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The Origins of Pain in Diverticular Disease: Peripheral or Central?

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BA MB BChir MRCS (Eng)

Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

August 2013

Dedication

In memory of George Sidney Davies.....

...and to my wonderful parents (Janet and John Smih), grandmother (Nancy Davies) and boyfriend
(HP Mok) for their endless encouragement.

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Smith J, Humes DJ, Spiller RC. Should we treat uncomplicated symptomatic diverticular disease with fibre? *BMJ.* 2011 May 25;342:d2951. PMID: 21613363

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Humes D, Smith JK, Spiller RC. Colonic diverticular disease. *Clin Evid (Online).* 2011 Mar 14;2011. PMID: 21401970

Acronyms and abbreviations

Abbreviation	Description
12-HETE	12-hydroxyeicosatetraenoic acid
2-AG	2-arachidonoyl glycerol
5-ASA	5-aminosalicylic acid
5HT	Serotonin
AA	Arachidonic Acid
ACC	Anterior Cingulate Cortex
ACTB	Beta-Actin gene
ADD	Asymptomatic diverticular disease
AEA	Arachidonoyl ethanolamide
aINS	Anterior Insula
ALOX12	12-Lipoxygenase
ALOX15	15-Lipoxygenase
ALOX15B	15-Lipoxygenase B
ALOX5	5-Lipoxygenase
ALOX5AP	Arachidonate 5-Lipoxygenase activating protein
AL-PFC	Anterolateral Prefrontal cortex
AMYG	Amygdala
ANS	Autonomic Nervous System
ASIC	Acid Sensing Ion Channels
ASL	Arterial Spin Labelling
ATP	Adenosine-5'-triphosphate
BA	Beta-Actin
BCL-2	B Cell lymphoma 2
BDGF	Brain Derived Growth Factor
BDKRB2	Bradykinin receptor 2
BMI	Body Mass Index
CALCA	Calcitonin-related polypeptide alpha
CALCB	Calcitonin-related polypeptide beta
CCL11	Chemokine (C-C motif) ligand 13 or eotaxin-1
CCL13	Chemokine (C-C motif) ligand 13 or MCP-4
CCL2	Chemokine (C-C motif) ligand 2 or Monocyte Chemotactic protein 1 (MCP-1)
CDH1	Cadherin-1

Abbreviation	Description
CGRP	Calcitonin gene related peptide
CHEPs	Contact Heat- Evoked Potential Stimulator
CMKLR1	Chemokine-like receptor 1 or ChemR23
CNR1	Endocannabinoid receptor 1
CNR2	Endocannabinoid receptor 2
Coag	Coagulation
CRHR	Corticotrophin receptor
CTPA	CT Pulmonary Arteriogram
CYP2J2	Cytochrome P450, family 2, subfamily J
DBS	Dorsal brainstem
DD	Diverticular Disease
DEPC	Diethyl pyrocarbonate
DL-PFC	Dorsolateral Prefrontal cortex
DNIC	Descending nociceptive inhibitory or facilitatory controls
DTI	Diffusion tensor imaging
ENS	Enteric nervous system
EPHX2	Epoxide hydrolase 2 (SEH)
EPI	Echo-planar imaging
F2RL1	Protease activated receptor 2 PAR2
F2RL3	Protease activated receptor 4 PAR4
FAAH	Fatty acid amide hydrolase
FBC	Full blood count
FC	Faecal calprotectin
FD	Functional dyspeptics
FDR	False Discovery Rate
FM	Fibromyalgia
fMRI	Functional magnetic resonance imaging
FPR2	Formyl peptide receptor 2
GALR1	Galanin receptor 1
GALR2	Galanin receptor 2
GLM	General linear model
GDNF	Glial cell line-derived neurotrophic factor
GP	General practitioner
HAD	Hospital Anxiety and Depression scale

Abbreviation	Description
HSDD	High somatising symptomatic diverticular disease
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HpTH	Hypothalamus
HTR3A	5HT 3A receptor
HTR3B	5HT 3B receptor
HTR4	5HT 4 receptor
IBS	Irritable bowel syndrome
IBS-D	Diarrhoea predominant IBS
ICAM1	Intercellular adhesion molecule 1
IFG	Inferior frontal gyrus
IFNG	Interferon, gamma
IHC	Immunohistochemistry
IL10	Interleukin 10
IL13	Interleukin 13
IL17A	Interleukin 17A
IL1B	Interleukin 1b
IL1RN	Interleukin 1 receptor antagonist
IL6	Interleukin 6
IL8	Interleukin 8
INS	Insula
IQR	Inter-quartile range
KITLG	KIT ligand
LA	Linoleic acid
LC	Locus coeruleus
LFTs	Liver function tests
IPFC	Lateral-Prefrontal Cortex
LTA4H	Leukotriene A4 synthase
LTB4R	Leukotriene B4 receptor
LTC4S	Leulotriene C4 synthase
LTE₄	Leukotriene-E ₄
MAdCAM1	Mucosal addressin adhesion marker-1
MCC	Mid Cingulate Cortex
MCL-1	Myeloid cell leukaemia sequence 1

Abbreviation	Description
MEG	Magnetoencephalography
MGLL	Monoglyceride lipase
mINS	Middle Insula
MMP	Matrix metalloproteinases
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
mPFC	Medial Prefrontal Cortex
MUC1	Mucin 1, cell surface associated
MUC3A	Mucin 3, cell surface associated
MWU	Mann Whitney U
MYD88	Myeloid differentiation primary response protein 88
N	Number of samples
NAPEPLD	N-acyl phosphatidylethanolamine phospholipase D
NCF	Nucleus cuneiformis
NFkB	Nuclear factor kappa B
NGF	Nerve Growth Factor
NGF	Nerve growth factor (beta polypeptide)
NGFR	Nerve growth factor receptor
NK1R	Neurokinin receptor 1 (Substance P receptor)
NOD2	Nucleotide-binding oligomerization domain containing 2
NOS	Nitric oxide synthase
NOS2	Nitric oxide synthase 2, inducible
NSAIDs	Non-steroidal anti-inflammatory drugs
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1
°C	Degrees centigrade
OEA	N-oleoyl ethanolamide
OFC	Orbitofrontal cortex
OR	Odds Ratio
PACAP	Pituitary adenylate cyclase activated protein
PAG	Periaqueductal gray
PARM 1	Prostate androgen regulated mucin like protein 1
PBN	Parabrachial nucleus
PCC	Posterior cingulate cortex
PCRN	Primary Care Research Network

Abbreviation	Description
PCS	Pain catastrophizing score
PDE4B	Phosphodiesterase 4B,
PDE4D	Phosphodiesterase 4D,
PEA	N-palmitoyl ethanolamide
PFC	Prefrontal cortex
pgACC	Perigenual ACC
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PHQ12	Personal Health Questionnaire 12
pINS	Posterior Insula
PLA2	Phosphatidolipase
PO	Parietal operculum
PPARG	Peroxisome proliferator-activated receptor gamma
PPAR-gamma	Peroxisome proliferator-activated receptor gamma
PRDM1	PR domain zinc finger protein
PTGER1	Prostaglandin E receptor 1
PTGER1	Prostaglandin E receptor 1
PTGER3	Prostaglandin E receptor 3
PTGES	Prostaglandin E synthase
PTGES2	Prostaglandin E synthase 2
PTGS1	Prostaglandin-endoperoxide synthase 1
PTGS2	Prostaglandin-endoperoxide synthase 2
RFX	Random effects
RPLPO	Ribosomal protein large PO
RPLPO	Ribosomal protein, large, P0,Gene
RQ	Relative quantification
RR	Relative Risk
RVM	Rostroventral medulla
S1	Primary somatosensory cortices
S2	Secondary somatosensory cortices
SDD	Symptomatic Diverticular disease
SELE	E-selectin

Abbreviation	Description
SES-CD	Simple Endoscopic Score for Crohn's disease
SLC6A4	Serotonin transporter
SMA	Supplemental Motor Area
SOD1	Superoxide dismutase
SOD1	Superoxide dismutase 1
SS-CRP	Super sensitive C-reactive protein
TACR1	Tachykinin receptor 1
TACR2	Tachykinin receptor 2
TBNS	Trinitrobenzene sulphonic acid
TBXA2R	Thromboxane A2 receptor
TBXAS1	Thromboxane synthase 1
TBXAS1	Thromboxane A synthase 1 (platelet)
TGFB1	Transforming growth factor beta
TGFBR1	Transforming growth factor, beta receptor 1
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)
Thal	Thalamus
TIMP	Tissue inhibitors to matrix metalloproteinases
TJP1	Tight junction protein 1 (zona occludens 1)
TJP2	Tight junction protein 2 (zona occludens 2)
TLR	Toll Like Receptor
TLR2	Toll like receptor 2
TLR4	Toll like receptor 4
TLR5	Toll like receptor 5
TLR7	Toll like receptor 7
TLR8	Toll like receptor 8
TLR9	Toll like receptor 9
TMS	Trans-cranial magnetic stimulation
TNF	Tumor necrosis factor
TNF-alpha	Tumor necrosis factor alpha
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
TNFSF10A	Tumor necrosis factor (ligand) superfamily, member 10A
TNFSF15	Tumour necrosis ligand superfamily 15
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15
TOLLIP	Toll interacting protein

Abbreviation	Description
TPH1	Tryptophan hydroxylase 1
TPH1	Tryptophan hydroxylase 1
TRKa	Neurotrophic tyrosine kinase receptor type 1 (Nerve Growth Factor receptor)
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1
TRPV4	Transient receptor potential vanilloid 4
TXB₂	Thromboxane-B ₂
UC	Ulcerative colitis
UCL	University College London
UE	Urea and Electrolytes
VAS	Visual Analogue Score
VCAM1	Vascular cell adhesion marker-1
VIP	Vasoactive intestinal peptide
VL-PFC	Ventrolateral prefrontal cortex
VM-PFC	Ventromedial prefrontal cortex
ZO-1	Zona occludens 1
ZO-2	Zona occludens 2

Abstract

This study was designed to identify the processes which underlie pain in symptomatic diverticular disease (SDD). Our hypothesis was that a spectrum of both peripheral and central pathologies were involved, with those that had a more peripheral problem having abdominal symptoms only while those with multiple symptoms throughout the body, having an altered central pain processing. The first study examining the brain response to cutaneous pain using functional magnetic resonance imaging (fMRI) has supported this hypothesis. Although a statistically significant difference in sensory pain threshold was not demonstrated between the groups, fMRI imaging has shown greater emotional processing during pain and reduced anticipatory inhibitory responses in the high somatising symptomatic diverticular disease (HSDD) groups. However this is not as clear cut as we had anticipated which may be due to subject selection and demonstrate a spectrum of mixed peripheral and central changes as well as those with only peripheral or central components.

In the second part we performed a randomized placebo controlled study of mesalazine 3gm versus placebo. Mesalazine significantly reduced expression of many genes associated with inflammation in SDD patients. A reduction in the median number of hours of pain per week was seen. The study was not designed to allow intention to treat analysis but has shown promising results which will need to be consolidated with future large scale studies.

Both these studies support a tailored approach to SDD patient treatment based on the underlying pain process which can be both central and peripheral. The Patient health questionnaire 12 (PHQ12) may be one simple measure of doing this, but again needs to be confirmed with further larger studies.

Chapter 1: Introduction

1.1 Definition and Incidence

Colonic diverticulosis is the most common structural abnormality of the colon, yet our understanding of it is rudimentary. It affects 5% of people in their 5th decade and up to 66% of the elderly population in the United Kingdom. It is responsible for substantial morbidity with 68,000 hospital admissions recorded per year in the UK and it contributes to about 2,000 deaths.

The definitions of diverticulosis and diverticular disease were established by the European Association for Endoscopic Surgery consensus development meeting in 1999¹:

“Colonic diverticular disease is a condition seen mostly in the sigmoid region. It is characterized structurally by mucosal herniation through the colonic wall, generally accompanied by muscular thickening, elastosis of the taenia coli, and mucosal folding. This condition may be asymptomatic (diverticulosis) or associated with “symptoms,” termed diverticular disease, which may be complicated or uncomplicated. The term diverticulitis is used to indicate superadded inflammation involving the bowel wall. Other pathologic complications include perforation, fistula, obstruction, and bleeding.”

Studies using national databases of hospital admissions suggest its incidence and/or complications are increasing²⁻⁴. A recent study from the United States reported a 26% increase in admission for acute diverticulitis between 1998 and 2005. The rise in admission rates were greatest in younger patients e.g. 45-64years and 18-44 years². In the 2004 National Hospital Discharge Survey in the United States America (USA), diverticular disease was responsible for 312,000 admissions and 1.5 million days of hospital care⁵ at a cost of 2.6 billion US dollars per year⁶. This makes diverticular disease the 5th most costly gastrointestinal condition in the USA after gastro-oesophageal reflux disease, gallbladder disease, colorectal cancer and peptic ulcer disease⁶. The changing burden and

complications of disease, changing management and subsequent cost is likely to increase further as western populations age^{4, 7, 8}.

1.2 Aetiology of development and symptoms

The mechanism by which diverticula develop is still not understood. A link with reduced dietary fibre has been identified since the 1960's⁹⁻¹¹. However the exact mechanism by which the mucosa herniates through the muscular wall of the colon, at the weak points where the blood vessels penetrate, to create the characteristic false diverticulum is still elusive. Several theories have been postulated, including:

1.2.1 Increased intra-luminal pressure

Based on the principle of Laplace's Law, decreased stool bulk leads to a reduced colonic diameter and requires greater wall tension to transmit the stool along the colon. The increased wall thickness in DD has been used to support this theory¹².

1.2.2 Segmentation

Excessive segmentation and uncoordinated contraction between the segments causes the raised intra-luminal pressure. Several motility studies have suggested that high pressure activity in the colon is more common in symptomatic DD patients and can be correlated with symptoms¹³. Electrophysiological activity has been reported to change with elevated activity in early and silent or low levels of activity in advanced DD cases¹⁴. When colonic muscle from DD patients is electrically and neurochemically stimulated, altered contraction and relaxation properties have been shown compared to controls¹⁵. However, the methodologies used between the studies vary and how well such models using resected muscle translate to clinical features is uncertain as the surgical manipulation and anaesthetic drugs used during surgery may well alter neuromuscular excitability. The alteration of the luminal contents of the gut, with bowel preparation, small numbers and poor

patient selection, different anatomical sites of measurement and limited duration of the studies also affect the ability to draw conclusions or extrapolate findings to the general population¹³. It has been suggested that the cause for bowel segmentation and altered colonic motility may be linked to age-related loss of nerves from the gastrointestinal tract¹⁶. However, conflicting results have been reported in the number of nerve fibres, ganglia and interstitial cells of Cajal in DD patients. Although animal models and histological studies in humans suggest decreased nerve density with age¹⁵, there is no evidence to link this directly with the development of diverticula.

1.2.3 Altered collagen and elastin deposition

Increased risk of diverticula has been linked to several connective tissue disorders such as Ehlers-Danlos¹⁷⁻¹⁹ and Marfans²⁰. Muscle wall thickening in DD is not due to hypertrophy of the longitudinal and circular muscle, but caused by deposition of elastin and collagen between the muscle fibres. Scarring from diverticulitis also changes the ratio of type I and III collagen²¹. Increased expression of matrix metalloproteinases (MMPs) and tissue inhibitors to matrix metalloproteinases (TIMPs) has been linked to disease severity^{21 22 23}. However, altered collagen and enzyme levels can also occur with inflammation and could be a complication rather than a cause of DD.

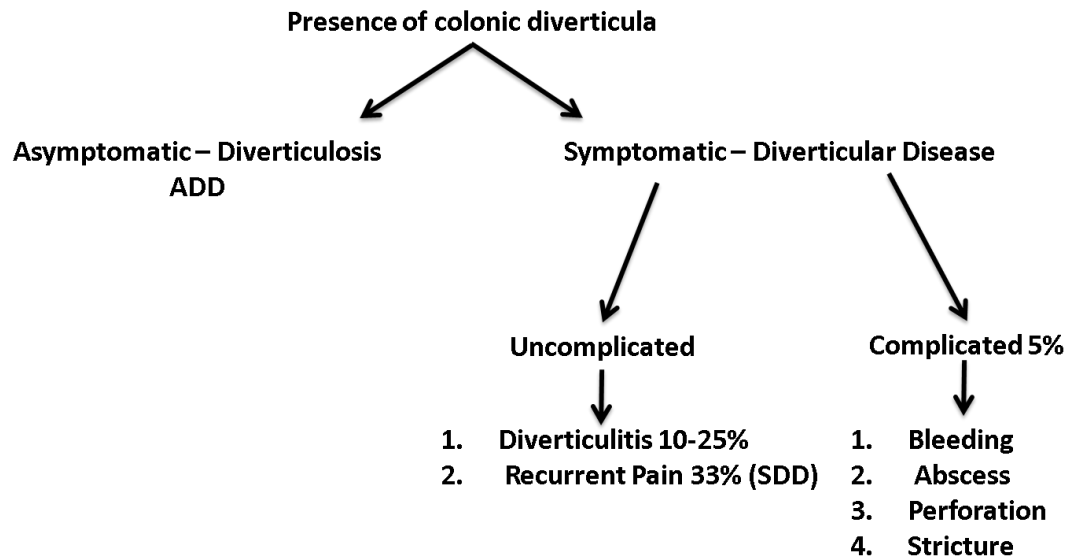
1.2.4 Genetics

A family history has been implicated by some groups. Recent studies using linked twin database and hospital records to show high concordance between monozygous twins, suggesting genetic link²⁴. However no gene linkage studies have so far been performed and are long overdue as these would be likely to throw light on disease mechanisms and encourage new lines of research.

1.3 Risk factors for developing diverticular disease

Diverticular disease can lead to a multitude of complications and has been classified into complicated and uncomplicated disease accordingly (see figure 1.1).

Figure 1.1 Complications of Diverticulosis and Diverticular disease²⁵⁻²⁷



In western countries it is most commonly found in the descending and sigmoid colon. Most research has concentrated on the 1-2% of the individuals with complicated disease, who often require hospital treatment. Research in this area has mainly focused on the epidemiology of these conditions or possible surgical treatments. However, for the vast majority of patients with uncomplicated diverticula, the condition is asymptomatic (ADD) and only a minority have recurrent episodes of chronic pain (SDD).

Little is known about the risk factors for developing symptomatic uncomplicated diverticular disease (SDD). In a study of patients diagnosed with diverticulosis on barium enema, a third of patients reported recurrent episodes of pain in the left iliac fossa lasting 1 or more hours and occurring on 3 or more days per month²⁶. The study suggested that a previous episode of inflammation, such as diverticulitis (RR 3.9), or psychological conditions can predispose people

with diverticular to develop chronic pain symptoms. The proportion of patients reporting pain was maintained over the 7 years between studies, demonstrating the prolonged morbidity, reduced quality of life and cost associated with this condition ^{28 29}.

Other risk factors for diverticular complications have been implicated from epidemiological studies, but there are no prospective studies so whether these are causative. These include: Low levels of physical activity, high BMI ^{30, 31 32}, and smoking ³³. Other suspected risk factors for diverticulitis include eating nuts, corn and pop-corn, but their association with complications has recently been questioned³⁴. NSAIDs, hypertension, hyperuricemia, steroids, use of calcium-channel blockers and anti-coagulants and patients with three concomitant metabolic diseases, including arteriosclerotic diseases, have increased the risk of diverticular bleeding^{35 36}. A genetic component is also suspected from epidemiological work³⁷. However it is not known if these also increase the risk of SDD.

It has been suggested that chronic pain in diverticular disease may in fact be a form of irritable bowel syndrome (IBS). Although there are many similarities between the conditions there are also several key differences as shown in figure 1.2

Figure 1.2 Similarities and differences between SDD and IBS

- | • Similarities | • Dissimilarities |
|---|---|
| <ul style="list-style-type: none">• Pain• Altered bowel habit<ul style="list-style-type: none">– e.g. frequency, consistency• Altered Psychological scores<ul style="list-style-type: none">– e.g. PHQ12-SS• Can be triggered by inflammatory event<ul style="list-style-type: none">– e.g. PI-IBS, diverticulitis• Visceral Hypersensitivity | <ul style="list-style-type: none">• Age – Peak onsets are different• SDD do not meet Rome Criteria• Structural alteration of bowel in DD• No evidence IBS leads to SDD |

There are several arguments against IBS and SDD being the same condition including:

- (i) The ROME criteria state that IBS is a diagnosis of exclusion occurring in a structurally normal bowel.
- (ii) Most patients with DD are much older than classical IBS patients, who are most frequently diagnosed in their 20s or 30s.
- (iii) DD patients' symptoms do not correspond to the precise ROME criteria, such as altered bowel habit corresponding with the pain, or relief with defecation.

For example, a questionnaire study from the USA has suggested an association between Rome II defined diarrhea predominant IBS and colonic diverticular disease³⁸, but only 5.6 - 14.2% of subjects met the Rome I criteria for this diagnosis in Humes et al's study²⁶. There is also no evidence that a prior history of IBS leads to the development of diverticula or chronic pain with diverticular disease. Thus although superficially the two conditions are similar and owing to their frequent occurrence may overlap, there are important differences which will be explored in the following text.

1.4 Pathophysiology of chronic pain in uncomplicated symptomatic diverticular disease

To understand how chronic pain may develop in diverticular disease it is first important to understand the normal pain pathways from the gut.

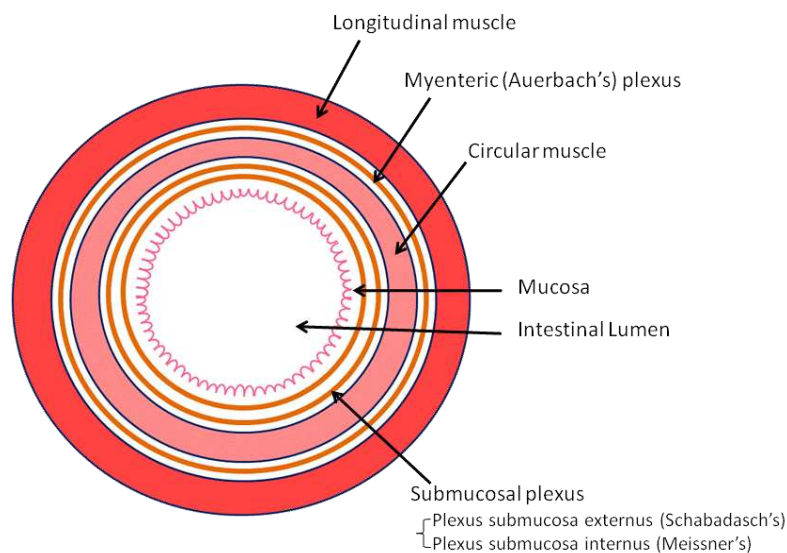
1.4.1 Anatomy of normal pain pathways

The pain pathways from the viscera and skin are similar and involve the cutaneous or enteric nervous system, afferent nerve fibres, spinal tracts and a variety of regions within the brain. These will be discussed in turn.

(i) The enteric nervous system

The nerve supply to the bowel is complex and poorly understood. Within the bowel, there are two plexus, the myenteric (Auerbach's)³⁹ and submucosal (Meissner's)⁴⁰. The submucosal plexus can be further subdivided into the internal (true Meissner's) and external (Schabadasch's)⁴¹⁻⁴⁴ (Figure 1.3).

Figure 1.3 Enteric nervous system of the bowel.



These interconnect extensively and provide sensation, through stretch and chemical receptors, and can control secretion of mucus and motility.

(ii) Afferent Fibres

Sensory information from the descending colon, sigmoid and rectum are relayed to the central nervous system by the sacral/pelvic and splanchnic afferent nerves. These innervations have endings which terminate in all layers of the bowel and can communicate extensively with the enteric nervous system, which makes identification of nociceptor transduction and modulation difficult⁴⁵ (reviewed in Knowles and Aziz⁴⁶). These afferent fibres can be classified further by⁴⁶⁻⁵⁰ (see table 1.1);

- (1) Trophic requirements (e.g. NGF, TrkA receptors, GDNF, BDNF),
- (2) Expression of neuro-chemical signaling and channels (e.g. Substance P, VIP, NOS, CGRP, ATP channels, TRP family, Sodium or potassium channels) and
- (3) Activity characteristics.

Table 1.1 Nerve afferent type and characteristics in visceral pain transmission.

(Based on review by Knowles and Aziz⁴⁶)

Name	Properties	Activation
Tonic	Wide Dynamic Range	Low threshold receptors which increase firing activity linearly with increasing bowel wall stretch.
High Threshold	Respond to noxious stimuli	Low firing activity at rest but increased firing rate at 'painful' bowel wall tensions.
Silent	Modified by inflammation	Not active unless exposed to mediators of inflammation.
Mesenteric and serosal	Respond to distortion of mesentery and serosa	Activated by high wall tensions involving the serosa or mesentery.

(iii) Dorsal Horn and Spinal Cord

Spinal afferents from the bowel synapse with dorsal root ganglia within the dorsal horn. This is also the site where somatic (cutaneous) afferents synapse and is often referred to as viscerosomatic convergence. Visceral afferents make up approximately 7-10% of afferents to the spinal cord. In animal models, where afferent nerve synapses have been studied in most detail, the visceral afferents synapse most commonly in laminae I, II, V and X of the spinal cord. However, unlike somatic afferents, visceral afferents on entering the spinal cord send projections up and down the spinal cord, synapsing at multiple levels, resulting in diffuse overlapping of spinal segments and poor localization of visceral pain. Visceral afferents are also thought to send projections to autonomic ganglia, which can influence local reflexes and blood flow to the bowel (Reviewed in Knowles and Aziz⁴⁶, See Figure 1.4 and 1.5)

Figure 1.4 Diagram of Pain Pathways between the gastrointestinal tract and the brain

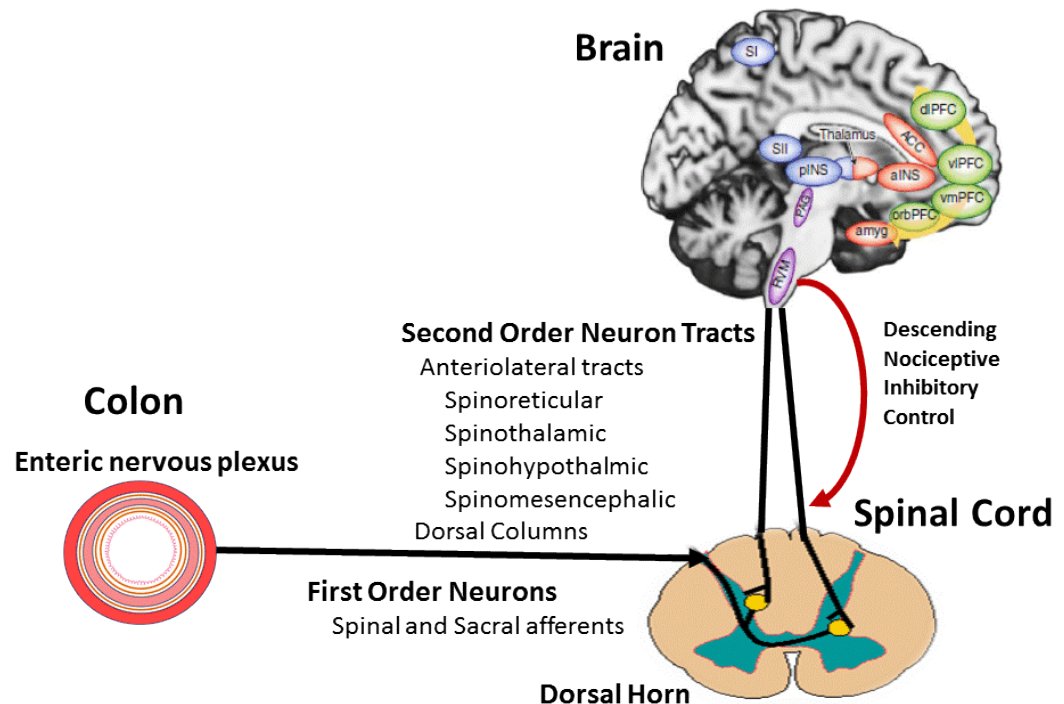
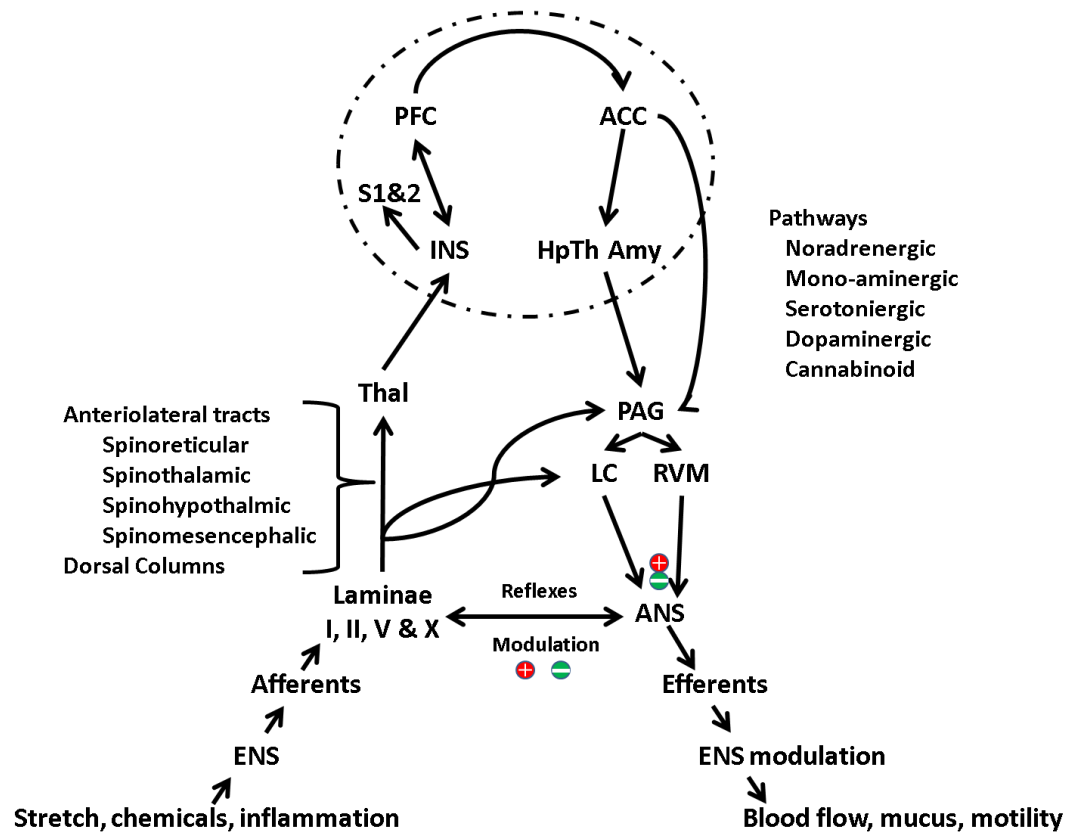


Figure 1.5 Overview of Pain Pathways (Based of review by Knowles and Aziz⁴⁶ and Mayer and Tillisch⁵¹).



