BIOMARKERS TO ASSESS AN ANTI-INFLAMMATORY TREATMENT FOR IRRITABLE BOWEL SYNDROME: MAST CELL ASSAYS AND MAGNETIC RESONANCE IMAGING

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Abstract

Irritable bowel syndrome (IBS) remains a heterogeneous condition and is a common condition. The causes of IBS remain poorly understood and there is a lack in biomarkers to distinguish this condition.

Recently, there have been reports on the release of immune mediators leading to symptoms of irritable bowel syndrome. Mast cells, which can be activated by allergy or stress, are thought to be important cause of symptoms in some IBS patients because they can release chemicals, which cause pain and diarrhoea. Currently, there are few effective treatments available to alleviate these symptoms. Recent small studies have shown that Mesalazine, an 'anti-inflammatory' drug, may be able to modify and reverse the symptoms of IBS with diarrhoea. One small study suggested Mesalazine reduced mast cell numbers. This current study is one of the largest studies looking at the use of Mesalazine as a form of treatment for IBS with diarrhoea. Unfortunately, this study did not show any beneficial effect of Mesalazine treatment in unselected patients with IBS and diarrhoea. Potentially, there is a subgroup of IBS patients who developed their symptoms following a bout of gastroenteritis who appeared to benefit from Mesalazine treatment but a larger study is needed to confirm this. In this study, the mast cell mediators released from mucosal biopsies was not a useful marker of disease since it failed to correlate with any symptoms.

Magnetic resonance imaging (MRI) is a potentially useful tool to assess the physiology of the gastrointestinal tract in patients with functional gut disorders as it does not involve radiation and is not invasive. So far, there is a lack of biomarkers to

assist in diagnosis and treatment of irritable bowel syndrome. The MRI marker pill used in the multiple studies in Chapter 3 to assess whole gut transit time is very promising as it is now applied, in the research setting, to patients with chronic constipation such as slow transit constipation and irritable bowel syndrome with constipation. Further use of the MRI and adding a stimulus such as laxative in patients with chronic constipation is helpful to distinguish between functional constipation and irritable bowel syndrome with constipation; thus helping with its medical management. The use of MRI as a biomarker for diagnosis of irritable bowel syndrome remains promising although it was not demonstrated in this thesis.

List of publications

Articles in peer reviewed conference:

1. **C. Lam**, W. Tan, M. Leighton, J. Williams, A. Agrawal, S. Sen, S. Foley, M. Rutter, A. Ramadas, P. Whorwell, A. Montgomery, and R. Spiller, 'OC-069 Mesalazine for Treatment of Diarrhoea-Predominant Irritable Bowel Syndrome (IBS-D): A Multi Centre, Parallel Group, Randomised Placebo Controlled Trial', *Gut,* 63 (2014), A34. Oral presentation in the British Society of Gastroenterology 2014, Manchester, United Kingdom

2. **C. Lam**, G. Chaddock, C. Hoad, C. Costigan, E. Cox, S. Pritchard, K. Garsed, L. Marciani, P. Gowland, and R. Spiller, 'Pwe-161 the Macrogol Drink Test to Distinguish Functional Constipation (Fc) and Constipation Predominant Irritable Bowel Syndrome (IBS-C): Underlying Mechanisms Demonstrated Using Mri', *Gut,* 63 (2014), A195. Poster presentation in the British Society of Gastroenterology 2014, Manchester, United Kingdom.

3. **Ching Lam**, Wei Tan, Matthew Leighton, Jessica Williams, Anurag Agrawal, Sandip Sen, Stephen Foley, Matt Rutter, Arvind Ramadas, Peter J. Whorwell, Alan Montgomery, and Robin C. Spiller, '712 a Multi-Centre, Parallel Group, Randomised Placebo Controlled Trial of Mesalazine for Treatment of Diarrhoea-Predominant Irritable Bowel Syndrome (Ibs-D)', *Gastroenterology,* 146 (2014), S-123-S-24. Oral presentation in the Digestive Disease Week 2014, Chicago, United States of America.

4. **C. Lam**, G. Chaddock, K. Garsed, C. Hoad, C. Costigan, E. Cox, S. Pritchard, L. Marciani, P. Gowland, and R. Spiller, 'P1531 Increased Small Bowel and Colonic Volumes with Impaired Motility in Chronic Constipation (Cc): Significant Difference between Slow Transit Constipation (STC) and IBS with Constipation (IBS-C) Shown by Novel Mri Technique', *United European Gastroenterology Journal* 1 (2013), 412. Poster Presentation in UEGW Week 2013, Berlin, Germany.

5. **C. Lam**, D. Sanders, P. Lanyon, K. Garsed, S. Foley, S. Pritchard, L. Marciani, C. Hoad, C. Costigan, P. Gowland, and R. Spiller, 'Contrasting Changed in Small Bowel Water Content in Patients with Diarrhoea: Coeliac Disease and Scleroderma Versus IBS and Healthy Controls', *United European Gastroenterology Journal* 1 (2013), A108. Oral Presentation in UEGW Week 2013, Berlin, Germany.

6. **Ching Lam**, Gemma Chaddock, Caroline L. Hoad, Carolyn Costigan, Eleanor Cox, Luca Marciani, Penny A. Gowland, and Robin C. Spiller, 'A New Validated Whole Gut Transit Time (WGTT) Measurement Using Magnetic Resonance Imaging (Mri-WGTT) Technique', *Gut,* 62 (2013), A14. Poster presentation in the British Society of Gastroenterology 2013, Glasgow, United Kingdom

7. **Ching Lam**, Gemma Chaddock, Caroline L. Hoad, Carolyn Costigan, Eleanor Cox, Luca Marciani, Penny A. Gowland, and Robin C. Spiller, 'Tu2072 a New Validated Mri Method for Measuring Whole Gut Transit Time', *Gastroenterology,* 144 (2013), S-920. Oral presentation in the British Society of Gastroenterology 2013, Glasgow, United Kingdom

8. **Ching Lam**, Gemma Chaddock, Caroline L. Hoad, Carolyn Costigan, Eleanor Cox, Luca Marciani, Penny A. Gowland, and Robin C. Spiller, 'Tu2072 a New Validated MRI Method for Measuring Whole Gut Transit Time', *Gastroenterology,* 144 (2013), S-920. Poster presentation in the Digestive Disease Week 2013, Orlando, United States of America

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1. D. Tooth, K. Garsed, G. Singh, L. Marciani, **C. Lam**, I. Fordham, A. Fields, R. Banwait, M. Lingaya, R. Layfield, M. Hastings, P. Whorwell, and R. Spiller, 'Characterisation of Faecal Protease Activity in Irritable Bowel Syndrome with Diarrhoea: Origin and Effect of Gut Transit', *Gut,* 63 (2014), 753-60.

2. K. Murray, V. Wilkinson-Smith, C. Hoad, C. Costigan, E. Cox, **C. Lam**, L. Marciani, P. Gowland, and R. C. Spiller, 'Differential Effects of Fodmaps (Fermentable Oligo-, Di-, Mono-Saccharides and Polyols) on Small and Large Intestinal Contents in Healthy Subjects Shown by Mri', *Am J Gastroenterol,* 109 (2014), 110-9.

3. L. Marciani, K. C. Garsed, C. L. Hoad, A. Fields, I. Fordham, S. E. Pritchard, E. Placidi, K. Murray, G. Chaddock, C. Costigan, **C. Lam**, J. Jalanka-Tuovinen, W. M. De Vos, P. A. Gowland, and R. C. Spiller, 'Stimulation of Colonic Motility by Oral Peg Electrolyte Bowel Preparation Assessed by Mri: Comparison of Split Vs Single Dose', *Neurogastroenterol Motil* (2014).

4. G. Chaddock, **C. Lam**, C. L. Hoad, C. Costigan, E. F. Cox, E. Placidi, I. Thexton, J. Wright, P. E. Blackshaw, A. C. Perkins, L. Marciani, P. A. Gowland, and R. C. Spiller, 'Novel Mri Tests of Orocecal Transit Time and Whole Gut Transit Time: Studies in Normal Subjects', *Neurogastroenterol Motil,* 26 (2014), 205-14.

5. K. Garsed, J. Chernova, M. Hastings, **C. Lam**, L. Marciani, G. Singh, A. Henry, I. Hall, P. Whorwell, and R. Spiller, 'A Randomised Trial of Ondansetron for the Treatment of Irritable Bowel Syndrome with Diarrhoea', *Gut* (2013).

6. R. Spiller, and **C. Lam**, 'An Update on Post-Infectious Irritable Bowel Syndrome: Role of Genetics, Immune Activation, Serotonin and Altered Microbiome', *J Neurogastroenterol Motil,* 18 (2012), 258-68.

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Terms and Abbreviations

Term/Abbreviation	Definition
5-ASA	5-Aminosalicylic Acid
5HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin
BRU	Biomedical Research Unit
BSFS	Bristol Stool Form Scale
CACE	Complier Average Causal Effect
CC2	Chymase
CDC HRQOL4	Centres for Disease Control and Prevention Health-Related Quality of Life 4
CDH1	Cadherin 1
CI	Chief Investigator
COX-2	Cyclooxygenase-2
CPA3	Carboxypeptidase A3
CRF1	Corticotrophin Releasing Factor 1
CRH	Corticotrophin Releasing Hormone
CTU	Clinical Trials Unit
DMEC	Data Monitoring Committee
EAR	Tryptase
EC cells	Enterochromaffin Cells
ELISA	Enzyme-Linked Immunosorbent Assay
EOT	End of Trial
EQ-5D	Euro-QoL
FC	Functional Constipation
FFE	Fast Field Echo
FITC	Fluorescein Isothyocyanate
FODMAPs	Fermentable Oligo-Di and Mono-saccharides and Polyl hydric alcohols
GI	Gastrointestinal
H+E	Haematoxylin and Eosin
HADS	Hospital Anxiety and Depression Score
HPLC	High Performance Liquid Chromatography
HV	Healthy Volunteers
IBS-C	Irritable Bowel Syndrome (IBS) with Constipation
IBS-D	IBS with diarrhoea
IBS-M	Mixed IBS
ICC	Intra-Class Correlation Coefficient
IELs	Intra-Epithelial Lymphocytes
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IMP	Investigational Medicinal Product
IQR	Interquartile Range
IRIS	Infrared Isotope
ITT	Intention-To-Treat
LC-MS	Liquid Chromatography Mass-Spectrometry

LHBT	Lactulose Hydrogen Breath Test
LU	Lactose Ureide
LUBT	13C-Labelled Glycosyl Ureides
MCT	Mast Cell Tryptase
MI	Motility Index
MRI	Magnetic Resonance Imaging
NFκB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NMR	Nuclear Magnetic Resonance
OCTT	Orocaecal Transit Time
PEG	Polyethylene Glycol
PHQ12SS	Patient Health Questionnaire 12 Somatic Symptoms Scale
PHQ-15	Patient Health Questionnaire
PI	Principal Investigator
PI-IBS	Post-infectious irritable bowel syndrome
PIS	Participant Information Sheet
PPAR	Peroxisome Proliferator-Activated
ROM	Radio-Opaque Markers
SBWC	Small Bowel Water Content
SD	Standard Deviation
SD	Somatization Disorder
SI	Sensitivity Index
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedure
SPMMRC	Sir Peter Mansfield Magnetic Resonance Imaging
SPMRC	Sir Peter Mansfield Magnetic Resonance Centre
TFE	Turbo Field Echo
TLR9	Toll-Like Receptor 9
TNF-α	Tumour Necrosis Factor Alpha
TSE	Turbo Spin Echo
UK	United Kingdom
USA	United States of America
VAS	Visual Analogue Scale
WGTT	Whole Gut Transit Time
WMC	Wireless Motility Capsule

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1.1. Definition of Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a chronic condition. The key features are abdominal pain /discomfort together with erratic bowel habit. The absence of biomarkers or gold standards for diagnosis of IBS has inhibited many physicians and general practitioners from labelling a patient with the diagnosis of IBS. This has led to many unnecessary investigations as a means of achieving a diagnosis of exclusion, thus leading to a substantial burden to the National Health Service (NHS). Throughout the years, there have been multiple attempts to define IBS using a set of criteria. Later, an international consensus group has developed the Rome criteria based on positive symptoms to standardise recruitment of patients with IBS in research. Table 1 shows the development of IBS diagnostic criteria throughout the years.

Table 1: Development of IBS diagnostic criteria

Criteria	Diagnostic criteria	Year	Sensitivity	Specificity
Manning[1]	At least 2 of these symptoms with abdominal pain: • Abdominal pain relief by defecation • Loose stool associated with onset of pain • Pain relief by passage of stool • Abdominal bloating • Passing of mucus • Incomplete evacuation	1978	0.78[2]	0.72[2]
Kruis[3]	Combination of symptoms such as: (for more than 2 years) • Abdominal pain • Flatulence • Irregular bowel habit alternating between diarrhoea and constipation • Excluding symptoms suggesting other organic disease including per rectum bleeding	1984	0.77[2]	0.89[2]

	• Normal physical examination • Normal laboratory tests • Normal haemoglobin • Normal leukocyte count • Erythrocyte sedimentation rate			
Rome I [2]	At least 3 months of continuous abdominal pain or discomfort relieved by defecation or associated with changes in stool frequency or consistency and at least 2 of the following (on at least ≥25% of occasions): • Altered stool frequency • Altered stool consistency • Alteration in stool passage • Passing of mucus per rectum • Bloating or distension	1990	0.71[2]	0.85[2]
Rome II [4]	Abdominal pain or discomfort of at least 12 weeks with preceding symptoms for 12 months along with ≥2 of these features: • Relief with defecation • Onset associated with a change in frequency of stool • Onset associated with a change in consistency of stool	1999	0.69[5]	0.66[5]
Vanner [6]	Combination of Rome criteria AND red flag syndrome such as • Weight loss • Nocturnal symptoms • Blood mixed with stool • Recent antibiotics use • Abnormal physical examination • Family history of colon cancer	1999	0.78[6] PPV = 98%[6]	0.35[6]
Rome III[7]	Recurrent abdominal pain or discomfort at least 3 days/month in the last 3 months with symptom onset 6 months prior to diagnosis At least 2 or more of the following • Improved with defecation • Onset associated with change in frequency of stool • Onset associate with change in consistency of stool	2006	0.75[8]	0.80[9]

IBS is further sub typed using the Bristol Stool Form Scale (BSFS)[7] (Figure 1):

Diarrhoea predominant IBS (IBS-D): ≥25% stools having consistency of 6 or 7 and <25% stools with consistency 1 or 2

Constipation predominant IBS (IBS-C): ≥25% or more of stools have consistency of 1 or 2 and <25% stools with a consistency 6 or 7

Mixed IBS (IBS-M): ≥25% or more of stools have consistency of 6 or 7 and ≥25% stools have a consistency 1 or 2

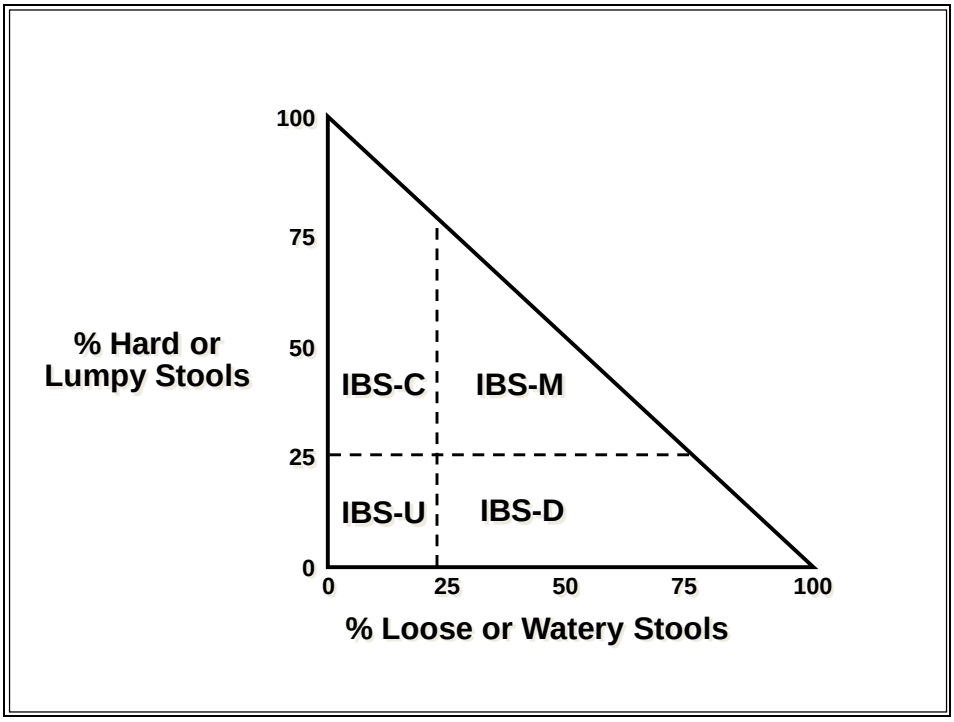


Figure 1: Subtyping IBS according to stool consistency

1.2. Epidemiology

IBS is a common chronic condition and accounts for up to 20% of gastroenterology referrals from the primary care to the secondary care in the United Kingdom (UK)[10]. In a large population survey by the Royal College of General Practitioners, 1 in 12 general practice consultations are due to digestive problems and up to 30% of all digestive problems are due to irritable bowel syndrome [11]. Most IBS patients are young to middle aged females. A recent study using the UK General Practice Research Database (GPRD) gave an overall female to male ratio of 3 to 1 in newly diagnosed IBS in primary care [12]. This suggested that females are more likely to report symptoms during consultations compared to males. The incidence of new IBS peaks in the 30's and 40's with only a few new diagnoses of IBS in the older age groups (Figure 2)[13]. Overall, the incidence of IBS in the United Kingdom (UK) appears somewhat similar to other European countries and the United States of America (USA) [14, 15]. Overall incidence of IBS may be under represented as many patients do not seek medical attention and one plausible reason may be the disillusionment with current treatment options.

IBS is a chronic condition that can impair patients' quality of life and their performance both at work and at home [15, 16]. Specific factors that impinged on their lifestyle were diet, concentration, long journeys, physical appearance, the ability to eat out and the ability to lead a 'normal life'[15]. Work productivity would be affected with more sickness days off work and more consultations with medical professions. Studies by Amouretti *et al.* [17] and Creed *et al.* [18] demonstrated that quality of life in IBS is significantly worse than the general population. Cost incurred by this disease for each patient could be approximately £1500 per patient/year [16, 18]

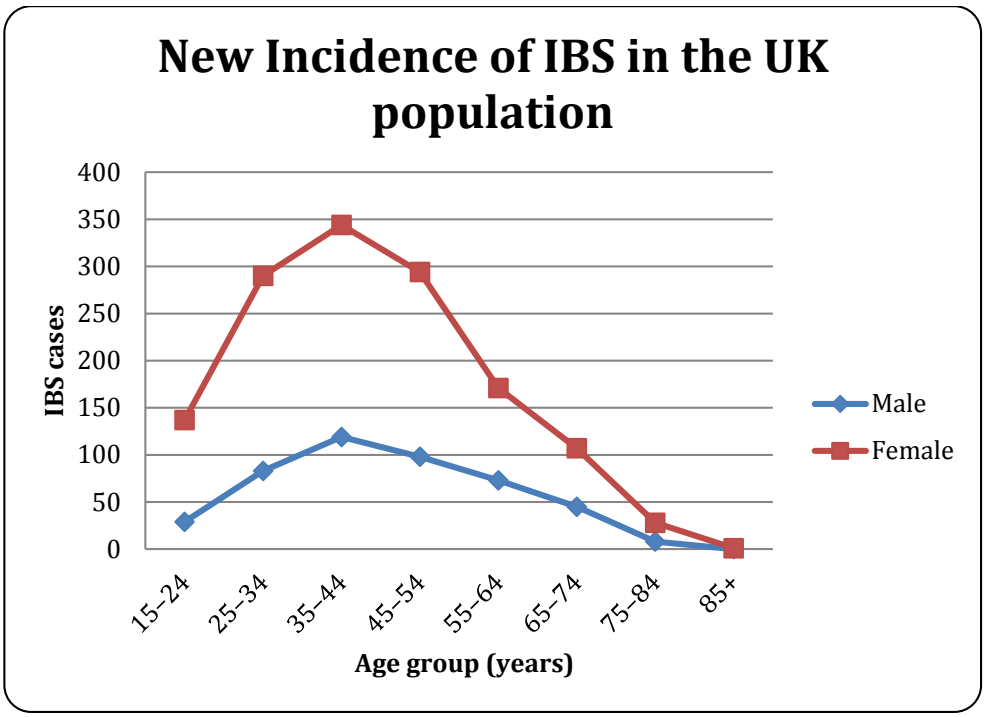


Figure 2: Incidence of newly diagnosed patients with IBS in primary care in the UK which peaked in the 3rd to 4th decade of life. Redrawn from Jones et al [12]

1.3. Pathophysiology

1.3.1. Genetic

A study on monozygotic and dizygotic twins in Australia suggested that there is a genetic link predisposing to IBS, with a heritability of 57%[19]. Later, Levy *et al.*[20] showed concordance for IBS is greater in monozygotic twins than dizygotic twins, which supports a hereditary component in IBS. On the contrary, IBS in twins could be due to social conditioning since having an IBS parent is an independent predictor of IBS and a stronger predictor than having a twin with IBS. These studies have their limitations since the diagnosis of functional bowel disease was not based on a set criterion such as the Rome I or II criteria. A study in 2004[21] showed the concordance rate of IBS in monozygotic and dizygotic twins are similar which may conclude that genetic factor has little influence on IBS development. So far, the only strong genetic link to IBS is the TNFSF15 gene, which is associated with Crohn's disease [22, 23]. A large cohort study in America and Sweden[22] showed that the TNFSF15 gene is strongly associated with an increased risk of developing IBS (OR=1.37). This was again confirmed with a British cohort of IBS-D associated with TNFSF15 and TNFα genetic polymorphism[23].

1.3.2. Stress/ life events

Childhood learning and conditioning played a role in determining whether one develops IBS. If a mother has IBS, the child independently reports more medical problems and school absences[24]. This correlated with having the diagnosis of IBS as an adult in later life[25]. High stress and anxiety levels in subjects are more prone to developing IBS after gastroenteritis[26]. A review article by Spiller and Garsed [27] succinctly summarised the psychological stressors that could influence one to develop IBS following a bout of gastroenteritis. These stressors are hypochondriasis (relative risk = 2.0), adverse life events in the preceding 3 months (relative risk = 2.0) and depression (relative risk 3.2).

Mechanistic study on stress:

It is now recognised that there is interaction between stress and the gut. The corticotrophin releasing factor (CRF) activation is the key to initiation of stress response via the hypothalamic-pituitary-adrenal axis in IBS [28, 29]. Recent evidence has shown that the CRF1 receptor interacts with CRF ligands and is involved in colonic motor response to various stressors [30, 31]. A study by Gue *et al.* [32] demonstrated the interaction between CRF and stress via the central CRF pathway, caused worsening of abdominal pain and activation of mast cells in rats. A human study by Santos *et al.*[33] showed activation of mast cells in the gut by releasing of mast cell mediators such as histamine and tryptase following immersion of a hand into cold water. This confirmed that stress could activate intestinal mast cells providing a possible mechanism for stress as a cause for accelerating small bowel transit.

1.3.3. Somatisation/psychological

One of the most difficult aspects of managing IBS are patients often have multiple co-morbidities. These include psychological disorders such somatisation disorder and panic attacks, urinary symptoms such as dysuria, nocturia, frequency and urgency of micturition, gynaecological symptoms such as dyspareunia and chronic pelvic pain and musculoskeletal problems such as chronic fatigue syndrome. Some may have undergone unnecessary invasive tests and treatment leading up to laparotomy, hysterectomy or cholecystectomy [12, 34, 35]. The rates of abdominal/pelvic surgery in IBS patients were reported twice as high as those of the normal population and there was as high as a 3-fold increase in gall bladder surgery in this group of patients [36]. 30-60% of patients with IBS symptoms have fibromyalgia and vice versa [37, 38]. Patients who have both of these conditions have worse quality of life and displayed significant hypersensitivity to pain compared to those with only either IBS or fibromyalgia alone[38].

Somatization disorder (SD) is a psychiatric disorder defined as multiple medically unexplained symptoms. These symptoms include psychiatric and neurological complaints. Although SD is rare with an incidence of around 1 per 1000, there is a near similar problem which is the "physical symptom disorder" found in as many as 1 in 10 of primary care consultations [39] . It may go unrecognized by physicians and general practitioners because training is focused on the identification and treatment of specific organic diseases[40]. Documenting the patient's complaints, co-morbidities and previous attendances to hospitals provide a helpful pointer towards the existence of SD. Patients with irritable bowel syndrome who manifest a degree

of somatisation often meet diagnostic criteria for other functional disorders[41]. It is important to recognize and identify these patients since they are more difficult to manage as they often report worse global IBS symptomatology with a poorer response to conventional IBS treatments [40].

The Patient Health Questionnaire 15 (PHQ-15) is a useful questionnaire, which documents somatic symptoms from different parts of the body. The PHQ15 contains 3 gastrointestinal symptoms which if deleted leave the PHQ12 Somatic Symptoms scale (PHQ12SS) as a useful measurement of non-gastrointestinal (GI) symptoms [42]. A PHQ12SS score >6 identify patients with IBS with a sensitivity of 66.4% and specificity of 94.7% and a positive likelihood ratio of 13.2. A low score is useful and should prompt a search for other diagnoses. Another tool that is useful is the 14-item Hospital Anxiety and Depression scale (HAD). This is a reliable tool to detect anxiety and depression [43] which is important since it contributes to the severity of the disease and if severe (score >15), it will warrant specific treatment such as antidepressants/anxiolytics.

1.3.4. Visceral hypersensitivity

Visceral hypersensitivity plays a pivotal role in the motor function of the gut and abdominal discomfort in IBS patients. Visceral pain is defined as reduction in threshold for pain and discomfort. The causes of visceral hypersensitivity are varied and may reflect the heterogeneity of IBS.

Over the past decade, visceral hypersensitivity can be measured using the rectal barostat to induce abdominal pain. Previous studies showed IBS patients have hypersensitivity to rectal distension [44-46]. One of the large studies by Mertz in 1995 [44] showed 94% of IBS patients have lower threshold of rectal distension, increased intensity of sensation or altered viscerosomatic referral. A more recent study by the Mayo clinic reported only 7.6% having thresholds for pain sensation below the 10th percentile and 13% having thresholds above the 90th percentile. The discrepancies between these 2 studies were probably due to the strict protocol adherence for studies performed in the Mayo clinic and differences in the definition of the word 'threshold'. In the United Kingdom, Agrawal and colleagues [47] showed IBS patients with bloating without distension have lower pain threshold and a bigger desire to defecate compared to IBS patients who have symptoms of bloating with abdominal distension.

Immune activation leading to activation of mast cells and its release of their mediators e.g. histamine and tryptase may play a part in altered sensation in IBS patients [46, 48]. (The role of inflammation in IBS will be discussed later.)

Review articles by Aspiroz *et al.*[49] and Larauche [50] have summed up visceral hypersensitivity in IBS clearly (see Figure 3). The central mechanism, which is the brain, plays a role in how one modulates the perception of afferent information/ visceral pain. The development of functional magnetic resonance imaging (fMRI) has helped us achieve a better understanding in brain activities during stimulation of pain/sensation. In IBS subjects, pain by rectal distension led to greater activation of anterior cingulate cortex, which is the main area in the central nervous system where the emotional aspect of pain is registered[51]. This postulated that IBS patients

might have abnormal brain pathways, which led to a low threshold of pain. Another study by Piche showed IBS patients have thermal cutaneous and visceral hypersensitivity, which may indicate abnormalities in the descending anti-nociceptive pathways [52].

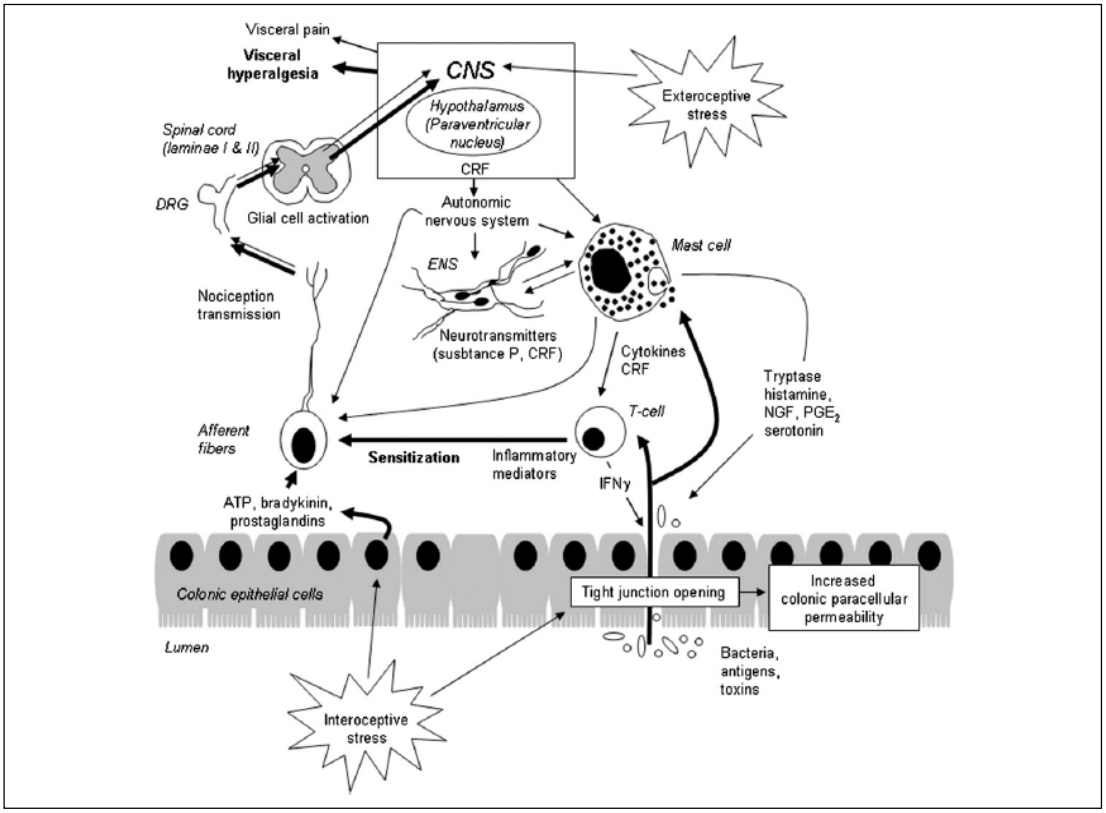


Figure 3: Putative role of central and peripheral CRF signaling pathways to influence immune processes and potential implications in stress-related IBD and IBS symptoms

1.3.5. Inflammation

Approximately 25% of patients who had acute bacterial gastroenteritis such as Campylobacter, Salmonella and Shigella;, develop IBS, which is often a continuation of the initial diarrhoeal illness and hence frequently meet the Rome criteria for IBS [53, 54]. Patients who developed post-infectious IBS (PI-IBS) have similar features as a

subgroup of IBS patients with diarrhoea. The relative risk of one developing IBS following a bacterial gastroenteritis in a year was 11.9 compared to the general population in the United Kingdom (UK) [55]. Risk factors that predispose this group of patients to IBS were prolonged illness during the acute gastroenteritis episode, female sex, the use of antibiotics and previous psychological disturbance [27, 54, 56, 57]. Therefore, PI-IBS has been used in many studies to further understand the pathophysiology of IBS in general. (Further discussion on PI-IBS and the role of inflammation will be continued in Section 2)

1.3.6. Intestinal permeability

Following the outbreak of gastroenteritis in Walkerton, Canada due to contamination of the municipal water supply, a significant number of patients developed PI-IBS after 2 years following the event [58]. In this group of subjects, there was increased intestinal permeability. This provided further evidence in the organic nature patient with functional bowel disorder. These finding were consistent with previous findings by Spiller and colleagues [59] where the gut permeability was increased in patients infected with *Campylobacter jejuni* gastroenteritis and in PI-IBS. Another study by Park *et al.* [60] showed increased intestinal permeability in unselected IBS patients. Further study by Dunlop and colleagues[61] showed increased intestinal permeability in both PI-IBS and IBS-C patients. IBS-D patients who have increased intestinal permeability seemed to correlate with abdominal pain severity and worsening IBS symptoms[62].

A study looking for genetic risk factors for PI-IBS had identified 3 genetic regions of interest i.e. Cadherin 1 (CDH1), Interleukin 6 (IL-6) and Toll-like receptor 9 (TLR9) which played a part in the intestinal barrier [63]. CDH 1 is a transmembrane glycoprotein that acts as a tight junction and is responsible for the intestinal barrier. IL-6, an inflammatory cytokine, was elevated in IBS patients more so in patients with diarrhoea and this raised the possibility that this pro-inflammatory cytokine may be involved in the integrity of intestinal barrier[64].

Numerous animal studies have showed stress increased gut permeability via mast cell activation. Piche *et al.* [65] showed all IBS subtypes have increased intestinal permeability to fluorescein isothyocyanate (FITC)-sulfonic acid. Biopsies from IBS patients had reduced zonulin-1 mRNA (Zonulin-1 is a modulator of intestinal barrier function). Supernatant from incubated IBS biopsies increased the permeability of Caco-2 monolayers to FITC-dextran, an effect not blocked by histamine receptor antagonists. A likely candidate for mediating this effect would be mast cell tryptase which other groups have showed increased in IBS biopsy supernatant [66]. In this study by Buhner *et al.*, resected colon specimens were used and loaded with a voltage sensitive dye to image the response of human myenteric plexus neurons to supernatant from IBS colonic biopsies. It evoked action potential discharges in submucosal plexus neurons when supernatants from the IBS specimen were applied but not on control supernatant samples. Furthermore, serotonin, histamine and tryptase antagonists were able to reduce these neurone responses.

Increased tryptase has recently been reported in IBS-D which suggested tryptase may mediate increased permeability in IBS[67]. Interestingly, tryptase levels were enhanced in IBS compared to controls. When tryptase inhibitor was added into the

rectal biopsy of the IBS patients, the permeability normalised[68]. This study found no increase in expression of protease-activated receptor 2; suggesting tryptase activity itself played a role in the increase of intestinal permeability of IBS patients.

Modulation of the intestinal barrier

Probiotics are living organisms that provide health benefits to the host. The mode of action of probiotics consist of (a) binding of intestinal epithelial cells and inhibiting adhesions of pathogen, (b) enhance intestinal barrier function, (c) acidification of colon fermentation, (d) immune-modulatory actions, (e) secretion of bacteriocins, (f) alteration in mucosal response to stress and (g) inhibition of visceral hypersensitivity [69]. A randomised clinical trial in China had demonstrated that the use of probiotics is beneficial in surgical patients undergoing colectomy[70]. This study demonstrated the use of probiotics would stabilise the integrity of tight junction proteins in colonic mucosa epithelium and the balance of gut microbiota thereby reducing postoperative infection. A mechanistic study by Zeng and colleagues [71] showed the use of probiotics, such as active lactic acid bacteria, decreased intestinal permeability in IBS-D patients along with improvement in their global well being and abdominal pain. This may be a promising treatment in IBS[72] but there is still a need to establish types of species, strains and the dose of probiotics which may be beneficial.

1.3.7. Transit

Assessing transit is an important part of characterising the subtypes of IBS as most studies showed faster transit in IBS-D. However transit through the bowel is intrinsically quite variable depending as it does on many factors including diet, emotion and menstrual cycle. Metcalf *et al.*[73] refined the method for whole gut transit that involved taking 20 radio-opaque pellets for 3 days and an abdominal x-ray (AXR) on the 4th day. This became a conventional method to assess whole gut transit and is currently used across all healthcare providers. In the past, we believed that different gut transit plays a predominant role in different subtype of IBS patients. The differences in gut motility between IBS patients and healthy controls are variable and may not be a good parameter to be used as a diagnostic tool for IBS [74-76]. An earlier study by Cann and colleagues [77] showed a relationship between gut dysmotility in IBS subtypes both affecting the small bowel transit and colonic transit. Later in 1999, Horikawa and colleagues [78] studied gut transit in IBS and found it to have accelerated whole gut and colonic transit in IBS-D but remain normal in IBS-C. When bowel transit was assessed along with symptoms, a study in the USA [79] demonstrated that patients with IBS-D have faster colonic transit and there was a correlation of abdominal symptoms with powerful contractions of the colon. In the IBS-C subtype, Agrawal *et al.* [80] demonstrated that the IBS-C patients have delayed colonic and orocaecal transit time. In that study, it showed significant correlation between colonic/ orocaecal transit with clinical symptoms such as abdominal distension, a symptom that most IBS patients suffer from. Also, this study demonstrated that abdominal bloating was inversely correlated with stool

consistency. The IBS 'bloaters' with alternating bowel habits demonstrated rapid small bowel transit without any difference in colonic transit when compared with healthy volunteers [81]. This finding was very different from one studied by Cann *et al.* [77] where the small transit was delayed in IBS patients who have predominantly pain and bloating. Overall, small and large bowel transit in IBS is variable and studies have confirmed that there is great heterogeneity in the healthy population and IBS patients.

1.3.8. Diet

Promoting a healthy diet, '5 a day', which includes dietary intake of 5 x '80g' portions of fruit and vegetables daily makes good sense from a public health perspective as it may well reduce the risk of cardiovascular disease, type-2 diabetes and obesity in the UK population. However the '5 a day' diet may not be beneficial to patients with an irritable and hypersensitive bowel since these foods typically have high content of fibre, fructose, fructans, and polyhydric alcohols.

A dietary history should distinguish soluble from insoluble "fibre" which is a misnomer since most "fibre" is not fibrous. However the term is widely used to describe non-starch polysaccharides often found in plants characteristically resistant to human digestive enzymes. Soluble fibres consist of pectins, gums and mucilages, best known as guar gum and psyllium. These soluble fibres characteristically form viscous solutions with water and can be prescribed in pure form. Insoluble fibre consists of harder structural components of plants including

celluloses, hemicelluloses and lignins. They are typically particulate and insoluble in water for example corn fibre and wheat bran. While soluble fibre can help IBS patients with constipation [82] insoluble fibre like bran can adversely affect symptoms and it is important that levels of dietary fibre intake are assessed. It is also important for symptom assessments during a reduced dietary fibre trial period.

Effect of bran

Patients who are symptomatic may modify their diet or increase their fibre intake prior to consulting their general practitioner. Some studies show that increasing fibre intake such as bran may be beneficial in constipation but not diarrhoea and in some IBS patients aggravates abdominal distension, flatulence and diarrhoea [81, 83, 84]. About half (55%) of IBS patients believe it worsens their symptoms while only small proportion (10%) report any symptom improvement [85].

FODMAPs

Recent work in Australia showed that a diet high in FODMAPs (Fermentable Oligo-Di and Mono-saccharides and Polyl hydric alcohols) could trigger abdominal symptoms such as flatulence, bloating, abdominal discomfort and changes in bowel habit in some IBS patients[86, 87]. FODMAPs consist of fructose, lactose, fructo-and galacto-oligosaccharides (fructans and galactans) and polyols (sorbitol, mannitol, xylitol and maltitol) (see Figure 4 for examples of foods with high FODMAPS content).

Fructose is a 6-carbon monosaccharide found in many foods and comes in 3 forms e.g. monosaccharide (free fructose), dissacharide (sucrose) or fructans (polymer of fructose). Free fructose is found in fruit and honey. Fructose may also be present in the diet as a constituent of the disaccharide sucrose or as fructans, which are

polymers of fructose with small amounts of glucose. Fructose is usually absorbed via two transporters in the small intestine epithelium: the GLUT5 fructose-specific transporter in the apical membrane and the GLUT2 transporter which carries glucose, fructose and galactose across the basolateral membrane [88]. GLUT2 transporters have also been seen on the apical membrane when glucose is present and being transported by a SGLT1 (sodium/glucose-galactose co-transporter) which increases the uptake of fructose [89] – in part explaining why malabsorption of fructose can be seen when there are lower levels of glucose present [90]. Absorption of fructose in the gut is less efficient than glucose therefore it is possible to exceed the absorptive capacity of the small bowel, leading to excessive fructose delivered to the colon. In the colon, it would be rapidly fermented by bacteria producing short-chain fatty acids, carbon dioxide, hydrogen and methane [86, 91]. The hydrogen and methane that are produced are expired through the breath or passed as flatus. When these osmotic changes and rapid gas productions occur, it induces bowel symptoms such as flatus, bloating, abdominal discomfort and erratic bowel habit [92, 93]. IBS patients do not appear to malabsorb more than normal controls but they seem to be more sensitive to the effects of these carbohydrates [94]. An MRI study recently showed that fructose increased small bowel water content and its effect was dampened with addition of glucose together with fructose[95].

Over recent years there has been a marked increase in consumption of fructose and fructans; particularly in the United States where high-fructose corn syrup is widely used as a sweetener in soft drinks, sugared fruit drinks, jams and baked goods [96, 97].

It is important to assess intake of such substances since two randomized controlled trials [87] demonstrated that fructose and fructans worsened IBS symptoms [98].

Lactose malabsorption affects up to 70% of adults worldwide and in some, it can cause IBS-like symptoms[99], although only about 1 in 3 would be aware of their intolerance. Severity of symptoms is very much dose dependent and the effect of this would be lessened if lactose is mixed with other foods ensuring slower delivery of chyme to the small intestine[100]. A mutation which arose in North Western Europe and Northern Nigeria prevented the normal post weaning reduction in lactase levels and thus led to high lactase levels throughout adult life (lactase persistence)[101]. The prevalence of this mutation is highest in Scotland and declines as one moves south and west[102].

Assessing dietary intolerance to FODMAPS by history is difficult since the effect of each FODMAPS component depends on what is consumed simultaneously [92, 103]. If fructose and sorbitol were given in a mixture, it seemed to cause more symptoms than when each of these components were given separately [92]. If each of these components were given along with glucose, the malabsorption process would be reduced[104]. This may explain why some sources of fructose with low glucose content; such as pears are less well tolerated than sources with high glucose content such as grapes[105] .

It is likely that some IBS patients may respond to a low FODMAPS diet, though with such a complex diet requiring intensive dietician input, placebo effects may cause similar positive responses. So far, the results of a low FODMAPS diet for IBS patients

remain promising[86, 87, 106] since most patients would rather modify their diet than to take medication(s) that may cause unnecessary adverse events.

FODMAP	Excess fructose	Lactose	Oligosaccharides (fructans and/or galactans)	Polyols
Problem high FODMAP food source	*Fruits:* apples, pears, nashi pears, clingstone peaches, mango, sugar snap peas, watermelon, tinned fruit in natural juice	*Milk:* cow, goat and sheep (regular & low-fat), Ice cream	*Vegetables:* artichokes, asparagus, beetroot, Brussels sprout, broccoli, cabbage, fennel, garlic, leeks, okra, onions, peas, shallots.	*Fruits:* apples, apricots, cherries, longon, lychee, nashi pears, nectarine, pears, peaches, plums, prunes, watermelon
	Honey	*Yoghurt* (regular & low-fat)	*Cereals:* wheat & rye when eaten in large amounts (e.g. bread, pasta, couscous, crackers, biscuits)	*Vegetables:* avocado, cauliflower, mushrooms, snow peas
	Sweeteners: fructose, high fructose corn syrup	*Cheeses:* soft & fresh (e.g. ricotta, cottage)	*Legumes:* chickpeas, lentils, red kidney beans, baked beans	*Sweeteners:* sorbitol(420), mannitol(421), xylitol(967), maltitol (965), isomalt (953) & others ending in '-ol'
	Large total fructose dose: concentrated fruit sources; large serves of fruit, dried fruit, fruit juice		*Fruits:* watermelon, custard apple, white peaches,	

Figure 4: Food sources containing high FODMAPS content[90]

1.4. Conclusion

IBS consists of a large heterogeneous group of patients where its pathophysiology remains to be elucidated. Immune activation is believed to play an essential role in developing IBS. IBS may possibly be sitting at the other end of a spectrum similar to inflammatory bowel disease; since it shares certain common pathways in its pathogenesis; for example genetic defects, increased gut permeability and exacerbation of symptoms following stress.

1.5. Aim of this thesis:

The main aim of this thesis was to assess the role of inflammation in a subgroup of patients who have IBS with diarrhoea. This thesis will describe the effect of Mesalazine, an anti-inflammatory drug, in the treatment of IBS-D with the aim of looking for relevant mediators or biomarkers. The second aim was to explore the use of magnetic resonance imaging (MRI) to look for potential biomarkers in IBS.

Role of inflammation in Irritable Bowel Syndrome

1.6. Post-infectious IBS

1.6.1. Epidemiology

Post-infectious irritable bowel syndrome (PI-IBS), a chronic condition, is defined by newly developed of IBS symptoms following an episode of acute infectious gastroenteritis. This subgroup of IBS patients has a normal bowel habit prior to this acute episode. The acute episode of infectious gastroenteritis is defined by having at least 2 or more clinical features such as fever, vomiting, diarrhoea and a positive stool culture [107].

A recent large community survey in the United Kingdom (UK) which involved over 6800 participants has revealed that the overall incidence of infective diarrhoea was 274 cases/ 1000 persons/ year with a maximum incidence in young children [108]. Viral gastroenteritis was the commonest cause with norovirus being the most frequent organism isolate. The most common bacterium was *Campylobacter* spp. with incidence rate of 11 cases/ 1000 persons/ year in 2009. Other common bacterial intestinal infections were *Salmonella* spp. and *Escherichia coli*. However, it is worth noting that less than 1 % of episodes of gastrointestinal infections in the community are reported to the national surveillance systems, therefore its true incidence would be grossly underestimated. Due to the under reporting of infectious gastroenteritis, the true incidence of PI-IBS may be greater than what is currently believed.

Epidemiological studies have suggested that enteric infection is one of the most important risk factors for developing IBS. These findings generally equate to those found for psychological risk factors, such as anxiety and increased levels of depression and sleeping disorders. Smoking, body mass index and alcohol excess

also show similar effects [109]. The proportion of patients developing IBS following gastrointestinal infections varies in different series of studies. This can be from 3.7% [110] to 36% with the highest incidence being seen in those with the most severe infection as judged by bleeding, fever and weight loss[111]. The most common causes of bacteria causing PI-IBS in the UK are *C. jejuni*, *Salmonella enteritidis* and *Shigella flexneri*. Most often, PI-IBS patients describe a persistence of their initial illness leading to multiple visits to the general practitioners or hospitals. Most of these patients meet the Rome criteria diagnosis for IBS with diarrhoea (IBS-D) [53]. Therefore, the similarities between PI-IBS and other subtypes of IBS may provide a better insight into the pathophysiology of all IBS especially when the onset and cause of symptoms in PI-IBS is clearly defined

1.7. Risk factors

In a meta-analysis, the overall effect of developing IBS following an infectious gastroenteritis gave a pooled odds ratio of 7.3 (CI 4.8-11.1)[112]. A review by Spiller and Garsed summarised succinctly risk factors and the relative risks with each component. See figure 5.

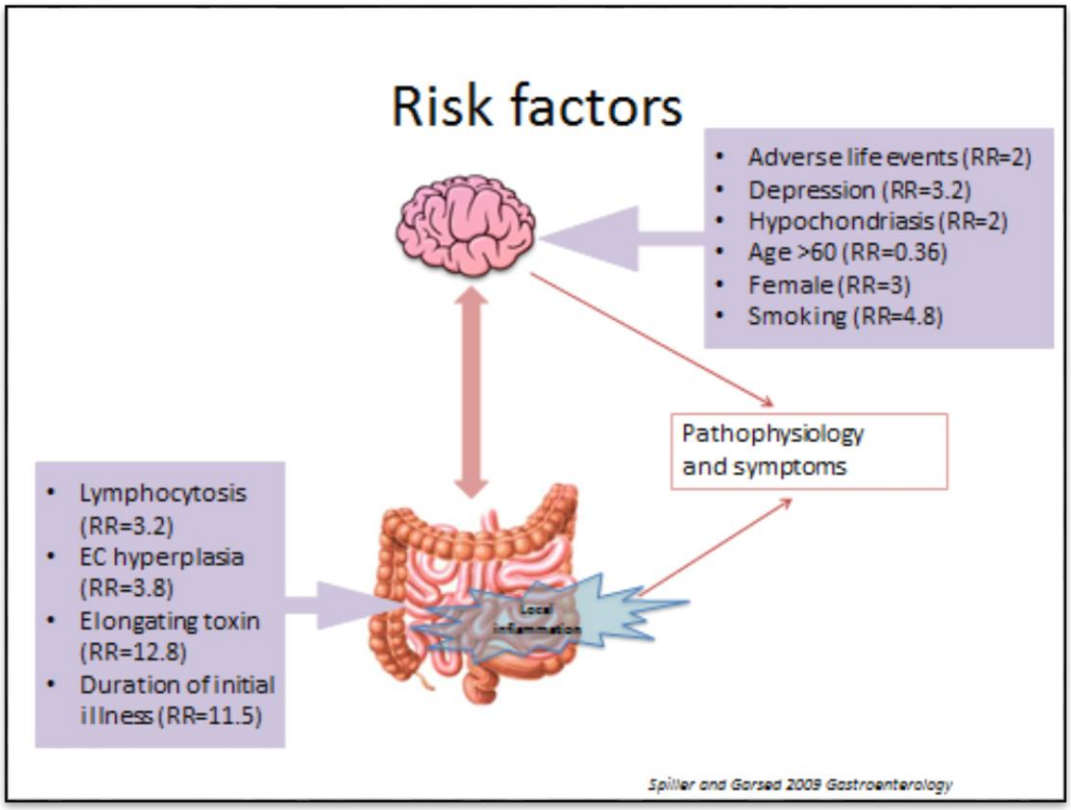


Figure 5: Summary of risk factors of developing PI-IBS

1.7.1. Genetics

Previous studies have demonstrated that there may be a familial tendency when studying monozygotic and dizygotic twins but social learning also is an important factor in a child developing IBS [19-21]. Recent studies on single nucleotide polymorphism (SNP) supports the idea of genetic influence contributing to IBS. A greater proportion of IBS patients are heterozygous for the -308 (G/A) SNP, which is a high producer of TNF-α [113]. A high producer TNF- α and low producer of IL-10-10-1082 A allele was more prevalent in IBS patients (9%) versus control (3%). Recently, a gene that is associated with Crohn's disease was identified and has demonstrated an increased risk of IBS (OR 1.37) in a cohort of IBS patient [22]. The gene that was

identified was the G allele of SNP rs4263839 in the TNFSF15 gene. Another study by Swan and colleagues [114] had identified a closely related SNP in the TNFSF15 gene, which increased the risk of developing IBS-D, and there was an increased prevalence of the TNF-α SNP rs1800629 genotype GA in PI-IBS. Other reports such as the Walkerton outbreak in 2007 have shown association between SNPs with PI-IBS. They identified 3 gene regions such as the Cadherin 1, IL-6 and Toll-like receptor 9 [63]. The limitation of this study was its small sampling size that did not withstand corrections for multiple testing. These associations would need to be reproduced again in another separate cohort.

1.7.2. Physical and Psychosocial

Studies have confirmed that high stress and anxiety levels, hypochondriasis, adverse life events occurring in the preceding 3 months and depression increase the risk of developing PI-IBS [26, 27, 57, 107]. Smoking increases the risk of developing PI-IBS to about 5 fold but its mechanism is yet unclear at present whilst age (>60 years) protects one from developing PI-IBS (RR 0.36) [27], either due to declining immune response as one becomes older or alternatively prior immunity reducing the severity of the initial illness.

Psychological stress can exacerbate pain syndromes such as IBS but how it increases the risk of developing PI-IBS is still unclear; although stress is known to alter immune function. In the animal models, corticotrophin-releasing hormone (CRH) seems to be a key mediator of stress acting via the hypothalamic-pituitary-adrenal

axis as well as locally in the gut. CRH acts via the CRF1 receptors that cause stimulation of colonic motility and watery diarrhoea in rats and mice [28, 30]. When CRH1 receptor antagonist was used, it prevented diarrhoea in rats, indicating that the brain CRF1 signalling pathway is important in colonic motor responses[115]. In another animal model, rats that were previously infected with *Citrobacter rodentium* showed raised level of corticosterone and epinephrine levels following chronic water avoidance stress. They also found increased peripheral nociceptive signalling from rectal distension and tissue proteases. These proteases are likely released from activated mast cells that can induce hyperexcitability in colonic dorsal root ganglia cells [116]. In humans, inducing stress such as immersion of the hand into cold water can cause pain and sympathetic activation. This could lead to activation of mast cells releasing mediators such as histamine and tryptase in the small bowel [33]. Santos and group have also demonstrated in their study that with stress, this could lead to an increase in human small bowel secretion [33]. The same group have shown evidence of increased numbers of mast cells and tryptase in jejunal biopsies of anxious IBS-D patients [117], which is replicated by the Nottingham group [118]. Therefore, there is a possible explanation that stress could increase human small bowel secretion and motility that leads to accelerated transit which is a characteristic of IBS-D.

1.8. Pathophysiology

The pathophysiological causes of PI-IBS are multiple. However there is evidence to show low grade 'immune activation' in PI-IBS and IBS patients. Changes in enteric nerves and altered microbiome may also be implicated in the pathophysiology of PI-IBS.

1.8.1. Immune activation

Enterochromaffin cells (EC cells) & lymphocytes

The EC cell is a subtype of neuroendocrine cells in the gut and contains a 90% proportion of its body store of serotonin (5-HT) [119]. EC cells act as a sensory transducer and play an important role in response to luminal pressure and contents such as nutrients and bacterial products by secreting peptides and amines that activate the enteric nerves and transmit information to the central nervous system. 5-HT strongly influences the gut's motility and secretion especially when exposed to toxins such as cholera. The 5-HT activates enteric reflexes via the 5-HT_{1p}, 5-HT_3, 5-HT_4 and 5-HT_7 receptors to stimulate secretion and propulsion of the gut [120]. In animal studies, mice infected with *Trichinella species* or *Trichuris muris* develop T-cell mediated immune response in the gut causing an increase in EC cells and 5-HT content [121-123]. Although acute inflammation resolves, following acute infection, there is persistent T lymphocyte-dependent EC cell hyperplasia. Other studies in IBS

patients have shown similar evidence. Serial rectal biopsies on patients following Campylobacter jejuni gastroenteritis who developed PI-IBS has shown raised 5HT containing EC cells, intra-epithelial lymphocytes(IELs) and T lymphocytes which could persist for many years [59, 107].

Cytokines

The cytokines are products of monocytes or macrophages. The monocytes and macrophages are parts of innate immunity and play an important part in mounting an acute inflammatory phase when there is invasion of infectious agent. Recent studies increasingly have shown B cell and T cell expressions are increased in IBS patients. A group from Sweden demonstrated isolated B cells in blood, showed increased expression of IgG and co-stimulatory molecules CD80 and CD86 [124]. Moreover, the gut homing integrin $\beta7^{+}$ B cells in IBS patients were higher than in the controls which may implicate that the source of B cell activation may be from antigens in the gut. Another study from the group has demonstrated increased T cell activation, CD4 and CD8, in IBS patients. Pro-inflammatory cytokine IL-1β is raised compared to the control and showed a weak correlation with dissatisfaction of bowel habit in IBS patients [125]. Neither of the two studies stated whether the IBS cohorts were PI-IBS patients.

Other studies have demonstrated similar results showing raised pro-inflammatory cytokines such as IL-1, TNF-α, IL6 and reduction in IL-10, which is an anti-inflammatory cytokine, in colonic biopsies or peripheral blood mononuclear cells in PI-IBS patients [54, 126, 127]. Other studies have showed imbalance in cytokines of different subtypes of IBS patients [23, 128]. Overall there is lack consistency with

findings on the cytokines and their methodologies and patient selection is varied which may explain the variability in the results.

Mast cells

Recently, there is evidence to show that mast cells may be implicated as one part of the pathogenesis of IBS. Recent studies have shown that mast cells are increased in the small and large bowel of all subtypes of IBS patients but especially in IBS-D patients [54, 117, 118, 129-131]. Mast cell products can activate enteric nerves within the lamina propria which may be relevant to IBS symptoms. The number of mast cells which lie in close proximity to the enteric nerves, <5 μm from the nerve, correlate with the severity and frequency of visceral pain in IBS [48, 132]. When mast cells are activated, mediators such as histamine, prostaglandin and proteases such as tryptase [133] are released, which can activate enteric nerves. Tryptase signals to the cells through proteinase-activated receptor 2 (PAR2 receptor) which can cause neuronal excitability [134, 135] leading to visceral hypersensitivity and increased gut motility. Recently, a study demonstrated that the down regulation of proteinase-activated receptors 4 (PAR4 receptor) may be implicated in the pathogenesis of IBS [136] although previous studies were mostly in animal models and in studies of inflammatory bowel disease. Histamine also activates the enteric nerves by interaction via H1 and H2 receptors [48]. These mast cell mediators such as histamine and tryptase are increased in the biopsy supernatants of IBS patients compared to healthy controls [48, 66, 137] and this release of mediators activate human enteric afferent nerves which likely play a role in visceral sensitivity but so far, there are no correlations between this and clinical symptoms.

1.9. Prognosis

Over time (years), there is a slow decline in the prevalence of PI-IBS once the initial diagnosis has been established. In a 5 year review following *Salmonella spp* infection, a study by McKendrick *et al* [138] showed 7 out of 11 patients had abnormal bowel habit but only 5 had diarrhoea more than once a week. Another study reported 43% of PI-IBS patients had recovered after a 6 year follow up [139] while a meta-analysis of PI-IBS reported steady reduction in PI-IBS symptoms. The odd ratios for those infected compared to healthy controls at 3 months was 7.6 and at 3 years it was 3.8 [140]. The long term follow up of the outbreak of gastroenteritis in the Walkerton outbreak showed a decline in the prevalence of PI-IBS from 28% to 15.4% after 8 years. These data are reassuring as the prognosis of PI-IBS is good.

1.10. Mesalazine

1.10.1. Background

Mesalazine is an anti-inflammatory drug commonly used to treat mild to moderate inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. It was first introduced in 1975 and it is a derivative of salicylate acid. Mesalazine is delivered as enteric-coated 5-amino-salicylate acid and exerts its effect mainly in the gastrointestinal tract. It is metabolised into N-acetyl-mesalazine by the intestinal mucosa and systemically in the liver. Some acetylation occurs through the action of colonic bacteria. It does not cross through the blood-brain barrier since the majority of the compound is protein bound. Mesalazine is excreted in urine and faeces. In general, the medication is safe.

1.10.2. Mode of action of Mesalazine/ Sulphasalazine in IBS:

Many studies in the past have showed Mesalazine can interfere with the activation of the inflammatory pathway. There is substantial evidence of low-grade immune activation in IBS particularly in those with diarrhoea following acute bacterial gastroenteritis[141]. The mucosal changes observed in the PI-IBS group were very similar to those in the IBS-D group[64, 139, 142]. Therefore, with these similar changes observed in both of these groups, it was worth using mesalazine as a treatment for the unselected group of patients with IBS-D.

Prior studies using mesalazine

The first anecdotal open label trial of 12 patients with resistant IBS-D, who responded to mesalazine [143], showed a benefit that took about 2-3 months to become apparent. There have since been three further reports of open label treatment [144, 145] and two small randomised control trials [146, 147]. All but the Corinaldesi trial [146] used patients with IBS-D. The Bafutto trial used mesalazine 800 mgs tds for 30 days in 61 IBS-D patients and showed benefit with a reduction in stool frequency, stool consistency and abdominal pain but was uncontrolled [145]. The Andrews study involved just 6 patients but this showed mesalazine decreased biopsy proteolytic activity. Both of the randomised control trials are rather too small to be sure of their significance with n=20 and 17 respectively. One study showed a significant reduction of mast cell numbers and an overall reduction in inflammatory cells [146].

1.10.3. Risk and benefits

Mesalazine has been widely used for more than 45 years and there is extensive data on side effects. In general, the drug is well tolerated. Nephrotoxicity is seen at a rate of about 1 per 100,000 prescriptions [148], more common but less serious side effects include diarrhoea, nausea, vomiting and abdominal pain together with headaches and rarely pancreatitis and blood disorders. Balancing this, irritable bowel syndrome patients suffer marked decrease in quality of life, similar to that of other chronic diseases like diabetes and heart failure. They also lose significant

amounts of time off work, and when they are at work; work less efficiently. A simple safe and effective treatment would be of undoubted benefit to what is a substantial subgroup of the population given that IBS with diarrhoea affects around 3% of the general population.

