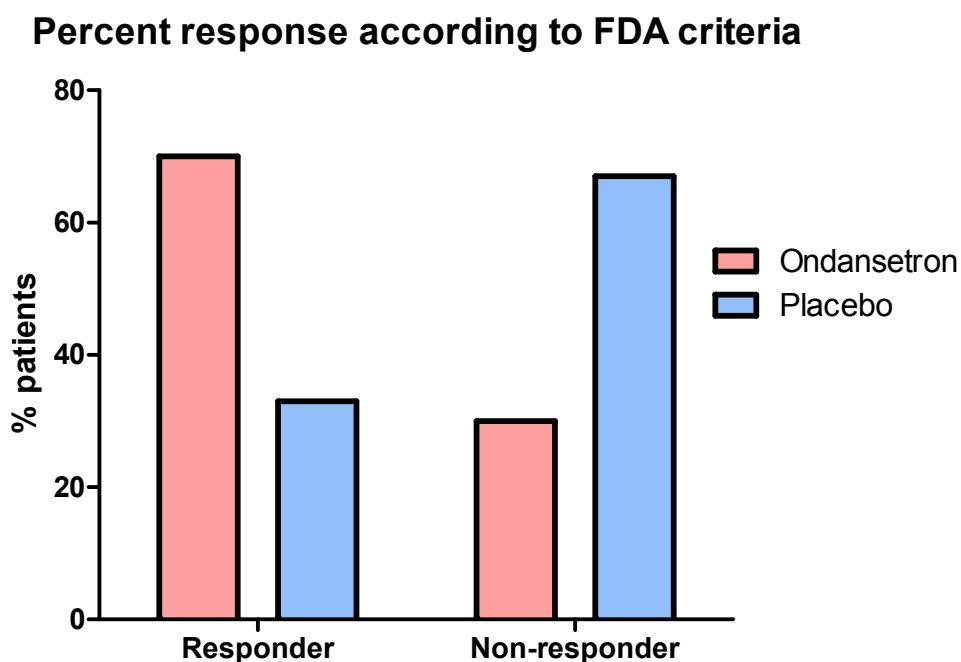
Figure 4-10 FDA response, 33% of patients had a response according to the FDA criteria to placebo with 67% not responding, whilst taking Ondansetron 70% responded and 30% did not giving a NNT of 3 (95% CI 2-4.2)

4.7 Effect of Ondansetron on measures of "unpredictability", stool form variability and EMR

It is clear that Ondansetron has an effect on mean weekly stool form frequency and urgency but in order to investigate the ability of Ondansetron to affect day to day symptoms I introduced the concept of stool form variability, EMR, and number of days with stool form>5, whilst reviewing the results of the baseline diaries. These alternative measures aim to capture

daily events that are responsible for patients distress and symptoms. The effect of Ondansetron on number of days per week with stool form >5 has been discussed in the preceding section.

In the patent group Ondansetron does not reduce stool form variability when compared to placebo, in fact the median change in stool form variability was 0 (IQ -1-1.5)with Ondansetron and 0 with placebo (IQ -1-1)$p=0.39$. Those with high stool form variability were not more likely to respond using the FDA criteria than those with low stool form variability, Fishers exact test $p=0.39$.

There was no reduction in number of days with EMR from baseline whilst taking Ondansetron compared to placebo, median reduction 0.0 for Ondansetron (IQR 0-1) and 0.0 for placebo (IQR 0-1).

4.8   Other considerations

4.8.1   The effect of Ondansetron on stool form in men

There are more females affected by IBS than men so it is natural to assume men will form a smaller proportion of subjects in this study. There were in fact 28 men in the intention to treat analysis, looking at this small subgroup there was still a significant improvement in stool form of 1.3 (IQ 0.6-1.8)points compared to 0 (IQ -0.2-0.3)points with placebo. The mean change in stool form for men when taking ondansetron was not significantly different from that in women, 1.2 (95% CI 0.84-1.50) compared to 1.50 (1.24-1.72)points on the BSF scale, $p=0.16$, suggesting there is not a sex dependent mechanism by which ondansetron has its effect.

4.8.2 Order effect

In a crossover study such as this it is important to consider whether there is an order efect i.e. more chance of experiencing a positive effect depending on the treatment period in which the active drug is given. Using a Freidmen test with Dunn's multiple comparisons there was no significant difference in decrease in baseline stool form between those receiving ondansetron first and those receiving ondansetron second p=<0.05

4.8.3 Dosing

The dose of Ondansetron taken in the final 2 weeks of treatment ranged between 4mg every 3 days- 8mg tds, the mode dose taken was 4mg every other day. The mode dose of placebo taken during the endpoint weeks was 6 tablets a day with a range between1 tablet alternate days and 6 a day.

5   The effects of Ondansetron on measures of whole gut function

5.1   Whole gut transit (WGT) in hours

Whole gut transit was measured in healthy volunteers and in patients at the end of each treatment period, i.e. once after placebo and once after Ondansetron as described in section 3.2.

5.1.1 WGT in healthy volunteers and patients

WGT was non-normally distributed (Shapiro-Wilks test). The median whole gut transit in healthy volunteers was 46 hours (IQR 12-58) whilst WGT in the patients taking placebo had a significantly shorter median transit of 16 hours (IQR 7-29) p= 0.004, Mann Whitney test, fig 5.1.

Whole gut transit time in Healthy Volunteers and Patients
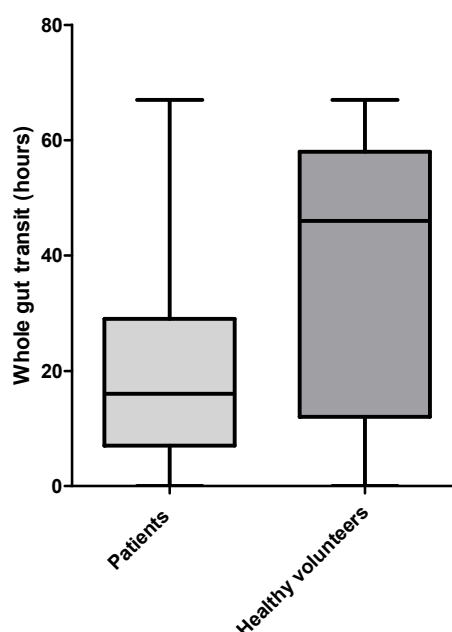
Figure 5-1 WGT transit time in healthy volunteers and patients, median WGT in healthy volunteers was 46 hours (IQR 12-58) significantly different from the 16 hours (IQR 7-29) in patients whilst taking placebo, p= 0.004.

Dividing the transit into 3 sections as detailed in section 3.2 gives values for segmental transit. Segmental transit was non-normally distributed (Shapiro-Wilks test). There was a significant shortening in left colonic transit time in patients which was 2.5 hours (IQR 0-7) vs. 12 hours (IQR 3-24) in healthy volunteers p=<0.05 similarly the rectosigmoid transit time was 4 (IQR 1-9) in patients vs. 7 (IQR 4-15) in healthy controls, p=<0.05. Segmental transit times in patients and healthy volunteers are shown in Table 5.1

	Transit time (hours) HV (n=19)	Transit time (hours)Placebo (n=106)	P value (Kruskall-Wallis)
Right colon	13 (IQR 5-18)	6 (IQR 2-12)	ns
Left colon	12 (IQR 3-24)	2.5 (IQR 0-7)	0.0001
Rectosigmoid	7 (IQR 4-15)	4 (IQR 1-9)	0.0001

Table 5-1 Segmental transit in patients (placebo) and Healthy volunteers.

5.1.2 Whole gut transit, stool form and stool frequency

There was no correlation between weekly average Bristol stool form in patients taking placebo (n=102) with whole gut transit r =-0.115 p=0.26 (Spearman) and stool form did not correlate with transit in healthy volunteers either r =0.25 p= 0.313 (Pearson).

Stool frequency whilst taking placebo did however weakly correlate with WGT in patients r =-0.293 p=0.003 (Spearman) but this was not seen in healthy volunteers r= 0.178 p= 0.47 (Spearman)

5.1.3 Whole gut transit, psychological distress and somatisation

It should be noted that this was a comparison of baseline psychological distress (anxiety + depression component of the HADS score) and whole gut

transit in patients when taking placebo (n=102). This did not demonstrate a significant correlation r=-0.185 p=0.065 (Spearman).

Baseline somatisation in patients as measured by the PHQ-12 score also showed no correlation with whole gut transit r=-0.143 p=0.151 (Spearman).

5.1.4 The effect of Ondansetron on whole gut transit

Ondansetron significantly increased whole gut transit time compared to placebo (n= 98) with a median transit time of 25 hours (IQR 13.5-47.5) compared to 16 (IQR 7-29), p=<0.0001 (Wilcoxon matched pairs), this remains markedly lower (by 21 hours) than the median transit time for healthy volunteers, fig 5.2.

Whole gut transit in patients taking Ondansetron vs. Placebo
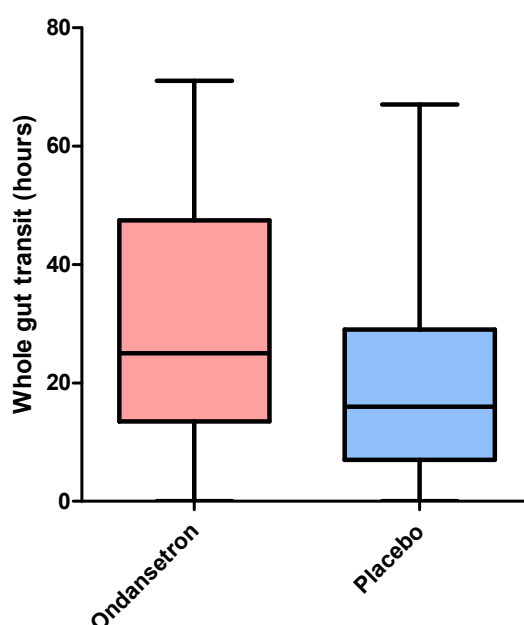
Figure 5-2 Whole gut transit in patients taking Ondansetron vs. Placebo with a median transit time of 25 hours (IQR 13.5-47.5) on Ondansetron compared to 16 (IQR 7-29) on placebo, p=<0.0001 (Wilcoxon Matched pairs).

5.1.5 The effect of Ondansetron on segmental transit

The effect of Ondansetron on colonic transit (n=96) was greatest in the left colon and rectosigmoid where the median transit times were 6 and 7 hours respectively compared to 2.5 and 4 hours whilst taking placebo, as summarised in table 5.2 .

	Transit time (hours)Placebo (n=106)	Transit time (hours)Ondansetron (n=102)	P value
Right colon	6 (IQR 2-12)	7 (3-16)	ns
Left colon	2.5 (IQR 0-7)	6 (0-17.25)	<0.05
Rectosigmoid	4 (IQR 1-9)	7 (2-13)	<0.05

Table 5-2 The effect of Ondansetron on segmental transit.

5.2 MRI Small bowel water

The method for calculating fasting SBWC is described in section 3.1.6.1

5.2.1 Small bowel water content in healthy volunteers

Fasting SBWC was non-normally distributed in patients and healthy volunteers (Shapiro-Wilk test). Fasting SBWC was significantly greater in healthy volunteers (n=20) 53.48 ml (IQR 36.22-125.6) compared to 35.55 ml (IQR 16.75-72.69) in patients (n=51) with IBS-D p=0.0335 (Mann Whitney U), fig 5.3. This is in keeping with previous findings [148] although it should be noted that the difference is smaller than previously described.

SBWC Patients (placebo) vs. Healthy volunteers
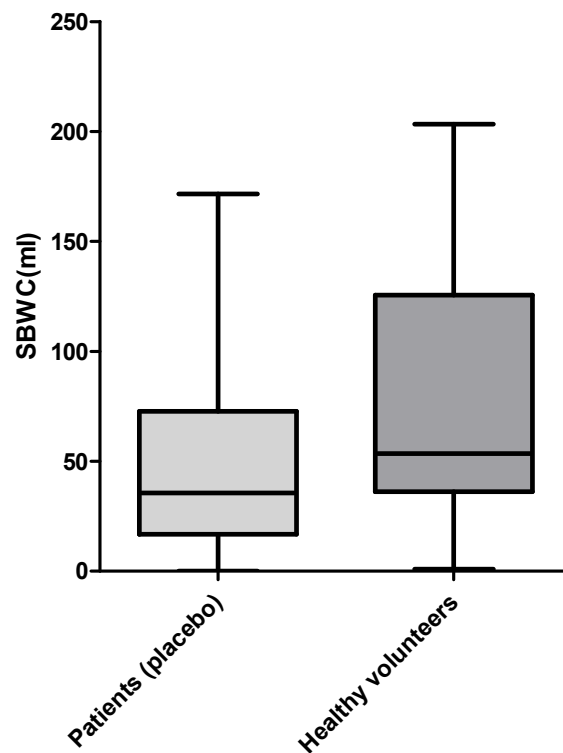
Figure 5-3 Median fasting SBWC is significantly greater in healthy volunteers, 53.48 ml (IQR 36.22-125.6) compared to 35.55 ml (IQR 16.75-72.69) in The effect of Ondansetron on small bowel water content

We hypothesised that Ondansetron would increase the amount of fasting SBWC by lengthening small bowel transit time. There was a trend towards increased fasting SBWC whilst taking Ondansetron 56.52 ml (IQR 24.03-87.17) compared to placebo 35.55 ml (IQR 16.75-72.69), p=0.21 (Wilcoxon Matched Pairs) (n=50). Although this did not reach significance it is interesting that the increase in water content results in values similar to the median SBWC seen in our age and sex matched healthy population.

5.2.2 Small bowel water content and transit

SBWC has previously been shown to correlate well with small bowel transit [148];

But in this study the correlation of SBWC in patients taking placebo (n=56) with WGT r=0.261 did not reach statistical significance p=0.067 (Spearman). Given that small bowel transit time is usually only a small fraction of WGT, it is not surprising that this link is seen to be less significant when making comparisons taking into account the transit time within the large bowel as well.

5.2.3 Small bowel water content and BSF diary data

Combining healthy volunteers and patients taking placebo we were able to show a weak but significant association between SBWC and bloating (n=63) r=-0.308 p= 0.014. There was however no correlation between SBWC and average urgency r=-0.07 p=0.584, average pain r= -0.164 p=0.199, average stool consistency r =0.103 p=0.424, average stool frequency r= -0.27 p= 0.106 (Spearman)

5.2.4 Small bowel water content psychological distress and somatisation

Again Combining healthy volunteers and patients taking placebo we were able to show a weak but significant association between SBWC and somatisation (n=68) r =-0.2 p= 0.049 (Spearman), but not between baseline measures of psychological distress r=-0.2 p= 0.1 (Spearman).

5.3   MRI parameters in the colon

5.3.1   MRI ascending colon water content (ACWC)

ACWC was non-normally distributed (Shapiro-Wilks). There was no increase in median free water in the ascending colon in patients (n=56) which was 0.5ml (IQR 0.0-2.4) compared to healthy volunteers (n=20) 1.0ml (IQR 0.2-3.1), difference not statistically significant with p=0.3 It is however clear that the variability was much greater in the patient group with a maximum value of 31.7 compared to 9.5mls in the healthy volunteers.

6 Markers of Serotonin function

6.1 5-hydroxytryptamine transporter linked polymorphic region(5-HTTLPR) identification in patients with IBS-D

6.1.1 Distribution of 5-HTTLPR polymorphisms

There was no difference in the distribution of 5-HTTLPR polymorphisms between the 122 patients and the 19 healthy volunteers accepting that the number of healthy volunteers is small, Chi squared p=0.64. This is summarised in Table 6.1.

5-HTTLPR polymorphism	Patients (%)	Healthy Controls(%)
Ll	40(33)	8(42)
Ls	53(43)	8(42)
ss	29(24)	3(16)

Table 6.1 Distribution of 5-hydroxytryptamine transporter linked polymorphic region (5-HTTLPR) group in the patient and control groups.

6.1.2 5-HTTLPR polymorphisms and baseline patient and volunteer characteristics

There was no difference in baseline stool form, frequency, pain, urgency, bloating, or quality of life, symptom severity and measures of somatisation, when grouped by 5-HTTLPR polymorphism as shown in Table 6.2.

5-HTTLPR polymorphism	ll	ls	ss	P=
Baseline stool form (1-7)	5.2 (95% CI 4.9-5.4)	5.1 (95% CI 4.8-5.3)	5.0 (95% CI 4.6-5.4)	0.52
Baseline stool frequency	2.6 (IQR 1.3-4.1)	2.4 (IQR 1.7-3.4)	2.4 (IQR 1.7-3.0)	0.77
Baseline pain score (0-3)	1.26 (95% CI 1.01-1.51)	1.18 (95% CI 0.95-1.42)	1.09 (95% CI 0.78-1.40)	0.72
Baseline urgency score (0-3)	1.57 (IQR 1.14-2.14)	1.50 (IQR 0.75-2.11)	1.43 (IQR 1.0-2.14)	0.76
Baseline bloating score (0-3)	0.86 (IQR 0.29-1.71)	1.14 (IQR 0.33-2.0)	1.43(IQR 0.43-2.0)	0.3
Baseline IBSQOL	400.4 (IQR 342.5-526.7)	450.0 (IQR 331.2-593.1)	485.8 (IQR 301.9-588.5)	0.7
Baseline IBSSSS	311.9 (95%CI 281.8-341.9)	307.2 (95%CI 284.2-330.3)	299.8 (95%CI 270.3-329.2)	0.85
Baseline PHQ-12	6.8 (95%CI 5.6-8.0)	6.6 (95%CI 5.6-7.5)	7.9 (95%CI 6.5-9.3)	0.27

Table 6.2 Baseline values for symptoms and psychological scores by of 5-hydroxytryptamine transporter linked polymorphic region group.