Key: ACC, anterior cingulate cortex (includes dorsal, rostral and midCC areas); AMYG, amygdala; ANS, autonomic nervous system; ENS, enteric nervous system; HpTH, hypothalamus; INS, insula (includes ant., mid. and post. areas); LC, locus coeruleus; PAG, periaqueductal gray; PFC, pre frontal cortex (includes dorsolateral and orbitofrontal areas); RVM, rostromedullary nucleus; S1&2, primary and secondary somatosensory cortices; Thal, thalamus;

(iv) Spinal Tracts

After synapsing, nociceptive information is transmitted by second order afferents to the brain. These neurons travel in several spinal tracts; anterolateral tracts e.g. spinothalamic, spinohypothalamic, spinomesencephalic, spinoreticular and dorsal columns⁵². It is thought the latter three spino-tracts are mainly involved in unconscious reflexes and autonomic responses. The

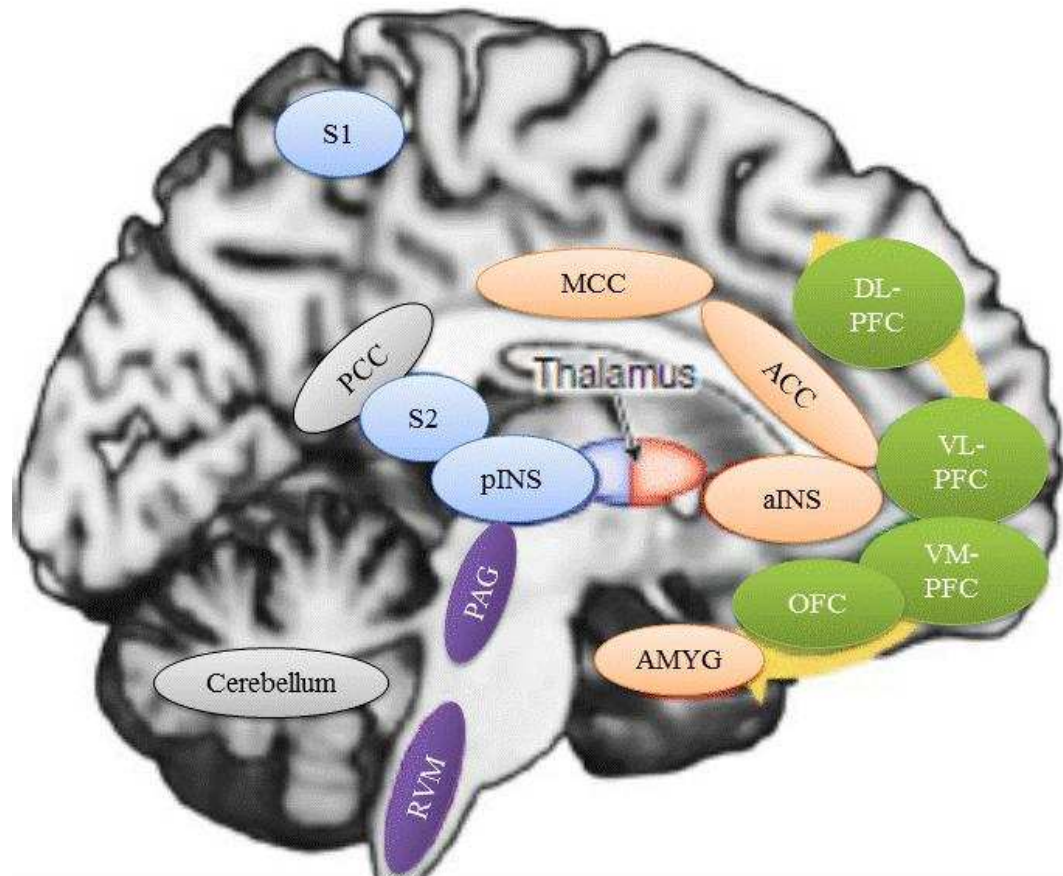
spinothalamic tract is thought to project to the thalamus where it is relayed to other areas involved in conscious pain perception and processing, such as the primary and secondary somatosensory cortices (S1 and S2), the insula (INS), the anterior cingulate cortex (ACC) and frontal cortices. Most of the information related to pain transmission in the dorsal columns comes from animal studies and its role in human pain is not fully understood. However it is thought that instead of transversing the spinal cord to run of the contralateral side, pain information is also transmitted by the ipsilateral dorsal columns to the contralateral ventral posterolateral nucleus of the thalamus^{46, 53}. (See Figure 1.4 and 1.5)

(v) The Brain

The primary response to visceral and somatic pain is complex and not fully understood. Two pathways have been identified; the lateral sensory-discriminative and medial emotional or affective pathways. Cortical regions include the anterior cingulate cortex (ACC), posterior, mid and anterior INS, PFC, S1 and S2. Subcortical regions involve the PAG, HpTHal, AMYG, Hippocampus, and cerebellum (Figure 1.6).

There are extensive connections between the ACC and INS, with co-activation occurring in most studies of emotion processing⁵⁴. Both receive lamina spinothalamic projections and are connected to the parabrachial and periaqueductal gray (PAG), part of the descending nociceptive inhibitory control (DNIC) network, and the PFC⁵⁴, producing a fronto-limbic regulatory network⁵⁵⁻⁵⁸.

Figure 1.6 Simplified diagram of some of the key brain regions involved in the modulation and perception of painful perception (modified from Lee and Tracey 2010⁵⁹)



Orange areas: affective areas of pain processing. Blue: somatosensory areas of pain processing, Green: regions of higher emotional control, Purple: areas important in descending inhibitory and/or facilitatory controls. Grey: other keys areas with less defined role

Key: ACC, anterior cingulate cortex; AMYG, amygdala; INS, insula (includes ant and post. areas); MCC, mid cingulate cortex; DL-PFC, dorsal lateral prefrontal cortex, VL-PFC, ventrolateral prefrontal cortex; VM-PFC, ventromedial prefrontal cortex; OFC, orbitofrontal cortex; PAG, periaqueductal gray; PCC, posterior cingulate cortex; PFC, pre frontal cortex (includes dorsolateral and orbitofrontal areas); RVM, rostroventral medulla; S1&2, primary and secondary somatosensory cortices; Thal, thalamus;

The INS is thought to integrate sensory and motor information from the viscera with the attentional and emotional centres. These involve the limbic system including the ACC and amygdala (AMYG)^{54, 60}. The insula plays an important role in risk perception, attention and anticipation^{54, 58, 61}. The pINS is also important, receiving input from the spinal thalamocortical pathway and being somatotopically organized to a range of stimuli⁶²⁻⁶⁵, but not imagined or remembered pain^{66, 67}.

The PFC is complex and involves several centres in pain processing and modulation^{55, 57}. The right lateral PFC is important in this process as it performs cognitive reappraisal of stimuli and inhibits limbic activity. The ventrolateral PFC also assists this process. It has been shown to be active in analgesia states (arising from the belief that pain can be controlled) and interacts with the nucleus accumbens to inhibit the activity of the AMYG⁶⁸⁻⁷⁰. Another part of the PFC, the ventromedial PFC, is involved with the fear of pain, although in some cases it can exacerbate anxiety and pain experience^{71, 72}.

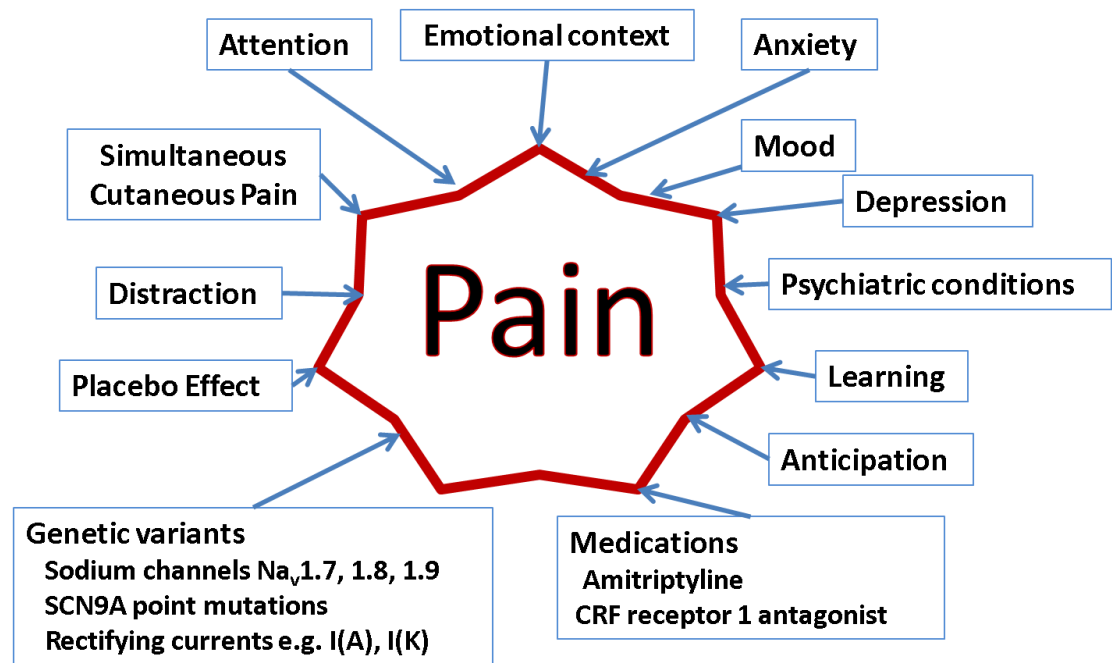
Brain responses to visceral stimulation in healthy subjects has been reviewed recently by Mayer et al⁷³. The review focused on visceral studies, which included papers on oesophageal, gastric, colonic and rectal stimulation using a variety of techniques. It identified consistently activated brain regions in all these studies. These included the posterior (pINS) and anterior (aINS) insula and the anterior cingulate cortex (ACC). Other regions with high consistency in reported activation included the primary somatosensory cortex (S1), regions within the Pre-frontal cortex (PFC) and thalamus (Thal). Direct anatomical connections between brain areas activated during rectal distension in healthy women (INS, ACC, THAL, S1, S2 and the PFC) have recently been observed using diffusion tensor imaging (DTI)⁷⁴.

It also should be noted that most studies look at brief episodes of acute pain. Owen et al⁷⁵ have addressed this issue by using arterial spin labeling (ASL) techniques to look at pain processing in a tonic muscular pain model in healthy volunteers. The 10 slices acquired covered from the thalamus to the somatosensory cortices only and did not include the cerebellum or the brainstem. Pain in the

first 5 minutes was associated with increased blood flow to the INS, bilateral putamen and the inferior frontal gyrus (IFG), the anterior MCC, Perigenual ACC, bilateral thalamus and contralateral SII. However, bilateral insula and thalamus activity was prominent in prolonged pain, while the anterior mid-CC rapidly returned the baseline, suggesting a preferential decrease in emotional pain processing.

Pain processing can also be modulated by several factors^{73, 76-79} which are shown in figure 1.7. These are possible processes that can be altered in disease states and will be discussed later in the introduction.

Figure 1.7 Factors affecting pain perception and processing.



1.4.2 Pain Pathways in Diverticular disease

(i) Enteric nervous system

Neuronal structure and neuro-chemical expression in resected acute and chronic diverticulitis specimens and endoscopic mucosal biopsies in asymptomatic and symptomatic diverticular disease patients have been investigated⁸⁰. The resected specimens demonstrated increases in tachykinins, substance P and galanin in the submucosal plexus and circular muscle⁸⁰. These findings are supported by other studies where altered muscular activity to acetylcholine, nitric oxide, endocannabinoids, tachykinins and substance P were found in resected DD specimens⁸¹⁻⁸⁸. In Simpson et al's study⁸⁰, nerve remodeling was also seen. In the SDD mucosal biopsies, the submucosal plexus also showed increased tachykinins, substance P and galanin as well as vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activated protein (PACAP) compared to the ADD group, but without any histological difference. The increase in galanin significantly correlated with frequency of defecation, supporting the role of neural changes in other gastrointestinal symptoms as well as pain⁸⁰.

An abnormal ENS has been found in other gastrointestinal conditions associated with pain and altered motility e.g. IBS, slow transit constipation^{89, 90}, and is thought to play a part in the development of diverticula and symptoms. This fits with multiple studies demonstrating altered contractile activity within the bowel, especially in the diverticula effected segment⁹¹⁻⁹⁶. The contractions occur most commonly after food, which often triggers pain in symptomatic patients⁹³.

However histological studies of diverticular subjects have reported conflicting results. This is possibly because many reports do not distinguish those with prior diverticulitis and those without which may make a key difference. Some studies have reported few changes within the ENS⁹⁷, others have decreases in myenteric plexus nerves⁹⁸ or increases in submucosal nerves⁹⁹. Sectioning the ENS using a cross-section is a poor way to visualize the myenteric plexus which is best evaluated using whole mounts. Therefore a study of patients with DD and 10 with rectal tumours, who underwent resection using this technique, is of interest. They showed that in the myenteric

plexus, the nerves were thinner and often 'interrupted' and there were a paucity of myenteric ganglia compared to the rectal tumour group¹⁰⁰. Unfortunately the methodology of these studies was not the same, with different resection specimens included in the diverticular and control groups and different sample preparations and staining methods used. Patient symptoms and reasons for resection were also not reported and no comparison was made between them and the neuronal changes, reducing the interpretation of the results.

However a recent high quality histological study in which tissue was carefully laid flat and cut along the plane of the circular muscle to optimally display the myenteric plexus of 27 DD, with documented symptoms of diverticulitis, abdominal pain, changed bowel habit and/or bleed, has also shown decreased neuronal density in all neuronal plexus. Decreased ganglia and glial cells in the mesenteric and submucosal plexus, but an overall high glia to neuronal ratio (oligo neuronal hypoganglionosis), were also found¹⁰¹. These glial changes may be just as important as the neuronal ones, as there is increasing evidence that colonic glial cells not only support and protect the neuron, but can also influence its gene expression, phenotype, and neuro-chemical expression¹⁰². Unfortunately there are no studies that have shown whether these neuronal and muscular changes are present before the development of diverticula and or if they lead to symptoms.

(ii) Functional impact on sensory function

Work at Nottingham and by other groups, suggests that SDD patients show visceral hypersensitivity to rectal barostat distension^{103, 104}. This phenomenon, which also occurs in IBS, is defined as increased sensitivity to a stimulation, so that pain is perceived at a lower stimulus (reduced pain threshold) and/or there may be increased pain to a stimulus (hyperalgesia) and/or pain to a stimulus that was previously not perceived as painful (allodynia)^{105, 106}. In Humes et al¹⁰⁴ (2012), rectal barostat distension showed significantly reduced pain threshold to stimulation in the SDD group compared to ADD and healthy age and sex matched volunteers (HV). Mucosal samples also showed elevation in RNA expression of tachykinins and galanin receptors (GALR1 and

NK1R), TNF-alpha and IL-6 in the SDD group, suggesting that the development of painful DD is associated with these neuro-chemical changes and low level chronic inflammation¹⁰⁴. The theory that chronic inflammation has a role in the development of painful DD is supported by epidemiological work^{26, 28, 107, 108}, which suggests that the development of visceral hypersensitivity in diverticular disease is related to a previous episode of diverticulitis (relative risk of 3.9)²⁶.

Similar post-inflammatory visceral hypersensitivity has been seen in animal models and humans with conditions such as IBS and IBD. In animal models, a controlled inflammatory event can lead to increased response to mechanical stimuli, with alterations in mechanosensitivity of afferents and changes in channel families such as transient receptor potential (TRP), purinergic receptor family (e.g. P2X3), acid sensing ion channels (ASIC), tetrodotoxin resistant sodium channels (e.g. NaV1.7-1.9) and rectifying potassium channels¹⁰⁹⁻¹¹². Animal models of TBNS induced inflammation have shown that these changes can be long standing and similar to those seen following diverticulitis, with raised galanin levels remaining above normal range for over 96 days post initial inflammatory insult⁸⁰. However it is important to note that the animal model used and the development of visceral hypersensitivity, through psychological, inflammatory or other methods, can produce different results which may not always be applicable to human¹⁰⁹. This is not a new phenomenon as post-inflammatory visceral hypersensitivity was first reported in the WWII after amoebic dysentery^{113, 114}. But it's only within the last 20 years that larger epidemiological and biochemical studies have shown a link between inflammation, occurrence of symptoms and neural changes¹¹⁵⁻¹¹⁷.

One of the most recent and well documented examples is the Walkerton Health Study. 2300 people in Walkerton (Ontario, Canada) became ill after bacterial contamination (E. coli O157:H7 and Campylobacter species) of the town's water supply occurred in 2000. Seven people died as a result and there was a documented increase in conditions such as gastritis and IBS. After 2 years the incidence of IBS in the general population who had not suffered from gastroenteritis was 10.1%, compared to 36.2% in the affected group¹¹⁸. Although spontaneous recovery occurred in most

people, some people developed long term problems, with 14.3% - 15.4% reporting symptoms at 6 and 8 years respectively¹¹⁹. There was also an increased incidence in children affected (OR 4.6, 95% CI 1.6 -13.3)¹²⁰ and an increased incidence of dyspepsia (OR 2.30, 95%CI 1.63-3.26)¹²¹. Risk factors for developing symptoms include female sex, young age and severity of the initial gastroenteritis. Psychological co-morbidity also played a role. In dyspepsia, smoking was also identified as a risk factor.

Genetic risk factors for PI-IBS in Walkerton residents were assessed. Seventy-nine gene variants were identified from potential pathophysiological pathways including mucosal barrier function, innate immune system, response to bacterial motifs and the 5HT pathways. These include two Toll-Like Receptor 9 variants, CDH1 (a tight junction protein) and IL6¹²². These findings fit with the observed increased intestinal permeability associated with irritable bowel syndrome, found in patients 2 years after the Walkerton gastroenteritis outbreak¹²³.

(iii) Gastrointestinal and peripheral immune functions

It has been suggested that altered intestinal flora and mucosal barrier function may influence low grade chronic inflammation, with altered cellular, cytokine profile and/or response to stimulation lead to and maintaining visceral hypersensitivity in uncomplicated SDD.

There is some evidence to support this theory as altered peripheral circulating immune cells and cytokines have been noted in IBS (IL-1beta, TNF-alpha, IL-6 and IL-8)¹²⁴⁻¹²⁹ and a small underpowered study in SDD¹³⁰. However there have been reported differences between the literature, which may be due to the different types of IBS recruited, gender, age, use of antibiotics and genetic differences between study populations.

Mucosal immune changes have also been noted in DD. In surgical specimens, significantly increased number of 5-HT producing cells have been reported¹³¹. But in a recent study, no differences were detected among a range of cytokines between 10 uncomplicated DD and 10 age

and sex matched controls¹³². However the study did not report any patient symptoms and with so few participants it is difficult to draw conclusions. Given the strong association with age it is important to note that there are colonic immunological changes that occur with increasing age, including fewer immunocytes in rectal mucosa¹³³ and reduced responses to antigen challenge from lymphocytes in the lamina propria¹³⁴ so any study of histology needs appropriately age-matched controls.

In IBS, colonic biopsies have shown increased numbers of mast cells, enterochromaffin cells and T lymphocytes¹³⁵⁻¹⁴¹. The most consistent changes relate to mast cells. One influential study showed that the number of activated mast cells located in close proximity to mucosal nerve fibres correlated with pain symptoms¹³⁹. Alteration of the immunological environment by infections or genetic predisposition can also influence the production of 5HT, expression of SERT and numbers of enterochromaffin cells^{142, 143}. Similar studies with *Trichinella spiralis* infections can also lead to altered nerve responses to stretch which can be inhibited by ondansetron¹⁴⁴, a 5HT₃ receptor antagonist.

Mast cell and gastrointestinal bacteria also produce proteases^{139, 145}. Protease producing cells and release of proteases from colonic biopsies are greater in IBS patients¹³⁹. These stimulate protease activated receptors, e.g. PAR-2, which can lead to intestinal inflammation¹⁴⁶, mucosal permeability¹⁴⁷ and neuronal excitability in animals^{148, 149}. Reduced PAR-4, which unlike the proinflammatory PAR-2 protects against inflammation, has also been reported in IBS mucosal biopsies¹⁵⁰.

(iv) **Molecular basis of inflammation and Post-infective Hypersensitivity in IBS**

Host predisposition to development of IBS after an inflammatory event has been focused recently after the discovery of SNPs in IL-6, TLR9 and CDH1¹²². In other genetic susceptibility studies several genes related to mucosal barrier function, such as mucin related genes (prostate androgen regulated mucin like protein 1, PARM 1; and MUC20)^{151, 152}, TLR-9¹⁵² and cytokines (tumour

necrosis ligand superfamily 15, TNFSF15 – especially in C-IBS¹⁵³, TNF-alpha G/A polymorphism and low IL10 producing phenotype^{154, 155}) have been discovered.

Altered mucosal barrier function, immunity and symptoms in IBS may be linked with observed changes in gastrointestinal flora¹⁵⁶. Mucosal permeability^{123, 136, 157, 158} and increased expression of pathogen recognition receptors (TLRs 4 and 5)¹⁵⁹ and innate immune activity (antibodies to flagellin and beta-defensin-2)¹⁶⁰⁻¹⁶² have also been found in IBS patients and experimental models. There is some evidence that symptoms, barrier and immunological function and nociception can be improved with probiotics (reviewed in ¹⁵⁶). In IBS, gastrointestinal microbiota has been reported to be unstable within individuals, compared to the general population¹⁶³, but may be due to differences in antibiotic use¹⁶⁴ or diet. Antibiotic use itself has been associated with developing IBS (adjusted OR 3.70; 95%CI 1.80-7.60)¹⁶⁵ or PI IBS after travelers' diarrhea (RR 4.13 95% CI 1.1-15.3)¹⁶⁶. Altered composition of gastrointestinal flora has also been reported in IBS, but with inconsistencies between studies¹⁶⁷⁻¹⁷¹. However the enteric microbiota-gut-brain axis has been postulated as a mechanism for chronic pain and functional gastrointestinal disorders¹⁷². Thus, there has been increased interest recently in modification of gastrointestinal flora with use of antibiotic (e.g. rifaximin) and pre and pro-biotic therapies in diverticular and IBS^{156, 173-177}. However no large RCTs have been published to date in DD or IBS.

Many of these changes in immunological and neuro-chemical receptors and transmitters have not been directly implicated in diverticular disease, but they may be relevant as suggested by animal models and other post inflammatory painful conditions, such as PI-IBS and IBD.

(v) **The Doral Horn and Spinal cord**

Spinal sensitization also occurs with up-regulation of neurotransmitters and receptors such as substance P, Galanin receptors, purinergic receptors and TLRs¹⁷⁸⁻¹⁸⁰. Although the mechanism by which these changes are produced and lead to altered pain processing and modulation is not well understood. There is also controversy over the role of astrocytes and microglia involvement in

visceral hypersensitivity. Increased microglial proliferation and activation to peripheral nerve injury or colonic inflammation or psychological stressors have been seen as well as visceral inflammation following thecal injection of microglial activators¹⁸¹⁻¹⁸⁴. It is also thought that descending central inhibitory or facilitatory modulation can also influence pain transmission at the spinal level.

(vi) Central Pain Processing

Although there have been no studies characterizing central brain responses in diverticular disease there is some evidence to suggest that alterations in pain processing may be present. Previous studies have shown significantly lower visceral sensitivity thresholds between symptomatic and asymptomatic patients with DD¹⁰³. Patient surveys have identified that in those who have an increased tendency to report short-lived recurrent abdominal pain also have increased anxiety scores on the Hospital Anxiety and Depression scale (HAD)²⁶ and higher scores on the Personal Health Questionnaire 12 (PHQ12)¹⁸⁵, a measure of somatisation. This suggests a role for altered central processing. Sensitization in diverticular disease may be similar to IBS^{186, 187}. Although, no fMRI or PET studies of patients with SDD have been reported, several studies in patients with chronic pain, such as IBS, have shown central alteration of pain processing¹⁸⁸⁻¹⁹².

In a review of imaging studies on patients with visceral pain, similar brain areas such as the INS and ACC were activated as reported in healthy subjects as in patients,⁷³. Unfortunately many of the studies were not controlled for confounders such as previous exposure to the scanner environment, anticipation, psychological problems and other co-morbidities, anxiety level and gender, which makes interpretation of the results difficult.

However in some studies in IBS, increased activity of the ACC, INS and emotional pain processing areas (amygdale, hypothalamus, infra-genua cingulate) were identified on anticipation of and on stimulation of the viscera^{190, 193-196}. In healthy controls the ACC activation has been shown to be correlated with unpleasantness of rectal distension and anxiety¹⁹⁷. In IBS patients great activation

was seen in the ACC and the number of pixels activated was increased¹⁹². This activity has been correlated with anxiety score while the PFC and cerebella areas correlate with depression score on the HAD¹⁹⁸.

In chronic back pain, which can fluctuate in intensity, fMRI studies have shown that during rapid increase in pain, active centres include the INS, ACC, parietal cortices, and cerebellum. However, during periods of sustained pain, activity was seen in mPFC, AMYG and ventral striatum. Intensity of the perceived pain correlated with mPFC activity, while INS activity was associated with pain duration in years¹⁹⁹. This suggested an engagement of internalized emotional processing regions (medial pain pathways) and long term maladaptive behavioral and psychological changes.

Recent evidence also suggests IBS patients fail to show the normal activity seen during anticipation of pain in the INS, ACC, amygdala and dorsal brain stem which is presumed to prepare normal subjects for pain and reduce the overall sensation^{190, 200}. Decreased activation of the dorsal pons region, which involved the periaqueductal gray (PAG), part of the DNIC pathways, has been reported in IBS and this might explain the visceral hypersensitivity^{188, 190, 200, 201}. Most IBS patients also show hypervigilance²⁰² possibly resulting from past experience. However repeated exposure to aversive stimuli can result in a habituated response so that IBS patients studied repeatedly over 1 year do show normalization of their initially abnormal response²⁰³. This is thought to be due to higher cerebral modulation and reduced emotional/amygdala excitation of attention centers²⁰⁴.

Several factors are thought to modulate pain perception. Many of these appear to be dysfunction in chronic pain conditions.

1.5 Modulation of pain pathways in healthy and chronic pain subjects

A variety of factors can influence perception of pain (see figure 1.7)

1.5.1 Descending inhibition and facilitation

This is a characterized and widely investigated brain network²⁰⁵, which appears to be important in many physiological processes²⁰⁶ and chronic pain conditions^{73, 207-214}. It involves several interconnected brain regions including the endogenous opioid system, hypothalamus, rostral ACC, AMYG, the periaqueductal gray (PAG), nucleus raphe magnus locus coeruleus (LC), mesencephalic pentane reticular formation and rostral ventromedial medulla (RVM)^{58, 205, 215-221}. Ethnic²²² and sex²²³ differences in descending inhibition of pain have also been described, and a recent meta-analysis suggests that males having more efficient descending nociceptive inhibitory controls (DNIC) than females²²⁴. In two rat models, activation of the descending inhibitory pathways after spinal nerve ligation protects against the development of chronic pain after an acute insult²¹⁵. This phenomenon may be important in human chronic pain development as pre-operative generalized hypersensitivity, as demonstrated by quantitative sensory testing, also appears to increase the risk of chronic pain following surgery^{214, 225-227}. Increasing evidence also suggests that descending facilitation can also occur^{206, 228-230}. Control of pain is through several mechanisms including opioid, serotonin, dopamine, noradrenaline and endocannabinoid pathways^{221, 231, 232}. It is thought that in some chronic pain conditions, descending inhibition of pain can switch to facilitation²²⁸ and may act to maintain a chronic painful state²³³. Activation of these pathways is thought to underlie the therapeutic effects of tricyclic antidepressants.

This is important in gastrointestinal pain as in a recent fMRI study in IBS showed a failure to decrease activation of the INS, supra-genual ACC, AMYG and dorsal brain stem in chronic pain groups^{190, 234}. In IBS, pain rating was significantly inversely correlated with the dorsal brain stem activity, suggesting that IBS patients fail to activate the descending inhibitory pathways during pain anticipation, resulting in a greater pain experience¹⁹⁰. It is not known if the descending

inhibitory pathways are affected in symptomatic DD or if these changes occur prior to or after an inflammatory event, such as diverticulitis.

1.5.2 Attention, Distraction and Counter-stimulation

Both visceral and cutaneous (somatic) pain can be influenced by attention. Attention to a painful stimulus increases pain reporting and fMRI demonstrates corresponding activation in the S1, aINS, PFC and ACC²³⁵⁻²⁴². The mid cingulate cortex (MCC) is thought to be essential for attention pain modulation^{236, 238, 239, 241}. There are few reports of attentional modulation in visceral stimulation in the lower gastrointestinal tract. Some studies involving oesophageal stimulation have suggested that the S1/S2, ACC, left MCC and right PFC are involved^{243, 244}. Stimulation with another painful event has been shown to reduce the pain experience. Studies suggest that although counter stimulation may have a distraction component other effects, possibly mediated through the descending inhibitory pathways, may also play a role²⁴⁵. Top-down modulation and reduced emotional engagement of attentional pathways are thought to underlie habituation to perceived pain in IBS. Similar circuit interaction may be present in diverticular disease and be amenable to pharmacological or psychological intervention²⁰⁴.

1.5.3 Anticipation, learned behavior and hypervigilance

In Pavlovian conditioning models, the expectation of pain activates the ACC and PFC^{73, 246}, which have connections to the descending inhibitory system described above. Anticipation of a painful stimulus results in difference in brain activity in IBS compared to controls, with increased activation of attention and emotional network areas such as the frontal and posterior parietal areas²⁰⁰. Berman et al (2008)¹⁹⁰ have shown altered activity to anticipated rectal distension in IBS. In healthy volunteers the INS, supra-genual ACC, AMYG, and dorsal brainstem (DBS) decreased. In IBS patients significant differences were found in the right posterior INS and DBS compared to healthy volunteers.

Several factors can influence anticipation of stimuli. Personality traits, such as neuroticism, have been shown correlated with activity in the INS, ACC, AMYG, parahippocampus and THAL during anticipation of pain²⁴⁷. Hyper-vigilance and altered response to the anticipation of pain are also thought to play a role in abdominal pain in IBS²⁴⁸.

Many of these activated and deactivated areas have been associated with coping and corticolimbic inhibition of pain²⁴⁹. These areas were classically involved in pain modulatory responses and are thought to be involved in mechanisms of chronic pain²⁴⁹. IBS patients have also shown dysfunctional inhibition of pain with heterotrophic stimulation and anticipation of pain²⁰⁰. There are no studies that have previously examined anticipation of pain in DD.

1.5.4 Emotion, Mood, Depression and Anxiety

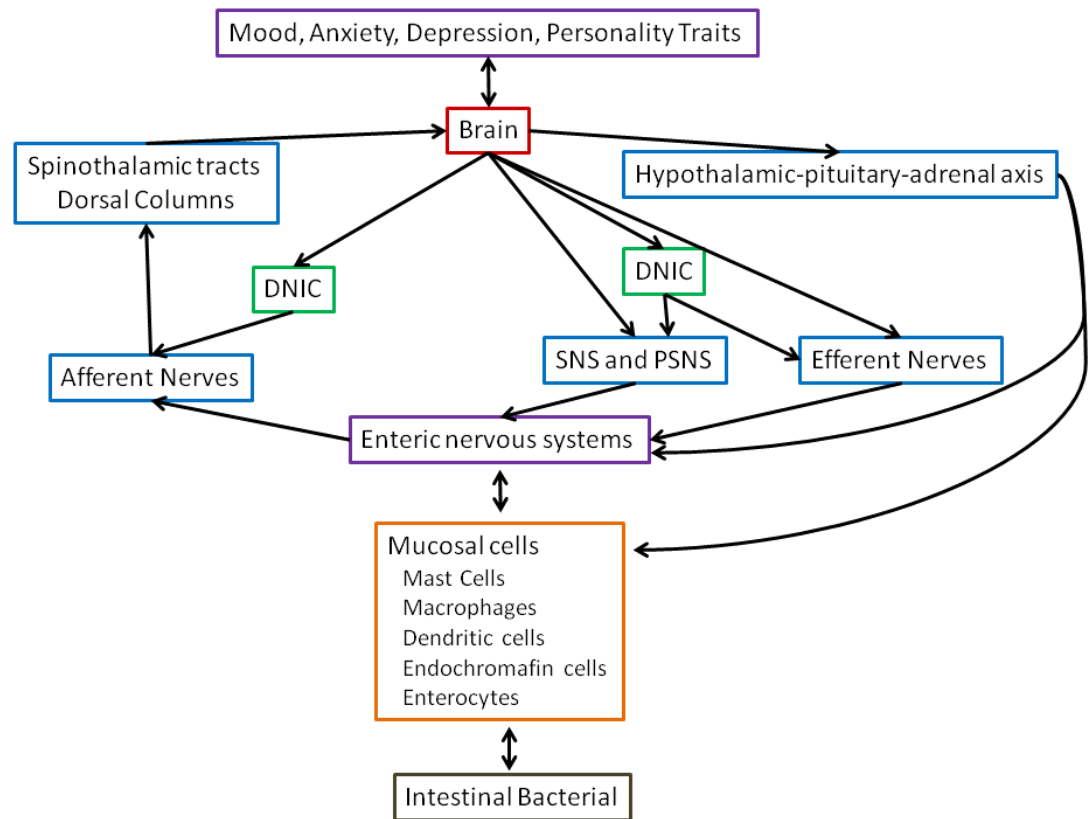
Emotional arousal appears to modulate both pain spinal reflexes as well as perception²⁵⁰. The INS is thought to play a role in integration of emotion and perception of pain, while the thalamus, PFC and AMYG may have a role in altering spinal reflexes. Other regions identified include the parahippocampal and brainstem regions²⁵¹. Depressed mood has been shown to increase pain perception, with increased activity in the hippocampus, PFC and the subgenual ACC. Significant correlation was also found between increased pain intensity and the AMYG and inferior frontal gyrus²⁵². Personality and anxiety trait also influence pain perception, with pre-stimulus functional connectivity between the brainstem and the INS determining if stimulus is perceived as painful or not⁵⁸. Other regions involved in anxiety and anticipation of pain include the entorhinal responses, which predicted activity within the mid INS and perigenual CC²⁵³.

In IBS anxiety and depressed mood have been found to correlate positively with pain ratings. Brain region activation has also been found to correlate. Anxiety score is associated with activation of the anterior mid-CC and pregenual anterior CC, while depression score associates with activity in the PFC and cerebella regions¹⁹⁸. During rectal stimulation, IBS patients also show more stress-induced activation of INS, MCC, VL-PFC, but reduced modulation of INS activity during

relaxation compared to health volunteers¹⁹⁴. This suggests that anxiety and depression may play a role in altered pain processing in chronic pain conditions but it is not known if these are primary or secondary effects.

The brain gut axis is bidirectional with the gut stimulating the brain and central brain processes also influencing the function of the gastrointestinal tract²⁵⁴⁻²⁵⁶. In human and animal studies, psychological stress can alter gastrointestinal flora and mucosal permeability and can affect the development of anxiety-like problems and pain²⁵⁷⁻²⁵⁹ (Figure 1.8).