1.10.4. Rationale for the current study

Studies in Nottingham over the last decade have identified the importance of inflammation in various subgroups of IBS. We have focused on the group of IBS patients who develop symptoms following acute bacterial gastroenteritis, the so called post infectious IBS. In this group, we have been able to show that the acute inflammatory insult associated with acute *Campylobacter jejuni* enteritis is followed by a more prolonged indolent phase with increased chronic inflammatory cells long after the infecting organism has left the body. In this subgroup of IBS we have demonstrated activated circulating peripheral blood mononuclear cells with increased cytokine production and an associated increase in inflammatory gene expression [141]. We also demonstrated the importance of anxiety and depression [107], which along with adverse life events that increase the risk of post infective IBS (PI-IBS) [57]. The changes observed in PI-IBS are very similar to those in IBS-D, the predominant bowel disturbance being diarrhoea with a similar prognosis [139]. This work has been supported by others who have shown inflammatory changes in IBS-D patients who did not have a background of previous infection [64, 142]. Such studies have also shown increased inflammatory cells and increased expression of inflammatory cytokines including IL-1β [127]. Increased gut permeability has also been

shown in IBS-D [61], making a trial of an anti-inflammatory treatment a logical choice. Safety is of pre-eminent importance in IBS drugs as can be seen by the recent withdrawal of Tegaserod [149] and the previous withdrawal of Alosetron [150]. Both drugs, which were therapeutically effective, had to be withdrawn owing to rare side effects (incidence < 1 per 700 patient treated). This leaves such patients bereft of effective treatments, a gap which mesalazine might well have filled. Our hypothesis was that mesalazine by virtue of its anti-inflammatory actions will alter the inflammatory mediators; leading over a number of weeks, to a reduction in the number of mast cells and a reduction in the release of inflammatory mediators. Previous studies have shown that 5-Aminosalicylic acid (5-ASA) inhibits the release of inflammatory mediators including histamine and prostaglandin D2[151]. It also inhibits activation of the transcription factor NFκB which is a major link in the inflammatory cascade [152]. More recently, it has been recognised that 5-ASA exerts an anti-inflammatory effect mediated via peroxisome proliferator-activated receptor-γ (PPAR-γreceptors)[153]. Whether directly or indirectly, 5-ASA has also been reported to inhibit inducible nitric oxide synthetase production and also prostaglandin production via its COX-2 inhibitory effects [154]. Mesalazine therefore both by virtue of inhibiting other inflammatory pathways and by directly inhibiting mast cell pathways may reduce mucosal immune activation.

We planned to investigate the effect of long term mesalazine on mast cell numbers, the chronic inflammatory cells and the mucosal production of inflammatory cytokines, IL-1β, TNF-α as well as mast cell specific tryptase.

1.10.5. Mesalazine product used for this study

The product that was used for this study (described below) was called Pentasa, manufactured by Ferring Pharmaceuticals Ltd. The followings are the pharmacology properties based on the summary of product characteristics provided by the company:

Pentasa sachet prolonged release granules consist of ethylcellulose coated microgranules of mesalazine. Recommended dose for adults is up to 4g / day in divided doses. Following administration, mesalazine is released continuously throughout the gastrointestinal tract in any enteral pH conditions. The microgranules enter the duodenum within an hour of administration, independent of food co-administration. The average small intestinal transit time is approximately 3 – 4 h in healthy volunteers. 30-50% of Pentasa is absorbed predominantly in the small intestine[155]. It reaches a steady state after 5 days following oral administration.

Manufacturer of Pentasa (including packaging): Ferring Pharmaceuticals Ltd.

The manufacturing, packaging and labelling of the placebo was identical to the active drug except for the active ingredient.

Manufacturer of placebo: QPharma AB (Sweden) and Ferring Pharmaceuticals Ltd.

1.11. Clinical trial to assess efficacy of Mesalazine in IBS-D

Title of trial: Efficacy and mode of action of Mesalazine in the treatment of diarrhoea predominant irritable bowel syndrome

This was a multi-centre, two-arm, parallel group, double blind, randomised placebo-controlled trial comparing mesalazine with placebo in patients with diarrhoea-predominant irritable bowel syndrome.

1.11.1. Aim of study

a) The purpose of this trial was to define the clinical benefit and possible mediators of the benefit of mesalazine in IBS-D.

b) Symptoms (primarily bowel frequency) and markers reflecting mast cell activation and small bowel tone were evaluated in this study.

1) The primary objective

Effect of mesalazine on stool frequency at end of study (weeks 11 and 12)

2) The secondary objectives

Effect of mesalazine on:

a) Overall IBS symptoms

b) Mast cell numbers, mucosal lymphocytes and faecal tryptases

c) Small bowel tone by measurement of fasting small bowel water content through MRI (discussed in the next chapter)

d) To assess ability of biomarkers (mucosal/ MRI parameters) to predict treatment response

1.11.2. Trial / study design

This was a multi-centre, two-arm, parallel group, double blind, randomised placebo-controlled trial comparing Mesalazine with placebo in patients with diarrhoea-predominant irritable bowel syndrome. Design of the study was modified after consultation with a selection of interested patients from the Nottingham Digestive Diseases Biomedical Research Unit patient advisory group who provided a lay member for the Trial Steering Committee.

1) Randomisation and blinding

This was a double-blind parallel group study. Neither participant nor supervising doctor nor study nurse, were aware of the treatment allocation.

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 Unit (CTU) in accordance with their standard operating procedure (SOP) and held on a secure server. The randomisation was stratified by the recruiting centre. The supervising doctor or study nurse obtained a randomisation

reference number for each participant by means of a remote, internet-based randomisation system developed and maintained by the Nottingham CTU.

The sequence and decode of treatment allocations were concealed until all interventions were assigned and recruitment, data collection, and all other trial-related assessments were complete.

2) Participants

a) Recruitment

Participants were recruited between April 2011 and May 2013 with the last patient completed in August 2013. Participants were recruited from IBS clinics at the investigator's hospital, or from lists of patients who had previously taken part in research studies and had indicated that they would like to be contacted about future relevant research projects. In addition, we had, in conjunction with the local Primary Care Research Network, approached GPs to ask them to search their databases for eligible participants and send out letters of invitation along with participant information sheet (PIS). This ensured that the initial approach to patients was from a member of the patient's usual care team or from appropriately authorised research nurses. We also advertised in the local newspaper due to slow recruitment and information about the study was on display in the relevant clinical areas. Ethical approval was sought for any adverts or posters displayed. Patients were seen in the research centres in participating hospitals and enrolled by research nurses or doctors.

Initial recruitment into this trial was slow and it was felt that the eligibility criteria for IBS-D, was too demanding. We therefore modified the eligible criteria for IBS-D

following registration with the clinicaltrials.gov to reflect the fact that, as others have found, the bowel habit of IBS-D patients is less abnormal than patient's recall suggests[156].

The patients were required to meet the modified Rome III criteria for IBS-D[7], defined as a stool frequency of ≥3 per day for more than 2 days per week and ≥25% of stools to be of type 5-7 and ≤25% type 1-2 according to the Bristol Stool Form Scale (BSFS)[157]. To exclude other causes of diarrhoea, we required normal colonoscopy and colonic biopsies, normal full blood count, serum calcium and albumin, C-reactive protein and negative serological test for coeliac disease. Lactose intolerance was tested by asking patients to consume 568 ml of milk/day and performing a lactose breath hydrogen test to see whether they developed diarrhoeal symptoms within 3 hours. If the stools were watery and frequent, the patient then underwent a 7-day retention of selenium75-labelled homocholic acid taurine test or a trial of cholestyramine to exclude bile acid malabsorption. If any of these tests were positive patients were excluded from the study.

All patients gave written consent.

During the screening period of 2 weeks, patients were only allowed a maximum 2 doses of 4mg Loperamide per week and discontinued any IBS medication. Once randomised, patients were allowed to take Loperamide (as required) to control their symptoms, as we hypothesised that Mesalazine would take at least 6 weeks to exert its effect on the gut. At the last 2 weeks of the trial, patients were not allowed Loperamide or any antibiotics.

Other inclusion and exclusion criteria were as stated below:

b) Inclusion criteria

i. Male or Female patients aged 18-75 years able to give informed consent.

ii. Patients should all have had a colonoscopy or sigmoidoscopy within the last 12 months to exclude microscopic or any inflammatory colitis. (If not, but they have had a negative colonoscopy within 5 years and symptoms are unchanged, then a sigmoidoscopy and mucosal biopsy of the left colon would be sufficient to exclude microscopic or any inflammatory colitis).

iii. IBS-D Patients meeting Rome III criteria prior to screening phase.

iv. Patients with ≥25% soft (score >4) and <25% hard (score 1 or 2) stools during the screening phase, as scored by the daily symptom and stool diary*.

v. Patients with a stool frequency of 3 or more per day for 2 or more days per week during the screening phase*.

vi. Satisfactory completion of the daily stool and symptom diary during the screening phase at the discretion of the investigator.

vii. Women of childbearing potential willing and able to use at least one highly effective contraceptive method throughout the study. In the context of this study, an effective method is defined as those which result in low failure rate (i.e. less than 1% per year) when used consistently and correctly such as: implants, injectables, combined oral contraceptives, sexual abstinence or vasectomised partner.

*If inclusion criterion 4 and/or 5 were not met but the results were considered atypical (as observed from medical history and patient recall) then the patient was allowed to re-screen on 1 occasion only. There had to be sufficient data completed during the screening phase to allow adequate classification.

Definition of IBS-D meeting Rome III criteria [7].

Abdominal pain or discomfort at least 2- 3 days/month in the last 3 months (criterion fulfilled for the last 3 months with symptom onset at least 6 months prior to screening) associated with two or more of the following:

- Improvement with defecation;
- Onset associated with a change of stool frequency;
- Onset associated with a change in form (appearance) of stool.

c) Exclusion criteria

i. Women who are pregnant or breast-feeding.

ii. Prior abdominal surgery which may cause bowel symptoms similar to IBS (note appendectomy and cholecystectomy will not be an exclusion).

iii. Patients unable to stop anti-muscarinics, anti-spasmodics, high dose tricyclic antidepressants (i.e. above 50 mg/day), opiates / anti-diarrhoeal drugs*, NSAIDs (occasional over the counter use and topical formulations are allowed), long-term antibiotics, other anti-inflammatory drugs or 5-ASA containing drugs.

iv. Patients on selective serotonin re-uptake inhibitors and low dose tricyclic antidepressants (i.e. up to 50 mg/day) for at least 3 months previous unwilling to remain on a stable dose for the duration of the trial

v. Patients with other gastro-intestinal diseases including colitis and Crohn's disease.

vi. Patients with the following conditions: Renal impairment, severe hepatic impairment or salicylate hypersensitivity.

vii. Patients currently participating in another trial or have been in a trial within the previous 3 months.

viii. Patients who in the opinion of the investigator are considered unsuitable due to inability to comply with instructions.

ix. Patients with serious concomitant diseases e.g. cardiovascular, respiratory, neurological etc.

x. Positive test for bile acid malabsorption

*(A full list of excluded or dose controlled medications can be found in Appendix 1)

*Loperamide was allowed as rescue medication throughout the trial, however if >2 doses / week were taken during the screening phase then they were not eligible, though they could be re-screened on 1 occasion only.

d) Expected duration of participant participation

Study participants participated in the study for 14 weeks.

e) Removal of participants from therapy or assessments

The following subject withdrawal criteria applied:

i. Non-compliance - if less than 75% of IMP doses* are taken between visits, at the investigator's discretion.

*as advised by the study doctor, taking into account that not all participants will be advised to take the full study dose due to intolerance.

ii. If the participant has remained on the initial lower dose of 2g once a day for 3 weeks and the medication is still not tolerated, at the investigator's discretion.

iii. Adverse reaction (serious and non-serious) with clear contraindications.

iv. Participant withdraws consent.

v. Safety reasons e.g. pregnancy**

vi. Lost to follow up.

vii. Participant develops an excluded/contraindicated condition.

viii. Investigator discretion. (e.g. Protocol violations)

ix. Un-blinding, at the discretion of the PI in conjunction with the CI.

Participants withdrawn from the study were replaced. The participants were told that withdrawal would not affect their future care. Participants were also made aware (via the information sheet and consent form) that should they withdraw the data collected up to their withdrawal cannot be erased and may still be used in the final analysis.

** In the event of a pregnancy occurring in a trial participant or the partner of a trial participant, monitoring shall occur during the pregnancy and after delivery to ascertain any trial related adverse events in the mother or the offspring. Where it is the partner of a trial participant, consent will be obtained for this observation from both the partner and her medical practitioner.

3) Summary of overall trial design:

Participants were identified from both primary and secondary care. They were required to meet the modified Rome III criteria and then underwent a 2-week screening with stool diary (see inclusion and exclusion criteria). If eligible, they were randomised (week 0) into taking either a 2g Mesalazine or placebo for the first week and an increment of 4g if they tolerated the medication after 7 days. A weekly stool diary had to be completed for 12 weeks. Participants had telephone call visits at week 1, 3 and 9 to assess for tolerance and compliance. They then returned, in the middle of the trial (week 6) to replenish their medication. They were required to fill in study questionnaires at the beginning and end of the study. Samples of stool, blood and serum were taken at the beginning and end of the study.

For participants in Nottingham (following consent), participants had a fasting baseline and end of study magnetic resonance imaging of their abdomen and sigmoid biopsy (Figure 6 and Table 2).

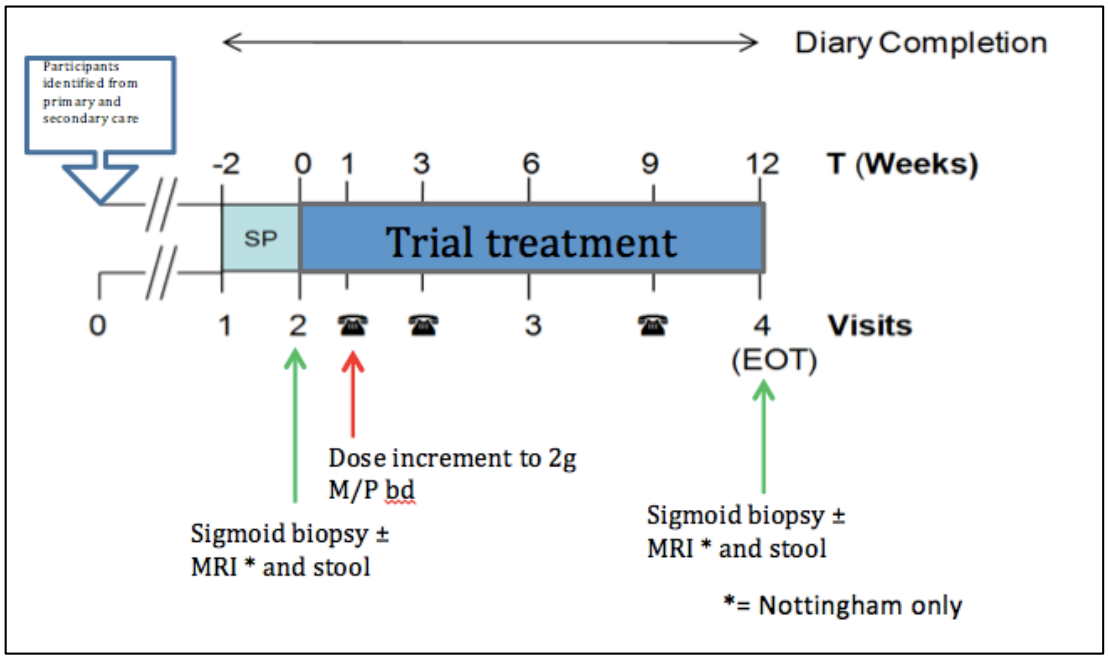


Figure 6: Schematic drawing of the study design

Table 2: Patient visits and contacts

Procedure (Time (T) in wk)		**Visit 1 Screening (T = -2)**	**Visit 2 Randomisation (T = 0, from 1st dose)**	☎ **(T = 1)**	☎[e] **(T = 3)**	**Visit 3 (T = 6)**	☎[e] **(T = 9)**	**Visit 4 Final visit (T = 12)**
Check eligibility		●	●	Check on diary completion, AE check, concurrent medication & treatment tolerance with particular reference to the step increase in IMP dose.	Check on diary completion, AE check, concurrent medication & treatment tolerance.		Check on diary completion, AE check, concurrent medication & treatment tolerance.	
Informed consent		●						
Demographics and bowel symptoms		●						
Physical examination and history		●						
Daily symptom and stool diary[a]		●	●			●		●
Sigmoidoscopy with biopsy to exclude microscopic colitis[b]		●						
Pregnancy test			●					
Randomisation			●					
Questionnaires[c]			●					●
Blood and stool sample			●			●		●
Flexible sigmoidoscopy and biopsies[d]			● (Nottingham only)					● (Nottingham only)
MRI scans[d]			● (Nottingham only)					● (Nottingham only)
IMP	Dispense		●			●		
	Return					●		●
Adverse reaction recording						●		●

[a] Daily symptom and stool diary was completed throughout the participant's involvement in the trial. These were reviewed at each visit

[b] Unless the participant had had a colonoscopy within the last 12 months that excluded microscopic or any inflammatory colitis.

[c] CDC HRQOL4, EQ-5D, HADS and PHQ-15.

[d] Only participants recruited at the Nottingham site underwent MRI scans and flexible Sigmoidoscopy with biopsies.

[e] Telephone contact was either by telephone or e-mail or if convenient at the hospital

1.11.3. Main outcome measure:

1) Clinical outcome:

a) Primary endpoint:

Daily mean stool frequency during weeks 11-12 of the treatment period

b) Secondary endpoint

(all assessed during weeks 11-12 of the treatment period)

i. Average daily severity of abdominal pain on a 0-10 scale.

ii. Days with urgency during weeks 11-12 post-randomisation.

iii. Mean stool consistency using Bristol Stool Form Score.

iv. Global satisfaction with control of IBS symptoms as assessed from the answer to the question "Have you had satisfactory relief of your IBS symptoms this week? Yes/No."

c) Ancillary secondary endpoints

i. EQ-5D

ii. CDC HRQOL4

iii. HADS

iv. PHQ-15

d) Safety endpoints

i. Adverse events related to the trial treatment

ii. Withdrawal from the trial treatment due to adverse events.

2) Mechanistic outcome:

a) Primary endpoint:

Mast cell numbers (mean % area stained per m^2) at week 12

b) Secondary endpoints:

i. Mast cell tryptase release during 6-hour biopsy incubation

ii. IL-1β, TNF-α, histamine and serotonin secretion during same incubation

iii. Small bowel tone assessed by volume of fasting small bowel water (Section 3)

iv. Faecal Tryptases and calprotectin

1.11.4. Sample size

Our previous study on diarrhoea predominant IBS patients gives a mean stool frequency of 3.1 (standard deviation 2.0). Tuteja and colleagues reported Mesalazine decreasing stool frequency by 1.4 bowel movements per day[147]. Our study had 80% power to detect such an effect at the 1% two-sided alpha level. We aimed to randomise at least 125 patients to allow for a 20% drop out rate but owing to recruitment ongoing at multiple sites and patient requests we actually recruited 136.

Much smaller numbers are needed to assess the effect of Mesalazine on mast cell numbers and tryptase release. Corinaldesi *et al* reported a 36% decrease in mast cell numbers from mean 9.2, (standard deviation 2.5) [146] that required just 12

patients to show such a decrease with a power of 90% at the 1% alpha level.

1.11.5. Data Analysis

(An independent statistician at the University of Nottingham performed all clinical primary and secondary analyses. The remaining analyses such as the mechanistic and post hoc analyses were carried out by myself.)

Analysis and presentation of data was in accordance with CONSORT guidance. The primary data set included stool diary filled out for at least 10 days out of 14. Balance between the trial arms at baseline was examined using appropriate descriptive statistics. For continuous variables, data was summarised in terms of the mean, standard deviation, median, lower & upper quartiles, minimum, maximum and number of observations. Categorical variables were summarised in terms of frequency counts and percentages.

The general approach for between-group comparisons was intention-to-treat (ITT). Appropriate regression modelling was used to evaluate the primary and secondary outcomes, and safety data, with due emphasis placed on clinical importance of 95% confidence intervals for between-group estimates.

No formal adjustment for multiple significance testing was applied.

Full details were given in a separate Statistical Analysis Plan and approved before data lock.

The safety monitoring functions of the trial were undertaken by the Data Monitoring Ethics Committee (DMEC). The DMEC meetings were held bi-annually and the committee members were happy with the progress of the trial.

Clinical

1) Assessment of efficacy

We used descriptive statistics to compare the randomised groups at baseline. The primary outcome was assessed using intention to treat without imputation. We used a generalised linear mixed model to compare Mesalazine group and placebo group for the primary outcome, with adjustment for the baseline value of the outcome, and study centre as a random effect. Additionally, we adjusted for any variables showing imbalance at baseline in secondary models. We compared the characteristics of participants who did and did not adhere with the study medication before estimating the treatment effect and if the medication was actually taken using Complier Average Causal Effect (CACE) analysis. We investigated the effect of missing primary outcome data using multiple imputations. The secondary outcomes were assessed using similar models as for primary outcome, or logistic or Poisson regression as appropriate dependent on outcome type.

We undertook subgroup analyses by including appropriate interaction terms in the linear mixed model for primary outcome according to baseline daily mean stool frequency, baseline mean abdominal pain score and baseline mean HADS anxiety score.

Secondary outcomes were treated similarly, after transformation if appropriate, while binary and count outcomes were handled by multiple logistic or Poisson regression as appropriate. All analyses were performed using the current version of Stata adopting the intention to treat principle without imputation for missing data (with a sensitivity analysis using multiple imputation for the primary outcome). .

We planned to conduct a number of pre-specified subgroup analyses.

For each of the following three outcomes:

a) Stool frequency during week 11-12
b) Number of days with any stool consistency scoring 6 or 7 during week 11-12
c) Average of worst pain for each day during week 11-12

We investigated whether there were any differences in between-group effects according to the following baseline variables: (1) anxiety; (2) stool frequency; (3) abdominal pain; (4) mast cell activation*

These sub-group analyses were conducted by including appropriate interaction terms in the regression models, and as the study has not been powered to detect any such sub-group effects, were considered as exploratory and would require confirmation in future research

*Mast cell activation will be defined as elevation of any of the inflammatory mediator components such as mast cell tryptase, IL-1β, TNF-α, histamine and serotonin in biopsy supernatant.

The primary mechanism hypothesis to be investigated was that treatment with Mesalazine reduces inflammation, which in turn reduces clinical symptoms. The aim of this type of analysis is to estimate how much of any observed treatment effect can be attributed to a variable that is thought to be an intermediate on the causal pathway, or mediator.

After summarising inflammatory markers at baseline and 11-12 weeks' follow up by trial arm using appropriate descriptive statistics, we will examine change in these markers (stool calprotectin, mast cell tryptase and mast cell % area stained) and change in stool frequency using a scatterplot.

2) Procedures for missing data

The effect of missing data will be investigated in sensitivity analyses by multiple imputations using the method of chained equations. Incomplete data is defined as stool diary completed for <10 out of 14 days during weeks 11-12.

3) Definition of populations analysed

Safety set: All randomised participants who received at least one dose of the study drug.

ITT set: All randomised participants for whom at least one post-baseline assessment of the primary endpoint is available

Mechanistic

The primary mechanistic hypothesis to be investigated was that treatment with Mesalazine reduces inflammation, which in turn reduces clinical symptoms. The aim of this type of analysis is to estimate how much of any observed treatment effect can be attributed to a variable that is thought to be an intermediate on the causal pathway, or mediator. After summarising inflammatory markers at baseline and 11-12 weeks' follow up by trial arm using appropriate descriptive statistics, the change in these markers (stool calprotectin, mast cell tryptase, mast cell % area stained) and change in stool frequency using scatterplots were examined.

The statistical analysis was carried out with the use of Prism 6 (GraphPad Software Inc, San Diego, CA). Normality of the data was tested by using the D'Agostino & Pearson omnibus normality test. Comparisons between 2 different groups were done using the two-tailed Mann Whitney test or unpaired t-test depending on normality. Comparisons within similar group were done using Wilcoxon matched-pairs sign rank test or paired t-test depending on normality. The sample size for further subgroup analyses were small, therefore no assumptions were made about the distribution of data, and non-parametric testing was used. The data are expressed as mean (± SD) when normally distributed and as median (interquartile range) when not normally distributed.

1) Sigmoid biopsy

Patients who consented to this had a sigmoid biopsy taken before and after treatment. This was performed in the unprepared bowel. 8 tissue biopsy samples were obtained from the sigmoid colon (30cm from the anus) using a '2.4 mm Captura biopsy forceps without spike' by Cook@ Medical. They were taken for:

Immunohistochemistry (H+E, CD3, CD68, 5-HT and mast cell tryptase)

Supernatants for tryptase, carboxypeptidase A3, chymase, histamine and serotonin

Results of the immunohistochemistry and supernatants for tryptase, carboxypeptidase A3, chymase, histamine and serotonin were compared against a group of healthy controls from a previous study. The preparation of the sigmoid biopsy samples was similar for the healthy control. Biopsy samples obtained from the healthy controls were processed in the same period of time as the IBS-D patients.

a. Immunohistochemistry

2 biopsy samples obtained were soaked in formalin until they are ready to be cut, fixed and embedded in paraffin wax. Samples were sent to the histopathology laboratory at Queen's Medical Centre, Nottingham University Hospitals Trust, for dissection, embedding and staining. Immunohistochemistry staining were for CD3, CD68, enterochromaffin cells containing serotonin (5-HT) and mast cell tryptase (MCT). Table 3 (below) shows a simplified protocol for these stains.

Table 3: Protocol for immunohistochemistry staining (courtesy from Immunohistochemistry laboratory in Nottingham University Hospitals Trust)

Antibody	Supplier (order code)	Dilution	Pretreatment
MCT	Dako (M7052)	1/500	Protease 1 for 4 min Primary antibody for 32 min Roche Ultraview detection kit plus Amplification
CD3	Leica (NCL-L-CD3-565)	1/50	SCC1 (EDTA based buffer) for 64 min Primary antibody for 32 min Ultraview detection kit plus Amplification
CD68	Dako (M0814)	1/2000	SCC1 for 64 min Primary antibody for 32 min Ultraview detection
5HT	Dako (M0758)	1/400	Protease1 for 4 min Primary antibody for 32 min Ultraview detection

The slides prepared were scanned into the computer using the nanozoomer and were magnified x40 for ease of portability. Cell counting was performed by a single person (LTX; fellow from the FRAME lab, University of Nottingham) who was blinded to the study. Detection of each stained cell type was checked for reproducibility (>95%) before cell counting began. At least 5-10 areas around lamina propria were drawn and CD68 cells were counted giving an average cell number per mm^2. CD3, which is a marker of lymphocytes, was assessed by counting the number of stained cells at the superficial epithelium per area drawn (mm^2) and an average obtained. The 5-HT cells were counted at the deep lamina propria and an average of number of cells/mm^2 obtained. Mast cell tryptase expression was detected in the

lamina propria using automatic software (i-Tem by Olympus) as some mast cells may be in a de-granulated state, thus making cell counting difficult. Results were presented as the percentage area stained for mast cell tryptase.

b. Tissue biopsy for supernatants:

Preparation:

2 biopsies were obtained and immediately processed to obtain biopsy supernatants. Initial biopsies were weighed before each was placed into a Falcon 3037. 1 ml sterile water was placed around the edge of each biopsy before adding 2 ml of Hanks balanced salt solution (LH-SIG2025E) into the centre of the Falcon 3037. This was then placed into the incubator for 30 minutes at 37^{o}C, 5% CO^{2}. After 30 minutes, the central solution (supernatant) was collected and placed into a cryovial for storage at -80^{o}C. A further 2 ml Hanks solution was added to the centre of the Falcon 037 and incubated for a further 30 minutes at the same temperature and CO^{2} setting. Following 30 minutes, the supernatant was collected and placed into a 2nd cryovial for storage at -80^{o}C. This process is repeated again for a third time. At the end of the 3rd incubation, the biopsy was weighed and stored at -80^{o}C.

The supernatants from the 1st incubation (0-30 min) were used to measure these contents:

i. Serotonin (5HT)
ii. Tryptase
iii. Chymase
iv. Carboxypeptidate 3 (CPA3) and

v. Histamine

Serotonin (5HT) supernatant assay protocol

The samples were processed and analysed by Dr Gulzar Singh (School of Medicine, University of Nottingham).

Preparation of samples for analysis

5HT release supernatant samples were freeze-dried initially.

The residue was dissolved in 400ul of methanol: water (50:50).

The samples were then vortexed for 30 seconds, and centrifuged at 25,000g for 10min.

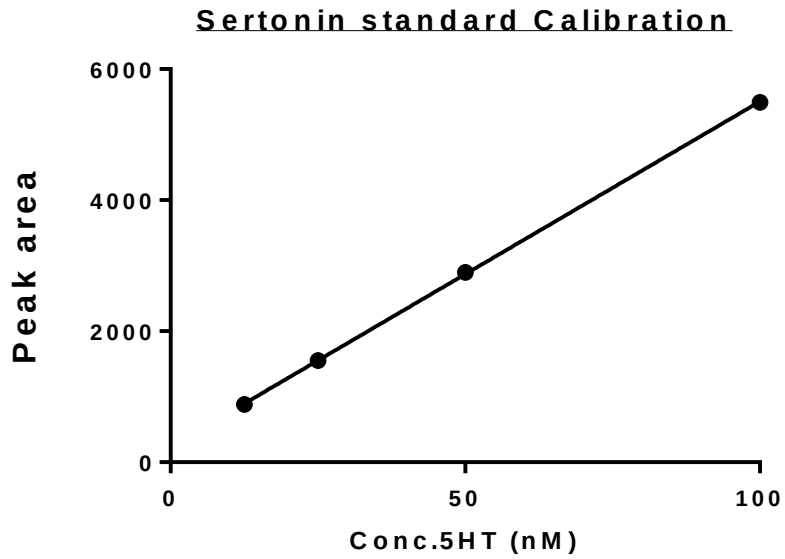
The 5HT-containing supernatant was filtered through cellulose acetate membrane with pore size 0.2um and analysed using liquid-chromatography mass-spectrometry system (LC-MS).

5HT assay using LC-MS

Reversed-phase HPLC was used, a Luna column C18 (3um, 2.1 x 75mm) chromatographed at 0.4mL.min^{-1} on a Jasco PU-2085 Plus semi-micro HPLC pump system using an isocratic system of 5% acetonitrile and 0.01% formic acid in water.

A Triple Quadrupole mass-spectrometer (Waters Quattro Ultima) was employed using positive-ion Electrospray ionisation with time-resolved MRM transitions for precursor and product ion analysis and using MassLynx 4.0 software to control all systems and data processing.

Analysers was mass-calibrated using a range of standard 5HT concentrations and alpha-methyl 5HT was be used as an internal standard.



Waters Quattro Ultima LC-MS/MS.

This is a triple quadrupole instrument with excellent performance for quantitative analysis. It is used for targeted metabolite profiling and readily capable of monitoring multiple analysates simultaneously. The Liquid Chromatography mass-spectrometry (LC-MS-MS) system consists briefly of:-

Waters 2700 Sample Manager autosampler.

Alltech degassing system.

Perkin –Elmer column oven.

Jasco PU-2085 Plus semi-micro HPLC pumps.

Waters MS-MS triple quadrupole Quattro Ultima

Mast cell mediators

Mast cell proteases (tryptase, chymase and CPA3) were measured by sandwich ELISA assays provided by the Immunopharmacology Research Group, the University of Southampton, as described previously [158-160]. Briefly, coating antibodies against tryptase (EAR), chymase (CC2) and CPA3 (CA2) were coated on 96 well ELISA plates (COSTAR) for overnight at +40C. Blocking with 2% BSA after three washes for one hour at room temperature; followed by adding samples or protein standards of tryptase, chymase or CPA3 which were extracted and purified by the same research group. The plates were incubated for 90 min; then detecting antibodies specifically against tryptase (AA1), chymase (CC5) or CPA3 (CA5) were used. Finally, the avidin-HRP and colorimetric subtract TMB system was used, and the absorbance was read at 450nm of the microplate reader, Thermo max (Molecular Devices). Prior to all assays, the validation to the specific body fluids and protein spiking were carried out. The assays were blinded.

Histamine was measured using a commercial kit called Histamine (Life science format) Elisa kit by Neogen® Cooperation. This was processed by the Immunopharmacology Research Group, the University of Southampton. See Appendix 4 for instructions.

c) Serotonin and 5HIAA content

Sigmoid biopsies obtained, were immediately snap frozen in liquid nitrogen before being stored in a -80^{o}C freezer prior to processing.

Preparation of samples for analysis

Preparation and analysis for Serotonin and 5HIAA content was performed by Dr Gulzar Singh (School of Medicine, University of Nottingham).

i. 5HT release supernatant samples were freeze-dried initially.
ii. The residue was dissolved in 400ul of methanol: water (50:50).
iii. The biopsies were homogenised for 10 s using MSE sonicator (Soniprep 150, MSE (UK) LTD., Worsley Bridge Rd. Lower Sydenham, London).
iv. The samples were then vortexed for 30 seconds, and centrifuged at 25,000g for 10min.
v. The 5HT-containing supernatant was filtered through cellulose acetate membrane with pore size 0.2um and analysed using liquid-chromatography mass-spectrometry system (LC-MS).
vi. The rest of the processing was similar as above for Serotonin supernatant assay analysis.

d) Inflammatory mediators

Levels of IL-1β and TNF-α was analysed by using a commercial kit V-Plex immunoassay by Meso Scale Discovery, Rockville, United States of America. This was processed by a clinical fellow from the Centre of Biomolecular Science, University of Nottingham. He was blinded to the study.

e) Stool:

i. Stool tryptase methodology

Stool samples were collected no more than 2 hours before it was handed over to the research staff for storage in the -80 ^{0}C freezer. All processing has been performed by BRU technicians (Melanie Lingaya and Yirga Falcone) and results obtained thereafter. Samples were analysed for faecal tryptase based on methods published recently by our group[161]. See Appendix 6 for methodology. Faecal protease activity is expressed in trypsin units per milligram of protein

ii. Stool calprotectin

Note: freezing stool samples may result in slight increased in calprotectin concentrations due to lysis of neutrophils in the sample.

The Buhlmann calprotectin ELISA kit was used for extraction and quantification of stool calprotectin (MRP8/14; S100A8/S100A9). Below (Figure 7) is the short protocol for calprotectin extraction

Full instruction in Appendix 5

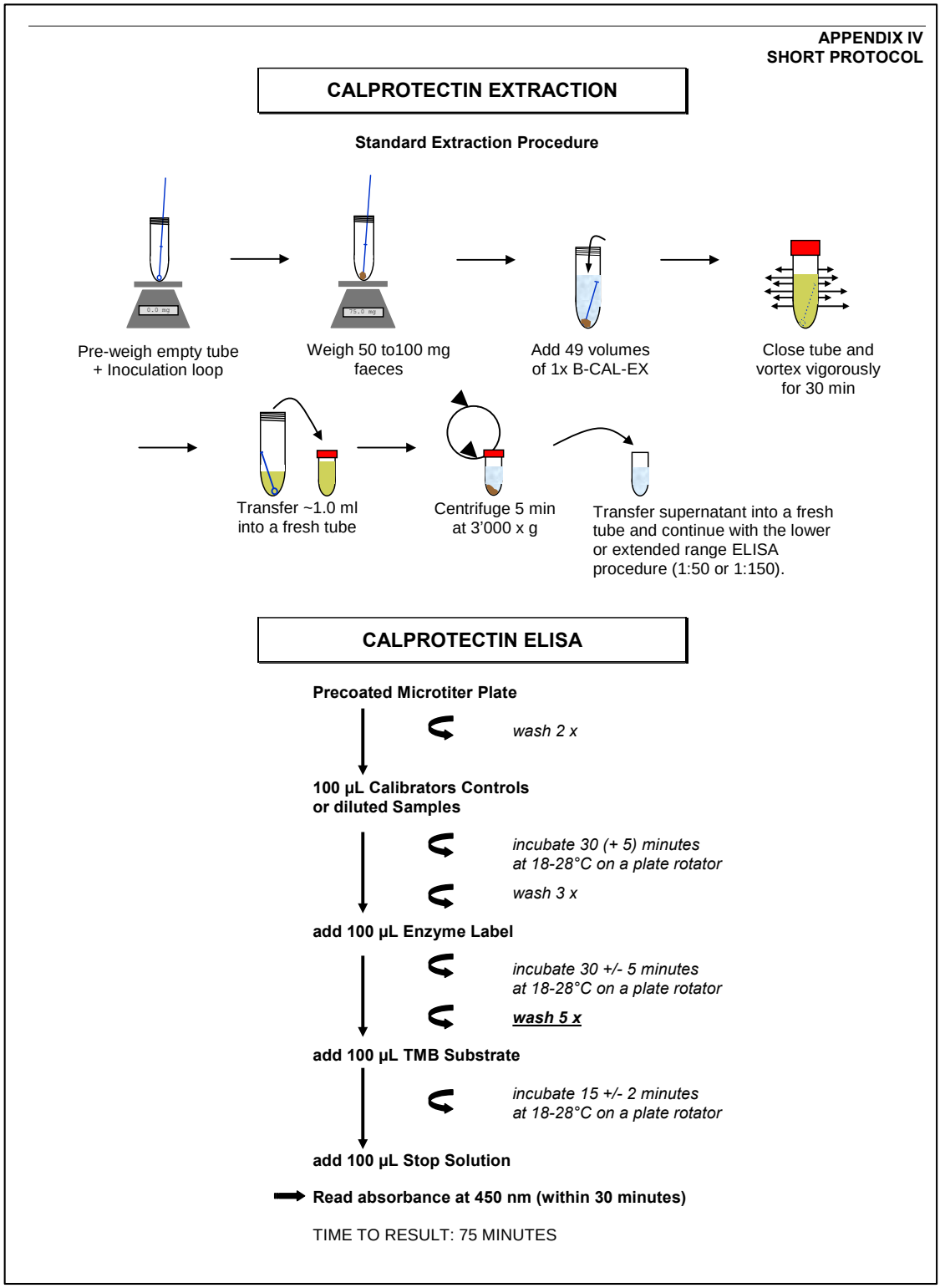


Figure 7: Short protocol on calprotectin extraction

1.11.6. Results

Of 221 initially screened, 185 were eligible and 136 were enrolled and randomised into the study (Figure 8). Follow up was completed in August 2013. The most frequent reason for exclusion was decline to participate. The commonest reason for not meeting inclusion criteria was that the patients' diaries during the 2 weeks run in period indicated that they did not have loose stools ≥25% of the time or stool frequency of 3 or more per day for 2 or more days per week.

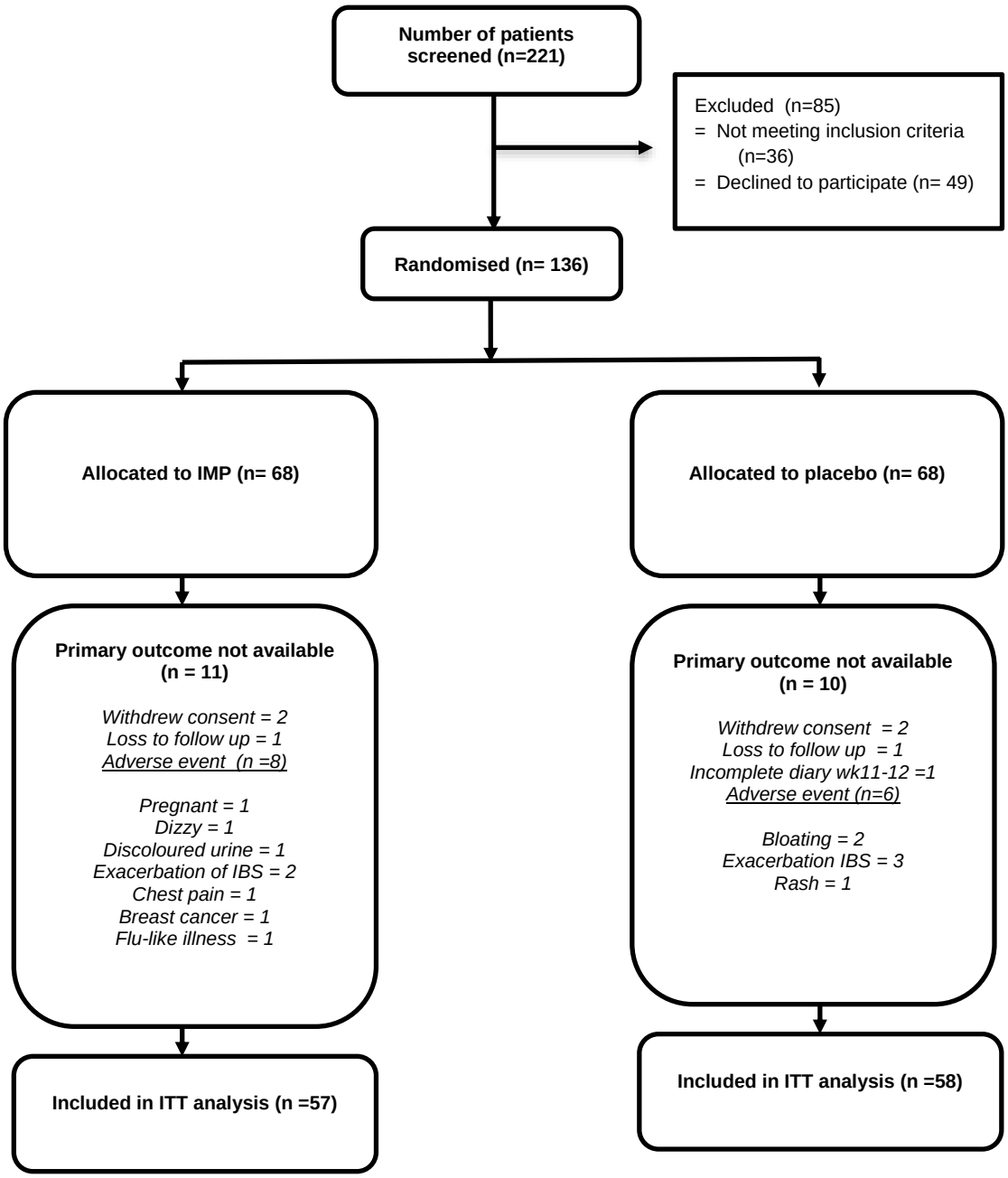


Figure 8: Patient flow diagram (CONSORT diagram)

1) Demographics:

There were a total of 8 sites that participated in this study. Table 4 and Supplementary tables 1 and 2 (Appendix 7) showed a summary of recruitment by site and by treatment arm.

Characteristics of enrolled patients in both groups were similar at baseline (Table 5).

Table 4: Summary of recruitment by site and by treatment arm

Site	Placebo	Mesalazine
Nottingham	38	40
Manchester	16	15
Derby	2	2
Mansfield	4	3
Doncaster	5	5
Stoke on Trent	1	1
South tees	1	1
North tees	1	1

Table 5: Summary of baseline data by treatment group

Characteristic	Mesalazine (n=68)	Placebo (n=68)	Total (n=136)
Age at enrolment			
Mean (SD)	42.6 (15.2)	47.1 (13.5)	44.8 (14.4)
Gender			
Male N (%)	26 (38.2%)	28 (41.2%)	54(39.7%)
Ethnicity			
White	66 (97.1%)	66 (97.1%)	132(97.1%)
Black	0	1 (1.5%)	1(0.7%)
Asian	1 (1.5%)	0	1(0.7%)
Mixed	1 (1.5%)	0	1(0.7%)
Other	0	1 (1.5%)	1(0.7%)
Daily mean stool frequency			
Mean (SD)	3.6 (1.6)	3.6 (1.8)	3.6 (1.7)
Daily mean abdominal pain score			
Mean (SD)	4.1 (2.2)	3.6 (2.0)	3.6 (1.7)
Number of days with urgency			
Median (IQR)	13 (10-14)	12 (9-14)	12.5 (9-14)
Stool consistency			
Mean (SD)	5.4 (0.7)	5.6 (1.0)	5.5 (4.4)
HADS score			
Mean (SD)	9.1 (4.5)	8.6 (4.3)	8.8 (4.4)
PHQ-15 score			
Mean (SD)	12.6 (5.2)	13.1 (5.6)	12.8 (5.4)

Symptoms are based on 14 days screening symptom diaries

Primary and secondary outcome data were collected for 115 (85%) and 116 (85%) participants respectively at 11-12 weeks of follow up.

2) Clinical primary outcome:

The primary intention to treat comparison showed no evidence of any clinically significant difference between Mesalazine and placebo for the primary outcome (Table 6). Additional adjustments for variables (age, abdominal pain score, number of days with urgency and PHQ-15 score) displaying imbalance at baseline did not materially change the results, nor did multiple imputation analysis or CACE analysis (Tables 7a-c).

Subgroup analyses (Table 8a) of the primary outcome by baseline daily mean stool frequency suggest that Mesalazine may be more effective among patients with greater baseline stool frequency which is associated with larger treatment effect but this could be a chance finding and would require confirmation in further studies. There was no evidence that treatment effect differed according to baseline pain or hospital anxiety and depression score (HADS) (Tables 8b and 8c).

Our sensitivity analysis using multiple imputation of missing data for the primary outcome showed no effect on primary outcome (Table 7b).

Table 6: Clinical primary outcome of daily mean stool frequency at week 11-12

Daily mean stool frequency [mean (SD)]			
	11-12 weeks	**Between group difference at 11-12 weeks (95% CI)***	**P value**
Placebo (N=58)	2.7 (1.9)	--	--
Mesalazine (N=57)	2.8 (1.2)	--	--
Mesalazine vs. Placebo	--	0.10 (-0.33,0.53)	0.66

Table 7a: Primary analysis with further adjustment of baseline covariates

Average Stool Frequency	**Adjusted* Diff. in mean frequency**	**P-value**	**95% C.I.**
Mesalazine (N=57) vs. Placebo (N=58)	0.13	0.56	(-0.31, 0.57)

**Adjusted by age, study centre and baseline daily mean stool frequency*

Table 7b: Primary analysis with multiple imputation

Average Stool Frequency	**Adjusted* Diff. in mean frequency**	**P-value**	**95% C.I.**
Mesalazine (N=57) vs. Placebo (N=58)	0.06	0.17	(-0.18, 0.99)

**Adjusted by baseline daily mean stool frequency and study centre*

Table 7c: Primary analysis (CACE)

Average Stool Frequency	Adjusted* Diff. in mean frequency	P-value	95% C.I.
Mesalazine (N=57) vs. Placebo (N=58)	0.16	0.67	(-0.58, 0.91)

**Adjusted by baseline daily mean stool frequency and study centre*

Table 8a: Primary outcome subgroup analysis by baseline stool frequency

	Placebo (N=58)	Mesalazine (N=57)
Daily mean stool frequency at 11-12 weeks by baseline frequency [mean (SD)]		
Baseline frequency ≤2.4	1.6(0.5)	1.7(0.4)
Baseline frequency >2.4 and ≤3.4	2.2(1.1)	2.2(0.5)
Baseline frequency >3.4 and ≤4.6	2.7(0.9)	3.1(1.3)
Baseline frequency >4.6	4.7(2.9)	4.1(1.1)
Estimates* for interaction in primary analysis model with 95% CI and P value		
Primary outcome by baseline stool frequency	-0.26 (-0.51, -0.01); p=0.04	

**adjusted by baseline daily mean stool frequency and study centre*

Table 8b: Primary outcome subgroup analysis by baseline abdominal pain score

	Placebo (N=58)	Mesalazine (N=57)
Daily mean stool frequency at 11-12 weeks by baseline abdominal pain score [mean (SD)]		
Baseline pain score ≤2.2	2.9(2.8)	2.7(0.9)
Baseline pain score >2.2 and ≤4.1	2.6(1.4)	2.4(0.7)
Baseline pain score >4.1 and ≤5.3	2.4(1.4)	3.0(1.6)
Baseline pain score >5.3	3.2(1.7)	2.9(1.3)
Estimates* for interaction in primary analysis model with 95% CI and P		
Primary outcome by baseline pain score	-0.03 (-0.10, 0.04); p=0.36	

**adjusted by baseline daily mean stool frequency and study centre*

Table 8c: Primary outcome subgroup analysis by baseline HADS score

	Placebo (N=58)	Mesalazine (N=57)
Daily mean stool frequency at 11-12 weeks by baseline HADS score [mean (SD)]		
Baseline HADS score ≤5.0	3.1(3.2)	3.0(1.4)
Baseline HADS score >5.0 and ≤9.0	2.3(1.3)	2.8(1.2)
Baseline HADS score >9.0 and ≤11.5	3.0(1.5)	2.9(1.3)
Baseline HADS score >11.5	2.0(0.9)	2.6(1.3)
Estimates* for interaction in primary analysis model with 95% CI and P		
Primary outcome by baseline HADS score	-0.01 (-0.04, 0.03); p=0.79	

**Adjusted by baseline daily mean stool frequency and study centre*

3) Clinical secondary outcomes:

No differences were apparent for any of the secondary outcomes, with the exception of number of days with urgency (Table 9), which were increased by about 20% on Mesalazine treatment compared to placebo.

Table 9: Secondary outcome results

	Baseline	11-12 weeks	Between group comparison at 11-12 weeks (95% CI)[1]	P value
Daily mean abdominal pain score [mean (SD)]				
Placebo	3.6(2.0)	2.2(2.1) (N=59)	--	--
Mesalazine	4.1(2.2)	2.8(2.1) (N=57)	--	--
Mesalazine vs. Placebo	--	--	0.07 (-0.54, 0.68)[2]	0.83
Number of days with urgency [median (IQR)]				
Placebo	12[9-14]	8(1-13) (N=59)	--	--
Mesalazine	13[10-14]	11(5-14) (N=57)	--	--
Mesalazine vs. Placebo	--	--	1.22 (1.07, 1.39)[3]	<0.01
Weekly mean stool consistency [mean (SD)]				
Placebo	5.6[1.0]	4.7(1.1) (N=59)	--	--
Mesalazine	5.4[0.7]	4.7(1.0) (N=57)	--	--
Mesalazine vs. Placebo	--	--	0.13(-0.21, 0.48)[2]	0.45
Number of days with consistency score 6 or 7 [median (IQR)]				
Placebo	11(8-13)	6(2-9) (N=59)	--	--
Mesalazine	11(8-13)	7(2-11] (N=57)	--	--
Mesalazine vs. Placebo	--	--	1.09(0.95, 1.27)[3]	0.21
Mean HADS score (SD)				

Placebo	8.6(4.3)	6.9(3.6) (N=59)	--	--
Mesalazine	9.0(4.5)	7.5(5.0) (N=57)	--	--
Mesalazine vs. Placebo	--	--	0.67(-0.38, 1.72)[2]	0.21
Mean PHQ15 score [mean (SD)]				
Placebo	13.1(5.6)	9.4(5.0) (N=59)	--	--
Mesalazine	12.6(5.2)	10.0(5.2) (N=57)	--	--
Mesalazine vs. Placebo	--	--	0.63(-0.93, 2.20)[2]	0.43
Number of people with satisfactory relief of IBS symptoms [N (%)]				
Placebo	0	24(40.7%) (N=59)	--	--
Mesalazine	0	25(43.9%) (N=57)	--	--
Mesalazine vs. Placebo	--	--	1.13(0.51, 2.47)[4]	0.76
EQ5D: 5 division components [N(%)] who have no problems				
	Baseline		**After treatment**	
	Placebo	**Mesalazine**	**Placebo**	**Mesalazine**
Mobility	46(67.6%)	53 (77.9%)	47(79.3%)	44(77.2%)
Self-care	66(97.1%)	63(92.6%)	57(96.6%)	52(91.2%)
Usual activity	39(57.4%)	44(64.7%)	44(74.6%)	45(78.9%)
Pain/ Discomfort	7(10.3%)	8(11.8%)	15(25.4%)	15(26.3%)
Anxiety/ Depression	39(57.4%)	39(57.4%)	37(62.7%)	35(61.4%)
EQ VAS score [mean(SD)]				
	Baseline	**After**	**Between group**	**P value**

		treatment	comparison	
Placebo	64.3(20.2)	69.7(18.3) (N=59)	--	--
Mesalazine	64.2(20.6)	72.6(19.2) (N=57)	--	--
Mesalazine vs. Placebo	--	--	2.39 (-3.24, 8.02)[2]	0.41

[1] *estimate depends on type of outcome variable and is adjusted by baseline value of the outcomes if appropriate*

[2] *difference in means*

[3] *incidence rate ratio*

[4] *odds ratio*

4) Compliance:

Compliance was defined, a priori, as taking ≥75% of the medication throughout the 12 weeks. Each patient was given 2 boxes of medication during the 12-week study, each box containing 100 sachets. The amount of medication taken was calculated by 200 minus the number of medication sachets returned at EOT. Compliance with medication (Table 10) and baseline characteristics of compliers (defined as taking ≥75% of the medication throughout the 12 weeks) (Table 11) were similar in both groups. Analysis of the primary outcome using CACE approach showed no difference between the two treatment arms [Mean difference (95% Confidence Interval)]: 0.2(-0.6,0.9).

Table 10: Summary of compliance with trial medication (Participants who completed 12 weeks of treatment)

	Placebo (n=59)	Mesalazine (n=57)
Compliance[1] **Mean (SD)**	72%[17%]	71%[19%]
Complier[2] **N (%)**	35(59%)	33(58%)

[1] *Calculated as 100 minus proportion of trial medication returned*

[2] *Complier is defined as compliance ≥75%*

Table 11: Summary of baseline data by complier and treatment group

	Non-complier (n=48)		Complier (n=68)	
	Placebo (N=24)	Mesalazine (N=24)	Placebo (N=35)	Mesalazine (N=33)
Age				
Mean (SD)	48.9(13.6)	41.4(14.1)	45.2(14.2)	40.6(14.7)
Gender				
Male N (%)	12(50.0%)	10(41.7%)	11(31.4%)	12(36.4%)
Ethnicity				
White N (%)	24(100%)	24(100%)	34(97.1%)	32(97.0%)
Asian N (%)	0	0	0	1(3.0%)
Other N (%)	0	0	1(2.9%)	0
Daily mean stool frequency, Mean [SD]	3.7(1.8)	4.2(1.7)	3.7(2.0)	3.3[1.5)
Abdominal pain score Mean [SD]	3.6(2.1)	4.3(1.6)	3.5(2.0)	4.5(2.4)
Number of days with urgency, Median [IQR]	13(10-14)	13(11-14)	12(7-14)	13(10-14)
HADS score, Mean (SD)	9.2(4.1)	7.9(3.8])	8.1(4.2)	9.9(4.9)
Stool consistency, Mean (SD)	5.4(0.7)	5.5(0.7)	5.7(1.2)	5.3(0.6)
PHQ-15 score, Mean (SD)	12.3(4.7)	12.0(3.9)	13.2(6.2)	13.8(5.4)

5) Adverse events

The most frequent occurring side effect was exacerbation of IBS symptoms, which could be worsening abdominal pain or diarrhoea. 2 patients (3%) from the Mesalazine and 3 patients (5%) from the placebo group complained of this and were withdrawn from the study. 1 patient was pregnant in the middle of the trial period although she had a negative pregnancy test at the start of the trial. She was withdrawn from study with no adverse consequence to herself or her newborn[162]. 1 patient from the Mesalazine group was found to have breast cancer and she was withdrawn from the study as her IBS symptoms and stool diary would be very difficult to interpret. All participants who developed these adverse events were withdrawn from the study and their symptoms settled on follow up (Table 12).

Table 12: Adverse events following randomisation

Adverse event	Mesalazine	Placebo
Exacerbation of IBS (worsening abdominal pain and/ or diarrhoea)	2	3
Bloating	0	2
Dizziness	1	0
Chest pain	1	0
Rash	0	1
Discoloured urine	1	0
Pregnant	1	0
Flu-like illness	1	0
Breast cancer	1	0

6) Mechanistic primary outcome:

(All tissue samples obtained from the IBS-D patients and healthy volunteers [from another study] were processed in the same period of time.)

The mast cell percentage area stained was elevated in IBS-D patients when compared to our normal range previously established in our laboratory. Median (IQR) for IBS-D were 4569 (3500-5884) while the normal range is 1936 (1453-3145) per m^2; (Figure 9). There was no reduction in mast cell % area stained following treatment with Mesalazine (Figure 10 and Table 13).

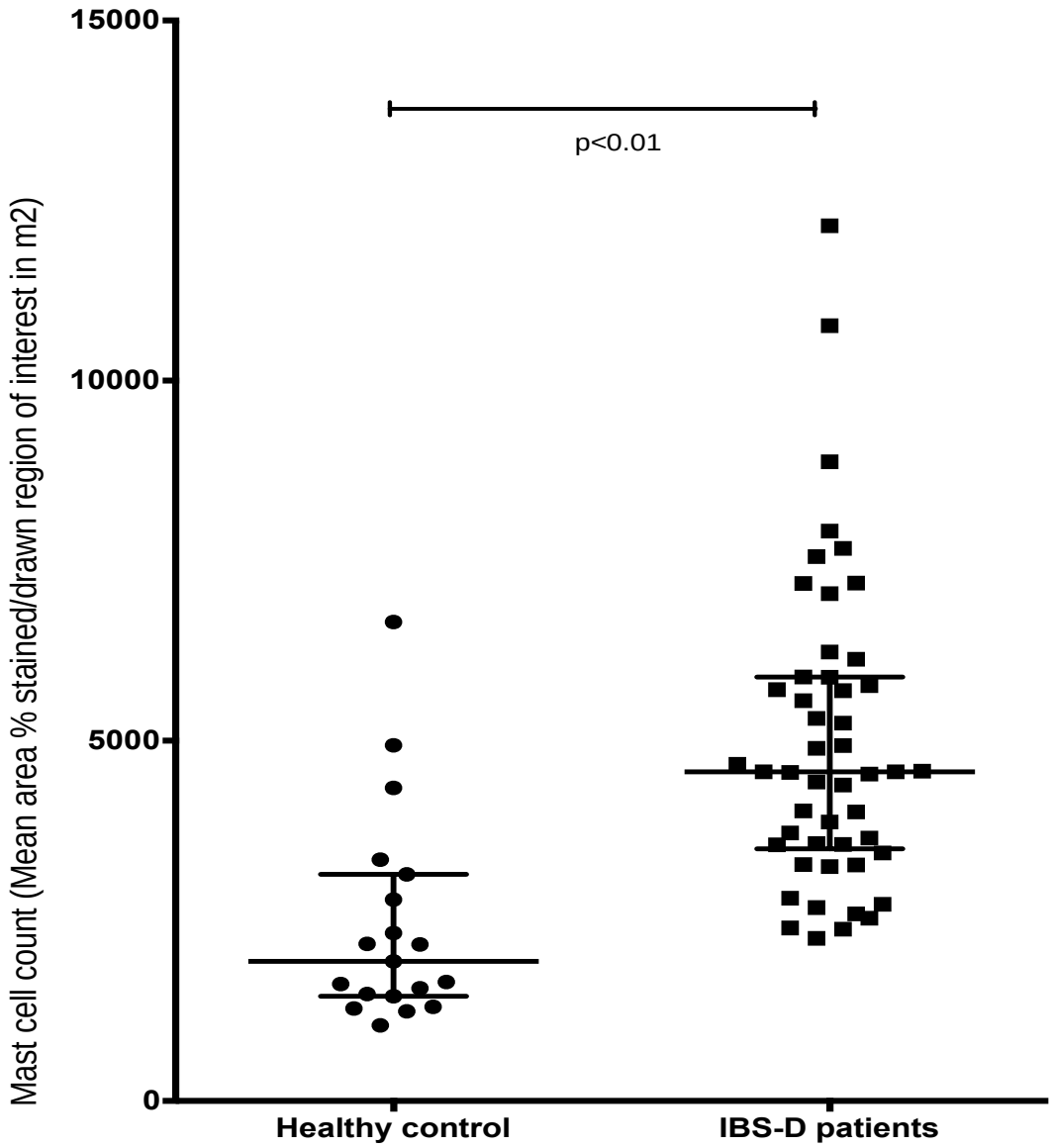


Figure 9: Mast cell count assessed from % area stained comparing healthy controls and patients with IBS-D

Table 13: Effect of Mesalazine vs. placebo on mast cell % area stained in patients with IBS-D

Mast cell percentage area stained/m2	Mesalazine baseline	Mesalazine after treatment	Placebo baseline	Placebo after treatment
Mean (SD)	5167 (2729)	5303 (2014)	4765 (1238)	3978 (1802)

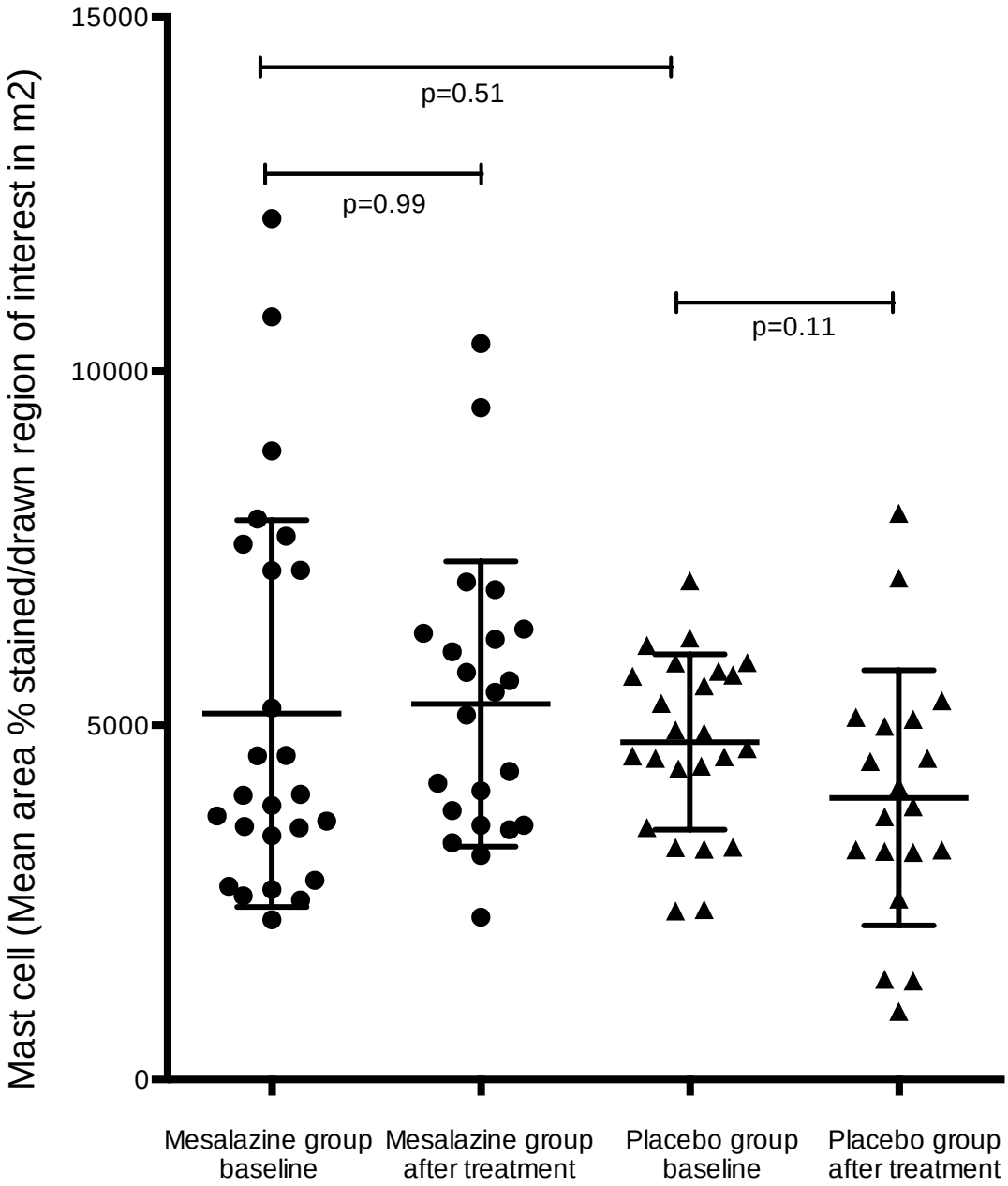


Figure 10: Effect of Mesalazine vs. placebo on mast cell % area stained in patients with IBS-D, (Mean,SD)

7) Mechanistic secondary outcome:

a) Mast cell tryptase and other mediator release during biopsy incubation

Baseline supernatant levels were compared between IBS-D and healthy volunteers. There was no significant increase in the baseline mediator levels except carboxypeptidase A3 (CPA3). See Figure 11 and Table 14.