There was a trend to an increase in baseline anxiety and depression scores in patients and volunteers with the ss polymorphism, though this did not reach significance, with a p value of 0.053 for anxiety scores and 0.24 for depression. These data are shown in Figure 6.1 and Figure 6.2.

Baseline Anxiety Score by 5HTTPLR Polymorphism
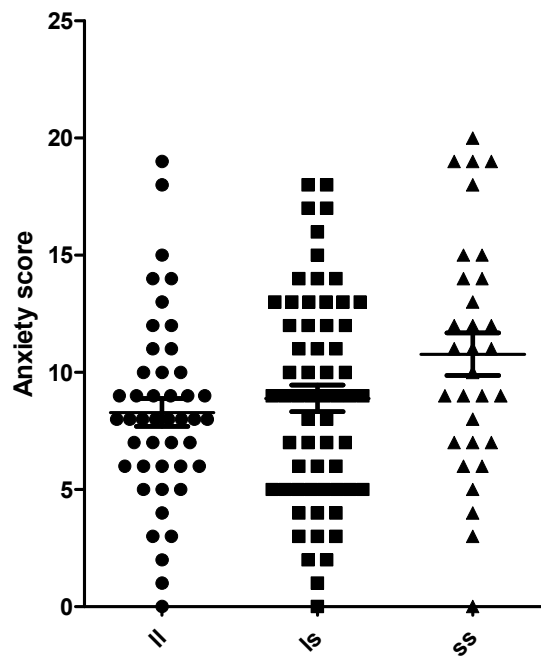
Figure 6-1 Baseline anxiety scores in each of the three 5-hydroxytryptamine transporter linked polymorphic region (5-HTTLPR) polymorphism groups. The mean anxiety score in the ll group was 8.3(95% CI 7.1-9.5), in the ls group 8.9 (95% CI 7.7-10.0) and in the ss group 10.8 (95% CI8.9-12.6) p=0.053.

Baseline Depression Score by 5HTTPLR Polymorphism
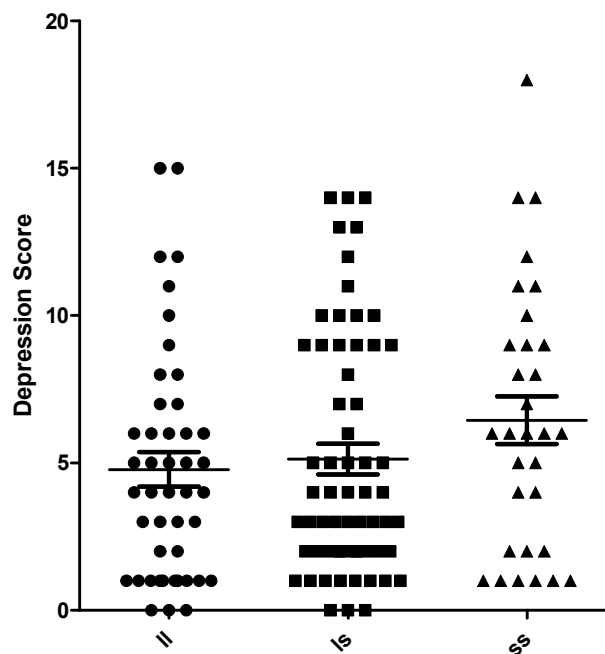
Figure 6-2 Baseline depression scores in each of the three 5-hydroxytryptamine transporter linked polymorphic region (5-HTTLPR) groups. The median depression score in the ll group was 4.0 (IQ 1-6.5), in the ls group 4.0 (IQ 2-9) and in the ss group 6.0 (IQ 2-9) p=0.24.

6.1.3 5-HTTLPR polymorphisms and response to Ondansetron

Using the draft FDA criteria for a stool form responder, (a reduction of 50% or more in the number of type 6 and 7 stools in a week), and dividing the patients into two groups, those who responded to Ondansetron and not to placebo or "true responders" and all others, non responders, there is a trend towards fewer responders in the groups with those with the most available serotonin. The ll (n=36) group had the greatest number of responders with 56% responding compared to 44% in the ls group (n=39) and 34% in the ss group (n=26), although this does not reach significance p=0.098 (chi-squared test for trend).

5-HTTLPR polymorphism	ll	ls	ss	P=
Delta stool form (1-7)	1.18 95% (CI 1.13-1.73)	1.43 95%(CI 1.12-2.02)	1.57 95%(CI 1.12-2.02)	0.3
Delta stool frequency	0.79 IQ (0.21-1.50)	0.97 IQ (0.24-2.22)	0.69 IQ (0.34-1.13)	0.59
Delta pain score (0-3)	0.30 95%CI (0.07-0.52)	0.32 95%CI (0.13-0.50)	0.10 95%CI (-0.20-0.40)	0.36
Delta urgency score (0-3)	0.67 95% CI(0.39-0.94)	0.58 95% CI(0.32-0.84)	0.63 95% CI(0.37-0.90)	0.88
Delta bloating score (0-3)	0.15 95% CI(-0.02-0.32)	0.12 95% CI (-0.11-0.32)	0.30 95% CI (-0.05-0.70)	0.5
Delta IBSSSS	62.53 95%CI (28.87-96.19)	93.77 95%CI (64.13-123.4)	92.28 95%CI (45.18-139.4)	0.35

Table 6.3 Change in mean weekly stool form, stool frequency, pain urgency and bloating scores from baseline whilst taking Ondansetron by 5-hydroxytryptamine transporter linked polymorphic region (5-HTTLPR) group.

5-HTTLPR polymorphisms do not exert a significant effect over mean

improvement in stool form, frequency, pain, urgency, bloating or IBSSSS

between groups as shown in table 6.3 above.

6.1.4 5-HTTLPR polymorphisms and WGT

Others have shown least improvement in gut transit times with Alosetron in the ls

group compared to the ll group although in this study the number of participants

with the ss polymorphism was too small to study [75]. We found no significant

difference in transit whilst taking placebo or Ondansetron between the groups. These

results are displayed in Table 6.4.

5-HTTLPR	ll	ls	ss	P=
WGT (hrs) with placebo	14 IQ (7-31)	14 IQ (6-24)	18 IQ (9-31)	0.25
WGT (hrs) with Ondansetron	23 IQ (12-35)	24 IQ (13-55)	28 IQ (22-48)	0.38

Table 6.4 Whole gut transit (WGT) taking placebo and Ondansetron by polymorphism.

6.1.5 5-HTTLPR polymorphisms and other markers of
serotonin function

5-HTTLPR polymorphisms did not significantly alter platelet paroxetine

binding kinetics, and the Bmax, and Kd were not significantly different

between groups. Baseline plasma 5HIAA levels were also not significantly

different between the ll, ls and ss groups. These results can be seen in Table

6.5.

5-HTTLPR polymorphism	ll	ls	ss	P=
Bmax (fMol/mg)	478.1 (IQR 272.0-738.7)	480.3 (IQR 237.2-783.3)	408.2 (IQR 312.6-564.9)	0.82
Kd (nMol/L)	0.9169 (IQR 0.3274-1.602)	0.6049 (IQR 0.3658-0.9705)	0.5559 (IQR 0.2515-1.001)	0.18
Plasma 5HIAA(nMol/L)	16.0 (IQR 12.4-20.0)	16.3 (IQR 13.0-20.0)	18.4 IQR 16.4-21.0)	0.3

Table 6.5 Effect of 5-hydroxytryptamine transporter linked polymorphic region (5-HTTLPR) polymorphisms on platelet Paroxetine binding kinetics and plasma 5-HIAA.

6.2 Paroxetine binding in patients and healthy controls

There was no difference in the number of SERT receptors on the platelet membranes of patients (n=70) compared to healthy volunteers (n=20) (Bmax) in addition the strength of binding to these receptors (kd) was also not different between the 2 groups.

	Patients	Healthy Contols	P=
Bmax (fMol/mg)	475.1 IQR (270.3-728.7)	458.3 IQR (213.0-683.2)	0.5
Kd (nMol/L)	0.61 IQR(0.28-1.24)	0.77 IQR(0.51-1.54)	0.24

Table 6.6 Paroxetine binding kinetics of patients and healthy controls.

6.2.1 Paroxetine binding and baseline patient and control characteristics

There was no correlation between Paroxetine binding bmax or Kd and plasma 5HIAA.There was also no correlation between bmax and Kd with any of the

other baseline measures contained in the stool diaries, HADS, PHQ-15 or IBSSS

scores.

6.3 Plasma 5-HIAA

6.3.1 Plasma 5HIAA in patients and healthy controls

Fasting plasma 5-HIAA was non-normally distributed in patients (n=71) and

normally distributed in healthy controls (n=20) (Shapiro Wilks). Median fasting

plasma 5HIAA was significantly higher in controls than patients, 20.75 (IQR

19.64-22.28) vs. 16.23 (IQR 12.22-19.21) nMol/L, p=>0.001 Mann Whitney U

test as seen in Figure 6.3.

Fasting 5HIAA in patients and controls
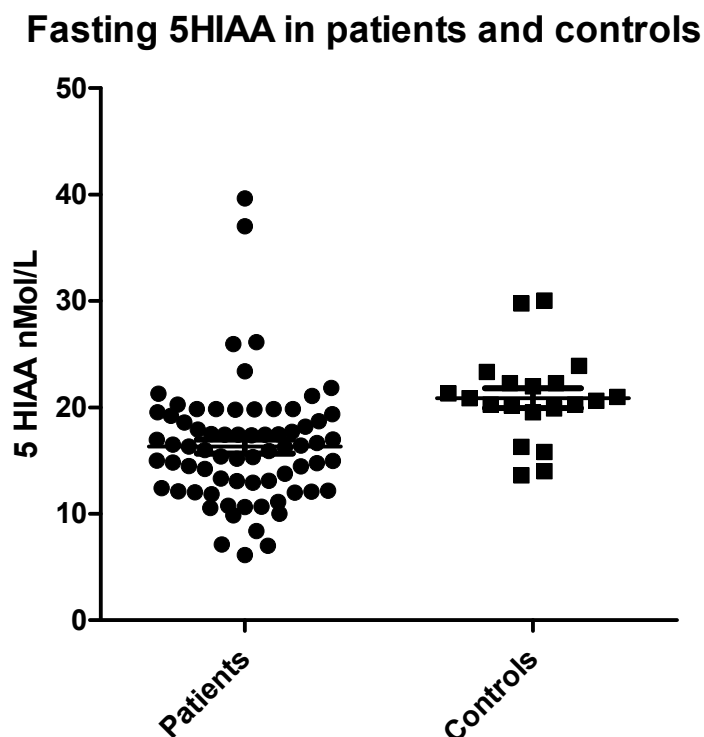
Figure 6-3. Fasting plasma 5-HIAA in patients is greater, 20.75 (IQR 19.64-22.28) than in controls 16.23 (IQR 12.22-19.21) nMol/L, p=>0.001.

6.3.2 Plasma 5-HIAA and baseline patient and control characteristics

71 patients and 20 age and sex matched healthy controls had their fasting plasma 5HIAA measured. There no correlation with fasting plasma 5HIAA and pain , urgency , bloating, stool form, and stool frequency There was no significant correlation between anxiety depression, PHQ-12, IBSQOL , IBSSS in patients.

	r= Spearman	P=
Average Pain	-0.057	0.642
Average Urgency	0.066	0.593
Average Bloating	0.003	0.978
Stool form	-0.085	0.486
Stool frequency	-0.130	0.284
Anxiety	-0.068	0.582
Depression	0.125	0.311
PHQ-12	0.074	0.540
IBSQOL	-0.003	0.978
IBSSSS	-0.104	0.390

Table 6.7 Correlation of baseline patient characteristics with plasma 5-HIAA.

Similarly there was no correlation with plasma 5-HIAA and any of the baseline

values in healthy volunteers.

6.4 Predicting the responder

	True responder	Non responder	P=
Age	38 (IQR 26-47)	45.0 (IQR 38-51)	0.0078*
Average pain (0-3)	1.19 (95% CI 0.97-1.42)	1.56 (95% CI 1.35-1.77)	0.0186*
Days with pain	5 (IQR 3-7)	6 (IQR 5-7)	0.0246*
Average urgency (0-3)	1.526 (95% CI 1.32-1.73)	1.774 (95% CI 1.59-1.95)	0.0675
Days with urgency	6 (IQR 5-7)	7 (IQR 5-7)	0.1162
Average Bloating (0-3)	1.0 (IQR 0.43-1.71)	1.43 (IQR 0.68-2.0)	0.1295
Days with bloating	5 (IQR 3-7)	6 (IQR 3-7)	0.1481
Stool form (0-7)	5.55 (IQR 4.85 -5.85)	5.46 (IQR 5.0-5.95)	0.5023
Stool frequency	2.64 (IQR 1.86-3.86)	2.62 (IQR 1.90-3.93)	0.9236
Anxiety	8.35 (95% CI 7.2-9.5)	10.38 (95% CI 9.2-11.6)	0.0158*
Depression	5 (IQR 3.0-7.25)	6 (3.0-9.0)	0.376
PHQ-12	6.7 (95% CI 5.8-7.6)	8.1 (95% CI 7.1-9.2)	0.0488*
IBSQOL	149.1 (95% CI 427.5-514.0)	170.5 (95% CI 399.1-490.4)	0.4134
IBSSSS	290.3 (95%CI 265.4-315.1)	321.3 (95% CI 298.5-344.1)	0.675

Table 6.8 Difference in baseline characteristics between true responders (those who responded only to Ondansetron and not to placebo , according to the FDA responder criteria) and non responders to Ondansetron(all others).

Using the FDA stool form responder criteria, a reduction in 50% or more in

number days a week with stool form >5, patients were dived into 2 groups.

Those who responded only to Ondansetron and not to placebo were termed true responders, and all others, who for the purpose of this analysis were termed non-responders. The difference in baseline characteristics between the two groups can be seen in table 6.8 above.

All the baseline parameters derived from questionnaires were slightly worse in those who did not respond to Ondansetron, with non responders being significantly older, having significantly more days with pain, greater average pain, anxiety, and somatisation as measured by the PHQ-12. However since these parameters were closely linked in a multivariate analysis (backwards logistic regression) only days with pain remained a significant independent predictor of response.

Markers of serotonin function were only analysed in the Nottingham group (n=71), there was no significant differences seen between those who responded and those who did not this can be seen in Table 6.9.

	Responders	Non responders	P=
Bmax (fMol/mg)	487.1(IQR 265.0-795.0)	429.3 (IQR 299.3-645.6)	0.7136
Kd (nMol/L)	0.8452 (IQR 0.33-1.25)	0.5983 (IQR 0.22-1.11	0.3
5HIAA(nMol/L)	16.97(IQR 13.09-19.82)	15.4 (IQR 12.02-17.50)	0.2821

Table 6.9 Difference in baseline measures of serotonin (taken in patients recruited in Nottingham only) between true responders and non-responders to Ondansetron.

7   Characterisation of faecal serine proteases

The results of a series of pilot experiments are presented first, followed by the analysis of faecal serine proteases (FSP) activity in the subjects included in the trial: Ondansetron in patients with diarrhoea predominant Irritable bowel syndrome "identifying the responder", the methods are detailed in Chapter 3. As noted in the methods section, assay's in this chapter were performed by Dr David Tooth and Dr Gulzar Singh, with the exception of the mast cell tryptase estimation which was done by the Queen's Medical Centre immunology department.

7.1   Study 1: FSP activity in patients and healthy volunteers

FSP activity was measured in baseline samples from 36 IBS-D and 9 healthy controls. FSP showed wide variability and was non-normally distributed in IBS-D (Shapiro Wilk, r=0.8, p 0.0003) but not in healthy controls. Values were significantly elevated in IBS-D with a median (IQR) of 451 units of trypsin/mg protein (61-963) versus 147(82-336), p=0.038, Mann-Whitney U test.

7.2   Study 2: In vitro inhibition of FSP activity in patient samples

The serine protease inhibitor, Aprotinin, was added to Trypsin standards and faecal extracts at a range of concentrations and showed a concentration dependent inhibition. At the higher dose (100 $\mu g.ml^{-1}$) all FSP activity from 7 IBS-D patients was inhibited.

7.3 Study 3: Proteomic techniques

A test mixture of bovine trypsinogen and equine myoglobin demonstrated Benzamidine-sepharose column selectivity (Figure 7.1a) giving a peak not seen in control samples (Figure 7.1b). Buffered faecal extracts, when chromatographed similarly yielded chromatograms showing non-retained components in flow-through followed by a stable baseline and retained components were then selectively eluted (Figure 7.1c and 7.1d).
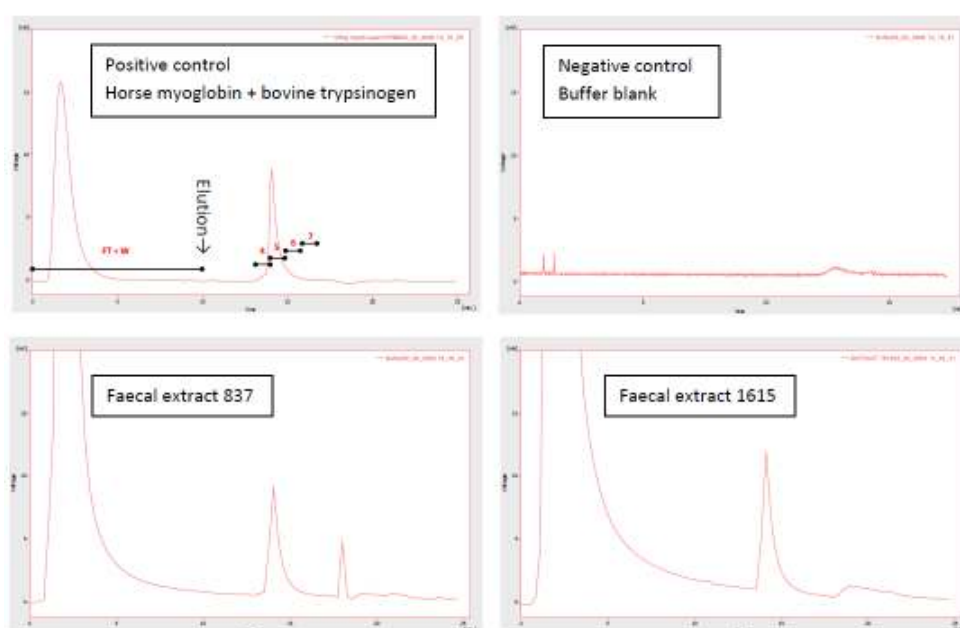
Figure 7-1 Chromatograms of Benzamidine affinity chromatographed faecal extracts.The top left chromatogram (7.1a) using bovine trypsinogen and horse myoglobin mixture shows a clear peak corresponding to trypsinogen which was not seen in the control sample (7.1b). 7.1c and 7.1d displays 2 IBS-D faecal extracts showing the eluting components peak in the same fraction as trypsinogen.