Figure 1.8 Central and peripheral pathways in the brain gut axis



1.5.5 Other changes in chronic pain

Other networks and structural changes have also been identified in chronic pain conditions. These include:

(i) Resting state networks

Activity of this default mode network (DMN) of nerves occurs at rest and is made up of the posterior CC, medial PFC and temporal regions²⁶⁰⁻²⁶². During responses to tasks or stimuli, regions within the network deactivate. However, this network is altered in several psychological²⁶³⁻²⁶⁶ and neurological conditions^{267, 268}. In fibromyalgia, the DMN has greater connectivity to the INS and the executive attention network than in healthy controls, suggesting that activity with these networks may contribute to spontaneous pain in this group²⁶⁹. There is some evidence to suggest that altered resting state networks also occur in gastrointestinal disorders. In functional dyspeptic patients, who underwent uncomfortable and sham gastric fundus distensions with H(2)(15)O-PET imaging, there was reduced posterior ACC activation and no or reduced deactivations in the AMYG and dorsal pons²⁷⁰. However, much of the current literature of DMN and pain has focused on back pain. In patients with chronic back pain, reduced deactivations have been shown in areas such as the medial PFC²⁷¹ and altered correlations with other networks such as the insular cortices, angular gyri and the middle frontal gyrus orbital region. These regions have been linked with executive control and may explain some of the associated problems with chronic pain, such as depression, sleep disturbances and altered decision making ability²⁷². Although it is likely that this network would also be disrupted, there are no previous studies examining it in irritable bowel syndrome or diverticular disease.

(ii) Structural brain changes

In patients with chronic pain the cortical thickness^{273, 274} and blood flow to this region is reduced, but can be increased by analgesia or symptom improvement. Grey matter changes have also been reported in other pain matrix regions in chronic pain conditions such as the AMYG, hippocampus, post central and superior frontal gyri, INS, prefrontal and ACC²⁷⁵⁻²⁷⁹. In fibromyalgia these changes

also correlate with disease duration and age²⁸⁰. In IBS changes in grey matter thickness have also been reported, with the hippocampus having thickened grey matter while the mid-cingulate cortex was thinned. The insular regions also showed altered thickness, with a reduction for IBS patients with a short duration of symptoms and increased in those who had long term pain²⁸¹. White matter changes have also been detected in thalamocortical tracts and insular regions^{276, 279, 282}. However similarities and differences in the regions affected have been seen between different conditions²⁸³⁻²⁸⁵. There is also suggestion that effective treatment may reverse these changes in some chronic pain²⁸⁶. It is not known if grey and white matter changes occur in diverticular disease and if these can be altered with treatment.

(iii) Pain catastrophizing

Pain catastrophizing is an altered response to pain associated with impaired coping. It has been characterized by heightened pain intensity^{287, 288}, increased disability and difficulty disengaging from pain²⁸⁹. It appears to reflect emotional instability and to be a stable trait²⁹⁰. Pavlin et al²⁹¹ have also shown that pre-surgery pain catastrophizing score (PCS) predict post surgery pain scores, suggesting that people with high PCS may be at risk of developing chronic pain. High PCS patients also exhibit increased pain vigilance²⁹²⁻²⁹⁴.

In a Functional MRI study by Seminowicz and Davis²⁹⁵, 22 healthy individuals underwent electrical median nerve stimulation and 2 pain intensity levels and completed pain catastrophizing questionnaires. Results showed that PCS was not correlated with the lateral discriminative pathway, such as the SSI or II. However the medial pathway centres, such as DL-PFC, INS, rACC pre-motor and parietal cortices, were associated and, during more intense pain. Activation of the PFC during pain was negatively correlated with PCS. The group suggested this fitted with an attention model of pain catastrophizing, with mild pain activating the cortical vigilance network. The PFC decrease at higher pain intensities suggesting a reduction in cortical modulation that impedes changing from this network and activating descending inhibitory pathways that suppress pain intensity. The study suggests that individual pain cortical responses and PCS are independent

of health, i.e. correlative even without the existence of a chronic pain state e.g. fibromyalgia²⁹⁶. However it has been increasingly identified in many chronic pain disorders and may be linked with continuation of pain and associated psychological problems²⁹⁷⁻²⁹⁹. It is not known if pain catastrophizing is important in pain perception in diverticular disease.

1.6 Aims and objectives

Diverticulosis (DD) commonly affects those over 60 years of age with increasing prevalence in younger age groups. It is responsible for substantial morbidity in the UK, which is increasing as the population ages. The reasons are uncertain, making research into this condition of utmost importance. Furthermore a recent survey found that there is considerable associated symptom burden with 36% suffering recurrent abdominal pain.

Visceral hypersensitivity plays a part in both IBS and DD⁴. It can occur because of:

- (i) Peripheral changes in afferent nerves causing increased firing
- (ii) Synaptic facilitation in the dorsal horn of the spinal cord
- (iii) Central hypervigilance⁵
- (iv) Impaired descending inhibitory or enhanced excitatory reflexes originating in the brain stem.

Painful DD patients have changes in enteric nerve neuro-chemical coding with increases in tachykinins and galanin^{80, 300}. This can be induced by inflammation in animal models and is seen in colonic resections for chronic complicated DD⁸⁰. The association of pain with previous episode of diverticulitis supports the theory of a peripheral nerve cause²⁶. However, a central component to abnormal pain processing, as occurs in IBS¹⁹⁵, has not been excluded in SDD patients³⁰¹. Identifying the level within the nervous system where sensitisation arises is the key to successful targeting of treatment to either the gut or the brain³⁰¹

While acute diverticulitis may be the initiating insult, a chronic low level inflammation and/or central changes in pain processing may be required to maintain visceral hypersensitivity. Whether anti-inflammatory agents acting at the level of the gut alone, such as mesalazine, could reverse this process is as yet unknown.

To investigate these areas, I plan to undertake two studies

1. Effects of somatic pain on cerebral activation using fMRI in symptomatic and asymptomatic DD and IBS patients
2. Mechanistic RCT of mesalazine in painful DD

Chapter 2: Abnormalities of central processing of somatic and visceral pain in Diverticular Disease and Irritable Bowel Syndrome using functional magnetic resonance imaging (fMRI)

2.1 Introduction

2.1.1 Visceral and somatic convergence

Visceral hypersensitivity has been demonstrated in many functional gastrointestinal conditions, including IBS^{302, 303} and recently in DD¹⁰⁴. The gold standard method for eliciting pain in patients with visceral hypersensitivity is with a rectal barostat³⁰⁴. In SDD the rectum has similar hypersensitivity to the sigmoid colon, where diverticular disease most commonly occurs¹⁰³. However this technique is invasive and has produced varied fMRI and PET results depending on the technique⁷³ used and analysis methods³⁰⁵. In a mature patient population, with significant associated anxiety and morbidity³⁰⁶, volunteering for a procedure that is not going to provide significant additional diagnosis, treatment or financial benefits may be reduced³⁰⁷⁻³⁰⁹. Thus studies involving an invasive procedure and discomfort are likely to have significant recruitment problems in this group.

In surgical conditions, such as cholecystitis or appendicitis, somatosensory changes in areas of referred pain have been demonstrated despite being pain free at assessment^{310, 311}. This demonstrates somatovisceral convergence, with cutaneous hyperalgesia developing in the areas associated with input from painful or inflamed viscera. However, a phenomenon of localized and/or wide spread hypersensitivity has also been identified in patients with functional gastrointestinal and other disorders, suggesting altered descending inhibitory pathways^{189, 201, 302, 312-321}. In IBS, visceral sensitivity has been significantly correlated to cutaneous thermal hypersensitivity³¹². This is thought to be maintained by central ‘top-down’ (e.g. anxiety, hypervigilance), spinal (e.g. pain inhibition/facilitation deficits) and peripheral ‘bottom-up’ mechanism^{221, 312, 322}. Similar chronic visceral and somatic hypersensitivity have been shown in

animal models following TNBS colitis^{323, 324}. However several differences in brain activation have been found between visceral and cutaneous painful events.

2.1.2 Differences in cutaneous and visceral pain processing in healthy subjects

Although many regions involved in somatic and visceral stimulation appear to be the same (secondary somatosensory cortex (S2), parietal cortices, THAL, basal ganglia and cerebellum), key differences have also been identified^{73, 235, 325-329}. These are thought to be due to the greater emotional and autonomic effects of visceral compared to somatic pain. This include a series of studies by Strigo et al^{329, 330} where a heat stimulus applied to the skin was shown to increase bilateral aINS and ventrolateral PFC when compared to activations produced by visceral mechanical stimulation within the same dermatome region. However greater activation was seen in the bilateral inferior S1 and primary motor cortices and rostral regions within the dorsal ACC with visceral compared to cutaneous stimulation. In a further study by Dunckley et al (2005), electric stimuli applied to the midline of the abdomen or the rectum showed similar activations in the PAG, parabrachial nucleus (PBN), nucleus cuneiformis (NCF) and the locus coeruleus (LCC)³²⁶. However PAG activation also correlated with anxiety rating on visceral stimulation. This area may have a greater role in the emotional unpleasantness of visceral compared to somatic pain³²⁶. Like visceral pain, somatic pain sensation is also influenced by modulators such as attention²³⁵, mood and emotion^{251, 331-335}.

Thus the use of cutaneous stimulation as a surrogate marker for visceral hypersensitivity may be useful in assessment of pain in SDD and prove more acceptable to the patient population allowing us to study a group of patients more representative of the whole population than if we had used rectal distension as our stimulus.

2.1.3 Localized or Global hypersensitivity

Difference in pain processing in IBS and other conditions has also identified possible 'pain signatures' for global hypersensitivity in the condition during anticipation and painful events^{73, 294}.

Although this has not been investigated in SDD, it is possible that localized and/or global cutaneous hypersensitivity may be present in this group. Several brain regions have been identified as important in pain processing in health and other chronic pain groups.

2.1.4 Background: brain regions involved in processing pain and anticipation of pain

There is a very large body of literature describing brain areas and brain networks involved in processing pain stimuli (delivered using a wide range of methods and paradigms) and anticipation of pain. Over 1600 publications to date relate just to fMRI and pain. This section provides a brief background description of some of the key areas involved in these processes with particular reference to visceral pain conditions and thermal somatic pain paradigms. This background information will be then referred to along the discussion of the results of this study.

(a) Cingulate cortex.

The ACC is of particular interest as the pACC is thought to be important in control of pain and its activity has been linked with the PAG and Pons³³⁶. As well as pain processing³³⁷⁻³⁴⁰, the cingulate cortex activity has also been linked with attention^{241, 341}, affective processing of painful stimuli reward probability and risk^{342, 343}, avoidance learning³⁴⁴ as well as anticipation of pain^{345, 346}.

It is thought that the cingulate cortex can influence other brain regions involved in pain processing. This has been demonstrated in animal and fMRI studies. In rats conditioned to expect a painful stimulus with an auditory cue, significant increased ACC activity was identified during the anticipation phase of the study, while during the noxious phase activity was present in the ACC, S1 and medial dorsal thalamus³⁴⁷. The correlations between these areas were also found to be increased during anticipated noxious stimuli compared to unanticipated events. 'Information flow' between the emotional and somatic brain areas was also enhanced³⁴⁷ by ACC anticipation activity. Uncertainty in a cue stimulus to an aversive stimulus has been shown to result in greater responses in the insula and amygdala compared to certain cues³⁴⁸. The activity of the ACC during the cued

anticipatory phase was found to be inversely associated with the insula and amygdala activity response to the aversive event³⁴⁸.

Altered activities of the ACC and associated regions have also been demonstrated in chronic pain groups. During cued anticipation of painful rectal distensions, deactivation of the insula, supragenual ACC, amygdala and brainstem have been shown using fMRI in controls but little deactivation in IBS patients¹⁹⁰. Increased activation of the dorsal ACC has highlighted its importance in IBS^{192, 349}. Treated IBS patients, who have reduced symptoms, demonstrate a reduction in dorsal ACC activation which supports this theory^{350, 351}.

Anticipation of pain can enhance synchronisation of activities of the ACC and associated regions involved in pain processing and that emotional areas can influence nociceptive processing in somatosensory areas³⁴⁷ in health and chronic pain groups²⁷⁰.

(b) Insula

The aINS is important in interoception (conscious sense of body condition)^{54, 352, 353}, emotional awareness^{54, 354}, magnitude of pain^{355, 356} and risk prediction⁶¹. The pINS is thought to be key in discriminative-sensory pain processing^{354, 357} and be somatotopically organised⁶². The INS and ACC have been identified in somatic^{358, 359} and visceral pain^{188, 303, 360} studies. Other areas are not as consistently activated in studies, such as the somatosensory cortices, thalamus and limbic/paralimbic areas⁷³. Although neuro-anatomic pathways are known^{361, 362}, the functional connectivity of these regions in pain anticipation and processing has not been fully elucidated³⁶³⁻³⁶⁵.

The INS and S2 (contained within the parietal operculum, PO), as well as the inferior frontal gyrus (IFG) have also previously been implicated in anticipation of and pain processing³⁶⁶. The IFG is a gyrus of the frontal lobe which includes the pars opercularis, triangularis and orbitalis (Brodmann's areas 44, 45 and 47). The IFG is important in recognizing environmental changes, which may lead to painful situations as well as pain discrimination and pain related anxiety^{68, 367, 368}. It is thought to

be important in the direction of attention towards pain perception³⁶⁹. The PO contains the S2 which interconnected with the insula, amygdala and hippocampus. The PO is thought to be important as a locus for pain perception and attention³⁷⁰⁻³⁷³ as well as being activated by pictures of painful events^{374, 375} and emotional modulation of pain during anticipation³⁷⁶. Functional connectivity has been identified between these areas during painful events³⁶⁶.

The IFG, which is close to the insula, and in which areas of activation and deactivation can often overlap, has also been implicated in pain discrimination, attention, anxiety and environmental ‘threat’ monitoring^{68, 368}. It has recently been shown to be more active when subjects have control over administration of painful stimuli than when they do not⁶⁸. The PO has been shown to be active during many painful and non-painful sensory stimulation³⁷⁷ as well as visual representations of pain^{374, 375}. The INS, IFG and PO network is thought to play a role in affective processing and has been linked to other limbic areas such as the hypothalamus^{369, 378}.

Anticipation to touch mainly occurs in the anterior insula, while the sensation itself results in mINS and pINS activity⁵⁴. Ploghaus et al have also demonstrated anticipation activity in the ACC, aINS and cerebellar cortices³⁴⁵, but this activity was anterior to the activity within these regions during noxious stimuli.

In patients with somatoform pain disorder who were given painful thermal stimuli, increased activity was found in the aINS, parahippocampus and amygdala while decreased activity was identified in the VM-PFC and OFC compared to healthy controls³⁷⁹. In patients with IBS and fibromyalgia, bilateral aINS activity has been linked with increasing reported somatic pain independent of attention²³⁵. While the S2 activity was correlated with increasing reported visceral pain³⁸⁰. Arterial spin labelling studies of chronic pain in OA patients have demonstrated increase blood flow to several pain matrix regions at rest, including the INS and cingulate cortices, amygdala, hippocampus, thalamus, S1 and S2 and the brainstem (PAG and nucleus cuneiformis). Over several sessions, changes in the perceived pain that participants were experiencing correlated

with activity in the rostral and subgenual cingulate cortex, PFC, mINS, aINS, and pre-motor cortex³⁸¹.

(c) Amygdala, hippocampus and parahippocampus.

The ACC and insula form part of the affective processing network along with the amygdala and hippocampus regions. Anxiety can influence perception of pain and the activity in the entorhinal cortex, which predicts the activity in the mINS (intensity coding) and perigenual cingulate (pgACC) (affective areas)³⁸².

Activation of the amygdala has also been found to be related to the passive duration of the anticipatory cue³⁸³. The Amygdala has been shown to deactivate during painful cutaneous^{371, 384, 385} and visceral stimuli^{188, 190, 386}. However, activation of the amygdala during pain has also been found in some studies^{195, 385}. Deactivation of the amygdala has been identified in animals and humans. In both, diversion of attention from the ‘fear of pain’ or ‘active coping’ strategies decreases the pain experience and emotion circuit activities. This has been seen in numerous studies in humans^{56, 239, 364, 387-391}. In a recent study³⁸³, lower amygdala activations and subjective pain experience were seen during active coping with a continuous performance task compared to inactivity (passive coping). This finding was most striking when using the reaction time as marker of engagement or attention with the task. Amygdala activity also increase by the duration of the anticipatory phase, which was independent of engagement in the task³⁸³.

The deactivation of the amygdala and its interconnectivity with other regions is thought to be important in chronic pain conditions^{392 393}. In a study of 28 patients with fibromyalgia (FM) and 14 health volunteers (HV), who underwent subjectively calibrated pressure pain, a reduction in connectivity between the rostral ACC, amygdala, hippocampus and brainstem was identified compared to the healthy volunteers³⁹³. The thalamus also showed little connectivity to other brain regions in the FM group, but significant connections to the orbito-FC brain regions to the thalamus was identified in the HV. The authors suggested this demonstrated decreased activation of the brain

pain inhibitory network in the FM patients and that this was important in pain maintenance in the group.

(d) Pre-Frontal cortex

Appraisal is the emotional evaluation of impending stimuli and can be divided into low and high levels. Low level appraisal is a non-conscious, hard-wire and pre-attentive, while high level appraisal is conscious controlled and requiring memory and attention³⁹⁴. Attenuated mPFC/ACC and increased lateral PFC activity during anxiety and anticipation of painful events is suggestive of high level appraisal³⁹⁴. The mPFC and ACC are important in evaluation of the self-relevance of stimuli, emotional awareness³⁹⁵ and attention to emotional stimuli^{395, 396}. Regional blood flow has been found to be decreased during anticipation of painful shocks in normal healthy volunteers in the mPFC (Brodmann's areas 10/32 and 24/25)³⁹⁵. This deactivation was inversely correlated with self-rated anxiety and correlated with midbrain activity³⁹⁵.

Control over events, such as self-controlled painful stimuli, can also influence the perceived stimulus and related anticipatory anxiety. In self-administered noxious stimuli, the ACC^{68, 397} and the dorsolateral (DL) and anterolateral (AL) PFC demonstrate higher activation⁶⁸. Activation in the AL-PFC during externally mediated stimuli also correlated with participants' general belief in control over their lives in healthy subjects⁶⁸.

Control and the nearness of threat are thought to be important in the modulation of key pain processing areas and the descending inhibitory pathways. Using PET in healthy volunteers with normal and capsaicin-treated skin, principal component analysis suggests that the DL-PFC also modulates activity in the thalamus, ACC, OFC and aINS and perception of pain⁵⁵. Also, in a maze pursuit paradigm, where healthy volunteers tried to evade a virtual predator which would capture and inflict pain, brain activity was found to alter from VM-PFC to the PAG on increasing proximity of the 'predator'. This shift was greater with increased anticipation of pain. PAG activity was also correlated with degree of dread and decreased confidence in escape from capture³⁹⁸.

Anticipation of a learned pain-stimulus decreases the activity in the ACC and VM-PFC³⁹⁹. This may be because less appraisal is needed for stimuli which have already been encountered.

The PFC is also important in expectation and, therefore, the placebo effect. The role of the DL-PFC in the placebo effect was examined using low level repetitive trans-cranial magnetic stimulation (TMS) to transiently inhibit the right or left DL-PFC in a heat-pain paradigm. Although pain experience was not affected the placebo effect was blocked, suggesting the DL-PFC is important in expectation and anticipation of pain⁴⁰⁰. Expectations of pain relief via a placebo during visceral stimulation also correlate with reduced activation of the thalamus, SS cortex, VL and DL-PFC during anticipation and in the thalamus during painful stimulation compared to the same participants when given a low expectation of pain relief. Participants who demonstrate a robust placebo effect have decreases in activation in the DL-PFC in anticipation and in the SS cortex, thalamus and PCC during painful events compared to participants with low placebo effects⁴⁰¹.

(e) Somatosensory cortex.

The somatosensory cortex has been linked with sensory-discriminative pain pathways. However ipsilateral S2 has been previously implicated in anticipation and is enhanced by expectation of pain³⁷⁶. Magnetoencephalography (MEG) has been used to assess the SS cortices and S1 has been implicated in sensory and attention to painful stimuli, while in comparison the S2 only occurred during the noxious phase⁴⁰². MEG has been used in healthy volunteers to assess the activity of the somatosensory cortices in anticipation of pain from distension of the oesophagus with an intraluminal balloon⁴⁰². S1 and S2 showed bilateral asymmetrical activations were seen in the Beta bands. In S1 this was a continued increase during anticipation which continued with the pain but at a different frequency. Somatotopic representations of touch have been mapped in S1, S2 and in the operculo-insula cortex. Multiple somatotopic pain representations have also been mapped in the operculo-insula cortex for hand and foot to heat and pin-prick sensations^{62, 63} and for muscular pain^{64, 403}. In a study, which characterised individual response to a range of sub-threshold, threshold and painful stimuli, areas in the somatosensory, amygdala and insula cortices showed linear

relationship between activity and increasing painful stimulus⁴⁰⁴. The aINS and S2 have also been identified as key pain processing areas in psoriatic arthritis. Mechanical pain stimulus results in activity in the insula, S1 and S2, MCC and thalamus⁴⁰⁵. After anti-inflammatory medication pain intensity was correlated with activity in the aINS and S2, suggesting these areas are important in processing pain intensity⁴⁰⁵.

(f) Cerebellum

Activity in the cerebellum has been demonstrated in many studies of pain^{73, 406-409}, pre-attentive detection⁴¹⁰ and anticipation of pain (ipsilateral posterior cerebellum)^{345, 406}. Initial fMRI studies suggested that cerebellar activations were related to the withdrawal behaviour and the motor pathways related to this response⁴⁰⁸ or to attention, verbal ratings and learning^{411, 412}. However, the cerebellum is now thought to modulate nociceptive processing with several pharmacological and electric stimulation in animal models producing exacerbation and attenuation of noxious stimuli⁴⁰⁶. fMRI studies have shown different pattern of cerebellar activity to non-painful and painful stimuli⁴¹³ and this is altered in patients with chronic pain states⁴⁰⁹. It is also thought to have a role in affective pain processing^{218, 329, 409}. In healthy volunteers, areas within the cerebellum involved in processing of aversive heat stimuli and pictures are similar and have been suggested to be involved in general aversive processing and may involve both sensory and emotional networks⁴¹⁴.

In a recent study in 15 IBS and 12 healthy women, depression score, calculated from the HAD questionnaire, correlated with non-painful rectal distension activity in the CRUS I, II and VIIB of the right cerebellum and in the vermal lobule V during painful stimulations⁴¹⁵. CRUS II and VIIB are thought to be involved in cognitive processing⁴⁰⁶. Anxiety was also correlated with CRUS II activity in non-painful distensions⁴¹⁵. The cerebellum has also been implicated in chronic pain and associated psychiatric disorders⁴⁰⁶.

(g) The ‘default mode’ network

Another theory for chronic pain is a failure of the brain to shift from the resting state default mode network to allow appropriate anticipation and modulation of emotional processing. This has previously been shown in IBS, where slow ramp tonic distensions result in increased activation of the insula, ACC and VM-PFC, and less deactivation in the VM-PFC, PCC and precuneus¹⁹⁶. This suggests that IBS patients have an inability to shift from the default mode network and modulate emotional responses to visceral distension, unlike healthy volunteers¹⁹⁶.

The pACC has previously been implicated in pain control³³⁶. In a recent study it has also been found to be correlated positively with activity of the DMN in resting states⁴¹⁶. In a study by Minassian et al 2012, 20 right handed subjects received electrical shocks to their right forearm⁴¹⁷. This group demonstrated several DMN areas which were deactivated during the anticipation phase of the study and subsequently became active during the pain phase. These areas include the bilateral precuneus, PCC, hippocampus region, bilateral angular gyri and VM-PFC.

In a separate study of 20 healthy subjects, the default mode network was assessed during rest anticipation and pain states. A CHEP Medoc system was used to give a 12 sec heat pulse to the right forearm in 61 patients. Deactivation of classical DMN areas such as the mPFC, parahippocampus, precuneus and lateral temporal cortex, as well as non classical areas such as the pre-motor area, contra-lateral S1 and M1 and the superior frontal gyrus were also seen on fMRI⁴¹⁸. Interestingly the group showed that there was greater range of deactivations at lower rather than higher pain thresholds and postulated that this was the result of ‘preparation for escape from pain,⁴¹⁸. However other groups have found the opposite, where increasingly demanding cognitive tasks and pain result in increased attention and decreased DMN activity⁴¹⁹. In healthy volunteers and in FD similar regions deactivate during gastric distensions include the amygdala/hippocampus, ACC, PCC and precuneus, dorsal and ventromedial PFC, occipital and posterior temporal lobes. These similarities suggest that in health and disease there is a shift from the ‘default-network’ to

attention on the pain stimulus. Anterior MCC has been shown to be activated in HV and IBS during intestinal distension^{195, 420} and has been correlated with anxiety and fear of pain⁴²¹.

2.2 Hypotheses and aims of this study

2.2.1 Aims

The aims of the study are to determine differences in cortical and sub-cortical pain processing to thermal cutaneous stimuli in painful DD with comparison to non-painful DD and IBS.

2.2.2 Hypotheses

- SDD participants with a past history of acute diverticulitis will show peripheral hypersensitivity as demonstrated by greater activation of the S1 and 2, THAL and pINS compared to asymptomatic DD and IBS participants.
- Painful DD with low PHQ12-SS scores will show enhanced response to painful stimulation of the foot (L5/S1) but not of the hand (C7/8), suggesting only localized hypersensitivity due to somato-visceral convergence
- Painful DD with high PHQ12-SS scores will show enhanced responses to painful stimulation in both regions similar to IBS participants, suggesting widespread or global hypersensitivity due to hyper-vigilance or derangement of the DNICs
- Participants with IBS will demonstrate greater activation of affective and arousal areas similar to SDD patients with high PHQ12-SS scores suggesting a greater emotional engagement in pain processing. They will also demonstrate global hypersensitivity to stimuli as suggested by previous studies.
- IBS and DD participants with high PHQ12-SS scores will have similar anticipatory responses to pain compared to non-painful and painful DD with low PHQ 12 scores with evidence to suggest derangement of DNIC
- A high PHQ12-SS and Pain catastrophizing score in participants will be associated with greater activation of affective and arousal areas to somatic stimulation

2.3 Methods

2.3.1 Study approvals

This study was assessed and approved by the Regional Ethics Committee (Nottingham committee 2: REC Number: **09/H0403/43.**) prior to commencing. The study was performed to GCP principles and sponsored by the University of Nottingham. The study was funded by a Wellcome Trust research training fellowship.

2.3.2 Power calculation

Based on the literature⁴²² and our previous work using rectal barostat distension³⁰⁵, n = 12 subjects are required to show a >30% functional MRI difference in visceral sensation between groups which we considered clinically significant. We therefore aimed to recruit 20 subjects in each group to allow for possible 25% dropout and for scans excluded because of motion artifact.

2.3.3 Participant recruitment

Study participants with IBS, ADD and SDD were identified and recruited from gastrointestinal medicine and surgery clinics, databases of patients held at the NDDC, who had previously expressed interest in participating in research and local newspaper and bus adverts using standardized adverts, letters and information sheets. Participants who responded to the initial approach were contacted by the author by phone. The participants' gastrointestinal diagnosis and initial screening for inclusion and exclusion criteria (See **Appendix 6.1**) was performed by structured telephone questionnaire and consultation of hospital and general practitioner records, after obtaining the participant's written consent. All suitable participants were invited to a study day. This one-off visit lasted 3 hours in duration and participants received an inconvenience allowance of up to £100.

Participants attended the Sir Peter Mansfield Magnetic Resonance Centre at the University of Nottingham on 1 occasion, having completed validated questionnaires on gastrointestinal habits, hospital anxiety and depression scores, PHQ15 and pain catastrophizing score at home the night

before. None of the participants' usual medications or food were withheld before the visit except for ondansetron (IBS participants), which has possible central effects and could have altered the brain responses⁴²³. Medical and MRI screening was rechecked and written consent was obtained on the day of the study. Participants' height and weight were also assessed.

2.3.4 MRI scanner and Medoc Peltier device

All MRI was carried out on a state-of-the-art, research dedicated Philips Achieva 3T MRI scanner, sited at the Sir Peter Mansfield Magnetic Resonance Centre (Figure M2.1a). A Medoc PATHWAY Pain & Sensory Evaluation System (Medoc, Israel) was used for thermal stimulation of the hand and the foot (Figure M2.1b). This was equipped with a fMRI-compatible CHEPs (Contact Heat-Evoked Potential Stimulator) thermode probe (Figure M2.1c), with a 27 mm diameter thermode provided with a 10 meter cable and a filter that could be passed through the Faraday cage walls of the scanner room. The thermode was placed on the back of the hand (or foot) of the patients and maintained in place with its own Velcro strap and an additional sized tubi grip bandage (NHS supplies: D, E and G sizes, Supplies Codes: 1437, 1434, 1439, NHS catalog code: EGA 017, EGA019, EGA023) as shown in Figure M2.1d.

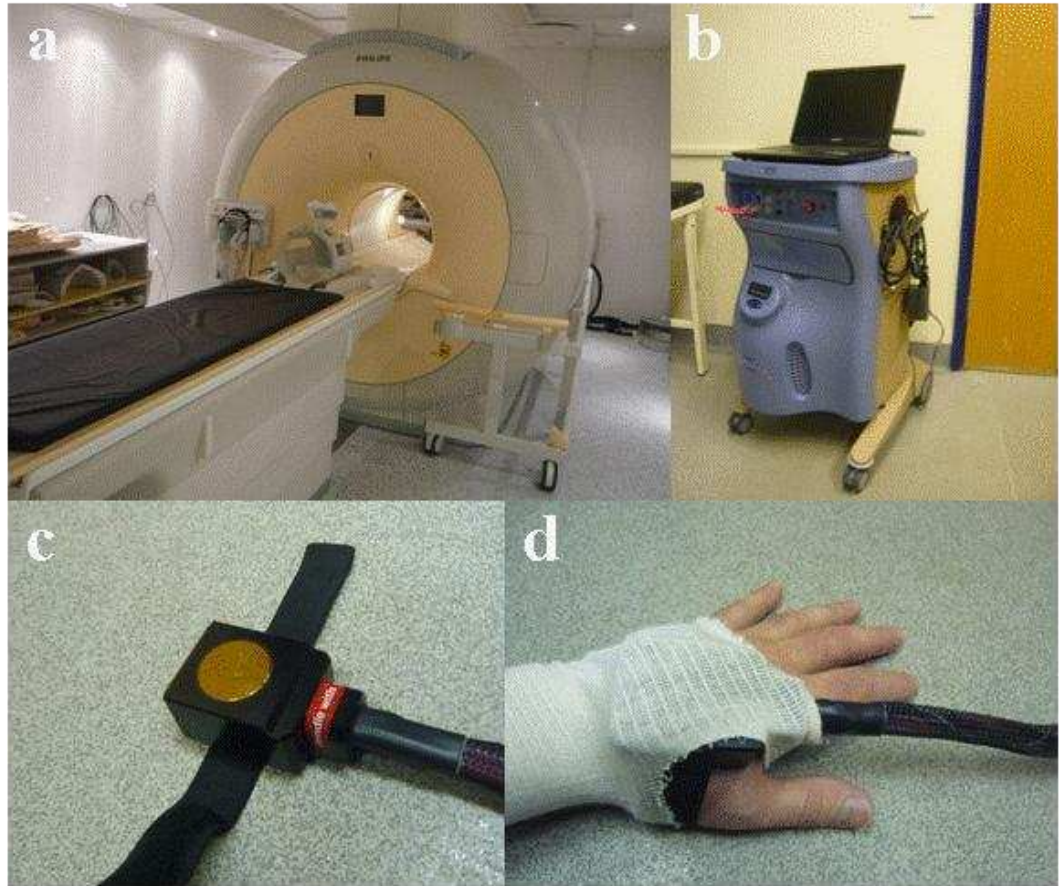


Figure M2.1: (a) The Philips Achieva 3T MRI scanner, sited at the Sir Peter Mansfield Magnetic Resonance Centre. On the scanner bed the 8-element parallel imaging head coil can be seen. On top of the coil there is the mirror that allowed patients to look at a screen where the cue signal was projected during the fMRI runs. (b) The Medoc peltier device. (c) The Medoc fMRI compatible CHEPs thermode. The copper disk is the part that was placed in contact with the patients' skin, held in place by the black Velcro strap and a tubigrip bandage as shown in (d).

2.3.5 Sensory testing and thresholds

Sensory testing was performed on the dorsum of the left hand and foot using the Medoc fMRI compatible pathway system. All testing was undertaken in the anteroom of the Philips 3T MRI scanner. Participants were positioned in a comfortable chair, orientated away from CHEPs computer screen to prevent confounding. Participants were made comfortable with their arm supported on a table padded with pillows.

To measure limits of sensory threshold, Medoc designed software was used. Patients were given verbal instructions with demonstration as the CHEPs probe increased temperature by 1°C/second from 32°C to a maximum of 55°C. Once the response unit was activated the temperature of the probe rapidly decreased to baseline (32°C). Reassurance that the probe would not damage the skin was also given. Initially, for the first 4 temperature trials, participants were asked to press the response unit when the temperature 'started to become painful'. Average temperature, variation and standard deviation and a visual analogue score (VAS) of the pain intensity out of a score of 10 were recorded after completion of the trials, with 0 being 'no pain', 1 'slight pain' and 10 'severe pain'. The 4 temperature trials were then repeated, but participants were asked to activate the response unit when they could 'no longer tolerate' the temperature increase and response data was recorded as before.

To measure responses at set temperature, participants were asked to score 3 grouped heat pulses. The heat pulses were all at a set temperature and 5 seconds in duration, with 5 seconds 'rest period' between each at a baseline of 35°C. After 3 heat pulses were delivered participants were asked to rate the pain intensity using the VAS. A further 3 pulses were then delivered at a different temperature and the scoring repeated. Participants were deliberately not told the temperature of each three pulses or if the next set of temperatures would be higher or lower than the preceding set. They were advised only that they could give a VAS score for each 3 pulses which was the same, greater or less than previous. The 3 pulse blocks testing was continued until a VAS score of 6-7 was given or the participant requested to stop i.e. they did not want higher heat pulses to be delivered.