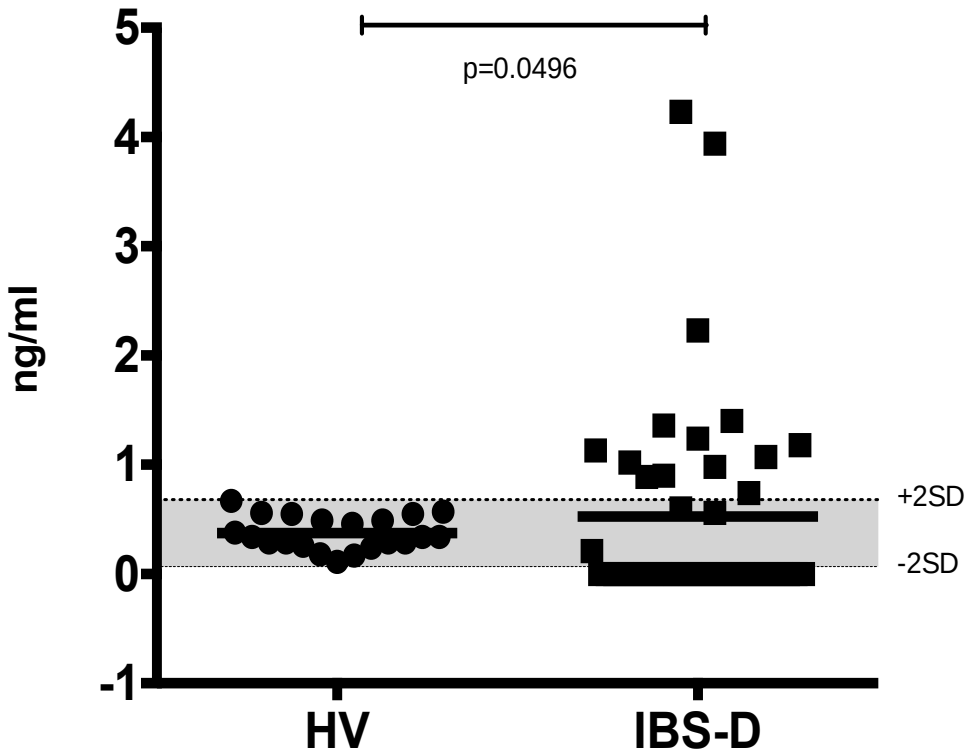


Figure 11: Baseline Carboxypeptidase A3 levels in IBS-D patients. Shaded area indicates normal range in healthy volunteers(HV), (Median,IQR).

Table 14: Baseline supernatant levels between HV and IBS-D patients

Baseline supernatant levels (ng/ml), Median (IQR)	Healthy volunteer (HV) N=21	IBS-D patient, N=45	P value
Tryptase	6.7 (3.8-11.4)	4.3 (1.8-8.9)	0.07
Chymase	0	0 (0-0.9)	0.14
CPA3	0.34 (0.28-0.52)	0 (0-0.9)	0.05
Histamine	1.6 (0.7-3.8)	0.7 (0-1.3)	<0.01

Following treatment of either Mesalazine or placebo in the IBS-D patients, there was no change in the mediators. See Table 15 and Figures 12-16.

Table 15: Supernatant mediators following treatment of Mesalazine or placebo

Supernatant mediators (ng/ml), Median (IQR)	Mesalazine baseline (n=21)	Placebo baseline (n=23)	Mesalazine after treatment	Placebo after treatment
Tryptase	4.3 (1.5-8.6)	4.6 (2.5-9.1)	4.9 (1.8-8.2)	5.8 (2.1-10.3)
Chymase	0 (0-0.3)	0 (0-0.8)	0 (0-1.7)	0 (0-0.4)
CPA3	0 (0-0.3)	0 (0-1.0)	0 (0-0.8)	0 (0-0.5)
Histamine	0.9 (0.3-1.4)	0.7 (0-1.4)	0.8 (0-1.2)	0.7 (0.2-1.0)
5-HT (pmol/mg)	9.4 (6.1-15.1)	6.3 (2.7-13.7)	10.7 (5.4-14.0)	9.3 (3.4-14.7)

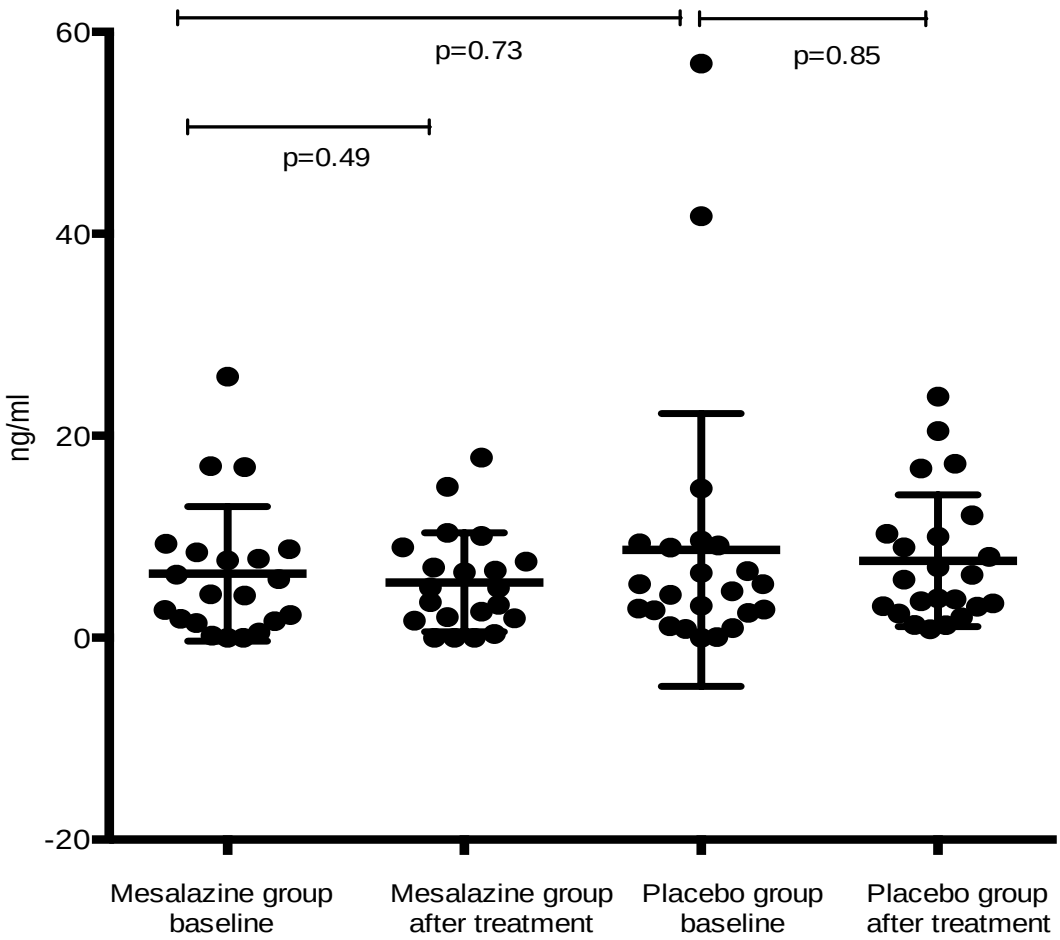


Figure 12: Tryptase levels before and following treatment with Mesalazine or placebo, (Median,IQR)

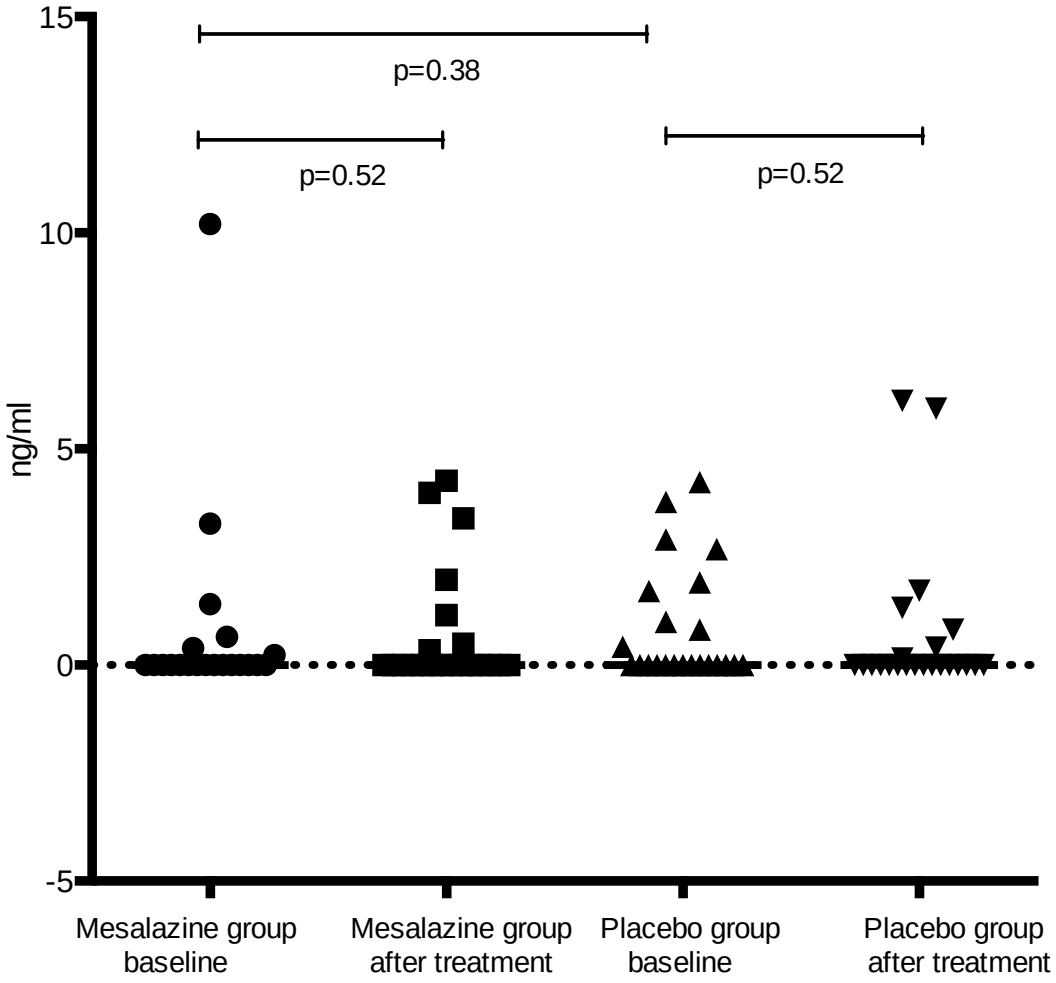


Figure 13: Chymase levels before and after treatment with Mesalazine or placebo, (Median,IQR).

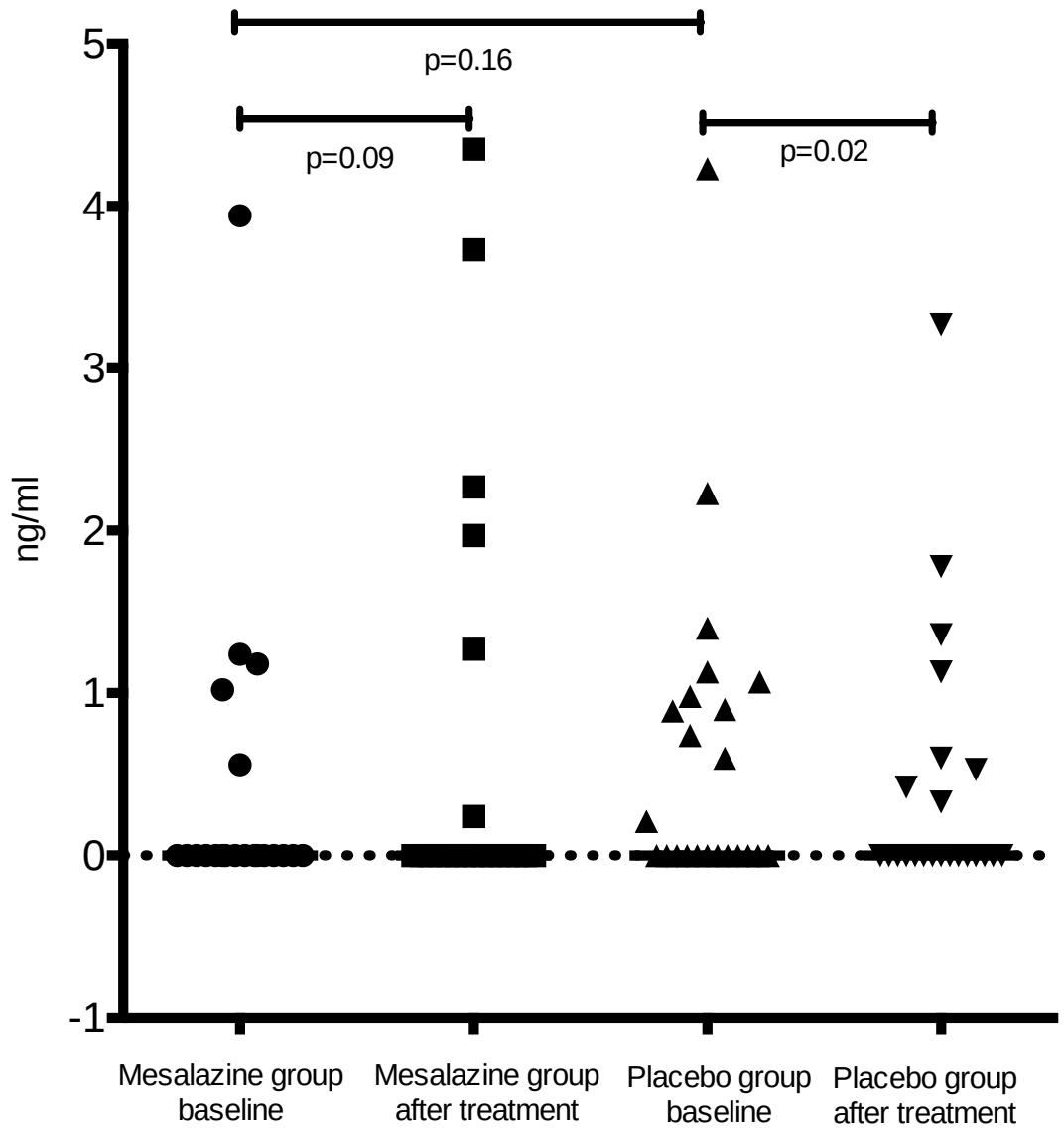


Figure 14: Carboxypeptidase A3 (CPA3) levels before and after treatment with Mesalazine or placebo, (Median,IQR).

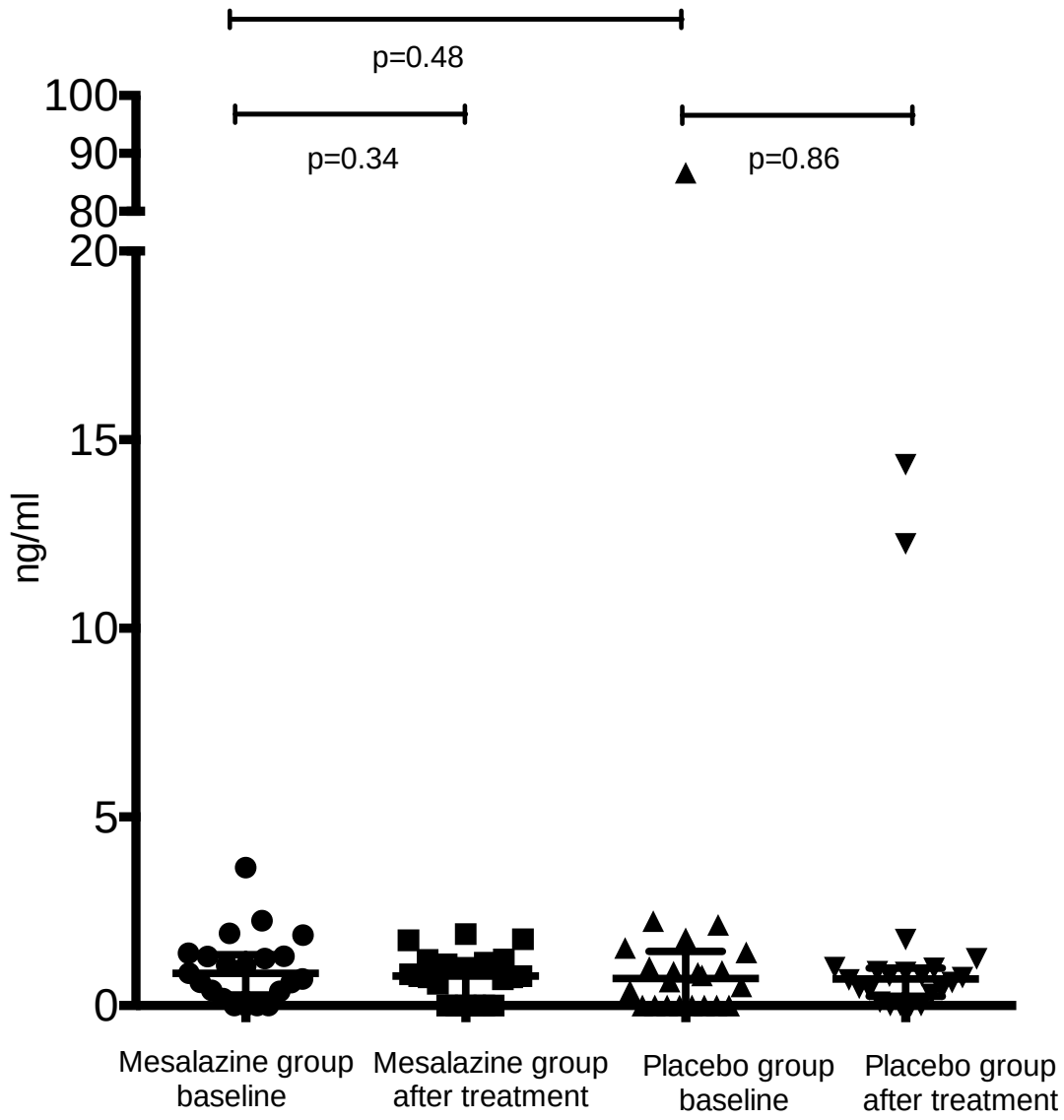


Figure 15: Histamine levels before and after treatment with Mesalazine or placebo, (Median,IQR).

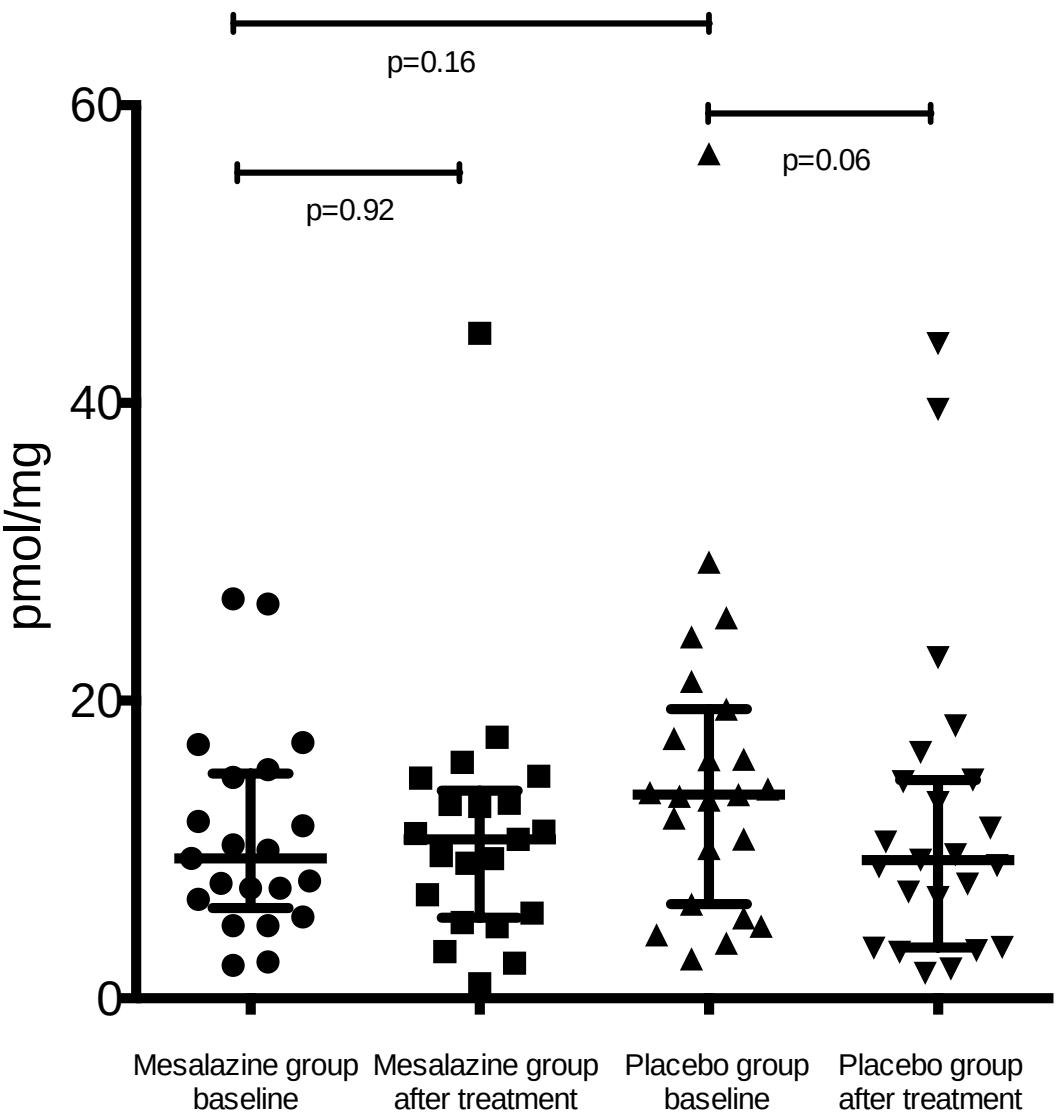


Figure 16: Serotonin (5HT) levels before and after treatment of Mesalazine or placebo (Median,IQR).

b) Serotonin and 5HIAA release (sigmoid biopsy)

The baseline 5HIAA and 5-HT ratios is significantly higher in the patient group compared to the healthy control with median (IQR) of 0.11 (0.06-0.27) and 0.02 (0.01-0.05) respectively with $p<0.01$. See Figure 17.

Following treatment of mesalazine/placebo, there were no significant changes in either group. See Figure 18. Mean differences (SD) following treatment for mesalazine was -0.06 (0.37) and placebo group was 0.02 (0.42); $p=0.43$.

There was no correlation between baseline 5HIAA: 5-HT ratio with baseline serotonin supernatant or serotonin cell count (Table 16).

There was no correlation between baseline 5HIAA: 5-HT ratio with clinical symptoms (Table 16).

Table 16: Correlation between 5HIAA:5-HT ratio with 5-HT supernatant/cell count and clinical symptoms

Correlation between baseline 5HIAA: 5-HT ratio	Correlation Spearman, r	P value
Mechanistic:		
Serotonin supernatant (pmol/mg)	-0.11	0.49
Serotonin cell count (per mm2)	-0.22	0.15
Clinical symptoms:		
Abdominal pain severity	-0.04	0.80
Urgency	0.07	0.64
Bloating	-0.23	0.14
Average daily bowel frequency	0.18	0.25
Average stool consistency	0.18	0.24
Anxiety	-0.06	0.69
Depression	0.03	0.86

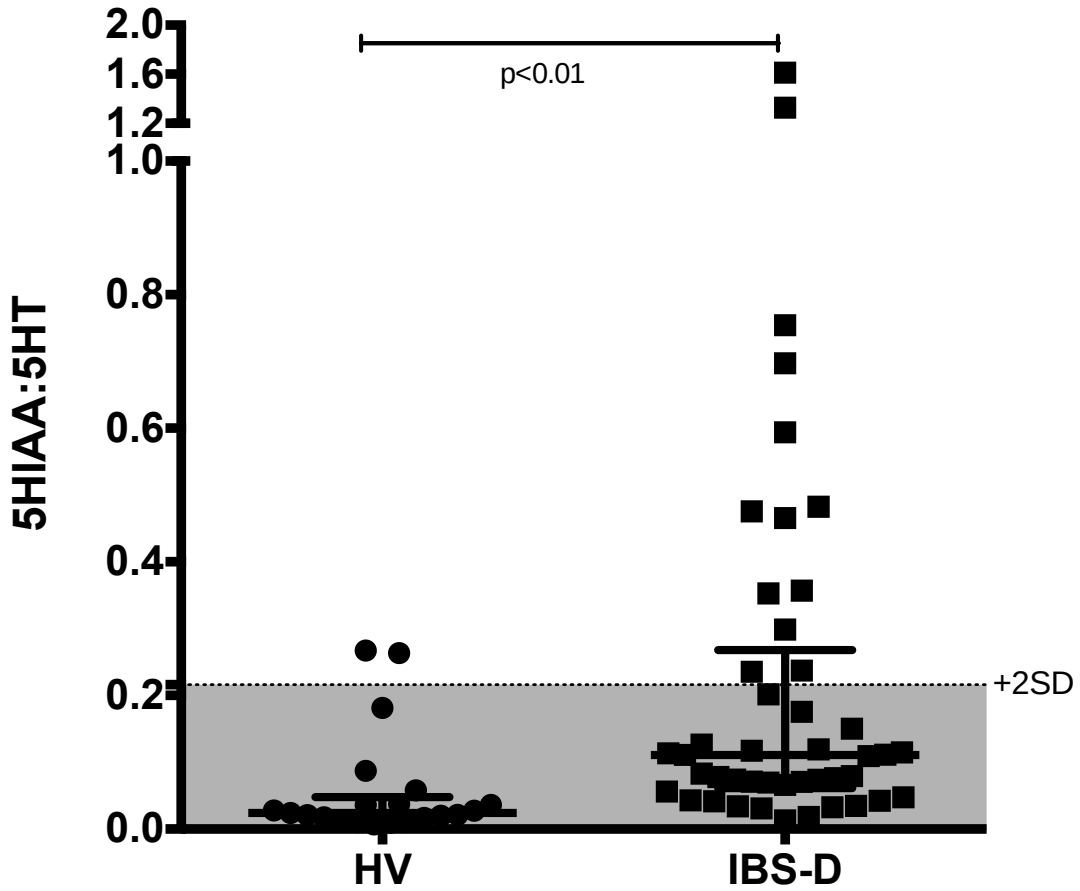


Figure 17: Baseline 5HIAA and 5HT ratio in healthy volunteer and IBS-D

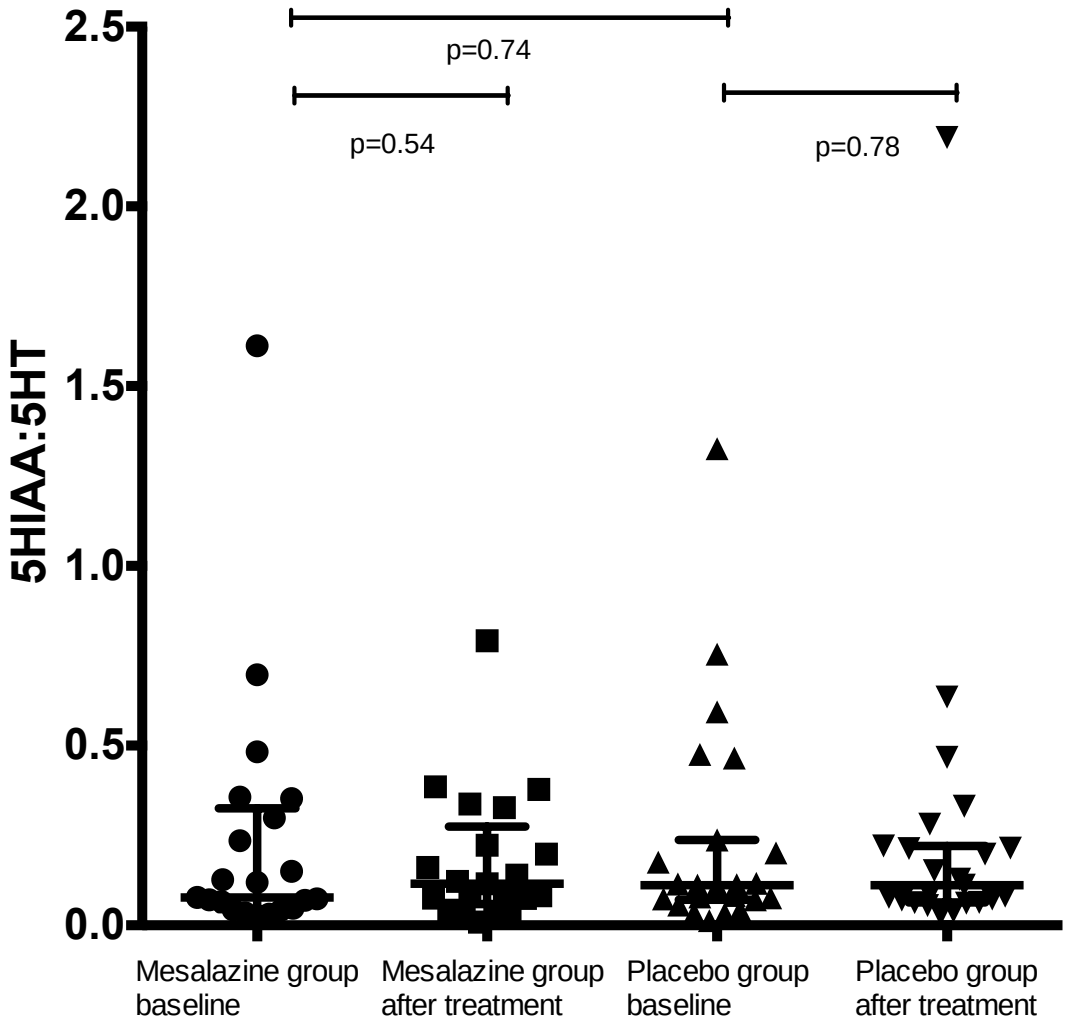


Figure 18: 5HIAA and 5HT ratio following treatment of either mesalazine or placebo, Median (IQR).

c) IL-1β, TNF-α

Levels of IL-1β and TNF-α in supernatant were below the level of detection.

d) Small bowel tone assessed by volume of fasting small bowel water

Further details/results (see Section 3.4)

e) Faecal Tryptases

27 and 30 pairs of stool samples were collected in the Mesalazine and placebo groups respectively. The baseline faecal tryptase level was in the range between 6.8 and 577.8 trypsin units/mg of protein, which was variable. There was a significant increase in faecal tryptase following treatment of Mesalazine (Figure 19 and Table 17). There was no correlation between baseline faecal tryptase and baseline supernatant tryptase level, Spearman r=0.13, p=0.41. There was no significant correlation between baseline faecal tryptase with anxiety, depression and bowel symptoms (Table 18).

Table 17: Faecal tryptase levels following treatment with Mesalazine or placebo

Faecal tryptase (trypsin units/ mg of protein), Median (IQR)	**Mesalazine baseline (n=30)**	**Placebo baseline (n=27)**	**Mesalazine after treatment**	**Placebo after treatment**
	61.2 (37.6-101.4)	66.5 (44.8-126.5)	82.7 (40.5-194.8)	70.9 (36.0-191)

Table 18: Correlation between faecal tryptase and anxiety, depression and abdominal symptoms

Correlation between baseline faecal tryptase (trypsin units/mg of protein)	Spearman r correlation	P value
Baseline abdominal pain severity	-0.19	0.14
Baseline urgency	-0.15	0.23
Baseline bloating	-0.13	0.31
Baseline average daily stool frequency	-0.08	0.54
Baseline average stool consistency	-0.15	0.23
Baseline anxiety score	-0.02	0.88
Baseline depression score	-0.02	0.85

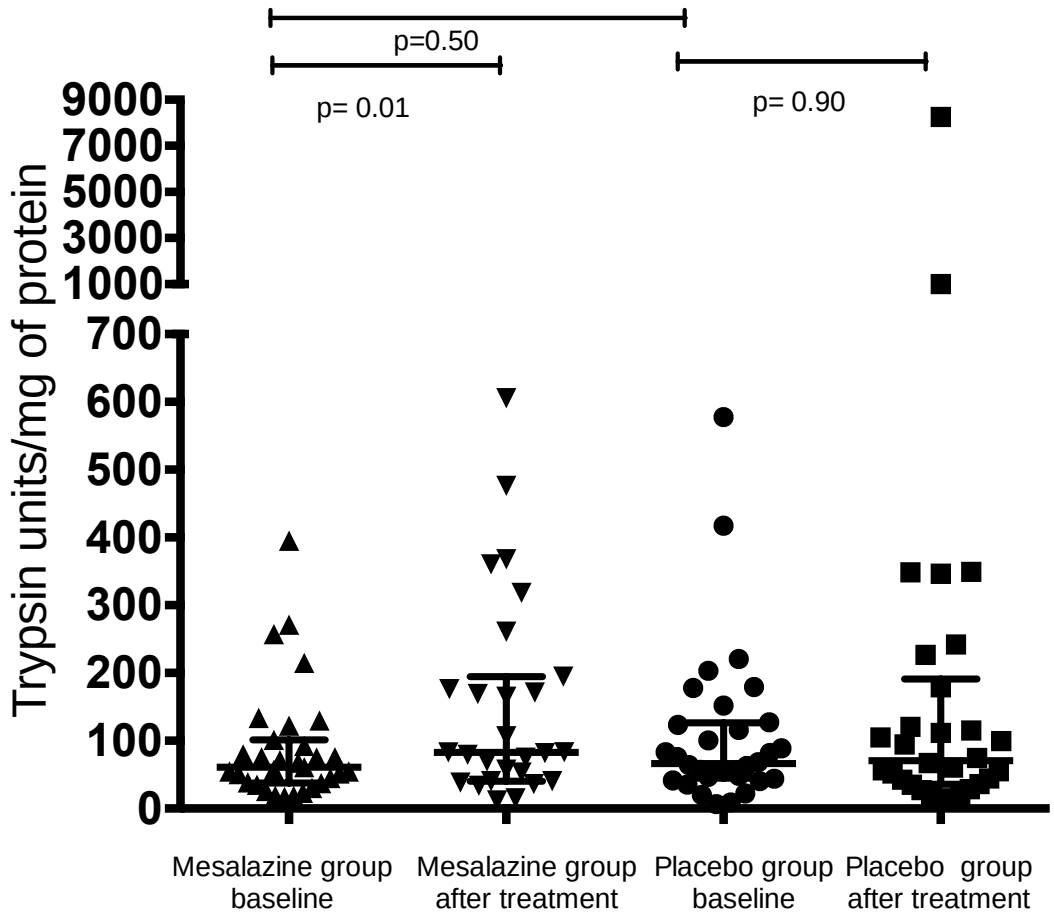


Figure 19: Change in faecal tryptase following treatment of Mesalazine compared with placebo, (Median,IQR).

f) Difference in primary outcome measure between those with different TNFSF15 polymorphism

Genotyping has yet to be done but given the predicted small numbers with the risk allele and the lack of evidence of immune activation we don't think there will be such a gene effect.

8) Post-hoc analysis

a) Mast cell % area stained

There was weak correlation for mast cell count with urgency score and stool consistency (Table 19) but no correlation with abdominal pain severity or bloating.

Table 19: Correlation between mast cell count with clinical symptoms

Clinical Symptoms			
No correlation of mast cell % area stained with abdominal pain severity, average stool frequency and bloating			
Correlation between mast cell count (n=44)	**Spearman r**	**95% CI**	**P value**
Urgency score (0-10)	0.27	-0.005 to 0.51	0.05
Stool consistency (Bristol Stool Form Scale)	0.30	0.01 to 0.5	0.04

There was no significant correlation of mast cell percentage area stained with objective measures of tryptase, chymase, CPA3 and histamine in biopsy supernatants.

Definition of 'normal' mast cell percentage area stained was 0-4936/m2 (cut off at 90th centile from healthy control). This study showed 9 out of 23 in the Mesalazine group have high mast cell percentage area stained. In the Mesalazine group who had high mast cell percentage area stained, there were no significant changes in clinical symptoms such as abdominal pain severity, bloating, urgency, average daily

bowel frequency and stool consistency following treatment compared to the 'normal' mast cell percentage area stained group. See Table 20 and Figures 20-24.

Table 20: Mean difference in symptoms between the 'normal' and 'high' mast cell percentage area stained IBS-D patients who were on Mesalazine

Mean difference in symptoms scores following treatment with Mesalazine (after-before), (SD)	'Normal' mast cell percentage area stained in IBS-D patients treated with Mesalazine (N=14)	'High' mast cell percentage area stained in IBS-D patients treated with Mesalazine (N= 9)	P value
Abdominal pain severity	-1.66 (1.44)	-1.88 (2.14)	P=0.77
Urgency symptoms	-2.26 (2.16)	-1.96 (1.52)	P=0.73
Bloating symptoms	-1.70 (1.58)	-0.41 (1.63)	P=0.07
Average daily bowel frequency	-0.91 (1.83)	-1.21 (0.73)	P=0.65
Average stool consistency	-0.94 (1.37)	-0.16 (0.48)	0.08

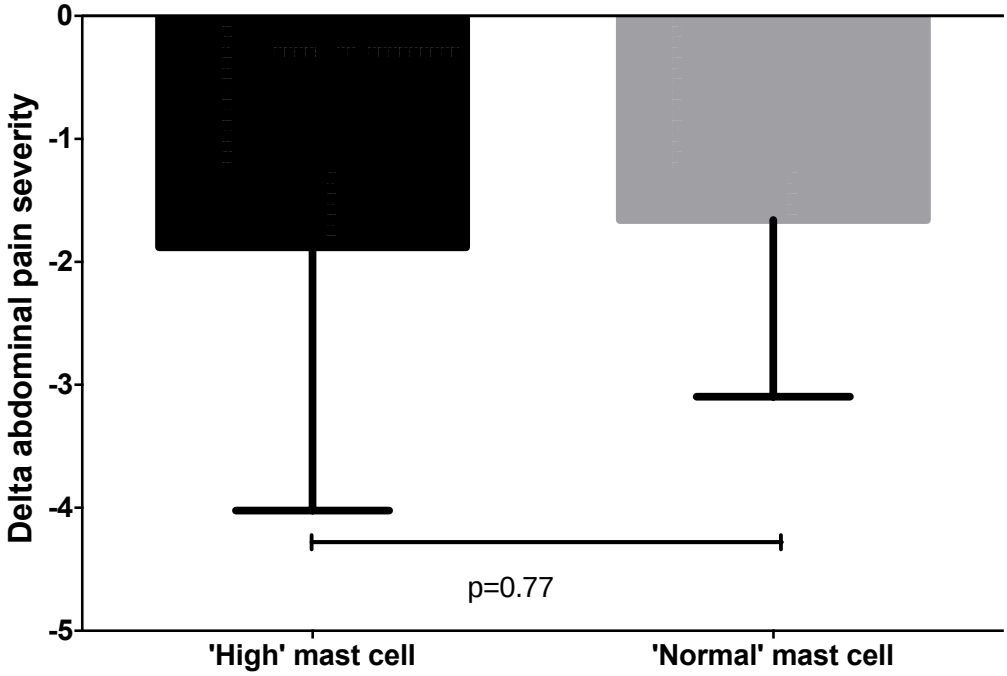


Figure 20: Differences in abdominal pain severity score following treatment of Mesalazine in the groups with 'high' and 'normal' mast cell percentage area stained

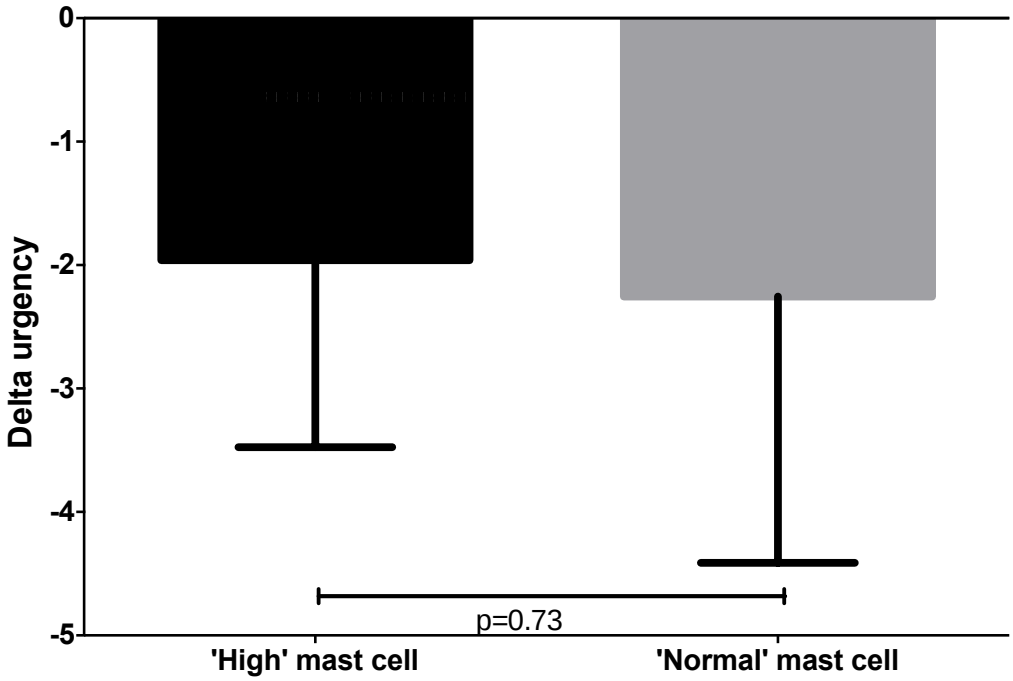


Figure 21: Differences in urgency score following treatment of Mesalazine in the groups with 'high' and 'normal' mast cell percentage area stained

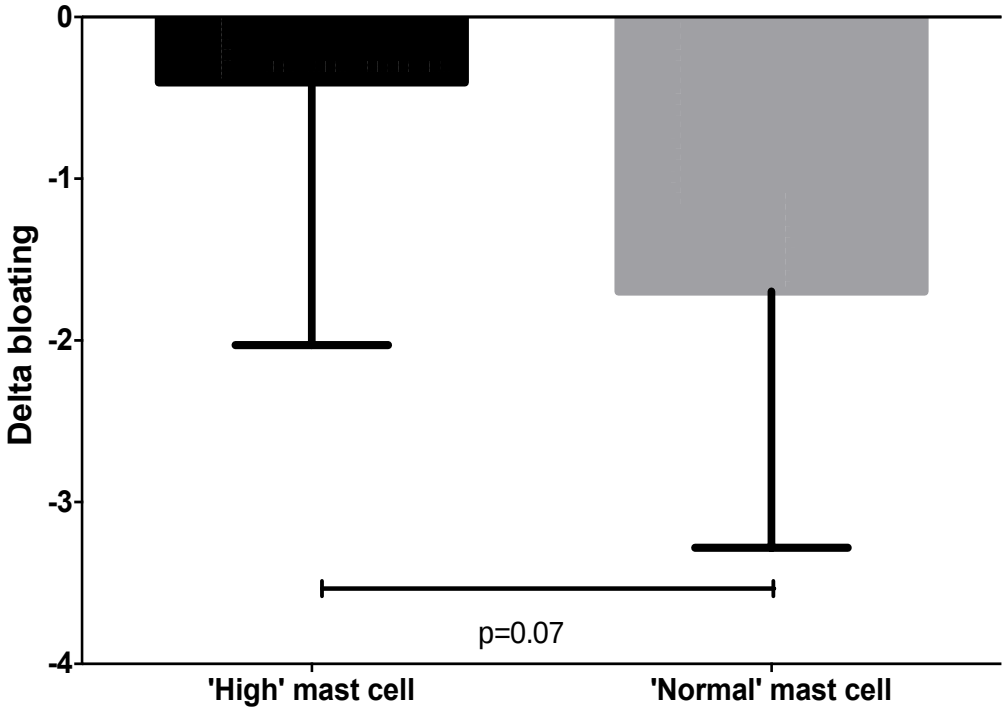


Figure 22: Differences in bloating score following treatment of Mesalazine in the groups with 'high' and 'normal' mast cell percentage area stained

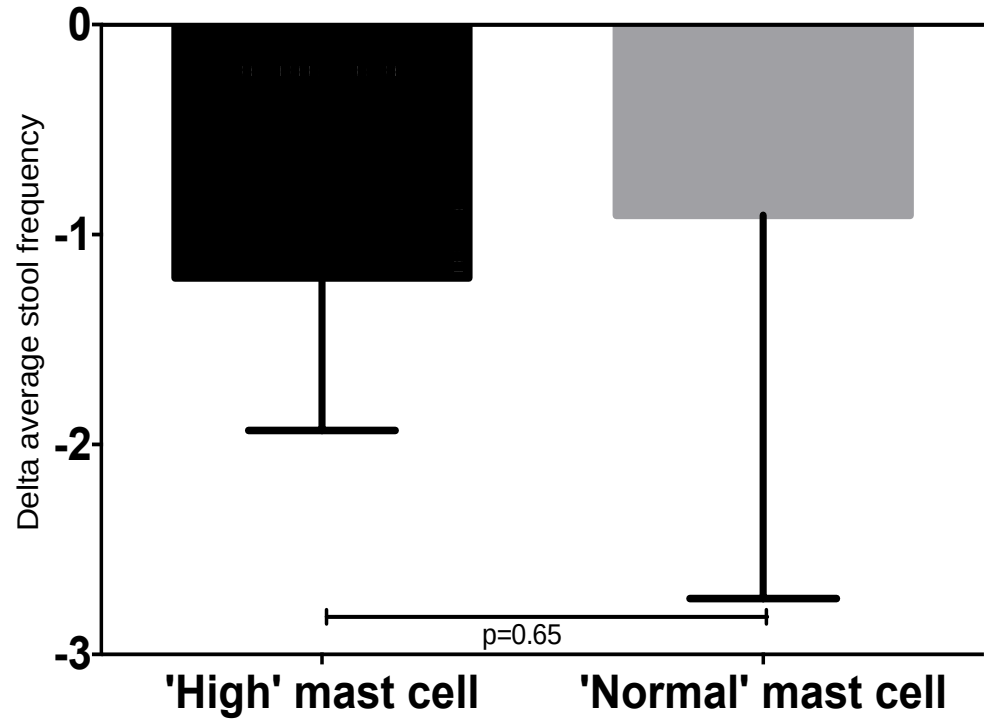


Figure 23: Differences in average daily stool frequency following treatment of Mesalazine in the groups with 'high' and 'normal' mast cell percentage area stained

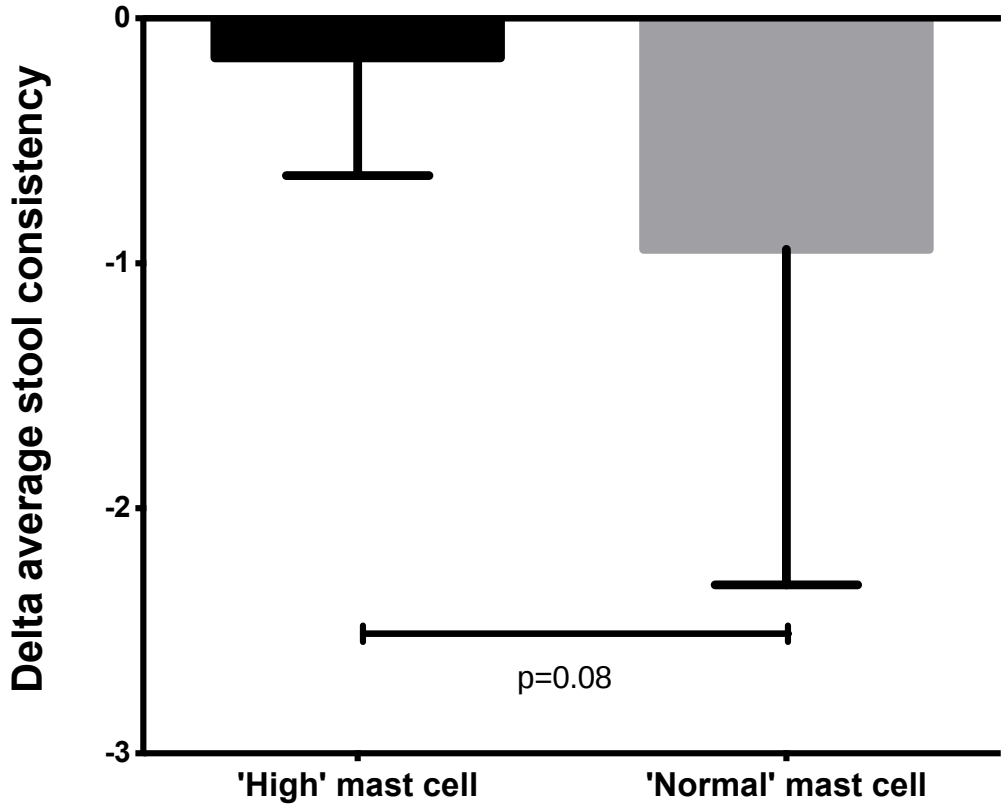


Figure 24: Differences in average daily stool consistency following treatment of Mesalazine in the groups with 'high' and 'normal' mast cell percentage area stained

b) Other immune cells e.g. CD3, CD68 and serotonin (5-HT) containing enterochromaffin cells

i. CD68

The CD68 is a marker of macrophage. Baseline CD68 is significantly lower in the IBS-D compared to healthy control. Median (IQR) were 1037 (836.8-1204) and 1326 (1257-1549); $p<0.01$. Figure 25.

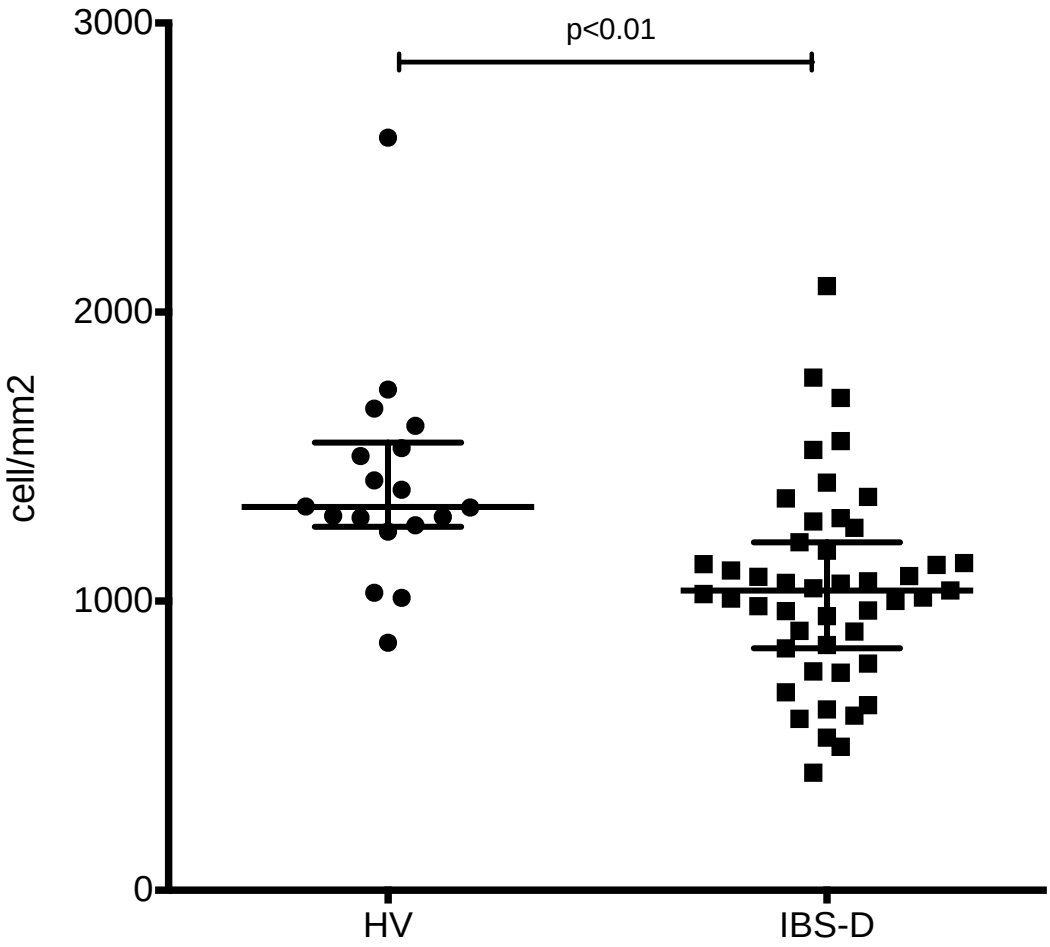


Figure 25: Baseline CD68 cell count comparing HV and IBS-D patients

Following treatment with mesalazine/placebo, there was no significant change in CD68. See Figure 26.

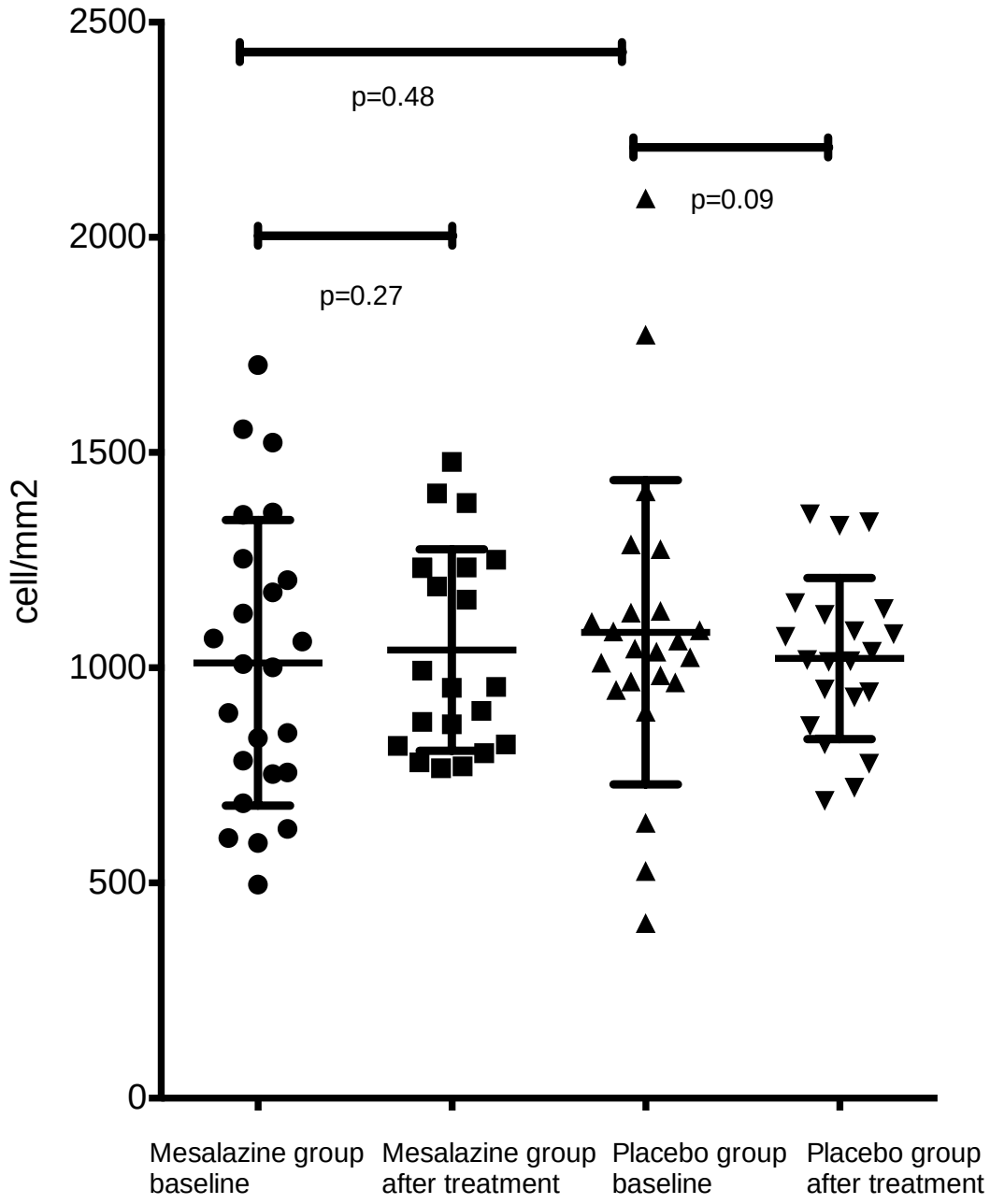


Figure 26: CD68 count following treatment with either Mesalazine or placebo

iii. Serotonin (5-HT) containing enterochromaffin cells

The 5-HT cell count in deep lamina propria is significantly lower in IBS-D patients compared to healthy control. Median (IQR) were 304.9 (189.2-480.9) for healthy control and 159.4 (109.1-221.0); p<0.01 (Figure 27). There was no change in 5HT cells numbers following treatment with mesalazine or placebo (Figure 28).