Micropreparative SDS-PAGE showed the flow-through and eluate fractions to be heterogeneous mixtures of components with a broad molecular size range (Figure 7.2). Several components were selectively retained and their relative

abundance was elevated in a sample pooled from 6 patients compared to a pooled sample from 6 healthy controls. Eleven gel bands were excised and proteins identified (Table 7.1) using Proteomic procedures. The major component bands were human pancreatic enzymes including trypsin, alpha-1 anti-trypsin, carboxypeptidase B1/A, peptidase S1, PRSS1/2 (tryptases), alpha-amylase, pancreatic elastase 3A as well as IgG light chain (kappa) together with various bacterial products including transmembrane protease M50, GADH and various GADPH bacterial membrane receptors.
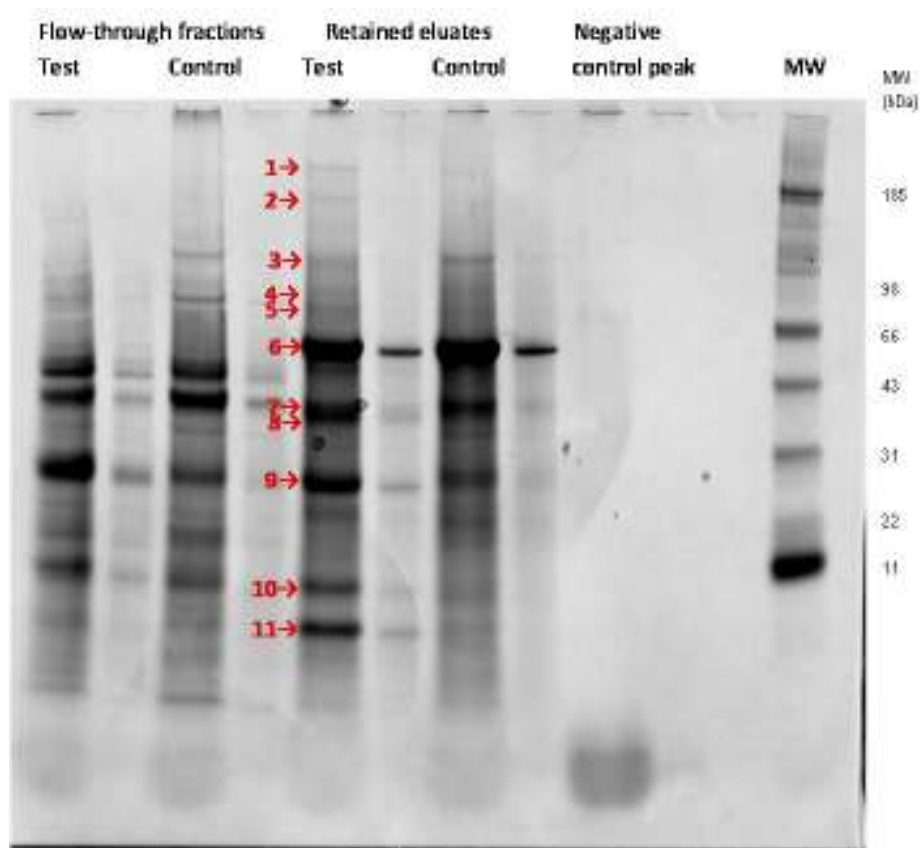
Figure 7-2 Showing SDS-PAGE profiles of non-retained and retained components from faecal extracts of (pools of) IBS-D (Test) and Healthy controls (Control) each at two gel loadings. MW shows protein calibrants at a range of molecular weights. Components 1 to 11 were excised and putatively identified (Table 7.1).

Experimental Ref(Band No.)	Components identified	Rationale
1	Porcine Trypsin	This is the protease used to 'digest' target proteins
2	Pancreatic amylase	Not unexpected in gut. Inexplicable interaction with affinity ligand.
	Human keratin	
	Porcine Trypsin	Common human derived contaminant and not unexpected in human samples
3	Human N-peptidase	Specifically retained low abundance protease
	Pancreatic amylase	
	Porcine Trypsin	
4	Pancreatic amylase	
	Porcine Trypsin	
5	Maltase glucoamylase	Probable amylase homologue. Unknown whether uniquely identified or data artefact
	Porcine Trypsin	
6	Human alpha-amylase	Amylase isoform
	other spp glycosyl hydrolases	Unknown if uniquely identified or data artefact
7	Human alpha-1 anti-trypsin	Kunitz domain Serine-Protease inhibitor, possible interaction partner with a target enzyme
	alpha-amylase	
	bacterial GADH	Probably an abundant microbial enzyme
8	Various spp GADPH	Probably an abundant microbial enzyme
	bacterial membrane receptors	Probably an abundant microbial surface protein
9	Human carboxypeptidase B1/A,	Specifically enriched protease
	Human Trypsin	Not unexpected in gut
	Human peptidase S1 (AKA tryptases; there are various spliced 25-31kDa homologues [90-98% homology], including mast-cell tryptase)	Possible Trypsin homologues
	IgG light chain (kappa)	
	Human pancreatic elastase 3A	
	Human alpha amylase	
	Bacterial transmembrane protease M50	
10	Human Trypsin (various homologues), Human mesotrypsin	
	bacterial GADPH	
11	Human Trypsin	
	Human PRSS1/2 (tryptases)	
	bacterial transmembrane protease M50	

Table 7-1 Putative identity of bands on SDS-PAGE chromatograms in Figure 7.2.

7.4 Study 4: Faecal elastase, amylase and mast cell tryptase quantification

Since our proteomic analysis suggested a major serine protease component was trypsin we also wanted to look at levels of 2 other commonly produced pancreatic enzymes within the stool, pancreatic amylase and elastase. While trypsin and amylase are degraded by faecal bacteria and so normally excreted in small amounts, elastase is more stable and readily detectable in stool. As such it is used widely to detect pancreatic insufficiency in clinical practice. Faecal elastase values were normally distributed but not significantly different in the IBS-D patients being 2.4 ± 0.2 (mean ± SEM) versus 2.47 ±0.36 units/mg of stool in healthy controls, p=0.85, N=36. Pancreatic amylase was also identified and quantitated (Figure 7.3). This was non-normally distributed, and somewhat higher in IBS with a median (IQR) of 122.4(0.48-325.2) versus 17.0 (10.3-102.8) units/mg for healthy controls but this did not achieve statistical significance owing to wide variability, p=0.07. However faecal amylase did correlated with FSP activity, r=0.04, p=0.006, n=45.
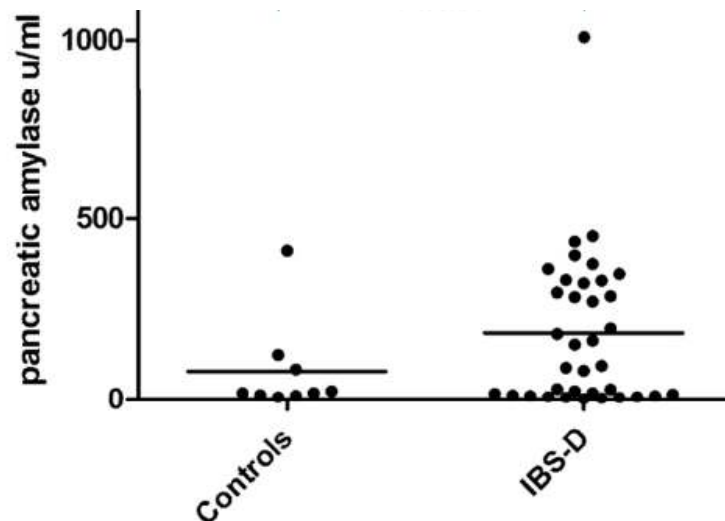
Figure 7-3 Faecal levels of amylase in IBS-D versus controls.

Using a specific well validated Phadia Immunocap assay for mast cell tryptase we found undetectable levels in stool even though spiking the sample with pure human tryptase (Sigma-Aldrich, Warrington,UK) did show that we could detect mast-cell tryptase (MCT) when present in stool extract using this method.

7.5 Baseline faecal serine protease activity in patients and healthy controls

FSP activity was measured in 21 healthy controls and 79 patients with IBS-D as part of the study Ondansetron in patients with diarrhoea predominant Irritable bowel syndrome "identifying the responder". As with the baseline values of the first 36 patients there was wide variability in the larger group from the placebo arm and although IBS values, median (IQR) 501(245-1421) trypsin units/mg protein were numerically greater than the matched healthy controls 302(147-4) trypsin units/mg protein the difference was not

significant. If we combine all the healthy controls from part 1 and part 2 the control value is 302(147-475) trypsin units/mg protein n=51 and the difference from IBS-D is significant, p=0.003 but as Figure 7.4 shows there is a wide variation in both groups.
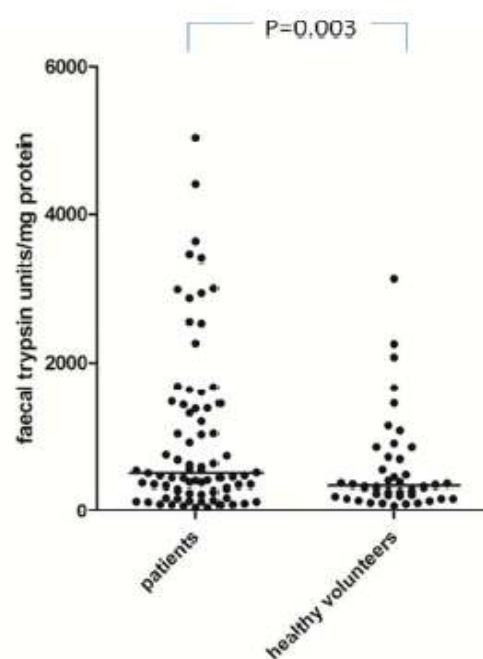
Figure 7-4 Faecal serine protease activity expressed in trypsin units/mg of protein showing IBS-D with significantly higher values than controls but with wide variability in both groups. Mann Whitney U, p=0.003.

7.5.1 Baseline faecal serine protease activity, anxiety, depression, and symptom severity.

Anxiety scores were normally distributed, whereas depression scores were non-normally distributed (Shapiro Wilks).There was a positive correlation between both anxiety and depression with FSP activity, Pearson r=0.26, p=0.018 and Spearman r =0.31, p= 0.0026 respectively. There was a negative correlation with FSP activity and

quality of life score, Pearson r=-0.25, p=0.029, but not PHQ12-SS, or IBSSS. The results are summarised in Table 7.2

	r=	P=
Anxiety	0.26	0.018*
Depression	0.31	0.0026*
IBSQOL (patients only)	-0.25	0.029*
PHQ-12	0.14	0.15
IBSSSS (patients only)	0.19	0.11

Table 7-2 Summary of correlation of FSP activity with baseline patient and volunteer baseline psychological and symptom severity scores.

These psychological variables are closely correlated and when entered into a backwards logistic regression model using the 72 cases with data for all variables only depression remained an independent predictor of FSP activity in the model, p=0.012.

7.5.2 Total FSP activity and stool form, frequency, pain, urgency and bloating

Mean weekly pain, urgency and bloating scores were all non-normally distributed (Shapiro Wilk) .There was a positive correlation between FSP activity in patients and controls and mean weekly pain scores Spearman r =0.16 p= 0.019, mean weekly urgency scores Spearman r=0.16, p=0.018, and mean weekly bloating scores Spearman r= 0.16, p=0.019. There was no correlation between serine protease activity and stool form or frequency. These results are summarised in Table 7.3.

	r=	P=
Stool form	-0.02	0.71
Stool frequency	0.04	0.53
Pain	0.16	0.019*
Urgency	0.16	0.018*
Bloating	0.16	0.018*

Table 7-3 Summary of correlation of FSP activity with mean weekly symptom scores.

7.5.3 Alteration in FSP activity with Ondansetron treatment compared to placebo

Change in FSP activity between baseline and Ondansetron and baseline and placebo was non-normally distributed (Shapiro Wilk). There was no significant reduction in FSP activity from baseline with Ondansetron compared to placebo with a median (IQR) fall of 20.61 (-366.8-409.9) vs. 26.74 (-349.6-414.7) units of trypsin/mg protein p= 0.9, Mann Whitney U test.

7.5.4 Faecal serine protease activity and WGT

WGT transit (hours) in patients taking placebo and controls was non-normally distributed (Shapiro-Wilk). WGT in this group was positively correlated with FSP activity Spearman r=0.31, p= 0.0047. The addition of data from the Ondansetron arm of the study (non-normally distributed data Shapiro Wilk) abolishes this effect Spearman r =-1.67, p=0.084.

In order to understand this variation we correlated transit and FSP activities measured on placebo in the IBS-D patients which showed a significant negative correlation, Figure 7.5 Spearman -0.32 95%CI (-0.51 - 0.09), p=0.005, n=79, as did

the healthy volunteers in whom we had transit measurements, Spearman r=-0.55 95%CI (-0.81—0.11), p=0.014, n=20. FSP activity also correlated significantly with the average urgency in IBS patients, Spearman r=0.2695%CI (0.03-0.47), p=0.02, n=73. Ondansetron slowed transit increasing transit time while decreasing FSP with a negative correlation between the rise in transit and the increase in FSP, r=-0.40, p=0.003, in the 55 patients with complete values for both FSP and transit at both time points.
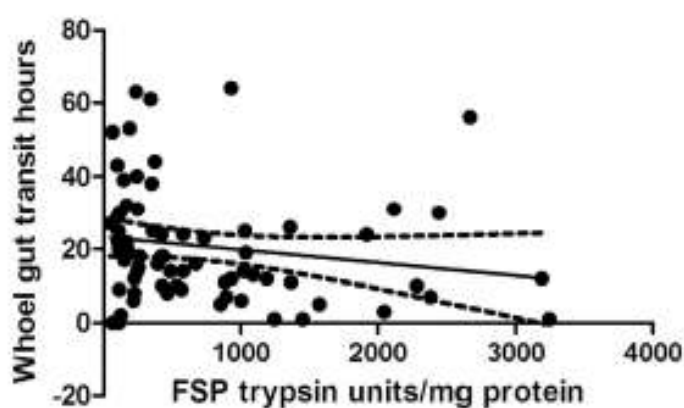
Figure 7-5 Inverse correlation between FSP activity and whole gut transit in IBS-D, Spearman -0.32, p=0.05 n=79.

As will be discussed in chapter 8, our interpretation was that increased bacterial degradation of endogenous trypsin, facilitated by the slowing of WGT, leads to a reduction in downstream stool FSP activity.

8 Discussion

Patients with IBS-D suffer markedly, not only from loose and frequent stools but particularly from the associated urgency and fear of incontinence which generates panic and anxiety. Relief from these symptoms therefore represents an important unmet need. The 5-HT$_3$ receptor antagonist Alosetron has been shown to increase stool consistency, decrease urgency and reduce abdominal pain leading to a global increase in satisfaction with treatment [1]. Its use is restricted following an increased incidence of ischaemic colitis and this agent is not available in Europe. The aims of this thesis were to assess the efficacy of the commonly prescribed 5-HT$_3$ antagonist Ondansetron, which has a good safety profile, in patients with IBS-D and to identify biomarkers that might allow us to predict response defining an Ondansetron responsive endophenotype of IBS:

The following hypotheses were tested:

5. The 5-HT$_3$ receptor antagonist Ondansetron is an effective and well tolerated treatment in patients with IBS-D with a low number of side effects.

6. Ondansetron acts to increase small bowel water and slow colonic transit.

7. Response to Ondansetron will be more effective in those with abnormally increased mucosal serotonin availability at baseline.

8. Faecal serine protease activity will be reduced by treatment with Ondansetron as a result of slower colonic transit.

This study has shown Ondansetron to be a well tolerated and effective treatment in patients with IBS-D, and that it significantly slows whole gut transit time, but does not increase small bowel water content. Ondansetron has a number needed to treat of only 3 using the FDA stool responder criteria and represents a potentially significant breakthrough in the treatment of this patient group. Defining an Ondansetron responsive phenotype in this study has been challenging, in part because of a small sample size and also as a result of the use of a dose titration model (see below). The discussion of the results obtained for baseline measures of serotonin turnover and the studies to identify the origin of faecal serine proteases and the effect of Ondansetron on faecal serine protease activity will follow the main discussion of the RCT of Ondansetron vs. placebo.