2.3.6 The 'VAS temperature'

The temperature at which the subjects rated the pain intensity at a VAS score of 6 or 7 was designated as the 'VAS temperature' and this temperature was used as individual threshold for the following study paradigm. This temperature threshold will be referred to throughout this dissertation as 'VAS temperature'.

A 2 minute 'test run' check was then performed to confirm that the VAS temperature scores were reproducible for that given individual and to assess if a standard temperature of 45°C could be used for the subjects a. This was achieved using a 2 minute protocol consisting of four 5 second stimuli (two VAS and two heat pulses at 45°C). These heat pulses were separated by a 25-30s second rest period. Participants were advised that the stimulus would be similar to those they would experience when in the scanner and that the stimulus was supposed to be 'painful'. They were told that in the scanner they would need remain still to reduce movement artifact and that this 'test run' was to ensure the temperatures used for the heat pulses could be tolerated. A VAS score was taken at the end of the 2 minute protocol for the participants overall pain intensity rating. If participants could not tolerate either temperature, they were adjusted down by 0.5°C and the protocol repeated until a tolerated temperature was selected. At least 0.5°C difference between the VAS temperature and the 45°C or adjusted temperatures was maintained for the study paradigm protocols.

2.3.7 Scanning protocol

(a) Participant positioning and instruction

Functional MRI images were obtained using a 3T Philips Achieva scanner and 8-channel SENSE dedicated brain imaging coil (Figure M2.1).

(b) Visual cues

All participants received standardized verbal information about the scanner and study and were shown the scanner. The receiver coil around the head of the subjects had an in-built mirror (Figure M2.1a) that allowed them, once positioned in the scanner, to see a projection screen in front of the magnet bore, which is commonly used to project visual stimuli and/or instructions during fMRI studies. In this study the participants were instructed to look at a small blue cross projected on the screen. This would change to white cross to give a visual 'cue' prior to any heat stimulus. The visual cue formed an important part of the paradigm design as would allow analysing the data for anticipation of pain.

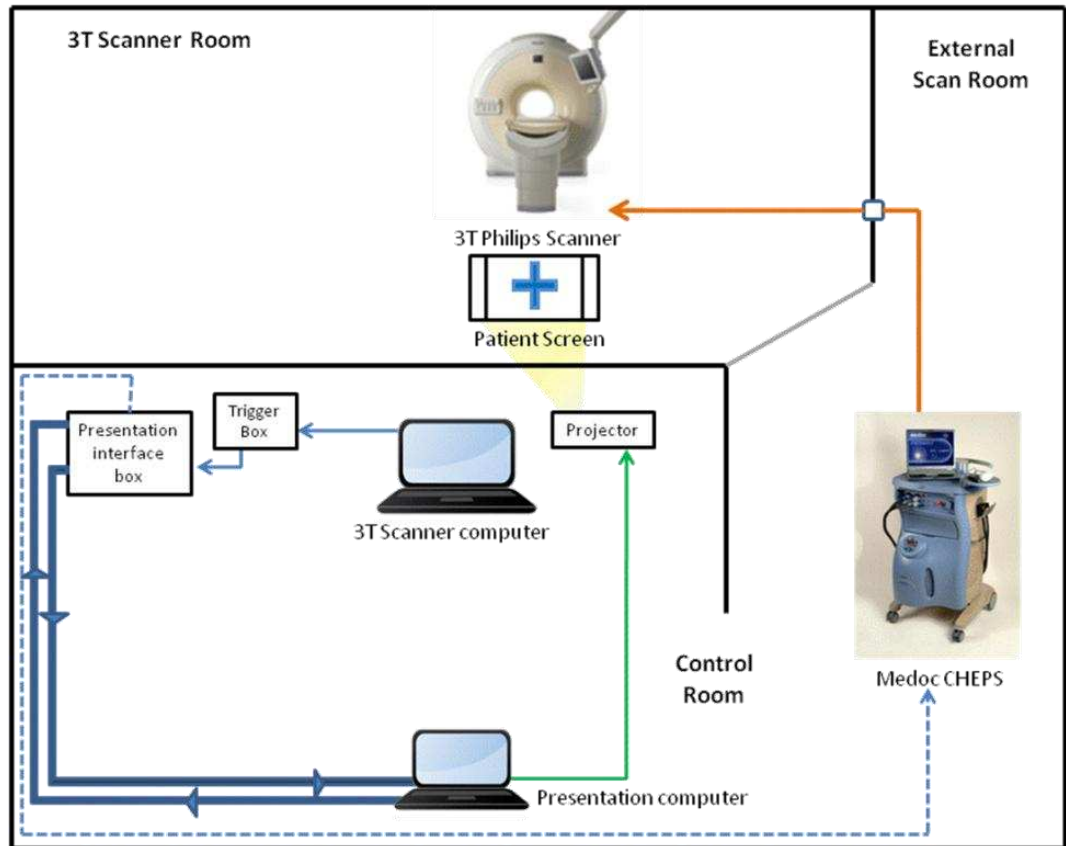
Participants were asked to pay attention to the screen and to the heat stimulus when delivered. Participants were given ear plugs and headphones to dampen down the loud noise arising from the running imaging sequence and positioned supine on the scanner bed with the CHEPs probe attached to the dorsum of the left hand or foot as for the sensory testing. The scanner ‘nurse call’ alarm button was placed in the participant’s right hand in case they needed to call for attention. No music or other verbal stimulus was provided during image acquisitions.

(c) Medoc Peltier paradigm and fMRI image acquisition





Firstly, a set of T1 weighted scout images were taken to allow planning of the imaging study and an automatic calibration scan was run to set up the 8-element parallel receiver head coil. After this, a 2 minute training paradigm was performed. This allowed the participant to become familiarized with the scanner environment, to see the ‘cues’ on the screen and to receive two 5 sec heat pulses, similar to what they would experience during the actual study experiment. This allowed also the research staff to confirm that the scanner, presentation computers and Medoc CHEPS Pathway system were set up and synchronized correctly (Figure M2.2). The participants were then asked to give a prediction of the VAS rating they would give at the end of the first peltier paradigm. The main study experiment was then commenced using 1 of 2 study paradigms as described below.

Two pseudo-randomized peltier paradigms were designed (Figure M2.3). The designs of the paradigms were based on our previous barostat paradigms³⁰⁵, other sensory studies^{79, 424} and sensory testing guidelines from the German research network on neuropathic pain^{425, 426}. Each contained five 5 second ‘VAS temperature’ heat pulses and five 5 second ‘45°C’ (or equivalent as described above) with 25-30 seconds ‘rest’ period between each ‘cue’ and heat pulse. Prior to each heat pulse a 5-12 second visual ‘cue’ was given. Within the paradigms there were also two additional blank (no temperature stimulus) 5 second periods preceded by a short 2 second ‘cue’. These ‘blanks’ were designed to reduce the participant’s ability to predict the subsequent heat pulses (Fig. M2.3). The sequence of timing of the cues and stimuli for each paradigm can be found in **Appendix 6.2**.

Figure M2.2 Set up and interconnections between computer systems



Key: Cable Connections

-  BNC cables
-  VGA cables
-  Parallel port cables
-  Optic cables

Each paradigm lasted 8-9 minutes. After each paradigm the Medoc CHEPs probe was repositioned to the other site, e.g. the hand or foot, and the either paradigm 1 or 2 commenced. After the 2 paradigms were completed, i.e. 1 on the hand and 1 on the foot, the participants were removed from the scanner and given a 15 minute break where they could mobilize around the department and drink water. They were then returned to the scanner for a further paradigm on the hand and foot and acquisition of a MRI structural image that would be later used for data processing.

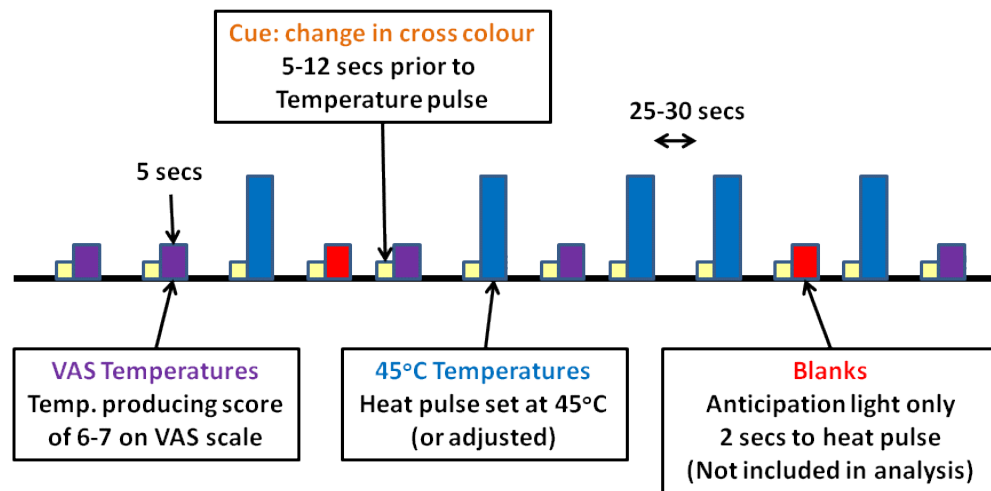
The imaging module used for the thermal stimulation study was a single-shot, double-echo echo-planar imaging (EPI), with a matrix of 80×77 and 40 slices covering the whole brain with no gaps between slices. The resolution was isotropic $3 \text{ mm} \times 3 \text{ mm} \times 3 \text{ mm}$ and 177 dynamic scans (7,080 images in total) were acquired during one single run on the foot or the hand. The images were originally from transverse plane but the stack was tilted along the AC-PC axis which helped minimising orbitofrontal artefacts from the nasal cavity. The scan parameters were repetition time $TR=3\text{s}$, echo times $TE= 25\text{ms}$ and 50ms , fat suppression SPIR and 80° flip angle. The anatomical images acquired at the end were sagittal T1 weighted MPRAGE, 256×256 matrix, 160 slices with no gap between them, $1 \text{ mm} \times 1 \text{ mm} \times 1 \text{ mm}$ isotropic resolution, repetition time $TR=8.2 \text{ ms}$, echo time $TE= 3.8 \text{ ms}$ and 8° flip angle. This sequence lasted 4-5 minutes. The order of the stimulation site (e.g. hand or foot) and the paradigms e.g. paradigm 1 or 2) for each participant were randomized prior to commencing the study to avoid order effects.

2.3.8 fMRI Image processing

All fMRI images were analyzed using SPM8 (Wellcome Trust Centre for Neuroimaging, University College London [UCL], UK). Details of analysis methods can be found in **Appendix 6.4**. Images were corrected for movement and slice timing and normalized to an EPI template, following by smoothing (8mm kernel). Box-Car general linear model (GLM) was used for the heat stimuli and cue. Each model was convolved with canonical haemodynamic response function (HRF). Individual motion parameters for each paradigm and subject were used as no interest covariates in the GLM. Blank stimuli were not included within the analysis. First level fixed effects analysis was performed for each participant. Motion parameter, image quality and questionnaire data were assessed for each participant and incomplete or poor quality datasets were removed from further analysis, leaving 14 subjects per group (see **Appendix 6.3** for further information on subject selection). Second level random effects (RFX) analyses at the group level for the 'VAS temperature' pulses (FDR [false discovery rate] corrected for multiple comparisons at $p < 0.05$, voxel threshold 5) and for the cue (uncorrected $p < 0.001$, voxel threshold 5) were performed.

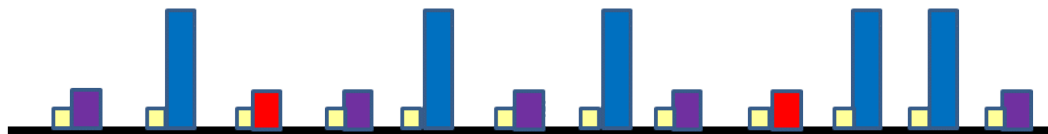
Figure M2.3 Paradigm design

(A) Basic Paradigm design and Key



(B) Paradigm designs

Paradigm 1:



Paradigm 2:

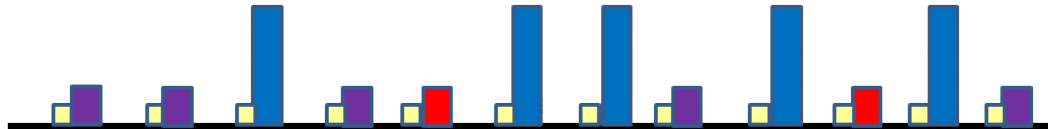
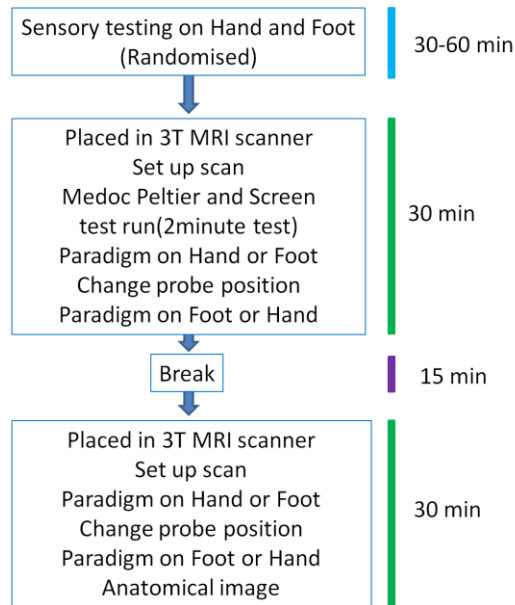


Figure M2.4 Study Sequence



2.3.8 fMRI Image processing (continued)

Further analyses were performed, including 2 sample t test of responsive areas between the hand and the foot within each group, and the ‘VAS temperature’ and ‘cue’ events on the hand or foot between groups. Analysis of covariates of interest, including VAS rating during the study, VAS temperature used, anxiety and depression scores on the HAD questionnaire, total pain catastrophizing score, age and body mass index (BMI), was also performed. Regions of interest were identified for each analysis using the PickAtlas (version 2.4).

2.3.9 Statistical Questionnaire analysis

Participant questionnaire data was stored on Microsoft Office Access 2007 (Microsoft USA) and transferred to SPSS (version 15, IBM, Portsmouth UK) and GraphPad Prism (Version 5, California USA) for further analysis. Continuous group data was compared using nonparametric t test (Mann-Whitney U). Significance of correlation between pain intensity ratings with questionnaire data was assessed using Spearman’s rank coefficient. Statistical significance was considered at a $p < 0.05$ level.

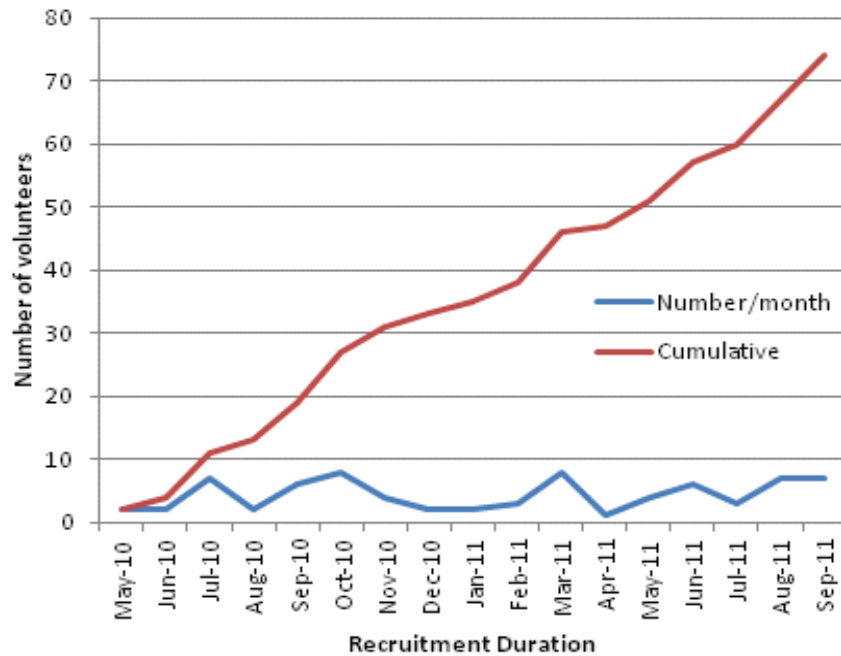
2.4 Results

2.4.1 Recruitment and demographic results

(a) Participant Recruitment

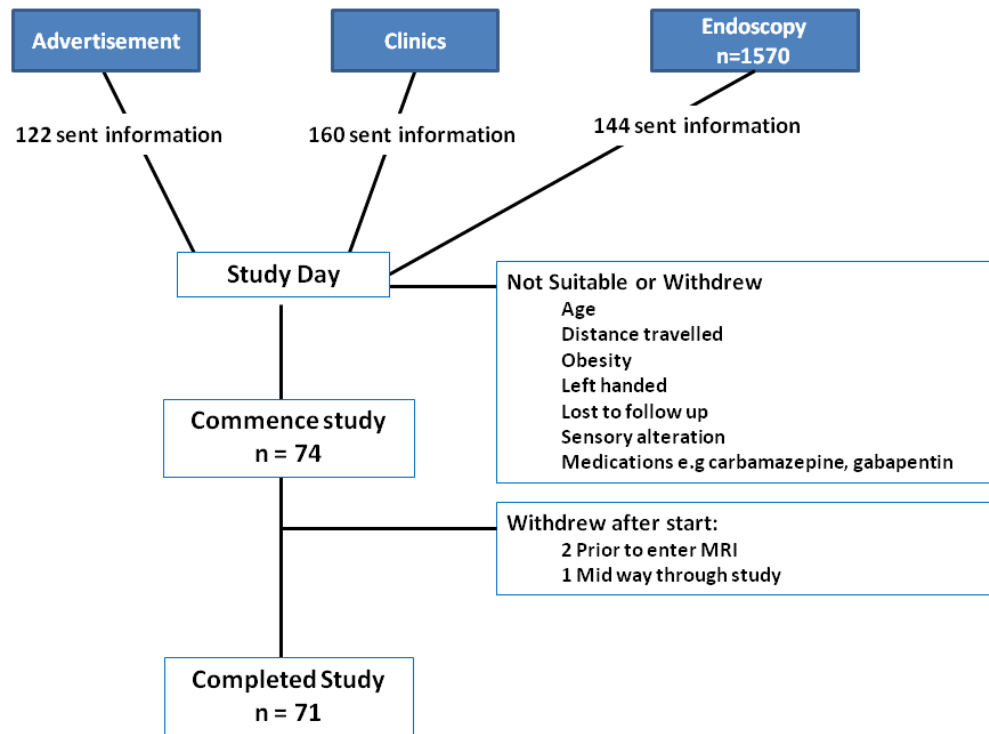
The study started recruiting in February 2010 with the first participant to be scanned in March 2010. Unfortunately the original Medoc Peltier CHEPS pathway system suffered a fault and a new Peltier was purchased from Medoc Israel. Therefore the first participant was scanned in May 2010. Participant recruitment was challenging and only 1-8 people recruited per month over a total of 19 months. Recruitment rate is shown in figure R2.1

Figure R2.1 Recruitment rate



426 Potential participants, identified from clinics, advertisements and endoscopy lists were sent standardized information about the study leading to several enquiries and 74 participants being recruited. Reasons for volunteers declining or being excluded from participating in the study can be seen in figure R2.2.

Figure R2.2 Flow diagram of participant recruitment



3 participants withdrew from the study: 2 after the sensory testing and before scanning and 1 at the break after the first scanning session.

(i) Demographics

74 participants took part in the study with 20 in the asymptomatic (ADD), 18 in the IBS and 36 in the SDD group. The distribution of PHQ12 scores within the SDD group was assessed and the cohort divided into 2 subgroups: one with a total PHQ12 scored less or equal to 6 (n=19, low symptomatic or LSDD) and one group with a score greater or equal to 7 (n=17, high symptomatic or HSDD). Demographic data for all the groups can be seen in table R2.1

Table R2.1 Demographics for all participants

Groups	ADD	LSDD (PHQ <6)	HSDD (PHQ >7)	IBS
All subjects	N=20	N=19	N=17	N=18
Female	50%	63.2%	70.6%	77.8%
Previous Diverticulitis	10%	36.8%	29.4%	0%
PMH psychiatric	25%	15.8%	17.6%	50%

After analysis of questionnaires, MRI motion plots and images derived from 1st level analysis for each participant, several participants were excluded from further analysis. Groups of 14 participants were created for each group based on the most complete data sets available. A table of subjects and reasons for exclusion can be seen in **Appendix 6.3**.

Demographic data for this subset, which underwent 2nd level RFX group analysis, is shown in table R2.2

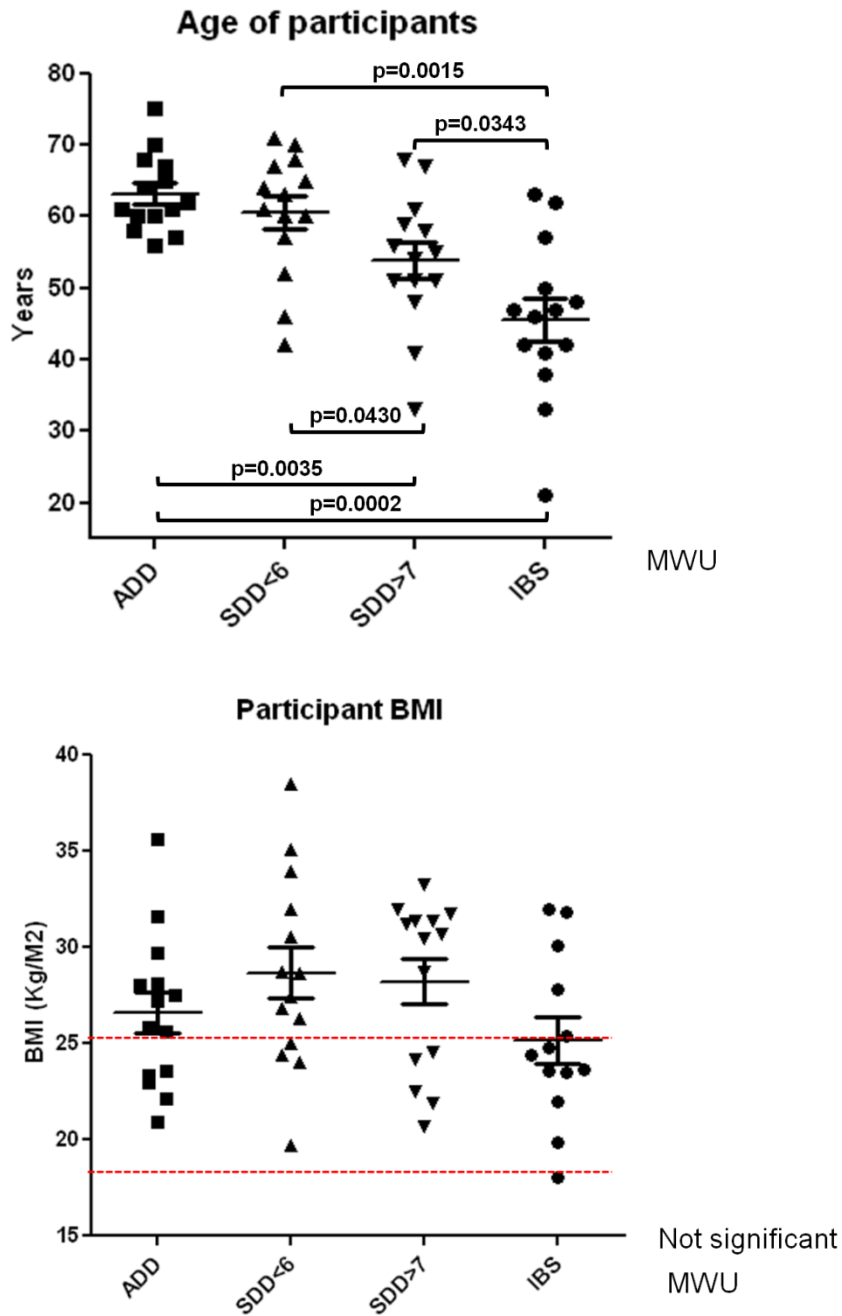
Table R2.2 Subset group demographics

fMRI Analysis groups (n=14)	ADD	LSDD (PHQ <6)	HSDD (PHQ >7)	IBS
Female	42.5%	57.1%	78.6%	78.8%
Previous Diverticulitis	0	50%	35.7%	0
PMH psychiatric	28.6%	7.1%	21.4%	42.9%

Further demographic analysis of the subset group included age and BMI and are shown in figure R2.3. Non-parametric t test (Mann Whitney U (MWU)) was used to confirm significant differences between groups.

Figure R2.3 Subset group age and BMI

(Red bars on the BMI graph represent normal BMI ranges according to World Health Organisation⁴²⁷)



2.4.2 Questionnaires results

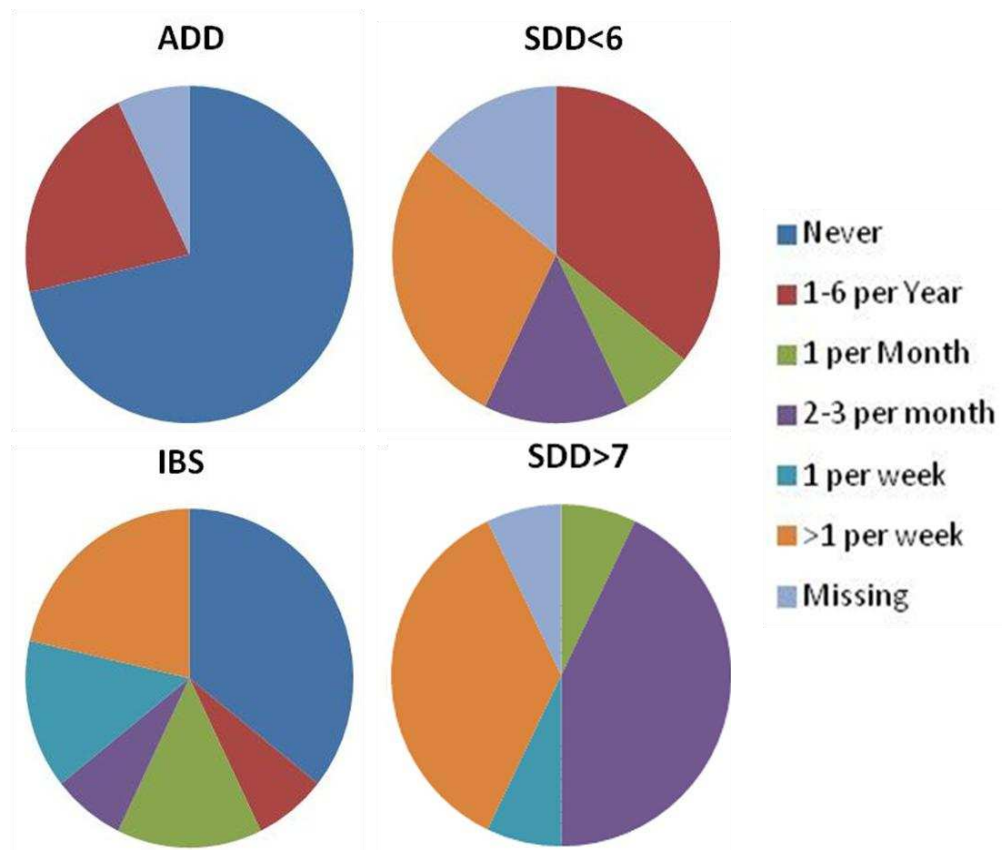
(i) Participant gastrointestinal symptoms

Pain duration was divided into pain lasting greater than 24 hours and pain lasting less than 24 hours.

(a) Pain lasting ≥ 24 hours

Both SDD groups reported pain lasting longer than 24hours more frequently than compared to the ADD and IBS groups (Figure R2.4). The incidence of pain lasting greater than 24hours (figure R2.4) was also increased in the High SDD (PHQ ≥ 7) compared to the Low SDD group (PHQ ≤ 6).

Figure R2.4 Graphical representation of Incidence of Pain lasting >24hours reported on questionnaire by group over 1 year.



(b) Pain lasting ≤24hours

For pain lasting less than 24 hours, there was a significant difference in the incidence and duration of pain between the ADD and both SDD and IBS groups (table R2.3)

Table R2.3 Incidence of Gastrointestinal symptoms reported on questionnaire per group over 1 year excluding pain lasting > 24 hours which was assumed to represent diverticulitis. Note the greater frequency and more prolonged pain in SDD compared to IBS.

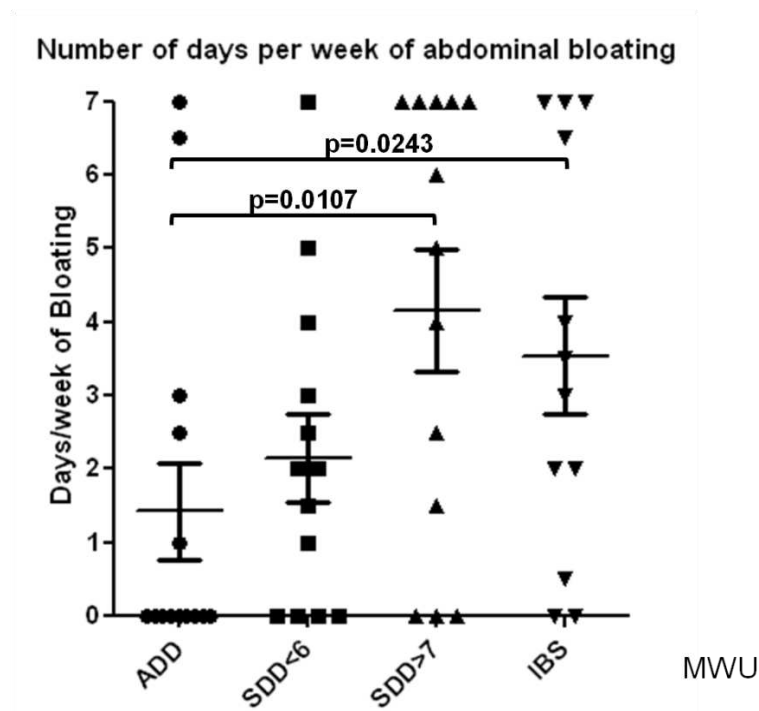
Groups	ADD	SDD (PhQ <6)	SDD (PhQ >7)	IBS
Median Days per month of pain (<24hrs) (iqr)	0	3 * (0-11)	15 *** (5-28)	7.5 *** (3.3-12)
Median Pain duration (hrs) (iqr)	0	1.5 * (0-5)	6 ** (3.4-24)	2.5 ** (0.8-12)
Median GP visits in last year for bowels (range)	1 (0-4)	0.5 (0-8)	2 (0-3)	0 (0-10)
Median: Bowels open/day (range)	2 (0.64-3)	1.5 (0.57-4)	2 (0-4)	1 (0.29-4)
Loose motions (days/week) (iqr)	1 (0-2.75)	0 (0-2)	2 (0-3)	1.5 (0-2)
Hard motions (days/week) (iqr)	2 (0-3.38)	1 (0-2)	1.75 (0-2.13)	1 (0-2.5)
Tenesmus (days/week) (iqr)	0 (0-1.5)	0 (0-2)	1 (1-2)	1 (0-2)
Blood per rectum	64.3%	14.3%	42.9%	28.6%

Add vs group * p<0.05 ** p<0.001 *** p< 0.0001

(c) Bowel habits

The median number of time the participants' bowels were opened and the incidences of hard or loose stool were similar between all the groups. There was a trend for tenesmus in the high SDD group, but it did not reach statistical significance. However the High SDD and IBS did report significantly more bloating compared to the ADD group (Figure R2.5), but there was no statistical difference between the SDD ($p=0.0818$) or IBS groups (Low SDD $p=0.2059$ and High SDD $p=0.5985$). The incidence of bleeding was also different between the groups, with 64.3% of participants in the ADD group reporting bleeding during the last year compared to only 14.3% in the low SDD and 42.9% and 28.6% in the high SDD and IBS groups respectively (table R2.3).

Figure R2.5 Graph representation of the incidence of bloating per week reported on questionnaire per group over the last year.



(d) GP visits

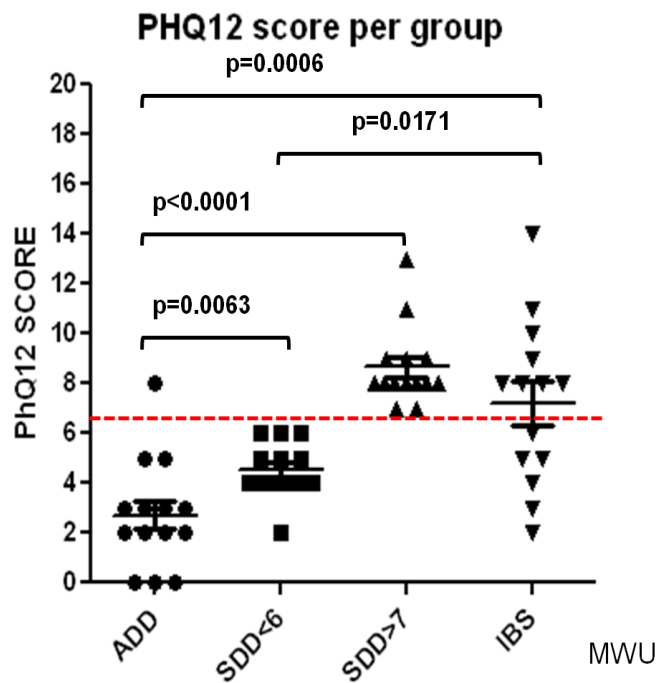
The median number of GP visits and the range is shown in table R2.3. No statistical difference was identified between the groups.