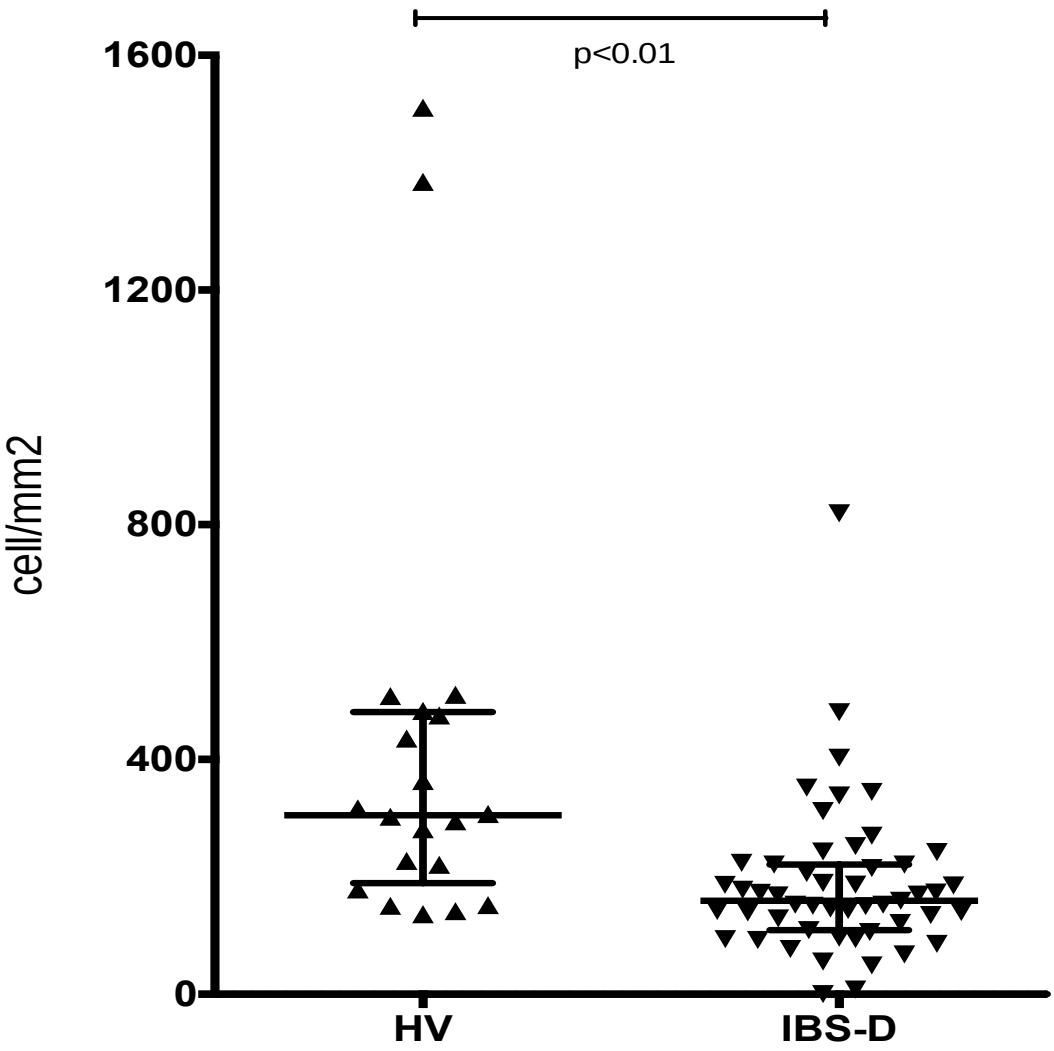


Figure 27: Baseline Serotonin (5-HT) cell count comparing HV with IBS-D patients

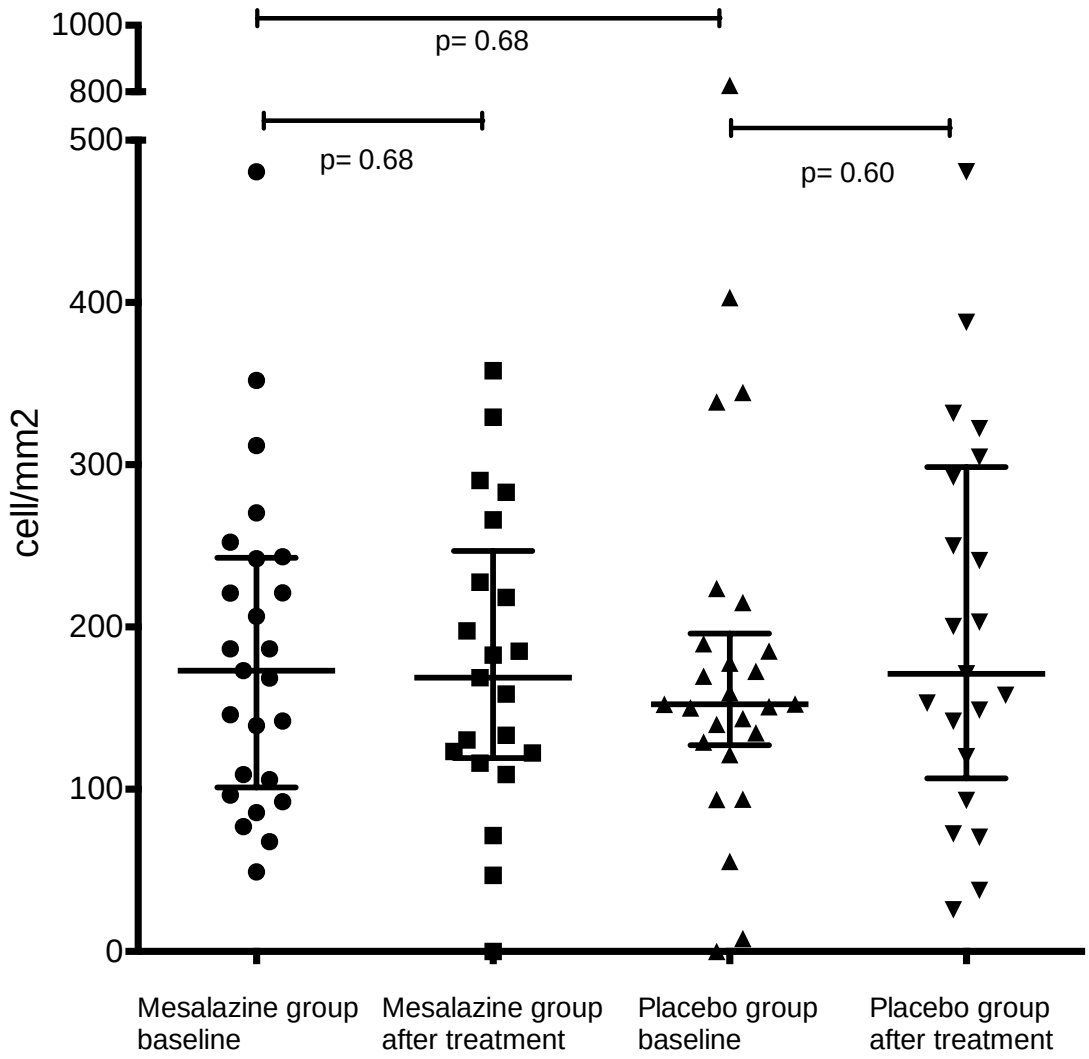


Figure 28: 5HT cell count following treatment of with Mesalazine or placebo

v. CD3

The baseline CD3 count is significantly lower in the IBS-D group compared to the healthy control. Median values were 51.2 (IQR 0-154.6) vs. 716.3 (IQR 460.1-1163) cell/mm2; p<0.01 (Figure 29).

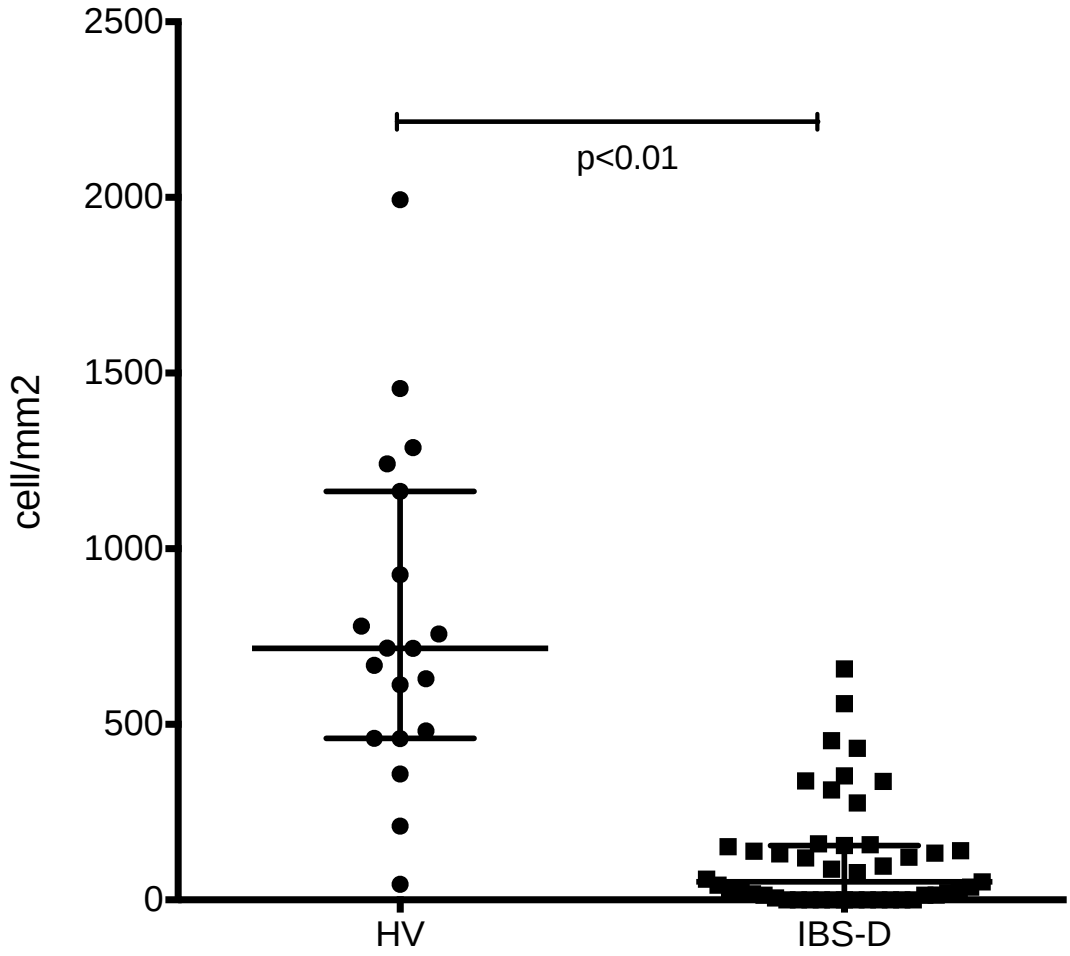


Figure 29: CD3 count between healthy control and IBS-D patients

There was a paradoxical increase in CD3 count following treatment with Mesalazine for reasons which are unclear. Given Mesalazine is an anti-inflammatory agent; it was surprising that there was an increase in CD3 count. See Figure 30.

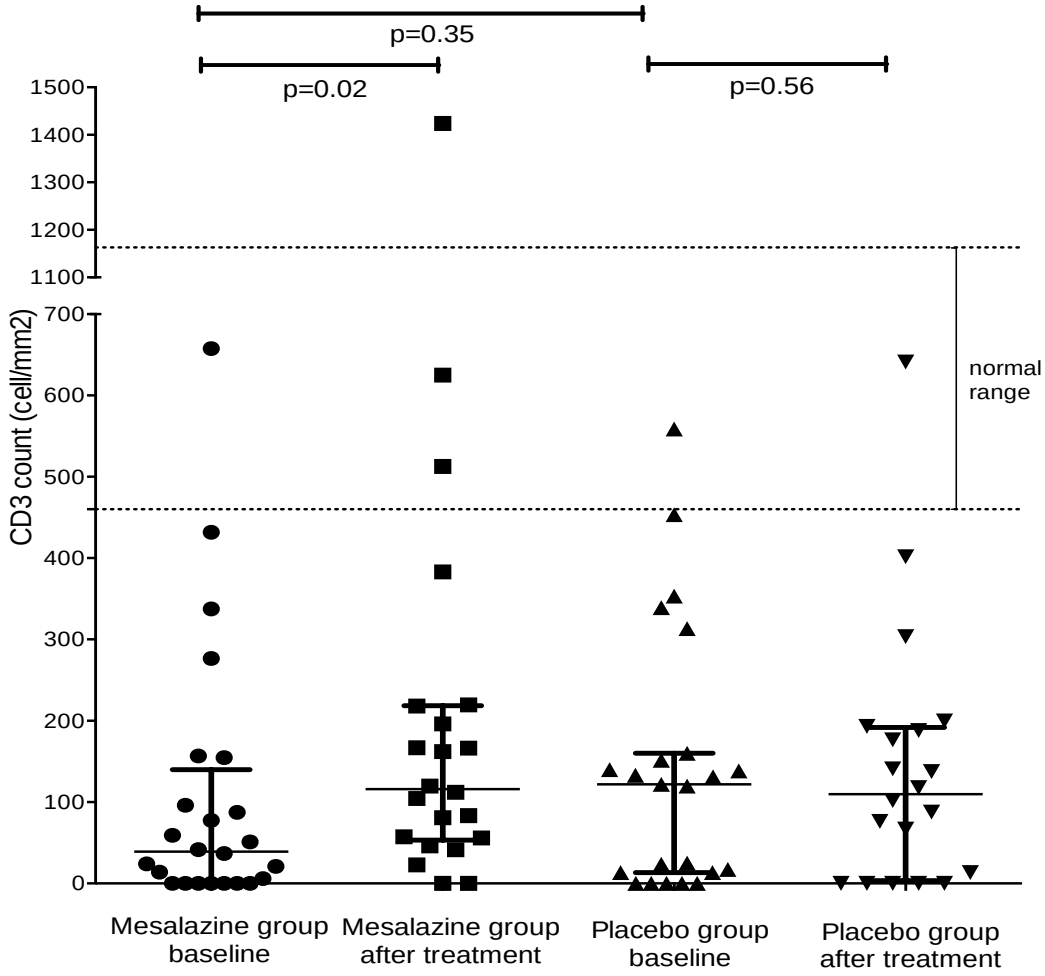


Figure 30: CD3 count before and after treatment of Mesalazine or placebo

c) Stool calprotectin

Samples were obtained in 53 patients (30 placebo, 23 Mesalazine). Baseline stool calprotectin levels varied widely ranging from undetectable to as high as 420µg/g. There was a negative correlation between calprotectin levels and baseline total hospital anxiety and depression scores (HADS) but this did not reach significance (r=0.25; p=0.07). See Figure 31.

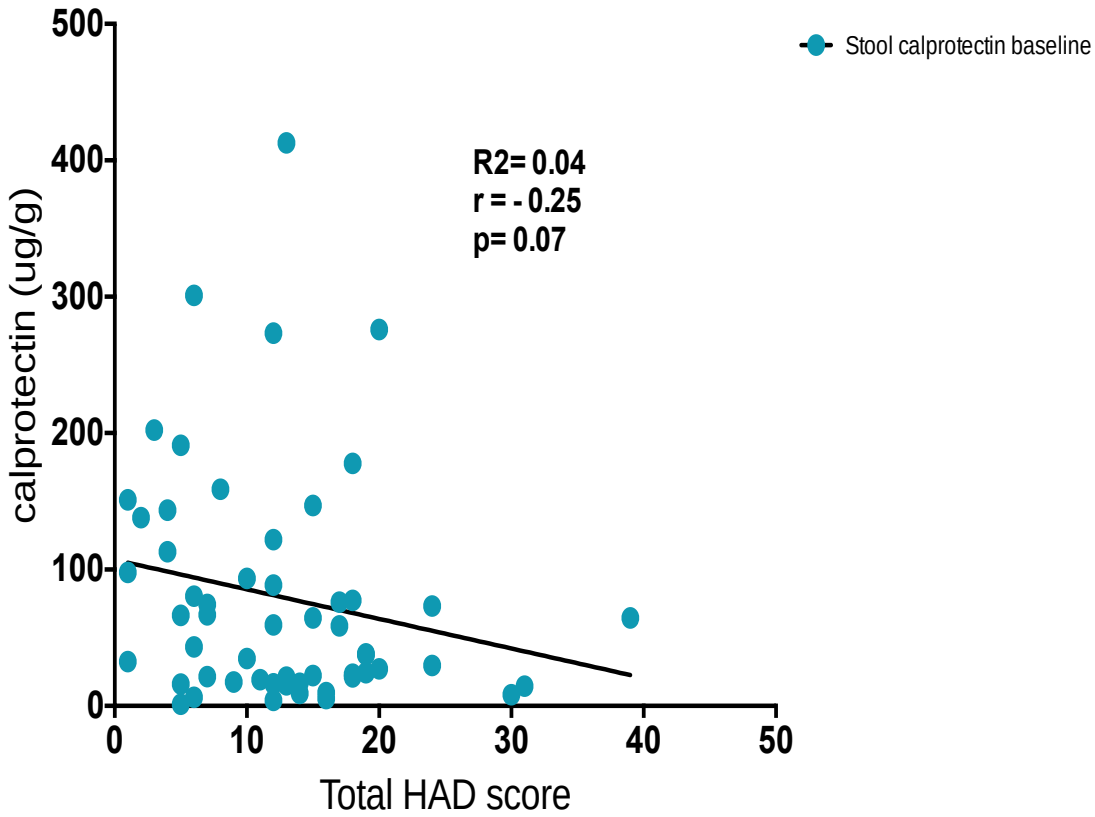


Figure 31: Correlation between baseline calprotectin levels (ug/g) and baseline total hospital anxiety and depression score (HADS)

Stool calprotectin levels were divided into 2 groups i.e. ≤100ug/g (Group B) and ≥101 ug/g (Group A). Between these 2 groups, there were no differences in their

baseline clinical characteristics such as abdominal pain severity, average daily stool frequency and stool consistency. Group A with higher calprotectin levels (≥100 ug/g) at baseline showed a significantly lower total HADS score (median = 7.0, IQR= 3.75-13.5) than Group B (Median= 13.0, IQR = 7.0-18.0); p=0.03. See Figure 32.

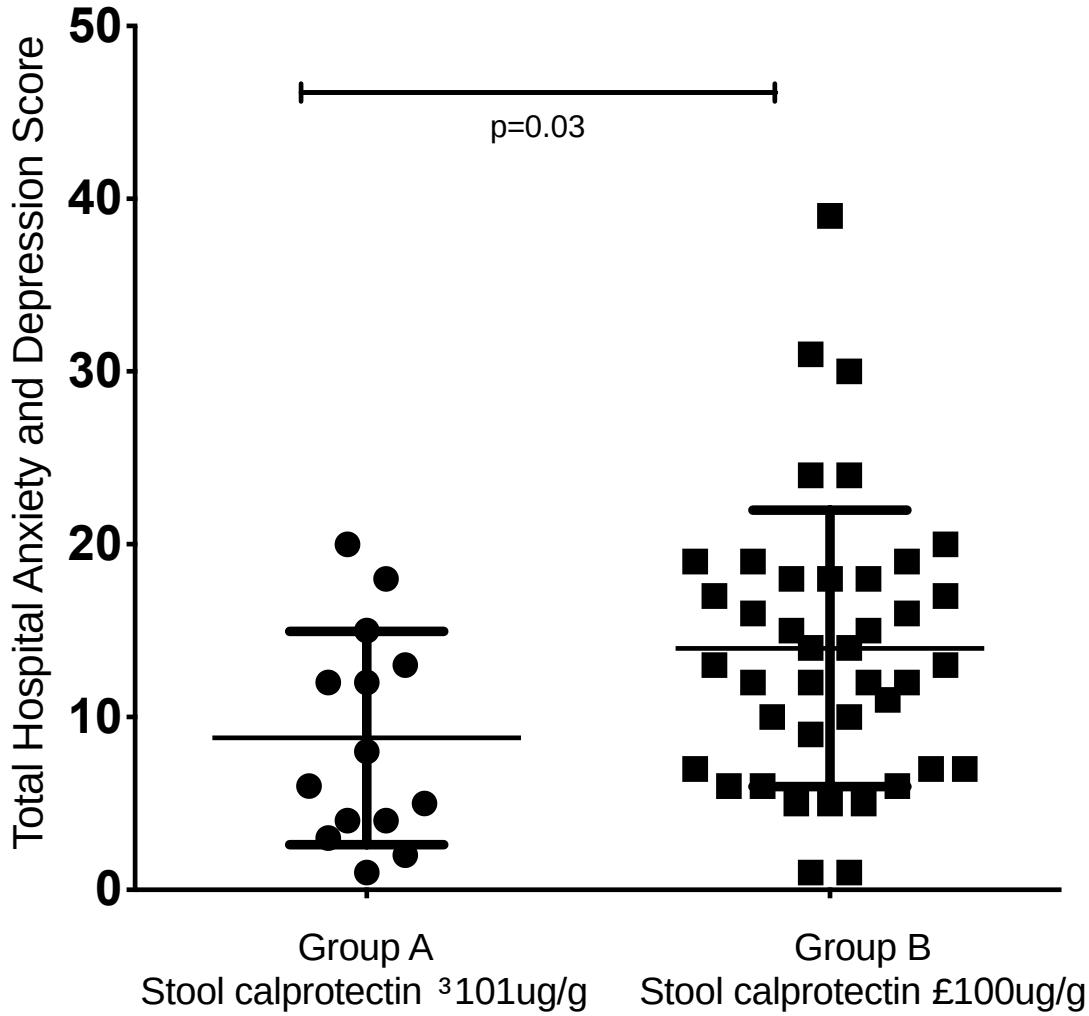


Figure 32: Baseline stool calprotectin levels when divided into 2 groups, (Median,IQR).

Overall Mesalazine did not alter calprotectin levels. Median (IQR) differences in the mesalazine and placebo group were 0.01 (-14.9-15.4) and -0.14 (-43.7 -17.2), p=0.99

(Figure 33). If the participants who have abnormal calprotectin levels (≥101 ug/g) were excluded in the analysis, there was no significant improvement in stool calprotectin levels following treatment with Mesalazine or placebo (Figure 34). There was no significant improvement of clinical symptoms following treatment with Mesalazine when compared with placebo. See Figures 35-40 below.

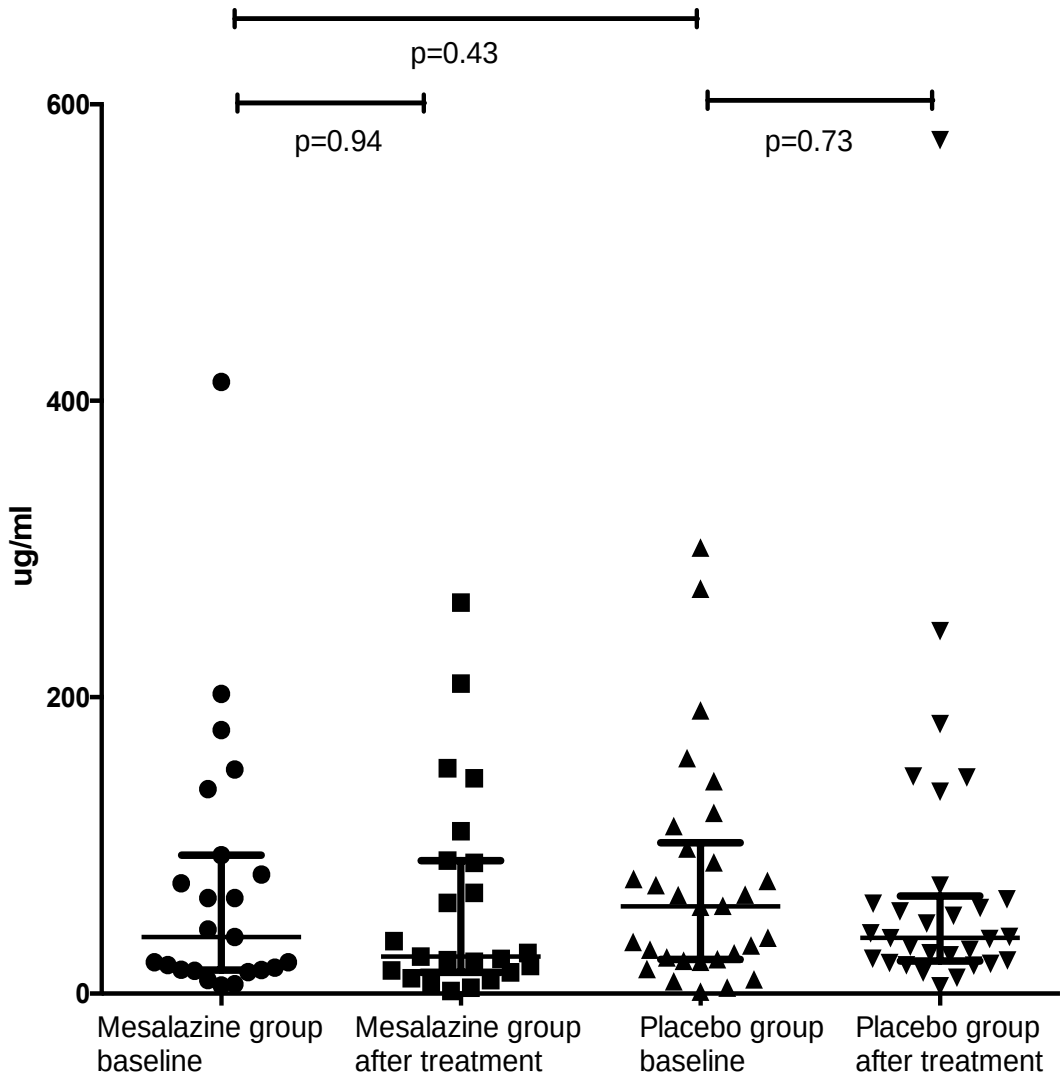


Figure 33: Stool calprotectin levels following treatment with Mesalazine or placebo, Median (IQR).

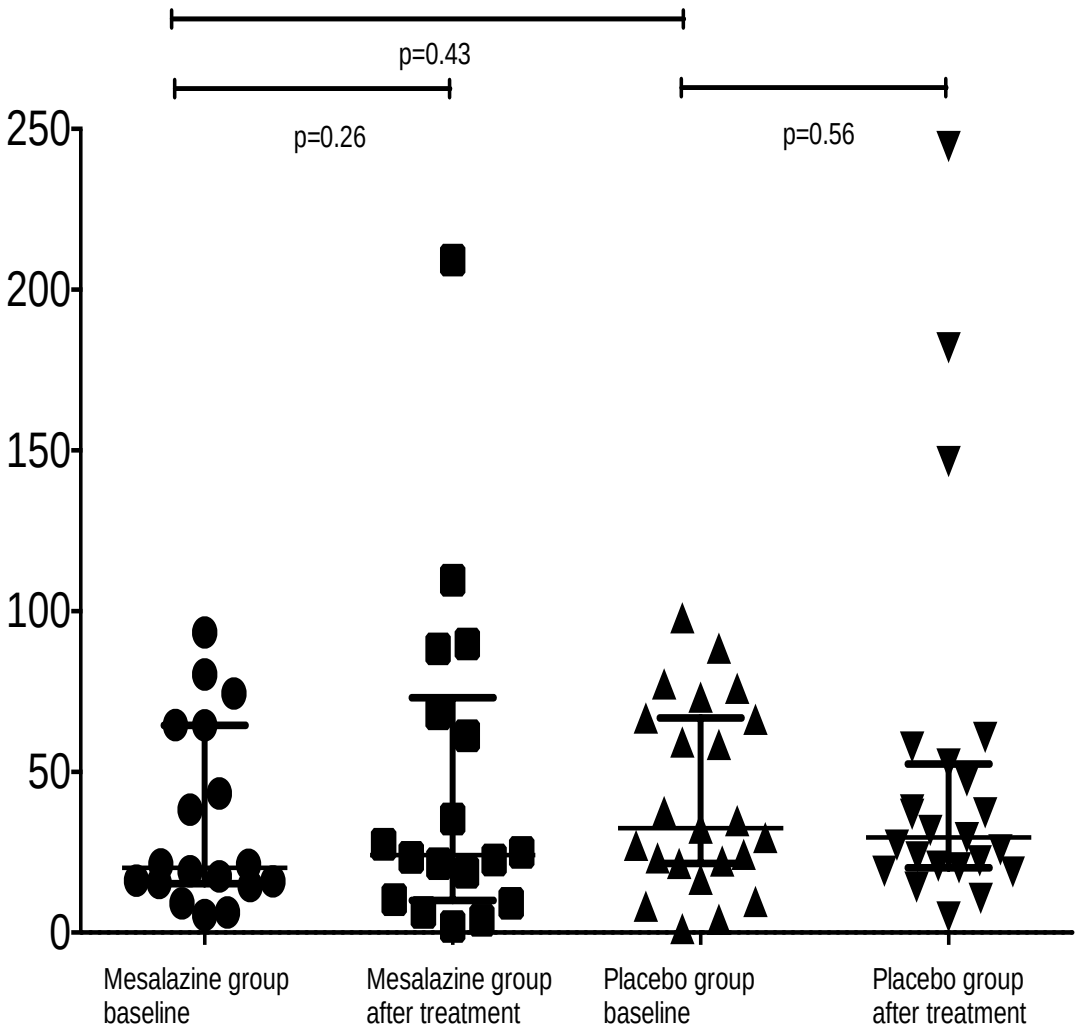


Figure 34: Stool calprotectin levels following treatment with Mesalazine or placebo (Following exclusion of stool calprotectin level (≥101 ug/g), Median (IQR).

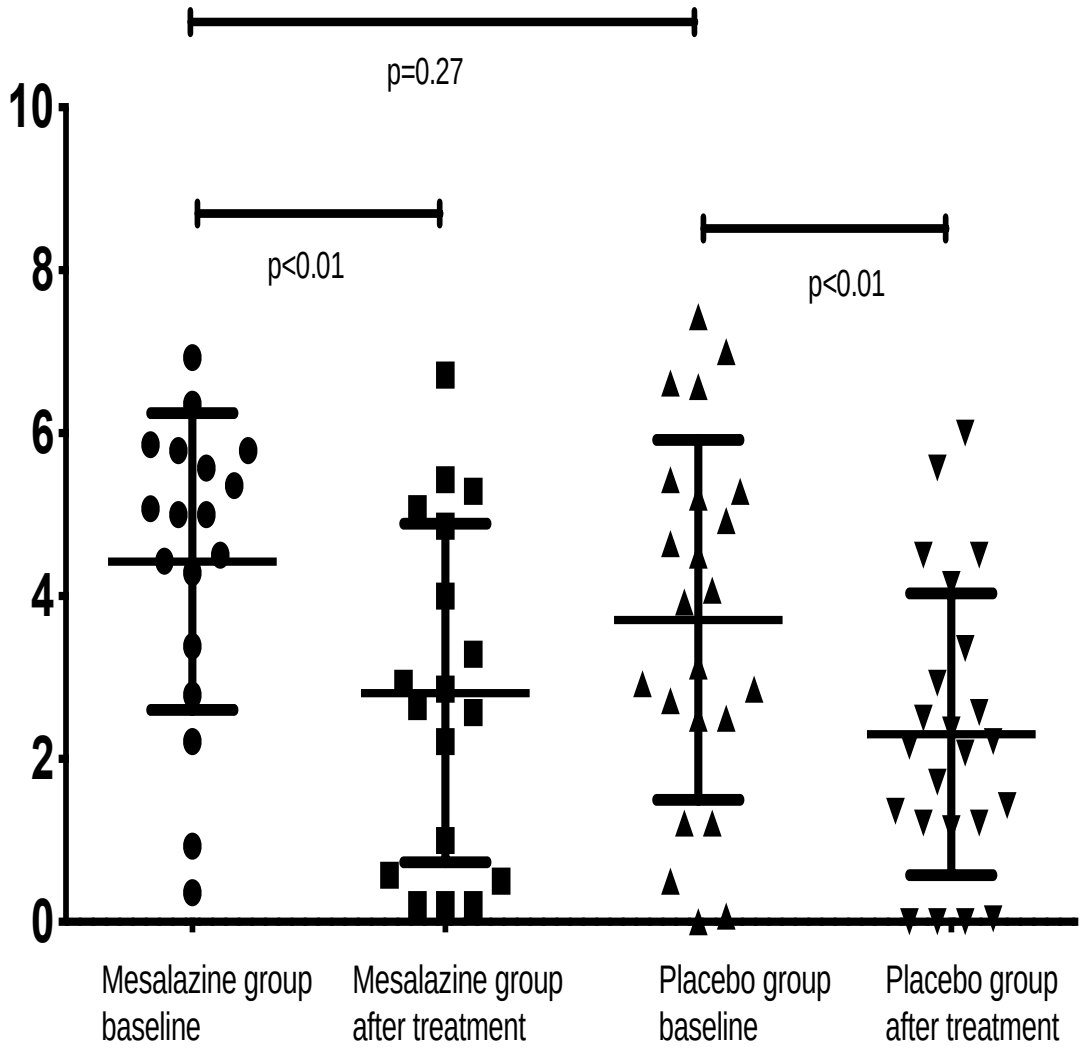


Figure 35: Abdominal severity score following treatment with either mesalazine or placebo (Following exclusion of stool calprotectin level (≥101 ug/g), Mean (SD).

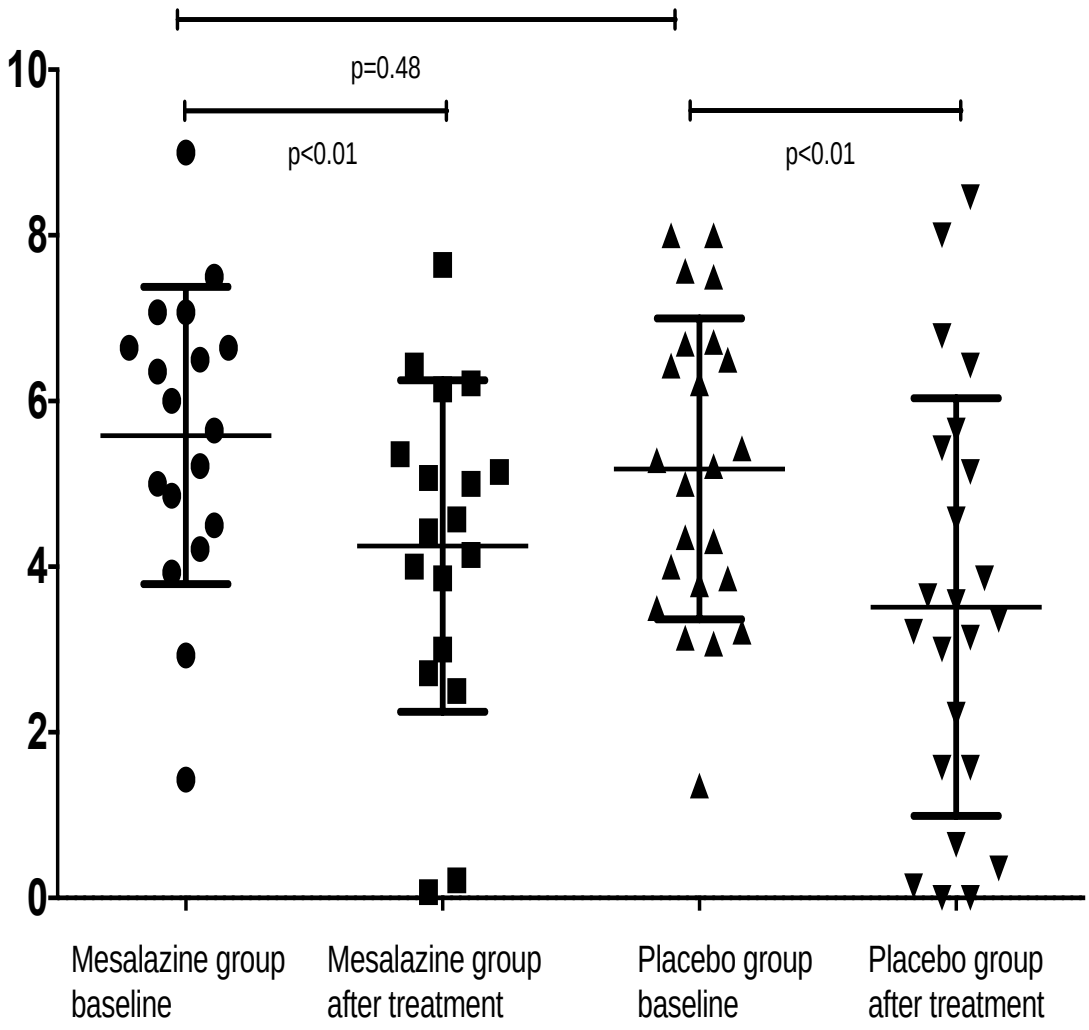


Figure 36: Urgency score following treatment with either mesalazine or placebo (Following exclusion of stool calprotectin level (≥101 ug/g), Mean (SD).

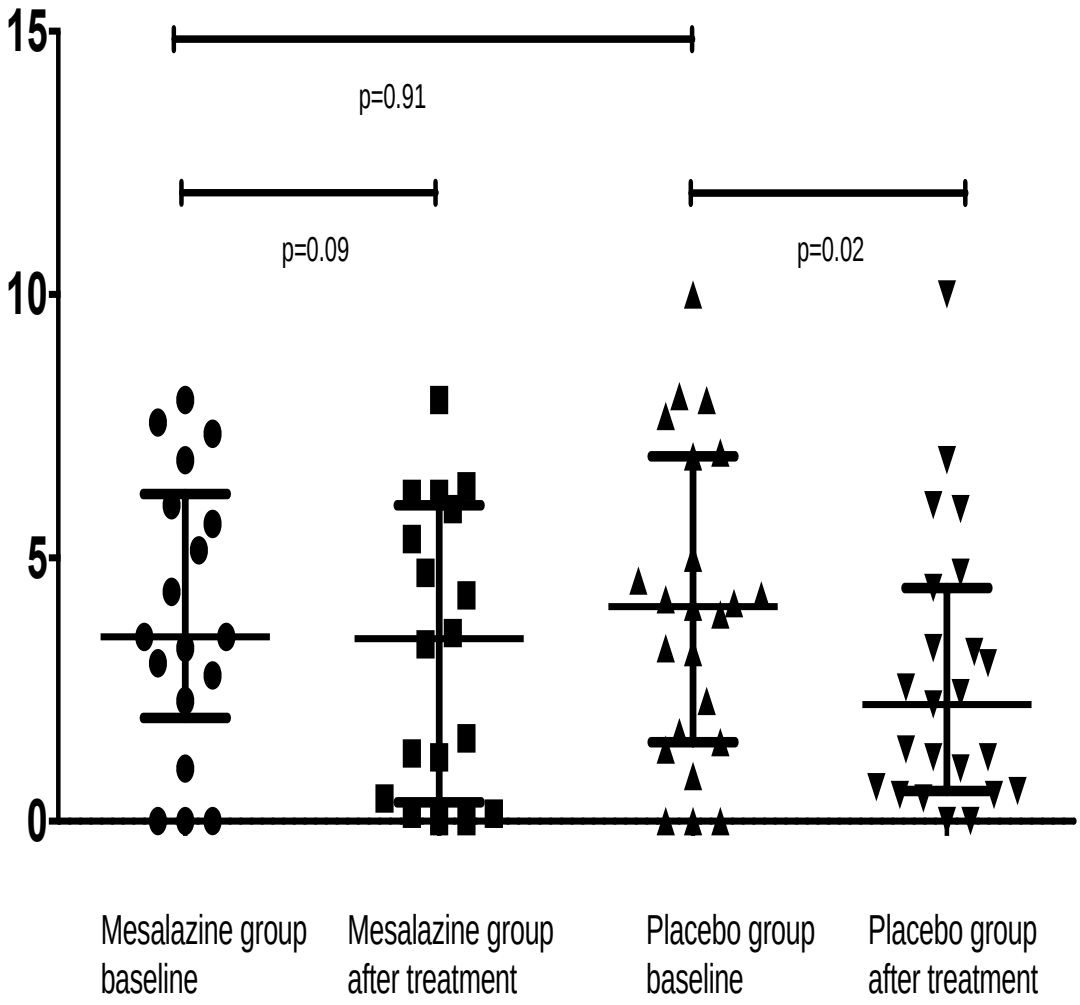


Figure 37: Bloating score following treatment with either mesalazine or placebo (Following exclusion of stool calprotectin level (≥101 ug/g), Median (IQR).

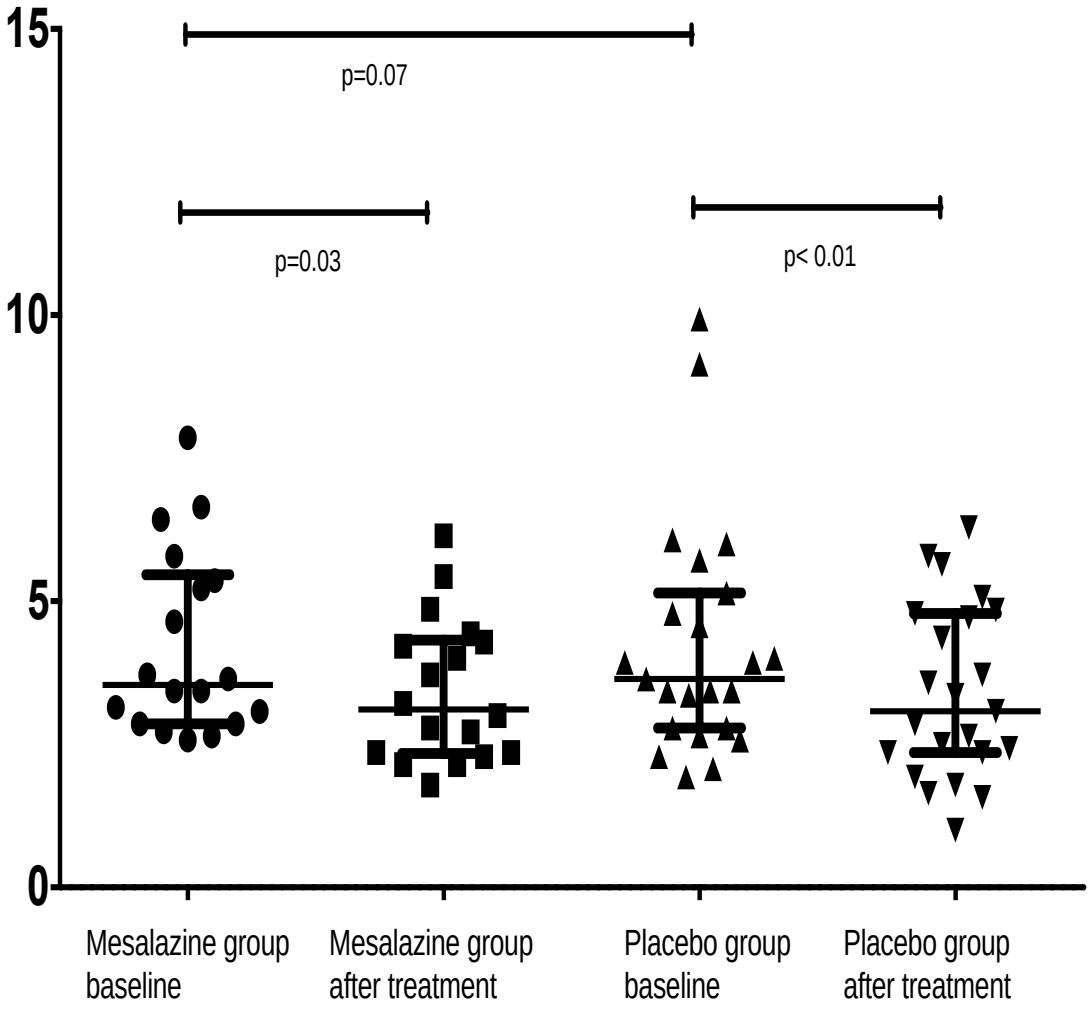


Figure 38: Average daily stool frequency following treatment with either mesalazine or placebo (Following exclusion of stool calprotectin level (≥101 ug/g), Median (IQR).

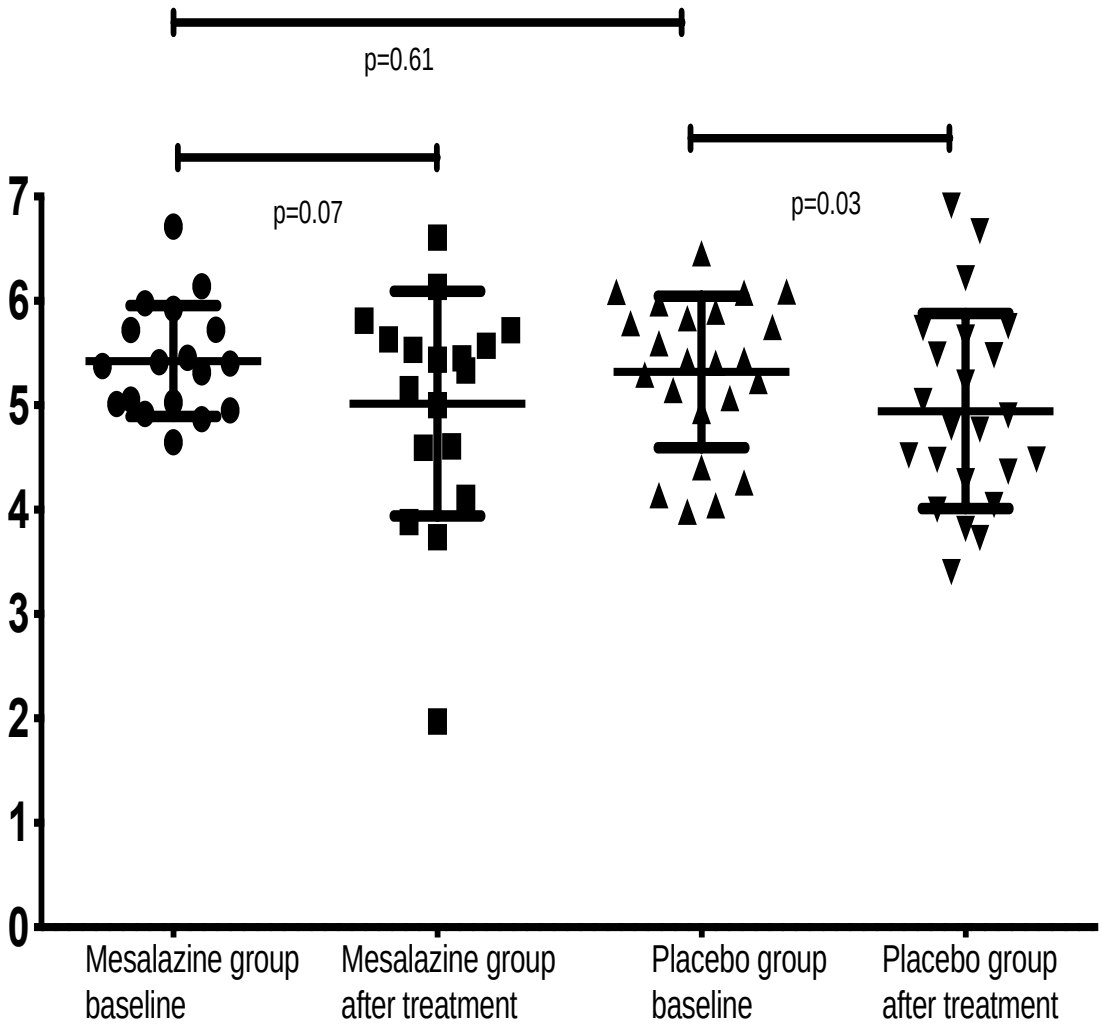


Figure 39: Average daily stool consistency following treatment with either mesalazine or placebo (Following exclusion of stool calprotectin level (≥101 ug/g), Mean (SD).

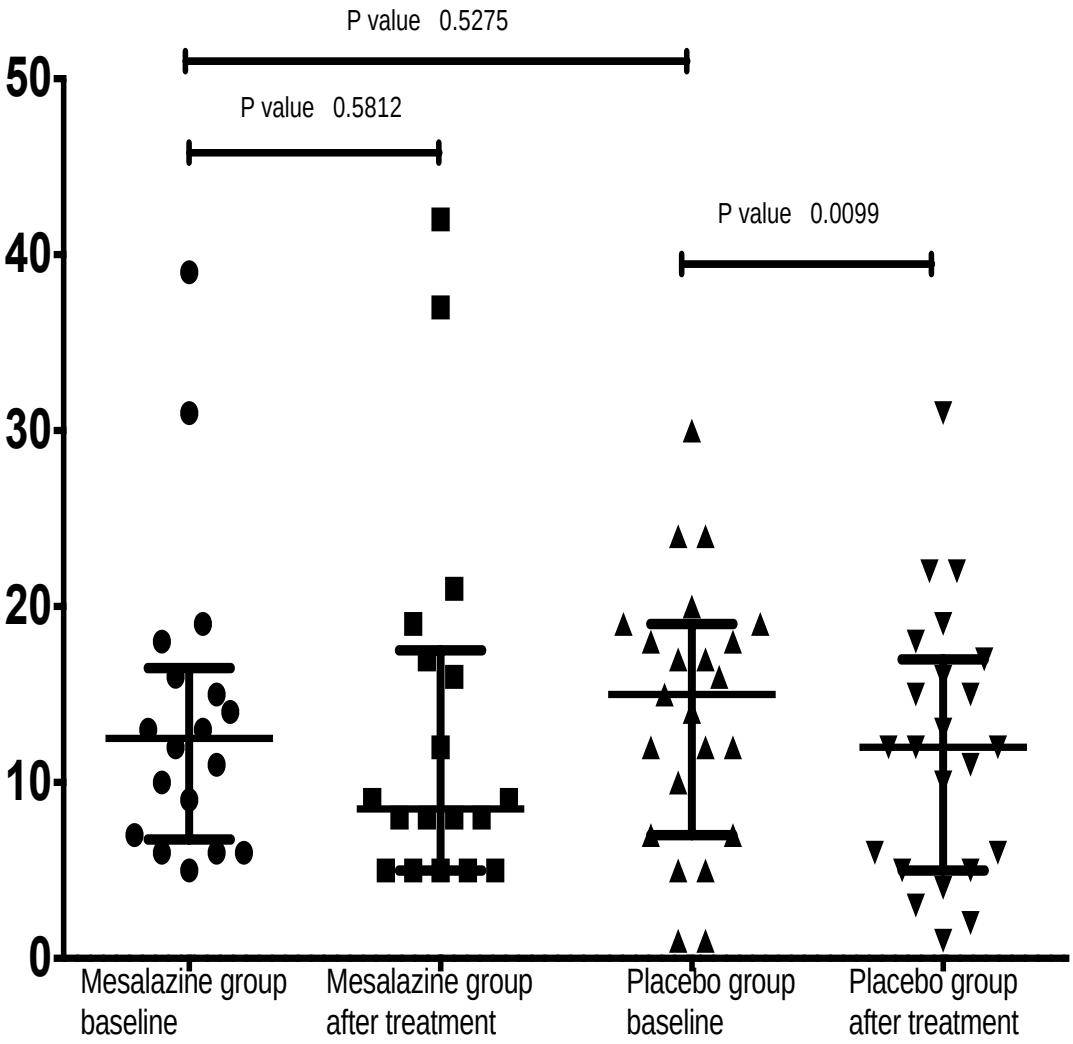


Figure 40: Total HAD score following treatment with either mesalazine or placebo (Following exclusion of stool calprotectin level (≥101 ug/g), Median (IQR).

Group with stool calprotectin levels> 100 ug/g

There were in total 12 IBS-D patients who have stool calprotectin levels of more than 100 ug/g. 5 patients were in the Mesalazine group and 7 in the placebo group. There was no significant change in stool calprotectin levels following treatment. Mean difference (SD) in the Mesalazine group was -98.13 (141.6) ug/g and 30.26 (158.3), p = 0.60 (Figure 41). There was no significant changes in the total HAD and clinical symptoms e.g. average abdominal pain, urgency, bloating, bowel frequency or stool consistency following treatment with either Mesalazine or placebo.

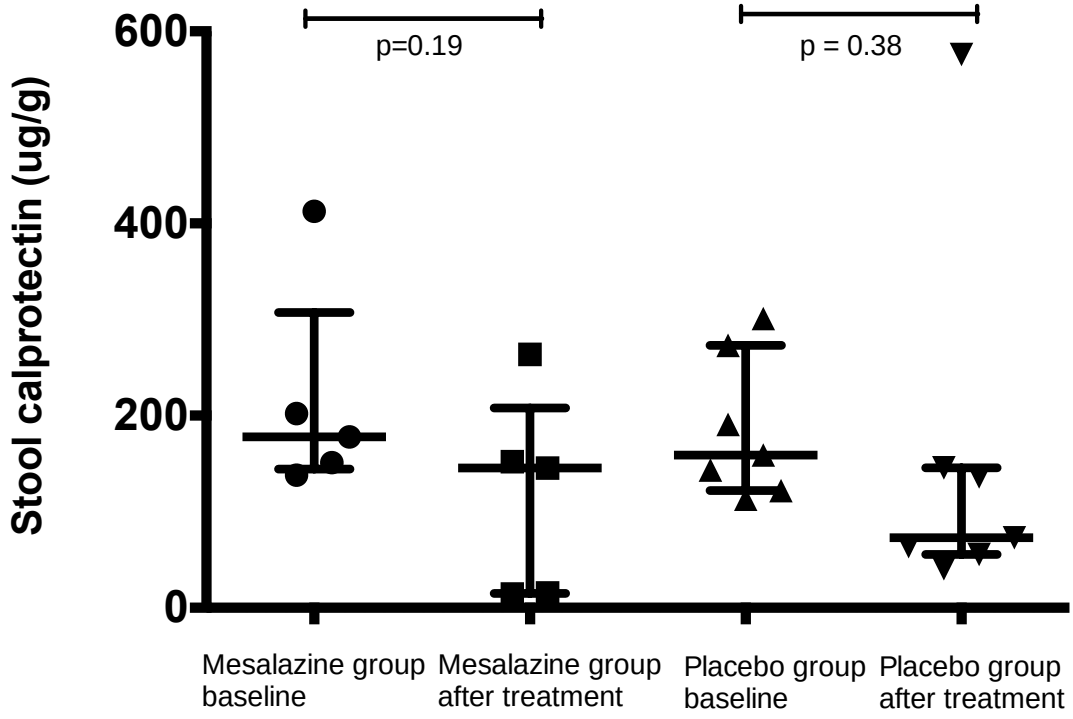


Figure 41: A subgroup of IBS-D patients who have high stool calprotectin levels (>100 ug/g) following treatment with Mesalazine or placebo.

d) Post infectious irritable bowel syndrome (PI-IBS)

Rome III criteria for irritable bowel syndrome following an episode of infectious gastroenteritis are characterised by ≥2 of the following symptoms: fever, vomiting, diarrhoea and positive stool culture [131]. 13 participants in the study met the criteria for PI-IBS. 8 participants were randomised into the Mesalazine group and 5 were allocated to the placebo. There was significant improvement in the clinical symptoms such as abdominal pain, urgency and stool consistency following treatment of Mesalazine. See figures 42-44.

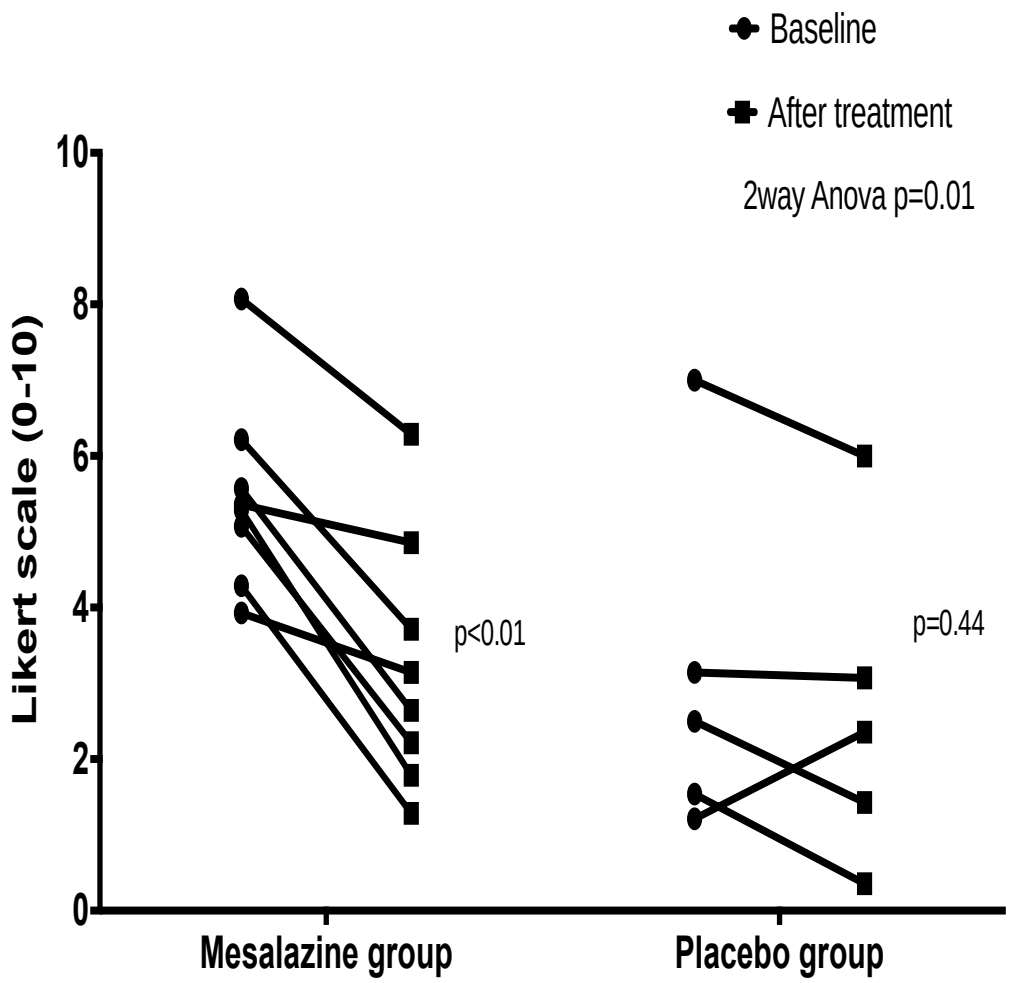


Figure 42: Abdominal pain severity before and after treatment of either Mesalazine or placebo

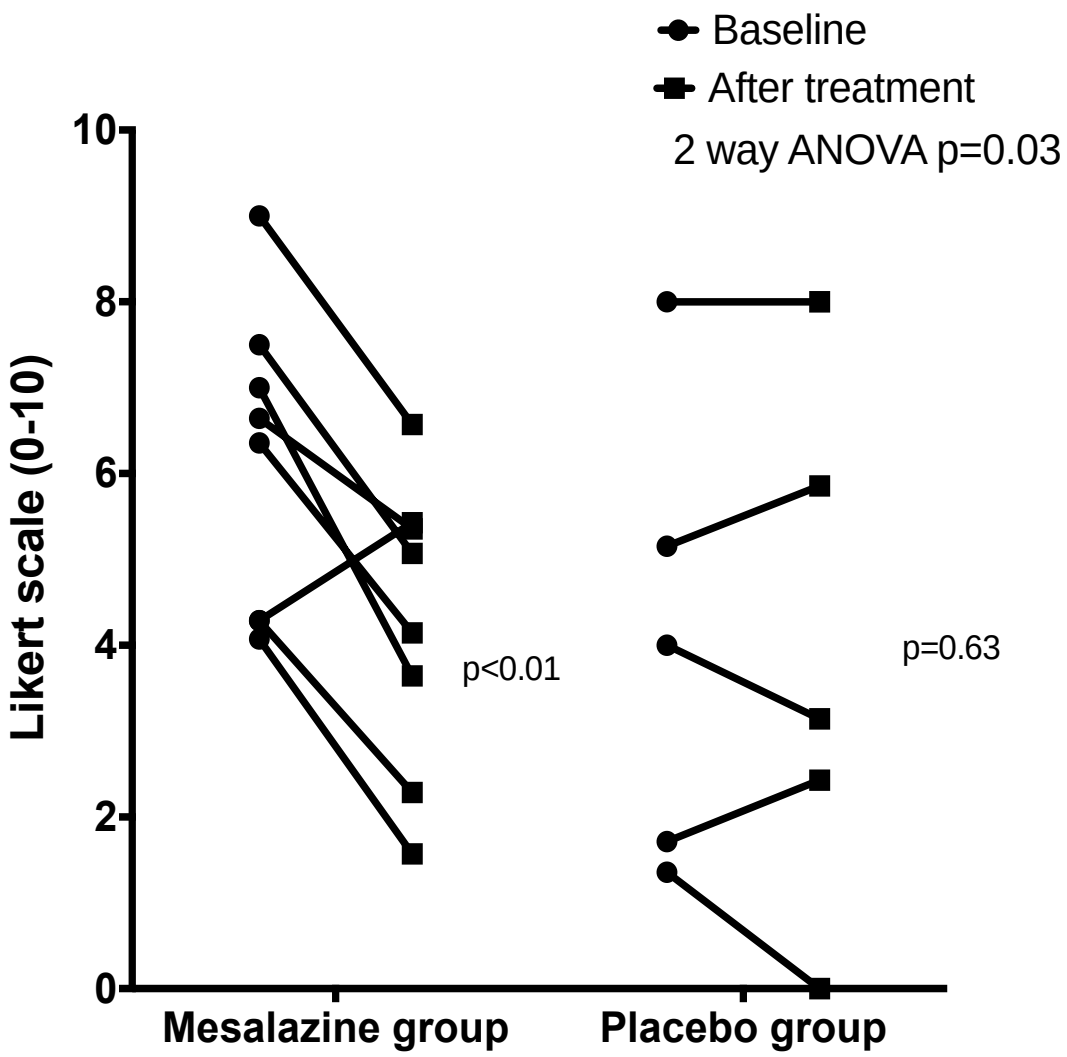


Figure 43: Urgency symptom before and after treatment of either Mesalazine or placebo

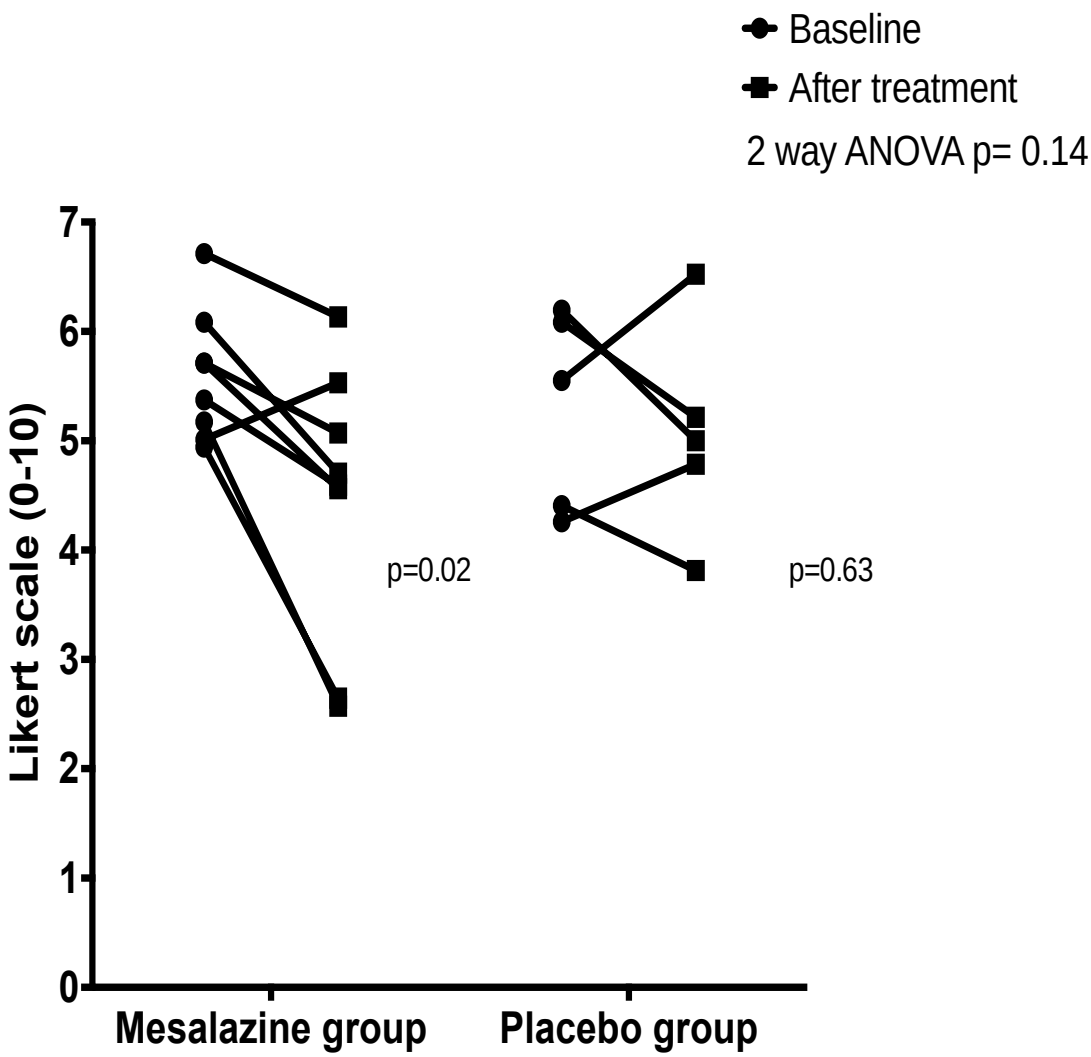


Figure 44: Urgency symptom before and after treatment of either Mesalazine or placebo

1.11.7. Discussion

Over the past decade, there have been several promising studies using 5-aminosalicylate acid for treatment of both IBS predominantly diarrhoea[143, 146, 163] and PI-IBS[164, 165] but sample sizes were small and their significance uncertain. These studies were motivated by recent findings of 'immune activation' in the gut mucosa of IBS patients, dominated by mast cells and T lymphocytes rather than the polymorphonuclear leukocytes characteristic of colitis. These studies were supported by several studies suggesting impaired mucosal barrier in IBS[166], which by allowing access of luminal bacterial products to the mucosal immunocytes might cause this activation[167]. These data suggested that Mesalazine, being an anti-inflammatory agent, might benefit this condition. Animal studies suggest Mesalazine improves barrier function in colitis but whether this is true in IBS is uncertain[168]. This study is one of the largest trials so far looking at the treatment of Mesalazine in IBS-D patients following best practice to ensure that both investigators and patients were blinded to the study and that data analysis was carried out by independent statisticians. The effect of Mesalazine was analysed only after 12 week treatment as it was felt that Mesalazine was a disease modifying rather than symptomatic treatment and early reports suggested benefit was most obvious after 2-3 months[143]. This study showed that Mesalazine did not improve bowel frequency after 12 weeks treatment when compared to placebo in unselected patients. As with other studies in IBS, this study showed a strong placebo effect on bowel symptoms and also on the total hospital anxiety and

depression and somatic scores suggesting that patients felt better in general after taking part in the trial.