8.1 Baseline data

8.1.1 Stool form variability

As part of this RCT we have learnt much from the information contained in the baseline stool diaries. As expected our patients were anxious and depressed, they had looser stool and more urgency than healthy volunteers but no difference in stool form variability (difference between minimum and maximum stool form within one day). Stool form variability was proposed as measure of the unpredictability of symptoms in IBS-D, a known major cause of

distress in patients. This measure in our patient population appears unhelpful and does not mirror the intra-individual variability or unpredictability in symptoms described, with no difference between healthy volunteers and patients and no correlation with symptoms or symptom severity. This is contrary to others who have shown variability to be greater in patients than controls in a smaller population (54 patients) but this may reflect the subtypes included. IBS-D may be more consistent than IBS-mixed type who probably form around half of most IBS series. Stool form variability has been noted to be a major predictor of discordancy between reported subtype and actual subtype [169] supporting the explanation that there is a tendency for patients to "catastrophise" and report the occasional severe symptom as being common when in reality it is rare enough in this case not to alter average data.

Stool form variability did not change with Ondansetron. Given that baseline was not different from the healthy population this is a reassuring finding, large swings from loose stool to constipated as often described by patients when for example taking Loperamide would be a potentially unacceptable symptom.

8.1.2 Baseline data: EMR.

A commonly recognised clinical phenotype and a major source of unpredictable symptoms and high stool frequency is that of the "early morning rush". Here the patient complains of multiple and frequent bowel motions (BM) soon after waking. In this study I have defined a morning rush

as 2 or more BM in less than 1 hour occurring after midnight and before12 midday. Greatly more patients than volunteers experience this EMR and a severe morning rush defined as more than 5 days a week with a morning rush represented a group with a greater mean stool frequency, higher IBSSS coupled with increased psychological distress and a more impaired quality of life. One might hypothesise that this morning frequency leads to difficulties with leaving the home and may impact on employment, a major source of stress making this an area worthy of further study. It is interesting to note that the group with SMR contained 4 of the 12 dropouts in this study, suggesting they are a more difficult group to study, either because of increased psychological abnormalities or perhaps difficulty attending study visits, many of which took place in the morning.

Ondansetron did not reduce the number of days with EMR, despite an overall reduction in loose stool and urgency and whole gut transit. It is possible that this has been influenced by the high number of dropouts in the SMR group, but also may be a reflection of increased psychological abnormalities in this subgroup of patients that is ongoing despite improvements in some aspects of their stool habit. In addition many patients have altered their behaviour over time with a need to open their bowels several times before leaving home to reduce fear of accidents. It is possible that this particular driver to bowel opening may improve over time as confidence in the effect of Ondansetron grows and that the 5 week study period was therefore not long enough to observe a change in EMR.

8.2 Primary end point

There was a significant change in stool form whilst taking Ondansetron compared to placebo. The BSF scale is not ordinal, but as seen in this study, an average improvement of more than 1 point on this 7 point scale, represents a clinically relevant change in stool form. A reduction in 1 point leads to significantly less loose stool. It is these loose stools which are associated with more urgency contributing to incontinence, a major fear and source of distress in our patient group.

The clinical relevance of this is also captured as part of the measure of satisfactory relief. This was 68% of patients whilst taking Ondansetron compared to 17% on placebo, telling us the improvements in symptoms were meaningful to the majority of patients taking part. As we did not find significant changes in pain and bloating it is assumed that the major contributors to this measure would be the improvement in stool form frequency and urgency.

Satisfactory relief is a subjective measure of treatment efficacy that captures the range of symptoms experienced. Debate as to the magnitude of change captured by this measure exists. Data from a study examining patients receiving standard care found that patients who at baseline had the mildest severity reported the highest proportion of satisfactory relief when this measure was repeated 6 months later. These findings suggest that satisfactory relief is confounded by IBS symptom severity [170] but this effect was abolished in a further study where those who reported adequate relief at

the start of the study were excluded [171]. Although vital to understand the true impact a drug is having on symptoms in a condition plagued with a high placebo response rate this measure of satisfactory relief does still capture what our patients want: to feel relieved of their symptoms. Therefore in a condition where there remains an absence of clinical markers this measure remains a useful outcome.

The mean weekly BSF whilst taking Ondansetron was 3.94, not dissimilar to the mean weekly stool form in healthy volunteers of 3.5, caution should be exercised however as this is by no means the whole story. It is important to be mindful of the multitude of symptoms reported by this patient group, including additional disturbances of defecation such as straining, urgency and a sensation of incomplete evacuation even when the stool form is normal [172], reminding us that factors other than altered stool form contribute to the generation of symptoms in IBS.

Comparison of the magnitude of this effect with other treatments acting on stool form is ideally done in a head to head study. Existing studies tell us that the other 5-HT$_3$ antagonists have a significant effect on stool form and Alosetron for example improves form by 0.6 on a 5 point scale, but importantly this reduction is coupled with a high incidence of constipation of 25% [156]. Loperamide is a commonly prescribed drug in IBS-D and is both cheap and readily available. It acts on peripheral opiate receptors to slow transit through the small and large bowel. This results in an increased capacitance of the gut and a delay in the passage of fluid through the intestine [173]. Direct comparison of the order of reduction in form is however difficult,

few trials have been done in IBS and there is methodological variation, with for example the use of visual analogue scales, and reporting of stool as watery, intermediate or shaped[174]. In a very small placebo controlled study stools were reported as formed in 72% of 11 patients taking Loperamide and 7% of 10 patients taking placebo[175]. The use of rescue Loperamide in this study was unfortunately not uniformly recorded but would have been an insight into understanding the relative effectiveness of these 2 preparations.

8.3   The effects of Ondansetron on symptoms

8.3.1   Urgency

We have seen a significant reduction in urgency whilst taking Ondansetron. This finding is important since urgency is one of the strongest predictors of reduced quality of life[176] and as others have reported, response to 5-HT_3RAs also correlates with improvement in quality of life [177]. Quality of life at baseline was measured as part of this study and follow up measurement after treatment with Ondansetron would be an important area for further study.

8.3.2   Pain

We have not been able to demonstrate a reduction in either average pain scores or number of days with pain with Ondansetron in this study. This is surprising when one considers that 5-HT_3 -receptors are present in peripheral and central nervous systems[178], and in animal models of visceral pain, the

5-HT$_3$ - receptor antagonists Granisetron and Ondansetron reduce vasoactive reflexes induced by gastrointestinal tract distension[179]. Early in vivo studies using Ondansetron at a dose of 16mg TDS gave conflicting results when it came to improvements in pain. One demonstrated a reduction in daily episodes of pain as well as changes in rectal sensitivity and compliance[180] whilst in contrast, another study of just 11 patients, Ondansetron showed no improvement in abdominal pain [80]. Alosetron and Cilansetron both relieve abdominal pain in patients with a pooled relative risk of 1.30: 95% CI, 1.22-1.39[1]. It is possible that these higher doses of Ondansetron are needed to affect pain sensation (the mode dose in patients was just 4mg on alternate days) but 68 % of our patients reported adequate relief from their symptoms with Ondansetron, compared to 17% with placebo, coupled with a significant reduction on overall symptom severity as measured by the IBSSS , so perhaps pain is less important than the urgency in this particular patient group.

New partial agonists of the 5-HT$_3$ receptor have been proposed to overcome the high incidence of constipation seen with Alosetron, a reduction in constipation with these agents may lead to higher dosing and perhaps a greater effect on pain[181]?

8.3.3 Bloating

Bloating is a notoriously difficult symptom in IBS and the mechanism of its generation disputed. Slowing of transit via 5-HT$_3$ antagonism might potentially

increase bloating and Tegasarod a 5-HT$_4$ receptor partial agonist and promotility agent does reduce bloating in patients with constipation[182] Findings that 5-HT$_3$ antagonism in the upper GI tract reduces the sensation of post prandial fullness, by perhaps acting on visceral afferent perception[183] and blunting of antral distension evoked increases in colonic tone[184]suggest a potential for an impact on bloating using Ondansetron. However no alteration in bloating was seen in our study or in other studies with the 5-HT$_3$ antagonist Alosetron [156]. Changes in gas handling, gas production, visceral sensation and posture have all been implicated in the generation of bloating in this patient group and it has been suggested a more complex management strategy combining dietary, pharmacological, bacteriological and even behavioural approaches may be needed to improve this difficult symptom[185].

It is interesting to note that studies of the effect of Loperamide, one of the most commonly prescribed drugs in this patient group, have not convincingly shown an effect on symptoms other than perhaps a reduction in stool frequency[186]. It is our experience that patients use this medication as a "rescue" therapy but its use in the long term appears not to be satisfactory, one could speculate this lack of demonstrated efficacy in reduction of other symptoms such as pain and bloating and even a reported increase in pain at night in one study[174] might be contributing to reduced patient satisfaction . The absence of a documented increase in pain with the use of Ondansetron may make its use more appealing to patients than Loperamide.

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8.4 Dose and Safety

Regrettably Alosetron , a 5-HT$_3$ RA for which there is substantial evidence of benefit[1] was withdrawn from widespread use because of an unacceptable incidence of severe constipation (around 25%) and a much lower incidence (0.7 per 1,000 patient-years) [187] of ischemic colitis . Our trial shows that Ondansetron can achieve useful results with a low incidence of side effects. Constipation occurred in just 9% and rapidly resolved on dose reduction giving a discontinuation rate due to constipation of just 2% with no incidents of ischemic colitis.

 The high incidence of constipation with Alosetron appears in retrospect to have been due to the use of too high a dose. Subsequent studies using lower doses suggest that 0.5 of a mg daily is associated with a much reduced incidence of constipation which was just 9% in the 0.5 mg group compared with 5% in the placebo group. However these low doses were sufficient to produce a response in 50.8% of patients compared with 30.7% in placebo [188]. Unfortunately ischemic colitis remains a concern despite careful monitoring with an incidence of 2 per 1000 [189]. Ondansetron's potency in blocking the 5-HT$_3$ receptor is between 3-10 times lower than Alosetron [190] which may explain the low incidence of side effects in our study. Ramosetron is another 5-HT$_3$ RA, proven to be effective in IBS-D but unfortunately only marketed in Japan. It has an affinity for the 5-HT$_3$ receptor 3 times Alosetron [191] but is given at a very low dose of 5ug, with Constipation rates of

7%[71] equivalent to 0.015 mg Alosetron again suggesting that lower doses of 5-HT$_3$ RAs might well be the best strategy in IBS-D.

The use of dose titration in this study was key to its success; patients were able to select the lowest effective dose, the mode being just 4mg every other day, leading to greatly less constipation than has been seen in other related preparations.

The further benefits of Ondansetron, particularly in Europe, where neither Alosetron nor Ramosetron are available, are that it is generic drug, available worldwide at a low price, with a very long experience of safe usage and, to our knowledge, no reports of ischemic colitis.

8.5 Number needed to treat

We have demonstrated that Ondansetron in our patient group is effective with a low risk of side effects, its effectiveness is summarised by its low NNT of just 3. This allows us to place this treatment alongside therapies that are currently available and speculate that the use of Ondansetron may lead to improved outcomes in this group of patients who are often dissatisfied with conventional treatment.

One of the most frequently used groups of agents are the tricyclic antidepressants. These are cheap and readily available, and a single meta-analysis has reported a number needed to treat of as low as 3 although the individual trial quality was variable [192]. In contrast attempts to compare tricyclics and a SSRI against placebo did not find either to be superior,

although Imipramine had a greater effect on the psychological distress experienced by patients [193]. Additionally further meta-analysis of antidepressants in IBS by Cochrane concluded that there was no evidence that antidepressants were effective in the treatment of IBS, it is notable that many larger studies were excluded because of methodological weakness and numbers were small [194]. Many of these studies used the global assessment of patients response to therapy, this comprises of a variety of dimensions each differently weighted by the individual, which despite the negative conclusions of the meta-analyses points to potential gains on a patient by patient basis. The FDA has attempted to address this deficiency in endpoint selection with its new symptom specific criteria which we have adopted in our current study. In practice tricyclic agents although frequently initiated have the potential to aggravate constipation and their other side effects including dry mouth and drowsiness often preclude their use even at low dose, with up to 40% discontinuing for these reasons [195].

The NNT of Ondansetron compares favourably with the other 5-HT$_3$ antagonists. Ramosetron has been shown to achieve global relief from IBS symptoms in 47% compared to 27% with placebo giving an NNT of 5, which is similar to Alosetron [71]. Cilansetron which was withdrawn at an early stage because of 4 cases of ischaemic colitis reduced stool consistency, stool frequency and urgency leading to satisfactory relief in 41% compared to 18% with placebo giving an NNT of 4.7 [196] and a metanalysis of 14 eligible RCTs of Alosetron (n=3024) or Cilansetron (n=1116) vs. placebo resulted in a NNT of 7.7 for relief of abdominal pain, 4.2 for global relief of symptoms [1].

The low NNT in this study is not likely to be a direct result of enhanced drug efficacy, indeed we have described the lower receptor affinity of Ondansetron compared to Alosetron [190] and Ramosetron in the preceding section [191], but of the dose titration model used. This model reflects clinical practice and patients were able to take a daily dose that did not result in constipation. It can be seen that individual dosing ranged from 4mg every 3 days, to 24mg daily demonstrating a very large variation in individual sensitivity to 5-HT$_3$ antagonism with Ondansetron

Comparison of Ondansetron with Loperamide is more difficult, studies are small and although use is widespread, evidence for efficacy beyond a reduction is stool frequency is lacking[186].

The place of Ondansetron in the treatment algorithm for patients with IBS-D is an area where further study is needed, but its quick onset of action and low NNT make it an attractive first choice in a condition where repeated trials of treatment are often needed, allowing the patient and clinician to move on quickly if it is not found to be efficacious.

8.6   Mechanisms of action

8.6.1 Transit

We have demonstrated a significant slowing of colonic transit in patients taking Ondansetron compared to placebo with the greatest effect seen in the left colon. This is a well documented effect of this class of medication and our findings compare favourably with earlier work. The first of these studies using

radio opaque markers in healthy volunteers and Ondansetron at high dose (16mg TDS) resulting in a mean total colonic transit time on placebo of 27.8 h, vs 39.1 h whilst taking Ondansetron. Transit times through the left colon and rectosigmoid were prolonged by the drug, but right colonic transit was not significantly altered leading the authors to postulate that the left colon may have the highest density of 5-HT$_3$ receptors in the large bowel[79]. In a second study of 11 patients taking 16mg tds of Ondansetron, colonic transit tended to be longer during drug therapy than during the placebo trial, but this difference was not significant. Small intestinal transit and orocecal transit were unchanged by the drug[80]. It is striking that these earlier studies used a much greater dose than in our patient group yet we are able to demonstrate a significant alteration in transit.

The effect of Alosetron on transit appears to be similar with no overall effect on the orocaecal transit time using the radiopaque marker technique, but an increase the whole gut transit time as a result of an increase in the left colonic transit time [197]. Both Alosetron and Ondansetron have a direct, selective inhibitory effect on the frequency of spontaneous MMC in isolated small and large intestine in C57BL/6 mice [198], but also may influence transit by changing rectal sensitivity. These changes in sensitivity have been demonstrated with the use of iv Granisetron [199] and also with Alosetron , this change in sensitivity may underlie slowing of left sided transit seen in our patients. Although rectal sensitivity was not measured here it could be inferred that the significant reduction in urgency may be in part secondary to changes in sensitivity.

Changes in receptor density and rectal sensitivity may important but in a patient group with fast transit caution should be exercised in interpreting the findings of a measurement taken at day 3. It may be that this cheap and patient acceptable method of measuring transit is not in fact sensitive enough and may miss subtle differences in right sided transit at 72 hour post marker ingestion. This may be a factor underlying the surprising finding that stool form in this study does not correlate with transit as has been shown by others[200].

8.6.2 Small bowel water

As in other work from Nottingham [148] we have again shown a reduction in SBWC in patients compared to healthy volunteers, the size of the effect is smaller, and we speculate this is a reflection of the heterogeneity of the measure.

We hypothesised that Ondansetron would slow small bowel transit and reduce MMC frequency leading to increased small bowel water in patients taking Ondansetron compared to placebo. This was not seen, with no difference in SWBC whilst taking placebo compared to taking Ondansetron. This might initially seem surprising given that we have shown a reduction in SBWC and the antroduodenal Motility Index with Ondansetron in healthy volunteers. The dosing in these subjects was however much higher with volunteers taking 8mg of Ondansetron 3 times a day on the day preceding scanning and 16mg of Ondansetron 1 hour preceding the scan[153]. This is consistent with other studies in which 5-HT administration increased the

frequency of the MMC[201, 202], and 5-HT3 antagonism reduces MMC frequency[198] .In our study the SBWC measurements were taken in the final week of treatment when because of the use of dose titration, some patients had been taking doses as low as 4mg every other day.

In contrast to changes in transit in the small bowel in volunteers receiving high doses of Ondansetron, it is interesting to note that 2 studies using 16mg of Ondansetron in patients showed a significant prolongation in whole gut transit but did not change mouth to caecum time[80, 203] Selective5-hydroxytryptamine receptor antagonism with Alosetron also exerts its effect on whole gut transit in the colon, not the small bowel when assessed by the radio opaque marker method used in conjunction with the breath hydrogen test to quantify small bowel transit time[197].

Despite the overall lack of effect of Ondansetron on SBWC we were able to show a weak but significant association between SBWC and bloating, and also between SBWC and somatisation. In the original work knowing both the total SBwC and the rate of transit an average Flow Index was calculated. This suggested that the faster transit seen was mostly due to a smaller intestinal diameter, or "spaghetti bowel," which may reflect increased circular muscle tone [148]. This increased tone was hypothesised to be related to anxiety and result in pain and bloating. The finding of a link with both bloating, and a measure of psychological abnormality in the form of somatisation in this study fits with this hypothesis and points to a small effect of Ondansetron on the small bowel.