(ii) Participant psychological questionnaire results

(a) Physiological health questionnaire 12 (PHQ12)

This questionnaire was used to divide the SDD group into low (≤ 6) and high (≥ 7) scorers for further analysis. The graph (figure R2.6) shows PHQ12 score for the IBS and the ADD groups as a comparison to the SDD groups. The red bar marks the cut off between the two SDD groups. Statistical significance was calculated using a non-parametric t-test (MWU).

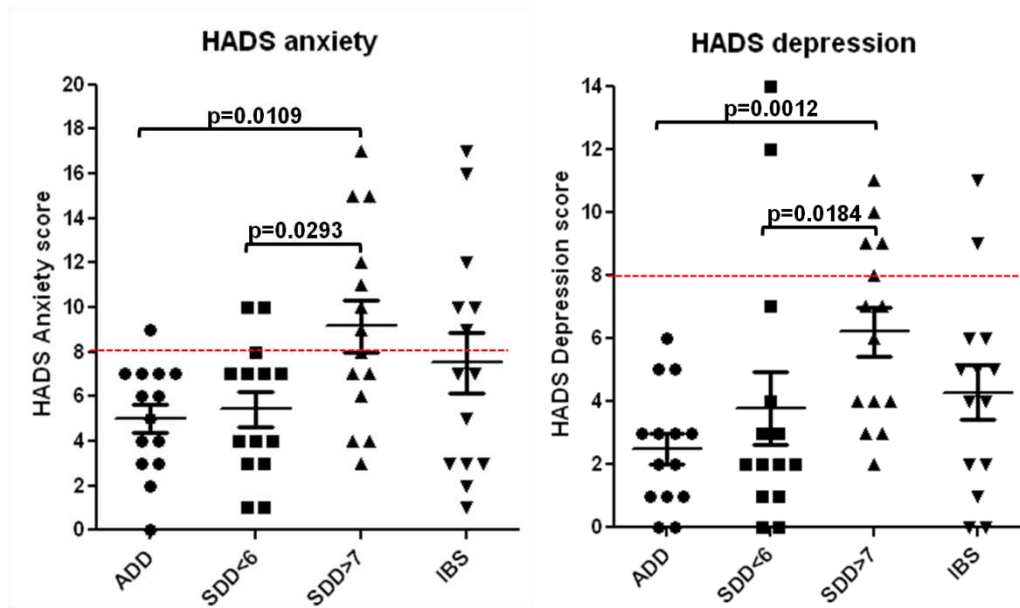
Figure R2.6 PHQ12 scores per Subset analysis groups



(b) Hospital Anxiety and Depression score (HADS)

Figure R2.7 Hospital Anxiety Depression Score

The graphs show the (A) Anxiety and (B) Depression Sub-score. The red broken line indicates the cut of between normal (below) and clinically significant anxiety and depression (above).



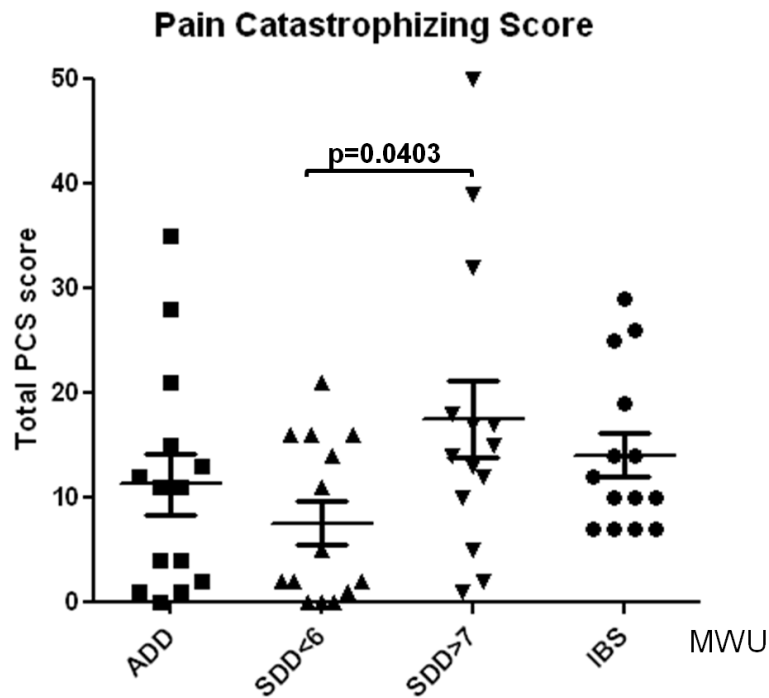
MWU

(c) Pain Catastrophizing Score (PCS)

Pain catastrophizing score distribution for each subset group analysis is shown in figure R2.8.

Significant difference in the total scale was identified between the low and high SDD groups.

Figure R2.8 PCS per Subset analysis groups



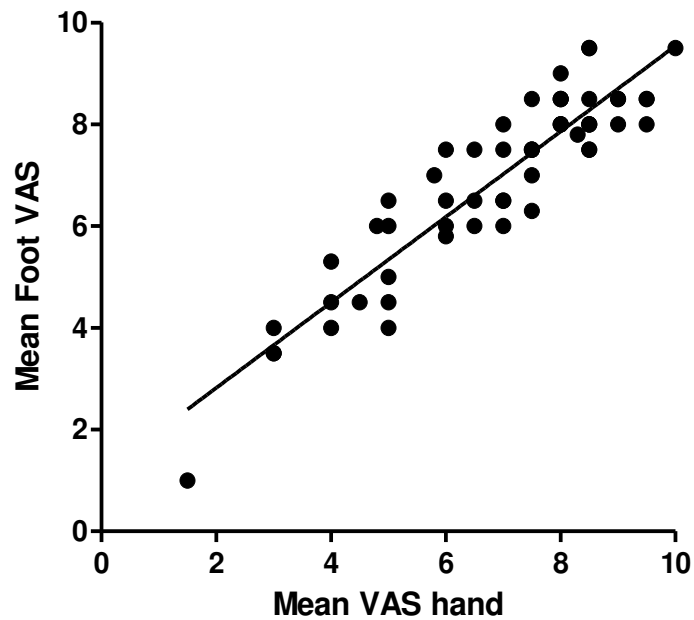
Multiple significant correlations were identified between the PHQ12, HAD, and PCS questionnaires and age of the subjects (Table R2.4).

Table R2.4 Correlations between questionnaires and ages of subjects

	PHQ12	HAD anxiety	PCS total
HAD anxiety	0.4531 ↑ p<0.0001	NA	NA
PCS total	0.2142 ↑ p=0.0024	0.2132 ↑ p=0.0049	NA
Age	0.3080 ↓ p<0.0001	0.1598 ↓ p=0.0195	0.07182 ↓ p=0.0423

Sensory testing prior to scanning paradigms resulted in identifying a 'VAS temperature' which consistently gave a VAS scores between 6 and 7. Despite this there was a variation in reported VAS at the end of the study paradigms themselves. This may have been to altered stress and anxiety caused by being in the scanner itself and was difficult to predict and control for. There was also strong correlation between the VAS scores given at the end of each paradigm for the hand and the foot (Figure R2.9: $r^2=0.8425$, $p<0.0001$)

Figure R2.9 Correlation between Foot and Hand VAS scores.



However there was no significant correlation between the 'VAS temperature' used and the VAS score for the hand or foot when compared with age, BMI, PCS score or depression component of the HAD questionnaire. There were no significant correlations either with BMI. Significant correlations are shown in table R2.5

Table R2.5 Correlation between Patient demographics, Questionnaires, VAS score during the paradigms and Vas Temperatures.

↑ positive correlation, ↓ negative correlation, n.s. not significant.

	Temp (°C)	PHQ12	HAD: Anxiety	HAD: Depression	PCS	Age
VAS Hand	$r^2=0.1025$ ↓ $p=0.0109$	$r^2=0.05660$ ↑ $p=0.659$	$r^2=0.05986$ ↑ $p=0.0403$	n.s.	n.s.	n.s.
VAS Foot	$r^2=0.1579$ ↓ $p=0.0043$	$r^2=0.05002$ ↑ $p=0.2247$	$r^2=0.04672$ ↑ $p=0.0759$	n.s.	n.s.	n.s.
HAD Anxiety	n.s.	$r^2=0.4531$ ↑ $p<0.0001$				
HAD Depression	n.s.	$r^2=0.2509$ ↑ $p<0.0001$	$r^2=0.3972$ ↑ $p<0.0001$			
PCS	n.s.	$r^2=0.2142$ ↑ $p=0.0024$	$r^2=0.1598$ ↑ $p=0.0195$	n.s.		
Age	n.s.	$r^2=0.3080$ ↓ $p<0.0001$	$r^2=0.1598$ ↓ $p=0.0195$	$r^2=0.09445$ ↓ $p=0.0112$	$r^2=0.07182$ ↓ $p=0.0423$	

2.4.3 Sensory testing results

Table R2.6 demonstrates the median and inter-quartile range (IQR) of the temperatures selected for the VAS temperature heat pulses and the mean VAS scores given at the completion of each study on the hand or foot. Although there was a trend for lower temperatures and higher VAS scores in the SDD and IBS groups for both the hand and foot, this did not reach significance. There were also no significant differences in VAS temperatures used or VAS scores between the hand and feet within each group.

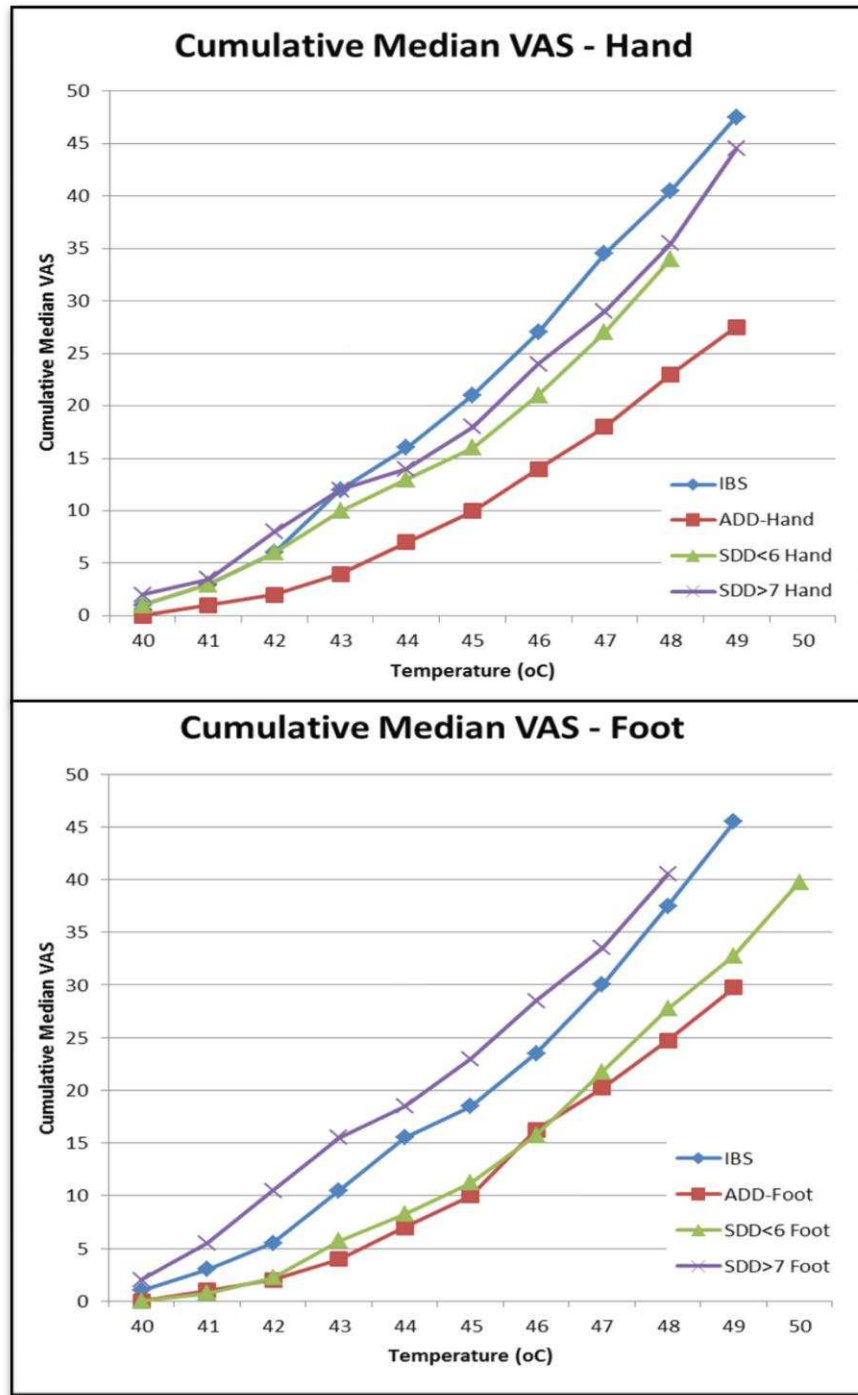
Table R2.6 Median VAS temperatures and scores per group

Group	Hand Median Temp. (°C) (IQR)	Hand Median VAS (IQR)	Foot Median Temp. (°C) (IQR)	Foot Median VAS (IQR)
ADD	45.40 (43.50-46.75)	6.0 (4.75-8.13)	45.50 (43.38-46.00)	7.25 (4.88-8.50)
LSDD	43.75 (42.50-47.00)	7.5 (4.95-8.00)	43.50 (42.00-46.63)	6.75 (6.00-8.50)
HSDD	43.75 (41.88-45.75)	8.25 (6.00-8.50)	43.75 (41.88-44.88)	7.50 (5.63-8.75)
IBS	43.75 (42.38-45.63)	7.25 (6.00-8.50)	44.50 (43.13-46.13)	7.75 (6.38-8.00)

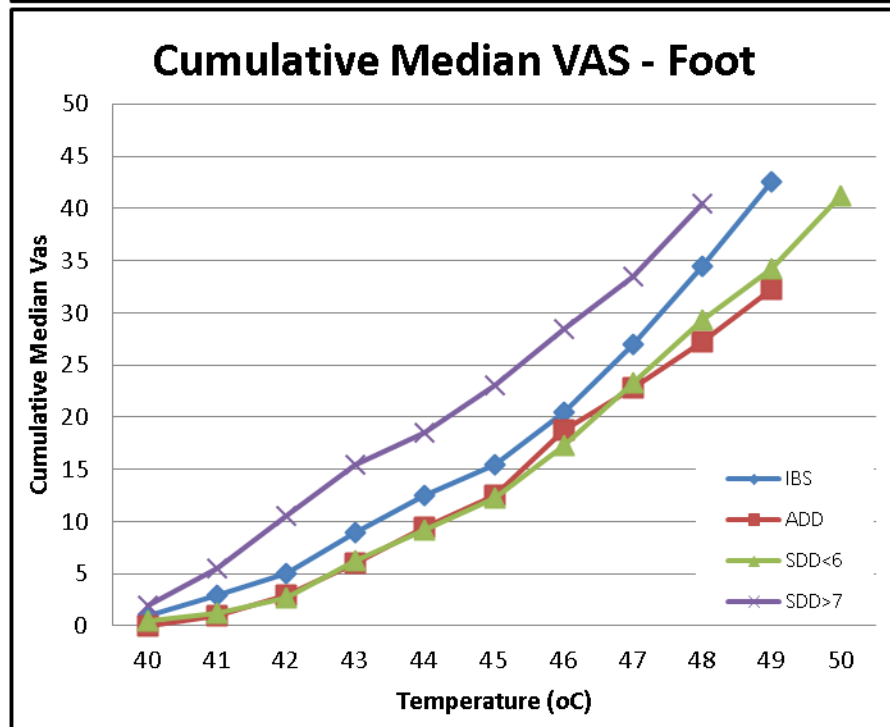
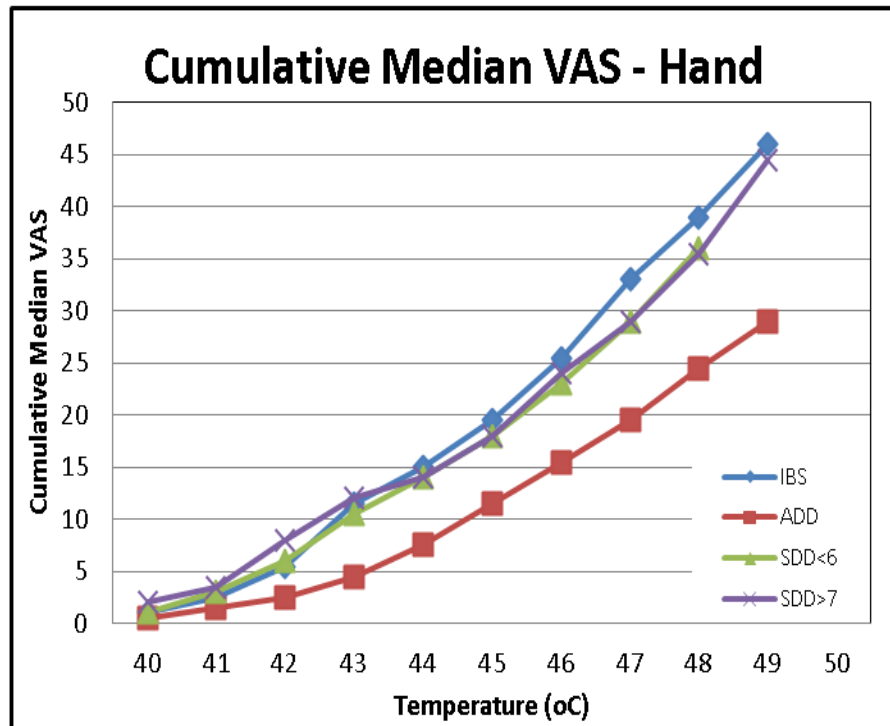
As participants stopped sensory testing once a VAS score of 6-7 was identified, the relationship between VAS score and temperature is difficult to represent graphically. Therefore, a cumulative median has been created where the previous VAS scores for lower temperatures are added to the subsequent VAS score for the next temperature tested i.e. (ADD: mean VAS 40 + mean VAS 41 + mean VAS 42 etc) (Figure R2.10 (a) and (b)).

Figure R2.10 Cumulative VAS score per temperature used in sensory testing per analysis group (a) and for all participants (b).

(a) Median Cumulative Vas score per temperature for Hand and Foot per analysis group (n=14 per group)



(b) Median Cumulative Vas score per temperature for Hand and Foot for all participants (IBS N=18, ADD N=20, LSDD N=19, HSDD N=17)



2.4.4 fMRI Results

Both the VAS temperature and the '45°C' temperature components of the paradigm were planned to be used in for the analysis. This would have given functional brain comparisons for the groups at a consistent level of pain (VAS temperature stimulus) and at a consistent temperature (45°C stimulus). Unfortunately during the duration of the study it was found that few subjects were able to tolerate the 45°C stimulus and this had to be adjusted to allow any imaging to be obtained. Thus the analysis performed is only of the VAS temperature.

Despite this the study still generated a very large amount of fMRI results and summarising these in a comprehensible format suited for this dissertation was a challenge in itself. For this purpose the main fMRI results were summarised here in tables subdivided by main functional (e.g. somatosensory, affective) and then anatomical (e.g. S1, anterior insula and left or right hemisphere) area. Activations and deactivations were then represented per patient group by arrows (↑ and ↓ respectively).

(i) Group map descriptions for hand and foot stimuli

Table R2.7 Group Maps

2nd level random effect analysis of 14 subjects per group (FDR [false discovery rate] corrected, Voxel Threshold 5). Below are simplified results showing activations (↑) and deactivations (↓) within different brain regions for the VAS heat stimulus to the left foot (A) or hand (B) for each group (FDR corrected $p < 0.05$) using a temperature which was rated 6-7 on 10 point visual analogue scale (VAS temperature. Areas which included both activations and deactivations are represented by ↑↓. Most consistently activated areas are highlighted in yellow.

Key: SMA: Supplemental Motor Area, PFC prefrontal cortex, ACC, anterior cingulate cortex, MCC mid cingulate cortex, PCC posterior cingulate cortex, S1 primary somatosensory cortex, S2 secondary somatosensory cortex, Motor, Primary Motor cortex. DNIC descending nociceptive inhibitory or facilitatory controls.

Table R 2.7 (A) Activations (↑) and deactivations (↓) for the VAS heat stimulus to the left foot using a temperature which was rated 6-7 on 10 point visual analogue scale (VAS temperature).

Area		Side	ADD	LOW SDD	HIGH SDD	IBS
Somatosensory	S1	L				
		R				
Somatosensory	S2	L	↑	↑ ↓	↑	↑
		R	↑	↓	↑	↑
Somatosensory	Post-Ins	L				
		R				
Somatosensory	Mid-Ins	L	↑	↑		
		R		↑	↑	↑
Affective	Ant-Ins	L	↑			↑
		R	↑	↑		
Affective	ACC	L	↓		↓	
		R		↑		
Affective	MCC	L	↑		↑	
		R	↑ ↓	↑	↑	↑
Affective	PCC	L			↑	
		R				
Affective	Medial PFC	L		↓	↓	↓
		R		↓		↓
DNIC	Lateral PFC	L	↑	↑ ↓	↑ ↓	↑ ↓
		R	↑	↑ ↓	↓	↓
DNIC	Orbito-FC	L		↓		↓
		R				
Somatosensory	Lentiform Nuclei and Thalamus	L	↑		↓	↑ ↓
		R	↑	↓	↓	↓
Affective	Amygdala (Hippocampus)	L		↓	↓	↓
		R			↓	↑ ↓
Somatosensory	Cerebellum	L	↑	↑	↑	↑ ↓
		R	↑		↓	↑ ↓
Somatosensory	Inferior Parietal	L	↑			↓
		R				
Somatosensory	Temporal	L	↑ ↓	↑ ↓	↑ ↓	↑ ↓
		R		↓	↑ ↓	
Motor	SMA	L				
		R				
Somatosensory	Post-central Gyrus	L	↓	↓	↓	↓
		R	↑ ↓	↓	↓	↓
DNIC	Subthalamic/ Brainstem		↑	↑		

Table R2.7 (B) Activations (↑) and deactivations (↓) for heat stimulus to the left hand using a temperature which was rated 6-7 on 10 point scale (VAS temperature stimulus.) Note the pattern is very similar to that seen with foot stimulus.

Area	Side	ADD	LOW SDD	HIGH SDD	IBS
Somatosensory S1	L				
	R				
Somatosensory S2	L		↑	↑	
	R	↑	↑	↑	↑
Somatosensory Post-Ins	L				
	R				
Somatosensory Mid-Ins	L		↑	↑	
	R		↑	↑	↑
Affective Ant-Ins	L	↑		↑	
	R	↑			↑
Affective ACC	L				
Affective MCC	R		↑		
	L	↑	↑		↑
Affective PCC	R			↑	
	L				
Affective Medial PFC	L	↓	↓	↓	↓
	R			↑	↓
DNIC Lateral PFC	L	↑↓	↑↓	↑↓	↓
	R	↑↓	↓	↓	↑↓
DNIC Orbito-FC	L	↓	↓		↓
	R		↓		↓
Somatosensory Lentiform Nuclei and Thalamus	L	↑↓		↓	↑
	R	↑↓		↑	↑
Affective Amygdala (Hippocampus)	L		↓	↓	↓
	R			↑	
Cerebellum	L	↑	↑	↑	↑↓
	R	↓	↑	↑↓	↑↓
Inferior Parietal	L	↑	↑	↑	
	R				
Temporal	L		↓	↓	↓
	R	↓	↑	↓	
Motor SMA	L				
	R				
Somatosensory Post-central Gyrus	L	↑		↓	↓
	R		↑	↓	↑
DNIC Subthalamic/ Brainstem	L	↓	↑↓	↓	↓
	R	↓	↓	↑	↑↓

(vi) Intra-Group Analysis: Differences between hand and foot VAS stimuli

Below are simplified fMRI results of 2 sample t test comparing activations (↑) and deactivations (↓) when the VAS temperature stimulus is applied to the left hand or left foot (R2.9 A and B; uncorrected p<0.05). Most consistently activated areas are highlighted in yellow.

Table R2.8 (A) Hand > Foot VAS Stimulus

Areas where activations and deactivations are greater in the left hand than in the left foot.

Area	Side	ADD	LOW SDD	HIGH SDD	IBS
Somatosensory	S1	L			
		R			
Somatosensory	S2	L	↑		↓
		R	↑	↑	↑
Somatosensory	Post-Ins	L			
		R		↑	
Affective	Mid-Ins	L			
		R			
Affective	Ant-Ins	L			
		R			
Affective	ACC	L	↑		↓
		R		↑	↓
Affective	MCC	L	↓		↑
		R	↑	↑	↑
Affective	PCC	L			↓
		R			
Affective	Medial PFC	L	↑		↓
		R			↓
DNIC	Lateral PFC	L	↑		↓
		R	↑	↑	↑
DNIC	Orbito-FC	L			↓
		R			
Somatosensory	Lentiform Nuclei and Thalamus	L			↓
		R		↑	
Affective	Amygdala (Hippocampus)	L	↓	↓	↓
		R	↓	↓	↓
	Cerebellum	L	↑↓	↑	↑
		R		↑	↓
	Inferior Parietal	L			↑
		R			
	Temporal	L	↓	↓	↓
		R	↓	↓	↓
Motor		L			
		R			
	SMA	L	↑		↑
		R	↓	↑	
Somatosensory	Post-central Gyrus	L			
		R	↑	↑	↑
DNIC	Subthalamic/ Brainstem		↓	↓	↓

Table R2.8 (B) Foot > Hand VAS Stimulus

Areas where activations and deactivations are greater in the left foot than the left hand.

Area	Side	ADD	LOW SDD	HIGH SDD	IBS
Somatosensory	S1	L			
		R			
	S2	L			
		R			↑
Somatosensory	Post-Ins	L	↑		↑
		R	↑		↑
	Mid-Ins	L			
		R			
Affective	Ant-Ins	L			↑↓
		R			↑
Affective	ACC	L		↑	↑
		R			↑
Affective	MCC	L		↑	↑
		R		↑	↑
	PCC	L			
		R			↓
Affective	Medial PFC	L	↓		↑
		R			↑
DNIC	Lateral PFC	L	↓	↓	↑
		R			↑
DNIC	Orbito-FC	L			↓
		R			↑
Somatosensory	Lentiform Nuclei and Thalamus	L		↑	↑
		R	↑	↑	↑
Affective	Amygdala (Hippocampus)	L		↑	↑
		R			↑
Cerebellum		L	↑	↓	↑
		R		↓	
Inferior Parietal		L			
		R			
Temporal		L	↑	↑	↑
		R	↑↓		↑
Motor	SMA	L			
		R			
		L			↑
		R	↑		↑
Somatosensory	Post-central Gyrus	L			↓
		R	↓	↓	↑↓
DNIC	Subthalamic/ Brainstem		↑		↑

(vii) Inter-Group Analysis: Differences between hand and foot response to heat rated 6-7 on a 10 point scale (VAS temperature stimulus)

Below are simplified fMRI results of 2 sample t test comparing activations (\uparrow) and deactivations (\downarrow) between the ADD and other groups in the Foot (A) or Hand (B) when the VAS temperature stimulus is applied (uncorrected $p < 0.05$) (Table R2.9). The table is split into two parts. The first three results central columns are areas where there is a significant probability that activations and deactivations are greater in the ADD group compared to the others groups (ADD $>$). In the following three results columns on the right are areas where there is a significant probability that activations and deactivations are less in the ADD group compared to the others groups ($>$ ADD).

Further below are simplified fMRI results of 2 sample t test comparing activations (\uparrow) and deactivations (\downarrow) between the IBS and SDD groups in the Foot (A) or Hand (B) when the VAS temperature stimulus is applied (uncorrected $p < 0.05$) (Table R2.10). The table is split into three parts. The first two results columns on the left are areas where there is a significant probability that activations and deactivations are greater in the IBS group compared to the SDD groups (IBS $>$ SDD). In the two central results columns are areas where there is a significant probability that activations and deactivations are less in the IBS group compared to the SDD groups (SDD $>$ IBS). The third two results columns on the right compare the significant differences between the SDD groups, where the probability of (de)activations is greater in the Low SDD group (LSDD $>$ HSDD) or the High SDD group (HSDD $>$ LSDD).

The brain regions that have been used to create these tables can be large and contain many smaller subdivisions e.g. the thalamus is made of multiple nuclei. Therefore in some comparisons between the groups significant activation can be identified within both groups when compared to the other. For example in table R2.9(A) activation are seen in the S2 region in all the groups. This means that different parts of the S2 region was significantly activated, which can be seen in the more detailed tabulated co-ordinate data presented in **Appendix 6.5**.

Table R2.9 Inter-Group Analysis: Differences between brain activations to VAS temperature stimulation of the left foot (A) or left hand (B) for different groups compared to ADD group. Many areas showed greater activation in SDD and IBS

(A) Foot Vas Temperature

Area	Side	ADD> LSDD	ADD> HSDD	ADD> IBS	LSDD> ADD	HSDD> ADD	IBS> ADD
SS	S1						
SS	S2	↓				↑	↑
		↑	↑	↑	↑	↑	↑
SS	Post-Ins			↓			
		↑					↑
SS	Mid-Ins						↑
							↑
Aff.	Ant-Ins				↑		↑
					↑	↑	
Aff.	ACC			↑		↑	↑
Aff.	MCC		↑		↑	↑	↑
					↑	↓	↓
Aff.	PCC					↑	↑
Aff.	Medial PFC		↓	↑	↓	↓	↓
			↓		↑↓		↑↓
DNIC	Lateral PFC		↓	↑↓	↑↓	↑↓	↑↓
		↑	↓		↑	↑	↑
DNIC	Orbito-FC			↑			↓
							↑
SS	Lentiform Nuclei and Thalamus		↑		↑	↑↓	
			↑	↓	↑	↑	↑
Aff.	Amygdala (Hippocampus)		↓	↓	↑	↑	↓
			↓	↓	↑		
	Cerebellum	↑	↑		↓	↓	↑
					↓	↓	
	Inferior Parietal		↑	↑↓			
					↑	↑	
	Temporal	↓	↓	↓	↑↓	↑	↓
		↑↓	↑↓	↑↓	↓	↑↓	↑↓
	Motor						
	SMA		↑	↑			
					↑		↑
SS	Post-central Gyrus	↓	↓	↓		↑	
		↓	↓		↑	↑	↓
DNIC	Subthalamic/Brainstem		↑		↑	↓	

Table R2.9 Inter-Group Analysis: Differences between hand and foot VAS stimuli compared to ADD group. Highlighted areas show increased activation in DNIC areas in ADD versus HSDD and IBS.