Despite lack of benefit in unselected patients, a sub-analysis of the primary outcome of stool frequency in patients divided according to severity was determined *a priori.* This suggested that a group of patients who had the greatest bowel frequency did benefit from Mesalazine (mean difference -0.26,p=0.04). Our clinical findings seem consistent with another recent report[169]. There was no significant improvement in other IBS symptoms such as abdominal pain, bloating and stool consistency. There is strong evidence from this study that Mesalazine treatment increases the number of days with urgency by about 20%. There have been previous case studies reported of Mesalazine worsening diarrhoea in colitis[170, 171]. This may represent an allergic response to the drug, as there was an increase in T lymphocytes.

Raised mast cells numbers in the gut mucosa have been implicated in all subtypes of IBS[172] but mainly in IBS-D. Mast cells contain many mediators including histamine, serotonin and proteases such as tryptase[66]. Recently, there has been an interest in tryptase release as it has been shown to activate proteinase-activated receptor 2 which is found on afferent nerves and can lead to increased sensitivity of bowel distension[48]. In this study, the average mast cell count in IBS-D patients was elevated compared to those in healthy subjects previously studied in our laboratory. While most IBS-D patients have normal mast cell count (below 90th centile of healthy control) there was a subgroup with elevated counts. However comparing their baseline IBS symptoms, there was no difference between the 2

groups. There were no gender differences in mast cell count of IBS-D patients as previously described by others[173], nor any gender effect on other immune cells such as CD3, CD68 and 5-HT containing enterochromaffin cells.

Although mast cell count was elevated in IBS-D patients compared to the healthy controls, the supernatant levels of tryptase in IBS-D patients were not significantly elevated. Median (IQR) tryptase levels for IBS-D vs. healthy control were 4.3 (1.8-8.9) and 6.7 (3.8-11.4) ng/ml; p=0.07. Surprisingly supernatant histamine levels in our study were lower in IBS-D patients compared to healthy control, being [Mean (SD)] 0.7 (0.6) and 1.1 (0.8) ng/ml, respectively, p=0.02. Supernatant levels of tryptase and histamine were not altered following treatment of Mesalazine. Disappointingly we found no apparent association correlation between mast cell count and supernatant levels of release of the mast cell mediators examined, whether those released by all mast cells (tryptase and histamine) or restricted to a subpopulation (chymase and carboxypeptidase A3). This suggests that the overall degree of mediator release from colonic mast cells is independent of mast cell numbers, tryptase and histamine suggesting factors other than mere numbers determine mediator release. When designing the study, we followed previous published methods by other authors for obtaining biopsy samples[48]. However, in retrospect, the process of taking a biopsy involves considerable trauma and this is unstandardized which may possibly account for the lack of correlation with release of mediators during normal bowel function.

This study also looked further into patients who were on active Mesalazine treatment. Those who had a high baseline mast cell count did not show greater

improvement in abdominal pain, urgency, bloating, bowel frequency and stool consistency when this was compared to the group who had 'normal' mast cell count. Although mast cell count was weakly correlated with urgency symptom, it did not predict response to Mesalazine. Again, this provides no support for the previous suggestion that Mesalazine can reduce mast cell numbers[146].

Stool collected in Nottingham was used to obtain calprotectin level at baseline and EOT. Although a small proportion of patients have raised calprotectin levels (≥101ug/g), organic diseases such as inflammatory bowel disease were excluded in gastroenterology clinics using standard tests prior to patients entering the study. Others have also reported up to a quarter of IBS patients have marginally elevated calprotectin though the origin of this is unclear[174, 175]. Interestingly, the subgroup of patients (Group A) who had raised calprotectin level (≥100ug/g), have significantly less psychological distress than the group with stool calprotectin level ≤100 ug/g (Group B). This is felt that subgroup A's symptoms are secondary to local gut inflammation while subgroup B's symptoms are driven primarily by distress which causes gut symptoms secondarily. Unfortunately numbers were too small to answer the question of whether subgroup A responded better to Mesalazine. Stool calprotectin could therefore be used as a screening tool to allow more detailed studies of the mucosa in IBS-D in the future.

One uncontrolled study has suggested that Mesalazine might be effective in treating PI-IBS patients[164] but the only randomised controlled trial of Mesalazine in this condition was negative though possibly underpowered[165]. In our post-hoc analysis, a small subgroup fulfilling criteria for PI-IBS appeared to benefit from

Mesalazine but our study was also underpowered. Confirming this would require a larger and more adequately powered study.

Although Mesalazine has been available to use for many decades with good safety profile, this adequately powered study have showed it did not help the majority of IBS-D patients. The fact that certain subgroups might benefit emphasises that there is still a need for better phenotyping of this heterogeneous group of patients when evaluating new treatments.

1.11.8. Limitations

Despite strict entry criteria the population in this study was still heterogeneous. In retrospect this could have been better stratified by postinfectious onset. This was considered during the initial set up of the study but felt that this would make the trial very difficult to recruit. This could be overcome in future studies by having a great many more recruitment sites and around 5 times as many participants given that PI-IBS accounts for only around 20% of all cases of IBS-D but this would require more resources than we had available for this study. It is worth noting that there was an appreciable loss to follow up (15.5%) but not out of line with other similar studies. Dropouts are mostly likely due to failure of treatment and so unlikely to account for the negative result.

1.11.9. Research recommendations

This data suggests that it is unlikely that future trials of Mesalazine in unselected IBS would be fruitful.

If, there is a subgroup of IBS-D patients that may benefit from mesalazine, it is likely to be those with post-infective IBS and patients who have severe diarrhoea.

The link between mast cells and urgency is weak and again future work on the role of mast cells needs to better characterise the patients since the majority of unselected IBS do not have elevated mast cell numbers. It may be that as others have reported it is the number of activated mast cells that are important[48] and better markers of activation would be useful rather than the current gold standard of electron microscopy which is expensive and time consuming.

Finally the release of mediators from biopsies does not link well to symptoms or mast cell numbers. The dominant factor for release is likely to be crushing and tissue injury by the biopsy process, which is not well standardised and may overwhelm other factors which would be of further interest. There is a need for a better way of assessing in vivo activity of the mucosal cells.

1.11.10. Conclusion

This randomised placebo controlled trial in 115 unselected IBS-D showed that Mesalazine 4g per day was no better than placebo in relieving the symptoms of abdominal pain or disturbed bowel habit. A subgroup of IBS-D patients had elevated mast cell percentage area stained which correlates weakly with urgency and stool consistency. However, contrary to the previous report in just 10 patients, Mesalazine did not reduce mast cell percentage area stained. Further post hoc analysis showed raised calprotectin was associated with less psychological distress implying a more gut centred abnormality. A small subgroup with PI-IBS appeared to benefit but this requires a larger adequately powered study to confirm this findings.

Further phenotyping of the heterogeneous group of patients with IBS and diarrhoea is needed to allow better evaluation of new treatment.

Role of Magnetic Resonance Imaging in the Gastrointestinal Tract

1.12. Introduction

Magnetic resonance imaging (MRI) is very widely used worldwide because of its ability to non-invasively assess internal structures of the human body without exposing one to ionising radiation. MRI was previously termed, nuclear magnetic resonance (NMR) in the 20th century and was initially used by chemists to study chemical, physical and biological matters before it was used to investigate blood products, blood flow, skeletal muscle and living subjects using a 1 dimensional view. It was in the 1970s where MRI was developed by Paul Lauterbur and Peter Mansfield to allow 2 dimensional imaging. In 1975, Richard Ernst perfected the reconstruction of 2D images, which now became the basis of MRI worldwide[176]. The first scan using MRI to obtain images of a live subject was by Sir Peter Mansfield and colleagues in Nottingham in 1977. The scan comprised of a cross section of a human finger showing for the first time a detailed image of the soft tissue of the finger[177]. Following from this, the use of MRI in obtaining images of internal organs of the human body had expanded and has been widely used in clinical practice complimenting other radiological modalities[176].

MRI uses electromagnetic fields to produce internal images of a subject scanned by manipulating the hydrogen protons within the body. A spinning proton produces a mild magnetic field as it has the properties of a positive electric charge, spin and mass. The biggest source of hydrogen/proton in a human body is water followed by body fat. When a subject is placed in the magnetic field in the MRI machine, the protons align and spin around their axis when an external magnetic field is applied.

This creates a magnetic vector, M_0. When radio wave energy (radio frequency) is added into the magnetic field, the magnetic vector is deflected. When this radio frequency is switched off, the M_0 will return to its resting state and during this period, it will re-transmit the radio frequency. This signal will produce the MR images. In a semi-classical description the return to the resting state can be decomposed in a component parallel to the static magnetic field (longitudinal magnetisation) and one perpendicular to it (transverse magnetisation). The M_0 return to its resting state has therefore two separate components, one in the longitudinal plane (T1 relaxation)* and one in the transverse plane where it reflects the process of de-phasing of the excited protons (T2 relaxation)[178]**. A short review article by Berger [179] summarised the principles of the MRI very succinctly. Different body parts / organs/ tissues will exhibit different relaxation times (T1/T2) which is what gives the richness of contrast in the MRI images.

**T1 relaxation, which is also called spin-lattice relaxation time, is when the excited magnetisation returns to its resting state in the longitudinal plane. This is when the radio frequency energy is released back into its surrounding (lattice). Therefore this recovery period follows an exponential curve which will be shown later in this chapter. The time course whereby the system returns to its equilibrium state is characterised by the time constant T1 which is unique to every tissue[178]. T1 relaxation time is influenced by the strength of its magnetic field.*

***T2 relaxation time is called spin-spin relaxation time. This is when the excited magnetisation which is initially composed of all protons spinning in phase, begin to get 'out of phase'. Slowly, the loss of phase results in the signal decay and this is how T2 relaxation time is measured. The signal decay is described mathematically by an exponential curve. An example of this will be shown later in this chapter. T2 values are also influenced by magnetic field strength but not as much as T1s are.*

Over the past decade, there has been an increase in the use of MRI in imaging the gastrointestinal tract especially in the diagnosis of inflammatory bowel disease in

the small bowel and staging of lower rectal cancer. The use of MRI in functional bowel disease such as IBS is very limited at this present time. The advantages of MRI are the ability to visualise soft tissue in detail. No ionising radiation is involved which is very advantageous for scanning children and young female patients, furthermore it is none invasive, hence an ideal test for repeated examination following treatment or in a research environment and lastly, potentially able to test the gastrointestinal tract function and motility such as gastric emptying[180] and anorectal function[181]. The disadvantages of MRI are the cost of MRI scans, prolonged length of time for image acquisition, difficulty in correcting motion artefacts such as intestinal motility and breathing, the use of the machine has a weight limit (for example, the machine used for this thesis had a weight limit of approximate 120 kg) and some patients might find the MRI scanner claustrophobic. These current disadvantages may not be relevant in the near future due to the continuous development in MRI imaging techniques.

The Sir Peter Mansfield Magnetic Resonance Centre (SPMMRC) at the University of Nottingham is one of the very few centres in the world with a team dedicated to study gastrointestinal physiology and its function using MRI. Throughout the years, there has been development in the use of MRI parameters to study the gastrointestinal tract in a fasted and fed state, gastrointestinal motility and sensory function. Therefore, the use of MRI in research has proved to be very advantageous as we are able to visualise and observe undisturbed gastrointestinal function without the use of contrast or bowel cleansing agents.

In this chapter, new techniques to assess small and large bowel transit will be discussed, together with the use of a laxative challenge test and MRI to look at functional bowel disorders such as constipation and potentially to look for an MRI biomarker in patients with irritable bowel syndrome with diarrhoea (IBS-D).

1.13. Gut transit

1.13.1. Introduction

Small and large bowel transit measurements are variable as it depends on the methodologies used. The discussion of the gut transit measurements will be divided into 2 sections. First the orocaecal transit time (OCTT), which is to assess transit time from mouth to terminal ileum/ caecum. Secondly, whole gut transit time (WGTT) measurement is to assess transit time of the whole gut from mouth to colon.

1) Orocaecal transit time (OCTT)

a) Barium studies

The barium meal is one of the earlier methods used as a transit test. Commonly, it is still used to evaluate or rule out mechanical obstruction, small bowel diverticula and motility disorders. Small bowel transit is measured by drinking 200-400 ml of barium before abdominal x-rays are taken at specific periods of up to 9 hours. OCTT is defined as the time of ingestion of barium to the time barium reaches the caecum. Previous studies to assess small bowel transit on healthy volunteers were not standardized thus the results for each study were variable[182, 183]. The barium meal is not widely used today to assess motility due to the amount of radiation involved in this test.

b) Lactulose hydrogen breath test (LHBT)

Lactulose is a synthetic sugar containing fructose and galactose. It is not digestible in the small bowel and when it reaches the large bowel, lactulose is fermented by colonic bacteria producing hydrogen, methane and short chain fatty acids. Hydrogen produced by the gut bacteria is excreted in the breath[184], and this can be measured to assess OCTT. Routinely, 10g of lactulose are ingested and breath collected every 10-15 min for approximately 10 hours. The definition of the OCTT would be a rise in breath hydrogen as sustained release of hydrogen more than 5 parts per million (ppm) compared to baseline[185]. Due to its hyperosmolar effect of lactulose, it can increase bowel transit as described by Miller et al[185], hence disturbing the parameter which is being measured. Despite this limitation it is still a common test to use for assessment of OCTT due to its availability, ease of use and low cost.

c) 13C-labelled glycosyl ureides (LUBT)

13C-labelled glycosyl ureides have been accepted and applied for measuring intestinal transit time. Their chemical properties and physiological significance have been studied in detailed in the past by Heine and colleagues in 1995[186]. It has been validated[187] and is a non-invasive method of measuring OCTT. These 13C-glycosyl ureides have properties of low intestinal absorption as it resists cleavage by brush border enzymes of the gastrointestinal tract. The 13C-glycosyl ureides is used in small amounts unlike lactulose therefore it does not cause osmotic secretion into the small bowel and hence does not accelerate small bowel transit[188]. Importantly enzymatic degradation of the sugar-urea bond in the small bowel does not occur with mammalian enzymes but in the colon by the bacteria flora (*Clostridium*

Innocuum[189]). Previous research papers[186-188] have established the safety and usefulness of the product to test for OCTT. It is tasteless and there is no problem integrating it into test meals, subjects tolerated the product well and caused minimal discomfort. There have been no reported adverse events following this investigation and this test has also been used in paediatrics for similar indication i.e. to assess small bowel transit[190]. This method involved the use of stable isotope ^{13}C which is non radioactive. Subjects need to be pre-conditioned with unlabelled lactose ureide (LU) e.g. 1g LU 3-5 times/day prior to the test to induce enzymatic activity in the colon[186-188] before a small dose of ^{13}C lactose ureide is given along with the test meal the next day. Breath collections are taken from baseline and every 10-15 minutes for the next 9 hours. Breath analysis is performed using a spectrometer; on average it takes 2 hours to complete analysis of a set of breath bags per participant. Although this might be a simpler test compared to scintigraphy, the high expense of purchasing ^{13}C lactose ureide compared to lactulose has discouraged its use as a routine clinical test. The cost to purchase 500 mg of ^{13}C lactose ureide and 3g of unlabelled lactose ureide for a patient would cost approximately £80.

d) Small bowel transit using scintigraphy

This is not widely used in a clinical setting as it is expensive, labour intensive and involves the use of special equipment. Either radiolabelled Indium or Technetium labelled material is ingested in either a liquid or solid meal followed by intermittent scans using a gamma camera. The small bowel transit time can be calculated as the time for 10 or 50% of the activity to arrive at the caecum[191-193] and subtracting the

time for the equivalent proportion (10 or 50%) to be emptied from the stomach in the gastric emptying. The Mayo group assess small bowel transit rate from the percentage of delivered radioactivity entering the caecum at 6 h[192]. Normal values for small bowel transit would be between 11-70%[184] of radioactivity in caecum at 6 h. Although scintigraphy may provide a reliable method of measuring small and large bowel transit (see below for colonic transit), cautious interpretations need to be taken into account of assessment of small bowel transit in patients with delayed gastric or colonic transit.

2) Whole gut transit time (WGTT)

a) Radio-opaque markers (ROM)

The colon functions include storage of faecal residue, absorption of water, propulsion and defaecation. Dysequilibrium of any of these factors would lead to diarrhoea or constipation. Colonic transit is defined as the time food transits through the colon and accounts for 70-90% of whole gut transit time (WGTT). There have been different methods of measuring WGTT. The downside of these tests as currently used is the exposure to radiation especially in young women where functional bowel is more common. Hinton et al[194] described the use of radio-opaque markers (ROM) in measuring the WGTT. It had a good recovery rate of the markers and did not alter transit time theoretically but the task was arduous as it was required to either take multiple abdominal imaging to assess progression of

ROM or observing the passing of these markers in the stool. Later, Metcalf et al simplified this methodology by taking multiple ROM consistently for 3 days before an abdominal imaging taken at day 4 +/- day 7 (to localised abnormal colonic segment) thus reducing the amount of radiation exposure to the patients[73]. This Metcalf method of assessing WGTT in now widely used worldwide and is currently the gold standard to assess colonic transit.

b) Colonic scintigraphy

Scintigraphic methods to assess colon transit require the use of radioisotope materials, specialised equipment such as gamma cameras and it is expensive even though it is non invasive and safe to use. It has the advantage that multiple images can be taken without increasing radiation exposure. The radioisotope is either mixed in a meal or encapsulated in an acid resistant material such as methylmethacrylate and given together with a meal. Once the capsule passes the duodenum, the rise in pH causes the capsule to start to dissolve which takes around 4 hours by which time the capsule is in the terminal ileum or caecum. A gamma camera, obtains anterior and posterior images of the bowel, and as for assessing gastric emptying, regions of interest are drawn and radioisotope count is measured using a dedicated nuclear medicine computer[195, 196]. The images are taken at different times after ingestion e.g. 4 h, 24 h, 48 h and 72 h depending on protocols. Dual isotopes are used to allow assessment of both liquid and solid phases, Technetium and Indium. The interpretation of colonic transit is based on the geometric centre, which is a weighted average of the radioactivity counted over specific parts of the large bowel. There are 2 different methods of assessment, one

by the Mayo clinic using 5 segmented regions of the large bowel and defecated stool, e.g. ascending, transverse, descending, rectosigmoid and defecated stool. The 2nd method using the Temple method is similar except there are 7-segmented regions of the large bowel including ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, rectosigmoid and excreted stool. The geometric centre (GC) is calculated as the sum of the fraction represented by the counts in each region divided by the total counts with the sum multiplied by the region number (Equation 1)[197]. A low value of GC indicates the radioisotope material is close to the caecum and a higher value indicated the radioisotope material is in the rectosigmoid/excreted stool.

$$\text{geometric center} = \sum_{i=1}^{7} \text{ROI}_i \times i/\text{instilled counts},$$

Equation 1: Formula to calculate the geometric centre. i= region of interest number, ROI_i = the number of counts in the region of interest number i.[197]

The normal values for geometric centre (Temple method) are 2 to 7 for 24h, 4.6 to 7 for 48 h and 6.2 to 7 for 72 h.

Colonic transit using scintigraphy has been shown to have positive correlation with stool consistency and bowel frequency and it is easily reproducible[198] though it is worth noting that the coefficient of variation of repeated measures is 28% of GC at 24 hours and 14 at 48 hours, a figure no method improves on and which in part represents the true underlying variability in transit

3) Other tests for measuring colonic transit

a) Wireless motility capsule (WMC or SmartPill Wireless Motility Capsule)

This capsule which measures 2.68 x1.17cm in size is a wireless recording device that provides real-time measurement of its surrounding. It is ingested orally to measure the whole gut transit giving information of gastric emptying time, small bowel transit time and colonic transit time and overall whole gut transit time. It has the ability to measure pH, temperature and pressure thus providing information when the pill transits through the gastrointestinal tract before it is expelled out from the body[199]. The patient needs to wear a receiver belt during the test that normally takes up to 5 days. The use of this novel wireless pill is very informative as it reduces the number of investigations needed to assess pan enteric dysmotility of the gut plus it is very patient acceptable due to it's non-invasive technique. The drawback to this although it might be minor would be the risk of pill retention, equipment malfunction or inability of patient to swallow the pill. The use of WMC has been validated against radio-opaque markers and scintigraphy with both showing good correlation with WMC[200, 201]. In the UK, the use of WMC here is limited by its cost and is not widely available in all NHS hospitals and it is not approved by the National Institute for Health and Care Excellence (NICE) for use on functional bowel disorders.

b) Fluorine marker using MRI

Recently, there have been some developments on the use of a fluorine (19F) marker to assess gut transit[202]. Although this might be at its developmental stage, it does not involve radiation and the size of the pill is smaller than the WMC, as it measures 1.15 x 0.72 cm. To image and track the position of the 19F pill, a transmitter coil is used. Studies so far are promising but at present hardware needed to carry out fluorine MRI is only available in a handful of research units [202, 203].

c) Magnetic pill

A magnetic pill to assess colonic transit/bowel transit is also in a developmental stage. It is non invasive and as for the WMC, the patient only needs to swallow a magnetic pill of 0.6 x 0.7 cm in size and to wear a sensor belt throughout until the pill is expelled. The sensor belt will pick up signals which are then digitalised and transmitted to a laptop nearby for processing and storage[204]. Magnetic pill movement in the large bowel correlated well with radio-opaque markers[205].

WGTT varies within individuals, genders, dietary habit, age[206, 207] and race[208]. Other studies have shown that females have prolonged WGTT compared to their male counter-parts[73, 76, 209]. Upper limit of normal value for WGTT should be less than 72 hours[73, 195] and if this were prolonged, this would be defined as slow transit.

In conclusion, there are a wide range of different methods for measuring gut transit in healthy volunteers and patients. Some involve exposure to radiation, which would not be ideal in a cohort of gastrointestinal dysfunction patients, as they

would comprise mostly of young women. Therefore, subsequence sub-chapters would be a discussion on optimising the measurement of small and large bowel transit using MRI and its application in patients with irritable bowel syndrome to ascertain biomarkers.

1.13.2. Optimising measurement of small and large bowel transit using MRI

1.13.2.1. Background

Secondary care referrals for further management of functional gastrointestinal disorders are common and account for nearly 40% of all referrals[10]. Irritable bowel syndrome, functional constipation and functional diarrhoea are very common and diagnosis is mainly based on patient reported symptoms that can be unreliable[210]. The gut transit is an objective measurement to assess motor function and it could be used to guide treatment and predict efficacy of drugs[211]. Gut transit tests described in the previous sub-chapter, have been developed to measure OCTT and colonic transit/ whole gut transit (WGTT). Each test has its advantages and limitations as described earlier. We would like to take advantage of our easy accessibility of MRI in research to look at gut transit and hopefully we could overcome some of the limitations mentioned.

Therefore, the aims of this study were to validate 2 novel MRI methods to assess OCTT and WGTT and to assess their reproducibility.

1.13.2.2. Aims of the study

To optimise measurement of small bowel and large bowel transit using MRI scanning in particular to assess the reliability and precision of different measurement tools for these parameters in healthy volunteers

Examine the relation between bowel habit, colonic volumes and transit times in healthy volunteers

1) Primary outcome of the study:

a) Correlation between OCTT measurements using the MRI method and the LUBT

b) Correlation between WGTT measurements using the MRI marker pills and ROM method

2) Secondary outcome of the study:

Reproducibility between each test methods

1.13.2.3. Methods

The participants took part in an open label study that involved 2 separate test-retest regimes. Study 1 was to compared small bowel transit i.e. orocaecal transit using MRI against lactose ureide breath test (LUBT) and Study 2 was to compare whole gut transit (WGT) using MRI against the commonly used Metcalf radio-opaque marker (ROM) method.

This study was approved by the National Research Ethics Service (REC number 11/EM/0245) and was registered with the ClinicalTrial.gov identifier: NCT01534507. All volunteers gave written informed consent following assessment of eligibility. The studies were carried out according to the Good Clinical Practice principles.

1) Subjects

21 healthy volunteers (12 males, 9 females) aged between 21-70 years were enrolled into both the studies. Participants who had a history of gastrointestinal disease and taking any medication known to alter bowel motility were excluded from the studies. All volunteers completed an MRI safety questionnaire, to exclude persons with contraindications to MRI, and a hospital anxiety and depression score (HADS) questionnaire. All 21 volunteers completed study 1, which was repeated after a 1-week washout period, to assess reproducibility and 20 of 21 completed Study 2. See Figure 45.

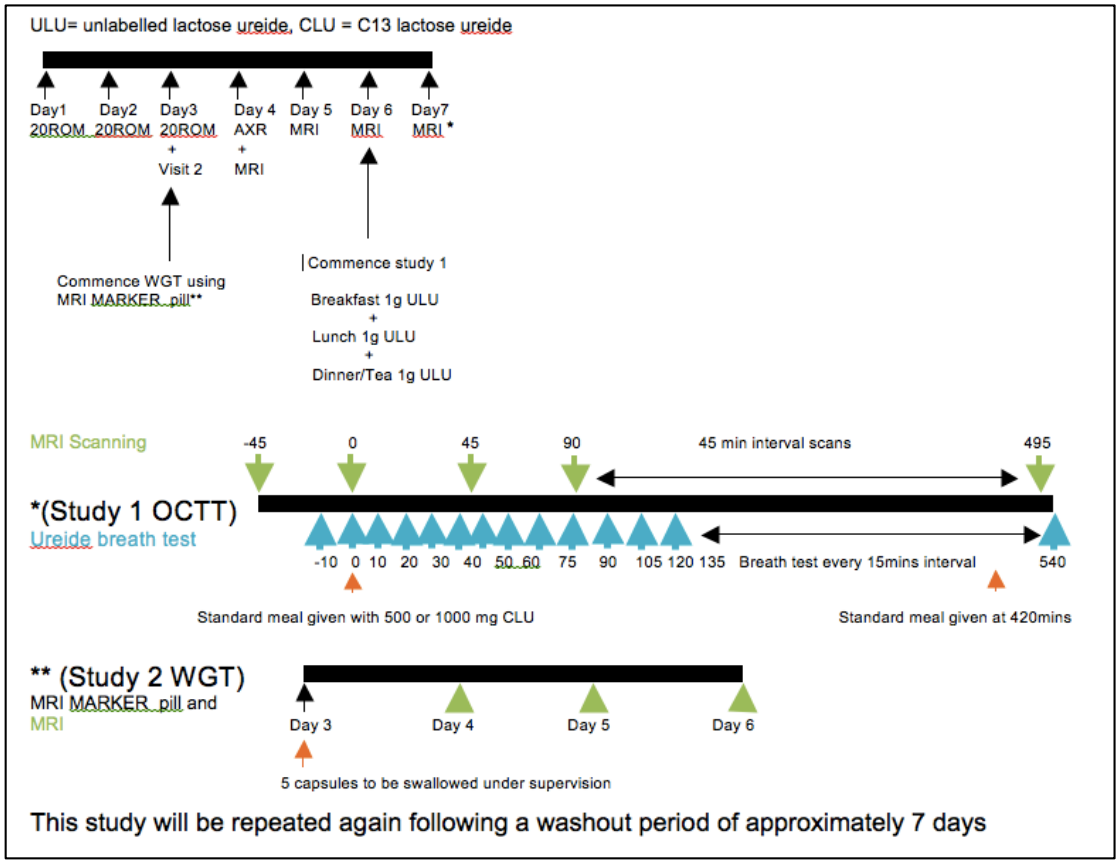


Figure 45: Schematic drawing showing how Study 1 and Study 2 were done cohesively

2) Study 1: Orocaecal transit time (OCTT)

a) MRI OCTT test

The healthy volunteers attended the 1.5 T Sir Peter Mansfield Magnetic Resonance Centre, University of Nottingham, at 8:00am after an overnight fast and underwent a baseline MRI scan before they were fed with a mixed meal which was used in a previous study [212]. This meal consisted of: 220 g creamed rice pudding (Sainsbury's, UK), 34 g seedless strawberry jam (Sainsbury's, UK), 15 g coarse wheat bran (Holland and Barrett, UK) and a glass of 100 ml orange juice from concentrate

(Sainsbury's, UK), altogether providing 362 kcal. The volunteers were scanned at 45 min intervals for a total of 8.5 hours. The second meal was provided at 6.5 hours. This consisted of: 400 g microwaveable macaroni cheese (Sainsbury's, UK), 100 g strawberry cheesecake (Sainsbury's, UK) and 250 ml still water (Sainsbury's, UK). In between each scan, the volunteers were sat in an upright position in the waiting room.

The MRI scanning was performed using a 1.5 T Philips Achieva scanner with a 16-channel XL torso coil. The arrival of the head of the meal into the caecum was determined from the MRI images using a dual-echo 2D multi-slice FFE sequence (Time [TR] = 212 ms, Flip Angle [FA] = 80°). 24 coronal images were acquired to cover the abdomen with an acquired voxel size of 2.01 x 2.87 x 7.00 mm^3 (reconstructed voxel size of 1.76 x 1.77 x 7.00 mm^3), a field of view [FOV] of 450 9 360 mm^2, and a slice thickness of 7 mm with no gaps (SENSE factor = 1.7). Images were acquired during a breath hold of 17 s [213]. An additional single shot turbo spin echo (TSE) sequence was acquired to measure small bowel water content (SBWC)[214] which meant subjects spent ~10 min inside the magnet for each time point. The arrival of the head of the rice pudding meal was assessed visually using the 2D FFE images. The OCTT was estimated as the time from the first scan to show entry of bolus of material giving a high intensity signal into the ascending colon. Prior to this event, most images were of low intensity. Measurement of the colonic volumes before (t=360min) and after (t=405min) a high calorie meal was based on the 2D FFE MRI images. This was measured as older studies have shown that clearance of the proximal colon may correlate with overall transit time[215]. Colonic volumes were

measured using a software called Analyze© 9.0 (Biomedical Imaging Resource, Mayo clinic, Rochester, USA).

b) Lactose Ureide Breath test (LUBT) for OCTT

LUBT was used to assess OCTT as this will be used to compare it with the MRI method for assessing small bowel transit. The LUBT protocol was based on the study by Geypens et al as the group had validated using LUBT with scintigraphy[187]. 24 hours before the test day, healthy volunteers ingested 1g (6mmol) of unlabeled lactose ureide (Euriso-top, Saint-Aubin Cedex, France) three times a day with their meals (morning, afternoon and evening). This was to stimulate bacterial enzyme activity to cleave the lactose ureide in the colon. On the test day, LUBT was performed alongside the MRI OCTT test (see Figure 45). The volunteer provided a baseline breath sample before being given the mixed meal (detailed above). The test meal was mixed with 500 mg ^{13}C labeled lactose ureide (Euriso-top, Saint-Aubin Cedex, France). Breath samples were taken every 10 min for an hour and then every 15 minutes for the next 9 hours. A second high calorie meal was given 6.5 hours later after the mixed meal. Breath samples were collected and analysed using an Infrared Isotope (IRIS) analyser machine (Wagner Analysen Technik GmbH, Germany). Results were expressed as delta over baseline, which is the difference between the ratio of $^{13}CO_2/^{12}CO_2$ in the post dose breath sample and the corresponding ratio in the baseline sample. The OCTT was taken at the time in which there was a significant increase from the background breath ^{13}C. This was defined as 2.5 times the SD of all previous above the running average of all previous points[187] (Figure 46). The OCTT was automatically determined from the data

obtained by using an in-house program written in Matlab (MathWorks, Natik, USA). The amount of lactose used in this test was 6 mmol and this would not exert a significant osmotic effect to alter the gastrointestinal transit.

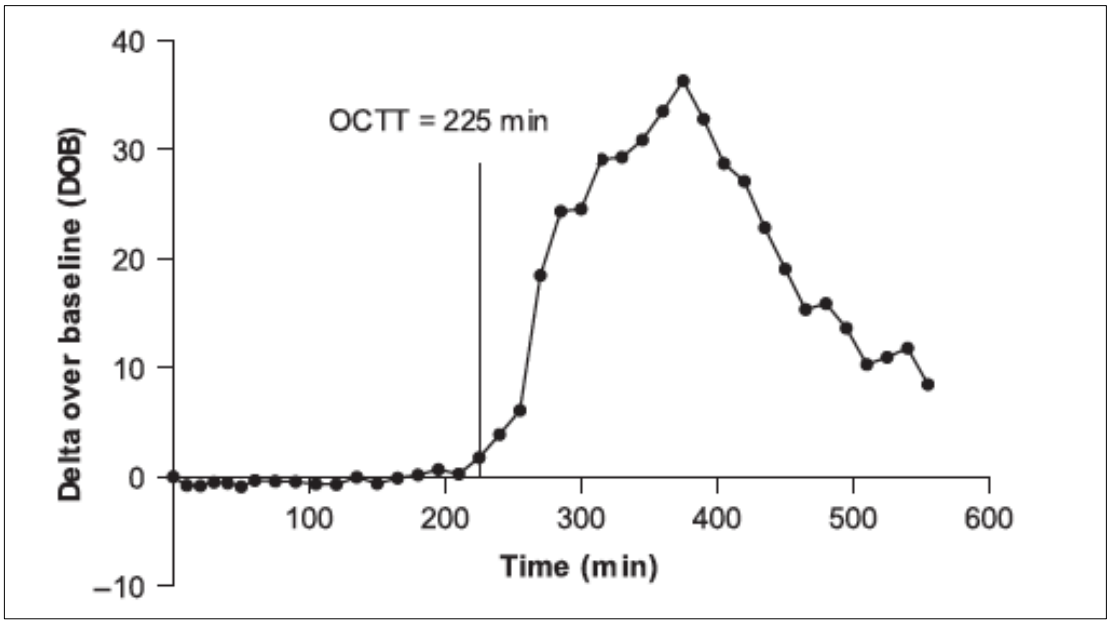


Figure 46: 13C breath excretion curve in one healthy volunteer

3) Study 2: Whole gut transit time (WGTT) test using MRI

Healthy volunteers swallowed 5 MRI marker pills, measuring 20x9 mm, at 09:00 am, 24 hours before having an MRI scan of the abdomen. The volunteers were given the pills and had to swallow them under direct observation.

a) MRI marker pills for whole gut transit

The MRI marker pills were manufactured in-house using a biologically inert polyoxymethelyne (Figure 47). The pill consisted of 2 half shells with a cuff that would allow the two shells to be glued together using cyanoacrylate glue. A small

hole had been drilled in the top of one half shell so the pill could be hand filled with 0.4 ml of 15 µM of Gadoteric acid (Gd-DOTA). A plastic screw was inserted into the hole and glued with cyanoacrylate glue to prevent leakage. To ensure there was no leakage, 20% of the pills that was produced for each batch were tested by adding blue dye, sealing and immersing in water at 37^{O}C for 48 hours. Dye leakage was detected by using a spectrophotometer reading at 400 nm. Any batches showing leakage were rejected (this was extremely rare).

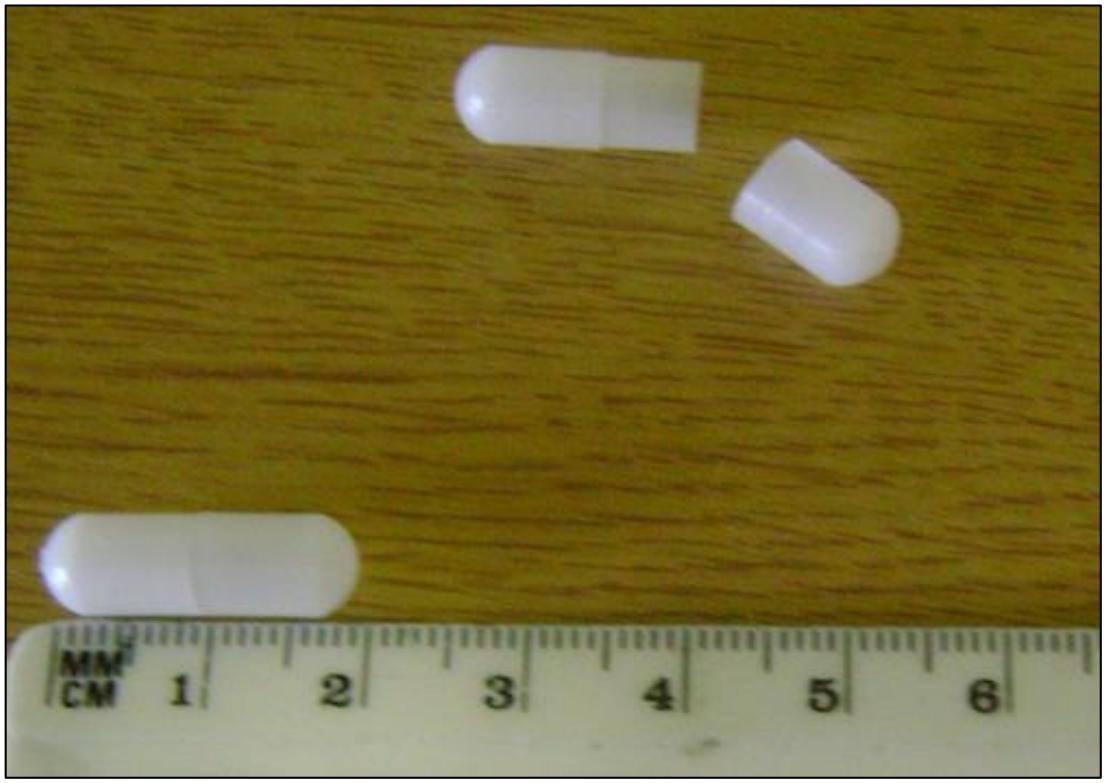


Figure 47: MRI marker pilled made of polyoxymethelene. 2 half shells were glued together and hand filled with 0.4 ml 15 µM Gd-DOTA. The pill has the dimensions of 20 x 7 mm.

The Gd-DOTA (Dotarem©, Guerbet, France) that was used to fill the pill is a complex of Gd^{3+} and the chelating agent DOTA. This is non-toxic, safe to use and has been routinely used in clinical practice as an MRI imaging contrast agent[216, 217]. This

agent shortens the T1 relaxation times of protons [218] thus increasing the signal on the T1 weighted images. There have been preliminary works to find the optimal concentration of Gd-DOTA at the Sir Peter Mansfield Magnetic Resonance Centre, University of Nottingham. 15 µM Gd-DOTA was used to give the maximum signal intensity from the capsule in the T1 weighted images [219]. The concentration of the Gd-DOTA was achieved by diluting 1 ml of Gd-DOTA, at a concentration of 280 ml/ml, with 33 ml of distilled water.

The volunteers were scanned in a 3T Philips Achieva MRI scanner using a multi-transmit body coil. The scan images and sequences can be found in the study by Chaddock et al[213]. Since a single scan cannot cover the entire abdomen, 2 scan images were obtained at 2 stations (with a 30mm overlap) in coronal view using two different sequences. Firstly, a T1 weighted 3D Turbo Field Echo (TFE) sequence (TE = 1.3 ms; TR = 2.9 ms, FA = 10°, FOV = 250 x 398 x 160 mm^3, Acquired resolution [AQR] = 2.3 x 2.3 x 4 mm^3), was used to count and locate the number of capsules remaining in the colon at 24 h (Figure 48). Secondly, a multi-echo FFE sequence[220] (TE1 = 1.07 ms; TE2 = 1.9 ms; TR = 3.0 ms, FA = 10°, FOV = 250 x 371 x 200 mm^3, AQR 1.8 x 1.8 x 3.6 mm3; SENSE factor = 2), using a 16 channel XL torso coil to receive the signal, was used to create a movie using the maximum intensity projections (MIP) of the water only images[213] (Figure 49).

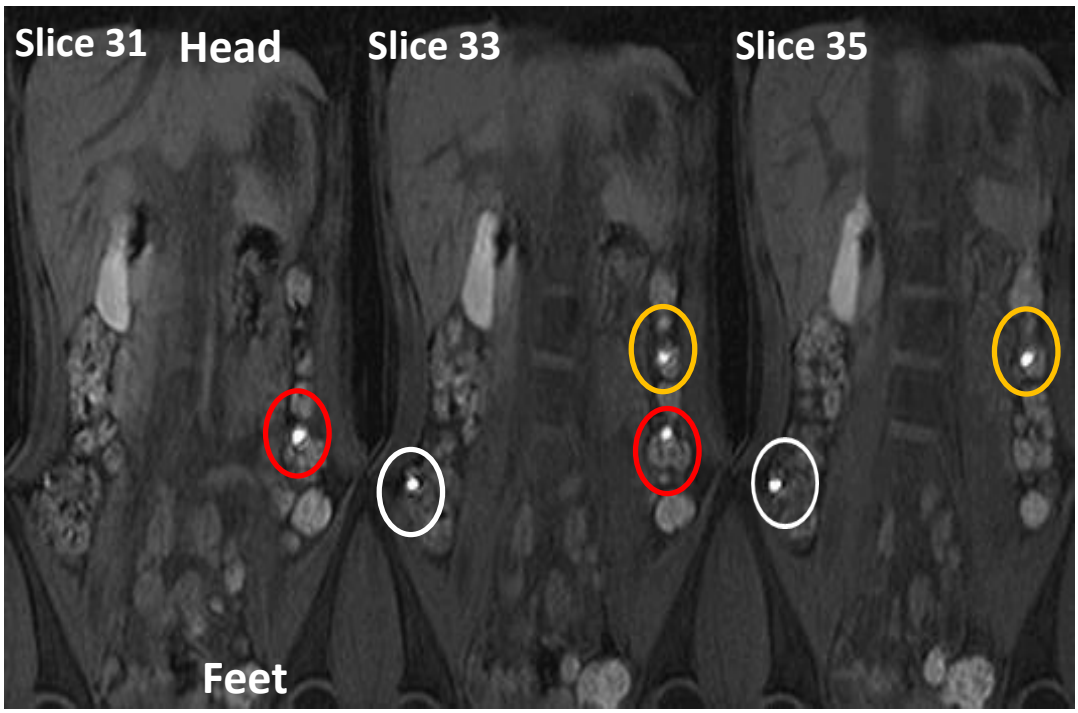


Figure 48: Coronal sections obtained at 2 stations with 30mm overlap using the 3T multi-transmit body coil

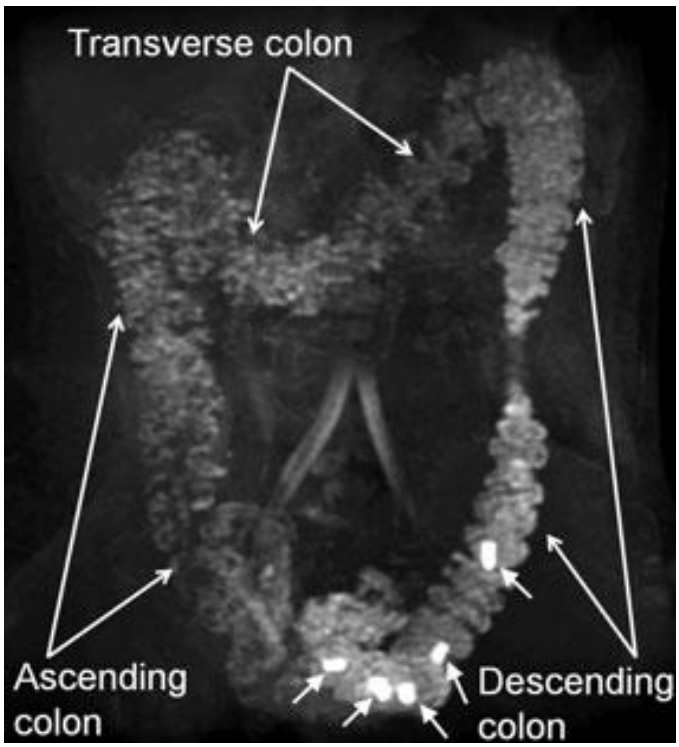


Figure 49: T1 weighted maximum intensity projection MR image showing 5 marker pills in the colon

The movies allowed rotation of the colonic image and were useful to clarify the position of the pills at 24 hours if the T1 weighted TFE images were not conclusive. Based on the MRI images, a transit score was calculated by dividing the distal small bowel and colon into 8 sections (Figure 50) and each pill was scored according to its position in the colon at 24 hours. On several data sets, 1 or 2 pills were separated in position by several segments from the rest of the pills (visualized together in a group). Since only 5 pills were used to calculate a transit score compared to the standard radio opaque marker tests (20 markers/day), a weighting factor was included into the calculation. This was to reduce the effect of outliers. The weighting factor was calculated for each capsule depending on the difference of the capsule score from the median capsule score. For a difference of 0 and 1 the weighting factor was 1, for all differences larger than 1 the weighting was the inverse of the difference. Finally, the weighted average transit score of the MRI marker pills was determined for each volunteer (Figure 51).

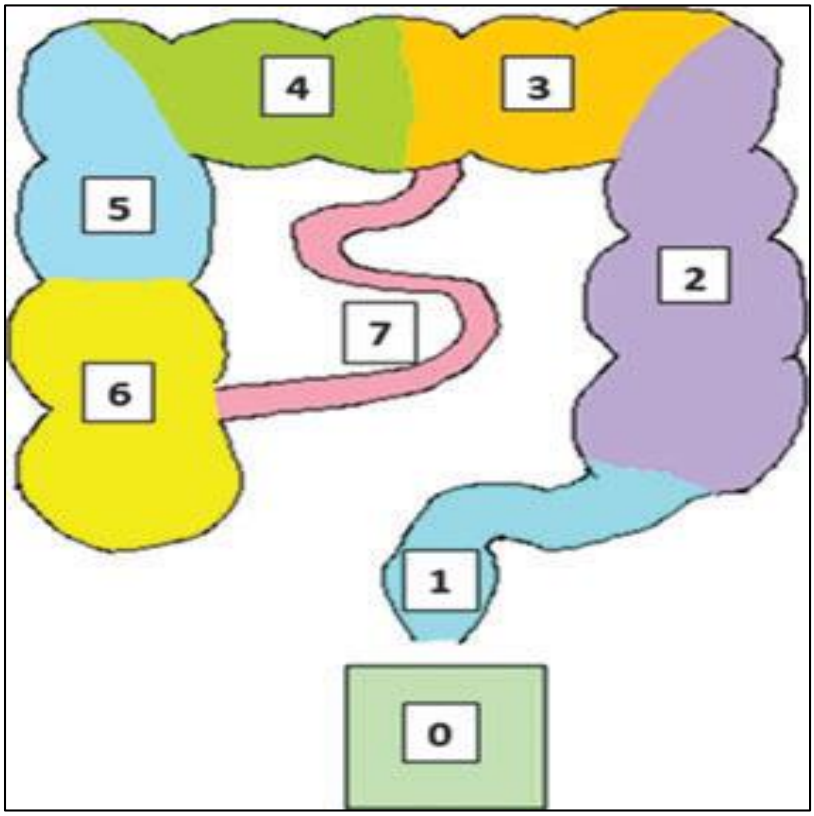


Figure 50: Segmented colon used to score the MRI marker pills 24 hour after ingestion. 0= not found (presume pill to be excreted), 1= sigmoid and rectum, 2 = descending colon, 3 = left transverse colon, 4 = right transverse colon, 5 = upper ascending colon, 6 = lower ascending colon and 7 = small bowel.

$$\text{Average transit score} = \frac{\sum\{no.of\ pills\ X\ no.of\ region\ X\ f\}}{\sum\ weighting\ of\ 5\ pill}$$

f = 1/ difference from median of pill position

Figure 51: Formula to assess average weighted transit score based on the position of the MRI marker pills

An example to calculate the average weighted transit score:

*There was 1 MRI marker pills in region 0, 2 in region 1 and 2 in region 2. The median position score was 1. The differences of each pill score from the median position score were -1, 0, 0, 1 and 1. All pills, which have a difference from median of 0 or 1, were given a weighting of 1. Those pills further away are weighted as 1/difference. Weightings for this example are: 1,1,1,1 and 1. Weighted average then becomes: (1*0+1*1+1*1+1*2+1*2)/(total weighting of 5 pills = 5) = 1.2*

A non-weighted least square fit was applied to the MRI marker pill scores and their corresponding ROM transit scores (based on the Metcalf study[73]) to determine a transit time in hours. This is based on the equation: y = mx+c. x is the average MRI marker position, m and c are unique coefficients determined from this study and y is the transit time in hours (Figure 52).

WGTT(hr) = (0.034 x weighted average transit score of MRI marker pills) - 0.124

Figure 52: Formula to calculate WGTT for the MRI marker pills

b) Radio-opaque markers (ROM) test

The ROM test used was based on the Metcalf study[73]. Volunteers swallowed 20 ROMs on 3 consecutive days (days 1, 2 and 3) and an abdominal x-ray was taken on day 4, immediately after an MRI scan was used to locate the MRI marker pills consumed the day before (day 3); see Figure 45. The ROM was made of silicone tubing that was impregnated with 13.5% barium. The dimension of each ROM was 2.42 x 5.09 mm (Altimex, Nottingham, UK). The WGTT was calculated by counting

the number of ROMs remaining on day 4 and multiplying by 1.2 to give WGTT in hours (Figure 53).

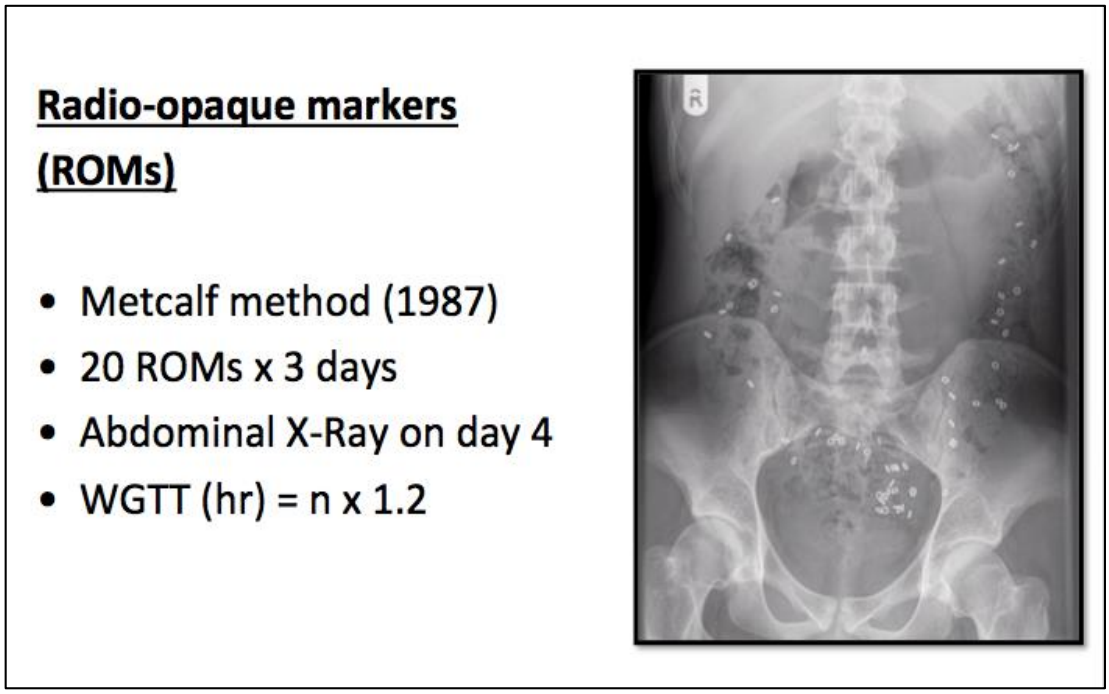


Figure 53: WGTT using the ROM method

1.13.2.4. Statistical analysis and power of studies:

1) Statistics:

All data analysis was carried out using Prism 5 software (GraphPad Software Inc, San Diego, USA). Data distribution was assessed using the D'Agostino and Pearson omnibus normality test. Since the data was not normally distributed, the results would be in median (IQR) and the Spearman's rank correlation coefficient test was used to assess correlations. To assess the reproducibility of the different methods as already described, intra-class correlation coefficient test (ICC) was used.

2) Power calculation:

Based on the study by Horikawa et al. [78], the mean colonic transit for healthy volunteers was 35.7 ± 12.9 h. For 80% power to detect a 25% difference in transit between the 2 methods, 19 participants would be needed for the study. 21 participants were recruited into the study to allow for dropouts and technical difficulties.

1.13.2.5. Results

All 21 healthy volunteers completed the study with no adverse events. 1 volunteer did not attend the x-ray appointments (Study 2) and 1 breath test data from study 1 was omitted due to high background noise within the data produced.

1) OCTT measurement using MRI and LUBT

The OCTT measured with LUBT was 225 min (165-278) and with MRI it was 225 (180-270). The correlation between these 2 methods to assess OCTT was weak with spearman, r = 0.28 (p=0.08) (Figure 54). The limit of agreement between the 2 methods for OCTT was shown using the Bland-Altman plot in Figure 55. This graph showed a small mean difference of -7.32 min between measures. The difference between measurements ranges from -197.6 min to 183.0 min. It appeared to show that the longer the transit time, there is a tendency for the difference between the 2 methods of measurements to increase. ICC test was used to assess repeatability

of these methods on two separate occasions. The ICC for repeat OCTT measurement using LUBT and MRI were 0.35 (p=0.058) and 0.45 (p=0.017) respectively. The inter-observer agreement for the MRI OCTT measurement gave an ICC of 0.44 (p=0.002). Inter-observer agreement for LUBT OCTT was not calculated as the OCTT results were generated automatically using an in house analysis program.

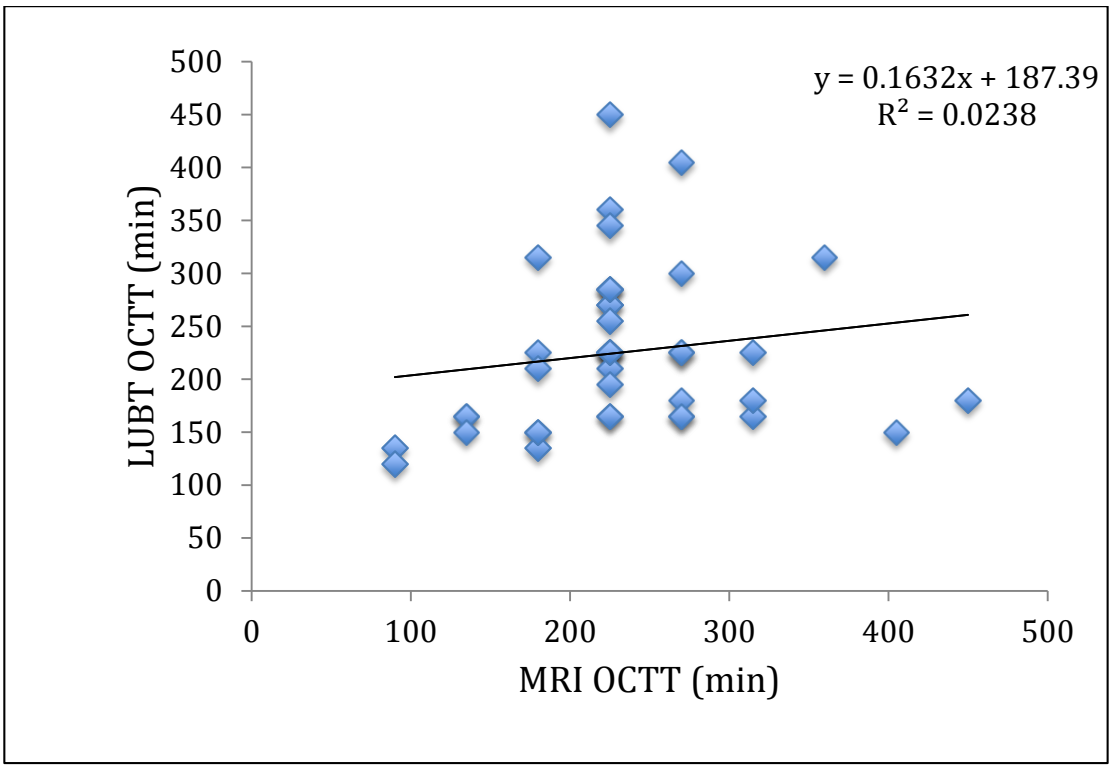


Figure 54: Correlation between MRI and LUBT tests for OCTT

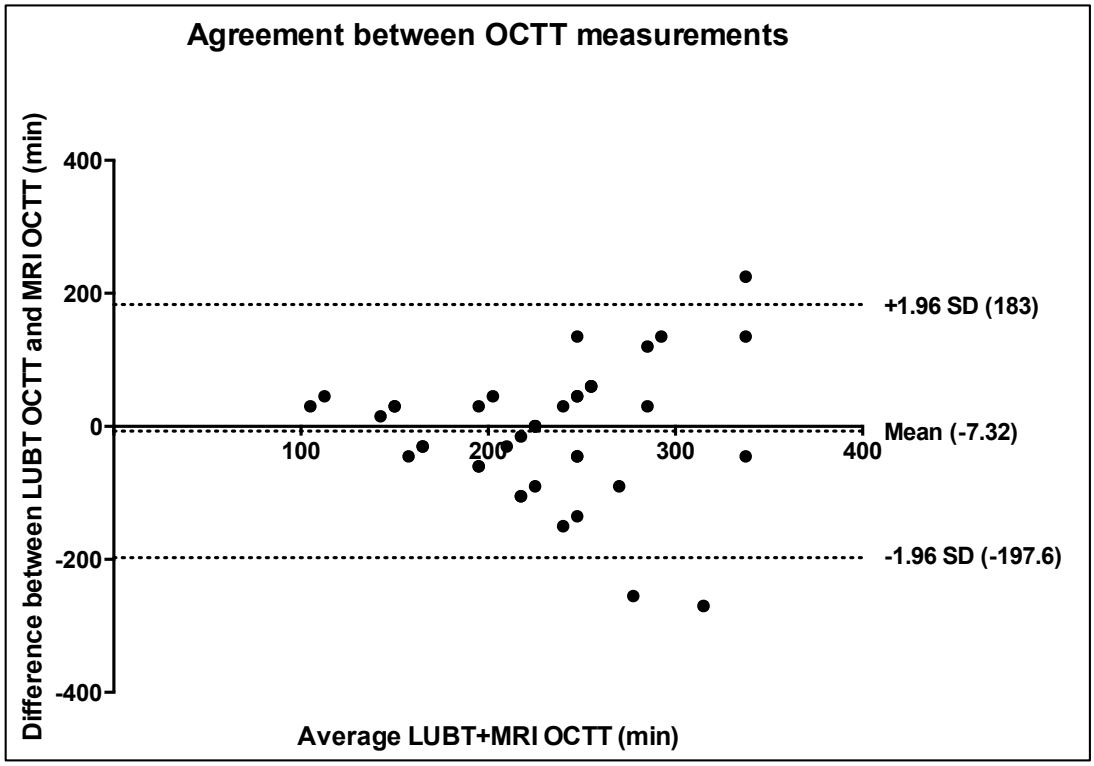


Figure 55: Bland-Altman plot to show agreement between the 2 OCTT methods

2) WGTT measurement using MRI marker pill and ROM

The mean (SD) WGTT measure using ROM was 31.2 (20.8) hours whilst the median average weighted transit score using the MRI marker pill was 0.8 (0-1.6). The average weighted transit score at 24 hour (post ingestion) was converted into WGTT in hours by using a regression equation linking these 2 techniques. Following this conversion, the WGTT for the MRI marker pills gave a median of 27.6 (3.7-50.0) hours. The correlation between these 2 methods was good giving a spearman r of 0.85 ($p<0.0001$), (Figure 56). The agreement between these 2 methods using the Bland-Altman plot showed the mean difference of 0.0045 hours but the 95% limits of agreement were from -25.69 to 25.68 (Figure 57). The repeatability test for MRI marker pill and ROM methods, which were obtained on 2 separate study days, were

assessed using ICC giving a reasonable score of 0.61 (p=0.001) and 0.69 (p≤0.001) respectively. The inter-observer agreement between WGTT measurement using MRI marker pill and ROM methods were assessed using ICC. The ICC for MRI marker pill method was good at 0.78 (p≤0.001) and as for ROM method; the ICC was very good at 0.995 (p≤0.001).