MRI of the bowel is a new technique; as yet many of the other drugs that alter gut transit have not been evaluated using this novel method, so a comparison of the effect on SBWC with other agents is not possible. It is interesting to note that Loperamide which we know acts to slow gut transit times has been evaluated in volunteers and does reduce SBWC [151] but its effect on this measure in patients is not yet known.

8.6.3 Colon water analysis

Colon water analysis took place in the ascending colon. It is not surprising that there was no effect downstream given that we were unable to demonstrate a change in SBWC. Additionally characterising the water content of the colon using MRI is fraught with difficulty. The T1 and T2 of colonic contents are very short and the reasons for this are not clear. Hypotheses include the presence of iron or the low free water fraction. Within the department (unpublished work, by Elisa Placidi) ex vivo MRI of stool that was subsequently freeze dried and then rehydrated revealed a very low free water content. The persistence of a short T1 and T2 after water addition may imply quenching of the signal is due to ferrous ions or bacterial products present in the faecal matter.

8.7 Markers of serotonin turnover

5-HT_3 antagonists are logical treatment in patients with IBS-D given the abnormalities of serotonin metabolism which have been demonstrated in this patient group. Post infective IBS, a subtype of IBS-D with very similar clinical features [204] has been shown to be associated with increased 5-HT-

containing enteroendocrine cells [35] and also increased post prandial 5-HT release [110] We examined 3 measures of serotonin function in this group;

1. The 5-HTTPLR polymorphism.

2. Platelet serotonin binding kinetics.

3. Plasma 5HIAA.

8.7.1 5-HTTPLR Polymorphism

Serotonergic action is terminated through reuptake from the synaptic cleft by the serotonin transporter protein (SERT-P). There is a 44 bp insertion ∕ deletion in the 5'-flanking promoter region (5-HTTLPR), which creates a short and a long allele. The presence of the short allele is associated with lower transcriptional activity and, as a consequence, lowers levels of SERT-P expression and reduced reuptake of serotonin [205]

We found no difference in 5-HTTPLR distribution between healthy volunteers and patients. Drawing a conclusion from this finding alone would be inappropriate given the small numbers involved but it is notable that there is considerable debate as to what the role of this polymorphism is in IBS. In a total of 1034 patients with the irritable bowel syndrome, and 1377 healthy controls, the presence of the short allele was not associated with an increased risk for the irritable bowel syndrome: OR 1.0; 95% CI: 0.7–1.4 for homozygous subjects, and OR 1.0; 95% CI: 0.8–1.2 for homozygous subjects and heterozygotes together[206].

In our study there was a trend to an increase in baseline anxiety and depression scores in patients and volunteers with the ss polymorphism, this did not reach significance but is in keeping with the finding that the 5-HTTPLR polymorphism accounts for 3 to 4 percent of total variation and 7 to 9 percent of inherited variance in anxiety-related personality traits [205]. There was however no difference in any of the other baseline parameters between groups despite the suggestion that 5-HTTPLR status might affect the severity of symptoms in patients with IBS. It has been hypothesised that S allele impairs the efficiency of 5-HT reuptake with a consequent prolonged and enhanced activation of serotonergic pathways mediating abdominal pain sensation. Camilleri et al found that the LS/SS genotypes are associated with increased pain sensation at rectal distension [207] and more recently LS and SS genotypes have been shown to be significantly correlated with IBS symptom severity across all IBS subtypes[208]. The same polymorphism predisposes to developing depression in response to life stressors [209]. Our patients had a high degree of satisfaction with Ondansetron treatment despite no significant difference from placebo on pain severity. As previously mentioned this group, all of whom had diarrhoea >25% of the time are often much troubled by the sensation of urgency, and may rate pain as a less troublesome symptom.

There is a suggestion from our data that there is a difference in response to Ondansetron between the 3 5HTTPLR polymorphisms with those in the ll group most likely to respond, numbers are too small to draw conclusions but comparison with others shows a variation in response according to

polymorphism has been demonstrated. Camilleri's group have shown an improvement in gut transit times with Alosetron in the ls group compared to the ll group, although in this study the number of participants with the ss polymorphism was too small to study. Here the hypothesis is that the drug can act more effectively in an environment with a lower synaptic concentration of 5-HT that needs to be competitively inhibited by the antagonist. In our population the greatest prolongation of transit with Ondansetron was also seen in the ls group. This was not significant even though we had 4 times the number of subjects and again this may reflect the far greater potency of Alosetron versus Ondansetron at the doses used.

5-HTTPLR polymorphism status did not significantly alter platelet paroxetine binding kinetics, or baseline plasma 5HIAA levels. Other work has shown platelet 5-HT uptake to be significantly higher in LL homozygotes compared with S carriers among a group of healthy individuals, but no significant difference was observed in transporter densities as measured by paroxetine binding between groups [210]. In alcoholic subjects Bmax did not differ in a statistically significant manner among LL, LS, and SS genotypes in, but they did find differences in platelet serotonin uptake [211]. The lack of difference in these measures of serotonin binding and breakdown may simply be a result of small numbers, or may suggest a lack of functional significance in this patient group.

8.7.2 Platelet paroxetine binding

The endogenous activity of 5-HT is controlled by the specific 5-HT transporter (SERT), which facilitates the intracellular reuptake of 5-HT and can be specifically blocked by selective 5-HT reuptake inhibitors (SSRIs), such as paroxetine and fluoxetine. SERT is widely expressed in intestinal epithelial cells, central or peripheral serotonergic neurons, and platelets, where it shares common molecular and physiological features [212]. It has therefore been hypothesized that changes in intestinal SERT function might also be present in SERT in platelets, which circulate systemically and are exposed to the same intestinal environment. Indeed previous work from our department has demonstrated binding of paroxetine to membranes of platelets from patients with IBS-D to be significantly greater than that from HVs and that this measure correlated inversely with platelet uptake of 5-HT and with mucosal SERT mRNA [121]. This inverse relationship between platelet 5-HT uptake and paroxetine binding led us to use paroxetine binding, an assay which can be performed on frozen samples, as a surrogate marker of impaired SERT in our patients with IBS-D.

In this study there was no difference in the number of SERT receptors on the platelet membranes of patients compared to healthy volunteers (Bmax) in addition the strength of binding to these receptors (kd) was also not different between the 2 groups.

The values obtained for Bmax and Kd in this study, performed in the same department, by the same operator, using the same methods and equipment,

are higher than in the study by Foley et al (Bmax = 475.1 IQR (270.3-728.7) vs. 226 IQR [92– 405] fmol/mg protein in patients and 458.3 IQR (213.0-683.2) vs. 109 IQR [69 –175] fmol/mg protein in healthy volunteers)[121]. A review of the literature suggests there is variability in this measure with mean values for Bmax in patients with IBS-d reported as 523 +/- 40 fmol/mg and healthy volunteers 1152 +/- 187 fmol/mg, in the only other study of this assay in an IBS population [120].

It is notable that in this piece of work that the healthy volunteers were age and sex matched and that the numbers were greater, 71 patients and 20 volunteers, compared to 12 subjects in each arm in the earlier Alosetron [120] work and 29 and 20 patients and healthy volunteers respectively in the previous study from this department[121].

There is evidence in fibromyalgia that Bmax correlates negatively with symptom severity but not Kd values [213], and after treatment with Alosetron, symptom severity score decreased significantly whereas B(max) and K(d) values did not change [120]. We did not however find an association with paroxetine binding and symptoms in our patients, and it may be that this convenient surrogate marker of SERT function is subject to too greater a degree of variability in this already heterogeneous population (in whom only some may have altered serotonin handling as a disease mechanism) to accurately reflect underlying changes gut mucosal serotonin handling.

8.7.3 Plasma 5 HIAA

5HIAA in platelet poor plasma is another surrogate marker of 5HT turnover, representing the relative activity of the enzyme monoamine oxidase not only in the platelets but also in other tissues such as the liver and lungs. Studies in IBS patients have found reduced fasting 5HIAA in patients with constipation, and normal 5HIAA in patients with diarrhoea but with an altered 5HIAA:5HT ratio after feeding, pointing to a reduced capacity for serotonin breakdown in this patient group [109].

Median fasting plasma 5HIAA was significantly higher in controls, which would fit with a reduced capacity for serotonin breakdown via impaired SERT function in patients as shown by others [121], however despite this there was no correlation with plasma 5HIAA levels and any of the baseline values in healthy volunteers or patients, including stool form and frequency suggesting this difference may not have a detectable functional consequence in this small number of subjects.

8.8 Predictors of response

Patients were selected to meet the Rome III criteria and so were necessarily similar at baseline. Only days with pain remained a significant independent predictor of response after multivariate analysis, but all the baseline parameters derived from questionnaires were slightly worse in those who did not respond to Ondansetron, with non responders being significantly older, having significantly more days with pain, greater average pain, anxiety, and

somatisation as measured by the PHQ-12. We found that those most severely affected were also more likely to dropout, indicating that the efficacy for treating severe diarrhoea with Ondansetron is limited, and perhaps the greater anxiety displayed is reflection of a different mechanism of diarrhoea unresponsive to 5-HT$_3$ modulation. The best response to Ondansetron is therefore likely to be in those with mild to moderate symptoms. However given its safety, low side effect profile and rapid onset of effect within 1 week in most cases, a trial of treatment would seem reasonable in most cases of IBS-D.

One of the stated aims of this thesis was to identify an Ondansetron responsive phenotype. The variable dosing regime mirrored clinical practice and gives a better idea of how the drug will perform in clinical practice. It undoubtedly improved response rate and had we chosen a fixed dose many patients would have developed constipation and probably dropped out or had worsening symptoms. It does however present a problem for analysis since only the stool diaries in the last 2 weeks are truly informative of the response meaning that we cannot input data to allow for incomplete diaries which would be normal practice. This led to a reduction in an already small sample (powered to detect a change in stool consistency) and this small number is likely to be a significant factor in preventing this aim from being achieved. Despite this the reduced response at the more severe end of the spectrum presents an interesting clue for further study.

8.9 Serine proteases

This thesis cofirms previous reports [94] that FSP activity is elevated in some patients with IBS-D but also shown that there is considerable overlap with normal subjects without IBS. Characterisation of the proteins responsible for the serine protease activity shows that most of this activity is likely due to human pancreatic enzymes.

While the pancreas is the putative source of this trypsin-like activity, enterocytes also contain trypsin mRNA and trypsin-like immunoreactivity [214], though the amounts are small relative to expression in the pancreas. Trypsin levels in colonic biopsies have been shown to be increased in IBS-D [143] so we cannot be certain how much of the observed increase in FSP is from enterocytes and how much pancreatic. Furthermore human epithelial cell lines secrete trypsinogen IV [215] the mRNA of which has also been found to be 1.5 fold increased in human small intestinal biopsies from IBS-D patients [216].

Trypsinogen IV is known to activate PAR2 receptors [215] and so may be important in activating afferent nerves, generating inflammatory changes and increasing permeability; all of which could contribute to IBS symptoms.

Using a Benzamindine affinity resin we extracted faecal serine proteases and characterised them by mass-spectrometric analysis of proteolysed components in gel electrophoresis. We identified several human enzymes including amylase, elastase, carboxypeptidase and trypsin. However it should

be noted that of these enzymes, only trypsin I is known to activate the PAR2 receptor and hence would be predicted to influence visceral sensitivity, gut barrier function and immune function. Surprisingly, using a highly specific immunoassay we found endogenous mast cell tryptase was undetectable in faecal extracts. Several papers have examined release of trypsin-like mediators from supernatants of IBS biopsies and most [143, 217, 218] but not all [219] have found increased release. Others have suggested that faecal serine proteases might be of bacterial origin but we found major components in purified faecal extract to be human derived. Earlier studies argued that because high levels of FSP activity were not seen in bacterial gastroenteritis this meant that fast transit was not important[94].However work not shown in this thesis done in our department using Moviprep to purge the colon of healthy volunteers shows a significant increase in FSP activity after accelerated transit. Most patients with gastroenteritis become anorexic which would be expected to reduce pancreatic enzyme secretion while our subjects in this purging study ate a 400 kcal low residue diet the night before the study which would be expected to adequately stimulate pancreatic secretion.

Since our data suggested a probable pancreatic origin for the serine protease activity we then examined other pancreatic enzymes in stool. Faecal amylase was assessed using a specific immunoassay to identify human pancreatic amylase and showed a similar trend to serine proteases with increased values which correlated with FSP activity. The amylase levels suggested two groups of patients and we found that IBS-D patients with higher faecal amylase had significantly higher FSP activity and greater anxiety scores and a tendency to

faster transit than those with lower values, suggesting to us that these increases might both be due to accelerated transit. Previous studies of faecal trypsin indicate that the very substantial amounts of pancreatic trypsin secreted each day are mostly degraded during transit through the colon since the 24 hour faecal output is approximately 1mg compared to an ileostomy output of 50-200mg [220]. Antibiotic therapy which inhibits bacterial degradation increases faecal trypsin and elastase in both rats [221] and humans [222]. The pancreas is the major source of human faecal elastase since very little is excreted in the faeces of patients with pancreatic insufficiency as assessed by intubated pancreatic function testing [223]. Elastase appears to be inherently more resistant to bacterial degradation. Thus after reducing the faecal bacterial content in humans by oral antibiotics, trypsin levels rose 100 fold while elastase levels rose only 2-3 fold[220] This may explain why elastase is not increased in IBS-D as little degradation occurs so faecal levels reflect pancreatic secretion which is not expected to be different in IBS-D compared to controls.

Slowing transit by any means would be predicted to reduce faecal tryptic activity and benefit symptoms. Although faecal serine protease activity whilst taking Ondansetron was not different compared to placebo there was a negative correlation between the rise in transit on Ondansetron and the increase in FP (r=−0.40, p=0.003) in the 55 patients with complete values for both FP and transit at both time points. . We have shown that pancreatic proteases are entering the colon as a function of rapid small bowel transit and this small bowel transit time may not be altered. This is supported by the

finding that SBWC is not altered leading us to believe that the changes in whole gut transit seen with Ondansetron are related to slowing of colonic transit. Higher doses of Ondansetron are likely to be needed to affect the small bowel but the effects on colonic transit are so potent that higher doses are precluded. Targeting the small bowel transit or directly influencing faecal serine protease activity may be the important in influencing difficult symptoms such as pain and bloating, whilst stool form and urgency can be successfully treated with Ondansetron. Direct targeting of FSP activity due to undegraded pancreatic enzymes may well be important since trypsin is a potent activator of PAR2 receptors. This activation can increase gut permeability [94] , which in some human studies is very clearly linked to abdominal pain and visceral hypersensitivity [224] Furthermore, sensitising the distal gut may aggravate diarrhoea and urgency.

8.10 Limitations and further work

8.10.1   Ondansetron in patients with IBS-D "identifying the responder"

There are 4 broad areas of limitation that should be taken into account when interpreting the findings of this study. The principal of these is the failure to define an Ondansetron responsive phenotype. This was a particularly important target as without a way to identify patients who might respond to a particular therapy physicians can contribute to patient dissatisfaction with treatment with multiple trials of ineffective therapy, with the additional risk of a patient experiencing side effects to a drug that has no impact on their

condition. As discussed this is most likely the result of a small sample size and also as a consequence of the use of a dose titration model.

 Secondly although patients were selected both form primary and secondary care and as such are likely to be a representative sample it is recognised that participants in a study scenario often gain benefit from the extra care and attention they receive whilst taking part in research, this factor may falsely enhance the efficacy of Ondansetron in this group.

Thirdly the duration of this study is short (6 weeks of treatment only), and is therefore not able to provide data on the longevity of effect, or incidence of side effects such as constipation over time. As we know patients symptoms naturally wax and wane and constipation might be expected to occur with longer term use.

Finally a number of treatments exist for patients with IBS-D, including dietary, psychological and pharmacological. The most commonly used drug therapies are Loperamide and the tricyclic antidepressants. This study is not able to demonstrate enhanced efficacy or tolerability of Ondansetron when compared to these readily available treatments.

Taking these factors into account it is clear that a further larger study is warranted. Given the low number needed to treat, the definition of a phenotype responsive to Ondansetron although still an important question might not be the best focus for a subsequent study. Additionally, acknowledging the small sample size, I would suggest the markers of serotonin system have not shown promise in this study. There are however

clinical clues which suggest that those with the most severe symptoms and greatest pain are least likely to respond. In my own practise this has led me to treating those who are more "biological" i.e. those with less anxiety and depression, less somatisation, better coping skills and perhaps a post infectious onset of their symptoms, with Ondansetron and those who are more "psychological" i.e. more anxious with more somatisation and less good coping skills with a TCA first line.

I would therefore suggest a study in which the place of Ondansetron in our current treatment algorithm was assessed whilst also garnering data in a larger number of patients over a longer follow up period. This should be a trial of Ondansetron vs. Amitryptiline, again using a dose titration method, with particular focus on collecting data on anxiety, depression, somatisation and coping skills as predictors of response.

A large study such as this is I believe a vital next step, but its execution will be costly and time consuming. A smaller study could explore the use of other biomarkers that may have promise. The changes seen in urgency in our patients led us to speculate that as well as changes in transit there may be changes in rectal sensitivity. Changes in rectal sensitivity as measured by barostat bag distension are well documented in patients with IBS and measurement pre and post the administration of Ondansetron has been demonstrated [225]. The presence of rectal hypersensitivity at baseline may predict response to treatment, and this could be explored in a smaller, mechanistic study. Work with in the department using MRI has demonstrated

a reduced capacity for the ascending colon to accommodate a postprandial inflow in patients with IBS-D as a potential driver to fast transit [226]. A test meal administered to patients on and off Ondansetron, would yield information not only on the effects of Ondansetron in the colon, but has the promise of a dynamic test that might be used to predict response to the drug.