(B) Hand Vas Temperature

Area	Side	ADD> LSDD	ADD> HSDD	ADD> IBS	LSDD> ADD	HSDD> ADD	IBS> ADD
SS	S1						
	R						
SS	S2		↑	↑	↑		
	R	↑	↑	↑			↑
SS	Post-Ins				↑		↑
	R						
SS	Mid-Ins						↑
	R						
Aff.	Ant-Ins	↑		↑	↑		↑
	R						
Aff.	ACC		↑				↑
	R	↓			↑		↑
Aff. +	MCC		↑	↑			↑
	R		↑		↑		↑
Aff.	PCC				↓		
	R				↓		
Aff.	Medial PFC	↑↓	↓	↑↓	↓	↓	↓
	R	↑	↓		↓	↑	↑↓
DNIC	Lateral PFC	↑	↑	↑	↓	↓	↓
	R	↑	↑↓	↑	↑	↓	↑
DNIC	Orbito-FC						
	R			↑	↑	↑	↑
SS	Lentiform Nuclei and Thalamus		↑↓	↓	↑	↑	↑
	R		↓	↓	↑	↑	↑
Aff.	Amygdala (Hippocampus)	↑↓	↓	↓	↑↓	↓	↓
	R	↓	↓	↓	↑↓	↑↓	
	Cerebellum		↑		↑	↑	↑
	R	↓		↑	↑↓		
	Inferior Parietal		↑				
	R				↑	↑	
	Temporal	↑↓	↓	↑↓	↑↓	↑↓	↑↓
	R	↑↓	↑↓	↑↓	↑↓	↑	↑
	Motor						
	R						
	SMA	↑	↑	↑	↑		↑
	R		↑		↑		↑
SS	Post-central Gyrus		↓	↓	↓		↓
	R	↓	↓		↓	↑	↑
DNIC	Subthalamic/Brainstem	↑	↑↓	↑			

Table R2.10 Inter-Group Analysis: Differences between activation in response to left foot (A) and left hand cutaneous heat stimuli compared to IBS and SDD

(A) Foot VAS Stimulus

Area	Side	IBS> LSDD	IBS> HSDD	LSDD> IBS	HSDD> IBS	
SS	S1	L				
		R				
	S2	L	↑	↑		
		R	↑	↑		↑
SS	Post-Ins	L	↑			
		R		↑		
	Mid-Ins	L		↑		
		R				
Aff.	Ant-Ins	L	↑			
		R	↑	↑	↑	
Aff.	ACC	L	↑			
Aff.	MCC	L				↑
		R	↑	↑	↑	↓
Aff.	PCC	L				
		R				
Aff.	Medial PFC	L		↓	↑↓	↑
		R	↓	↓		
DNIC	Lateral PFC	L	↑	↑↓	↓	↑
		R	↑↓	↑↓	↑	↑
DNIC	Orbito-FC	L		↑	↑	
		R	↑	↑		↑
SS	Lentiform Nuclei and Thalamus	L				
		R		↑	↓	↑↓
Aff.	Amygdala (Hippocampus)	L		↓	↑↓	↑↓
		R		↓	↑↓	↑↓
	Cerebellum	L	↑	↑		
		R				↓
	Inferior Parietal	L		↓		
		R			↑	↑
	Temporal	L	↑	↓	↓	↑↓
		R	↑	↓	↑↓	↑↓
	Motor	L				
		R				
	SMA	L		↑	↑	
		R		↑		
SS	Post-central Gyrus	L	↓	↓		↓
		R	↓	↑↓	↑	↑
DNIC	Subthalamic/ Brainstem		↑	↑		↑

Table R2.10 Inter-Group Analysis: Differences between hand and foot VAS stimuli compared to IBS and SDD

(B) Hand VAS Stimulus

Area	Side	IBS> LSDD	IBS> HSDD	LSDD> IBS	HSDD> IBS	
SS	S1	L				
		R				
	S2	L				
		R			↑	↑
SS	Post-Ins	L				
		R				
	Mid-Ins	L				
		R			↑	
Aff.	Ant-Ins	L	↑	↑		
		R	↑	↑	↑	↑
Aff.	ACC	L		↑	↓	
Aff.	MCC	L		↑		↓
Aff.	PCC	L	↑	↑		↑
		R				↓
Aff.	Medial PFC	L	↓	↓	↓	↓
		R		↑↓	↑↓	↑
DNIC	Lateral PFC	L	↑	↓		↑↓
		R	↑	↑	↑	↑
DNIC	Orbito-FC	L			↑	↑
		R		↑	↓	
SS	Lentiform Nuclei and Thalamus	L	↓	↓	↑	↑
		R		↑	↑↓	↑↓
Aff.	Amygdala (Hippocampus)	L		↑↓	↓	↑↓
		R	↓	↓	↓	↑↓
	Cerebellum	L		↑	↑	
		R		↓	↑↓	↑
	Inferior Parietal	L		↓		↑
		R				
	Temporal	L	↑	↑↓	↑↓	↑↓
		R	↑	↑↓	↑↓	↑↓
	Motor	L				
		R				
	SMA	L		↑	↑	↓
		R	↑	↑		↓
SS	Post-central Gyrus	L		↑↓	↓	
		R	↑↓	↑↓		↑
DNIC	Subthalamic/ Brainstem		↑	↑↓	↑	

Table R2.11 Inter-Group Analysis: Differences between activation in response to left foot (A) and left hand cutaneous heat stimuli between the SDD groups

(A) Foot VAS Stimulus

	Area	Side	LSDD> HSDD	HSDD> LSDD
SS	S1	L		
		R		
	S2	L	↑	
		R		↑
SS	Post-Ins	L		
		R	↑	↑
	Mid-Ins	L		
		R		
Aff.	Ant-Ins	L		
		R		
Aff.	ACC	L	↑	
Aff.	MCC	R	↑	
		L	↑	↓
	PCC	R		
		L		
Aff.	Medial PFC	L	↓	
		R	↑↓	
DNIC	Lateral PFC	L	↑↓	
		R	↑	↑
DNIC	Orbito-FC	L		
		R		
SS	Lentiform Nuclei and Thalamus	L	↑	
		R	↑	
Aff.	Amygdala (Hippocampus)	L		
		R	↑↓	
	Cerebellum	L		↑
		R	↓	
	Inferior Parietal	L		
		R	↑	
	Temporal	L	↓	↑
		R	↑↓	↑
	Motor	L		
		R		
	SMA	L	↑	
		R	↑	
SS	Post-central Gyrus	L	↓	
		R	↑↓	↓
DNIC	Subthalamic/ Brainstem		↑	

Table R2.11 Inter-Group Analysis: Differences between left hand and left foot VAS stimuli between the SDD groups

(B) Hand VAS Stimulus

	Area	Side	LSDD> HSDD	HSDD> LSDD
SS	S1	L		
		R		
	S2	L	↑	
		R	↑	
SS	Post-Ins	L	↑	
		R	↑	
	Mid-Ins	L		
		R		
Aff.	Ant-Ins	L	↑	
		R		
Aff.	ACC	L	↑	
		R	↑	
Aff.	MCC	L	↑	
		R	↑	
	PCC	L		
		R	↓	
Aff.	Medial PFC	L	↑↓	↓
		R	↑↓	
DNIC	Lateral PFC	L	↑↓	↓
		R	↑	↑
DNIC	Orbito-FC	L		
		R		
SS	Lentiform Nuclei and Thalamus	L	↑	
		R	↑	↓
Aff.	Amygdala (Hippocampus)	L	↑	↓
		R	↓	↓
	Cerebellum	L	↑	↓
		R		
	Inferior Parietal	L	↑↓	↑
		R		
	Temporal	L	↓	↓
		R	↑↓	
	Motor SMA	L		
		R		
		L		
		R	↑	
SS	Post-central Gyrus	L	↓	
		R	↑↓	↑
DNIC	Subthalamic/ Brainstem		↑	

Tables R2.9 - R2.11 show that during painful stimulation of the left foot, the ADD had less affective pain processing compared to other groups. Comparison between the LSDD and ADD groups showed few areas of significant differences, but included the somatosensory processing and DNIC areas such as the right pINS and bilateral PCG and the right lateral PFC. In contrast the LSDD groups had greater activity in affective areas such as the bilateral aINS, right ACC and MCC, bilateral amygdala and hippocampal regions and PFC.

During painful stimulus of the hand, the IBS group showed greater activation of the aINS compared to both SDD groups. The LSDD group demonstrated greater deactivation of the brainstem, orbito-PFC, cerebellum, and amygdala compared to the IBS group. Greater activity in the ACC and cerebellum was also seen in the IBS group compared to the HSDD group. However in the IBS and HSDD group both demonstrated increased activation and deactivation within different regions of the PFC, thalamus and amygdala suggesting wide spread but not identical activity within these regions.

(viii) Covariates analysis of VAS hand and foot stimuli.

In **Appendix 6.6** are simplified fMRI results comparing activations (↑) and deactivations (↓) correlating with participant's VAS score (reported pain intensity out of 10 at the end of the scanning paradigm) or VAS temperature (temperature at pre-scanning sensory testing at which subjects gave a VAS score of 6-7) used as the stimulus on the foot (A) and hand (B) during the stimulus (uncorrected $p < 0.01$ voxel 5). Other covariates used include the hospital anxiety and depression score, PCS and PHQ questionnaire. All effects were identified using group maps as a masked for the data.

(ix) Anticipation: group maps for the visual Cue stimulus

Below are simplified fMRI results showing activations (↑) and deactivations (↓) within different brain regions for the visual cue for both the left hand and foot stimuli (A) (Uncorrected $p < 0.001$,

voxel threshold 5) (Table R2.12). Areas which included both activations and deactivations are represented by ↑↓.

Table R2.12 shows that during anticipation of pain by group the ADD group showed activity in the insula cortex. A similar smaller activation was also seen in the LSDD group in the pINS and activations in the left ant and mid INS. In the HSDD and IBS group the right ant and mid INS and the left aINS were activated respectively. This contrasted with the cingulate cortex where only the right MCC was activated in the ADD group compared to the mid and ant cingulate cortices in the other groups. In the PFC the ADD group showed increased activity in the bilateral lateral PFC. This was similar to the other groups. Greater deactivation was also seen bilaterally in the amygdala in the ADD group but not the other groups. Activations and deactivations in other regions were similar between the different groups.

Table R 2.12 Group Maps

(A) Activations (↑) and deactivations (↓) for cue visual stimulus for both left hand and foot

Area	Side	ADD	LOW SDD	HIGH SDD	IBS
Somatosensory	S1				
		L			
		R			
	S2				
	L				
Somatosensory	Post-Ins				
		L			
		R	↓	↓	
	Mid-Ins				
	L	↓	↑		
	R	↓		↑	
Affective	Ant-Ins				
		L	↑	↑	↑
	R	↑		↑	
Affective	ACC		↑	↑	↑
	L				
Affective	MCC		↑	↑	↑
		L	↑	↑	↑
	R				
	PCC				
	L				
	R				
Affective	Medial PFC				
		L	↓	↓	↑↓
	R	↓	↓	↓	↑↓
DNIC	Lateral PFC				
		L	↑↓	↑↓	
	R	↑↓	↓	↑↓	↑↓
DNIC	Orbito-FC				
		L	↓	↓	↑↓
	R	↓			
Somatosensory	Lentiform Nuclei and Thalamus				
		L	↑	↑↓	↑
	R	↓	↑↓	↑	↓
Affective	Amygdala (Hippocampus)				
		L	↓		
	R	↑↓	↓		↑↓
	Cerebellum				
		L	↑	↑↓	↑↓
	R	↑↓			↑↓
	Inferior Parietal				
		L		↑	
	R				
	Temporal				
		L	↑↓	↑↓	
	R	↑	↑↓	↑	↑↓
Motor					
		L			
		R			
	SMA				
	L	↑		↑	↑
	R	↑		↑	↑
Somatosensory	Post-central Gyrus				
		L			
	R	↓			↓
DNIC	Subthalamic/ Brainstem				

(x) Intra-Group Analysis: Differences for the cue stimulus

Below are simplified fMRI results of 2 sample t test comparing activations (↑) and deactivations (↓) for the visual cue (R2.14. A and B; Uncorrected $p < 0.05$, voxel threshold 5).

Table R2.13. (A) Hand > Foot Cue Stimulus

Areas where activations (↑) and deactivations (↓) are greater in the hand than the foot.

Area	Side	ADD	LOW SDD	HIGH SDD	IBS
Somatosensory	S1	L			
		R			
	S2	L			
		R			
Somatosensory	Post-Ins	L	↑		↓
		R	↑	↓	
	Mid-Ins	L	↑		
		R	↑		
Affective	Ant-Ins	L	↑		
		R	↑		
Affective	ACC	L	↑		↑
		R			
Affective	MCC	L	↑		
		R	↑		↑
	PCC	L			
		R			
Affective	Medial PFC	L	↓	↓	↓
		R	↓	↓	↑
DNIC	Lateral PFC	L	↑↓	↑	↓
		R	↑		↑
DNIC	Orbito-FC	L			↓
		R			
Somatosensory	Lentiform Nuclei and Thalamus	L		↑↓	↑
		R	↑	↑	↑
Affective	Amygdala (Hippocampus)	L	↓	↓	
		R	↓		
	Cerebellum	L		↑↓	↑
		R		↑↓	
	Inferior Parietal	L			
		R	↑		
	Temporal	L	↑↓	↑	↑↓
		R	↑↓	↓	↑
Motor	SMA	L			
		R			
	Post-central Gyrus	L	↑		
		R		↓	
DNIC	Subthalamic/ Brainstem		↓		

Table R2.13 (B) Foot > Hand Cue Stimulus

Areas where activations (↑) and deactivations (↓) are greater in the foot than the hand.

Area	Side	ADD	LOW SDD	HIGH SDD	IBS	
Somatosensory	S1	L				
		R				
	S2	L				
		R				
Somatosensory	Post-Ins	L	↓		↓	
		R			↓	
	Mid-Ins	L			↑	
		R			↓	
Affective	Ant-Ins	L		↑		
		R				
Affective	ACC	L			↑	
Affective	MCC	R		↑	↑	
		L				
Affective	PCC	R			↓	
		L				
Affective	Medial PFC	L			↓	
		R	↓		↓	
DNIC	Lateral PFC	L	↓		↑ ↓ ↓	
		R		↓	↑ ↓ ↓	
DNIC	Orbito-FC	L			↑ ↓ ↓	
		R		↓		
Somatosensory	Lentiform Nuclei and Thalamus	L				
		R				
Affective	Amygdala (Hippocampus)	L			↓	
		R		↓		
	Cerebellum	L			↓	
		R			↓	
	Inferior Parietal	L		↑		
		R				
	Temporal	L	↑ ↓		↑ ↓	
		R	↑		↓	
Motor	SMA	L				
		R				
		L		↑	↑	
		R		↑	↑	
Somatosensory	Post-central Gyrus	L				
		R		↑		
DNIC	Subthalamic/ Brainstem					

(xi) Inter-Group Analysis: Differences between cue stimuli

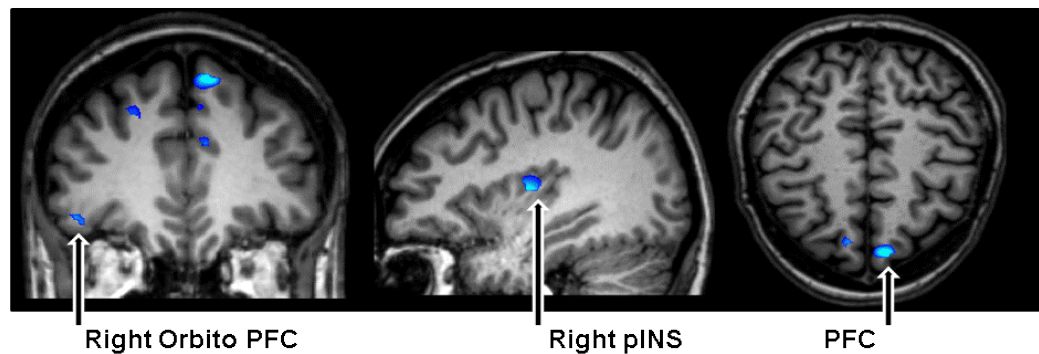
Below are simplified fMRI results of 2 sample t test comparing activations (↑) and deactivations (↓) between the ADD and other groups during cue stimulus on both the left hand and foot combined (uncorrected $p < 0.05$, voxel threshold 5) (Table R2.14 and figure R2.11). The table is split into two parts. The first columns are areas where there is a significant probability that activations and deactivations are greater in the ADD group compared to the others groups (ADD>). In the second columns are areas where there is a significant probability that activations and deactivations are less in the ADD group compared to the others groups (>ADD). These show that during anticipation, the ADD group showed consistently decreased activity within the PFC, including the orbito-PFC compared to the other groups suggesting greater preparatory activity for pain stimulus.

Table R2.15 are simplified significant results of 2 sample t test comparing activations (↑) and deactivations (↓) between the IBS and SDD groups for the visual cue (Uncorrected $p < 0.05$, voxel threshold 5). These are also represented in figure R2.12. The columns show areas where there is a significant probability that activations and deactivations are greater in the LSDD group compared to the HSDD groups (LSDD>HSDD). Differences between in IBS and SDD groups during the Cue stimulus can also be seen in **Appendix 6.7**.

In table R2.15 and appendix 6.7, again mixed activation and deactivation throughout the PFC was seen during both hand and foot stimulation, when the SDD and IBS groups were compared. However during anticipation the IBS group had significant greater right L-PFC deactivation compared to the SDD groups while the SDD groups had greater M-PFC deactivation compared to the IBS group. When the SDD groups were compared greater activity was seen in the LSDD PFC compared to the HSDD. This suggests some preparatory activity in the IBS group.

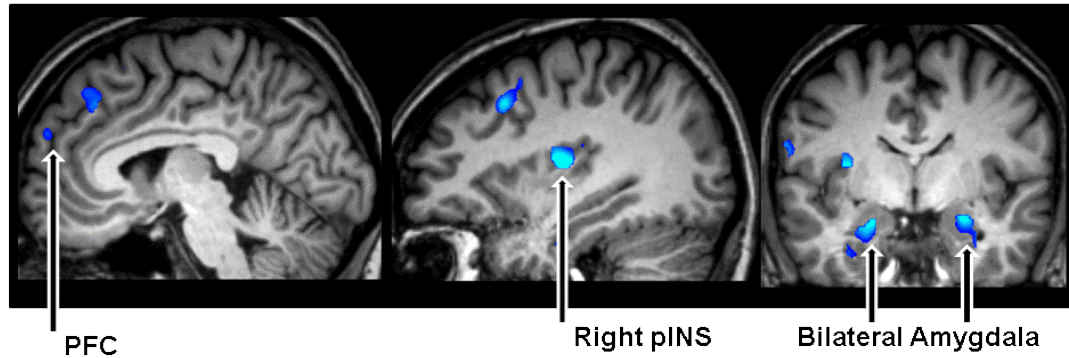
Figure R2.11 Inter-Group Analysis: Differences between groups during the Cue stimuli on the left hand and foot combined compared to ADD group. Deactivations are depicted in the blue colour spectrum while activations are show in the red-yellow spectrum. Figure (A) depicts the deactivations in the ADD group which are statistically more significant than those in LSDD group while (B) shows the same comparison between the ADD and HSDD groups. Figure (C) shows the areas in the LSDD and HSDD groups which have statistically more significant activation than those in ADD group.

(A) Greater deactivations in the ADD compared to LSDD group during cue stimulus



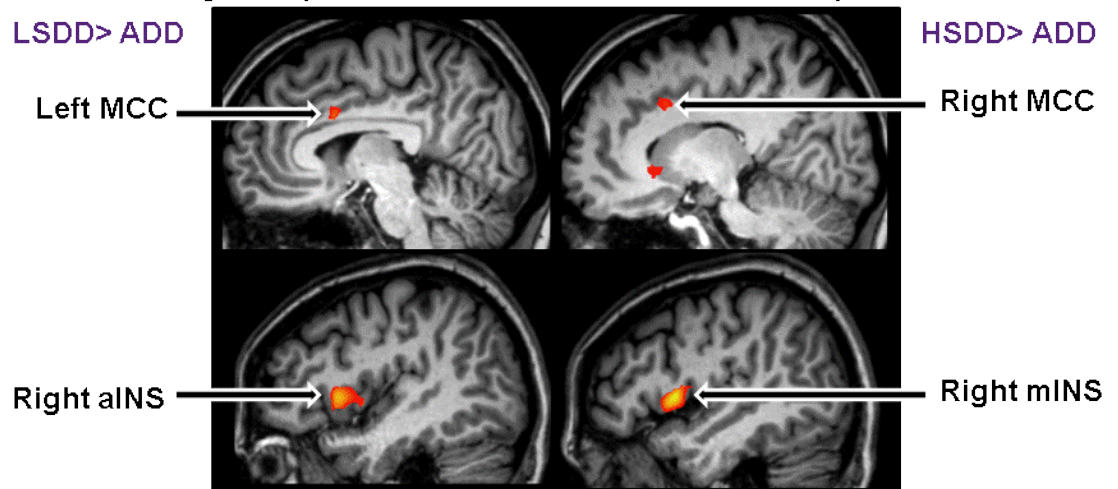
	Co-ordinates (x,y,z)	T Score	p Value
Bilateral Prefrontal	-10, 40, 52	3.02	0.001
	10, 32, 52	2.68	0.004
R Orbito-PFC	48, 40, -14	2.39	0.008
R pINS	36, -8, 8	3.02;	0.001

(B) Greater deactivations in the ADD compared to HSDD group



	Co-ordinates (x,y,z)	T Score	p Value
Bilateral Prefrontal	14, 44, 46	3.36	< 0.001
	- 2, 58, 30	2.05	0.020
Bilateral Orbito-PFC	44, 42, -8	2.05	0.020
	-38, 38, -6	2.49	0.006
Bilateral pINS	38, -10, 8	4.03	< 0.001
	-34, -28, 4	2.87	0.002
Bilateral Amygdala	22, -6, -16	3.04	0.001
	-26, -6, -16	2.95	0.002

(C) Greater activations in the LSDD and HSDD groups compared to the ADD group

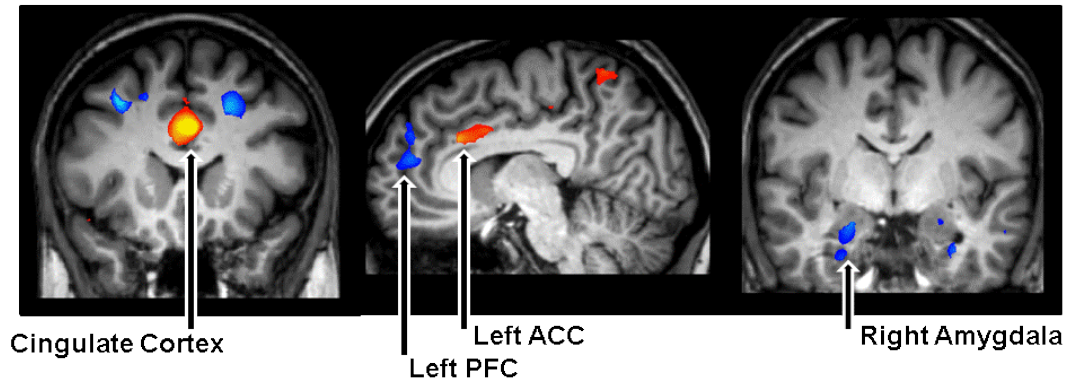


	Co-ordinates (x,y,z)	T Score	p Value	
LSDD > ADD	Bilateral Insula	-30, 10, -8	2.43	0.008
		50, 2, 6	2.85	0.002
	Bilateral Cingulate Cortex	-8, 22, 32	2.11	0.018
HSDD > ADD		6, 12, 26	2.19	0.014
	Right Insula	50, 12, 2	2.78	0.003
	Right Cingulate cortex	14, 16, 36	2.43	0.007

Table R2.14 Inter-Group Analysis: Differences between cue stimuli for ADD group.

Area	Side	ADD> LSDD	ADD> HSDD	ADD> IBS	LSDD> ADD	HSDD> ADD	IBS> ADD	
SS	S1	L						
		R						
	S2	L				↑		
		R				↑	↑	
SS	Post-Ins	L		↓				
		R	↓	↓	↓		↑	
	Mid-Ins	L			↓	↑		
		R						
Aff.	Ant-Ins	L		↓		↑	↑	
		R				↑		
Aff.	ACC	L		↓				
Aff.	MCC	L			↓	↑		
		R			↑↓	↑	↑	
	PCC	L						
		R						
Aff.	Medial PFC	L	↓	↓	↓	↓	↑	
		R	↓	↓	↓	↓	↑	↑↓
DNIC	Lateral PFC	L	↓	↑↓				
		R	↓	↑↓	↓	↑	↑	↑
DNIC	Orbito-FC	L		↓	↓			
		R	↓	↓	↓			↑
SS	Lentiform Nuclei and Thalamus	L		↓		↑↓	↑	↑
		R				↑	↑	
Aff.	Amygdala (Hippocampus)	L		↓	↓			
		R		↓	↓			
	Cerebellum	L	↑	↑	↑	↓	↓	
		R	↑			↓		↓
	Inferior Parietal	L		↑				
		R						
	Temporal	L	↑↓	↑↓	↑↓			
		R	↑	↑↓	↑↓	↑	↑	
	Motor	L						
		R						
	SMA	L	↓	↑↓	↑↓	↑		↑
		R				↑	↑	↑
SS	Post-central Gyrus	L	↑	↑				
		R	↓	↓				
DNIC	Subthalamic/Brainstem					↓		

Figure R2.12 Inter-Group Analysis: Differences between Cue stimuli on both the left hand and foot combined between the LSDD and HSDD groups. Deactivations are depicted in the blue colour spectrum while activations are show in the red-yellow spectrum. The Figure depicts the statistical comparison of significant difference in activations and deactivation in the LSDD compared to the HSDD groups.



LSDD > HSDD	Co-ordinates (x,y,z)	T Score	p Value
Cingulate Cortex (LACC, R MCC)	-2, 20, 30	3.18	0.001
Left Prefrontal	4, -26, 48	2.06	0.020
Left Orbito-PFC	-10, 60, 20	2.48	0.006
Bilateral Amygdala	-38, 38, -6	1.90	0.028
	22, -6, -16	2.50	0.006
	-24, -8, -16	1.78	0.038

Table R2.15 Inter-Group Analysis: Differences between cue stimuli for the SDD groups.

Area	Side	LSDD> HSDD	HSDD> LSDD	
SS	S1	L		
		R		
	S2	L		
		R		
SS	Post-Ins	L		
		R		
	Mid-Ins	L		
		R		
Aff.	Ant-Ins	L		
		R		
Aff.	ACC	L	↑	↑
Aff.	MCC	R	↑↓	
		L		↑
	PCC	R	↑	
		L		
Aff.	Medial PFC	L	↓	
DNIC	Lateral PFC	R	↓	
		L	↑↓	
DNIC	Orbito-FC	R	↑↓	
		L	↓	
SS	Lentiform Nuclei and Thalamus	R	↑↓	↑
		L		
Aff.	Amygdala (Hippocampus)	R	↓	
		L	↑↓	
	Cerebellum	R	↓	
		L		
	Inferior Parietal	R		
		L		
	Temporal	R	↑↓	
		L	↓	
	Motor	R		
		L		
	SMA	R	↑	
		L		
SS	Post-central Gyrus	R		
		L		
DNIC	Subthalamic/ Brainstem			↓

(xii) Covariates analysis of cue stimulus

In the **Appendix 6.8** are simplified fMRI results comparing activations (↑) and deactivations (↓) correlating with participant's HAD anxiety and depression scores and for the PCS and PHQ12 scores (uncorrected $p < 0.01$). All effects were identified using group maps as a mask for the data.

2.5 Discussion

2.5.1 Difference in responses to pain between the hand and foot within each group.

The hypothesis that pain processing between the hand and the foot would be different in the LSDD group due to peripheral nerve changes but similar in the other groups due to normal or alteration of central pain processing could not be proven using this somatic thermal pain paradigm. There was no significant difference in the temperature used for stimulation of the hand or foot within or between the groups (Table R2.7). However, altered pain processing was seen (Table R2.8). Overall in the ADD group there appeared to be a greater emotional response to hand stimulation compared to the foot with significant activity in the cingulate and PFC during hand stimulation. However there was associated increased activity of the DNIC with deactivations in brainstem and appropriate amygdala region deactivations associated with painful stimulation. In comparison there was greater affective activity associated with stimulation of the foot compared to the hand in the HSDD and IBS groups, with increased activity in the amygdala region, and cingulate cortices, and in the HSDD the PFC. Little difference was seen in the emotional pain processing areas in the LSDD group suggesting similar responses for both the foot and hand stimuli.

The hand is particularly important for function and greater emotional input and fear of injury can be attached hence the response in ADDs was not surprising. Interestingly in groups with altered pain states, a similar or greater affective pain component to foot stimulation was seen. This may be because the foot is sensitised due viscera-somatic conferences of sensory fibres within the same region of the spinal cord^{302, 314, 315, 320, 428} or may be suggestive of an altered DNIC^{188, 200, 312, 429, 430} and/or inability to adjust to threat of injury and emotional context^{242, 431}. There is evidence of greater emotional processing to visceral or muscular compared to cutaneous stimulation^{73, 303, 329, 432}. Chronic pain groups also have greater affective pain processing, altered DNIC and responses to fear and threat than normal individuals^{221, 433, 434}. This could explain the differences in pain processing within different body areas seen in this study.

2.5.2 Responses to pain between groups

There are marked similarities and differences between the groups during the pain (VAS temperature) experience (Tables R2.9-2.11). No significant differences were found in the VAS or temperature scores between each group (Table R2.6 and Figure R2.10). However there was a suggestion of increased thermal sensitivity in the cumulative VAS scores (Figure R2.10). In the tabulated group maps for the foot and hand painful stimulus activations and deactivations were seen in areas consistent with known pain pathways such as the anterior and mid insula, pre-frontal cortex, thalamus, cerebellum and in some groups the brainstem (Foot: ADD and LSDD, Hand: All) and amygdala (Foot and hand, LSDD, HSDD, IBS).

When comparing the ADD to other groups during foot stimulation, there were interesting differences, especially in the affective pain processing. There were few areas of increased activity in the ADD compared to LSDD group. These are mainly present in somatosensory processing and DNIC areas such as the right pINS and bilateral PCG and the right lateral PFC. In comparison, the LSDD groups had greater activity in affective areas such as the bilateral aINS, right ACC and MCC, bilateral amygdala and hippocampal regions and PFC. Similar differences were found in the HSDD and IBS groups compared to the ADD group. Hippocampal activity has also been seen in patients with somatoform pain disorders even at moderate thermal pain levels³⁷⁹.

During painful stimulation of the hand increased activation was seen in the ADD group in the S2, lateral PFC, thalamus, SMA and brainstem and deactivation in the right amygdala and hippocampal regions compared to the other groups. The LSDD group demonstrated greater deactivation in the PCC and medial PFC and PCG, and greater activation in the thalamus compared to the ADD group. The deactivation in the PCC may suggest movement of the SDD group away from the default pain network and greater attention to the pain stimulus, which will be further discussed below. The IBS group demonstrated increased activation in affective processing areas such as the mid INS, ACC and MCC compared to the ADD group. This suggested greater affective processing in the IBS group compared to the ADD group. The HSDD group showed little difference in activity

in the affective areas compared to the ADD group suggesting similar emotional processing of pain but potentially reduced activation of the descending inhibitory system as suggested by lack of brain stem activity^{221, 249}.

When comparing the chronic pain groups, the IBS group shows increased emotional processing with increased activity in the bilateral aINS and right aINS compared to the LSDD and HSDD respectively. Increased activity in the cingulate cortices and PFC cortices was also demonstrated compared to both groups. However compared to the HSDD group there is increased deactivation of the bilateral amygdala or hippocampal regions and somatosensory areas such as the right pINS, right thalamus, and right PCG. The LSDD and HSDD group demonstrate a variety of areas of activation scattered throughout the affective and DNIC areas.

In our study, increased activation was seen in the cingulate cortex in the LSDD, HSDD and IBS groups compared to the ADD group during pain and anticipation. This activation was greater in the IBS and LSDD group compared to the HSDD group during pain stimulation and anticipation. In a H₂¹⁵O-PET study²⁷⁰ in which functional dyspeptics (FD) were compared to healthy volunteers³⁹², the group found that during distension the FD group failed to activate the pACC which correlated negatively with anxiety levels. This supports the suspected role in attention and threat-association and its suspected modulation of amygdala and emotional circuits. Anxiety also correlated to activity in the dorsal pons/midbrain, which has been reported in IBS studies³⁹². These findings may fit with our findings which showed a lack of significant ACC activation in the HSDD group in comparison to the LSDD and IBS groups. Tack and colleagues speculated that activations in the locus coeruleus-parabrachial nucleus²⁷⁰, which is known to have projections to the cortices, including the pACC may be involved in pain processing. IBS patients' anxiety scores have been correlated with anterior MCC and pregenual ACC activity during painful rectal distension, while depression scores correlate with activation of the PFC and cerebellum¹⁹⁸. Altered aINS activation has also been found in other patient groups^{190, 435, 436} which support our findings. In IBS patients anticipating rectal stimulation, less deactivation was identified in the insula, supragenual ACC,

amygdala and brainstem compared to healthy controls¹⁹⁰. In anorexia nervosa, greater activation of the aINS, as well as the DL-PFC and cingulate was found compared to healthy women undergoing anticipated painful heat stimuli⁴³⁵. Subsequent greater activation of the DL-PFC and decreased activation of the pINS during painful stimulation was also found. This influence of the aINS over subsequent pINS and caudate activation has previously been demonstrated and correlated with the perceived touch intensity⁴³⁷. In MDD, decreased activity of the aINS was found along with increased activation in the VM-PFC, dorsal ACC, PCC and deactivation in the DL-PFC, SMA, mINS and cerebellum during anticipation of changes in stimulus intensity and/or cognitive demand was found compared to healthy controls⁴³⁶. This suggested that MDD patients were unable to effectively prepare to anticipated changes in environment. Similar depressive and anxiety symptoms may be influencing the activities found in the HSDD and IBS groups.

The reported amygdala responses agree with the findings in our study which showed greater significant activation of the amygdala regions in the LSDD, HSDD groups compared to the ADD group during foot pain stimulation. Greater deactivations in the ADD compared to the HSDD and IBS group during pain stimulation of the foot and also during anticipation of pain.

In our study the PFC was split into lateral, medial and orbito-PFC activity to allow ease of analysis between groups. We found greater deactivation in the M- and L-PFC in the ADD compared to the HSDD but mixed activation and deactivation in the SDD and IBS groups when compared to the ADD group during foot stimulation. Again the hand was more complicated and this may be due to the greater level of emotional processing of hand stimuli by the ADD group.

Alteration in 'effective connectivity of the emotional arousal circuitry' (rostral and subgenual cingulate cortex, amygdala and locus coeruleus) rather than afferent sensory processing (insula, thalamus, OFC, dACC) or cortical modulation (PFC and parietal cortex) are thought to underlie the symptoms and perceived pain in IBS⁴³⁸. Our finds suggest the amygdala and hippocampus regions may also be important in SDD symptoms as well, especially for those with high PHQ12 scores.

In our simplified tables the cerebellum has been presented as a single area and therefore some of the detail in specific areas of activation and deactivation will be lost. In general the ADD group demonstrated greater left sided activation of the cerebellum during foot stimulation, while the SDD groups had significant deactivation bilaterally. However during hand stimulation, this effect seemed to be reverse. This may be related to the altered emotional processing for hand stimulation in the ADD group which has been suggested by other studies^{218, 329, 409, 414}. This altered cerebellum activity agrees with studies in other chronic pain groups, such as lower back pain subject, when shown pictures of potentially painful events⁴³⁹.