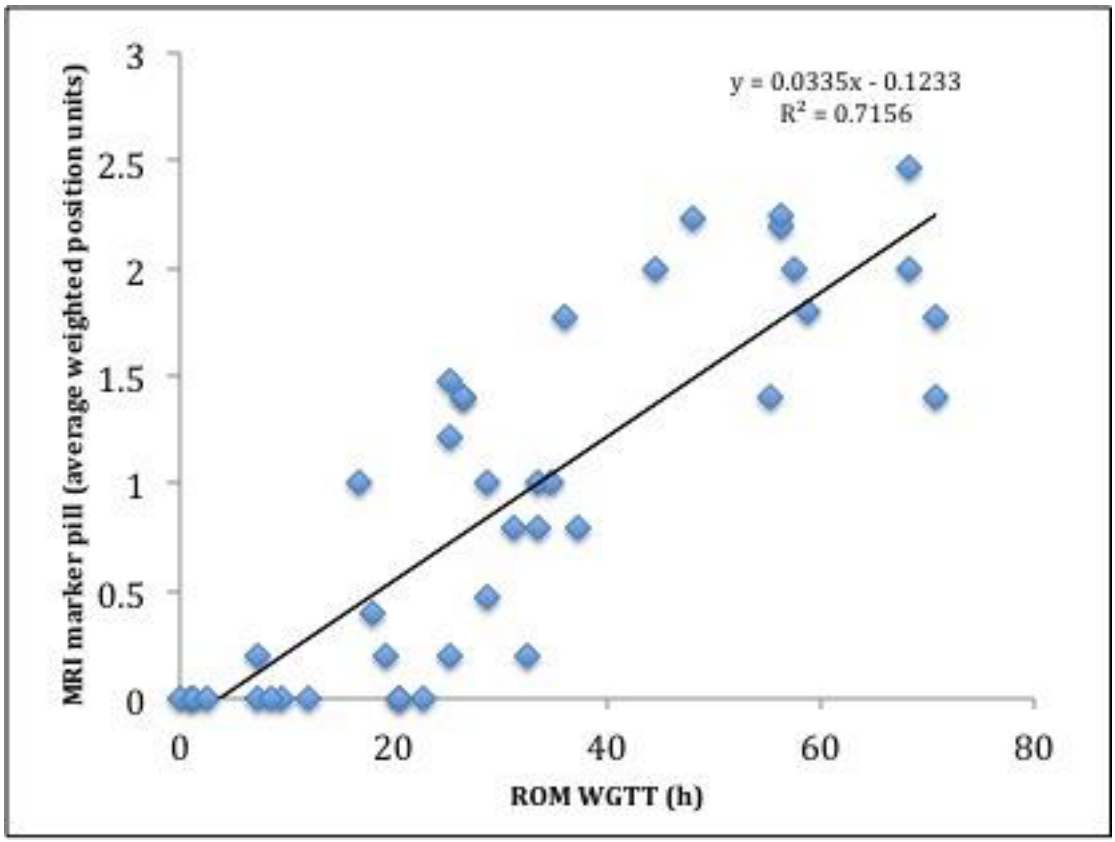


Figure 56; Correlation between MRI marker pills and ROM for WGTT

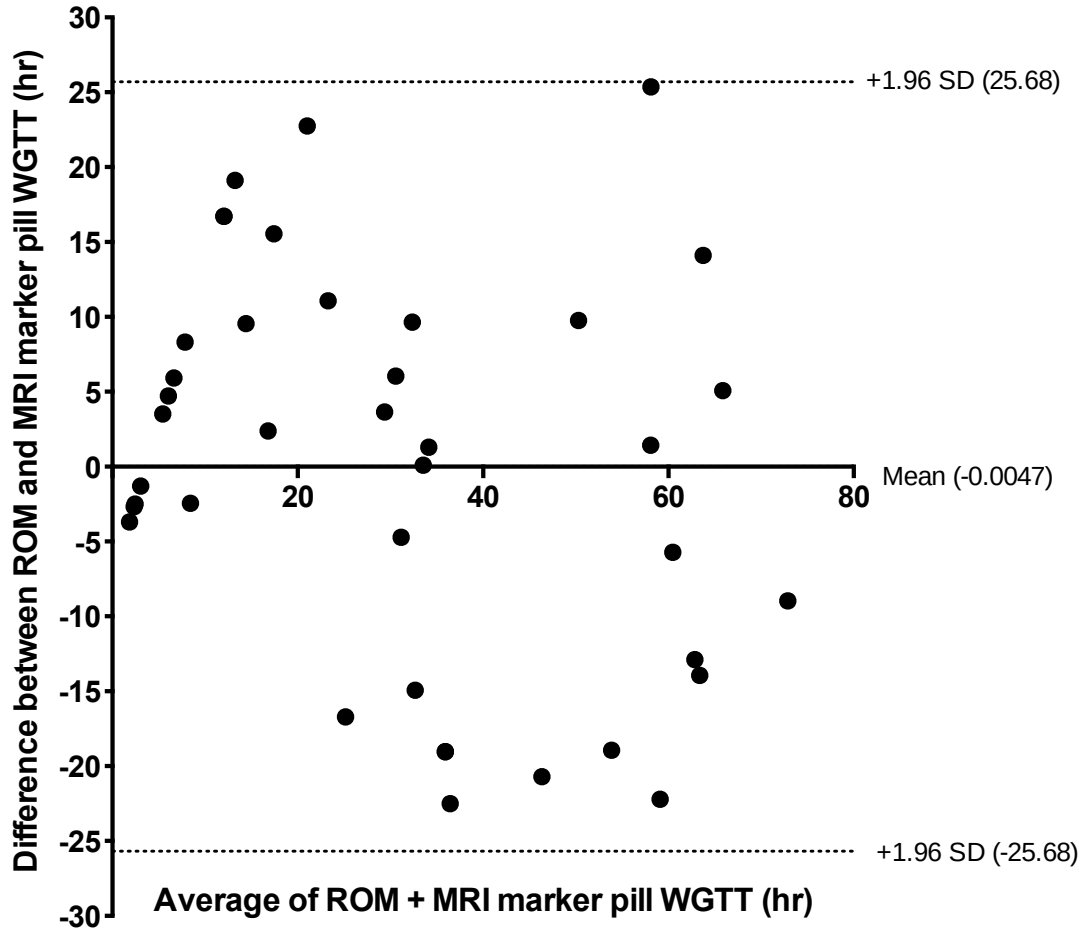


Figure 57: Agreement between WGTT measurements

3) Other results:

Extra information was obtained during the MRI scans of the healthy volunteers. These were used to measure their regional and total colonic volumes at t=460 min (before meal) and at t=405 min (after meal) and small bowel water content (SBWC). The scanning methods used were coronal dual echo fast field echo sequence images for colonic volumes [221] and single shot fast spin echo images used to assess SBWC[214].

There was no correlation between colonic volume, SBWC and transit time (both OCTT and WGTT). There was no significant correlation between transit time (OCTT and WGTT) with BMI, anxiety and depression score (Table 21). There was a weak correlation between OCTT and age. There was no significant difference in the colonic volumes between genders. Baseline total colonic volumes for male and female were 568 (139) and 616 (217) ml respectively, p=0.55. There was no correlation between baseline colonic volumes with weight, height, BMI and age (Table 22). Both female and male healthy volunteers have similar transit times with median of 27.6 (3.7- 45.4) for male and 25.6 (3.7-58.8) for female; p=0.7.

Table 21: Correlation between MRI parameters and healthy volunteers' demographic

	OCTT (min)*	**WGTT (hr)****
Age	r = 0.36 (p=0.02)	r = -0.08 (p=0.61)
Height (m)	r = -0.04 (p=0.81)	r = -0.11 (p=0.50)
Weight (kg)	r = 0.09 (p=0.56)	r = -0.21 (p=0.18)
BMI (kg/m^2)	r = 0.13 (p=0.41)	r = -0.23 (p=0.14)
Anxiety score	r = -0.29 (p=0.08)	r = 0.42 (p=0.16)
Depression score	r = -0.15 (p=0.38)	r = 0.16 (p=0.31)
Total colonic volume at t=360min (ml)	r = 0.26 (p=0.10)	r = 0.16 (p=0.33)
Ascending colon volume at t=360min (ml)	r = 0.13 (p=0.41)	r = 0.13 (p=0.41)
Transverse colon volume at t=360min (ml)	r = 0.02 (p=0.89)	r = 0.13 (p=0.42)

Descending colon volume at t=360 min (ml)	r = 0.29 (p=0.07)	r = 0.17 (p=0.27)
Δ t=405-t=360 change in ascending colonic volume (ml)	r = -0.19 (p=0.23)	r = -0.10 (p=0.90)
Δ t=405-t=360 transverse colonic volume (ml)	r = -0.11 (p=0.50)	r = -0.16 (p=0.31)
Δ t=405-t=360 change in descending colonic volume (ml)	r = -0.04 (p=0.81)	r = -0.13 (p=0.43)
Fasted small bowel water content (SBWC) (ml)	r =0.17 (p=0.28)	r = -0.08 (0.61)
AUC SBWC (ml/min)	r = -0.01 (p=0.97)	r = 0.08 (p=0.65)
r = spearman's rank correlation coefficient; * = OCTT measured using LUBT; ** = WGTT measured using MRI marker pill		

Table 22: Correlation between total baseline colonic volumes and healthy volunteers' demographic

Correlation between baseline total colonic volume (ml)	Spearman, r	P value
Age	0.07	0.77
Weight	0.04	0.88
Height	0.33	0.15
BMI	-0.18	0.44

1.13.2.6. Discussion

There was no difficulty in identifying the MRI marker pills in the MRI images. If the T1 weighted TFE image was inconclusive, the 3D rotating MIP movie was used. It showed the exact position of each of the pills in the large bowel. This method is simpler compared to the Metcalf ROM method as the latter can be difficult to identify the precise site of ROMs located in the pelvic region on a plain abdominal x-ray. The method quantifying WGTT in this MRI marker pill was similar to that of colonic transit using scintigraphy. It is based on the use of geometric mean and colonic segments as previously described by Krevsky *et al.*[197]. However, the novel aspect of this study using MRI marker pill WGTT is the formula used to calculate the transit time takes into consideration of the spread of MRI markers position along the gut. This was calculated by looking at the difference of each pill position from the median pill position and using this to apply to a weighting factor to each pill score. In the majority of the healthy volunteers, the MRI marker pills travelled along the gut as a group. In a few healthy volunteers, a few of the MRI marker pills separated substantially from the group, which heavily affect the mean position score. Thus a weighting factor was applied to each pill score to reduce this dispersion factor. The use of weighting factor for the MRI marker pills scores made only a small change to the average median pill position unit, 0.97 (non-weighted) versus 0.8 (weighted) but if this was applied to a slow transit bowel, this would make a large change to the transit time. Also, if the weighted score was used, the Spearman's rank correlation coefficient with the ROM method improves from 0.7 ($p<0.01$) for non-weighted to 0.85 ($p<0.01$) for weighted.

This study was built on a previous MRI feasibility study by Buhmann *et al.*[222] where the study used 5 small eppendorf tubes (2.4 x 0.6 cm) filled with Gadolinium-DPTA and normal saline solution as markers of transit, giving a transit time of 41±9 h in women and 31±10 h in men. As methodology and analysis were different from our current study, it would be very difficult to compare results. Based on this study, the SPMMRC had designed and optimised the MRI marker pill to be used in clinical practice, comparing the method against the widely used Metcalf ROM method and using the MRI scans at a single 24 h time point instead of 6 time points over 60 h. The optimal assessment period for measuring colonic transit using scintigraphy was 24 h [223] and had showed lowest short-term intra-subject variation. This methodology was adopted into this study. However, other studies have suggested that 48 h assessment does better for slow transit[198], therefore it would be worthwhile to include a 48 h MRI scan to address this point.

In this study, there was a strong correlation between the WGTT measure by MRI and the ROM method. Other studies have suggested that ROM, which are normally 2mm in diameter, and the MRI marker pills, which are larger (2.0 x 0.7cm), may travel through the different regions of the bowel at different rates. Small pellets < 2mm diameter empty from the stomach during the digestive phase whilst larger capsules will empty slower after a meal during the phase III of the migrating motor complexes[224, 225]. This is confirmed with another recent study using scintigraphy and the WMC to assess gut transit[201]. However, once pills/markers are in the small bowel and mixed with chyme, the movement is unaffected by the size of markers[226, 227]. There has been suggestion that larger capsules would move ahead of smaller

markers in the larger bowel[228], but a study by Rao *et al.*[229] showed similar WGTT between ROM (29.7 [IQR 22.4-45.7] h) and the WMC. This result was similar to that observed in this study.

The MRI method to assess WGTT allows us to easily and accurately assess the position of the MRI pills and assign accurately to the upper or lower half of the ascending, transverse and descending colon. However, the sigmoid is more convoluted making it difficult to assign markers with such precision, therefore the sigmoid and rectum, were grouped together.

The reproducibility for WGTT was better than for OCTT, similar to other studies[76], giving an ICC value of 0.69 ($p<0.01$) for the ROM method and 0.61 ($p<0.01$) for the MRI marker pill method. In this study, there was an interest in developing a purely MRI method to quantify both WGTT and OCTT. MRI and LUBT were used for OCTT assessment. The median OCTT value using LUBT was 225 min (IQR 165-278 min), which was slightly faster compared with another study which gave an OCTT value of 292±58 min[187]. The slight difference in OCTT could likely be accounted to the type of meal used which in the study by Geypens et al, the breakfast consisted of one scrambled egg and 2 slices of bread, which was smaller than the one used in this study. The ICC values for repeated measurements of LUBT and MRI were both poor at 0.35 ($p=0.06$) and 0.46 ($p=0.02$), suggesting OCTT depends on other uncorrected/uncontrolled factors as others have reported[198]. The individual variability is similar to both techniques and this suggests the variability reflects intrinsic biological variability rather than methodological variability[76].

There is no gold standard of measuring OCTT. Current tests such as the lactulose breath hydrogen test are used commonly. It is known to have an osmotic effect on the unabsorbable lactulose, which accelerates transit when compared with scintigraphy[185], thus making it unsuitable for assessment. Interpretation of breath hydrogen is quite complex especially in patients since bacterial overgrowth is common and would give spuriously short OCTT time. It is also true for the LUBT test but as the dose used in this study was small, it would not alter the transit in the way lactulose does. The WMC can be used to assess OCTT (as discussed earlier) by measuring the time the pH rises on entering the duodenum to the time it falls on entering the colon[230]. The median OCTT using a standard eggbeater meal (196 kcal) in the study by Sarosiek *et al.* showed 276 min. The disadvantages of this method are limited by cost and the risk of the pill getting stuck in the small bowel (e.g. Crohn's disease and radiation enteritis). So far, the 'best' standard used for OCTT measurement would be scintigraphy but at present, the techniques used are varied and normal range is wide[231]. Measuring OCTT using our MRI technique has been described in the past[212] and involved looking at the arrival of the high intensity head of a 362 kcal rice pudding meal in the caecum. The median value for OCTT was 225 min (IQR 180-270), which were in very close agreement with values reported[212]. In this study, the OCTT values using LUBT and MRI were similar but showed poor agreement between the two methods (Figure 55). The limitation to the MRI technique compared with LUBT was the prolonged scanning time interval, which was every 45 min, where as the LUBT breath test sampling time interval was every 10-15 min. The other limitation to the MRI technique for OCTT was the difficulty in interpretation of the arrival of the head of the meal on the MRI images, especially in

cases where bright residues appeared in the caecum before or soon after eating the rice pudding meal, which could cause confusion with the later actual arrival of the head of meal. There was poor inter-observer agreement for measuring OCTT using MRI, giving an ICC of 0.44 ($p=0.002$), which suggests that this would not be a useful technique for OCTT as it is very operator dependent.

On the other hand, the MRI marker pills described here are simple. It involved 1 visit for a set of MRI scans which takes around 5 min to perform. The images are easy to interpret due to the detailed anatomical information provided. The MRI scanning sequences are similar and available to any clinical MRI scanner platforms. For these reasons, it could be widely adopted in clinical use. By using the algorithm in this study, the results of this test can be interpreted easily by a clinician. Furthermore, there was a very strong inter-observer agreement between measurements using this method giving an ICC of 0.78 ($p<0.01$). In this study, a 3T MRI scanner was used but this method can be used in a 1.5T MRI scanner (Unpublished, see subsequent chapter). Also, as the T1 weighted 3D TFE sequence does not require the use of a dedicated torso coil to receive the radio-frequency signal, this increases the portability and simplicity of the method for use in clinical practice. If a dedicated torso coil is used, it can acquire images that can be converted into a 3-D rotating movie and these provide good spatial resolution, which can be very useful for further confirmation of the exact position of the MRI marker pills. Since the images are easy to interpret a trained research assistant could report scans.

The cost of MRI is falling and this possibly could be an advantage for cost effectiveness if other tests could be avoided. Using this technique, not only does it measures WGTT, MRI scans could provided further information of the gut such as colonic transit and small bowel water content of patients with various gastrointestinal dysfunctions. This method is non-invasive, does not expose the patient to radiation allowing the use of it for repeated tests in young women and children following treatment and is also a very patient acceptable test.

1.14. Clinical use of MRI testing on IBS

1.14.1. Background

Community surveys indicate that over 25% of the population report suffering from constipation at least some of the time [232]. When patients are asked what specific symptoms trouble them most the commonest complaint is straining to pass stool, followed closely by hardness of stool and infrequent bowel movements. About 2/3 also complain of abdominal pain [233]. Abdominal pain associated with constipation is a key feature of irritable bowel syndrome (IBS) with constipation (IBS-C) which distinguishes it from functional constipation (FC) in which pain is either absent or not prominent[7]. The other symptoms of these two conditions like hard stools and straining overlap extensively and if one suspends the requirement for FC patients to not have IBS, then 44% of patients with functional constipation also meet Rome III IBS-C criteria while 85% of IBS-C meets the criteria for functional constipation[234]. However making the distinction may be worthwhile since as we show below the underlying pathophysiology and response to treatments differ in important ways. While IBS is frequently associated with rectal hypersensitivity as assessed by rectal barostat [235], rectal hyposensitivity with lack of urge to defecate has been reported in 23% of unselected patients with constipation[236]. When compared to healthy volunteers, slow transit constipation sufferers show reduced sensitivity and higher threshold for urge while IBS-C have lowered threshold to pain perception[237]. Furthermore motility differs in important ways, with some studies showing delayed transit in “painless constipation” while painful constipation (now called IBS-C) was

associated with reduced pain threshold but variable transit within the normal range[238]. More recent prolonged (24 h) ambulatory manometry recordings in FC with severe slow transit have shown reduced motility, with reduction in the normal stimulatory effect of waking and eating [239]. In contrast studies using radiotelemetry it is shown that IBS-C patients have increased contractions, particularly in the distal quartile of colonic transit compared to both FC and healthy controls[240]. These different underlying mechanisms have implications for treatment since prokinetic agents such as Prucalopride, which benefit FC, are not licensed for IBS-C in whom clinical experience shows a higher incidence of pain and diarrhoea. Likewise bulk laxatives like polyethylene glycol (PEG) electrolyte which treat FC well do not alleviate the pain of IBS-C even though they stimulate more frequent defecation[241]. The need for bowel cleansing for both ambulatory manometry and the rectal barostat test significantly alters the underlying pathology and the techniques introduce many other variables including psychological distress which may account for why their results correlate poorly with other clinical features. Furthermore not all patients will agree to such invasive tests making the observations biased in unpredictable ways. There are limitations of pure observational studies since many patients change both lifestyle and diet in order to minimise symptoms making it more difficult to distinguish underlying abnormalities of function, which might be more clearly shown by assessing the response to a standardised intervention. We have recently developed a non-invasive magnetic resonance imaging (MRI) technique which allows measurement of intestinal water content[214] , colonic volumes [221], motility and transit [213] in a highly patient acceptable way. We have used these techniques to create a test of colonic function by giving a large dose of

the osmotic laxative, Moviprep®, a combination of PEG and electrolyte to distend the ascending colon and allow observation of the colonic response which, as our results show, differ in the two conditions of IBS-C and FC.

1.14.2. Aim of the study

To assess:

1) The fasting small bowel water
2) The whole gut transit time (WGTT)
3) Colonic volumes at baseline and during the 4 h MRI scanning following 1L Moviprep®
4) Motor function of the ascending colon using the motility index score

1.14.3. Methods

This was an open label study examining the response of the small and large intestine to acute ingestion of 1 litre of polyethylene glycol (PEG) and electrolyte solution (Moviprep®). We used a virtually identical protocol to that already reported in healthy controls (HV)[242] to study patients with constipation. These studies were approved by the National Research Ethics Service, United Kingdom (10/H0906/50 and 11/EM/0440) and by the Medicines and Healthcare products

Regulatory Agency (MHRA CTA reference number 03057/0045/001-0002). This study was registered with the ClinicalTrials.gov (Identifier NCT01622972) and the EU clinical trials register with EudraCT number 2010-021879-85. There were no changes to the protocols from that published at registration. All participants gave written informed consent and the studies were carried out according to the Good Clinical Practice principles.

1) **Subjects**:

48 (45 females, 3 males, 21-68 years old) patients with constipation were recruited from general gastroenterology clinics in the Nottingham University Hospitals Trust, Nottingham. These comprised two groups classified according to the Rome III criteria into functional constipation (FC) or irritable bowel syndrome predominantly constipation (IBS-C)[7]. Since this was a secondary referral practice these patients had all failed at least 1 simple laxative in the past before entry into the study. Participants were required to stop any laxatives and medications that would affect the gut motility for at least 7 days prior to the allocated study day. All participants completed a safety questionnaire to exclude contraindications to MRI such as metal implants in the body. They also all completed the Hospital Anxiety and Depression Score (HADS) questionnaire and Patient Health Questionnaire 12 Somatic Symptom scale (PHQ12SS) to assess psychological and somatic distress. Data from 12 HV (6 females, 6 males, 20-50 years old) free from gastrointestinal diseases and medications that could alter the gut motility who were also part of a previous study

involving an identical protocol except for omission of the whole gut transit measurement[242] were used to compare with the patient data.

2) Study design:

The HV group protocol was as follows. Subjects attended on the study day following a minimum of 4 hours fasting. They had the baseline MRI scan before being given 1 litre of Moviprep® containing 100 g PEG (mean molecular weight 3350 Daltons), 7.5g Na_2SO_4, 2.7g NaCl, 1g KCl, 4.7g ascorbic acid, 5.9g Na ascorbate which they were required to consume within 60 minutes. Following this, they had hourly MRI scans and completed bowel symptom questionnaires throughout the 5-hour study period. The bowel symptom questionnaire consisted of a 100mm VAS scale on each of the following symptoms: abdominal pain, bloating, abdominal distension, abdominal fullness and nausea. They also filled in a stool diary based on the Bristol Stool Form Scale chart a week before the study day (whilst off laxatives to assess the baseline bowel symptoms), during the study day and 6 days after the study day. Particular note was made of the time to first defecation following ingestion, which often occurred within a few hours.

The constipated patient group followed the same protocol but also had an MRI assessment of whole gut transit time (WGTT), which required them to swallow 5 MRI marker pills (20mm x 7mm) at 8 am, 24 hours before the study day with imaging the next morning. This method of whole gut transit time (WGTT) using the

magnetic resonance imaging correlates well with the standard radio-opaque marker test [73, 213]. The patients were required to fast overnight before the study day. Following their baseline scans to assess transit markers and make baseline volume measurements, they ingested 1 litre of Moviprep® within 60 minutes before undergoing hourly MRI scans for 4 hours. We were able to reduce the study time to 4 hours as our HV results indicated all the important responses could be observed in 4 hours. Patients completed the bowel symptom questionnaire and stool diaries similar to the ones used on the HV group. Both groups were allowed to drink ad libitum after 60 minutes into the study.

3) MRI scanning protocol

All MRI scans were carried out in a 1.5T Philips Achieva scanner (Philips, Best, The Netherlands), using a 16-channel XL torso coil. All participants were scanned in a supine position for approximately 13 minutes while between scans they were sitting in an up-right position in the waiting room. A turbo spin echo single shot sequence (TR/TE = 8000/320 ms, FA = 90^{o}, FOV = 400x362x168 mm^{3}, ACQ res = 1.56x2.90x7.0 mm^{3}) was used to acquire T2 weighted coronal images for measurement of small bowel water content (SBWC) as previously validated [212, 214, 243]. This sequence gives high intensity signals from areas with free fluid and little signal from body tissues. Assessment of WGTT required coronal section images, which, as a single scan would not cover the entire abdomen, were obtained at 2 stations with a 30 mm, overlap using 2 different sequences. A T1 weighted 3D TFE sequence was used to count and

locate the number of pills remaining in the colon after 24 hours of pill ingestion. Secondly, to confirm the location of the pills, a multi echo FFE sequence was used to create a movie using the maximum intensity projection of the water only images as previously used in another study [213]. This movie allowed a 3D visualisation of the colon and the position of the pills in the uncommon event that the T1 weighted image scan was not conclusive. A coronal dual echo fast field echo sequence ($TR/TE_1/TE_2$ = 157/2.3/4.6 ms, FA = 80^o, FOV = 450x362x168 mm^3, ACQ res = 2.01x2.87x7 mm^3) was used to assess colonic volumes[221]. This was performed during an expiration breath hold of 13s and a transverse dual echo FFE sequence under a 20s expiration breath hold. Lastly, motility scans of the ascending colon involved a single sagittal cross sectional slice, using a balanced turbo field echo sequence (TR/TE = 3/1.52 ms, FA = 70^o, FOV = 330x228x15 mm^3, ACQ res = 1.5x1.5x15 mm^3), through the ascending colon which was scanned repetitively every second for 2 minutes during which time the participants were allowed to breathe freely. These images could be played at higher speed as a 'colonic motility movie' and were saved as Windows Media Video files.

1.14.4. Data analysis

All results in patients except WGTT were compared with the previously published healthy controls[242]. WGTT in patients were compared with previous values in healthy volunteers [213] from another study because the MRI marker pills were not optimised for usage at the time the HVs were studied. Sensitivity index (SI) score is

defined as the bloating score divided by the ascending colon volume, units per litre ($^{-}$l). Motility index (MI) was calculated as the duration of each contraction (in seconds) multiplied by the number of sections of the AC (proximal, mid or distal) involved, summed over all contractions in the 2-minute scanning interval. See Figure 58.

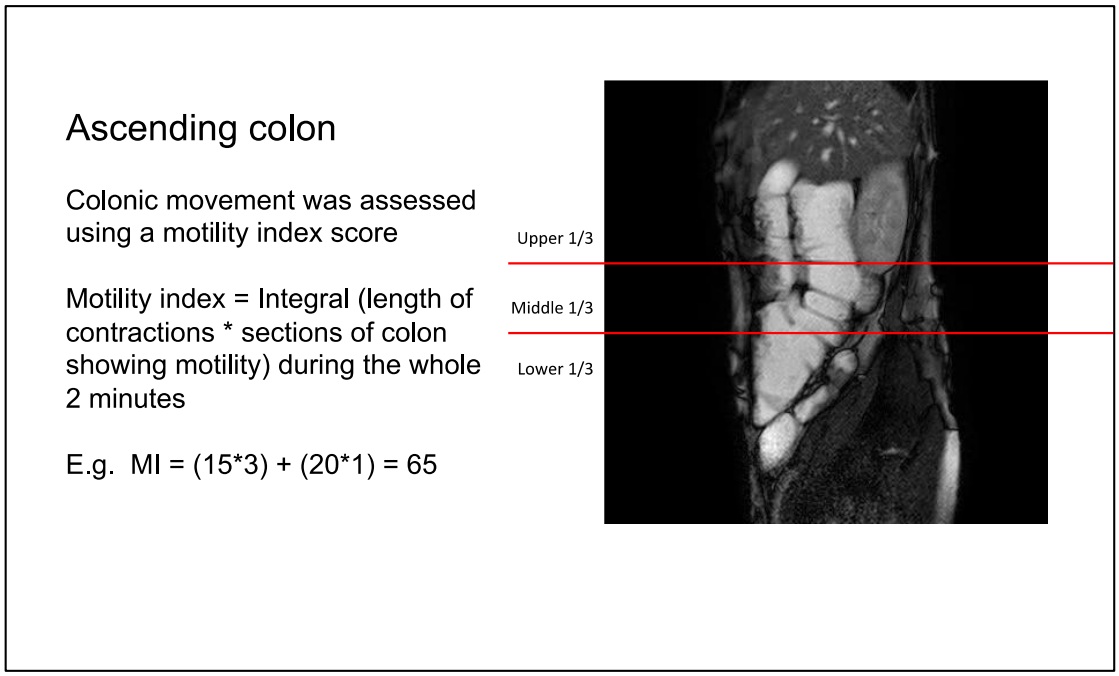


Figure 58: Example showing how MI was calculated

1.14.5. Statistics

All statistical analysis was carried out using the GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla California USA). D'Agostino and Pearson omnibus normality test was used to assess distribution of data. Normal distributed data is expressed as mean ± standard deviation (SD) and non-normal distributed

data is expressed as median (interquartile range; IQR). Normally distributed data was analysed using the unpaired t-test, 1 way ANOVA and 2 way ANOVA as appropriate while non-normally distributed data was analysed using Mann-Whitney test and Kruskal-Wallis test.

1) Power calculation

This used previous data of the effect of another non-absorbable osmotic laxative, mannitol[212]. The mean (SD) change in SBWC at 40 minutes postprandially after ingesting 300ml glucose was 6 (39.5) and using n=12, we calculated we could detect an increase of 55 ml with 90% power which was very much less than predicted from theoretical considerations which suggest a change of >1000ml. The plan was to use 24 per group to ensure the secondary endpoints were met, for which there is no data to perform a power calculation. There was no previous study using MRI to assess small and large bowel motility/function in IBS-C and FC so the power calculation was not done for these parameters.

1.14.6. Results

60 participants were recruited into the study (see Figure 59). 11 HV, 23 FC and 20 IBS-C were included in the intention to treat analysis following a total of 6 withdrawals.

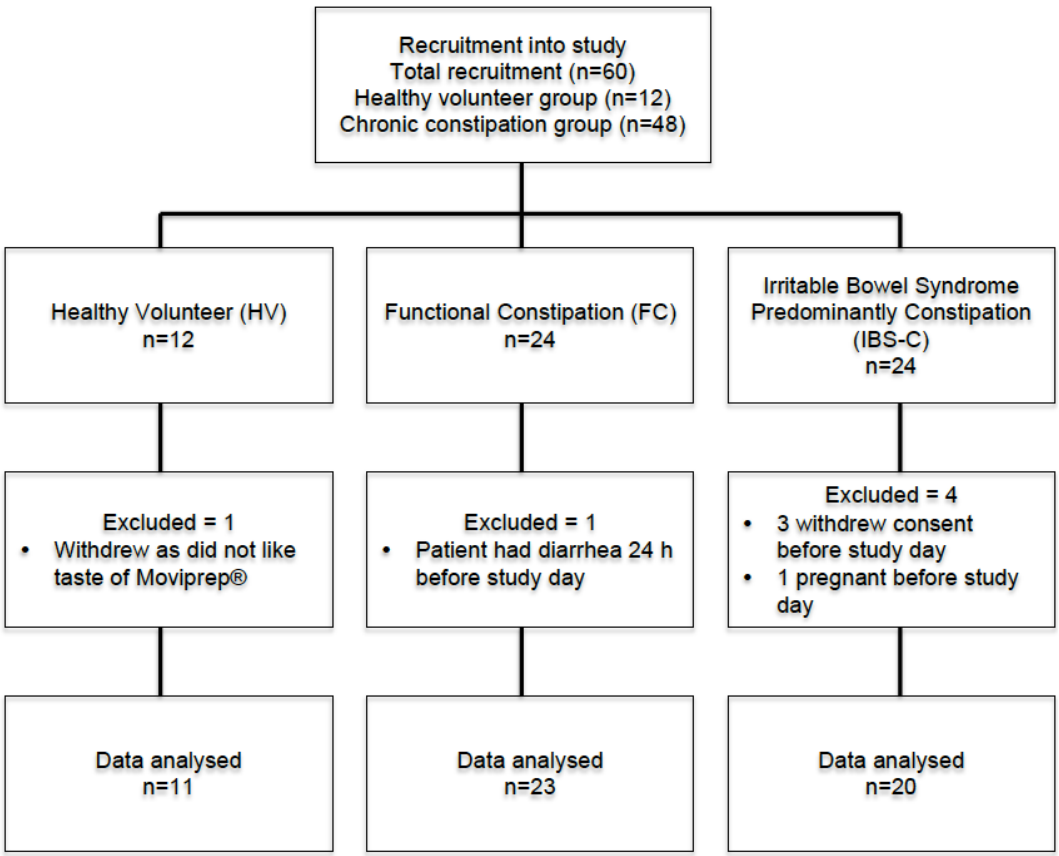


Figure 59: Recruitment flow chart

1) Demographics

Median age for HV was 25 (20-29), significantly younger than the FC = 47 (35-51), $p<0.001$ and IBS-C = 39 (27-52.8), $p<0.02$. The male to female ratio was by design 5:6 for HV, with a female predominance in patients, 2:21 for FC and 0:20 for IBS-C. As expected the FC and IBS-C groups had significantly higher psychological distress as assessed by the total HAD scores compared to HV (1way ANOVA, $p<0.01$) with means of 4.09 (2.63) for HV, 15.23(10.38) for FC and 12.25 (8.18) for IBS-C. The FC and IBS-C groups also had significantly higher somatic symptoms as assessed by the PHQ12SS scores compared to HV (1way ANOVA, $p<0.01$) with means of 2.73 (1.49) for HV, 6.61 (3.86) for FC and 7.05 (4.47) for IBS-C.

2) MRI parameters

a) Transit

WGTT was significantly greater in both the FC and IBS-C groups when compared with HV (Kruskal-Wallis $p<0.01$). The median WGTT was 109.7 (79.4-129.6) h for FC, 63.3 (47.8-100) h for IBS-C and 27.5 (3.7-50.0) h for HV. The WGTT for FC was significantly greater than IBS-C, $p<0.01$. Figure 60.

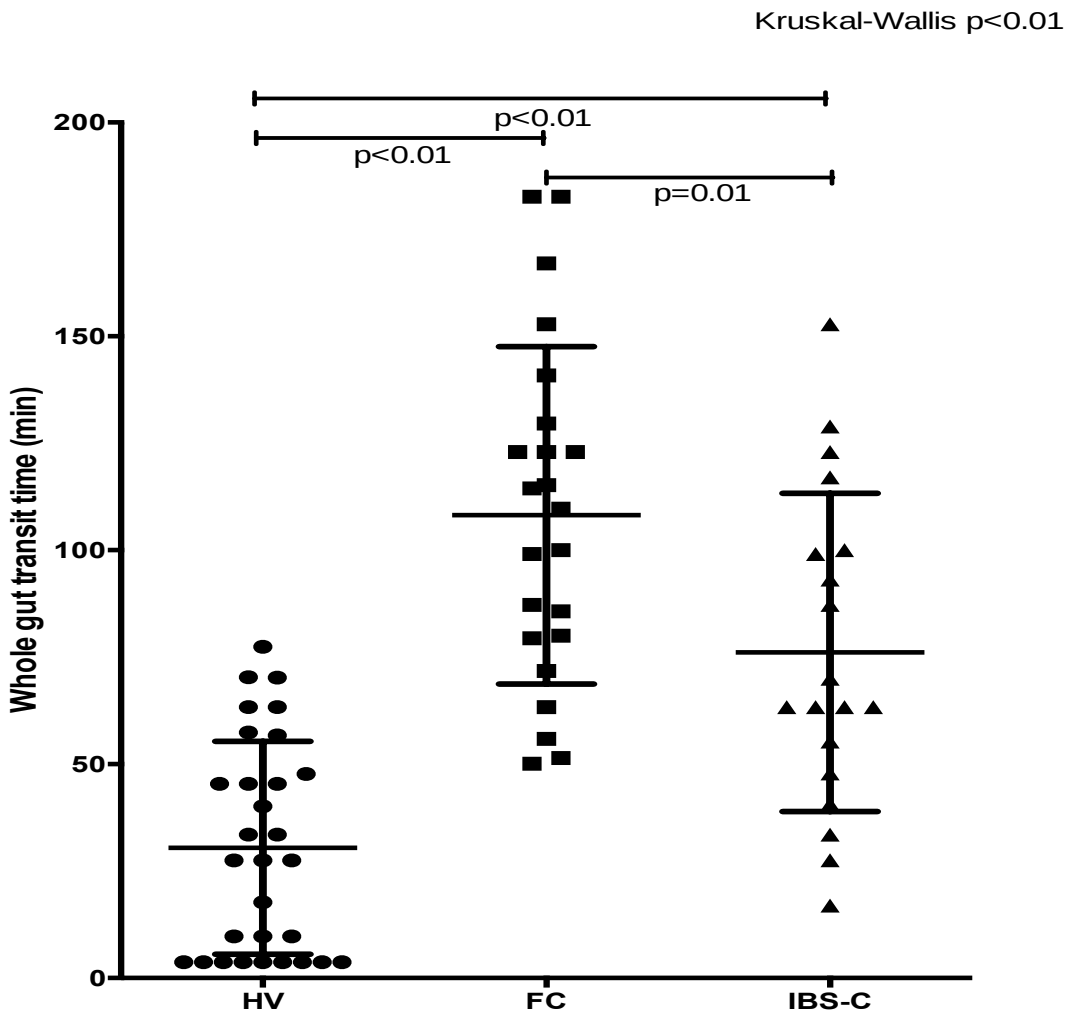


Figure 60: Whole gut transit time (WGTT)

b) Intestinal volumes

Fasting SBWC was significantly higher in FC compared to IBS-C with 82.46 (63.37-141.9) ml and 38.45 (14.61-70.20) ml respectively, p <0.01. Figure 61.

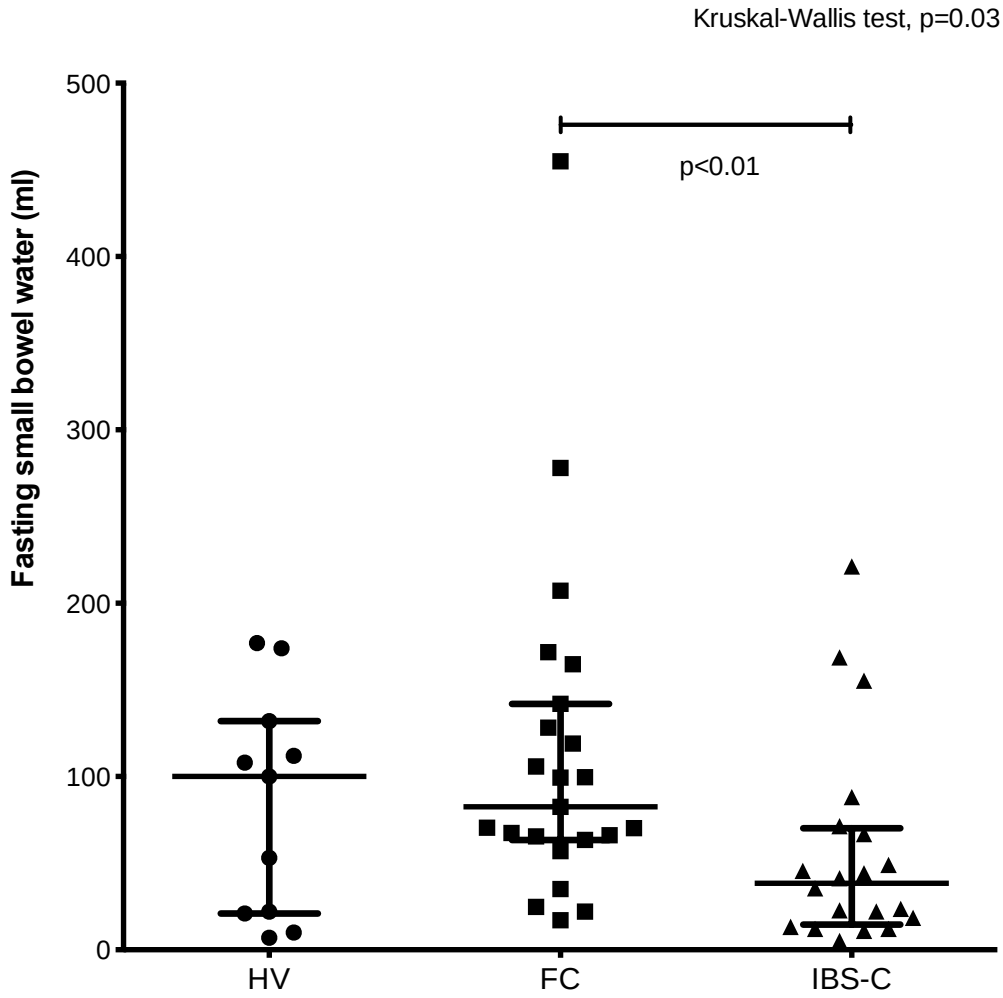


Figure 61: Fasting small bowel water (SBWC)

Baseline ascending colon volume was significantly higher in the FC groups compared to HV and IBS-C (Table 23 and Figure 62). 2 h after ingestion of Moviprep®, the AC volumes significantly increased in the FC group compared to HV and IBS-C (Table 23 and Figure 62).

When the total colonic volume was measured, FC had significantly higher total colonic volume compared to HV and IBS-C (Table 23). As can be seen in Figure 63

the total colonic volume for FC nearly doubled from baseline at 2 hours following ingestion of 1 litre Moviprep®, and remained significantly higher during the subsequent 3 hours when compared to HV and IBS-C (2-way ANOVA p <0.01). There was no significant differences in the total colonic volumes for the IBS-C group at baseline and subsequent total colonic volumes when challenged with 1 litre of Moviprep® when compared with HV.

Table 23: Colon volumes Mean (SD)

	HV	**FC**	**IBS-C**	**P value;** **1 way ANOVA**
Baseline AC volume (ml)	193 (84.1)	314 (100.8) [a,b]	226.2 (70.9)	<0.01
AC volume at 2 h following ingestion of Moviprep®	356.5 (153.3)	596.9 (170.2) [c,d]	389.2 (163.3)	<0.01
Baseline total colonic volume (ml)	589.5 (260.5)	847.2 (279.8) [e,f]	662.2 (239.5)	0.0151

[a] significantly different from HV, p=0.0016; [b] significantly different from IBS-C, p<0.01
[c] significantly different from HV, p=0.0004; [d] significantly different from IBS-C, p<0.01
[e] significantly different from HV, p=0.0152; [f] significantly different from IBS-C, p=0.03

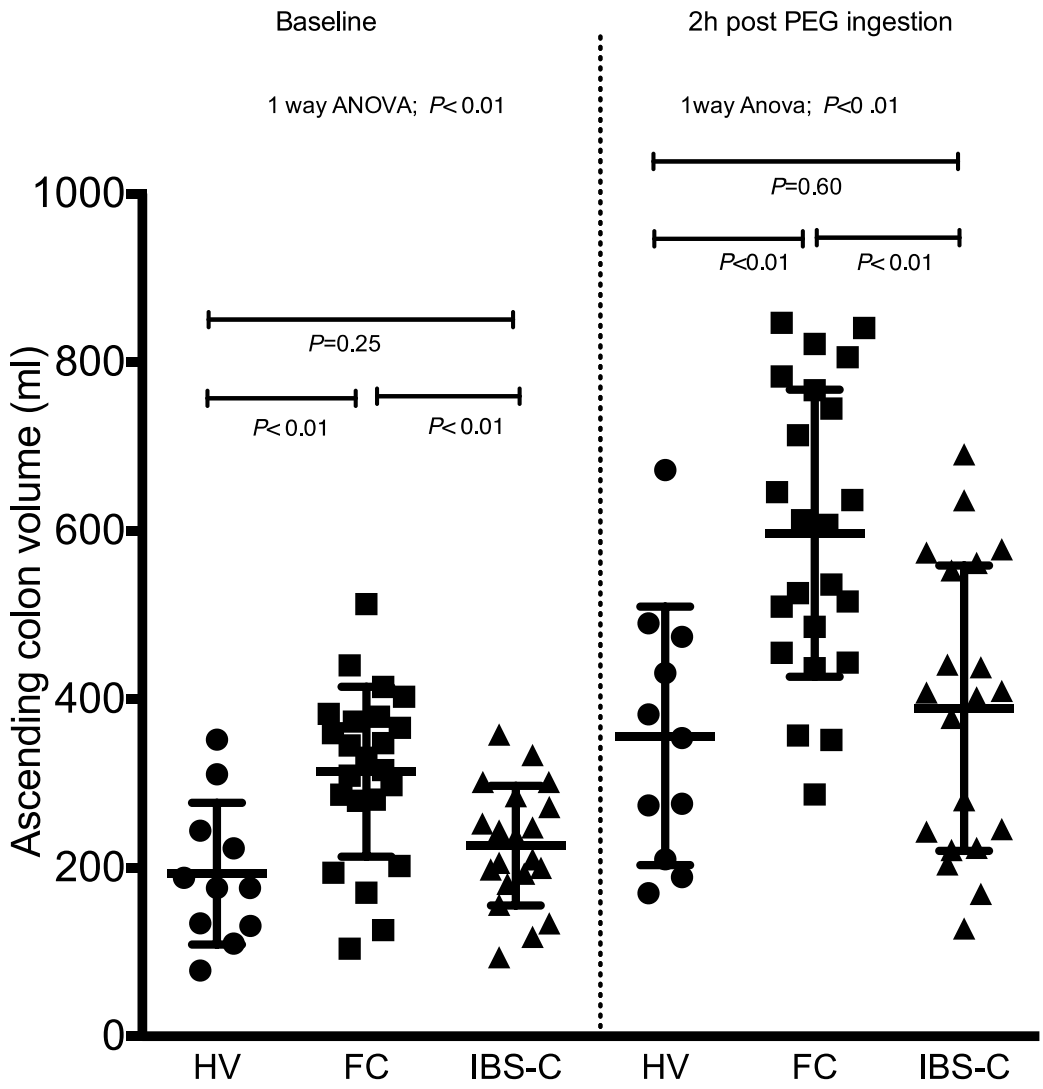


Figure 62: AC volume at baseline and 2h after Moviprep© ingestion

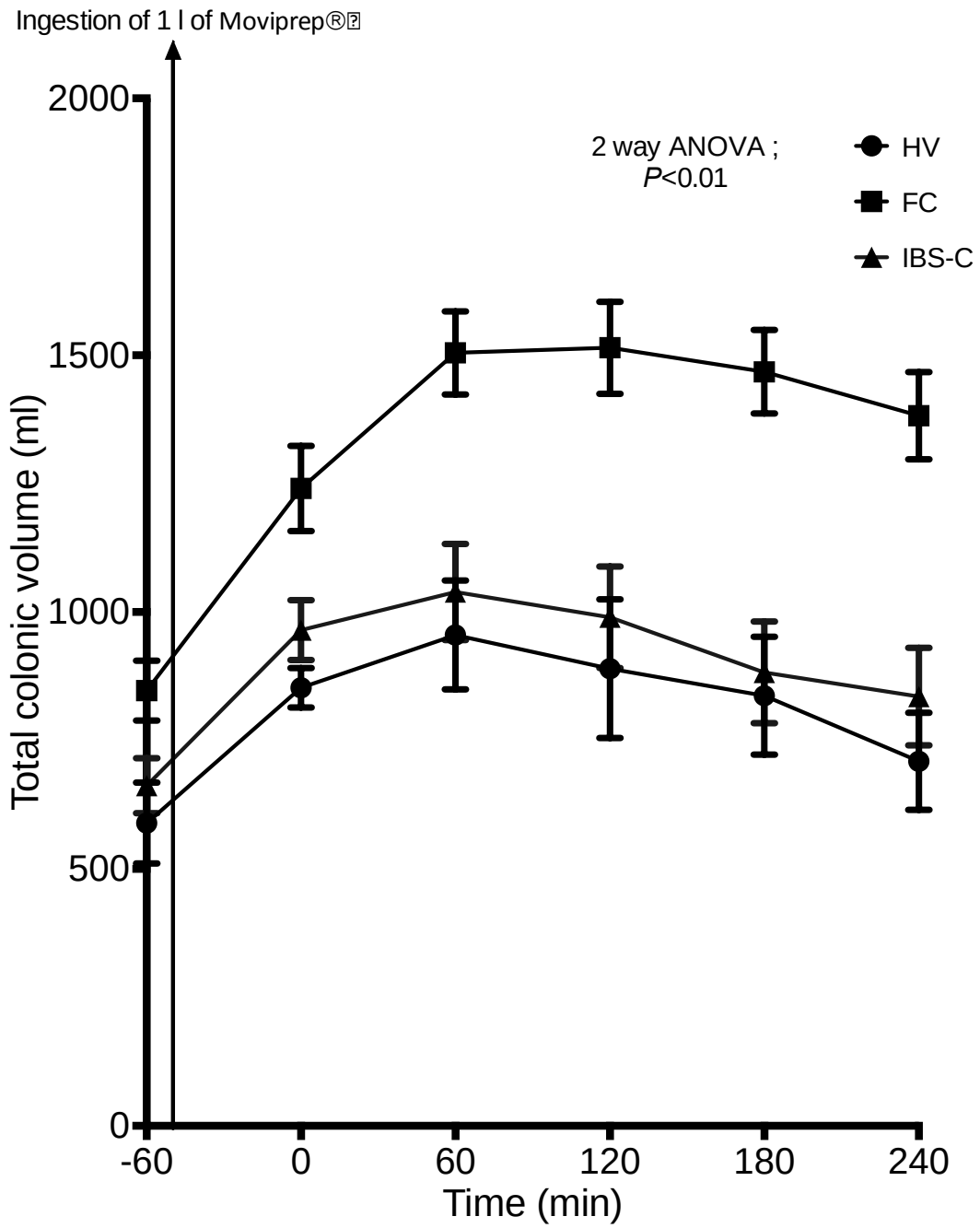


Figure 63: Total colonic volumes during the study day

c) Motility and sensitivity indices

The motility index (MI) of the ascending colon was significantly lower in FC compared to both HV and IBS-C at 2 hours (Table 24 and Figure 64). The SI at 2

hours was significantly higher in both FC and CC group compared to HV (Table 26) but not different between the patient groups (Figure 65).

Table 24: Motility and sensitivity indices at 2h post ingestion of Moviprep©

2 h post ingestion of PEG **[Median (IQR)]**	**HV**	**FC**	**IBS-C**	**Kruskal-Wallis, p value**
MI	82 (48-111)	15.5 (0-49.5)*	58.5 (20.3-84)	<0.01 *P<0.01 compared to IBS-C
SI	4.18 (1.34-8.47)	12.55 (7.15-16.91)	15.0 (5.84-21.42)	0.02

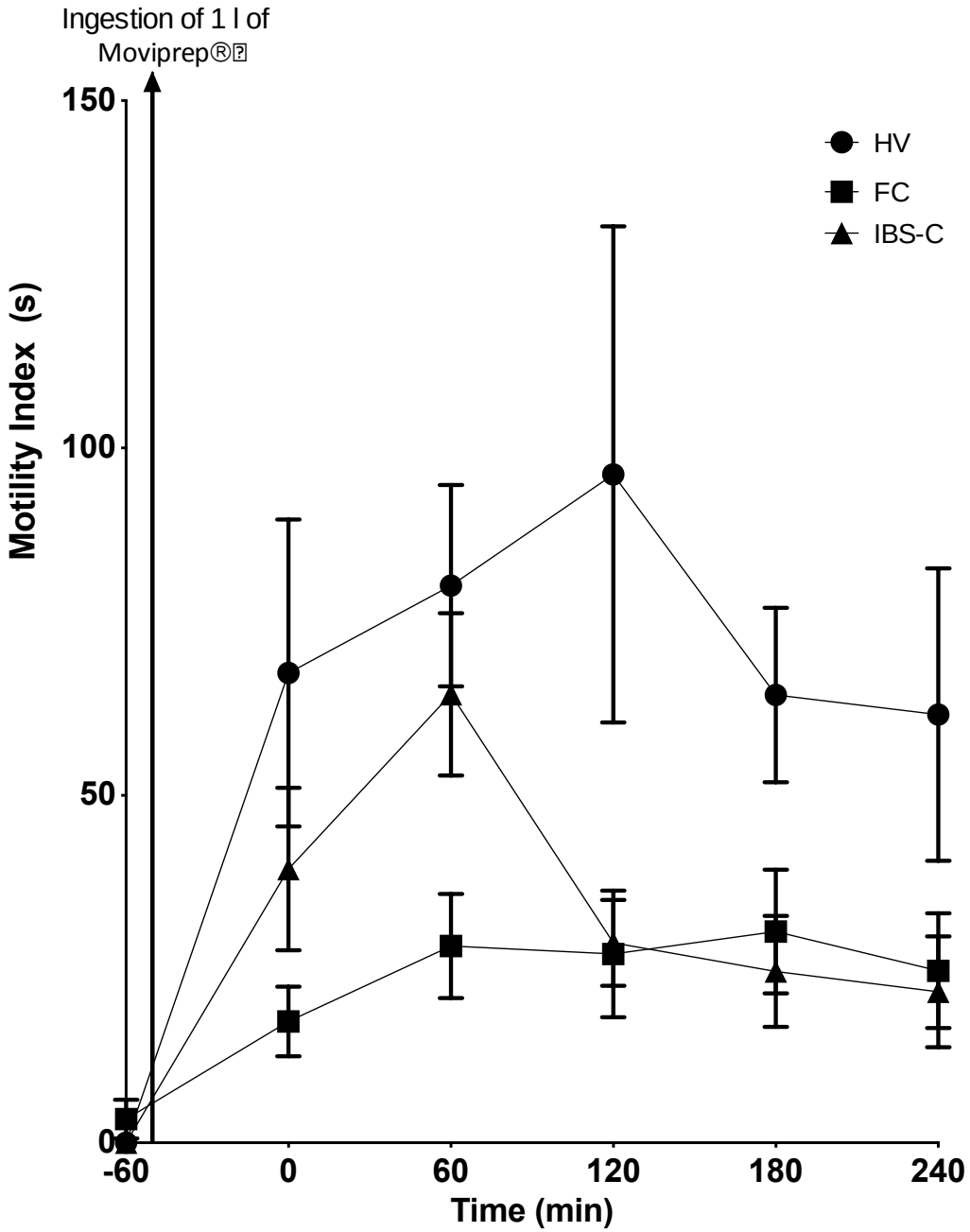


Figure 64: Motility index throughout the study day

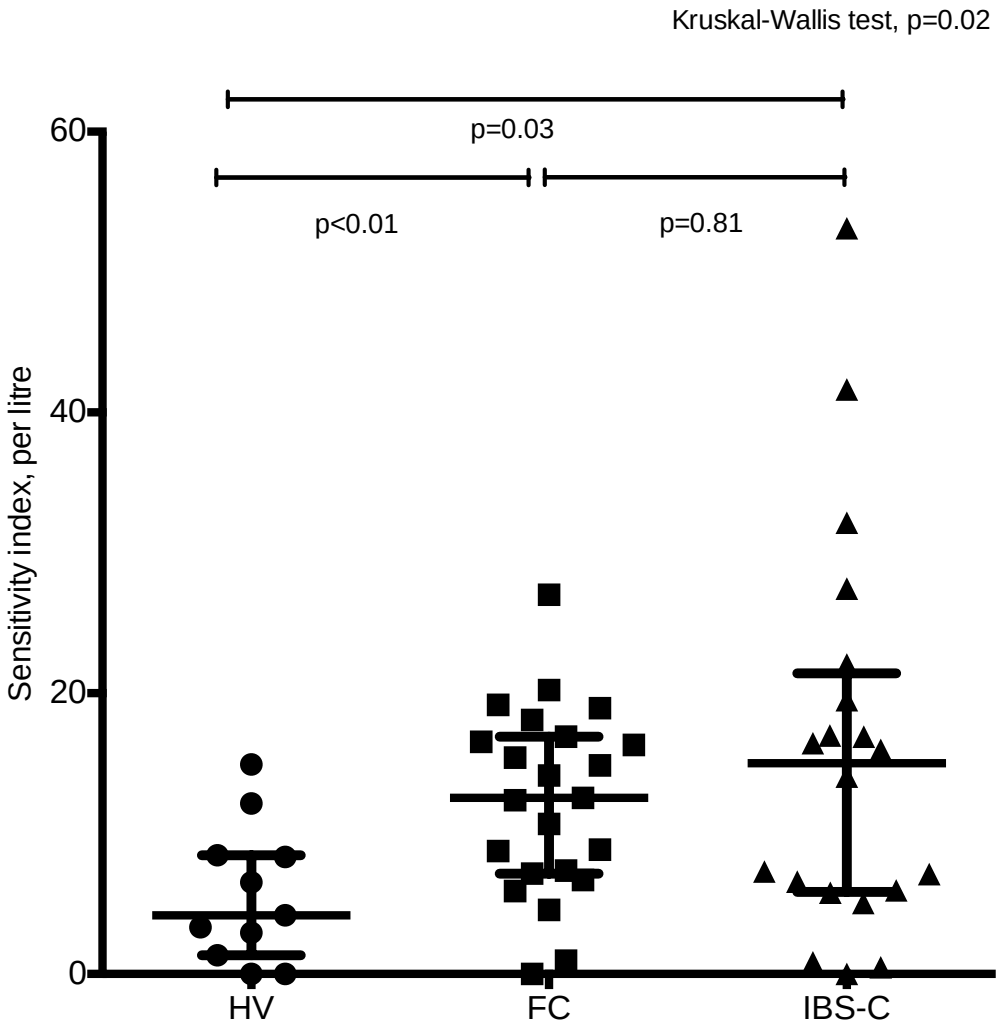


Figure 65: Sensitivity index 2 h after Moviprep ingestion

d) Bowel habit

The FC patients had significantly fewer bowel movements in the 24 hour period following ingestion of Moviprep® with only 3 (2-5) bowel movements compared to HV and IBS-C which was 7 (6-10) bowel movements/24 h in both of these 2 groups, Kruskal-Wallis p<0.01. The time to the first bowel movement following ingestion of Moviprep® was significantly longer in FC group compared with HV and IBS-C,

Kruskal-Wallis p<0.01, being 106 (67.5-175.0) min, 295 (116.3-526.3) min and 84 (48.8-111.3) min in HV, FC and IBS-C respectively (Figure 66).