Other smaller scale and no less valuable work could also increase our understanding of the effect of Ondansetron in patients. Baseline quality of life data was collected from all participants and a follow up study with an assessment of quality of life whilst taking Ondansetron would be of great interest. This would also collate data on the duration of efficacy, and medium to long-term tolerability of Ondansetron. It would be particularly exciting to see if there is ongoing resolution of symptoms and perhaps an improvement in pain over a greater study period as longer term changes transit may result in changes in gut flora and thus sensitivity.

As is often the case when such a large volume of information is collected as well as ideas for future work, there remains much potential for use of already collected data. Further analysis of the wealth of data contained in the stool diaries at baseline might identify other clinical biomarkers. For example it would be interesting to compare those who were ever constipated vs. never constipated as a predictor of response. Further diary work is also suggested following the identification of an early morning rush. Although the data is not recorded here, future diaries could contain the timing of meals to identify a

post-prandial rush, a very commonly reported symptom in patients. This would link well with a future study looking at colonic volumes post feeding.

8.10.2 Faecal serine proteases

We have identified the pancreas as the putative source of increased serine protease activity in patients with IBS-D, and suggest this increased activity is related to changes in transit. Ondansetron did not have a significant effect on small bowel transit in this study and it is clear that further work looking at small bowel transit time and FSP activity is needed. Other measures of small bowel transit, either using scintigraphy or the hydrogen breath test in patients and healthy volunteers would be appropriate, although we have already shown that rapid transit during purging leads to a large increase in FSP activity[227].

The correlation of FSP activity with urgency also suggests that new therapies targeting faecal serine protease could be useful in the treatment of this most debilitating symptom of IBS-D. Finally, if these changes in FSP are due to changes in gut microbiota it opens the possibility that modifying the microbiota by diet or probiotics might benefit IBS symptoms by degrading FSPs.

8.11 Conclusion

Reviewing the original aims of this thesis, I have been able to demonstrate that:

a. The 5-HT$_3$ receptor antagonist Ondansetron is an effective and well tolerated treatment in patients with IBS-D with a low number of side effects.

b. Ondansetron slows whole gut transit, but without a demonstrable difference in small bowel water content.

9. Markers of mucosal serotonin activity were not in this study predictors of response to Ondansetron.

10. Faecal serine protease activity was reduced by treatment with Ondansetron in proportion to the change in colonic transit.

In addition other important findings include:

Confirmation of previous reports that FSP activity is elevated in some patients with IBS-D but also that there is considerable overlap with normal subjects without IBS. We have increased our understanding of this phenomenon by characterising the proteins responsible for the serine protease activity, showing that most of this activity is likely due to human pancreatic enzymes.

Our study suggests that clinical rather than biochemical indicators predicted responsiveness to Ondansetron best. Patients with less severe symptoms are more likely to respond well to Ondansetron which should prove a useful addition to the current rather limited therapies available for this important group of patients.

9 Appendices

Appendix I

HAD questionnaire

Please complete each of the following questions, checking the one response that comes closest to how you have been feeling **in the past week.**

1. I feel tense or 'wound up':
   - 1 ☐ Most of the time
   - 2 ☐ A lot of the time
   - 3 ☐ Sometimes
   - 4 ☐ Never

2. I still enjoy the things I used to enjoy:
   - 1 ☐ Definitely as much
   - 2 ☐ Not quite as much
   - 3 ☐ Only a little
   - 4 ☐ Hardly at all

3. I get a sort of frightened feeling as if something awful is about to happen:
   - 1 ☐ Definitely and quite badly
   - 2 ☐ Yes, but not too badly
   - 3 ☐ A little, but it doesn't worry me
   - 4 ☐ Never

4. I can laugh and see the funny side of things:
   - 1 ☐ As much as I always could
   - 2 ☐ Not quite as much now
   - 3 ☐ Definitely not as much now
   - 4 ☐ Never

5. Worrying thoughts go through my mind:
   - 1 ☐ All of the time
   - 2 ☐ A lot of the time
   - 3 ☐ Sometimes, but not too often
   - 4 ☐ Rarely

6. I feel cheerful:
    - 1☐ Never
    - 2☐ Not often
    - 3☐ Sometimes
    - 4☐ Most of the time

7. I can sit at ease and feel relaxed:
    - 1☐ Definitely
    - 2☐ Usually
    - 3☐ Not often
    - 4☐ Never

8. I feel as if I am slowed down:
    - 1☐ Nearly all the time
    - 2☐ Very often
    - 3☐ Sometimes
    - 4☐ Never

9. I get a sort of frightened feeling like 'butterflies' in the stomach:
    - 1☐ Never
    - 2☐ Occasionally
    - 3☐ Quite often
    - 4☐ Very often

10. I have lost interest in my appearance:
    - 1☐ Definitely
    - 2☐ I don't take as much care as I should
    - 3☐ I may not take quite as much care
    - 4☐ I take just as much care as ever

11. I feel restless, as if I have to be on the move:
    - 1☐ Very much
    - 2☐ Quite a lot
    - 3☐ Not Very much
    - 4☐ Never

12. I look forward with enjoyment to things:
    - 1☐ As much as I ever did
    - 2☐ Somewhat less than I used to
    - 3☐ Definitely less than I used to
    - 4☐ Hardly at all

13. I get sudden feelings of panic:
    1☐   Very often
    2☐   Quite often
    3☐   Not very often
    4☐   Never

14. I can enjoy a good book or TV program:
    1☐   Often
    2☐   Sometimes
    3☐   Not often
    4☐   Rarely

Thank you very much for taking the time to fill in this questionnaire.

Please check that you have answered all the questions.

PATIENT IBS SEVERITY SCORE QUESTIONNAIRE

INSTRUCTIONS

This form is designed to enable us to record and monitor the severity of your IBS. It is to be expected that your symptoms might vary over time, so please try and answer the questions based on how you currently feel (ie over the last 10 days or so). All information will be kept in strict confidence.

1. For questions where a number of different responses are a possibility please circle the response appropriate to you.

2. Some questions will require you to write in an appropriate response.

3. Some questions require you to put a cross on a line, which enables us to judge the severity of a particular problem.

For example:

How severe was your pain?

Please place your cross (X) anywhere on the line between 0-100% in order to indicate as accurately as possible the severity of your symptom.
This example shows a severity of approximately 90%

0%	_____X___	100%
no pain	not very quite severe	very
	severe severe	severe

SEVERITY SCORE

1. *a) Do you currently suffer from abdominal (tummy) pain?* Yes ☐ No ☐

   b) If yes, how severe is your abdominal (tummy) pain?

   0% _____ 100%

   no pain   not very   quite   severe   very
               severe   severe   severe

   c) Please enter the number of days that you get the pain in every 10 days. For example if you enter 4 it means that you get pain 4 out of 10 days. If you get pain every day enter 10

   Number of days with pain ☐   x10

2. *a) Do you currently suffer from abdominal distention* Yes ☐ No ☐ (bloating, swollen or tight tummy) (*women, please ignore distention related to your periods)*

   b) If yes, how severe is your abdominal distention/tightness

   0% _____ 100%

   no pain   not very   quite   severe   very
               severe   severe   severe

3. *How satisfied are you with your bowel habit?*

   0% _____ 100%

   very   quite   unhappy   very
   happy   happy   unhappy

4. *Please indicate with a cross on the line below how much your Irritable Bowel syndrome is affecting or interfering with your life in general*

   0% _____ 100%

   not at all   not much   quite   completely
                            a lot

   IBS SEVERITY SCORE

5. *How many visits have you made to your doctor for your bowel symptoms in the past 6 months? (write in)* _____ *visits*

PHQ 15: Volunteer's Health Questionnaire

During the past 4 weeks, how much have you been been bothered by any of the following problems?	Not Bothered at all	Bothered a little	Bothered a lot
Stomach pain			
Back pain			
Pain in your arms, legs, or joints (knees, hips, etc)			
Menstrual cramps or other problems with your periods (Women only)			
Headaches			
Chest pain			
Dizziness			
Fainting spells			
Feeling your heart pound or race			
Shortness of breath			
Pain or problems during sexual intercourse			
Constipation, loose bowels, or diarrhoea			
Nausea, gas, or indigestion			
Feeling tired or having low energy			
Trouble sleeping			

10 References

1.   Andresen, V., et al., *Effects of 5-hydroxytryptamine (serotonin) type 3 antagonists on symptom relief and constipation in nonconstipated irritable bowel syndrome: a systematic review and meta-analysis of randomized controlled trials.* Clin Gastroenterol Hepatol, 2008. **6**(5): p. 545-55.

2.   Drossman, D.A., W.E. Whitehead, and M. Camilleri, *Irritable bowel syndrome: a technical review for practice guideline development.* Gastroenterology, 1997. **112**(6): p. 2120-37.

3.   Wells, N.E., B.A. Hahn, and P.J. Whorwell, *Clinical economics review: irritable bowel syndrome.* Aliment Pharmacol Ther, 1997. **11**(6): p. 1019-30.

4.   Simren, M., et al., *Quality of life in patients with irritable bowel syndrome seen in referral centers versus primary care: the impact of gender and predominant bowel pattern.* Scand J Gastroenterol, 2001. **36**(5): p. 545-52.

5.   Hammer, J. and N.J. Talley, *Value of different diagnostic criteria for the irritable bowel syndrome among men and women.* J Clin Gastroenterol, 2008. **42**(2): p. 160-6.

6.   Banerjee, R., et al., *Rome I criteria are more sensitive than Rome II for diagnosis of irritable bowel syndrome in Indian patients.* Indian J Gastroenterol, 2005. **24**(4): p. 164-6.

7.   Manning, A.P., et al., *Towards positive diagnosis of the irritable bowel.* Br Med J, 1978. **2**(6138): p. 653-4.

8.   Ford, A.C., et al., *Will the history and physical examination help establish that irritable bowel syndrome is causing this patient's lower gastrointestinal tract symptoms?* JAMA, 2008. **300**(15): p. 1793-805.

9.   Kruis, W., et al., *A diagnostic score for the irritable bowel syndrome. Its value in the exclusion of organic disease.* Gastroenterology, 1984. **87**(1): p. 1-7.

10.   Drossman, D.A., *The functional gastrointestinal disorders and the Rome II process.* Gut, 1999. **45 Suppl 2**: p. II1-5.

11.   Longstreth, G.F., et al., *Functional bowel disorders.* Gastroenterology, 2006. **130**(5): p. 1480-91.

12.   Cash, B.D., P. Schoenfeld, and W.D. Chey, *The utility of diagnostic tests in irritable bowel syndrome patients: a systematic review.* Am J Gastroenterol, 2002. **97**(11): p. 2812-9.

13.   Jellema, P., et al., *Systematic review: accuracy of symptom-based criteria for diagnosis of irritable bowel syndrome in primary care.* Aliment Pharmacol Ther, 2009. **30**(7): p. 695-706.

14.   Drossman, D.A., et al., *International survey of patients with IBS: symptom features and their severity, health status, treatments, and risk taking to achieve clinical benefit.* J Clin Gastroenterol, 2009. **43**(6): p. 541-50.

15.   Cremonini, F., et al., *Meta-analysis: the relationship between Helicobacter pylori infection and gastro-oesophageal reflux disease.* Aliment Pharmacol Ther, 2004. **19**(1): p. 145.

16.   Saito, Y.A., et al., *A comparison of the Rome and Manning criteria for case identification in epidemiological investigations of irritable bowel syndrome.* Am J Gastroenterol, 2000. **95**(10): p. 2816-24.

17.   Thompson, W.G., et al., *Functional gastrointestinal disorders in Canada: first population-based survey using Rome II criteria with suggestions for improving the questionnaire.* Dig Dis Sci, 2002. **47**(1): p. 225-35.

18. Heaton, K.W., et al., *Symptoms of irritable bowel syndrome in a British urban community: consulters and nonconsulters.* Gastroenterology, 1992. **102**(6): p. 1962-7.

19. Hungin, A.P., et al., *The prevalence, patterns and impact of irritable bowel syndrome: an international survey of 40,000 subjects.* Aliment Pharmacol Ther, 2003. **17**(5): p. 643-50.

20. Wilson, S., et al., *Prevalence of irritable bowel syndrome: a community survey.* Br J Gen Pract, 2004. **54**(504): p. 495-502.

21. Andrews, E.B., et al., *Prevalence and demographics of irritable bowel syndrome: results from a large web-based survey.* Aliment Pharmacol Ther, 2005. **22**(10): p. 935-42.

22. Halder, S.L., et al., *Natural history of functional gastrointestinal disorders: a 12-year longitudinal population-based study.* Gastroenterology, 2007. **133**(3): p. 799-807.

23. Husain, N., et al., *A population-based study of irritable bowel syndrome in a non-Western population.* Neurogastroenterol Motil, 2008. **20**(9): p. 1022-9.

24. Drossman, D.A., et al., *A prospective assessment of bowel habit in irritable bowel syndrome in women: defining an alternator.* Gastroenterology, 2005. **128**(3): p. 580-9.

25. Ford, A.C., et al., *Irritable bowel syndrome: a 10-yr natural history of symptoms and factors that influence consultation behavior.* Am J Gastroenterol, 2008. **103**(5): p. 1229-39; quiz 1240.

26. Neal, K.R., L. Barker, and R.C. Spiller, *Prognosis in post-infective irritable bowel syndrome: a six year follow up study.* Gut, 2002. **51**(3): p. 410-3.

27. Longstreth, G.F., et al., *Characteristics of patients with irritable bowel syndrome recruited from three sources: implications for clinical trials.* Aliment Pharmacol Ther, 2001. **15**(7): p. 959-64.

28. Neal, K.R., J. Hebden, and R. Spiller, *Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients.* BMJ, 1997. **314**(7083): p. 779-82.

29. Spiller, R.C., et al., *Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome.* Gut, 2000. **47**(6): p. 804-11.

30. Parry, S.D., J.R. Barton, and M.R. Welfare, *Factors associated with the development of post-infectious functional gastrointestinal diseases: does smoking play a role?* Eur J Gastroenterol Hepatol, 2005. **17**(10): p. 1071-5.

31. Mykletun, A., et al., *Smoking in relation to anxiety and depression: evidence from a large population survey: the HUNT study.* Eur Psychiatry, 2008. **23**(2): p. 77-84.

32. Chadwick, V.S., et al., *Activation of the mucosal immune system in irritable bowel syndrome.* Gastroenterology, 2002. **122**(7): p. 1778-83.

33. Lee, K.J., et al., *The alteration of enterochromaffin cell, mast cell, and lamina propria T lymphocyte numbers in irritable bowel syndrome and its relationship with psychological factors.* J Gastroenterol Hepatol, 2008. **23**(11): p. 1689-94.

34. Gwee, K.A., et al., *The role of psychological and biological factors in postinfective gut dysfunction.* Gut, 1999. **44**(3): p. 400-6.

35. Dunlop, S.P., et al., *Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS.* Gastroenterology, 2003. **125**(6): p. 1651-9.

36. Gwee, K.A., et al., *Increased rectal mucosal expression of interleukin 1beta in recently acquired post-infectious irritable bowel syndrome.* Gut, 2003. **52**(4): p. 523-6.

37. Wang, L.H., X.C. Fang, and G.Z. Pan, *Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis.* Gut, 2004. **53**(8): p. 1096-101.

38. Barbara, G., et al., *Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome.* Gastroenterology, 2004. **126**(3): p. 693-702.

39. Weston, A.P., et al., *Terminal ileal mucosal mast cells in irritable bowel syndrome.* Dig Dis Sci, 1993. **38**(9): p. 1590-5.

40. Dinan, T.G., et al., *Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker?* Gastroenterology, 2006. **130**(2): p. 304-11.

41. Liebregts, T., et al., *Immune activation in patients with irritable bowel syndrome.* Gastroenterology, 2007. **132**(3): p. 913-20.

42. Boyce, P.M., et al., *Epidemiology of the functional gastrointestinal disorders diagnosed according to Rome II criteria: an Australian population-based study.* Intern Med J, 2006. **36**(1): p. 28-36.

43. Dunlop, S.P., D. Jenkins, and R.C. Spiller, *Age-related decline in rectal mucosal lymphocytes and mast cells.* Eur J Gastroenterol Hepatol, 2004. **16**(10): p. 1011-5.

44. Moss-Morris, R. and M. Spence, *To "lump" or to "split" the functional somatic syndromes: can infectious and emotional risk factors differentiate between the onset of chronic fatigue syndrome and irritable bowel syndrome?* Psychosom Med, 2006. **68**(3): p. 463-9.

45. Nicholl, B.I., et al., *Psychosocial risk markers for new onset irritable bowel syndrome--results of a large prospective population-based study.* Pain, 2008. **137**(1): p. 147-55.

46. Whitehead, W.E., et al., *Modeling and reinforcement of the sick role during childhood predicts adult illness behavior.* Psychosom Med, 1994. **56**(6): p. 541-50.

47. Gwee, K.A., et al., *Psychometric scores and persistence of irritable bowel after infectious diarrhoea.* Lancet, 1996. **347**(8995): p. 150-3.

48. Spence, M.J. and R. Moss-Morris, *The cognitive behavioural model of irritable bowel syndrome: a prospective investigation of patients with gastroenteritis.* Gut, 2007. **56**(8): p. 1066-71.

49. Alonso, C., et al., *Maladaptive intestinal epithelial responses to life stress may predispose healthy women to gut mucosal inflammation.* Gastroenterology, 2008. **135**(1): p. 163-172 e1.

50. Israeli, E., et al., *The effect of restraint stress on the normal colon and on intestinal inflammation in a model of experimental colitis.* Dig Dis Sci, 2008. **53**(1): p. 88-94.

51. Levy, R.L., et al., *Irritable bowel syndrome in twins: heredity and social learning both contribute to etiology.* Gastroenterology, 2001. **121**(4): p. 799-804.