2.5.3 Covariates with pain processing

During painful stimuli the VAS score showed little correlation between the groups with any specific region (see **Appendix 6.6**). However in the foot, the temperature used did significantly correlate with pINS activation in all groups. In the HSDD and IBS groups increased correlation of cortical activity was seen in the cingulate cortices and in the LSDD, HSDD and IBS groups in the PFC.

During the pain stimulus for the foot there was little correlation of cortical activity with anxiety score for the ADD or LSDD groups, except the MCC and cerebellum and, in the ADD group, the PFC. The number of correlated areas increased in the ADD for the pain stimulus in the hand especially in the cingulate cortex and PFC. This compared to the HSDD and IBS groups which had correlated activity with anxiety score during pain stimulus in the insula, cingulate and prefrontal cortices. Some correlation was detected in the cerebellum and amygdala regions as well.

In the ADD group, there were only a few areas which correlated with depression. Other groups showed greater number of active regions. For the pain stimulus to the foot increased correlated activity was mainly seen with the HSDD and IBS groups in the PFC and cerebellum and in the IBS group the MCC and aINS. In the hand painful stimulus more areas were correlated in the LSDD and HSDD groups including the aINS and mINS, PFC, amygdala (HSDD) and cerebellum.

The PHQ12 questionnaire score is a marker of somatisation while the PCS is a measure of catastrophizing. Somatisation is described by Brown et al as ‘the tendency to experience somatic or visceral sensations as more intense, noxious or disturbing’ or ‘somatosensory amplification’^{369, 440}. Pain catastrophizing is a ‘negative cognitive–affective response to anticipated or actual pain’⁴⁴¹. Both can be associated with anxiety and depression hence assessing possible correlation of cortical responses to pain with these scores was valuable. Little significant correlations were found for the ADD group and PCS score. However significant correlations were found for the ADD group and PHQ12 score especially in the hand stimulus. In comparison increased correlation was observed in LSDD with PHQ12 and PCS scores, especially in the cingulate cortex and PFC during pain. The HSDD and IBS groups also showed greater areas of activity correlated with PCS and PHQ12 score. In the pain stimulus this included the insula, cingulate cortex, PFC amygdala and cerebellum. Catastrophizing, when controlling for depressive symptoms, has been linked with activity in the cerebellum and mPFC (anticipation), dorsolateral PFC and dACC (attention) and lentiform nuclei²⁹⁶.

2.5.4 Responses to anticipation of pain

During the cue stimulus, significant differences were seen throughout the insula, anterior and mid cingulate and SMA when anticipation of pain in the hand was compared to anticipation of pain in the foot (i.e. hand > foot) (Table R2.13). However similar activity in the PFC was seen in all groups except the HSDD. The ADD group also showed deactivation within the amygdala and brainstem which was not seen in others groups. This suggested that although the ADD group had a greater emotional anticipatory response to pain in the hand compared to the foot, deactivation of the amygdala and the brainstem, which is thought to be part of the descending inhibitory system, occurred correctly. When anticipation of pain in the foot was compared to anticipation of pain in the hand (e.g. foot > hand), significant activity was seen in the posterior (deactivation) and mid insula (activation) and the ACC (activation) compared to other groups. Greater deactivation was also seen in the PFC in the HSDD group. This suggested again a greater emotional anticipation of foot stimulation in the HSDD group compared to the other groups. However, unlike the anticipated

hand stimulation of the ADD group, compensatory deactivation of the amygdala or the brainstem was not seen. The lack of significant difference between the LSDD and IBS group anticipated stimulation of the foot and hand suggests similar anticipated responses in both limbs.

Comparison between the groups is quite complex (Tables R2.14-15 and Figures 2.11-12), but the overall suggestion is that there is a range of emotional processing in the SDD and IBS groups compared to the ADD groups which may explain some of the mechanisms of anticipation and pain in SDD.

Compared to the other groups the ADD group showed greater deactivations in the right pINS and PFC. Significant differences in deactivation were also seen in the ADD group in the amygdala regions compared to the HSDD and IBS groups and right PCG compared to the LSDD and HSDD groups. Increased activity in affective areas such as the MCC in the LSDD and right ACC and aINS activity was found in all groups compared to the ADD group. Increased activation of the thalamus and SMA and deactivation of the cerebellum was also seen in all in groups compared to the ADD group. This suggests again a greater emotional response to anticipated pain and reduced preparatory and modulatory activity.

When comparing the SDD groups with the IBS group significant differences in deactivations of the pINS, right medial and lateral PFC and activation of the cerebellum were identified in the IBS compared to the HSDD group. However significant activations of the right ant and mid insula and deactivations in the pINS and MCC were found in the LSDD group compared to the IBS group. Significant differences in deactivations in affective areas such as the amygdala region, mPFC, as well as temporal lobe and activations in the SMA were also found in the LSDD compared to the IBS group. The HSDD had fewer differences, but also included deactivations in the mPFC.

During anticipation of pain, the LSDD group showed much greater differences in activation of affective areas such as the ACC, mPFC and amygdala as well DNIC areas such as the lateral PFC

and right thalamus compared to the HSDD group (Table R2.15 and figure R2.12). There were few areas of significantly greater activity in the HSDD group compared to the LSDD group, but these included the thalamic regions and brainstem.

Thus in anticipation of pain there are several key regions found to have altered activity during anticipation between the groups including the insula, cingulate cortices, PFC, amygdala and hippocampal cortices, somatosensory cortices, thalamus and brainstem/PAG^{345, 442-445}. The functional connectivity between these areas, especially the aINS and brainstem before the stimulus is applied, is thought to be important in the subsequent experience of pain⁵⁸.

In our study greater insula activation was found in the LSDD, HSDD and IBS groups compared to the ADD group, where deactivation of the pINS was identified. Greater insula activation was also identified in the aINS and pINS in the IBS compared to the LSDD and HSDD groups. This suggests that in the SDD and IBS groups greater emotional awareness of the anticipated stimulus was present, which may have influence subsequent stimulus perception. This is supported by a study by Tracey's group⁴³¹. Their study of perceived threat of painful stimuli in healthy volunteers showed that the in high threat conditions more threshold stimuli were perceived as painful and this could be predicted by activity in the aINS during the anticipation phase⁴³¹. This study also showed increased functional connectivity between the aINS and MCC suggesting a 'salience network'⁴³¹. High confidence in pain beliefs also correlated with right aINS, post MCC and inferior parietal activity during pain anticipation⁴⁴⁶. Correlation between expected pain and activity in the ACC, PFC, INS and thalamus has also been identified, with manipulation of anticipated pain affecting subsequent activity in the INS, S1 and ACC⁴⁴⁷.

Anticipation and expectation is thought to be important in IBS. Patients with IBS can be normal or hypersensitive to rectal distensions. When comparing these groups using fMRI similar patterns of brain activation are found between normo-sensitive and controls groups, while hypersensitive individuals have greater INS activation and decreased deactivation of the pgACC during rectal

distension. However during the anticipatory phase greater activation of the right hippocampus was found in normo-sensitive IBS patients compared to controls. The differences between the IBS groups may be due to differences in expectation and rectal afferent input⁴⁴⁸. fMRI has also shown that the hippocampus is activated during anticipation of a painful event by a visual cue³⁸². Correlation between individual anticipation rating and hippocampal activation during anticipation has also been identified⁷⁹.

There was greater deactivation in the amygdala and hippocampal regions in the LSDD and ADD groups during anticipation when compared to the HSDD and IBS groups. This suggests better coping strategies in the ADD and partially in the LSDD compared to the other groups^{383, 449}.

As part of the DNIC pathway, deactivation of the brainstem would be expected during the anticipatory phase¹⁹⁰. Deactivation in the ADD group anticipatory phase was not associated with a significant deactivation in the brainstem region in our study, as has been described in other studies¹⁹⁰. This may be because of: (1) whole brain rather than ROI analysis; (2) no respiratory and cardiac gating was used when images were acquired; and (3) difficult resolution of activity within the region when whole brain images are being acquired. However increased significant activation in the subthalamic regions and brainstem was seen in comparisons between the IBS, LSDD and HSDD groups during pain stimulation (Table R2.9-R2.11). Interestingly deactivation was also seen in the HSDD group in this region during comparison with the ADD, IBS and LSDD during anticipation (Table R2.12, Figure R2.11 and Appendix 6.7). The exact region involved is difficult to elucidate due to the problems mentioned above and further investigation of DNIC pathways in SDD may be warranted.

During the anticipation phase greater left sided cerebellum activation was seen in the ADD group compared to the other groups. In the IBS group greater activation was also seen during anticipation compared to the HSDD group. In the LSDD group greater mixed activation and deactivation was seen when compared to the IBS and HSDD groups during anticipation.

In our study we were unable to show significant differences using our analysis method for evidence of altered PCC and precuneus deactivation to make ‘default mode network’ inferences. Further analysis with ROI may be necessary to identify this potential mechanism in SDD.

2.5.5 Covariates with anticipation of pain

Temperature and VAS score were not used as covariates for the cue stimulus as the patients were unaware of the temperature they would receive. Therefore, the HAD scores, PHQ12 and pain catastrophizing scores were used as correlates for anticipation of pain (Appendix 6.8).

During the anticipation phase, little correlation with anxiety scores was seen for the ADD group except deactivations in the lateral PFC and amygdala. However many more areas in the LSDD group correlated, especially in the ACC and PFC. This compared to the HSDD group, which had activation in the right mINS, right MCC and amygdala and deactivation in the PFC. The IBS had similar correlated activity to the HSDD group except anxiety also correlated with deactivation in the pINS and right amygdala. These findings suggested that anxiety has an influence over key areas in anticipation and pain processing. Many of the regions described above have been previously implicated in anxiety effects during anticipation of unpleasant events. Activity within the S2 and insula regions was most prominent in the IBS and HSDD groups, but not correlated with activity found in the ADD or LSDD groups. This finding is supported by a study in which anticipation of aversive images in anxiety prone and normal anxiety subjects demonstrated bilateral aINS activity when cued for adverse images, but this was greater in the right insula in the anxiety prone group⁴⁵⁰. Using functional connectivity analysis the insula was involved in a network consisting of frontal and parietal lobes in the anxiety prone group. This suggested that anxiety could lead to ‘greater anticipatory reactivity’⁴⁵⁰. This again supports the frontal and parietal region anxiety correlated activity seen here in the LSDD, HSDD and IBS groups. In a study of 17 IBS subjects the brain responses underlying the placebo effect were assessed during rectal distensions using fMRI. A new ‘drug’, which was in fact saline, was infused into patients and HV. A similar number of IBS and HV had placebo effect to the infused saline. However, a high HADS anxiety score was

predictive of a weak placebo effect. During the placebo effect greater activity was identified in the affective areas such as the INS, MCC, and VL-PFC in IBS patients compared to healthy controls. VLPFC was also increased during anticipation of pain in the IBS patients⁴⁵¹.

Other regions have also been implicated in anticipation of pain. In the LSDD and HSDD group, activity in the ACC and MCC was identified. The cingulate cortices are thought to be important in anticipation of pain and influences activity within others regions. In one study, uncertainty in the anticipation of potentially painful events resulted in greater intensity in the ACC and cluster size in the PO and pINS activation during non-painful stimuli in healthy volunteers³⁷⁶. Participants reported these non-painful stimuli as being more unpleasant suggesting the ACC and the PO and pINS and important in the 'modulation of affective aspect of sensory perception' when there is uncertainty about an expected stimulus³⁷⁶. Connectivity between the insula and the amygdala has also been identified in anxious anticipation of auditory stimuli⁴⁵². In the amygdala and hippocampal regions significant anxiety correlated deactivation were identified in the ADD and IBS groups while significant activation was seen in the HSDD group and no activity was correlated in the LSDD group. The right insula has been correlated with individual subjective experience for any type of stimulus, while the amygdala was mainly active during anticipation of aversive stimuli⁴⁵². The entorhinal complex is connected to affective areas such as the perigenual cingulate cortex and the mINS and is thought to prime affective responses to anticipated events³⁸². Activation of the hippocampus during anticipation has also been shown to be associated with activation of the INS/IFG/PO network during the following painful event³⁶⁹.

The hippocampal network has been shown to respond differently to painful thermal stimuli depending on the preceding anxiety of an anticipated painful stimulus. In our study, cued deactivations in the right amygdala/hippocampal region were correlated with anxiety in the ADD and IBS groups but activation was found to be correlated in the HSDD group. In healthy volunteers anticipation of a painful shock resulted in activity in the hypothalamus, PAG, caudate, precentral gyrus, insula, VL-PFC, DM-PFC, ACC and thalamus⁴⁵³. Although greater correlations

were found in the Insula, PFC thalamus and brainstem in the other groups, the ADD group did have an anxiety correlated deactivation in the PCG similar to this study. The group also found that a linear relationship between activity over the safe and strong anticipatory trials in the bilateral INS, ACC and IFG.

Anxiety and anticipation of pain have also been studied in patient groups. High neuroticism individuals had exaggerated anxiety and reduced brain activation to high and medium anticipatory trials⁴⁵³. In an study of anticipation of hyperventilation tasks, which resulted in unpleasant physiological symptoms, all participants activated the aINS and OFC and rostral and dorsal ACC and DM-PFC⁴⁵⁴. However in participants with a high fear of unexplained symptoms a greater activation of these areas was demonstrated⁴⁵⁴. This study showed similar brain regions are activated in anticipation of internal as well as external threats⁴⁵⁴. Similar anxiety correlated activity in the ACC was found in our LSDD group, and in the mPFC in our LSDD and HSDD group.

Stress results in changes in activity in the INS, MCC and VL-PFC in IBS subjects, suggesting altered emotional modulation of visceral sensation¹⁹⁴. These has been partly accounted for by higher anxiety levels in IBS patients, except for the PFC and insula¹⁹⁴. Anticipation of pain, and anxiety associated with it, has also been investigated in 8 healthy volunteers using midazolam⁴⁵⁵. Volunteers were cued with different coloured lights to expect a painful heat stimulus or warm non painful stimulus. At baseline, when only saline was given, the pain stimulus itself produced greater activity in the ACC, bilateral aINS and pINS, thalamus, S1, motor cortex, pre-frontal cortex, cerebellum and brainstem than to warm stimuli. During the anticipation phase of the pain versus the warm stimulus, the ACC, contra-lateral aINS, ipsilateral S2 and pINS were activated. Midazolam effected the activation to pain anticipation especially in the aINS, ACC and S2 on region of interest analysis but did not affect the activations during the pain itself⁴⁵⁵. These studies support the role of the insula in sensory and affective aspects of touch^{437, 455} and that alterations in anticipation can result in altered affective and perceived sensation.

In the anticipation phases correlated activity included significant activations in affective areas such as the bilateral ACC with anxiety and depression in the LSDD group. Scattered activity was found for the HSDD group in the insula, cingulate cortices, PFC, amygdala and brainstem for both anxiety and depression. In the IBS group significant activity was mainly associated with anxiety score, but significantly correlated activations were seen in the right S2 and left aINS and deactivations found in the left MCC and right PCG with depression score. Depression and chronic pain are closely associated with 75% of patients who suffer with depression reporting chronic pain⁴⁵⁶, while 30-60% of patients with chronic pain have depressive symptoms⁴⁵⁷. Depression is associated with passive coping mechanisms including helplessness, lack of control and rumination which may influence the emotional processing of chronic and experimentally induced pain⁴⁵⁸⁻⁴⁶¹.

A recent study in MDD patients demonstrated increased activity on ROI analysis in the amygdala and on whole brain analysis in the aINS, IFG, dorsolateral ACC, and dorsolateral PFC during anticipation of a painful event while control subjects showed greater activity in the caudate, precuneus, PCC and ventral brainstem. This activity correlated with depression is similar to our study, which showed activity within the aINS in the IBS and HSDD groups. Activity in the OFC was seen in the LSDD group only, while the LSDD and HSDD demonstrated activity within the cingulate cortices. In the HSDD group, cerebellar activity was also associated with depressive symptoms, which has also been found to be correlated with depressive symptoms in IBS patients undergoing rectal distensions⁴¹⁵. However we did not find a similar correlation with anticipation of pain and depressive symptoms in our IBS group.

Significant correlations were found for the ADD group and PHQ12 score in the anticipation phase in the right INS, bilateral OFC and thalamus and deactivation in the right amygdala/hippocampal regions. In comparison increased correlations between LSDD and PHQ12 and PCS scores, especially in the cingulate cortex and PFC during pain and anticipation phases.

The HSDD and IBS groups also showed areas of correlated activity with PCS and PHQ12 score in the anticipation phase with scattered activity detected in the pINS (IBS), MCC (HSDD) and PFC. The correlation of hippocampal region deactivation in the IBS group with PCS and in the right amygdala/hippocampal region in the ADD and LSDD and bilaterally in the IBS groups with PHQ12 fits with other studies. Hippocampal activity has been shown to be increased during expectation of pain and during the painful event itself and correlated with the participant's 'sensitivity to expectancy'⁴⁶². However in another study, participants with a range of somatisation score on the symptom checklist 90 (revised SCL-90-R) were subjected to low and high anxiety visual cued shocks. fMRI imaging identified that there were smaller differences in hippocampal activation in those with a high somatisation score for different level of cues compared to low somatisation participants³⁶⁹. This suggests that high hippocampal activity even in low anxiety situations may influence pain expectation and processing.

The association of PCS and PHQ12 with anxiety is also thought to be important. The inability to differentiate different levels of activity in the Insula, IFG and PO during high and low anxiety anticipation of pain was related to reported daily physical symptoms of participants in Gondo et al's 2012 study³⁶⁹. This suggests that participants with high number of reported symptoms may be continuously 'anxious' and have a high background activity within these key areas, resulting in minimal change in activity during pain anticipation. This may be why there are few areas which correlate with PCS and PHQ12 in the ADD group, who have low anxiety state, and in the HSDD who may have a continued high anxiety state.

Although there are no studies of pain catastrophizing in diverticular disease, other chronic pain groups have been studied. An fMRI study of 12 subjects with fibromyalgia and 14 healthy controls was used to identify the association of anticipation of pain, catastrophizing and altered cerebral pain processing. They found that catastrophizing behavior was increased in FM patients but not during the anticipation of experimental pain. Also FM patients showed increased activation of the PAG, posterior parietal cortex and DL-PFC during anticipation of pain⁴⁶³.

Although not assessed in our study, other personality traits can also influence pain perception. Neuroticism is also correlated with depression and anxiety disorders. During anticipated painful oesophageal distension positive correlation was found between the levels of neuroticism and brain areas involved in cognitive and emotional processing such as the parahippocampus, thalamus, ACC and insula. However during the pain stimulus negative correlation was found with these areas, suggesting potential maladaptive coping strategies in neurotic individuals²⁴⁷. These areas are similar to those found with PCS and PHQ12 scores covariates in the LSDD and HSDD groups. This suggests that many of the questionnaires and traits that have been used so far often have significant correlations between them and may be measuring similar traits and cerebral mechanisms underlying chronic abdominal pain.

2.5.6 Summary of findings

Enteric infection or long lasting mucosal inflammation in inflammatory bowel patients is not always associated with development of abdominal pain or IBS like symptoms⁴⁶⁴⁻⁴⁶⁷. Development of IBS like symptoms is probably a combination of altered gut flora, genetic susceptibility, immune modulation and personality trait^{464, 468}. Differences between IBS and Diverticular disease have recently been highlighted by Spiller (2012)⁴⁶⁹. However similar mechanism for the development and maintenance may exist between IBS and SDD. Our theory suggests that the LSDD group were peripherally sensitised while the HSDD group were centrally sensitised like the IBS group.

In our study there was increase activity in the pINS in the ADD and HSDD group but not the LSDD group in the foot compared to the hand, suggesting some increased sensory input for the foot. However this finding was not significantly different in the group comparisons and our theory that increased peripheral signals due to sensitisation of pain fibres within the bowel in the LSDD cannot be confirmed from our results. This is similar to previous studies which have been performed between 8 patients with ulcerative colitis (UC), 7 with IBS and 7 healthy volunteers showing activations were identified for all groups in the aINS and dACC. However there are few subjects in our study groups which may reduce the power in identifying this increased peripheral

input. Also our study gave stimuli at the same VAS score and not the same temperature which may have reduced the activations seen.

In Mayer's study¹⁹⁵ the IBS group did show greater activation of affective processing areas such as the amygdala, hypothalamus, rostral ACC and dorsal medial PFC. In our study both the IBS and HSDD showed greater affective pain processing compared to the ADD group with greater activity in the INS (IBS), ACC and MCC, and less amygdala deactivation during pain. However when comparing the IBS group to the SDD groups, greater activation of the INS and MCC were seen. During anticipation phases the HSDD and IBS groups were similar while increased activity in the right INS, MCC and deactivation in the amygdala was seen in the LSDD compared to the IBS group. This suggesting although similar, the altered central activities found in IBS are not identical to those in SDD groups

In our study the LSDD and ADD groups were also different with greater PFC activity in the ADD group and increased amygdala activity in the LSDD group in anticipation and increased activation of the PFC and deactivation of the amygdala in the LSDD during pain. This suggests that although the LSDD group has greater similarity in activation and deactivation to the ADD than the other IBS and HSDD group, it is not identical suggesting some element of altered central pain processing. Thus due to the artificial splitting of the SDD group according to their PHQ12 score, we in fact may have a heterogeneous mix of subjects in each group: The LSDD group with a predominance of peripheral sensitised subjects but with some with central components as well and; The HSDD group mainly central sensitised subjects in the HSDD group but also potentially a few peripheral sensitised subjects as well. The difference between the HSDD and IBS group may also be explained by this theory and also by the fact that many of the IBS subjects had received prior treatment for IBS by gastroenterology specialists while the HSDD group had not.

2.5.7 Limitations with the study

There were some limitations to the study.

(i) Patient selection

(a) Diagnosis of GI disorder

DD participants who took part in the study had diverticulosis confirmed on endoscopy, barium enema or CT scan. Of those who were symptomatic, some reported having had a previous episode of diverticulitis with either GP diagnosis or admission to hospital. However not all participants with diverticulitis had had it confirmed with CT or other imaging or biochemical tests. A prior episode of diverticulitis, which is hypothesised to cause a peripheral ‘sensitization’ resulting in some patients in having chronic pain, was not used as a part of the inclusion criteria due to experience of recruitment difficulties from prior studies. Visceral and cutaneous hypersensitivity is known only to affect a subset of patients with IBS^{316, 317}. However no investigations to confirm the extent of the DD or visceral hypersensitivity in IBS or DD¹⁰⁴ were performed. This may explain why no significant thermal hypersensitivity was identified in this study.

The PHQ12 score was used to divide the SDD group into 2 groups. The PHQ12 scores gave a bell shaped distribution and the cut off to give equal numbers in the LSDD and HSDD was between 6 and 7. Although the PHQ12 has previously been used to successfully divide patients in IBS and DD with suspected peripheral and central, the cut off was different³⁰⁶. The SDD may potentially be a heterogeneous mix of subjects which become similar towards the cut off mark. Thus in the SDD groups there is potential for some participants to have a peripheral, central or mixed pain processing picture which may reduce the contrast seen between the groups in terms of images and VAS scores with temperature changes.

As mentioned above, IBS patients were recruited from gastroenterology clinics. Many had experience of undertaking studies and had been treated with a range of medications. Thus the IBS subjects who were recruited may not demonstrate the expected pain processing as more medical naive counterparts such as those with SDD.

(b) Gender

Pain processing is different between men and women and has been hypothesised as why there is a greater female predominance in chronic pain disorders. However, women have greater anxiety sensitivity, compared to men, which may influence pain perception and processing. In our study this is important as there were a greater proportion of female participants within the symptomatic groups compared to the asymptomatic DD group.

Differences between the structure and function of male and female brain have been found in healthy subjects with experimental pain⁴⁷⁰ and/or in individuals with chronic pain conditions such as migraine⁴⁷¹. In healthy volunteer studies of visceral pain, similar rectal sensory thresholds and pain ratings have been found. In ROI analysis similar activations in the SS, INS DL-PFC and thalamus have been seen. However, on whole brain analysis women have been found to have greater activity in the cerebellum, and medial frontal gyrus during stimulation and in the DLPFC and middle temporal gyrus during anticipation of pain compared to men⁴⁷². Animal studies have suggested that oestrogen may affect the ACC resulting in greater pain sensitivity⁴⁷³. Similarly, in visceral distension pain similarities were identified within the DL-PFC, INS, SS and thalamus on ROI analysis. However on whole brain analysis increased activation in the dorsolateral PFC and middle temporal gyrus as found in women during anticipation and in the cerebellum and medial frontal gyrus during pain⁴⁷².

However, using nociceptive flexion reflexes and somatosensory evoked potentials, Goffaux et al suggest that variations in gender pain perception can be contributed to changes in thalamocortical processing which effect appraisal and emotional pain processing⁴⁷⁴. When they controlled for trait anxiety, they found that differences in cortical activity between men and women were lost⁴⁷⁴. A recent review also suggests that studies of biopsychosocial factors influencing pain difference in men and women are mixed and that further study is needed to try and explain any underlying causes⁴⁷⁵.

(c) Age

In our study the IBS and HSDD were significantly younger than the ADD and LSDD group. Although this is due to the nature of the conditions, with IBS mainly occurring in patients within their 20-40yrs and DD in the over 60yrs, it may still have influence our results. Age related reasons for confounding in our results include the increased risk of cardiovascular disease which may alter blood flow dynamics. Although we tried to control for this by excluding potential participants with a cardiovascular disease history, there is still potential for undiagnosed participants to have entered the groups. Also older people tend to be on multiple medications, many of which the effect of blood flow dynamics is not known or are only now being elucidated^{476, 477}.

Age itself is also thought to influence pain perception. Age related changes in brain volume in areas involved in pain processing have been identified⁴⁷⁸. DNIC response has also been found to negatively correlated with age⁴⁷⁹. However the implication of these changes are not known, but caution should be used when interpreting our results.

(ii) Group Size

Although this study overall had 74 participants with 14 subjects in each group, it is still a small study and it is difficult to take the conclusions formed here and apply them to the population at large. The areas identified do fit with the current theories of which areas of the brain should be activated and deactivated during anticipation but further study of this group is required or inclusion of this data into large meta-analysis but more substantial generalisations can be made.

(iii) Perception

No significant differences in cutaneous perception were seen between the study groups. This is despite known altered visceral sensation previously documented in DD and altered cutaneous^{314-316, 322, 480} and visceral sensitivity^{315, 322, 349} been demonstrated by several groups in IBS and animal models³²⁴. Studies by Verne and colleague has suggests that viscera-somatic overlap of hypersensitivity may also occur in IBS as we hypothesised in DD^{302, 317}. However in this study 78

patients were assessed with 57 controls to identify this, which is greater than the numbers used in our study. This may be one reason why our sensory testing results were not significant between the groups³¹⁷. Also the scale we used from 1 to 10 may have been limiting. A continuous, patient operated or variable system, which allows rating of each individual pain stimuli, may have been more useful.

It's also important to note that our fMRI results are of VAS temperature stimulus. This is a subjective reported pain stimulus which aimed to be consistent across all study groups. This was assessed in the scanner anteroom, before entering the scanner. It is possible that subjective rating of this temperature may have changed due to individual anxiety or other factors once placed in the scanner and may be the reason for altered VAS scores at the end of each paradigm. Unfortunately we were unable to perform further analysis of fMRI brain responses to a consistent temperature of 45°C as mentioned in the results. This additional analysis may have aided interpretation of our VAS temperature results and allowed a better assessment of group thermal sensitivity at a cerebral level rather than subjectively reported.

(iv) Analysis and resolution

As mentioned previously, this study used whole brain secondary level analysis without masking within the brain. This allowed identification of regions that we would not have hypothesised and looked for on other techniques, such as ROI analysis. However this technique did give greater risk of false discovery and made the data less robust to withstand correction for multiple comparisons. When analysis beyond the group maps was performed, few of the results withstood correction for multiple comparisons. Thus it is possible that some of our activated regions may be false. Also we did not gate our image collection to compensate for respiratory and cardiovascular movement which could have affected the images obtained from smaller areas such as the brainstem.

This is why we have only discussed larger regions of activation in areas which have previously been identified by several studies. Thus we feel that despite this potential flaw our findings are still valid.

2.5.8 Future directions

The study is important as it is the first to suggest both peripheral and central sensitisation in symptomatic diverticular disease patients. The PHQ12 questionnaire may also be a helpful simple tool to try and divide patients into those with peripheral and central components. This would help treatment in this group by allowing selection of peripherally acting, such as mesalazine, or centrally acting, as amitriptyline, medications. However further trials are needed to confirm the use of the PHQ12 for this purpose.

The PHQ12 score may also be useful in other general surgical and gastroenterology conditions and an adjunct to clinical judgement in complex cases. In patients who are requesting surgery for symptoms, such as pain, the PHQ12 may be helpful in deciding in those where there is a central component to their symptoms and that surgery may not be as beneficial. This would help in the counselling process for surgery and potential exploration of other treatment options. It would also help in consent process. This is important as evidence of central sensitisation in patients has been shown to influence the outcomes of surgery²¹⁴. Thus future studies in a range of different conditions could use modified versions of the PHQ15 or 12 score.

The MRI techniques used in this study are not new. However there is potential to use them to further research diverticular disease. This includes using MRI data and correlating altered pain processing with genetic variables. Correlation of pain processing changes and gene SNPs or alleles or binding of key receptors is starting to explore chronic pain processing beyond structural and functional MRI. These techniques may also identify genetic⁴⁸¹ or neuro-chemical changes^{482, 483}. In trigeminal neuralgia and FM decreased mu-opioid receptor binding in the nucleus accumbens^{482, 484}, amygdala and dorsal cingulate⁴⁸⁴ has been shown to be altered in patients compared to controls.

However increase levels of glutamate and glutamine in the posterior gyrus and lower myo-inositol levels in the sensori-motor and hippocampal areas have also been identified in FM patients⁴⁸⁵. Similar studies could also be performed in SDD and may lead to development of better assessment tools, research options and potential treatments. There is also potential to investigate the central and peripheral action of known and new medications to identify those that may have beneficial effects. One study in IBS patients has already shown that amitriptyline reduces activation of the ACC during painful rectal distensions and stress⁴⁸⁶. Pregabalin and SSRI have also been shown to influence the activity in amygdala, ACC and insula during anticipation of emotive visual images^{487, 488}. Similar studies with cognitive behavior therapies may also be helpful, and are starting to be performed in other conditions⁴⁸⁹. In IBS, anticipatory activity in bilateral orbito-PFC and medial temporal gyrus, predicts greater symptom improvement after 3 weeks of 5HT3R antagonist Alosetron⁴⁹⁰. Thus some anticipatory and descending control may be important in success of medication in chronic pain conditions. Our findings in SDD and IBS groups may therefore help in the development of further studies to look at the effects of both central and peripherally acting medications in these groups.

In our study, identification of the S1 and S2 regions were challenging and it was difficult to reliably identify these areas. Further analysis is needed to assess them.

Further research is also needed to identify the underlying causes of diverticular disease and the development of SDD to try and understand the genetic and psychological predisposition that may influence its development, such as have been undertaken for IBS after the Walkerton outbreak¹²². Other influences such as obesity can also be studied using clinical and imaging techniques. Although this study has started to describe the heterogeneity of SDD, there is still a long way to go to fully understand how pain is processed in this condition and what techniques we may use to diagnose and treat it successfully.

Chapter 3: Mechanistic randomised controlled trial of Mesalazine in symptomatic diverticular disease

3.1 Introduction

Although painful diverticular disease is relatively common, there has been little research in the underlying mechanisms and treatment of pain. Recent studies by Humes et al and Simpson et al have demonstrated an association between increased mucosal galanin and cytokines in DD^{13, 26, 80, 491}. However the mechanisms underlying this chronic low level inflammation are not fully understood. Nevertheless, several treatment options have been traditionally suggested. These include:

3.1.1 Surgery

Previous surgical interest on DD has focused on prevention of complications following acute diverticulitis or for the surgical treatment of complicated disease. In diverticulitis, surgery was aimed at preventing recurrence, future complications and to improve quality of life. However, recent studies failed to show an improvement in the quality of life in those post-surgery⁴⁹²⁻⁴⁹⁷.

The experience of pain depends in most cases on both a peripheral organ based pathology and the transmission of pain impulses from the organ to the central nervous system and ultimately the cerebral cortex. Some studies in IBS suggest an improvement of pain symptoms on local anaesthetic administration to the rectum, and suggest that tonic impulses from the affected organ maintain symptoms and global hypersensitivity⁴⁹⁸. Other studies on chronic pain groups suggest that effective treatments such as hip replacement for painful osteoarthritis may resolve central associated structural changes such as grey matter atrophy^{499, 500}. However in DD, one study suggests that painful symptoms persist even after surgery to remove the affected segment, implying that the central component predominates in at least some DD patients⁴⁹³. Further study is required in this area along with improvement in classification and reporting of DD patient groups. However it may

suggest that in some patients, the surgery itself, previous DD inflammatory events or the individual predisposition may result in persistent ‘phantom’ pain, driven primarily by central abnormalities in pain processing.