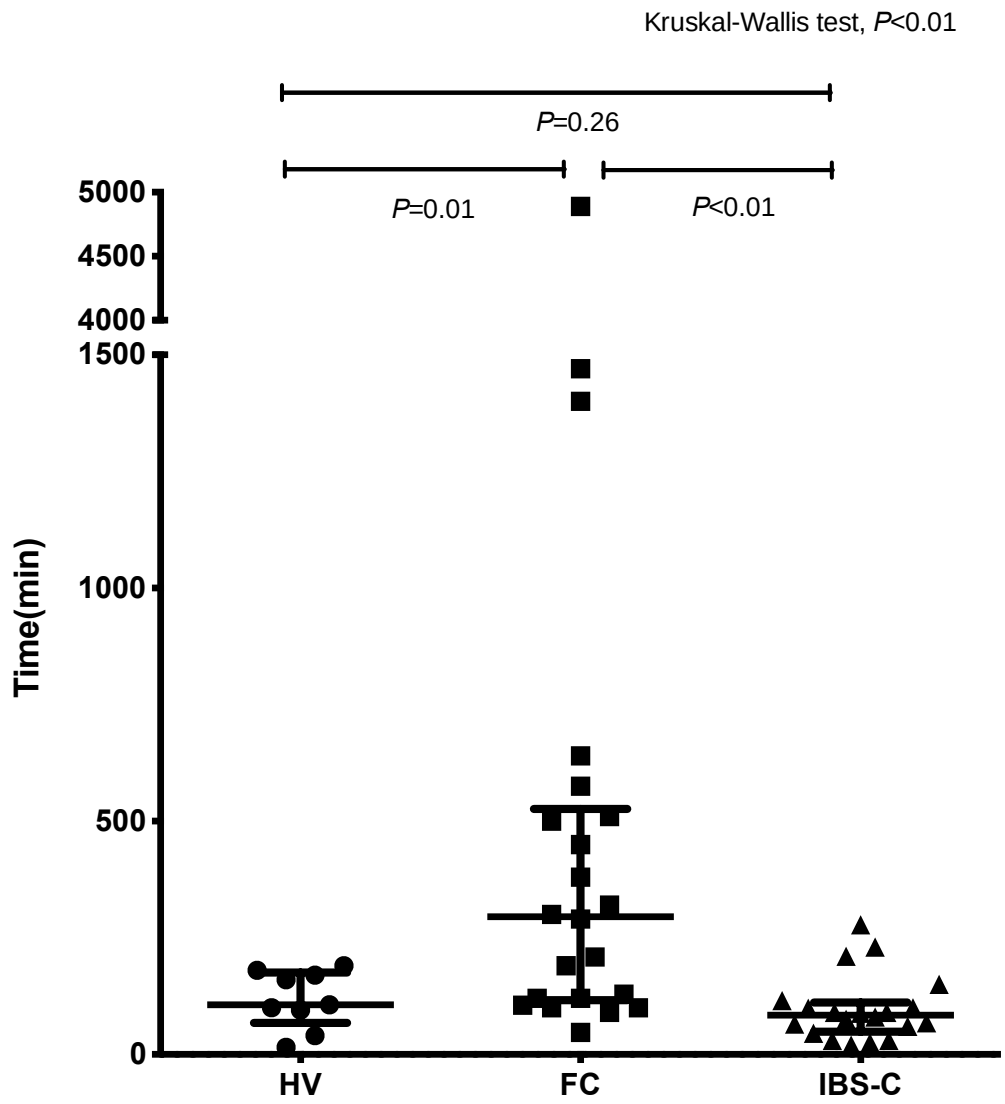


Figure 66: Time to first bowel movement (min)

e) Correlation between time to first bowel movement and MRI parameters

Time to first bowel movement correlated positively with ascending colon volume at 2h post Moviprep®, Spearman r= 0.44, p<0.01 and the fasting SBWC, Spearman r=0.34, p=0.04. If a cut off time at 230min was used in the time to first bowel movement, the sensitivity of this test to distinguish IBS-C from FC was 55% and specificity of 95%. SI correlated weakly with total HAD scores, Spearman r=0.23, p=0.09 (Figure 67).

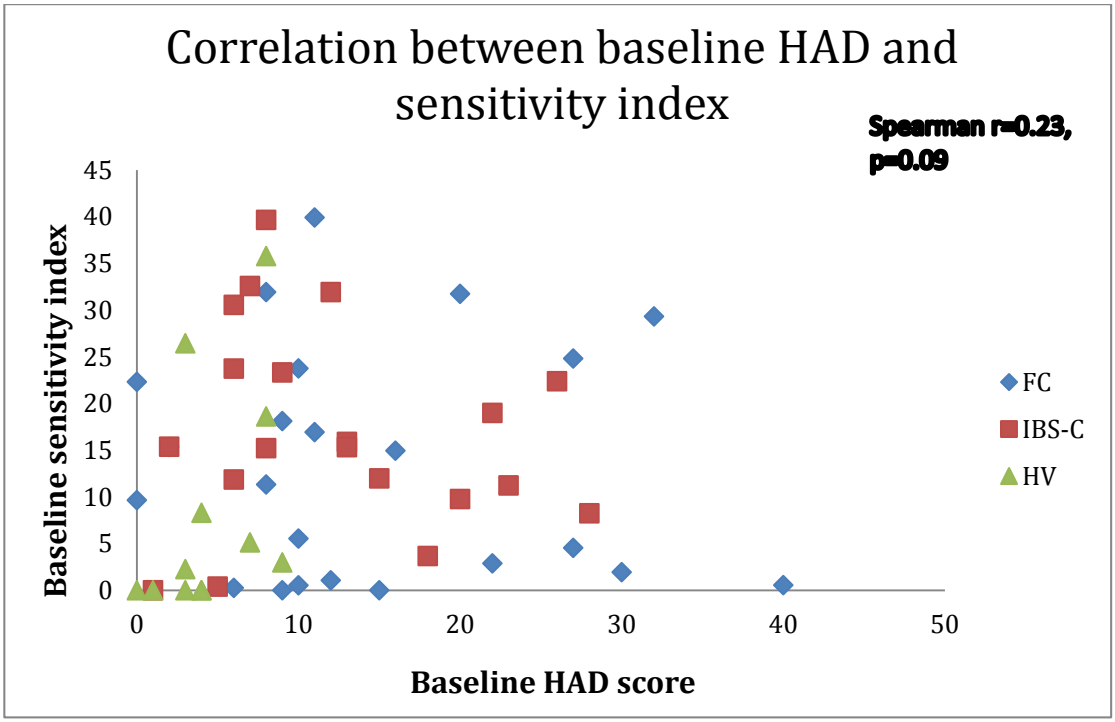


Figure 67: Correlation between baseline total hospital anxiety and depression (HAD) score and sensitivity index (SI)

1.14.7. Discussion

This is the first report of a non-invasive assessment of colonic function using MRI in constipated patients and as such provides much new data. Very early studies using X-rays had provided details of colonic motility and response to eating but only in an anecdotal way [244] and the realisation of the dangers of irradiation brought such studies to a rapid end. Subsequent studies have used transit of radio-opaque markers [73] and clearance of isotope from the colon [193] as surrogate markers of motility which do correlate reasonably well with symptoms[223]. Constipation is associated with slow transit[245] and diarrhoea with fast whole gut transit[77] and accelerated clearance of the ascending colon[215] but in each case the overlap with normal is substantial as is the day to day variability [76] at around 25%. This is undoubtedly because transit depends on many uncontrolled factors such as diet, microbiota, psychological factors as well as motility. Direct measurement of colonic motility has been possible but requires bowel preparation and is arduous for both patient and investigator, since given motility's substantial diurnal variation it requires very prolonged (up to 24 hours) recording to characterise[239]. This has limited its use despite the exquisite detail it provides[246]. The technique in this study is by contrast extremely easy to administer and very patient acceptable. There was no difficulty in getting volunteer patients who were keen to see how their bowel functioned.

By providing a large distending stimulus, this study demonstrated distinct motor responses, which are impaired in FC throughout the 4 hours of the study. IBS-C patients in contrast show a normal initial response but in the later half of the study this appears to tail off significantly while it is maintained in HV. Whether these

responses are useful clinically to predict response to therapy requires further study but anecdotally this has been used in the clinic in Nottingham and the results do separate out IBS-C from FC quite well and do guide therapy. The hypomotile colon in FC seems to respond well to prokinetics such as Prucalopride while the active IBS-C patient tends to get pain and diarrhoea without benefit.

The analysis of motility used in this study, though blind as to the subject, is very subjective and time consuming. The SPMMRC is working on automation to make this less subjective and to make it feasible to analyse longer time periods though the strength of the stimulus does mean that less time is required when waiting for spontaneous contractions.

All the images are analysed by an operator blind as to the patient details to avoid bias but our reproducibility studies are reassuring. The inter-observer variability for colonic volumes is 5%. For colonic transit, the inter-observer variability is good with intra-class correlation coefficient = 0.78; while the day to day repeatability of transit is acceptable with intra-class correlation coefficient of 0.61[213] .

In this study, visceral hypersensitivity was measured, non-invasively, by looking at the symptomatic response to the Moviprep® stimulus, which distends and causes vigorous contractions in both healthy volunteers and IBS-C subjects. Unfortunately there was substantial overlap possibly because the FC group starts from a much larger initial volume, which may make the arrival of large volumes of fluid more painful than in IBS patients who started with a relatively normal ascending colon.

While expense will limit the use of this test to specialist centres, its use in very severe cases in whom colectomy or sacral nerve stimulator implantation is contemplated, could be easily justified if it prevented an IBS-C patient from undergoing unnecessary and ineffective treatments.

Even for those without access to MRI for such patients, the Moviprep® challenge could be used without imaging since defecation within 230 minutes identifies 95% of IBS while only being found in 45% of FC. This is very useful since it should prevent the use of strong stimulant laxatives and suggest that an agent with both laxative and pain reliving properties such as Linaclotide might be the preferred treatment[247,248].

In summary this is a patient acceptable, technically undemanding colonic function test, which defines the differing underlying pathophysiology of FC and IBS-C, two common causes of constipation that require rather different treatments.

1.15. Imaging the gastrointestinal tract in IBS

1.15.1. Introduction

At present, there are no biomarkers using the MRI as a tool to objectively measure and evaluate symptoms of irritable bowel syndrome. MRI is still perceived as an expensive tool for this although it is patient acceptable and a non-invasive method to assess undisturbed bowel.

At the University of Nottingham, we have collaborated with the research MRI centre, Sir Peter Mansfield Magnetic Resonance Centre (SPMMRC) and took advantage of its availability/ speciality to evaluate MRI parameters in IBS patients. The SPMMRC had validated the quantification of small bowel water content using MRI[214] and this had been used to measure fasting small bowel water content (SBWC) on healthy controls and IBS-D patients[212]. This study by Marciani et al showed that fasting SBWC was lower than healthy controls which was confirmed by previous studies reporting faster orocaecal transit time (OCTT) and increased motility[249-251]. The migrating motor complex is more frequent in IBS-D and this has probably led to increased delivery of water into the ascending colon[251]. We also showed in IBS-D patients, a good correlation between anxiety and small bowel transit time which was assessed on the magnetic resonance (MR) images by the arrival of the bright fluid from the small bowel into the ascending colon.

Other new parameters to image the small and large bowel are slowly being developed here at the University of Nottingham. For example, T1 and T2 imaging

sequences have been used as part of a research tool to look into the chyme of the ascending colon. In a recent study in Nottingham, T1 sequence in the ascending colon in the untreated cohort of IBS-D patients was significantly higher than normal subjects (unpublished). The reason behind this difference may be the effect on the change in the colonic microbiota but it is still in the exploratory phase. In this section, further MRI imaging parameters on IBS-D will be explored and discussed.

1.15.2. Small bowel tone by measurement of fasting small bowel water content and other parameters through MRI

(Result from the MIBS study: Chapter 2)

1.15.2.1. Aim of the study:

1) To assess the effect of Mesalazine on the small bowel tone by measurement of fasting small bowel water content
2) To assess the ability of MRI parameters (T1/T2 sequence) to predict treatment response

1.15.2.2. Method:

Participants with IBS-D who met the modified Rome III criteria were recruited into the Mesalazine for irritable bowel syndrome with diarrhoea (MIBS) trial. This was a double blind randomised placebo controlled trial. See chapter 2. For participants in Nottingham, following consent, they had additional tests including the magnetic resonance imaging (MRI) of the abdomen at the beginning and end of the study visit. The MRI scan was performed in the 1.5T Sir Peter Mansfield Magnetic Resonance Centre at the University of Nottingham. Participants fasted overnight prior to MRI scans for both visits. To ensure safety, they filled in a MRI safety questionnaire prior to each visit.

1) Subjects:

40 patients (16 males, 24 females) aged between 19-65 years consented to have MRI scans of the abdomen during the trial.

2) MRI scanning protocol:

All MRI scans were carried out in a 1.5T Philips Achieva scanner (Philips, Best, The Netherlands), using a 16-channel XL torso coil. All patients were scanned in a supine position for approximately 13 minutes in total. An initial survey scan was to locate the position of the abdominal organs before a range of MRI scans were taken. This scan consists of a dual gradient echo imaging sequence (dual-echo fast field echo, FFE) with TE1 = 2.3 ms, TE2 = 4.6 ms and TR = 158 ms. This comprised 24 coronal

plane and 45 transverse images with in-plane resolution 1.76 mm × 1.76 mm and a slice thickness of 7 mm, with no gap between slices[243] (Figure 68).

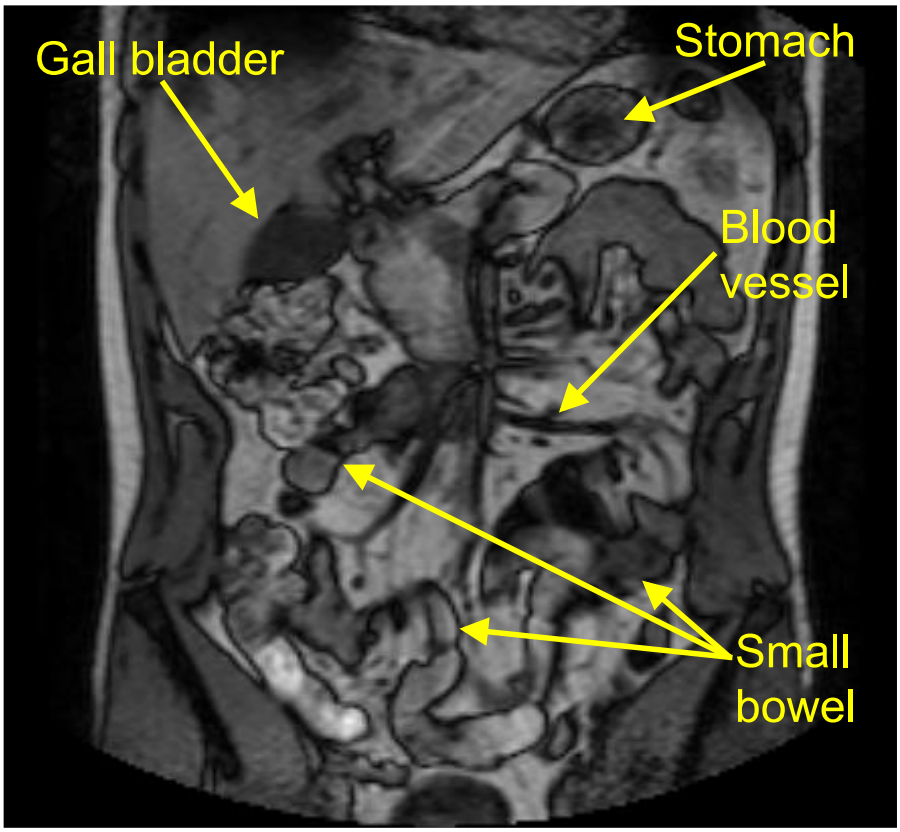


Figure 68: Initial survey scan to look at anatomy of abdominal organs using a dual gradient echo imaging sequence

A turbo spin echo single shot sequence (TR/TE = 8000/320 ms, FA = 90^{o}, FOV = 400x362x168 mm^{3}, ACQ res = 1.56x2.90x7.0 mm^{3}) was used to acquire T2 weighted coronal images for measurement of small bowel water content (SBWC) as previously validated [212, 214, 243] (Figure 69).

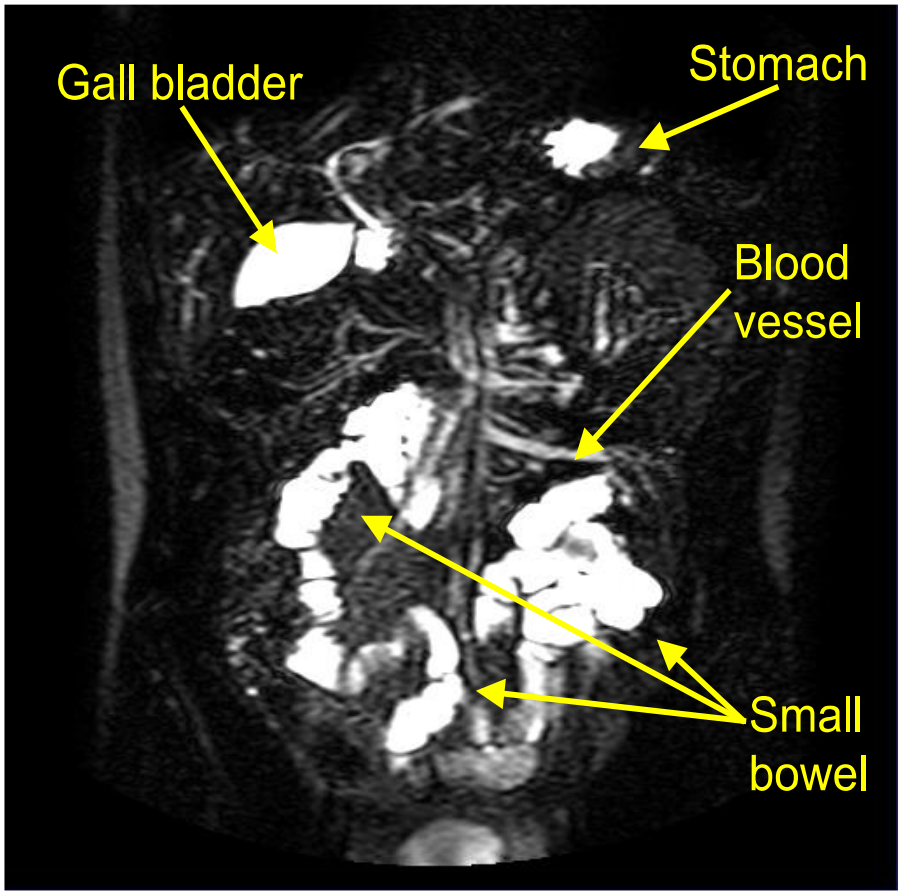


Figure 69: Single shot fast spin echo to analyse small bowel water content

The MRI sequences such as T1, reflecting the spin lattice relaxation time, and T2, reflecting the spin-spin relaxation times, were used in this protocol. The methodology for TI sequence was similar to a recently published study by Marciani et al [242]. The longitudinal relaxation time T1 of the ascending colon chyme was measured from a single, sagittal slice through the ascending colon using an Inversion Recovery Balanced Turbo Field Echo sequence with the following parameters: 1 sagittal slice, TR/TE= 3.0/1.5 ms, field of view = 400 x 400 mm, matrix size 256x256, slice thickness of 10mm and 8 different inversion times (TI) ranging

from 100-5000 ms. Each image was acquired during a breath-hold with 15 s of free breathing between each different TI to allow for full relaxation of the MRI signal.

As for T2 MRI sequence, the MRI protocol was similar to a previous study by the MRI research group at the University of Nottingham[252]. The relaxation time for T2 of the ascending colon was measured from a single sagittal slice through the ascending colon based on a T2-prepared bTFE sequence (TR/TE = 3.0/1.5 ms, TEprep values (ms): 20, 29, 43, 63, 93, 137, 201, 295, 434, 637, resolution 1.56 x 1.56 mm and a slice thickness of 7 mm)[252, 253]. Each image was acquired during a breath-hold with intermittent free breathing between each different T2.

3) Data Analysis

All data analysis was performed by a single person (CL) and the analyser was blinded to the study treatment.

a) SBWC

Fasting SBWC was analysed using in-house semi-automatic extraction and quantification software (Figure 70). This method was validated in the past using mannitol infusion into the small bowel via the nasoduodenal tube[214].

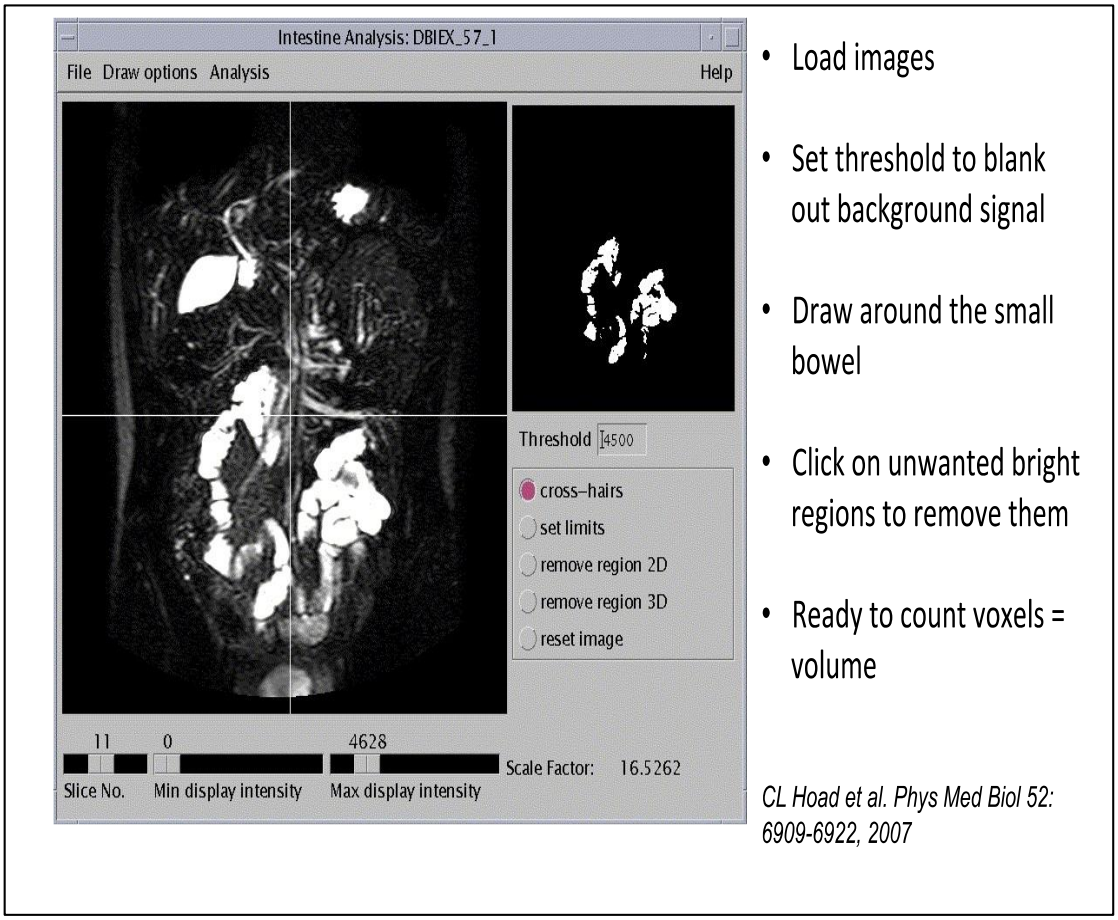


Figure 70: Method for quantification of small bowel water content (ml)

b) T1 and T2

T1 and T2 were calculated using an in house software program developed by the SPMMRC, University of Nottingham[253]. 3 regions of interest (top, middle and bottom of the ascending colon) were drawn to obtain either T1 or T2 values for each region. A mean of these regions were used to represent an overall T1/T2 relaxation time for the whole ascending colon. See figures 71 and 72 as an example of T1 and T2 quantification of the ascending colon.

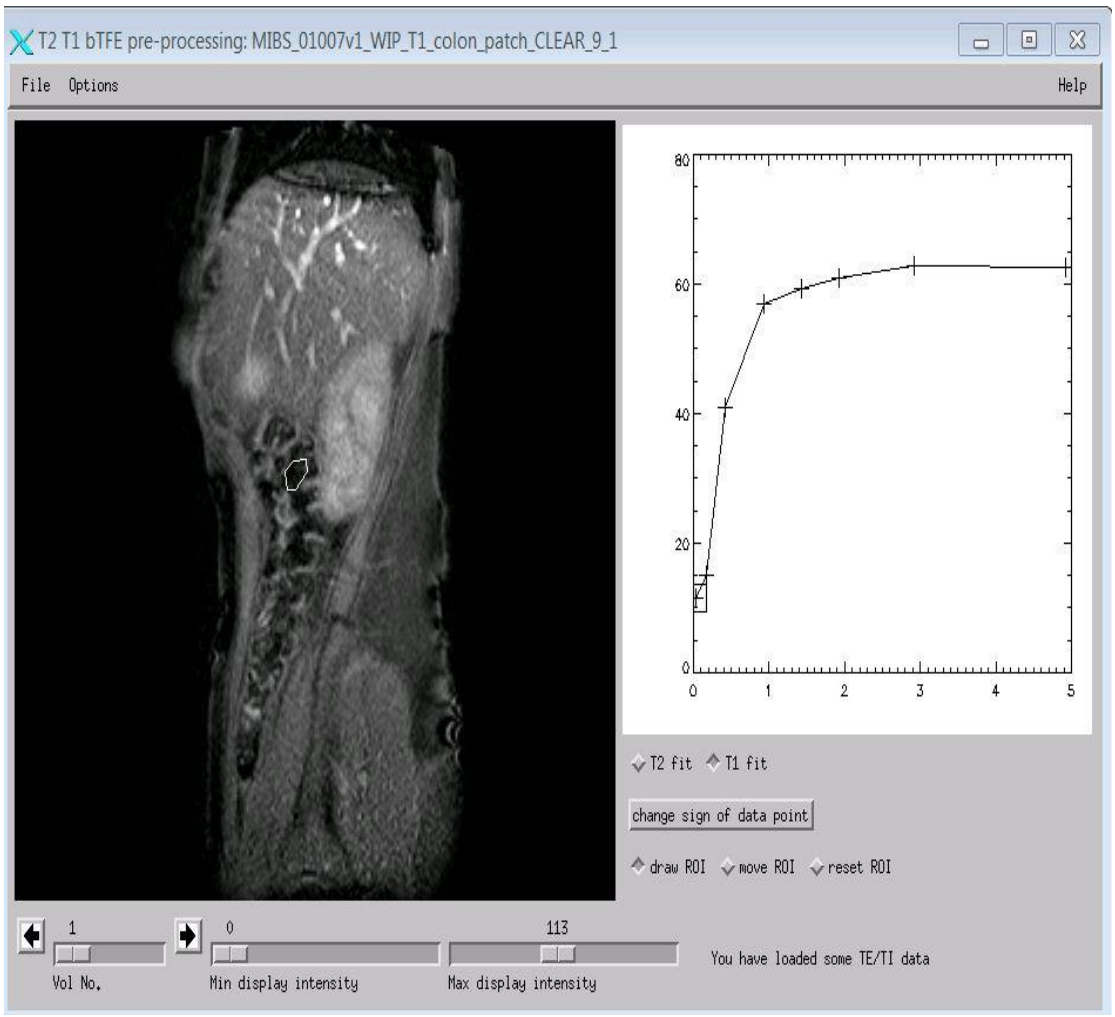


Figure 71: T1 analysis showing a region of interesting, top of the ascending colon. The exponential curve on the right showing the 'recovery period'.

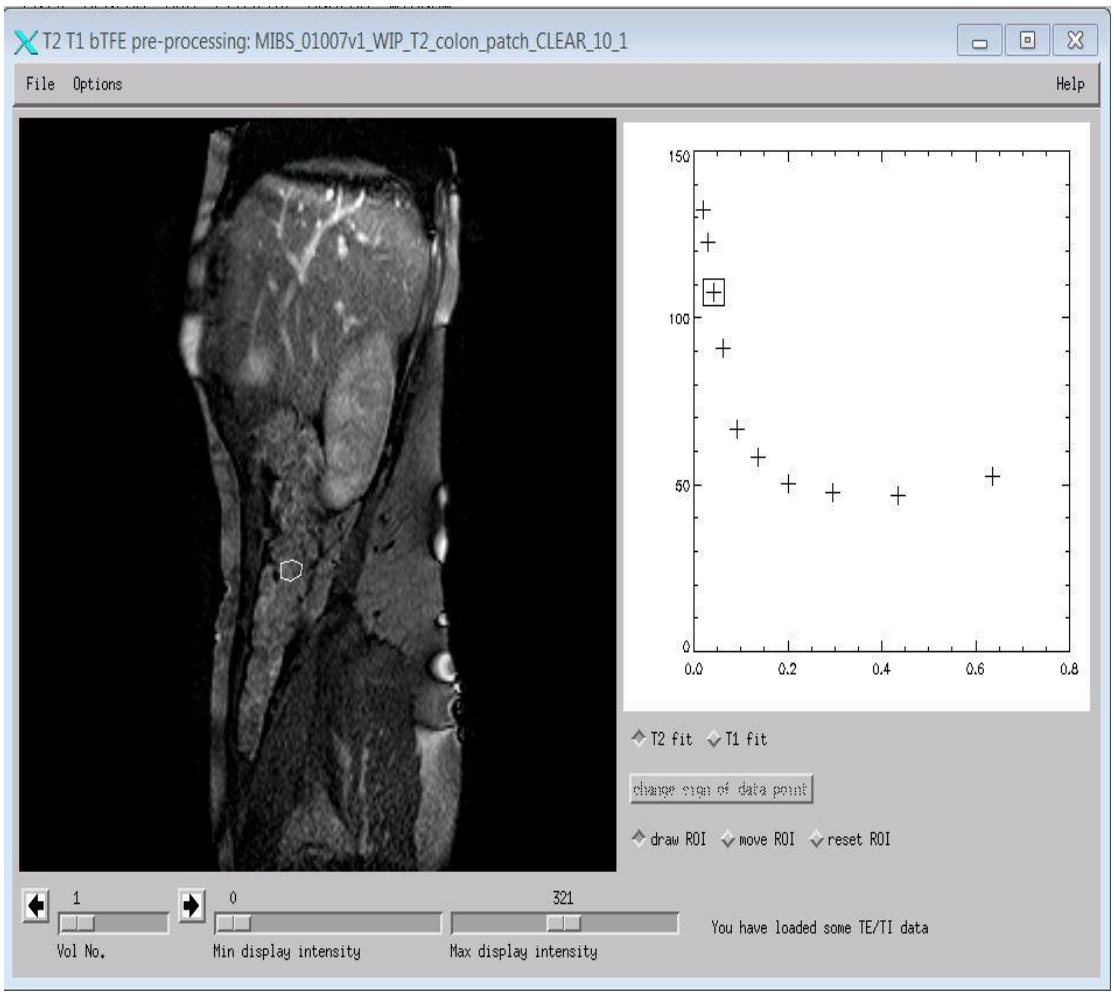


Figure 72: T2 analysis showing the decay curve in 1 region of interest, middle section of the ascending colon

1.15.2.3. Statistical analysis

1) Statistics

All statistical analyses were carried out using the GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla California USA). D'Agostino and Pearson omnibus normality test was used to assess distribution of data. Normal distributed data is expressed as mean ± standard deviation (SD) and non-normal distributed

data is expressed as median (IQR). Normally distributed data was analysed using the paired t-test, 1 way ANOVA and 2 way ANOVA while non-normally distributed data was analysed using Mann-Whitney/Wilcoxon test (for paired values) and Kruskal-Wallis test.

2) Power calculation

Power calculation was based on the primary end point of stool frequency (See chapter 2). There was no study using MRI parameters to assess the gastrointestinal tract following treatment of Mesalazine so we were not able to perform a power calculation for this.

1.15.2.4. Results

1) Baseline characteristics

20 participants were equally allocated to each arm. Baseline characteristics between the two treatment groups were similar. See table 25.

Table 25: Baseline characteristics between Mesalazine and placebo group

Median (IQR)	Mesalazine (n=20)	Placebo (n=20)	P value
Age	40.9 (16)	42.7 (12.5)	
Female (%)	14 (70%)	10 (50%)	
Anxiety score	9 (5-12)	8 (5-10)	0.45
Depression score	4 (2-9)	4 (2-7)	0.52
Total HAD score	13 (8-19)	12 (7-17)	0.60
PHQ12SS	6 (2-11)	6 (4-8)	0.97

2) Fasting SBWC

Baseline fasting SBWC average (SD) was 73 (56) ml for all 40 IBS-D patients. There was no significant change in fasting SBWC following treatment with Mesalazine, mean difference (SD) of -0.42 (0.67) ml compared with placebo, mean difference (SD) of -5.1 (53.8) ml, p=0.41 (Figure 73). There was no correlation between baselines fasting SBWC with other clinical parameters (Table 26).

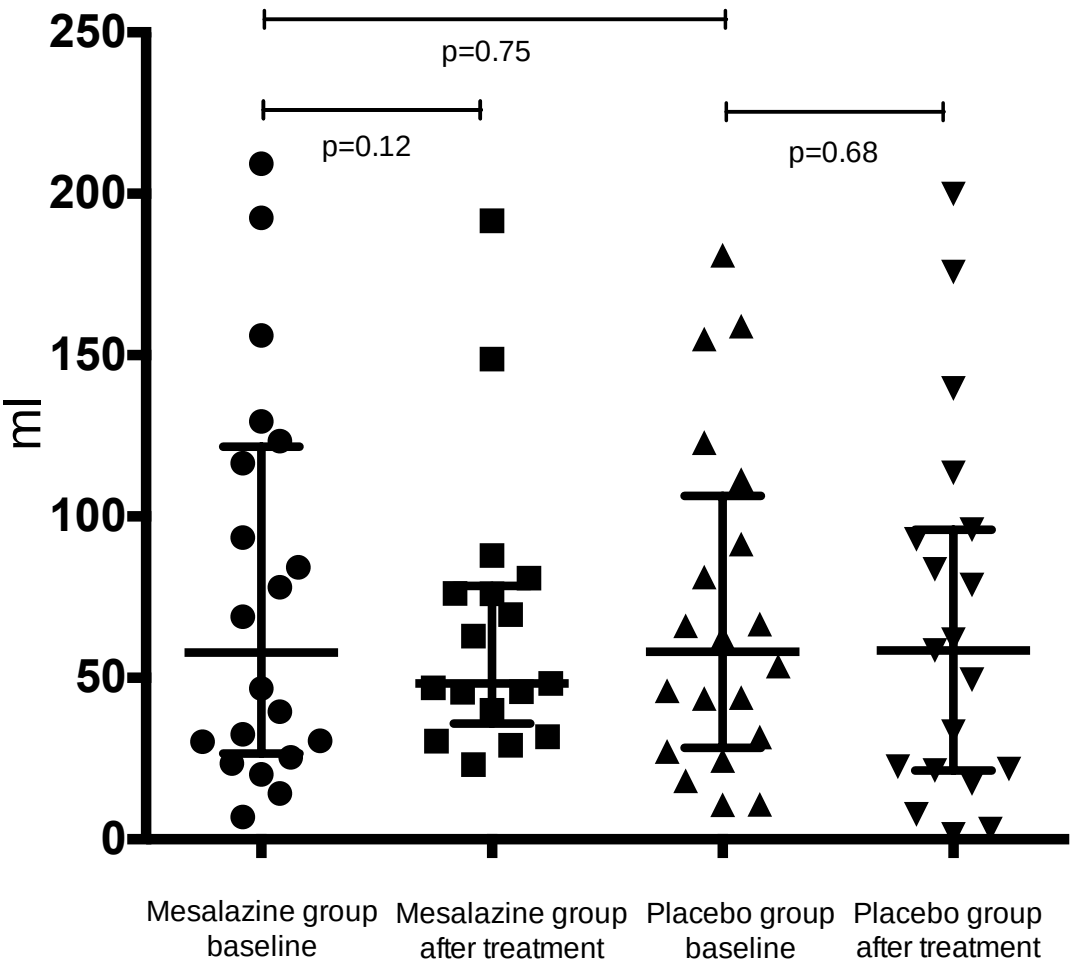


Figure 73: Fasting SBWC following treatments with Mesalazine and placebo

Table 26: Correlation between fasting SBWC with clinical parameters

Fasting SBWC	Correlation Spearman, r	P value
Abdominal pain severity (0-10)	0.14	0.38
Urgency (0-10)	0.16	0.34
Bloating (0-10)	-0.02	0.90
Average stool frequency	0.15	0.37
Average stool consistency	-0.09	0.59
Total HAD score	0.17	0.28
Anxiety score	0.16 (Pearson, r)	0.31
Depression score	0.12	0.45
PHQ12SS score	0.17	0.30

3) T1 relaxation

Following treatment with Mesalazine, T1 relaxation time was reduced significantly when compared with placebo (2way ANOVA, p=0.02), Figure 74, Table 27.

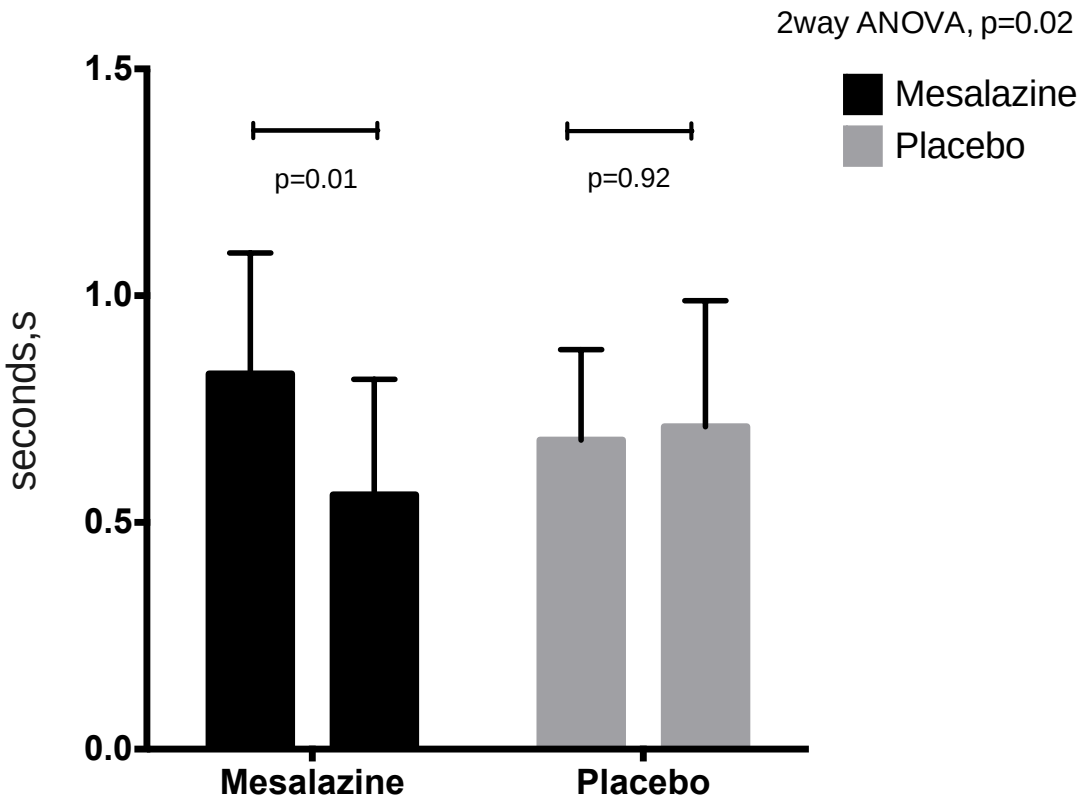


Figure 74: T1 relaxation time (s) significantly reduced following treatment with Mesalazine

Table 27: Comparison showing significant difference in T1 relaxation time following treatments with Mesalazine and placebo.

Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Adjusted P Value
Before treatment - After treatment			
Mesalazine	0.2663	0.05208 to 0.4805	0.0132
Placebo	-0.02914	-0.2222 to 0.1639	0.9236

Mesalazine group only:

There was no significant correlation between the baseline T1 relaxation time and baseline clinical parameters such as abdominal pain severity, urgency, bloating, average bowel frequency and stool consistency.

Mean differences in T1 relaxation time did not correlate with either mean difference in bowel frequency or stool consistency following treatment with Mesalazine (Table 28).

Table 28: Mean differences for T1 relaxation time, bowel frequency and stool consistency.

Correlation between T1 changes (s) following treatment with Mesalazine	Pearson, r	P value
Change in average daily bowel frequency (After treatment – baseline)	0.11	0.71
Average stool consistency (After treatment – baseline)	0.29	0.33

4) T2 relaxation time

T2 relaxation time did not significantly change following treatment with either Mesalazine or placebo, Figure 75. There was no significant correlation between T2 relaxation time and bowel frequency and stool consistency (Table 29).

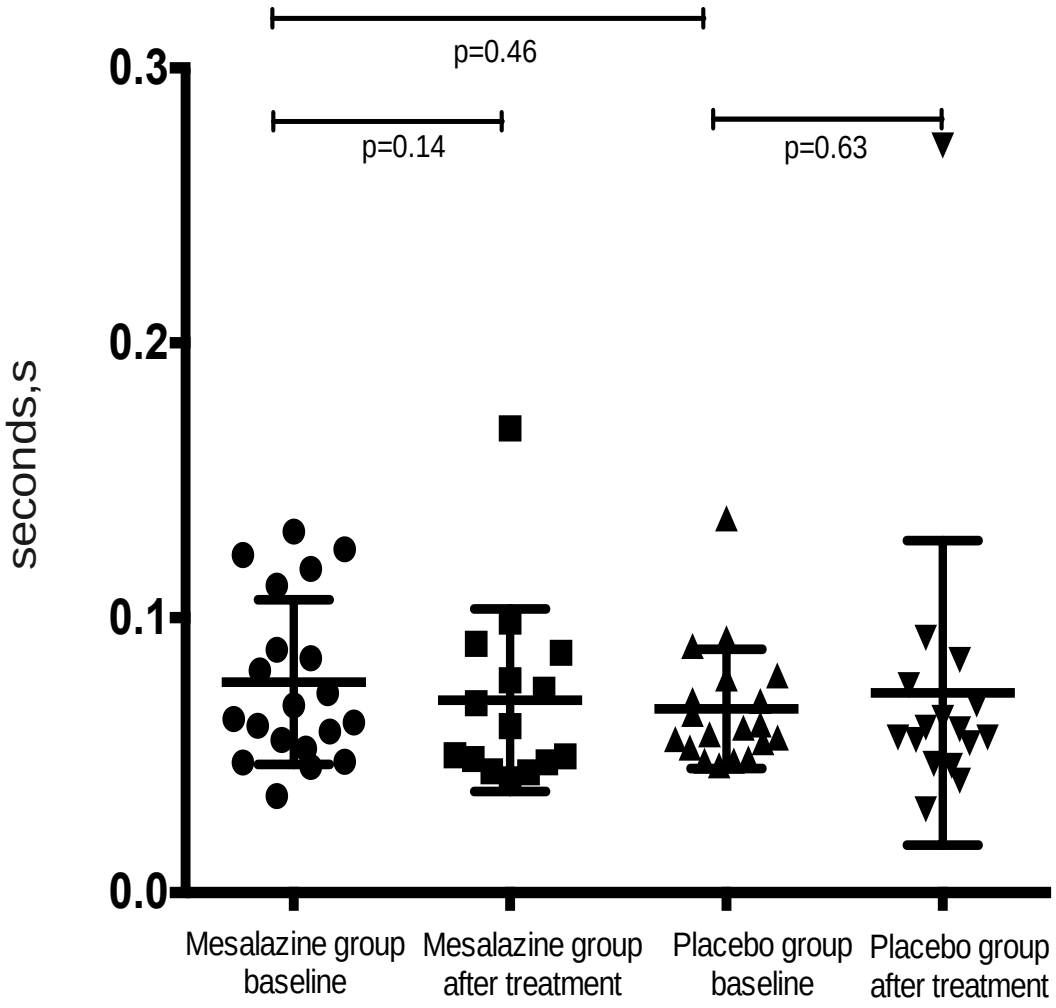


Figure 75: T2 relaxation time following treatments with Mesalazine and placebo

Table 29: Correlation between T2 relaxation time with bowel frequency and stool consistency

T2 relaxation time (s)	Correlation, Spearman r	P value
Average bowel frequency	0.004	0.98
Average stool consistency	-0.12	0.48

1.15.2.5. Discussion:

This study looked at the use of MRI as a tool to search for a biomarker for IBS. Previous studies looking at small bowel water content have reported IBS-D patients have lower fasting SBWC with median values of 42 (IQR 28-62)[212] and 36 (17-77) ml [unpublished data from a previous study looking at SBWC in IBS-D]. In this study, the fasting SBWC was higher with median values of 58 (IQR 28-115) ml. The possibility could be that the patients may not be compliant to fasting instructions but this would have shown on MRI scanning. The other possibility that might explain this may be the heterogeneity of IBS-D and the cohort of IBS-D patients used in the previous two studies compared to the current studied group may be different altogether. Further analysis of fasting SBWC did not differ between male and female which gave a mean (SD) fasting SBWC of 72 (51) and 74 (59) ml respectively, p=0.92. There was no correlation with fasting SBWC and anxiety unlike previous report[212] and no correlation between fasting SBWC with clinical parameters such as bowel frequency and stool consistency. A previous study had suggested IBS-D patients have significantly lower fasting SBWC that correlated with anxiety. In this cohort of patients, 15 of 40 patients had fasting SBWC content less than 35 ml (25^{th} centile healthy control), which may indicate increased gut transit. In this small group of patients, there was no significant difference in their baseline characteristics such as anxiety, depression, bowel frequency and stool consistency when compared with patients whose fasting SBWC was greater than 35 ml. Furthermore, there were no correlation between fasting SBWC with anxiety,

depression, bowel frequency or stool consistency in the group of patients who had very little fasting SBWC.

Analysis of T1 and T2 relaxation time of the ascending colon are still in the research phase. There have been no previous studies visualising the colon and its colonic chyme in undisturbed bowel. In this study, we use T1 and T2 relaxation times to assess the mode of action of Mesalazine in the ascending colonic chyme. Analysis of T1 and T2 relaxation times were analysed using semi-automated software. As mentioned in the method section, T1/T2 were taken as an average of 3 sections in the ascending colon (top, middle and bottom). Not all T1 and T2 images of the ascending colonic chyme were analysed. This was mainly due to high 'background' noise leading to poor fitting of the relaxation curves. Factors that could cause the high 'background' noises were the motion artefacts and the acute angle the sagittal image was taken at during MRI scans. Furthermore, some ascending colons were full of gas or had collapsed which limited further analysis of T1/T2 relaxations times. For these reasons, this may be a limitation to use this test as a potential biomarker.

T1 relaxation following treatment with Mesalazine showed a significant change when compared to placebo. Unfortunately this does not correlate with clinical symptoms. Mean (SD) T1 relaxation time in this study showed 0.79 (0.26) s compared to another cohort of IBS-D patients (Ondansetron for IBS-D) 0.78 (0.29) s, $p=0.9$. When T1 relaxation time in this cohort of IBS-D patients was compared with healthy controls, T1 relaxation time in IBS-D is significantly longer giving a mean (SD) 0.79 (0.26) in IBS-D and 0.45 (0.17) in healthy controls, $p < 0.01$. Therefore, T1

may be a marker to assess IBS-D but at present its utility is unclear and this will need further research.

T2 is a measure of how protons interact with each other following magnetisation. Pure water has a long T2 relaxation time approximately 2s and tissue/fat would have a shorter T2 relaxation time. In this study, T2 analysis did not showed any significant changes following treatment with either Mesalazine or placebo. There was no clinical correlation between T2 and clinical symptoms. Again, when comparing the baseline T2 results with another cohort of IBS-D patients from previous study (Ondansetron for IBS-D), the T2 results were consistent. The median T2 relaxation times for this study vs. another cohort of IBS-D patients were 0.06 (0.05-0.09) and 0.06 (0.05-0.07) s respectively, p=0.07.

Overall, the use of MRI to image functional bowel is promising. Although this study did not show much promising results for small bowel water, this is still in the early stages of research. There have been some developments of using MRI to assess colonic volumes[221] and gas[95]. This would give a better understanding of patients with IBS-D as the majority of patients complain of bloating along with abdominal pain and erratic bowel habit. Analysis of bowel gas and colonic volumes using MRI and correlating with their symptoms may be useful. Recently, MRI has been used to study patients with scleroderma and coeliac disease. This showed increased fasting SBWC in untreated coeliac disease patients[254] and the severity of the coeliac disease based on the Marsh grading tool, correlated with fasting total colonic volumes. Therefore, the use of MRI as a tool to visualise small and large bowel in functional gastrointestinal disorder is encouraging.

Conclusion

The introduction of this thesis gave a general view of irritable bowel syndrome and its multiple pathophysiology. IBS is common and remains heterogeneous. It is a waxing and waning condition. IBS is an important condition since it affects the quality of life of patients and is a substantial burden to the health service worldwide. The Rome Foundation had taken the challenge of forming a set of diagnostic criteria for IBS. The criteria for irritable bowel syndrome were based on symptoms and lacked the evidence-based approach and remained applicable mainly in the research setting. The sensitivity of the current Rome III criteria for IBS remained modest at approximately 70% with specificity of 80%[9]. Therefore many physicians in secondary care would have put patients through many investigations, as IBS remained a diagnosis of exclusion. Due to its multiple pathophysiology, treatment for this condition remained symptom based. Therefore, this has left patients bereft of an effective treatment for their condition and thus led to dissatisfaction in patients during consultations.

IBS is an interaction between a disturbed central pain processing pathway and local gut pathology. In chapter 2, I have focused mainly on pathology of the gut leading to symptoms of IBS. The introduction to this chapter mainly discussed the pathophysiology of post-infectious IBS as it clearly defines the onset and effect of IBS. Recently, there have been conflicting reports of 'immune activation' in the gut mucosa of IBS patients. The use of a local anti-inflammatory drug treatment, such as 5-ASA, targeting the gut mucosa of IBS was promising but these studies were few, small in numbers of participants and the studies were not blinded. Therefore, this chapter described one of the largest trials in the use of Mesalazine (5-ASA) in a

subgroup of IBS patients who have diarrhoea. The purpose of this trial was to define the clinical benefit of Mesalazine in IBS-D patients and possible mediators/ biomarkers in IBS-D. Disappointingly, this study did not show any clinical benefit on the use of Mesalazine in unselected patients with IBS-D but potentially may be of benefit for patients with PI-IBS. On the mechanistic side of the study, mast cell percentage area stained were elevated in IBS-D patients but had no correlation with mast cell tryptase supernatant or other clinical symptoms. The reason may be that the measurement of mast cells overall, included activated and 'latent' mast cell that may not be pertinent in correlating with clinical symptoms. Lymphocyte CD3 counts seemed to be significantly higher following treatment of Mesalazine. The reason for this is unclear but it is a possibility that the side effect of Mesalazine may be the cause of this elevation. Basic mechanism for Mesalazine/ 5-ASA is still unknown but based on this study, 5-ASA did not influence the 5-HT pathway as all three markers of serotonin e.g. 5-HT supernatant, 5-HT cell count and the ratio of 5HIAA and 5-HT showed no significant changes following treatment with mesalazine. Otherwise, I was not able to demonstrate any potential mediators or biomarkers to predict or evaluate the response of Mesalazine in IBS-D. Although this was a negative study, it had shed some light in the use of Mesalazine in IBS-D and the likelihood of 'immune activation' in the gut mucosa of IBS patients may be an epiphenomenon.

Gut transit measurements are variable and very often involve exposure to ionising radiation. The third chapter explored the use of magnetic resonance imaging in the gut transit. The use of the MRI marker pills for whole gut transit time is very promising. It had good correlation with the gold standard radio-opaque marker with

abdominal x-ray. The optimisation of WGT using the MRI marker pills had been applied to assess patients with chronic constipation. This was the first report using MRI to assess colonic function in patients with functional constipation and IBS-C in a non-invasive manner. These two conditions overlap significantly thus targeting treatment for these conditions remained quite difficult. This study described in Section 3.3 provided insightful information on the resting colonic volumes and its sensory motor function that differ between functional constipation and IBS-C following bowel distension with a stimulus such as 1 litre of Moviprep®. Other MRI parameters such as T1 and T2 image sequence to look into colonic chyme may be beneficial in the study of functional gastrointestinal disorder but so far, it had limited evidence especially in the IBS-D cohort. It would be interesting to assess T1 and T2 relaxation time in the cohort of IBS-C patients and compare these findings with IBS-D patients. The vast amount of information gained e.g. colonic volumes, small bowel water and colonic gas, following MRI scans of the abdomen especially after a standardized stimulus like macrogol or lactulose may hopefully be applicable in future clinical settings.

In conclusion, there is still a need to unravel the pathophysiology of IBS, as it will lead to novel treatments for IBS-D. Potential new treatment for IBS-D that might emerge in the near future are Eluxadoline[255] (currently in Phase 2 trial, a mixed μ-opioid receptor agonist and δ-opioid receptor antagonist), Ebastin[256] (Histamine 1 receptor antagonist), Ibudotant[257] (currently in Phase 3 trial, neurokinin type 2 receptor antagonist) and Asimadoline[258] (currently in Phase 3 trial, κ-opioid agonist). The role of MRI in the search for biomarkers in IBS remains promising.

While fasting measurements have limited value, assessment of both motility and sensation when used in conjunction with a standardized stimulus such as dietary provocation, prokinetics or distension such as that provided by macrogol, could be a valuable way to screen new drugs.

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Appendix

1.16. Appendix 1: Excluded Medication

Excluded medication and dose controlled medication. Please use in conjunction with the exclusion criteria definition.

Excluded Medication

NSAIDS
Aceclofenac
Acemetacin
Azapropazone
Celecoxib
Dexibuprofen
Dexketoprofen
Diclofenac Sodium
Etodolac
Etoricoxib
Fenbufen
Fenoprofen
Flurbiprofen
Ibuprofen
Indometacin
Ketoprofen
Mefenamic Acid
Meloxicam
Nabumetone
Naproxen
Piroxicam
Sulindac
Tenoxicam
Tiaprofenic Acid
Aspirin

Long-term antibiotics
Please refer to the latest version of BNF

Antispasmodics
Alverine Citrate
Mebeverine Hydrochloride
Peppermint Oil

Antimuscarinics
Atropine Sulphate
Dicycloverine Hydrochloride
Hyoscine Butylbromide Propantheline Bromide

Opiates / Anti-diarrhoeal
Codeine
Loperamide
Morphine

Anti-inflammatory
Prednisolone
Budesonide
Hydrocortisone
Azathioprine
Mercaptopurine

5-ASA containing
Balsalazide Sodium
Mesalazine
Olsalazine Sodium
Sulfasalazine

Dose Controlled Medication

SSRIs
Citalopram
Escitalopram
Fluoxetine
Fluvoxamine Maleate
Paroxetine
Sertraline

TCAs
Amitriptyline Hydrochloride
Clomipramine Hydrochloride
Dosulepin Hydrochloride
Doxepin
Imipramine Hydrochloride
Lofepramine
Nortriptyline
Trimipramine

1.17. Appendix 2: Clinical questionnaires

1. Hospital Anxiety and Depression Scale 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.

2. **CDC HRQoL-4 questionnaire**

Participant ID: _____ Date: __/__/__

Mesalazine for the treatment of IBS-D

Healthy Days Core Module (CDC HRQoL-4)

1. Would you say that in general your health is:

Please Read	
a. Excellent	1
b. Very good	2
c. Good	3
d. Fair	4
or	
e. Poor	5

Do not read these responses	
Don't know/Not sure	7
Refused	9

2. Now thinking about your physical health, which includes physical illness and injury, for how many days during the past 30 days was your physical health not good?

a. Number of Days	_ _	
b. None	8 8	
Don't know/Not sure	7 7	
Refused	9 9	

3. Now thinking about your mental health, which includes stress, depression, and problems with emotions, for how many days during the past 30 days was your mental health not good?

a. Number of Days	_ _	
b. None	8 8	**If both Q2 AND Q3 ="None", skip next question**
Don't know/Not sure	7 7	
Refused	9 9	

4. During the past 30 days, for about how many days did poor physical or mental health keep you from doing your usual activities, such as self-care, work, or recreation?

a. Number of Days	_ _	
b. None	8 8	
Don't know/Not sure	7 7	
Refused	9 9	

:

3. PHQ15 questionnaire

Participant ID: _____ Date: __/__/__

Mesalazine for the treatment of IBS-D

Patient Health Questionnaire

During the *past 4 weeks,* how much have you been bothered by any of the following problems?	Not bothered at all	Bothered a little	Bothered a lot
a. Stomach pain	☐	☐	☐
b. Back pain	☐	☐	☐
c. Pain in your arms, legs, or joints (knees, hips, etc)	☐	☐	☐
d. Menstrual cramps or other problems with your periods [Women only]	☐	☐	☐
e. Headaches	☐	☐	☐
f. Chest pain	☐	☐	☐
g. Dizziness	☐	☐	☐
h. Fainting spells	☐	☐	☐
i. Feeling your heart pound or race	☐	☐	☐
j. Shortness of breath	☐	☐	☐
k. Pain or problems during sexual intercourse	☐	☐	☐
l. Constipation, loose bowels, or diarrhoea	☐	☐	☐
m. Nausea, gas, or indigestion	☐	☐	☐
n. Feeling tired or having low energy	☐	☐	☐
o. Trouble sleeping	☐	☐	☐

4. EQ5D questionnaire

Participant ID: _____ Date: __/__/__

Mesalazine for the treatment of IBS-D

Health Questionnaire

English version for the UK
(validated for Ireland)

By placing a tick in one box in each group below, please indicate which statements best describe your own health state today.

Mobility

I have no problems in walking about ❑

I have some problems in walking about ❑

I am confined to bed ❑

Self-Care

I have no problems with self-care ❑

I have some problems washing or dressing myself ❑

I am unable to wash or dress myself ❑

Usual Activities (*e.g. work, study, housework, family or leisure activities)*

I have no problems with performing my usual activities ❑

I have some problems with performing my usual activities ❑

I am unable to perform my usual activities ❑

Pain/Discomfort

I have no pain or discomfort ❑

I have moderate pain or discomfort ❑

I have extreme pain or discomfort ❑

Anxiety/Depression

I am not anxious or depressed ❑

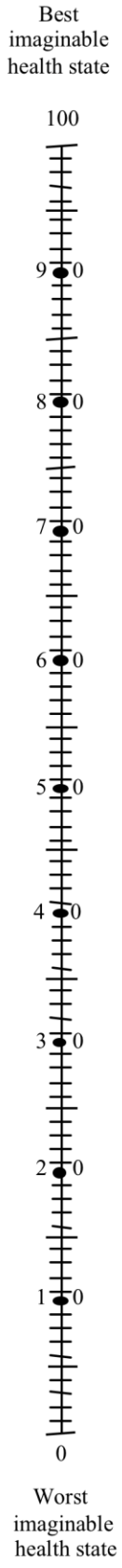
I am moderately anxious or depressed ❑

I am extremely anxious or depressed ❑

To help people say how good or bad a health state is, we have drawn a scale (rather like a thermometer) on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

We would like you to indicate on this scale how good or bad your own health is today, in your opinion. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your health state is today.

Your own health state today



1.18. Appendix 3: Stool diary used during the study period

Day	Date (e.g. Monday 05/Jun/10)	Hours of Pain? (If no pain enter '0')	Score each 0-10 using the scale below: Abdominal Pain Severity? 0 1 2 3 4 5 6 7 8 9 10 None----------Moderate----------Severe	Urgency?	Bloating?	Number sachets taken today? (If none enter '0')	STOOL FORM AND TIME (Form = score 1-7 from 'Stool Form' list below; Time = time of stool)								
								1st	2nd	3rd	4th	5th	6th	7th	8th
1							Form								
							Time								
2							Form								
							Time								
3							Form								
							Time								
4							Form								
							Time								
5							Form								
							Time								
6							Form								
							Time								
7							Form								
							Time								

Continue on back if >8 bowel movements for 1 particular day

Stool Form
1=Separate hard lumps, like nuts
2=sausage shaped but lumpy
3=like a sausage or snake, but with cracks on its surface
4=like a sausage or snake, smooth and soft
5=soft blobs with clear cut edges
6=fluffy pieces with ragged edges, a mushy stool
7=watery, no solid pieces

Answer the following after completion of day 7:

Have you had satisfactory relief of your IBS symptoms this week?

YES ☐(1) **NO** ☐(0)

1.19. Appendix 4: Histamine measurement using commercial kit (Neogen)

NEOGEN® CORPORATION ISO 9001

Histamine (Life Science Format) ELISA Kit Instructions

Please read all instructions carefully before beginning this assay

PRODUCT #409010
For research use only.