52. Adkin, D.A., et al., *Echo-planar magnetic resonance imaging to assess water volume in the distal small bowel.* Pharm Res, 1995. **12**(8): p. 1134-9.

53. Maxwell, P.R., et al., *Antibiotics increase functional abdominal symptoms.* Am J Gastroenterol, 2002. **97**(1): p. 104-8.

54. Mendall, M.A. and D. Kumar, *Antibiotic use, childhood affluence and irritable bowel syndrome (IBS).* Eur J Gastroenterol Hepatol, 1998. **10**(1): p. 59-62.

55. Whitehead, W.E., O. Palsson, and K.R. Jones, *Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications?* Gastroenterology, 2002. **122**(4): p. 1140-56.

56. Crean, G.P., et al., *A database on dyspepsia.* Gut, 1994. **35**(2): p. 191-202.

57. Svedlund, J., et al., *Upper gastrointestinal and mental symptoms in the irritable bowel syndrome.* Scand J Gastroenterol, 1985. **20**(5): p. 595-601.

58. Levy, R.L., et al., *Intergenerational transmission of gastrointestinal illness behavior.* Am J Gastroenterol, 2000. **95**(2): p. 451-6.

59. Bijkerk, C.J., et al., *Systematic review: the role of different types of fibre in the treatment of irritable bowel syndrome.* Aliment Pharmacol Ther, 2004. **19**(3): p. 245-51.

60. Cann, P.A., et al., *Irritable bowel syndrome: relationship of disorders in the transit of a single solid meal to symptom patterns.* Gut, 1983. **24**(5): p. 405-11.

61. Spiller, R., et al., *Guidelines on the irritable bowel syndrome: mechanisms and practical management.* Gut, 2007. **56**(12): p. 1770-98.

62. Rao, S., et al., *A 12-Week, Randomized, Controlled Trial With a 4-Week Randomized Withdrawal Period to Evaluate the Efficacy and Safety of Linaclotide in Irritable Bowel Syndrome With Constipation.* Am J Gastroenterol, 2012.

63. Lembo, A.J., et al., *Two randomized trials of linaclotide for chronic constipation.* N Engl J Med, 2011. **365**(6): p. 527-36.

64. Drossman, D.A., et al., *Clinical trial: lubiprostone in patients with constipation-associated irritable bowel syndrome--results of two randomized, placebo-controlled studies.* Aliment Pharmacol Ther, 2009. **29**(3): p. 329-41.

65. Ford, A.C., et al., *Efficacy of antidepressants and psychological therapies in irritable bowel syndrome: systematic review and meta-analysis.* Gut, 2009. **58**(3): p. 367-78.

66. Pimentel, M., et al., *Rifaximin therapy for patients with irritable bowel syndrome without constipation.* N Engl J Med, 2011. **364**(1): p. 22-32.

67. Camilleri, M., et al., *A placebo-controlled trial of prucalopride for severe chronic constipation.* N Engl J Med, 2008. **358**(22): p. 2344-54.

68. Corinaldesi, R., et al., *Effect of mesalazine on mucosal immune biomarkers in irritable bowel syndrome: a randomized controlled proof-of-concept study.* Aliment Pharmacol Ther, 2009. **30**(3): p. 245-52.

69. Dorofeyev, A.E., et al., *Clinical, endoscopical and morphological efficacy of mesalazine in patients with irritable bowel syndrome.* Clin Exp Gastroenterol, 2011. **4**: p. 141-53.

70. Sweetser, S., et al., *Do corticotropin releasing factor-1 receptors influence colonic transit and bowel function in women with irritable bowel syndrome?* Am J Physiol Gastrointest Liver Physiol, 2009. **296**(6): p. G1299-306.

71. Matsueda, K., et al., *A randomized, double-blind, placebo-controlled clinical trial of the effectiveness of the novel serotonin type 3 receptor antagonist ramosetron in both male and female Japanese patients with diarrhea-predominant irritable bowel syndrome.* Scand J Gastroenterol, 2008. **43**(10): p. 1202-11.

72. Matsueda, K., et al., *A phase II trial of the novel serotonin type 3 receptor antagonist ramosetron in Japanese male and female patients with diarrhea-predominant irritable bowel syndrome.* Digestion, 2008. **77**(3-4): p. 225-35.

73. Cremonini, F., S. Delgado-Aros, and M. Camilleri, *Efficacy of alosetron in irritable bowel syndrome: a meta-analysis of randomized controlled trials.* Neurogastroenterol Motil, 2003. **15**(1): p. 79-86.

74.   Chang, L., et al., *A dose-ranging, phase II study of the efficacy and safety of alosetron in men with diarrhea-predominant IBS.* Am J Gastroenterol, 2005. **100**(1): p. 115-23.

75.   Camilleri, M., et al., *Serotonin-transporter polymorphism pharmacogenetics in diarrhea-predominant irritable bowel syndrome.* Gastroenterology, 2002. **123**(2): p. 425-32.

76.   Grundy, D., P. McLean, and R. Stead, *Impact of 5-HT3 receptor blockade on colonic haemodynamic responses to ischaemia and reperfusion in the rat.* Neurogastroenterol Motil, 2007. **19**(7): p. 607-16.

77.   Painsipp, E., A. Shahbazian, and P. Holzer, *Alosetron, cilansetron and tegaserod modify mesenteric but not colonic blood flow in rats.* Br J Pharmacol, 2009. **158**(5): p. 1210-26.

78.   Emmanuel, A.V. and M.A. Kamm, *Laser Doppler flowmetry as a measure of extrinsic colonic innervation in functional bowel disease.* Gut, 2000. **46**(2): p. 212-7.

79.   Talley, N.J., et al., *GR 38032F (ondansetron), a selective 5HT3 receptor antagonist, slows colonic transit in healthy man.* Dig Dis Sci, 1990. **35**(4): p. 477-80.

80.   Steadman, C.J., et al., *Selective 5-hydroxytryptamine type 3 receptor antagonism with ondansetron as treatment for diarrhea-predominant irritable bowel syndrome: a pilot study.* Mayo Clin Proc, 1992. **67**(8): p. 732-8.

81.   Scolapio, J.S., et al., *Ascending colon response to feeding: evidence for a 5-hydroxytryptamine-3 mechanism.* Scand J Gastroenterol, 1995. **30**(6): p. 562-7.

82.   Zighelboim, J., et al., *Visceral perception in irritable bowel syndrome. Rectal and gastric responses to distension and serotonin type 3 antagonism.* Dig Dis Sci, 1995. **40**(4): p. 819-27.

83.   Maxton, D.G., J. Morris, and P.J. Whorwell, *Selective 5-hydroxytryptamine antagonism: a role in irritable bowel syndrome and functional dyspepsia?* Aliment Pharmacol Ther, 1996. **10**(4): p. 595-9.

84.   Lembo, A.J., et al., *Use of serum biomarkers in a diagnostic test for irritable bowel syndrome.* Aliment Pharmacol Ther, 2009. **29**(8): p. 834-42.

85.   Kellow, J.E. and S.F. Phillips, *Altered small bowel motility in irritable bowel syndrome is correlated with symptoms.* Gastroenterology, 1987. **92**(6): p. 1885-93.

86.   Rao, S.S., et al., *Investigation of colonic and whole-gut transit with wireless motility capsule and radiopaque markers in constipation.* Clin Gastroenterol Hepatol, 2009. **7**(5): p. 537-44.

87.   Metcalf, A.M., et al., *Simplified assessment of segmental colonic transit.* Gastroenterology, 1987. **92**(1): p. 40-7.

88.   Horikawa, Y., et al., *Gastrointestinal motility in patients with irritable bowel syndrome studied by using radiopaque markers.* Scand J Gastroenterol, 1999. **34**(12): p. 1190-5.

89.   Mayer, E.A., et al., *Functional GI disorders: from animal models to drug development.* Gut, 2008. **57**(3): p. 384-404.

90.   Deiteren, A., et al., *Performance characteristics of scintigraphic colon transit measurement in health and irritable bowel syndrome and relationship to bowel functions.* Neurogastroenterol Motil, 2010. **22**(4): p. 415-23, e95.

91.   Camilleri, M., et al., *Effect of renzapride on transit in constipation-predominant irritable bowel syndrome.* Clin Gastroenterol Hepatol, 2004. **2**(10): p. 895-904.

92.   Camilleri, M. and L. Chang, *Challenges to the therapeutic pipeline for irritable bowel syndrome: end points and regulatory hurdles.* Gastroenterology, 2008. **135**(6): p. 1877-91.

93.   Salonen, A., W.M. de Vos, and A. Palva, *Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives.* Microbiology, 2010. **156**(Pt 11): p. 3205-15.

94.   Gecse, K., et al., *Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity.* Gut, 2008. **57**(5): p. 591-9.

95.   Alfieri, A.B. and L.X. Cubeddu, *Effects of inhibition of serotonin synthesis on 5-hydroxyindoleacetic acid excretion, in healthy subjects.* J Clin Pharmacol, 1994. **34**(2): p. 153-7.

96.   Bulbring, E. and A. Crema, *Observations concerning the action of 5-hydroxytryptamine on the peristaltic reflex.* Br J Pharmacol Chemother, 1958. **13**(4): p. 444-57.

97.   Faerber, L., et al., *The neuronal 5-HT3 receptor network after 20 years of research--evolving concepts in management of pain and inflammation.* Eur J Pharmacol, 2007. **560**(1): p. 1-8.

98.   Tecott, L.H., A.V. Maricq, and D. Julius, *Nervous system distribution of the serotonin 5-HT3 receptor mRNA.* Proc Natl Acad Sci U S A, 1993. **90**(4): p. 1430-4.

99.   Lecrubier, Y., et al., *A randomized double-blind placebo-controlled study of tropisetron in the treatment of outpatients with generalized anxiety disorder.* Psychopharmacology (Berl), 1993. **112**(1): p. 129-33.

100.   Johnson, B.A., et al., *Ondansetron reduces the craving of biologically predisposed alcoholics.* Psychopharmacology (Berl), 2002. **160**(4): p. 408-13.

101.   Khan, N.A. and A. Hichami, *Ionotrophic 5-hydroxytryptamine type 3 receptor activates the protein kinase C-dependent phospholipase D pathway in human T-cells.* Biochem J, 1999. **344 Pt 1**: p. 199-204.

102.   Mousavizadeh, K., et al., *Anti-inflammatory effects of 5-HT receptor antagonist, tropisetron on experimental colitis in rats.* Eur J Clin Invest, 2009. **39**(5): p. 375-83.

103.   Vega Lde, L., et al., *The 5-HT3 receptor antagonist tropisetron inhibits T cell activation by targeting the calcineurin pathway.* Biochem Pharmacol, 2005. **70**(3): p. 369-80.

104.   Saria, A., et al., *5-HT3 receptor antagonists inhibit sensory neuropeptide release from the rat spinal cord.* Neuroreport, 1990. **1**(2): p. 104-6.

105.   Loisy, C., et al., *Effects on migraine headache of MDL 72,222, an antagonist at neuronal 5-HT receptors. Double-blind, placebo-controlled study.* Cephalalgia, 1985. **5**(2): p. 79-82.

106.   McCleane, G.J., R. Suzuki, and A.H. Dickenson, *Does a single intravenous injection of the 5HT3 receptor antagonist ondansetron have an analgesic effect in neuropathic pain? A double-blinded, placebo-controlled cross-over study.* Anesth Analg, 2003. **97**(5): p. 1474-8.

107.   Piche, T., et al., *Effect of ondansetron, a 5-HT3 receptor antagonist, on fatigue in chronic hepatitis C: a randomised, double blind, placebo controlled study.* Gut, 2005. **54**(8): p. 1169-73.

108.   Bearcroft, C.P., D. Perrett, and M.J. Farthing, *Postprandial plasma 5-hydroxytryptamine in diarrhoea predominant irritable bowel syndrome: a pilot study.* Gut, 1998. **42**(1): p. 42-6.

109. Atkinson, W., et al., *Altered 5-hydroxytryptamine signaling in patients with constipation- and diarrhea-predominant irritable bowel syndrome.* Gastroenterology, 2006. **130**(1): p. 34-43.

110. Dunlop, S.P., et al., *Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome.* Clin Gastroenterol Hepatol, 2005. **3**(4): p. 349-57.

111. Houghton, L.A., et al., *Increased platelet depleted plasma 5-hydroxytryptamine concentration following meal ingestion in symptomatic female subjects with diarrhoea predominant irritable bowel syndrome.* Gut, 2003. **52**(5): p. 663-70.

112. Clarke, G., et al., *Tryptophan degradation in irritable bowel syndrome: evidence of indoleamine 2,3-dioxygenase activation in a male cohort.* BMC Gastroenterol, 2009. **9**: p. 6.

113. Christmas, D.M., et al., *Increased serum free tryptophan in patients with diarrhea-predominant irritable bowel syndrome.* Nutr Res, 2010. **30**(10): p. 678-88.

114. Tack, J., et al., *Influence of acute serotonin reuptake inhibition on colonic sensorimotor function in man.* Aliment Pharmacol Ther, 2006. **23**(2): p. 265-74.

115. Chen, J.J., et al., *Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters.* J Neurosci, 2001. **21**(16): p. 6348-61.

116. Saito, Y.A., et al., *The genetics of irritable bowel syndrome.* Clin Gastroenterol Hepatol, 2005. **3**(11): p. 1057-65.

117. Kim, H.J., et al., *Association of distinct alpha(2) adrenoceptor and serotonin transporter polymorphisms with constipation and somatic symptoms in functional gastrointestinal disorders.* Gut, 2004. **53**(6): p. 829-37.

118. Yeo, A., et al., *Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women.* Gut, 2004. **53**(10): p. 1452-8.

119. Jarrett, M.E., et al., *Relationship of SERT polymorphisms to depressive and anxiety symptoms in irritable bowel syndrome.* Biol Res Nurs, 2007. **9**(2): p. 161-9.

120. Bellini, M., et al., *Platelet serotonin transporter in patients with diarrhea-predominant irritable bowel syndrome both before and after treatment with alosetron.* Am J Gastroenterol, 2003. **98**(12): p. 2705-11.

121. Foley, S., et al., *Impaired Uptake of Serotonin by Platelets from Patients with Irritable Bowel Syndrome Correlates with Duodenal Immune Activation.* Gastroenterology, 2011.

122. Coates, M.D., et al., *Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome.* Gastroenterology, 2004. **126**(7): p. 1657-64.

123. Camilleri, M., et al., *Alterations in expression of p11 and SERT in mucosal biopsy specimens of patients with irritable bowel syndrome.* Gastroenterology, 2007. **132**(1): p. 17-25.

124. Sjolund, K., et al., *Endocrine cells in human intestine: an immunocytochemical study.* Gastroenterology, 1983. **85**(5): p. 1120-30.

125. Dunlop, S.P., D. Jenkins, and R.C. Spiller, *Distinctive clinical, psychological, and histological features of postinfective irritable bowel syndrome.* Am J Gastroenterol, 2003. **98**(7): p. 1578-83.

126. Wheatcroft, J., et al., *Enterochromaffin cell hyperplasia and decreased serotonin transporter in a mouse model of postinfectious bowel dysfunction.* Neurogastroenterol Motil, 2005. **17**(6): p. 863-70.

127. Shen, B., et al., *Enterochromaffin cell hyperplasia in irritable pouch syndrome.* Am J Gastroenterol, 2008. **103**(9): p. 2293-300.

128. Motomura, Y., et al., *Enterochromaffin cell and 5-hydroxytryptamine responses to the same infectious agent differ in Th1 and Th2 dominant environments.* Gut, 2008. **57**(4): p. 475-81.

129. Bercik, P., et al., *Visceral hyperalgesia and intestinal dysmotility in a mouse model of postinfective gut dysfunction.* Gastroenterology, 2004. **127**(1): p. 179-87.

130. Keating, C., et al., *Afferent hypersensitivity in a mouse model of post-inflammatory gut dysfunction: role of altered serotonin metabolism.* J Physiol, 2008. **586**(Pt 18): p. 4517-30.

131. Miwa, J., et al., *Patients with constipation-predominant irritable bowel syndrome (IBS) may have elevated serotonin concentrations in colonic mucosa as compared with diarrhea-predominant patients and subjects with normal bowel habits.* Digestion, 2001. **63**(3): p. 188-94.

132. Costedio, M.M., et al., *Serotonin signaling in diverticular disease.* J Gastrointest Surg, 2008. **12**(8): p. 1439-45.

133. Hedstrom, L., *Serine protease mechanism and specificity.* Chem Rev, 2002. **102**(12): p. 4501-24.

134. Bustos, D., et al., *Colonic proteolysis following pancreatic duct ligation in the rat.* Int J Pancreatol, 1994. **16**(1): p. 45-9.

135. Macfarlane, G.T., et al., *Contribution of the microflora to proteolysis in the human large intestine.* J Appl Bacteriol, 1988. **64**(1): p. 37-46.

136. Gibson, S.A. and G.T. Macfarlane, *Studies on the proteolytic activity of Bacteroides fragilis.* J Gen Microbiol, 1988. **134**(1): p. 19-27.

137. Ossovskaya, V.S. and N.W. Bunnett, *Protease-activated receptors: contribution to physiology and disease.* Physiol Rev, 2004. **84**(2): p. 579-621.

138. Cenac, N., et al., *Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2.* Am J Pathol, 2002. **161**(5): p. 1903-15.

139. Cenac, N., et al., *Proteinase-activated receptor-2-induced colonic inflammation in mice: possible involvement of afferent neurons, nitric oxide, and paracellular permeability.* J Immunol, 2003. **170**(8): p. 4296-300.

140. Jacob, C., et al., *Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins.* J Biol Chem, 2005. **280**(36): p. 31936-48.

141. Cenac, N., et al., *PAR2 activation alters colonic paracellular permeability in mice via IFN-gamma-dependent and -independent pathways.* J Physiol, 2004. **558**(Pt 3): p. 913-25.

142. Vergnolle, N., et al., *Proteinase-activated receptor-2 and hyperalgesia: A novel pain pathway.* Nat Med, 2001. **7**(7): p. 821-6.

143. Cenac, N., et al., *Role for protease activity in visceral pain in irritable bowel syndrome.* J Clin Invest, 2007. **117**(3): p. 636-47.

144. Hamm, L.R., et al., *Additional investigations fail to alter the diagnosis of irritable bowel syndrome in subjects fulfilling the Rome criteria.* Am J Gastroenterol, 1999. **94**(5): p. 1279-82.

145. Francis, C.Y., et al., *Does routine abdominal ultrasound enhance diagnostic accuracy in irritable bowel syndrome?* Am J Gastroenterol, 1996. **91**(7): p. 1348-50.

146. Accarino, A., et al., *Abdominal distention results from caudo-ventral redistribution of contents.* Gastroenterology, 2009. **136**(5): p. 1544-51.

147. Hoad, C.L., et al., *Non-invasive quantification of small bowel water content by MRI: a validation study.* Phys Med Biol, 2007. **52**(23): p. 6909-22.

148. Marciani, L., et al., *Postprandial changes in small bowel water content in healthy subjects and patients with irritable bowel syndrome.* Gastroenterology, 2010. **138**(2): p. 469-77, 477 e1.

149. Froehlich, J.M., et al., *Small bowel motility assessment with magnetic resonance imaging.* J Magn Reson Imaging, 2005. **21**(4): p. 370-5.

150. Froehlich, J.M., et al., *Aperistaltic effect of hyoscine N-butylbromide versus glucagon on the small bowel assessed by magnetic resonance imaging.* Eur Radiol, 2009. **19**(6): p. 1387-93.

151. Placidi, E., et al., *The effects of loperamide, or loperamide plus simethicone, on the distribution of gut water as assessed by MRI in a mannitol model of secretory diarrhoea.* Aliment Pharmacol Ther, 2012. **36**(1): p. 64-73.

152. Lordal, M. and P.M. Hellstrom, *Serotonin stimulates migrating myoelectric complex via 5-HT3-receptors dependent on cholinergic pathways in rat small intestine.* Neurogastroenterol Motil, 1999. **11**(1): p. 1-10.

153. Marciani, L., et al., *Effects of a 5-HT(3) antagonist, ondansetron, on fasting and postprandial small bowel water content assessed by magnetic resonance imaging.* Aliment Pharmacol Ther, 2010. **32**(5): p. 655-63.

154. Ford, A.C. and P. Moayyedi, *Meta-analysis: factors affecting placebo response rate in the irritable bowel syndrome.* Aliment Pharmacol Ther, 2010. **32**(2): p. 144-58.

155. Irvine, E.J., et al., *Design of treatment trials for functional gastrointestinal disorders.* Gastroenterology, 2006. **130**(5): p. 1538-51.

156. Camilleri, M., et al., *A randomized controlled clinical trial of the serotonin type 3 receptor antagonist alosetron in women with diarrhea-predominant irritable bowel syndrome.* Arch Intern Med, 2001. **161**(14): p. 1733-40.

157. Julious, S., *Sample size redetermination for repeated measures studies.* Biometrics, 2004. **60**(1): p. 284; author reply 284-5.

158. Bjelland, I., et al., *The validity of the Hospital Anxiety and Depression Scale. An updated literature review.* J Psychosom Res, 2002. **52**(2): p. 69-77.

159. Kroenke, K., R.L. Spitzer, and J.B. Williams, *The PHQ-15: validity of a new measure for evaluating the severity of somatic symptoms.* Psychosom Med, 2002. **64**(2): p. 258-66.

160. Cohen, S., T. Kamarck, and R. Mermelstein, *A global measure of perceived stress.* J Health Soc Behav, 1983. **24**(4): p. 385-96.

161. Hahn, B.A., et al., *Evaluation of a new quality of life questionnaire for patients with irritable bowel syndrome.* Aliment Pharmacol Ther, 1997. **11**(3): p. 547-52.

162. McRobbie, D., *MRI from Picture to Proton*. 2005: Cambridge university press.

163. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.

164. Hellman, U., et al., *Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing.* Anal Biochem, 1995. **224**(1): p. 451-5.

165. Basseri, R.J., et al., *Importance of diarrhea in evaluating constipation in irritable bowel syndrome clinical studies.* J Clin Gastroenterol, 2011. **45**(9): p. 790-3.

166. Leigh, R.J. and L.A. Turnberg, *Faecal incontinence: the unvoiced symptom.* Lancet, 1982. **1**(8285): p. 1349-51.

167. Francis, C.Y., J. Morris, and P.J. Whorwell, *The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress.* Aliment Pharmacol Ther, 1997. **11**(2): p. 395-402.

168. (CDER), U.S.D.o.H.a.H.S.F.a.D.A.C.f.D.E.a.R., *Guidance for Industry Irritable Bowel Syndrome-Clinical evaluation of Drugs for Treatment*, U.S.D.o.H.a.H.S.F.a.D.A.C.f.D.E.a.R. (CDER), Editor. 2012.

169. Coletta, M., et al., *Discrepancy between recalled and recorded bowel habits in irritable bowel syndrome.* Aliment Pharmacol Ther, 2010. **32**(2): p. 282-8.

170. Schoenfeld, P. and N.J. Talley, *Measuring successful treatment of irritable bowel syndrome: is "satisfactory relief " enough?* Am J Gastroenterol, 2006. **101**(5): p. 1066-8.

171. Passos, M.C., et al., *Adequate relief in a treatment trial with IBS patients: a prospective assessment.* Am J Gastroenterol, 2009. **104**(4): p. 912-9.

172. Bharucha, A.E., et al., *Insights into normal and disordered bowel habits from bowel diaries.* Am J Gastroenterol, 2008. **103**(3): p. 692-8.

173. Schiller, L.R., et al., *Mechanism of the antidiarrheal effect of loperamide.* Gastroenterology, 1984. **86**(6): p. 1475-80.

174. Efskind, P.S., T. Bernklev, and M.H. Vatn, *A double-blind placebo-controlled trial with loperamide in irritable bowel syndrome.* Scand J Gastroenterol, 1996. **31**(5): p. 463-8.

175. Lavo, B., M. Stenstam, and A.L. Nielsen, *Loperamide in treatment of irritable bowel syndrome--a double-blind placebo controlled study.* Scand J Gastroenterol Suppl, 1987. **130**: p. 77-80.

176. Wiklund, I.K., et al., *An irritable bowel syndrome-specific symptom questionnaire: development and validation.* Scand J Gastroenterol, 2003. **38**(9): p. 947-54.

177. Cremonini, F., et al., *Randomised clinical trial: alosetron improves quality of life and reduces restriction of daily activities in women with severe diarrhoea-predominant IBS.* Aliment Pharmacol Ther, 2012. **36**(5): p. 437-48.

178. Kilpatrick, G.J., B.J. Jones, and M.B. Tyers, *Binding of the 5-HT3 ligand, [3H]GR65630, to rat area postrema, vagus nerve and the brains of several species.* Eur J Pharmacol, 1989. **159**(2): p. 157-64.

179. Moss, H.E. and G.J. Sanger, *The effects of granisetron, ICS 205-930 and ondansetron on the visceral pain reflex induced by duodenal distension.* Br J Pharmacol, 1990. **100**(3): p. 497-501.

180. Goldberg, P.A., et al., *Modification of visceral sensitivity and pain in irritable bowel syndrome by 5-HT3 antagonism (ondansetron).* Digestion, 1996. **57**(6): p. 478-83.

181. Moore, N.A., et al., *Partial agonism of 5-HT3 receptors: a novel approach to the symptomatic treatment of IBS-D.* ACS Chem Neurosci, 2013. **4**(1): p. 43-7.

182. Muller-Lissner, S.A., et al., *Tegaserod, a 5-HT(4) receptor partial agonist, relieves symptoms in irritable bowel syndrome patients with abdominal pain, bloating and constipation.* Aliment Pharmacol Ther, 2001. **15**(10): p. 1655-66.

183. Wilmer, A., et al., *Effect of the 5-HT3 receptor antagonist ondansetron on gastric accomodation and on perception of gastric distension after a meal.* Gastroenterology, 1996. **110**(4): p. A781-A781.

184. Bjornsson, E.S., et al., *Differential 5-HT3 mediation of human gastrocolonic response and colonic peristaltic reflex.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 1998. **275**(3): p. G498-G505.

185. Agrawal, A. and P.J. Whorwell, *Review article: abdominal bloating and distension in functional gastrointestinal disorders--epidemiology and exploration of possible mechanisms.* Aliment Pharmacol Ther, 2008. **27**(1): p. 2-10.

186. Ford, A.C. and P.O. Vandvik, *Irritable bowel syndrome.* Clin Evid (Online), 2010. **2010**.

187. Chang, L., et al., *Incidence of ischemic colitis and serious complications of constipation among patients using alosetron: systematic review of clinical trials and post-marketing surveillance data.* Am J Gastroenterol, 2006. **101**(5): p. 1069-79.

188. Krause, R., et al., *A randomized, double-blind, placebo-controlled study to assess efficacy and safety of 0.5 mg and 1 mg alosetron in women with severe diarrhea-predominant IBS.* Am J Gastroenterol, 2007. **102**(8): p. 1709-19.

189. Chang, L., K. Tong, and V. Ameen, *Ischemic colitis and complications of constipation associated with the use of alosetron under a risk management plan: clinical characteristics, outcomes, and incidences.* Am J Gastroenterol, 2010. **105**(4): p. 866-75.

190. Clayton, N.M., et al., *The pharmacological properties of the novel selective 5-HT3 receptor antagonist, alosetron, and its effects on normal and perturbed small intestinal transit in the fasted rat.* Neurogastroenterol Motil, 1999. **11**(3): p. 207-17.

191. Hirata, T., et al., *Evaluation of the pharmacological profile of ramosetron, a novel therapeutic agent for irritable bowel syndrome.* J Pharmacol Sci, 2007. **104**(3): p. 263-73.

192. Jackson, J.L., et al., *Treatment of functional gastrointestinal disorders with antidepressant medications: a meta-analysis.* Am J Med, 2000. **108**(1): p. 65-72.

193. Talley, N.J., et al., *Antidepressant therapy (imipramine and citalopram) for irritable bowel syndrome: a double-blind, randomized, placebo-controlled trial.* Dig Dis Sci, 2008. **53**(1): p. 108-15.

194. Quartero, A.O., et al., *Bulking agents, antispasmodic and antidepressant medication for the treatment of irritable bowel syndrome.* Cochrane Database Syst Rev, 2005(2): p. CD003460.

195. Clouse, R.E., et al., *Antidepressant therapy in 138 patients with irritable bowel syndrome: a five-year clinical experience.* Aliment Pharmacol Ther, 1994. **8**(4): p. 409-16.

196. Caras s, K.G., Biesheuvel E, Steinborn C, *cilansetron shows efficacy in males and female non-constipated patients with irritable bowel syndrome in a united states study.* Gastroenterology, 2001. **120**: p. a217.

197. Houghton, L.A., J.M. Foster, and P.J. Whorwell, *Alosetron, a 5-HT3 receptor antagonist, delays colonic transit in patients with irritable bowel syndrome and healthy volunteers.* Aliment Pharmacol Ther, 2000. **14**(6): p. 775-82.

198. Bush, T.G., et al., *Effects of alosetron on spontaneous migrating motor complexes in murine small and large bowel in vitro.* Am J Physiol Gastrointest Liver Physiol, 2001. **281**(4): p. G974-83.

199. Prior, A. and N.W. Read, *Reduction of rectal sensitivity and post-prandial motility by granisetron, a 5 HT3-receptor antagonist, in patients with irritable bowel syndrome.* Aliment Pharmacol Ther, 1993. **7**(2): p. 175-80.

200. Shim, L., et al., *Stool characteristics and colonic transit in irritable bowel syndrome: evaluation at two time points.* Scand J Gastroenterol, 2013. **48**(3): p. 295-301.

201.   Lordal, M., et al., *Concentration-dependent stimulation of intestinal phase III of migrating motor complex by circulating serotonin in humans.* Clin Sci (Lond), 1998. **94**(6): p. 663-70.

202.   Gorard, D.A., G.W. Libby, and M.J. Farthing, *5-Hydroxytryptamine and human small intestinal motility: effect of inhibiting 5-hydroxytryptamine reuptake.* Gut, 1994. **35**(4): p. 496-500.

203.   Gore, S., et al., *Colonic transit in man is slowed by ondansetron (GR38032F), a selective 5-hydroxytryptamine receptor (type 3) antagonist.* Aliment Pharmacol Ther, 1990. **4**(2): p. 139-44.

204.   Spiller, R. and K. Garsed, *Postinfectious irritable bowel syndrome.* Gastroenterology, 2009. **136**(6): p. 1979-88.

205.   Lesch, K.P., et al., *Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region.* Science, 1996. **274**(5292): p. 1527-31.

206.   Van Kerkhoven, L.A., R.J. Laheij, and J.B. Jansen, *Meta-analysis: a functional polymorphism in the gene encoding for activity of the serotonin transporter protein is not associated with the irritable bowel syndrome.* Aliment Pharmacol Ther, 2007. **26**(7): p. 979-86.

207.   Camilleri, M., et al., *Candidate genes and sensory functions in health and irritable bowel syndrome.* Am J Physiol Gastrointest Liver Physiol, 2008. **295**(2): p. G219-25.

208.   Colucci, R., et al., *Influence of the serotonin transporter 5HTTLPR polymorphism on symptom severity in irritable bowel syndrome.* PLoS One, 2013. **8**(2): p. e54831.

209.   Caspi, A., et al., *Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene.* Science, 2003. **301**(5631): p. 386-9.

210.   Greenberg, B.D., et al., *Genetic variation in the serotonin transporter promoter region affects serotonin uptake in human blood platelets.* Am J Med Genet, 1999. **88**(1): p. 83-7.

211.   Javors, M.A., et al., *Platelet serotonin uptake and paroxetine binding among allelic genotypes of the serotonin transporter in alcoholics.* Prog Neuropsychopharmacol Biol Psychiatry, 2005. **29**(1): p. 7-13.

212.   Lesch, K.P., et al., *Primary structure of the human platelet serotonin uptake site: identity with the brain serotonin transporter.* J Neurochem, 1993. **60**(6): p. 2319-22.

213.   Bazzichi, L., et al., *Alteration of serotonin transporter density and activity in fibromyalgia.* Arthritis Res Ther, 2006. **8**(4): p. R99.

214.   Koshikawa, N., et al., *Expression of trypsin by epithelial cells of various tissues, leukocytes, and neurons in human and mouse.* Am J Pathol, 1998. **153**(3): p. 937-44.

215.   Cottrell, G.S., et al., *Trypsin IV, a novel agonist of protease-activated receptors 2 and 4.* J Biol Chem, 2004. **279**(14): p. 13532-9.

216.   Kerckhoffs, A.P., et al., *Trypsinogen IV, serotonin transporter transcript levels and serotonin content are increased in small intestine of irritable bowel syndrome patients.* Neurogastroenterol Motil, 2008. **20**(8): p. 900-7.

217.   Barbara, G., et al., *Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome.* Gastroenterology, 2007. **132**(1): p. 26-37.

218.   Buhner, S., et al., *Activation of human enteric neurons by supernatants of colonic biopsy specimens from patients with irritable bowel syndrome.* Gastroenterology, 2009. **137**(4): p. 1425-34.

219. Klooker, T.K., et al., *The mast cell stabiliser ketotifen decreases visceral hypersensitivity and improves intestinal symptoms in patients with irritable bowel syndrome.* Gut, 2010. **59**(9): p. 1213-21.

220. Bohe, M., et al., *Determination of immunoreactive trypsin, pancreatic elastase and chymotrypsin in extracts of human feces and ileostomy drainage.* Digestion, 1983. **27**(1): p. 8-15.

221. Genell, S. and B.E. Gustafsson, *Impaired enteric degradation of pancreatic endopeptidases in antibiotic-treated rats.* Scand J Gastroenterol, 1977. **12**(7): p. 801-9.

222. Borgstrom, A., S. Genell, and K. Ohlsson, *Elevated fecal levels of endogenous pancreatic endopeptidases after antibiotic treatment.* Scand J Gastroenterol, 1977. **12**(5): p. 525-9.

223. Goldberg, D.M., *Proteases in the evaluation of pancreatic function and pancreatic disease.* Clin Chim Acta, 2000. **291**(2): p. 201-21.

224. Zhou, Q., B. Zhang, and G.N. Verne, *Intestinal membrane permeability and hypersensitivity in the irritable bowel syndrome.* Pain, 2009. **146**(1-2): p. 41-6.

225. Goldberg PA, Kamm MA, Setti-Carraro P, van der Sijp JR, Roth C, *Modification of visceral sensitivity and pain in irritable bowel syndrome by 5-HT3 antagonism (ondansetron).* Digestion. 1996 **57**(6):478-83.

226. Pritchard SE, Marciani L, Garsed KC, Hoad CL, Thongborisute W, Roberts E, Gowland PA, Spiller RC, *Fasting and postprandial volumes of the undisturbed colon: normal values and changes in diarrhea-predominant irritale bowel syndrome measured using serial MRI.* Neurogastroenterol Motil. 2014 **26**(1):124-30.

227. Tooth D, Garsed K, Singh G, Marciani L, Lam C, Fordham I, Fields A, Banwait R, Lingaya M, Layfield R, Hastings M, Whorwell P, Spiller R. *Characterisation of faecal protease activity in irritable bowel syndrome with diarrhoea:origin and effect of gut transit.* Gut. 2014 **63**(5): 753-60.