Storage Conditions:
Do not freeze kit components
All other kit components: 4°C

DESCRIPTION

Histamine is a heterocyclic primary amine derived from decarboxylation of the amino acid histidine. It is a mediator of inflammation closely associated with the initial phase of immediate hypersensitivity response (anaphylaxis). Histamine is synthesized by the enzyme histidine decarboxylase and is present in most cells, but typically stored in metachromatic granules of basophils and mast cells (granulocytes) (1). Histamine in the intracellular granules is bound to proteins and inactive until it is released from the cells.

During anaphylactic response, an antigen-IgG antibody complex formed *in vivo* activates the complement cascade and cleaves bioactive complement associated peptides called anaphylatoxins. Among anaphylatoxins, C_{3a}, which is derived from the complement component C_3, and C_{5a} derived from C5 releases histamine from mast cells (2). In IgE-mediated immediate hypersensitivity response, an IgE antibody is produced by B lymphocytes upon stimulation by an allergen and under the control of IL-13 and IL-4. Such IgE antibodies are secreted from B lymphocytes and bound to a high affinity receptor (FceR I High binding IgE receptor) on mast cells in the tissue or on basophilic leukocytes in the peripheral blood leukocytes (3). When IgE bearing mast cells or basophils encounter allergen to which the IgE antibody was directed, the allergen (antigen) binds to the cell bound IgE and agglutinates on the surface of these cells. This event triggers the release of granules into the blood stream. Degranulation of the mast cell involves release of mediators such as leukotriene $C_4/D_4/E_4$, thromboxane A_2, PGD_2, Platelet Activating Factor, histamine, heparin, tryptase, kallikrein, ECF-A, IL-8 and other cytokines. Histamine released from mast cell acts on smooth muscle and blood vessels, causing bronchoconstriction, vasodilation and increased vascular permeability (erythema) (4).

Histamine exerts its biological effects through three distinct receptors on various tissues and cells; H_1, H_2 and H_3. Among these histamine receptors, H_2 receptor is best recognized as associated with secretion of acid in the stomach leading to peptic ulcer. Thus, an H_2 receptor antagonist is used for treatment of peptic ulcers (5).

Tissue bound mast cells (such as in the skin, nasal mucosa) respond to incoming allergen and manifest as erythema (e.g. in skin test) and wheezing response. In various research areas, it is important to study *in vitro* histamine release from peripheral blood basophils. When whole blood from a sensitized animal is exposed to a given allergen, basophils respond to the allergen by releasing histamine into the incubation mixture. Using a whole blood sample, one can assess ex vivo response to a sensitizing antigen as a function of histamine released from the basophils (6).

Other than histamine being an important mediator of immediate hypersensitivity, histamine is found in decaying fish meat, especially of scombroid fish such as tuna. For this reason histamine is called "Scombrotoxin" (7). Histamine is also found in wine (8) and cheese (9).

1

INTENDED USE

This kit is designed for *in vitro* quantification of histamine in various biological fluids by competitive direct enzyme-linked immunosorbent assay (CD-ELISA). This kit is intended for use in investigative research only and not for human clinical diagnostic use.

This Histamine ELISA kit (Life Science Format) should not be used for determining histamine levels in scombroid fish. Neogen offers a separate kit for determination of histamine in fish and other foods.

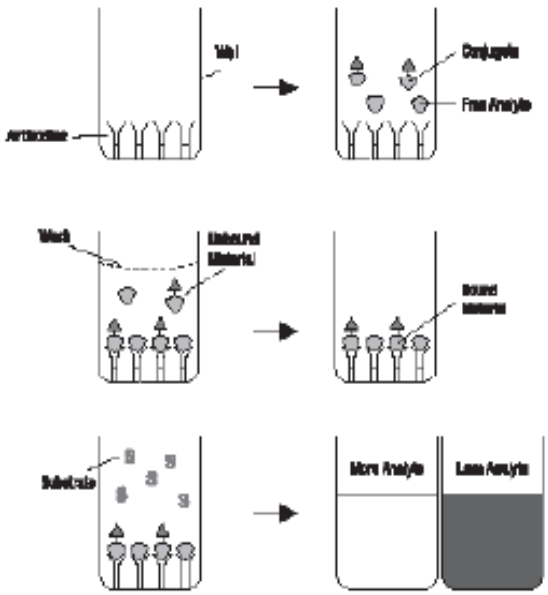
Intended User: Researchers in biomedical fields.

PRINCIPLE OF ASSAY

Neogen's Histamine ELISA test kit (Life Science Format) is a competitive direct ELISA (Enzyme-Linked Immunosorbent Assay) in a microwell format that allows users to obtain exact concentrations of histamine in nanograms per milliliter.

The microwells in this assay kit are pre-coated with a monoclonal antibody to histamine. The sample or standard solution is first added to the antibody coated microplate. Next, the enzyme conjugate is added and the mixture is shaken and incubated at room temperature for 45 minutes. During the incubation, unbound (free) histamine in the samples or standards is allowed to compete with enzyme (horseradish peroxidase: HRP)-labeled histamine (conjugate) for antibody binding sites. The plate is then washed, removing all the unbound material. The bound enzyme conjugate is detected by the addition of a one-component peroxidase substrate which generates color by horseradish peroxidase. An optimal color is generated after 30 minutes. A microplate reader is then used to take an absorbance reading at 650 nm.

Quantitative test results may be obtained by measuring and comparing the absorbance reading of the sample wells against the standard curve using a log-logit curve fitting model. The extent of color development is inversely proportional to the amount of histamine in the sample or standard. For example, the absence of histamine in the sample will result in a bright blue color, whereas the presence of histamine will result in decreased or no color development.



MATERIALS PROVIDED

1. **WASH BUFFER (25X):** 30 mL. Dilute 25-fold with deionized water. Diluted wash buffer is used to wash all unbound enzyme conjugate, samples and standards from the plate after the 45-minute incubation.
2. **K-BLUE SUBSTRATE:** 20 mL. Stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H_2O_2) in a single bottle. It is used to develop the color in the wells after they have been washed. **LIGHT SENSITIVE**. Keep substrate refrigerated.
3. **PBS SAMPLE DILUENT:** 1 foil pouch of dry powder yields 1 Liter of 10mM Phosphate Buffered Saline. Once prepared, this buffer is used for diluting extracted and non-extracted samples.
4. **HISTAMINE ENZYME CONJUGATE:** 6 mL. Histamine horseradish peroxidase conjugate, ready-to-use.
5. **HISTAMINE STANDARDS:** 6 vials containing 500 µL per vial. Ready-to-use Histamine standards at the following concentrations: 0, 2.5, 5, 10, 20, 50 ng/mL.
6. **HISTAMINE ANTIBODY-COATED MICROPLATE:** A 96 well Dynex microplate with a monoclonal antibody against Histamine. The plate is ready for use. **DO NOT WASH!**

Storage Requirement: The kit can be used until the expiration date on the label when stored at 2°-8°C.

MATERIALS NEEDED BUT NOT PROVIDED

1. Deionized water for diluting wash buffer and extraction buffer.
2. Precision pipettes that range from 10 µL-1000 µL and disposable tips.

NOTE: If all or several strips are to be used at one time, it is suggested that a multichannel pipette be used.

3. Reagents required for preparation of samples. Since wide varieties of samples can be assayed with this kit, each investigator should use the optimum conditions for extracting histamine with this kit.
4. Disposable reagent boats.
5. Graduated cylinders to dilute and mix wash buffer and extraction buffer.
6. Microplate reader with 650 nm filter.
7. Plate cover or plastic film to cover plate during incubation.

OPTIONAL MATERIALS:

8. 1 N HCl or Neogen's Red Stop Solution (product #301474).
9. Microplate shaker.

WARNINGS AND PRECAUTIONS

1. **DO NOT** use components beyond expiration date.
2. Store test kit between 2-8°C (35-46°F). Avoid prolonged storage of the kit at ambient temperatures. Do not freeze the test kit.
3. Glassware should not be used for extraction purposes. As Histamine may adhere to glass, using glassware may affect test results.
4. The samples to be analyzed with this kit must have a pH of 6-8. Excessively acidic or alkaline samples should be adjusted using 0.1 N NaOH or HCl.
5. **DO NOT** mix any reagents or components of this kit with any reagents or components of any other kit or lot number. This kit is designed to work properly as provided.
6. **DO NOT** pipette reagents by mouth.
7. Use of incubation times other than those specified may give inaccurate results.
8. Always pour substrate out of the bottle into a clean test tube or reagent boat. **DO NOT** pipette out of the bottle. An unclean tip could contaminate the entire contents of the substrate.
9. All specimens should be considered potentially infectious. Exercise proper handling precautions.
10. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
11. Use aseptic technique when opening and removing reagents from vials and bottles.
12. Keep plate covered except when adding reagents, washing or reading.
13. Kit components should be refrigerated at all times when not in use.

PROCEDURAL NOTES

1. The test kit should be brought to 18-30°C (64-86°F) prior to use.
2. Desiccant bag must remain in foil pouch with unused strips. Keep zip-lock pouch sealed when not in use to maintain a dry environment.
3. Always use new pipette tips to pipette the buffer, enzyme conjugate, standards and samples. This will help to eliminate cross contamination.
4. Before pipetting a reagent, rinse the pipette tip three times with that reagent (i.e. fill the tip with the desired amount of reagent and dispense back into the same vial. Repeat 2 times). Now the tip is properly rinsed and ready to dispense the reagent into your well or test tube.
5. When pipetting into the wells, **DO NOT** allow the pipette tip to touch the inside of the well, or any of the reagents already in the well. This can result in cross contamination.
6. Standards and samples should be assayed in duplicate.
7. To quantitate, always run a standard curve when testing samples.
8. Gently mix specimens and reagents before use. Avoid vigorous agitation.
9. When using only partial amounts of a kit, it is recommended to transfer the appropriate volume of each reagent to a clean vessel for repeated dispensing. This will reduce reagent contamination caused by repeated sampling from the original container.
10. Before taking an absorbance reading, wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.
11. Before opening the enzyme conjugate and standard vial, tap vial in an upright position to remove any liquid in the cap.
12. Substrate: Neogen's TMB substrate is ready-to-use. Do not dilute. Determine volume needed to perform the test. For each strip of 12 well, 2 mL of substrate is required or approximately 16 mL or substrate solution is required to run a 96 well microplate. Measure out the required amount of substrate into a clean reagent boat. Do not return unused substrate to the bottle. Keep the substrate protected from light until needed by covering the reagent boat.
13. PBS Sampe Diluent: Prepare by adding foil pouch of extract buffer to 1.0 L of deionized or distilled water. Swirl to mix. Store remaining buffer covered at room temperature.

SAMPLE PREPARATION

This assay is non-species specific. The amount of Histamine in your samples may differ. It is recommended that you conduct a preliminary test to determine the optimum dilution for your samples. Typically, tissue culture media, tissue extracts, cell and cell-free extracts can be used.

The samples to be analyzed with this kit must have a pH of 6-8. Excessively acidic or alkaline samples should be adjusted using 0.1 N NaOH or HCl.

TEST PROCEDURES

1. Allow reagents to warm to 18-30°C (64-86°F) prior to use.
2. Determine the number of wells to be used.
3. Determine the number of standards to be used. Six standard concentrations are provided with this kit. These standards are ready to use. Neogen recommends using 0, 2.5, 5. 10, 20, 50 ng/mL standards and suggests using a combination of at least 5 standards with each assay.
4. All standards and unknown samples should be assayed in duplicate. See Scheme I for suggested template design.

SCHEME I

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0	0.0	2.5	2.5	5.0	5.0	10	10	20	20	50	50
B	U1	U1	U2	U2	U3	U3	U4	U4	U5	U5	U6	U6
C	U7	U7	U8	U8	U9	U9	U10	U10	U11	U11	U12	U12
D	U13	U13	U14	U14	U15	U15	U16	U16	U17	U17	U18	U18
E	U19	U19	U20	U20	U21	U21	U22	U22	U23	U23	U24	U24
F	U25	U25	U26	U26	U27	U27	U28	U28	U29	U29	U30	U30
G	U31	U31	U32	U32	U33	U33	U34	U34	U35	U35	U36	U36
H	U37	U37	U38	U38	U39	U39	U40	U40	U41	U41	U42	U42

5. Add 50 µL of standards (S) or unknown (U) (some samples may require diluting) to the appropriate wells in duplicate. Change pipette tips for each standard and unknown solution.
6. Mix each reagent by inverting the reagent bottle prior to use.
7. Add 50 µL of the ready-to-use enzyme conjugate to each well. Use 8-channel pipette or 12-channel pipette for rapid addition.
8. Mix by shaking plate gently. A microplate shaker may be used.
9. Cover the plate with plastic film or plate cover and incubate at room temperature (18-30°C) for 45 minutes. ***NOTE: Keep plate away from drafts and temperature fluctuations.***
10. Dilute concentrated wash buffer with deionized water. The wash buffer is supplied as a 25X concentrate. Prepare by mixing wash buffer concentrate (30 mL) to 720 mL of deionized or distilled water. Swirl to mix. Do not shake. Store remaining wash buffer at room temperature.
11. After the conjugate incubation, dump out the contents of the plate. Tap out contents thoroughly on a clean lint-free towel.
12. Fill each well with 300 µL of the diluted wash buffer and then dump out contents. Repeat this step for a total of three times, then turn the wells upside down and tap out the remaining liquid on a clean lint-free paper towel. If available, an automated plate washer can be used for the wash step. With an automated plate washer, increase the number of wash steps from 3 to 5.
13. Pour the needed volume of substrate from the amber bottle into a reagent boat (not supplied). Add 150 µL of substrate to each well. Use new pipette tips. Neogen recommends using a multichannel pipette for best results. Mix by shaking plate gently. Please note, the remaining substrate in the reagent boat should be discarded.
14. Allow the plate to incubate at room temperature for 30 minutes.
15. Before reading the plate, gently shake the plate by sliding it back and forth on a flat surface. This will ensure uniform color throughout each well. Also use a dry cloth or towel to wipe clean the bottom of the microwells. Air bubbles should be eliminated, as they could affect analytical results.
16. The plate is read in a microplate reader using a 650 nm filter. If a dual wavelength is used, set W_1 at 650 nm and W_2 at 490 nm.
17. If accounting for substrate background, use 2 wells as blanks with only substrate in the wells. (150 µL/well). Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

NOTE: Some microplate readers can be programmed to do these subtractions automatically when reading the plate. Consult your instrument manual.

18. If the microplate can not be read after the 30 minute substrate incubation, add 50-100 µL of 1 N HCl or Neogen's Red Stop Solution to each well to stop enzyme reaction.
19. Read plate at 450 nm, if 1 N HCl solution was used to stop the reaction. Read plate at 650 nm if Neogen's Red Stop Solution was used. If a dual wavelength is used, set at W_1 450 nm and at W_2 650 nm.
20. Plot the standard curve and estimate the concentrations of the samples from the curve. See "CALCULATIONS."

Note: Absorbance readings will approximately double when stopped with acid. If absorbance readings are too high for measuring with your microplate reader, decrease the substrate incubation from 30 minutes to 15 or 20 minutes.

CALCULATIONS

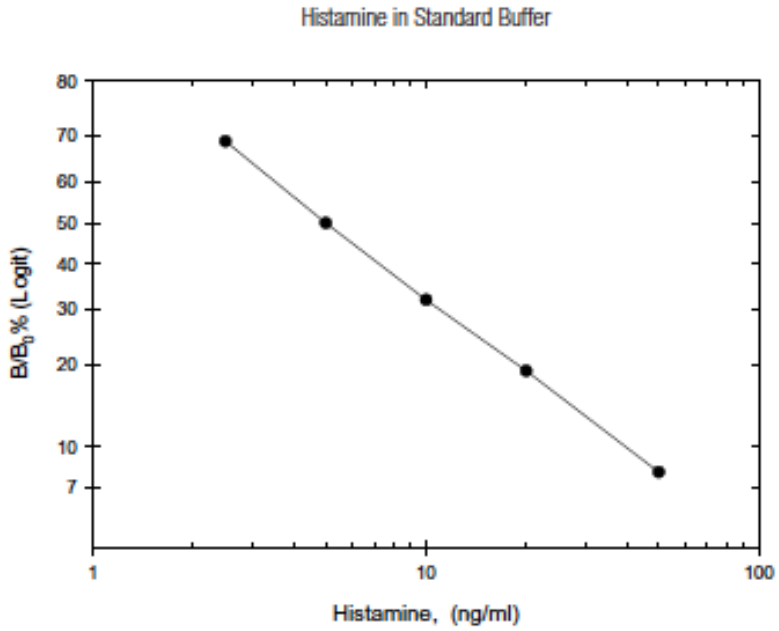
1. A log-logit curve fitting model for calculating the histamine levels of unknown samples is recommended.
2. Subtract the average substrate background absorbance from all standard and sample absorbance values. Average duplicate well absorbance values.
3. Calculate the percent of maximal binding (%B/B_0 value) of each standard by dividing the absorbance of the standard by the 0 standard absorbance and multiply by 100. Transform the ratio into the logit function, where logit = ln (%B/B_0/(100-%B/B_0)).

   Example: 0 Standard Absorbance = 1.800 = B_0
   10 ng/mL Standard Absorbance = 1.000
   %B/B0 = 1.000/1.800 x 100 = 55.5
   Logit = ln(55.5/(100-55.5) = 0.221
4. Repeat for all of the standard curves.
5. Graph your standard curve by plotting the logit for each standard concentration on the ordinate (y) axis against the log of the standard concentrations on the abscissa (x) axis. Draw a curve by using a curve-fitting routine (i.e. 4-parameter or linear regression). A log-logit curve is recommended for this assay.
6. Determine the %B/B_0 and logit values for each sample:

   Example: 0 Standard Absorbance = 1.800 = B_0
   10 ng/mL Standard Absorbance = 0.600
   %B/B_0 = 0.600/1.800 x 100 = 33.3
   Logit = ln(33.3/(100-33.3) = -0.695.
7. Using the standard curve, the concentration of each sample can be determined by comparing the logit value of each sample to the corresponding concentration of Histamine standard.
8. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor. If the absorbance values of a sample fall outside the values of the 2.5 or 50 ng/mL standard, concentrate or dilute the sample as appropriate and retest.
9. To convert mass based concentration of Histamine into molarity the following equation can be used: ng/mL x 9.005 = nmole/L (nM). E.g. 1.0 ng/mL = 9 nM.

TYPICAL STANDARD CURVE

A typical standard curve expressed as log-logit format is shown below.



TYPICAL DATA

NOTE: ***"Typical data" is a representation. Variances in data will occur. Optical density readings may fluctuate during the shelf-life of the kit, but the %B/B_0 should remain comparable. Measuring wavelength: 650 nm***

Standard	*Standard Concentration (ng/mL)*	*Optical Density (Absorbance Value)*	*%B/B_0*
S_0 (B_0)	0	2.186	100
S_1 (B_1)	2.5	1.498	69
S_2 (B_2)	5.0	1.089	50
S_3 (B_3)	10	0.700	32
S_4 (B_4)	20	0.413	19
S_5 (B_5)	50	0.185	8

PERFORMANCE CHARACTERISTICS

Limit of quantification: 2.5 ng/mL. Described as the lowest concentration point on the calibration curve that this test can reliably detect Histamine.

Range of quantification: 2.5 - 50.0 ng/mL. For quantitating samples above 50.0 ng/mL, contact Neogen Technical Services for dilution instruction if required.

Intra-assay Precision: ≤ 10%

Inter-assay Precision: ≤ 10%

Validated matrix: No application is developed for use with tissue culture, cell extracts, etc.

CROSS REACTIVITY

The monoclonal antibody used in Neogen's Histamine Kit is highly specific to Histamine as shown below.

HISTAMINE	100.0%
HISTIDINE	0.008%
CADAVERINE	0.003%
TYRAMINE	<0.01%
SPERMINE	<0.01%
PUTRESCINE	<0.01%
TRIMETHYLAMINE	<0.01%

DISCUSSION

Histamine release reactions *in vivo* and *in vitro* are investigated by various researchers. Some investigators use HPLC with fluorimetric detector, radioimmunoassay, and enzyme immunoassay to determine histamine contents of biological fluids. One attractive feature of studying histamine release using ELISA is that one can use whole blood to activate cells with stimulants and measure histamine in the same reaction mixture. The normal plasma level of histamine is less than 1 ng/mL, and 3-7 ng/mL is found in animals or patients with allergic response. Histamine contents of whole blood from human are between 20 to 200 ng/mL. In clinical situations, arterial hypotension is observed in patients whose plasma histamine reached 6 –8 ng/mL, bronchospasm at 7 – 12 ng/mL. If plasma histamine exceeds 100 ng/mL, it is lethal. Animal and fish tissues contain 1 –100 µg/g tissue.

Ferrer et al (10) showed that histamine can be released from whole blood of patients in response to antigenic response. Histamine can also be released from mouse mast cell line. Histamine release is modulated by addition of tetracosahexaenoic acid in the culture media (11). Eugenol (a major component of clove) reduced Compound 48/80-induced systemic anaphylaxis in rat. Eugenol also inhibited cutaneous anaphylaxis in response to anti-DNP-IgE and reduced serum histamine levels (12). Demoly et al used histamine release to predict allergic response to therapeutic drugs (13). In this paper, drug specific histamine release from venous blood (whole blood) was compared with the total histamine released by freeze-thawing the cells. The total histamine release by freeze-thawing was 61 ng/mL (median value).

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TECHNICAL ASSISTANCE

Technical assistance is available Monday-Friday, between 8:00 a.m. and 6:00 p.m. EST.

NEOGEN CORPORATION ISO 9001

944 Nandino Blvd • Lexington KY 40511-1205 USA
859/254-1221 or 800/477-8201 USA/CANADA

1.20. Appendix 5: Stool calprotectin measurement

ENGLISH

INTENDED USE

The BÜHLMANN Calprotectin ELISA kit is designed for the extraction and quantitative determination of human Calprotectin (MRP8/14; S100A8/S100A9) in stool samples (1-3).

PRINCIPLE OF THE ASSAY

After a short extraction procedure using one volume of faeces and 49 volumes of Extraction Buffer, the test allows for the selective measurement of Calprotectin-antigen by sandwich ELISA. A monoclonal capture antibody (mAb) highly specific to the Calprotectin heterodimeric and polymeric complexes (4-5), respectively, is coated onto the microtiter plate. Calibrators, controls and patients extracts are incubated at room temperature for 30 minutes. After a washing step a detection antibody (Ab) conjugated to horseradish peroxidase (HRP) detects the calprotectin molecules bound to the monoclonal antibody coated onto the plate. After incubation and a further washing step, tetramethylbenzidine (TMB) will be added (blue color formation) followed by a stopping reaction (change to yellow color). The absorption is measured at 450 nm.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Extraction Buffer	6 bottles 125 mL	B-CAL-EX	Ready to use
Microtiter Plate precoated with anti-Calprotectin mAb	2x12 x 8 wells	B-CAL-MP	Ready to use
Plate Sealer	6 pieces		
Wash Buffer Concentrate (10x) with preservatives	2 bottles 100 mL	B-CAL-WB	Dilute with 900 mL of deionized H_2O
Incubation Buffer with preservatives	3 bottles 125 mL	B-CAL-IB	Ready to use
Calibrators A to E[1) 2)] Calprotectin in a buffer matrix with preservatives	5 vials 1 mL	B-CAL-CASET	Ready to use
Control Low / High[3)] human serum with preservatives	2 vials 1 mL	B-CAL-CONSET	Ready to use
Enzyme Label Anti-Calprotectin Ab conjugated to HRP	2 vials 12 mL	B-CAL-EL	Ready to use
TMB Substrate TMB in citrate buffer	2 vials 12 mL	B-TMB12	Ready to use
Stop Solution 0.25 M sulfuric acid	2 vials 12 mL	B-STS12	Ready to use **Corrosive agent**

Table 1

[1)] The actual Calprotectin concentration of the standards A to E are 4, 12, 40, 120 and 240 ng/mL, respectively. During extraction a 1:50 sample dilution occurs followed by an additional 1:50 dilution of the extracts for the measurement in the ELISA. To take these dilution steps into account for the final calculations the of calibrators A to E the following concentrations have to be used for the lower range ELISA procedure (refer to page 4): **10, 30, 100, 300 and 600 µg/g of Calprotectin.**

[2)] If you choose the extended range ELISA procedure (refer to page 5) the following calibrator concentrations have to be used in the respective ELISA protocol: **30, 90, 300, 900 and 1800 µg/g of Calprotectin**

[3)] The controls contain lot specific amounts of native human Calprotectin. Refer to the additional QC data sheet for actual concentrations.

REAGENTS SUPPLIED UPON REQUEST

Fecal Extraction Devices

Smart-Prep	50 tubes, spatulas, and base caps	B-CAL-RD
Schebo® Quick-Prep™	50 tubes consisting of tube, cone & dosing tip	B-CAL-SO50
	1.3 mL, ready to use	

Table 2

STORAGE AND SHELF LIFE OF REAGENTS

Unopened Reagents	
Store at 2-8°C. Do not use past kit expiration date printed on the labels.	
Opened / Reconstituted Reagents	
Extraction Buffer	Store at 2-8°C until expiration date.
Microtiter Plate	Return unused strips immediately to the foil pouch containing the desiccant packs and reseal along the entire edge of zip-seal. Store until expiration date at 2-8°C.
Diluted Wash Buffer	Store for up to 6 months at 2-8°C.
Incubation Buffer	Store at 2-8°C until expiration date.
Calibrators	
Controls	
Enzyme Label	
TMB-Substrate	
Stop Solution	

Table 3

MATERIALS REQUIRED BUT NOT PROVIDED

Extraction Procedure

- 10 µL disposable inoculation loops
- 15 mL polypropylene tubes with screw caps required for standard extraction procedure; extraction devices (see above).
- Laminar flow work station
- Multi tube vortex mixer
- Precision balance (10-150 mg)
- Micro centrifuge (≥3000 g)

ELISA Procedure

- 10, 100 and 1000 µL precision pipettes with disposable tips.
- Disposable polystyrene or polypropylene tubes for the preparation of sample dilutions.
- 1000 mL cylinder for the dilution of the Wash Buffer Conc.
- Microtiter plate washer (see Technical Precautions) or squeeze bottle for Wash Buffer.
- Microtiter plate rotator (see Technical Precautions).
- Blotting paper.
- Microtiter plate reader for measurement of absorbance at 450 nm.

PRECAUTIONS

SAFETY PRECAUTIONS

- The microtiter-plate, calibrators and controls of this test contain components of human origin. Although tested and found negative for HBV surface antigen, HCV and HIV1/2 antibodies, the reagents should be handled as if capable of transmitting infections and should be handled in accordance with good laboratory practices using appropriate precautions.
- **Substrate and Stop Solution**: Substrate and Stop Solution: The Substrate TMB (B-TMB12) contains Tetramethylbenzidine, and hydrogen peroxide (H2O2). The Stop Solution (B-STS12) contains sulfuric acid (0.25 M). Each of those reagents is irritant to eyes, skin and

mucous membranes. Avoid contact with Eyes, skin and cloths Wear suitable protective clothing, gloves and eye protection. After contact with eyes or skin, wash immediately with plenty of water.
- Unused solution should be disposed of according to local State and Federal regulations.

TECHNICAL PRECAUTIONS

Kit components

- **Residues in the microtiter plate** wells result from the production process. They are removed in the washing step (Assay procedure step 3) and do not affect the results.
- Read carefully the instructions prior to carrying out the test. Test performance will be adversely affected, if reagents are incorrectly diluted, modified or stored under conditions other than those as detailed in this instruction for use.
- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Every effort should be made to ensure that no cross contamination occurs between reagents, samples or between wells.
- Let the reagents adjust to reach room temperature. Mix well (vortex) the reagents before use.
- Microwells cannot be re-used.

Extraction:

- To receive quantitative results it is important to homogenate the entire weighted stool sample in the extraction buffer. Avoid contamination at the top of the tube - insoluble (undigested) components can still be in the tube after extraction.
- The short centrifugation step of 5 minutes during the extraction can cause a turbid solution. Turbidity can be avoided by longer centrifugation but it shows no influence on the quantitative determination in the ELISA.

ELISA Procedure:

- In the ELISA procedure the **washing step is essential** to guarantee reproducible results. A **minimal incubation time** of the Wash Buffer in the wells **of at least 20 seconds** must be ensured each time.
- When using **automated washer,** BÜHLMANN strongly recommends using "plate mode" i.e. each process step (dispense/aspiration) is performed on all of the strips sequentially, before processing to the next process step. Thus, the minimal incubation time is guaranteed.
- The indicated no. of **washing cycles is mandatory** to ensure reproducible results.
- Set the **Plate rotator** (shaker) at 400 - 600 rpm (<10 Hz). Higher rotation frequency may cause poor dilution linearity at values between 300/900 and 600/1800 µg/g. Orbital rotation instead of reciprocal shaking should be used.
- It is recommended determining calibrators, controls and samples in duplicate. A new standard curve must be generated each time the assay is performed. Vertical alignment is recommended.
- If the initial concentration of an unknown sample reads higher than the top Calibrator, the sample must be further diluted with Incubation Buffer and assayed again according to the assay procedure. The resulting dilution factor must be accounted for the calculation of results.

SPECIMEN COLLECTION AND STORAGE

50 to 100 mg of native stool sample is needed for the extraction procedure.

Collect stool samples into plain tubes and store them refrigerated at 2-8°C for at least 6 days.
The extracts are stable for at least 7 days at 2-8ºC and for at least 24 months at -20ºC.
Important: The sample must be collected without any chemical or biological additions in the collection device.

STANDARDIZATION

The test is calibrated against purified MRP8/14 from human granulocytes.

ASSAY PROCEDURE

Extraction

Standard extraction procedure (refer to page 39)

1. Label and weigh (tare) the empty polypropylene tube together with the inoculation loop.
2. Take out 50 to 100 mg of the stool sample by means of the inoculation loop and place it into the pre-weighted tube.
3. Estimate the net amount of sample, break off the inoculation loop and leave the lower part of the loop in the tube.
4. Add Extraction Buffer (49 times the weight volume) to the tube and close the tube:

Weight [mg] Stool	Volume [mL] Extraction Buffer
50	2.5
55	2.7
60	2.9
65	3.2
70	3.4
75	3.7
80	3.9
85	4.2
90	4.4
95	4.7
100	4.9

5. Homogenize the sample on a multi tube vortexer by vigorous shaking (at highest speed) for 30 minutes.
6. Transfer the homogenate into a 2 mL Eppendorf tube and centrifuge in a microcentrifuge for 5 minutes at 3'000 x g.
7. Take the supernatant into a fresh, labeled tube and continue with the ELISA procedure.

Extraction procedures using fecal extraction devices:
The extraction procedure is described and illustrated in the instruction for use delivered with the respective extraction device.

1. Fecal Extraction Device Roche (Code 10745804 322) or BÜHLMANN **Smart-Prep** (Code: B-CAL-RD): The extraction time (vortexing) can be reduced to 1 minute.
2. **ScheBo® Quick-Prep™** (Code B-CAL-SO50): The extraction tubes are prefilled with extraction buffer. The extraction time is about 10 minutes (vortexing).

After extraction, centrifuge the tubes for 5 minutes at 3'000 x g. Alternatively, transfer the homogenate into a 2 mL Eppendorf tube and centrifuge it in a microcentrifuge for 5 minutes at 3'000 x g.

Decant the supernatant into a fresh, labeled tube and continue with the ELISA procedure.

The respective extraction procedures are published on the website:
http://www.buhlmannlabs.ch/core/inflammation/calprotectin/

ELISA PROCEDURES

The assay can be performed according to the following procedures – lower or extended range ELISA procedure. Which procedure is to be chosen depends on the expected Calprotectin concentration of the samples. For samples up to 600 µg/g choose the lower range procedure using a sample dilution of 1:50 (working range 10 – 600 µg/g) (refer to page 4, 5). If the samples tend to exceed 600 µg/g, choose the extended range procedure using a sample dilution of 1:150 (working range 30 – 1800 µg/g) (refer to page 5).

LOWER RANGE ELISA PROCEDURE

WORKING RANGE 10 – 600 µg/g
Allow the reagents to equilibrate to 18-28°C prior to use

1. Dilute the stool extracts 1:50 with Incubation Buffer (e.g. 20 µL extract and 980 µl incubation buffer) and mix well. Let the samples equilibrate for at least 5 minutes at 18-28°C prior to proceeding to step 4c.
2. Prepare a plate with sufficient strips to test the required number of calibrators, controls and diluted samples. Remove excess strips from the holder and re-seal them in the foil pouch together with the desiccant packs **without delay**. Store refrigerated.
3. Wash the coated wells twice using at least 300 µl of Wash Buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

Important: For every of the three wash steps a minimal **incubation time of at least 20 seconds of the Wash Buffer** in the wells must be ensured (see Technical Precautions – ELISA Procedure).

4a. Pipet 100 µl of Incubation Buffer (Blank)

Pipet 100 µl of Calibrator A-E into the respective wells.

4b. Pipet 100 µl of the Low and High Controls into the respective wells.

4c. Pipet 100 µl of each diluted sample into the subsequent wells.

5. Cover the plate with a plate sealer, and incubate for 30 + 5 minutes on a plate rotator set at 400-600 rpm at 18-28°C (see Technical Precautions - ELISA Procedure).
6. Remove and discard the plate sealer. Empty the wells and wash three times using at least 300 µl of Wash Buffer per well (see Technical Precautions – ELISA Procedure). Empty the wells and tap the plate firmly onto blotting paper.
7. Pipet 100 µl of Enzyme Label to all wells.
8. Cover the plate with a plate sealer, and incubate for 30 ± 5 minutes on a plate rotator set at 400-600 rpm at 18-28°C.
9. Remove and discard the Plate Sealer. Empty the wells and **wash five times** using at least 300 µl of Wash Buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

Important: Allow the TMB Substrate Solution to equilibrate to 18-28°C.

10. Pipet 100 µl of the TMB Substrate Solution to all wells.
11. Cover the plate with a plate sealer, protect the plate from direct light and incubate for 15 ± 2 minutes on a plate rotator set at 400-600 rpm at 18-28°C.
12. Pipet 100 µL of Stop Solution to all wells. Remove air bubbles with a pipette tip. Proceed to step 13 within 30 minutes.
13. Read the absorbance at 450 nm in a microtiter plate reader.

RESULTS & CALCULATION

WORKING RANGE 10 – 600 µg/g

Standard Curve: Record the absorbance at 450 nm for each calibrator and the blank. Substract the blank OD. Plot the absorbance (vertical axis) versus the Calprotectin concentration of the calibrators (horizontal axis) using a semi logarithmic lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four parameter logistic.
Samples and Controls: Record the absorbance at 450 nm for each sample and control. Subtract the blank value. Locate the corrected absorbance value of the sample on the vertical axis, draw a horizontal line intersecting the standard curve and read the Calprotectin concentration from the horizontal axis.
If you choose the lower range ELISA procedure, the following calibrator concentrations have to be used in the respective ELISA protocol: **10, 30, 100, 300 and 600 µg/g of Calprotectin.**
Additional dilution factors have to be multiplied with the results to obtain the final results.

Refer to Table 19 and Figure 1 for typical data (results and standard curve). *These results and standard curve are provided for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.*

PERFORMANCE CHARACTERISTICS

Assay performance characteristics have been established in duplicates.

WORKING RANGE: 10 – 600 µg/g

Intra-Assay Precision: 4.7 %. The intra-assay precision was calculated from 20 pairs of values from 3 extracted stool samples assayed in a single run according to the assay procedure. The values are presented in Table 20.
Inter-Assay Precision: <15 %. The inter-assay precision of the ELISA was calculated from 5 extracted stool samples. The aliquots were tested according to the assay procedure in 10 different runs by three technicians using 2 kit lots in two different labs. The values are presented in Table 21.
Detection limit (LoB): <10 µg/g. Twenty duplicates of Incubation Buffer were assayed in a single run. Mean and standard deviation were calculated for the absorbance values. The minimal detectable dose of Calprotectin was calculated to be clearly below Calibrator A (10 µg/g) by adding two standard deviations to the mean absorbance and intersecting this value with the standard curve obtained in a new run.
Detection limit (LoQ): <10 µg/g. Ten stool samples with values between 5.2 and 1254 µg/g Calprotectin were assayed 20 times in duplicates in one assay. The %CV and the mean values were calculated for each sample. The functional sensitivity was observed at 15 % CV. The resulting precision profile (Figure 2) allows the precise measurement within the whole standard range between 10 and 600 µg/g.
Dilution Linearity: 103 %. Seven stool samples with elevated Calprotectin values were extracted according to the assay procedure. The extracts were diluted with Incubation

Buffer and subsequently assayed according to the assay procedure. The expected values were calculated from the observed value found with the first dilution. The results are presented in Table 22.

Spiking Recovery: 100 %. Two extracted stool samples were spiked with different amounts of diluted, Calprotectin containing human serum. The samples were measured before and after spiking according the assay procedure. The results are presented in Table 23.

Crossreactivity: <0.1 %. Incubation Buffer spiked with different amounts of recombinant MRP8 and MRP14 were measured according to the assay procedure. The values are presented in Table 28.

EXTENDED RANGE ELISA PROCEDURE

Working Range 30 – 1800 µg/g
Allow the reagents to equilibrate to 18-28°C prior to use
The working range can be extended by a factor of 3, if you dilute the samples 1:150 instead of 1:50. This procedure is recommended, if high Calprotectin concentrations are to be expected. Precision and linearity of the assay allow for this extension of the kit range.

1. Dilute the stool extracts 1:150 with Incubation Buffer (e.g. 20 µL extract and 2980 µL incubation buffer) and mix well. **NB: Only dilute stool extracts. Standards and controls are ready to use.** Let the samples equilibrate for at least 5 minutes at 18-28°C prior to proceeding to step 4c.
2. Prepare a plate with sufficient strips to test the required number of calibrators, controls and diluted samples. Remove excess strips from the holder and re-seal them in the foil pouch together with the desiccant packs **without delay**. Store refrigerated.
3. Wash the coated wells twice using at least 300 µL of Wash Buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

Important: For every of the three wash steps a minimal **incubation time of at least 20 seconds of the Wash Buffer** in the wells must be ensured (see Technical Precautions – ELISA Procedure).

4a Pipet 100 µL of Incubation Buffer (Blank).

Pipet 100 µL of Calibrator A-E into the respective wells.

4b. Pipet 100 µL of the Low and High Controls into the respective wells.

4c. Pipet 100 µL of each diluted sample into the subsequent wells.

5. Cover the plate with a plate sealer, and incubate for 30 + 5 minutes on a plate rotator set at 400-600 rpm at 18-28°C (see Technical Precautions – ELISA Procedure).
6. Remove and discard the plate sealer. Empty the wells and wash three times using at least 300 µL of Wash Buffer per well (see Technical Precautions – ELISA Procedure). Empty the wells and tap the plate firmly onto blotting paper.
7. Pipet 100 µL of Enzyme Label to each well.
8. Cover the plate with a plate sealer, and incubate for 30 ± 5 minutes on a plate rotator set at 400-600 rpm at 18-28°C.
9. Remove and discard the Plate Sealer. Empty the wells and **wash five times** using at least 300 µL of Wash Buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

Important: Allow the TMB Substrate Solution to equilibrate to 18-28°C.

10. Pipet 100 µL of the TMB Substrate Solution to each well.
11. Cover the plate with a plate sealer, protect the plate from direct light and incubate for 15 ± 2 minutes on a plate rotator set at 400-600 rpm at 18-28°C.
12. Pipet 100 µL of Stop Solution to each well. Remove air bubbles with a pipette tip. Proceed to step 13 within 30 minutes.
13. Read the absorbance at 450 nm in a microtiter plate reader.

RESULTS

WORKING RANGE 30 – 1800 µg/g

Standard Curve: Record the absorbance at 450 nm for each calibrator and the blank. Substract the blank value. Plot the absorbance (vertical axis) versus the Calprotectin concentration of the calibrators (horizontal axis) using a semi logarithmic lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four parameter logistic.

Samples and Controls: Record the absorbance at 450 nm for each sample and control. Subtract the blank value. Locate the corrected absorbance value of the sample on the vertical axis, draw a horizontal line intersecting the standard curve and read the Calprotectin concentration from the horizontal axis. If you choose the extended range ELISA procedure, the following calibrator concentrations have to be used in the respective ELISA protocol: 30, 90, 300, 900 and 1800 µg/g Calprotectin.

Refer to Table 24 and Figure 3 for typical data (results and standard curve). *These results and standard curve are provided for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.*

PERFORMANCE CHARACTERISTICS

Assay performance characteristics have been established in duplicates.

WORKING RANGE: 30 – 1800 µg/g

Intra-Assay Precision: 4.0 %. The intra-assay precision (mean) was calculated from the results of 20 duplicates from 3 extracted stool samples assayed in a single run according to the assay procedure. The values are presented in Table 25.

Inter-Assay Precision: <15 %. The inter-assay precision of the ELISA was calculated from 5 extracted stool samples. The aliquots were tested according to the assay procedure in 10 different runs by three technicians using 2 kit lots in two different labs. The values are presented in Table 26.

Detection limit (LoQ): <30 µg/g. 18 stool samples with values between 10.8 and 2080 µg/g Calprotectin were measured 20 times in one assay. The % CV and the mean values were calculated for each sample. The LoQ was observed at 15 % CV. The resulting precision profile allows the precise measurement within the whole standard range from 30 to 1800 µg/g. The results are presented in Figure 4.

Dilution Linearity: 102 %. Five stool samples with elevated Calprotectin concentrations were extracted according to the assay procedure. The extracts were diluted with Incubation Buffer and subsequently assayed according to the assay procedure. The expected values were calculated from the observed value found with the first dilution. The results are presented in Table 27.

EXPECTED VALUES

Estimation of faecal Calprotectin is a reliable and easy way to distinguish organic from functional gastrointestinal diseases.

In a clinical study 401 symptomatic patients scheduled for colonoscopy were investigated. Endoscopy examination showed 273 patients with functional diseases whereas 128 patients had various organic diseases (colitis, Crohn's, ulcers, diverticulitis, polyps, adenomas, cancer, or infectious diseases).

ROC curve analysis (AUC: 0.935) resulted in an optimal clinical cut-off at 50 µg/g. Applying this cut-off, a clinical sensitivity and specificity of 84.4% and 94.5%, respectively can be reached in the differentiation between organic and functional diseases (see Table 29).

Faecal Calprotectin levels from adults and children are comparable, whereas levels of newborns can be significantly increased (8).

These data support the following recommendation for interpretation of results:

Normal values below 50 µg/g:

Calprotectin values <50 µg/g are not indicative of inflammation in the gastrointestinal tract. Patients with low Calprotectin levels are likely not to be in need of invasive procedures to determine the inflammation cause (12).

Elevated values between 50 and 200 µg/g:

Calprotectin values between 50 and 200 µg/g can represent mild organic disease such as inflammation caused by NSAIDs, mild diverticulitis and IBD in remission phase. The low inflammatory response shown within this range may suggest repeating the measurement and performing further investigations.

Elevated values above 200 µg/g:

Calprotectin values > 200 µg/g are indicative of active organic disease with inflammation in the gastrointestinal tract. Appropriate further investigative and curative procedures by specialists are suggested.

The cut-off level suggested for adults (50 µg/g) can also be used for children aged from 4 to 17 years regardless of sex (9).

QUALITY CONTROL

A thorough understanding of this instruction for use is necessary for the successful use of the product. Reliable results will be obtained only by using precise laboratory techniques (current GLP guidelines) and accurately following this instruction for use.

Since there is no control for Calprotectin commercially available, we recommend using a pool of positive stool extractions for internal quality control.

The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the Controls are lot-specific and printed on the additional QC data sheet.

If the precision of the assay does not correlate with the established limits and repetition excludes errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices ii) ELISA reader settings iii) expiration dates of reagents iv) storage and incubation conditions v) TMB Substrate Solution should be colorless vi) purity of water.

PERFORMANCE LIMITATIONS

- Reagents delivered with this kit are being optimized for the determination of Calprotectin from human stool samples.
- Test results should be interpreted in conjunction with information available from clinical assessment of the patient and other diagnostic procedures.

APPENDIX I

TABLES/ TABELLEN/ TABLES/ TABELLE/ TABLAS

LOWER RANGE PROCEDURE 10 - 600 µg/g

Table 19: **Example of Results**

	Conc. [µg/g]	Absorb. [OD]	Calc. Conc. [µg/g]	CV Conc [%]
Blank Avg.		**0.096**		
Cal A	10	0.073		
Cal A	10	0.066		
Cal A Avg.	**10**	**0.069**		**7.2**
Cal B	30	0.143		
Cal B	30	0.153		
Cal B Avg.	**30**	**0.148**		**4.8**
Cal C	100	0.465		
Cal C	100	0.456		
Cal C Avg.	**100**	**0.460**		**1.4**
Cal D	300	1.121		
Cal D	300	1.135		
Cal D Avg.	**300**	**1.128**		**0.9**
Cal E	600	1.658		
Cal E	600	1.671		
Cal E Avg.	**600**	**1.664**		**0.6**
Ctrl Low		0.201	41	
Ctrl Low		0.189	39	
Ctrl Low Avg.		**0.195**	**40**	**4.4**
Ctrl High		0.598	134	
Ctrl High		0.583	130	
Ctrl High Avg.		**0.590**	**132**	**1.8**

Figure 1: **Example of a Standard Curve**

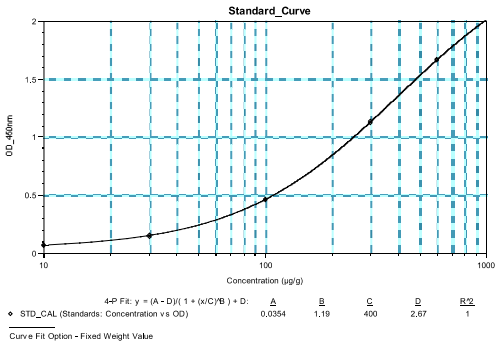


Table 20: **Intra-Assay Precision**

Sample	Mean [µg/g]	SD [µg/g]	CV [%]
low	52.5	1.4	2.7
medium	173.8	5.6	3.2
high	408.5	33.0	8.1
Mean			**4.7**

Table 21: **Inter-Assay Precision**

Sample	Mean [µg/g]	SD [µg/g]	CV [%]
Sample 1	18.1	2.5	13.5
Sample 2	44.5	6.4	14.5
Sample 3	74.3	7.9	10.7
Sample 4	227	15.0	6.6
Sample 5	520	57.8	11.1
Mean			**11.3**

Figure 2: **Precision Profile**

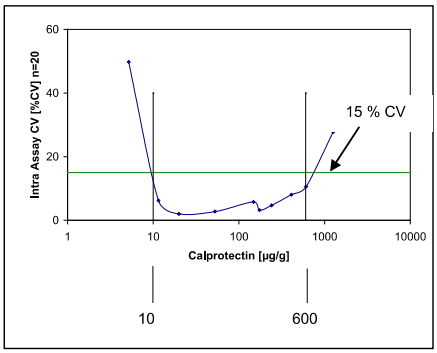


Table 22: **Dilution Linearity/Parallelism**

Sample	Dilution	Observed [µg/g]	Expected [µg/g]	O/E [%]
S 1	1:200	405	-	–
	1:400	182	202	90
	1:800	95	101	94
	1:1600	49	51	98
	1:3200	25	25	99
	1:6400	15.6	12.7	123
	1:12800	6.6	6.3	104
	Mean			**101**
S 2	1:50	232	-	–
	1:100	124	116	107
	1:200	61	58	106
	1:400	28	29	96
	1:800	12	15	86
	Mean			**98**
S 3	1:400	290	-	–
	1:800	145	145	100
	1:1600	73	72	100
	1:3200	39	36	106
	1:6400	19	18	103
	Mean			**103**
S 4	Mean			**100**
S 5	Mean			**124**
S 6	Mean			**88**
S 7	Mean			**106**
Mean				**103**

Table 23: **Spiking Recovery**

Sample	µg/g	spiked with [µg/g]	Observed [µg/g]	Expected [µg/g]	O/E [%]
S 1	5.3	10	13.6	15.3	88.9
		30	33.6	35.3	95.2
		100	101	105	96.1
		150	147	155	94.5
		300	287	305	94.1
		400	468	405	115.4
	Mean				**97.4**
S 2	24.6	10	32.9	34.6	95.1
		30	49.2	54.6	90.1
		100	139	125	111.5
		150	176	175	100.5
		300	358	325	110.3
		400	467	425	109.9
	Mean				**103**
Mean					**100**

Table 28* **Cross Reactivity**

Spiked with	**MRP8** [ng/mL]	**MRP14** [ng/mL]
100 µg/mL	26.0	38.7
10 µg/mL	8.0	3.4
1 µg/mL	<4.0	<4.0
100 ng/mL	<4.0	<4.0
10 ng/mL	<4.0	<4.0

Table 29* **Clinical Study**

	Calprotectin (EK-CAL)	Lactoferrin
n	401	391
cut-off	50 µg/g	3 µg/mL
Sensitivity	84.4%	74.2%
Specificity	94.5%	91.0%
PPV	87.8%	79.3%
NPV	92.8%	88.4%
LR+	15.4	0.17
LR-	8.25	0.28

* Data have been established with the lower range ELISA procedure

1.21. Appendix 6: Methodology for stool tryptase

1. Stool supernatant

   a) Before processing starts, stool sample was defrosted for an hour prior

   b) 1g of stool sample were transferred into a Falcon Tube before adding 5 ml of Tris Buffer and vortexed until stool has dissolved. Samples had to be sonicate if samples were not dissolved initially by vortexing.

   c) These samples was placed into a centrifuge at 1000 x g at room temperature for 15 minutes

   d) Supernatants obtained was stored in the -80 ^{0}C freezer until further processing

2. Stool filtration

   a) Stool supernatant was spun at 15000 x g for 5 minutes at room temperature.

   b) The spun stool supernatants was transferred to a 0.45 ul filter (Spin-X centrifuge tube filters pore size 0.45ul by SIGMA CLS8163)

   c) Filtrate was spun at 10 000 x g for 2 minutes

   d) The stool supernatant was transferred to a 0.22 ul filter (Spin-X centrifuge tube filters pore size 0.22 ul by SIGMA CLS8161) and was placed into the centrifuge at 10 000 x g for 2 minutes.

   e) This processed was repeated until all stool supernatants have passed through the 2 filters

3. Procedure for Serine Protease Assay

   a) 0.1mL TBS was added into each (Reaction) microplate well.

b) 0.1mL Trypsin (at 10-fold excess of previously optimised upper concentration) was added into wells E1, F1 and G1). It was diluted into 2-fold serially to column-12 and residual 0.1mL discarded.

c) 0.1mL was added into each test stool extract to A to C, 1, 5 and 9 and then diluted each serially by 2 fold 1-4, 5-8 and 9-12 respectively, discarded residual 0.1mL.

d) 0.1mL 2% (w/v) azo-casein was added into each well and it was mixed briefly using an orbital shaker or tapping by hand.

e) Wells were sealed using an adhesive film-seals and transfer to a 37^{o}C incubator for 30 minutes.

f) Film seal was removed and 0.1 mL 10% (v/v) TCA added into each well. Solution was mixed briefly using an orbital shaker or tapping by hand.

g) The well were re-sealed and incubated for 2 minutes at room temperature.

h) The microplates were centrifuged.

i) 150ul supernatant was transferred into the corresponding wells of the analytical microplate.

j) The absorbance was read at 440nm.

k) Protease activity was expressed as units per mg of protein against activity elicted by 1Unit of standard trypsin.

4. Procedure for Bradford Assay

a) 150ul water was added into to each microplate well, allowing 1 plate per 18 (or 6 triplicate) test samples

b) 150ul 1mg/ml BSA was added into to wells 1A and 1B. Dilute 2-fold serially to column 12 and discard residual 150ul to waste

c) 150ul distilled water was added into C1, C5 and C9

d) 150ul was added into each test stool extract and then the solution was diluted each serially 2-fold and residual 150ul discarded. E.g. test 1 in D1-D4, test 2 to D5-D8, test 3 to D9-D12

e) 150ul Bradford reagent was added into each well and mixed briefly using an orbital shaker or by tapping by hand.

f) The plate was incubated at room temp for 15 min.

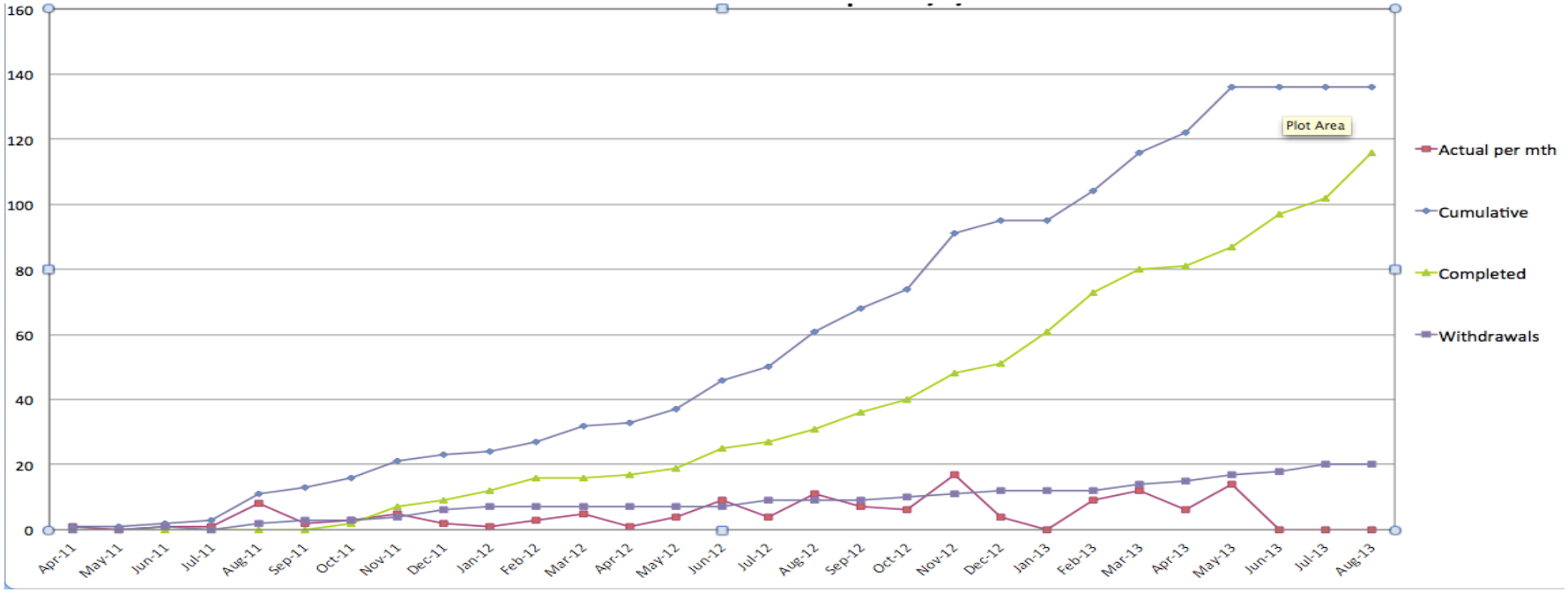
g) Whilst incubation was happening, 100ul of plate contents was transferred into a new plate (flat bottom ELISA style).

h) Absorbance was read at 595/600nm

1.22. Appendix 7: Supplementary results

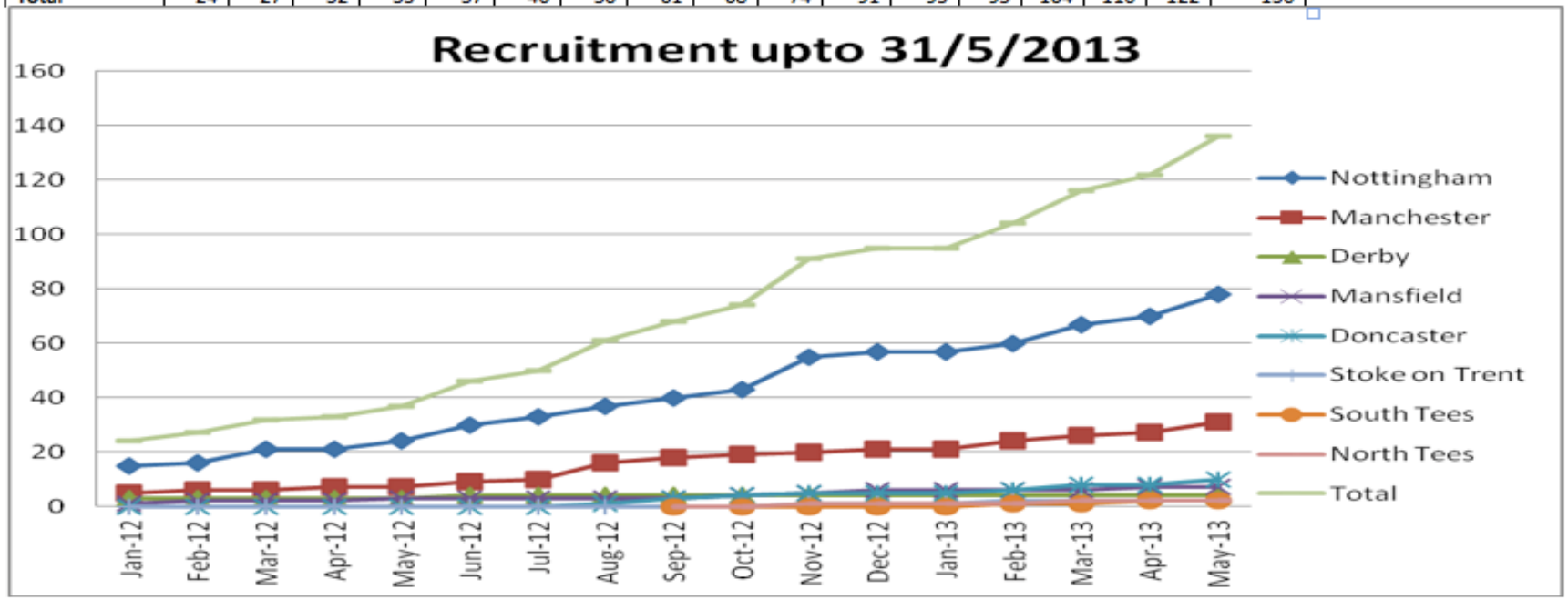
Supplementary table 1: Recruitment into study

MONTH	Apr 11	May 11	Jun 11	Jul 11	Aug 11	Sep 11	Oct 11	Nov 11	Dec 11	Jan 12	Feb 12	Mar 12	Apr 12	May 12	Jun 12	Jul 12	Aug 12	Sep 12	Oct 12	Nov 12	Dec 12	Jan 13	Feb 13	Mar 13	Apr 13	May 13	Jun 13	Jul 13	Aug 13
Actual per mth	1	0	1	1	8	2	3	5	2	1	3	5	1	4	9	4	11	7	6	17	4	0	9	12	6	14	0	0	0
Cumulative	1	1	2	3	11	13	16	21	23	24	27	32	33	37	46	50	61	68	74	91	95	95	104	116	122	136	136	136	136
Completed	0	0	0	0	0	0	2	7	9	12	16	16	17	19	25	27	31	36	40	48	51	61	73	80	81	87	97	102	116
Withdrawals	0	0	1	0	2	3	3	4	6	7	7	7	7	7	7	9	9	9	10	11	12	12	12	14	15	17	18	20	20


160
140
120
100
80
60
40
20
0
Plot Area
Actual per mth
Cumulative
Completed
Withdrawals
Apr-11
May-11
Jun-11
Jul-11
Aug-11
Sep-11
Oct-11
Nov-11
Dec-11
Jan-12
Feb-12
Mar-12
Apr-12
May-12
Jun-12
Jul-12
Aug-12
Sep-12
Oct-12
Nov-12
Dec-12
Jan-13
Feb-13
Mar-13
Apr-13
May-13
Jun-13
Jul-13
Aug-13

Supplementary table 2: Recruitment for each site

MONTH	Jan-12	Feb-12	Mar-12	Apr-12	May-12	Jun-12	Jul-12	Aug-12	Sep-12	Oct-12	Nov-12	Dec-12	Jan-13	Feb-13	Mar-13	Apr-13	May-13
Nottingham	15	16	21	21	24	30	33	37	40	43	55	57	57	60	67	70	78
Manchester	5	6	6	7	7	9	10	16	18	19	20	21	21	24	26	27	31
Derby	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4
Mansfield	1	2	2	2	3	3	3	3	3	4	5	6	6	6	6	7	7
Doncaster	0	0	0	0	0	0	0	1	3	4	5	5	5	6	8	8	10
Stoke on Trent	0	0	0	0	0	0	0	0	0	0	1	1	1	2	2	2	2
South Tees									0	0	0	0	0	1	1	2	2
North Tees									0	0	1	1	1	1	2	2	2
Total	24	27	32	33	37	46	50	61	68	74	91	95	95	104	116	122	136



Supplementary table 3: Summary of number of days with stool diary entered at baseline and 11-12 weeks

	Number of days with stool diary recorded: **Mean (SD)** **Median [IQR]**
Baseline	13.9 (0.3) 14 (14,14)
11-12 weeks	13.8 (1.2) 14 (14,14)