3.1.2 Medications

A variety of dietary supplements and medications are prescribed for DD though the evidence base for most is very weak when judged by modern standards^{501, 502}. They include:

(i) Fibre e.g. bran, ispaghula and methylcellulose

There has been only one small cross over randomized control trial for the effect of bran, ispaghula husks or placebo in symptomatic DD⁵⁰³ and only one small RCT of methylcellulose compared to placebo in symptomatic DD⁵⁰⁴. No significant effect was reported in the improvement of abdominal pain, evacuation of stool or general symptoms over the duration of the studies. There was also a significant withdrawal rate from the studies. Although fibre has been a traditional treatment for diverticular disease, and has implication in its aetiology, there is no evidence that it relieves the symptoms in symptomatic disease⁵⁰⁵.

(ii) Laxatives

The effect of lactulose has been compared to high fibre diet in one small RCT⁵⁰⁶. Unfortunately the study was not placebo controlled and outcomes were not clearly defined. So although fibre and lactulose may help with constipation there is no strong evidence to support their use in treating pain or other symptoms⁵⁰¹.

(iii) Antispasmodics

Although antispasmodics are commonly used in patients with recurrent abdominal pain, there have been no RCTs to support their use in diverticular disease.

(iv) Antibiotics

Rifaximin is a non-absorbable antibiotic that has received particular interest in the treatment of symptomatic diverticular disease and is thought to act on gastrointestinal flora as well as mucosal inflammation. There have been 4 RCT studies of rifaximin and dietary fibre, but no RCTs of rifaximin alone vs. placebo. In 3 of the studies⁵⁰⁷⁻⁵⁰⁹, the rifaximin was given in 7 day per month pulse treatments with daily fibre over 12 months. All showed some benefit in symptoms, which included pain. However, although randomized, these studies were not blinded and were not placebo controlled. There has been a small double-blind cross over study in 64 symptomatic DD patients using 20g/day of dietary fibre, with 1200mg/day of rifaximin or placebo. The study found that global symptoms scores, abdominal pain, bowel habit and bloating were improved with rifaximin. However the medications were only taken for 14 days with a 30 day washout in-between, which reduced interpretation of results and the long term benefits. Unfortunately none of these trials provide adequate evidence for the use of pulsed or long term antibiotics in SDD.

(iv) Mesalazine (5-aminosalicylic acid, 5-ASA)

There has been increasing interest in the use of mesalazine to eliminate symptoms in symptomatic diverticular disease and to prevent recurrences of diverticulitis though good quality studies are still lacking (Table I3.1).

Mechanism of action

The mechanism of action of mesalazine is not fully understood. It is thought to act mainly within the colon being delivered to the colon where around 1/3rd is absorbed by the mucosa. Its effectiveness is dependent on its mucosal concentration, with very little systemic distribution⁵¹⁰⁻⁵¹². It appears to have anti-inflammatory, anti-oxidant, anti-tumour and bacterial effects^{513-521 516, 522} but the key to its clinical benefits is unclear.

In the mucosa, 5-ASA anti-inflammatory mechanisms include immune-regulation by inhibiting nuclear factor kappa B (NFkB), RelA/p65, Ikb degradation and other signaling pathways⁵²³⁻⁵³²,

inhibiting production of cytokines, eicosanoids, TNF-alpha and interferon binding^{533, 534}. Inhibition of cellular proliferation and invasion and induction of apoptosis has also been identified in diverticular disease⁵³⁵ and models of malignancy⁵³⁶⁻⁵³⁸ as well as inhibition of lipid peroxidation. 5-aminosalicylates also act as free radical scavengers^{539, 540}. 5-ASA has been shown to inhibit epidermal growth factor receptor signalling in colorectal cancer (CRC) cell lines⁵⁴¹ and can increase cell death in non-adherent CRC HT-29 cell suspensions, by caspase dependant and independent pathways⁵⁴². It has been shown to alter inflammatory cells⁵⁴³ and mediators, such as TNF-alpha, IL-1Beta and TGF-beta^{512, 544-548}, PPAR-gamma⁵⁴⁹, cyclooxygenase/prostaglandin pathways^{515, 550}, platelet activating factor⁵⁵¹, matrix metalloproteinases⁵⁵², Toll-like receptor pathways⁵⁵³, superoxide dismutase⁵⁵³, trefoil factors⁵²⁹, heat shock proteins, heme oxygenase 1 activity⁵⁵⁴ and mucosal barrier function⁵⁵³.

The mucosal barrier function has been implicated in several gastrointestinal diseases such as celiac IBD^{555, 556}, IBS⁵⁵⁷, during normal aging process⁵⁵⁸ and stressful events⁵⁵⁹. Thus mesalazine has the potential to act on peripheral immune and barrier function which have been implicated in IBS and diverticular disease. It has also been shown to reduce fecal bacteria number⁵⁶⁰ and this antibacterial action may also contribute to its beneficial effects in both colitis and DD.

Clinical studies

Although there have been no robust RCT of mesalazine in SDD, there has been a study of mesalazine in IBS. In a small open label prospective study by Andrews et al (2011)⁵⁶⁰, 12 women with diarrhoea predominant IBS received 1.5g BD of mesalazine for 4 weeks. The study found that 67% of patients had a favorable response on global relief score and that faecal bacteria decreased. However this returned to baseline during the 4 week wash out period after the medication was stopped. There was an increase in bacterial species such as Firmicutes and bacteroidetes, especially in responders. This suggests that changes in gut bacterial populations may influence mucosal immune functions and contribute to pain relief. A separate cross-sectional study looking at the

colonic mucosa showed decreased mucosa-associated bacteria in IBD patients treated with mesalazine, despite ongoing mucosal inflammation¹.

Clinical studies of Mesalazine plus other agents

Other clinical studies looking at the effectiveness of mesalazine have used it in combination with other medications⁵⁰² (Table I3.1).

(a) Probiotics

There have been several studies, using *L. casei* or VSL#3 450billions/day treatment for 10 days/month with and without mesalazine^{561, 562} or balsalazide⁵⁶³. Although benefit was found with mesalazine and probiotics, the results of these studies are difficult to interpret. This is because the studies were open-labeled, the treatment regimes were pulsed and patients had also been previously treated with a course of mesalazine and rifaximin prior to commencing the study.

(b) Rifaximin

Several studies have used a combination of rifaximin and 5-ASA with comparison to rifaximin alone or to varying doses of rifaximin or mesalazine^{502, 564-566}. Treatment was given in pulses of 7 to 10 days per month. All reported improvement in global symptom scores or bowel habit and reduction in the occurrence of diverticulitis. Although many of these studies have high numbers of participants, the study designs make it difficult to compare results between trials and identify the true effect of mesalazine alone. Many trials use pulsed treatments of 10 to 15 days per month rather than continuous, even though a study suggested greater efficacy of treatment in the continuous rather than pulsed groups (per protocol: 77.8% vs. 56.3%)⁵⁶⁷. Interestingly 5% of participants broke protocol in the 'pulsed' medication group and were withdrawn. Some trials did not use randomization of participants and failed to perform endoscopy at the beginning of the study to confirm the presence of diverticular disease and exclude any other gastrointestinal conditions⁵⁶⁸.

All the above studies have focused on symptoms reported by the participants. There have been few mechanistic studies to compliment the change in reported symptoms in diverticular disease. However there has been a recent mechanistic double blind placebo controlled RCT of mesalazine in IBS patients⁵⁴³. This Italian study involved 20 patients with Rome II criteria IBS to placebo or mesalazine (800mg TDS) for 8 weeks. Colonoscopy and mucosal biopsies from the proximal descending colon were performed at the beginning and end of the study. The results demonstrated a decrease in inflammatory cells in the mesalazine treated group. On subtype analysis the only significant decrease was found in the TRYP+ mast cells. Significant decreases in inflammatory mediators IL-1beta, tryptase and histamine were also demonstrated. There was no significant reduction in reported symptoms except general wellbeing and treatment satisfaction

It is important to note that this study was small, with only 10 participants in each treatment arm so the study was not powered to detect differences in symptoms. The IBS patients included with all subtypes i.e. mixed, diarrhoea and constipation predominant IBS. Although these factors could have weakened the study's power, Corinaldesi et al's study⁵⁴³ does suggest potential benefit in IBS and encouraged us to explore its use in diverticular disease. This review of the literature shows that better evidence is required such as can only be obtained with a well designed placebo controlled double blinded RCT of mesalazine in symptomatic diverticular disease.

Table I3.1 Tabulated open labelled non-placebo controlled studies of mesalazine in SDD

	Number	Treatment	Follow up and Results
Gatta et al 2011 ⁵⁶⁸	Total: 149 Mesalazine = 67 Controls = 82	Pulsed 10 days per month Non randomised	Duration: 5 years M = 50 completed study C = 75 completed study No significant difference in development of diverticulitis. Symptom changes not reported.
Tursi et al 2008 ⁵⁶²	Total 71	M1: Mesalazine 800mg 10days/month M2: Mesalazine 1.6g 10days/month LM1: Mesalazine 800mg + lactobacillus casei 16 billion/day 10days/month LM2: Mesalazine 1.6g + lactobacillus casei 16 billion/day 10days/month L: lactobacillus casei 16 billion/day 10days/month	Duration: 24 months 88% symptom free. Not significant difference between groups.
Comparato et al 2007 ⁵⁶⁶	Total: 268 R1 = 66 R2 = 69 M1 = 67 M2 = 69	R1 Rifaximin 200mg BD R2 Rifaximin 400mg BD M1 Mesalazine 400mg BD M2 Mesalazine 800mg BD	Duration: 12 months M1 vs. R1 p=0.04 M2 vs. R2 p<0.0001 V0 vs. Vend significant for all except R1 Improvements on global symptom score, tenesmus, bloating, diarrhoea, bleeding, frequency and wellbeing after 12 months. Overall mesalazine had greater symptom improvement than rifaximin
Tursi et al 2007 ⁵⁶⁷	Total 40 Randomised to Grp A or B 1:1	Grp A: Mesalazine 1.6g/day Grp B: Mesalazine 1.6g/day for 10 days per month	Duration: 8 weeks treatment and 21 months follow up 34 completed study At 24 months: Symptom free p<0.05 77.78% in Grp A 56.25% in Grp B Symptom Recurrence p<0.005 5.56% in Grp A 31.25% in Grp B

Table I3.1 continued. Tabulated open labeled non-placebo controlled studies of mesalazine in SDD

	Number	Treatment	Follow up and Results
Tursi et al 2006 ⁵⁶¹	Total: 90	M: Mesalazine 1.6g/day L: L. Casei DG 16 billion/day 15 days per month LM: Mesalazine 1.6g/day + L Casei DG 16 billion/day 15 days per month	Duration 12 month 85 patients completed study 88.2% symptom free [IIT analysis] M = 76.7% L = 76.7% LM = 96% p<0.05 Symptom Recurrence = 11.1%
Di Mario et al 2005 ⁵⁶⁹	Total: 170 R1: 39 R2: 43 M1: 40 M2: 48	R1: Rifaximin 200mg BD R2: Rifaximin 400mg BD M1: Mesalazine 400mg BD M2: Mesalazine 800mg BDFor 10 days per month	Duration: 3 months Global symptom score used decreased in all groups but R1 p<0.0001. Greatest decrease in symptoms in mesalazine groups p<0.001

3.2 Aims

To undertake a pilot mechanistic, 2-group parallel design, randomized controlled trial of anti-inflammatory treatment (Mesalazine) in individuals with symptomatic diverticular disease to identify biomarkers to assess the relationship between inflammation and symptoms.

3.3 Methods

3.3.1 Trial Design

This mechanistic double blinded, randomised [1:1], parallel group pilot study of Mesalazine in symptomatic DD was initially designed in 2007 and approved by the Regional Ethics Committee and Medicine and Healthcare Regulatory Authority (REC reference: 07/Q2403/83 and EudraCT Number: 2006-006198-26). The protocol was published on ClinicalTrials.gov (NCT00663247) in April 2008. There were no deviations from the original study protocol. The trial was conducted in accordance with Good Clinical Practice (GCP) guidelines.

3.3.2 Participants

(i) Recruitment methods

Participants were identified from colorectal surgery and gastroenterology outpatient clinics at Nottingham University Hospitals and Royal Derby Hospital (UK), endoscopy lists at Nottingham University Hospitals and from databases of individuals with diverticular disease who had previously expressed an interest in participating in clinical research, held at the National Institute of Health Research Nottingham Digestive Disease Biomedical Research unit (NIHR NDDC BRU). Additional recruitment was achieved via approved advertisements on hospital notice boards and local newspapers (Nottingham Evening Post, Recorder and Metro). All potential participants were contacted using a standardized letter and study participant information sheet. Written consent was gained prior to any further contact or in accessing potential participants hospital or general practitioner (GP) records to confirm a diagnosis of diverticular disease. Only participants who had 1 or more diverticulum present in the descending or sigmoid colon on barium enema, flexible sigmoidoscopy or colonoscopy or on CT scan were eligible for the study. Structured telephone or face-to-face interviews and patient Hospital and GP records were also used to confirm other eligibility criteria (Table M3.1).

Table M3.1 Inclusion and exclusion criteria

Inclusion Criteria

1. Symptomatic diverticular disease with short lived recurrent abdominal pain for 1 hour or longer on 3 or more days a month for 3 or more months.
2. 18 – 85 years of age.
3. Signed informed consent
4. Presence of at least one diverticulum in the left colon

Exclusion Criteria

1. Pregnant or lactating women.
2. Severe co-morbidity, alcoholism or drug dependence or inability to give informed consent.
3. Contraindications to use of Mesalazine, including
 - a. Renal failure
 - b. Liver failure
4. Inability to stop NSAIDs (non-steroidal anti-inflammatory agents) or long term antibiotics.
5. The use of specific concomitant medications:
 - a. Immunosuppressants, e.g. azathioprine, 6-mercaptopurine, methotrexate, cyclosporine or any other experimental drugs
 - b. Non-steroidal anti-inflammatory drugs (NSAIDs) for more than 2 weeks cumulatively (exceptions: acetylsalicylic acid \leq 100 mg/d and paracetamol for analgesic use are allowed)
 - c. Oral, rectal or intravenous corticosteroids
 - d. Oral antibiotics: e.g. metronidazole, ciprofloxacin (exceptions: these medications are allowed for a 7 to 10 day course only, if deemed necessary for conditions unrelated to study disease)
 - e. Mesalazine-containing or -releasing drugs (e.g. mesalazine, olsalazine,

- sulfasalazine, balsalazide)
- f. Laxatives, anti-diarrheal or anti-spasmodic drugs as permanent treatment (i.e. > 1 week)
 - g. Analgesics as permanent treatment (i.e. > 1 week), except if deemed necessary for conditions unrelated to study disease
6. Presence of other gastrointestinal inflammatory conditions such as ulcerative colitis, Crohn's disease and Coeliac disease.

3.3.3 Study Setting and interventions

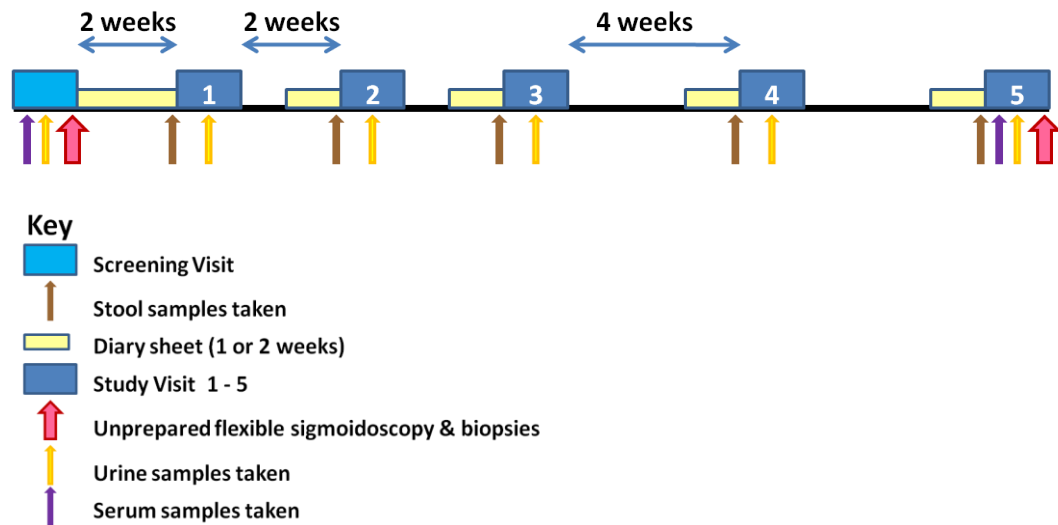
The study took place between September 2008 and January 2011 at the NIHR NDDC BRU, based at the Queen's Medical Centre (Nottingham University Hospitals, Nottingham, UK). Eligibility criteria were confirmed prior to obtaining written consent from all participants. Participants received up to £150 to cover out of pocket expenses for the duration of the study. The study lasted 3 months and was divided into screening (2 weeks pre-medication) and medication (12 weeks, 5 visits) periods (Figure M3.1).

Visit 0 - Screening and Baseline measurements

Participants completed previously validated questionnaires regarding their bowel habit, abdominal pain, somatic symptoms (PHQ-15: patient health questionnaire 15)⁵⁷⁰ and anxiety and depression (Hospital Anxiety and Depression score (HADS))⁵⁷¹. Clinical history and examination, with measurements of pulse rate, rhythm and character, blood pressure, temperature, saturations, height and weight, were performed. Blood samples were taken for baseline full blood count (FBC), urea and electrolytes (UE), liver function tests (LFTs), coagulation (Coag) and for super sensitive C-reactive protein (SS-CRP) and peripheral blood inflammatory cytokines. Blood for SS-CRP and cytokines was centrifuged at 25°C for 10 minutes at 2000g to allow separation of the serum. The serum was removed using sterile pipette (Eppendorf, Research Physio Care Concept, Germany) and the sample stored in eppendorfs at -20°C immediately to prevent degradation. Urine sample

was also collected and tested using URS-100 and/or Combur 7 dipsticks (Teco Diagnostics, CA, USA, and Roche, Switzerland). A 2.5 - 5ml urine aliquot was stored at -80°C.

Figure M3.1 Schematic of interventions and follow up during Trial.



Participants also had the option to undergo an unprepared flexible sigmoidoscopy either at the screening visit or at the subsequent visit 1, in 2 weeks time. The flexible sigmoidoscopy was performed in dedicated research facilities within the NIHR NDDC BRU by accredited practitioners using an Olympus scope (CF 240L Olympus, Essex UK) and Stack (CV 260 SL EVIS, Olympus, Essex UK). Six biopsies were taken from the sigmoid around the ostia of diverticula and six from the rectum using forceps (2.4m Cold Captura Biopsy Forceps, DBF-2.4-230-20-S G5606, Limerick, Ireland): Two were immediately placed in cyrotubes (NUNC CyroTubes [363401], Thermo Fisher Scientific, Langenselbold, Germany) and frozen in liquid nitrogen, two placed in 1.5ml of RNA later (R0901, Sigma-Aldrich, Dorset, UK) for 24-48 prior to being frozen at -80°C (as per manufacturer's instructions), and 2 placed in formalin pots (4% Formaldehyde, Genta Medical, York, UK) for histological assessment.

During the subsequent 2 weeks, participants completed daily diaries documenting the duration and intensity of their abdominal pain, general wellbeing, bloating and bowel habits, including scores of

bowel frequency and consistency, using the Bristol Stool Chart (Please see **Appendix 6.9** for example of diary sheets). They also collected a stool sample 24hrs prior to attending for their next visit. If a sample was not obtained in time, participants were able to return a sample using secure pre-paid envelopes by post. Stool samples were stored at -20°C upon receipt.

Medication: Visit 1

Medication was dispensed on study visit 1, approximately 2 weeks after the screening visit, and after participant diaries were checked. Three grams of medication, either mesalazine or placebo, were taken each morning by participants for the duration of the study.

All participants had 24hr access to a medical health professional during their participation and follow up. Adverse events were treated and recorded as per GCP and study protocols.

Medication: Visits 2-5

Subsequent follow up visits for monitoring and data collection were performed at 2 or 4 weekly intervals as per the protocol (Table M3.2 and Figure M3.1), to allow early detection of rare mesalazine complications, such as renal or liver impairment, and aplastic anaemia or pancytopenia. Prior to each visits participants completed a 7 day diary of bowel function and abdominal pain and collected and stored a stool sample. Participants returned any used medication at each visit and were issued with further medication to last until their next visit (+6 days in case of in-adverted delays in returning). This allowed recording of left-over trial medication returned at the follow-up visits and the final visit as well as by checks of the diary by the investigator as a measure of compliance. At each study visit, apart from visit 1, urine was collected and tested to detect any renal impairment. An aliquot of urine was stored at each visit in -80°C freezer. Participants were also asked at each visit if they had satisfactory relief from their diverticular symptoms.

At visit 5, additional questions were included if they thought they were taking the mesalazine or placebo and if they would continue the medication. If participants did wish to 'continue' the

medication, their care was transferred to National Health Service Gastroenterology Department or their general practitioner, where they were prescribed mesalazine and appropriate monitoring could also take place. Also on the final visit, additional blood was taken for final SS-CRP and cytokines and a further unprepared flexible sigmoid endoscopy with 6 biopsies taken from the sigmoid and rectum, as for baseline. At the end of the study, all unused medication underwent documented destruction by the Clinical Trials Pharmacy according to local policy. Receipts of medication destruction were set to Dr Falk Pharma for confirmation.

Table M2.2. Visit schedule.

Visit	Visit no.	Day no.	Week no.	Time window [days]
Screening visit	Visit 0	Day -14	Week -2	NA
Baseline	Visit 1	Day 0	Week 0	NA
Interim visit	Visit 2	Day 14	Week 2	± 6
Interim visit	Visit 3	Day 28	Week 4	± 6
Interim visit	Visit 4	Day 56	Week 8	± 6
Final visit	Visit 5	Day 84	Week 12	± 6

3.3.4 Laboratory methods

(i) RNA Methods

RNA was extracted from 1 sigmoid colorectal tissue biopsy in RNA later, which had been stored since collection in dedicated -80°C tissue storage facilities at the FRAME laboratory (Nottingham Medical School, Nottingham UK). All samples were extracted within a 2 week period after completion of follow up of all the trial participants. TRI reagent® (Sigma Aldrich USA Pcode101078497 T9424) extraction of RNA, Qiagen column clean up of RNA (Qiagen USA Cat No 74106) and creation of cDNA were performed in the FRAME laboratory under the supervision of Dr A Bennett, using standardized protocols, which can be found in **Appendix 6.10**.

(ii) Gene card

Custom made 96 gene micro-fluidic gene cards (Format 96a; P/N 4342259, Applied Biosystems, California USA) were constructed after review of potential molecular pathways involved in chronic inflammation and nociception in diverticular disease, irritable bowel syndrome and inflammatory bowel disease. Each card was designed to analyze 96 genes with no repeats, from 4 cDNA samples simultaneously. Housekeeper genes, which were chosen to be consistently expressed during the trial duration, included 18s, Beta-Actin, ribosomal protein large PO (RPLPO) and hypoxanthine phosphoribosyltransferase 1 (HPRT1). A full list of genes selected for the gene cards can be found in **Appendix 6.11**.

Micro-fluidic cards were loaded and analysed as per manufacturer's instructions⁵⁷². In brief, 60ul of cDNA was created using 1ug of RNA and Superscript III reverse transcriptase (Invitrogen USA Cat No 18080-093), according to manufacturer's instructions. 50ul of DEPC (Diethyl pyrocarbonate: Sigma-Aldrich USA D5758) treated HPLC grade water and 110ul of Universal Taqman master mix (P/N 4304437, Applied Biosystems, California USA) was added to create a final volume of 220ul. The micro-fluidic gene card has 8 wells, with 2 wells for each of the 4 samples. 100ul of the samples were placed in their corresponding wells and the plate centrifuged twice at 1200rpm for 1 minute using a Sorvall ST40 centrifuge (Thermoscientific, Loughborough UK). The plate was sealed with a plate sealer (Model 4331770 Rev A.5, Applied Biosystems California USA) and loaded into the 7900HT Fast Real Time PCR system analyser (Applied Biosystems California USA).

Initial analysis to generate CT values was undertaken using RQ manager software (Version 1.2 Applied Biosystems California USA). As more than 10 cards were used, this analysis was undertaken in two stages. Corrections to the automated analysis were performed to ensure that all thresholds and baselines were identical between the analysis groups.

CT values were then exported as text files and incorporated into an Excel spread sheet (Microsoft) to allow assessment of the housekeepers and calculation of the geometric mean and relative quantification (RQ). The sample used as the baseline for RQ analysis was RXB05 sigmoid baseline, as this sample demonstrated good expression with only 2 genes failing to amplify.

(iii) Histology

Sigmoid colon and rectal biopsies preserved in formalin histology pots were transferred to the Histology Department at Nottingham University Hospitals. All samples were preserved in paraffin blocks and 3µm sections cut and mounted on slides. The samples were dried for 20 minutes at room temperature and then at 60°C for 20 minutes. Samples were stained by Dr Claire Hawkes in dedicated Immunohistochemistry (IHC) Laboratories at the Nottingham University Hospitals for lymphocytes (CD3), macrophages (CD68), proliferating cells (KI67) and endochromaffin cells (Serotonin, 5HT) using a Bond Max automated staining processor (Leica Microsystems) (Please see Table M2.3 for antibodies and dilutions).

Stained slides were scanned at *20 (5HT) or *40 (CD3, CD68, KI67) magnification using a NanoZoomer 2.0-HT (Hamamatsu, Japan) in the Photography Division of the Pathology Department at Nottingham University Hospitals. Tissue architecture was also examined with haematoxylin and eosin (H & E) stained sections by a consultant pathologist, Dr A Zaitoun.

TableM2.3 Immunohistochemistry antibodies and dilutions.

Target	Antibody	Dilution	Notes
KI67	Clone MIB1, NCL-L-KI67-MM1, Leica Microsystems, Milton Keynes, UK.	1:50	Antigen retrieval for 30 minutes with ER2 (AR9640), a ready-to-use EDTA based pH9.0 solution
CD68	Clone KP1, M0814 Dako UK Ltd.	1:3000	Antigen retrieval for 20 minutes with ER1 (AR9961), a ready-to-use citrate based pH6.0 solution
CD3	NCL-L-CD3-565, Leica Microsystems, Milton Keynes, UK.	1:100	Antigen retrieval for 20 minutes with ER2 (AR9640), a ready-to-use EDTA based pH9.0 solution
5HT	clone 5HT-H209, M0758, Dako UK Ltd.	1:200	Enzyme pre-treatment for 10 minutes

(iv) Histology assessment

All scanned slides were assessed using NanoZoomer digital pathology virtual slide viewer software (Hamamatsu, Japan). Coefficients of variation were verified to be <0.1 and reproducibility >90% for each IHC stain prior to assessment of the samples (see **Appendix 6.12**). All slides were anonymised by the Pathology Department prior to staining. This prevented participant identification by the researchers performing the cell counts. The protocols for cell counting for each stain are:

CD3

CD3 positive cells appeared dark brown. Only cells with positive staining and an identifiable nucleus were counted. Cells within the epithelium and lamina propria were assessed, with areas adjacent to lymphoid follicles being excluded (Figure M3.2A). Up to 15 randomly selected areas of epithelia and lamina propria were assessed and number of cells per mm² calculated and used for

further analysis. Care was taken to include equal amounts of superficial (sub-epithelial) and deep lamina areas when measuring the lamina propria.

CD68

CD68 positive cells appeared dark brown. Only cells with positive staining and an identifiable nucleus were counted. Cells within the lamina propria were assessed, again with areas adjacent to lymphoid follicles being excluded (Figure M3.2B). Up to 15 randomly selected areas of lamina propria were assessed and number of cells per mm² calculated and used for further analysis. Again, when measuring the lamina propria, care was taken to include equal amounts of superficial (sub-epithelial) and deep areas.

KI67

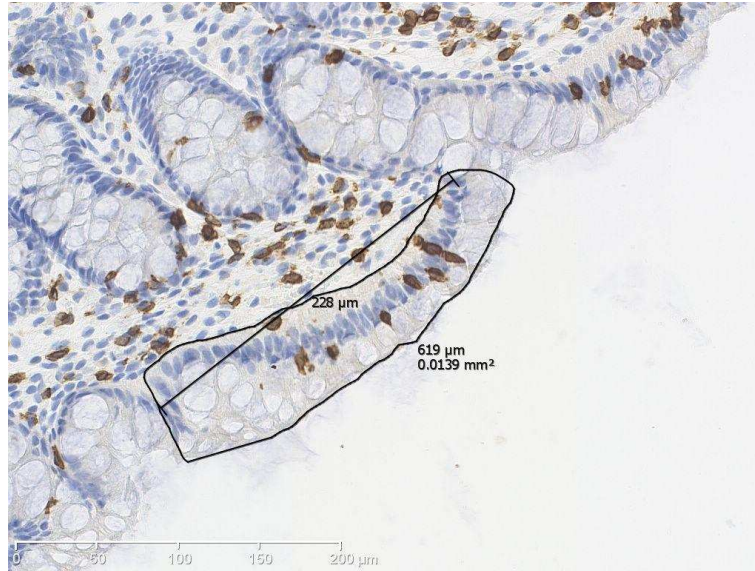
KI67 positive cells appeared dark brown. Only cells with positive staining and an identifiable nucleus within the epithelium were assessed, with areas adjacent to lymphoid follicles being excluded. Up to 15 randomly selected areas of epithelia were assessed and number of cells per mm² calculated and used for further analysis.

5HT

Only cells within the crypt mucosa were assessed. 5HT positive cells appeared dark brown. Up to 15 crypts per slides were measured and divided into deep and superficial segments (Figure M3.2C). The number of positively stained cells in each area was recorded along with the deep and superficial areas, the perimeter of each segment and the length of the crypt. The number of cells per mm² per segment was calculated and used for further analysis.

Figure M3.2 Histological assessment of (A) CD3, (B) CD68 and (C) 5HT using NanoZoomer digital pathology virtual slide viewer software at *20 Magnification.

(A) CD3 epithelial measurements



(B) CD68 lamina propria Measurements

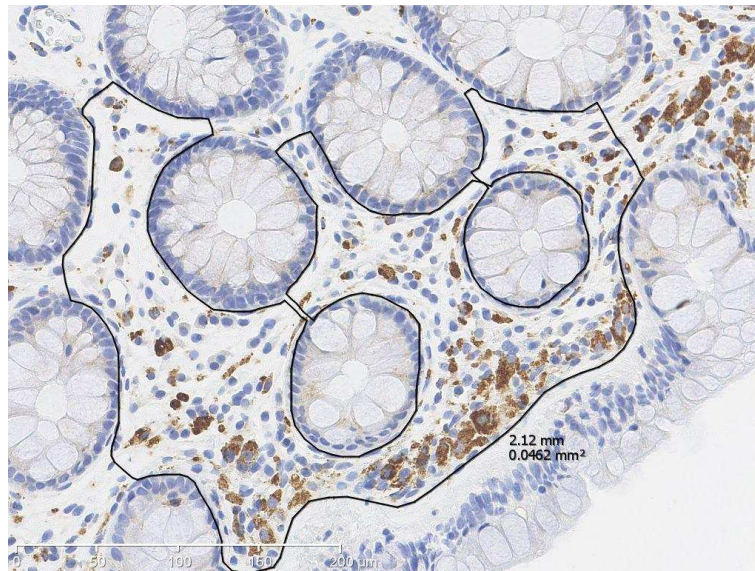
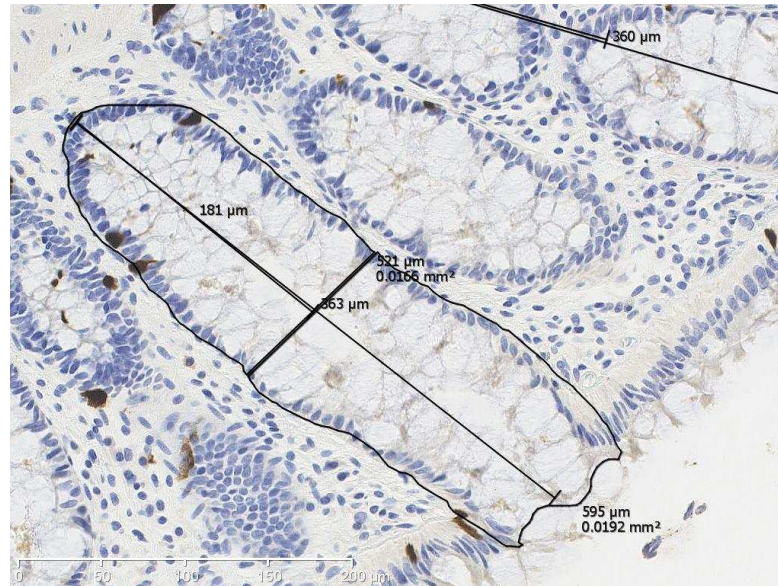


Figure M3.2 continued. Histological assessment of (A) CD3, (B) CD68 and (C) 5HT using NanoZoomer digital pathology virtual slide viewer software at *20 Magnification.

(C) 5HT crypt epithelial Measurements



(v) Faecal calprotectin

The assessment of Faecal Calprotectin was performed by Dr Louise Hawke at the Department of Pathology (Nottingham City Hospital, Nottingham University Hospitals, UK). The baseline and final visit stool samples were extracted using the device for stool collection (Calprest collection device, Code 9062, Eurospital, Trieste, Italy). Faecal Calprotectin analysis was carried out by ELISA (Calprest kit, Code 9031, Eurospital Trieste, Italy) according to the manufacturer's instructions.

(vi) Super Sensitive CRP

Serum samples were frozen at -20°C and transferred to Leeds Teaching Hospital for processing, via to Biochemistry Laboratories at Nottingham University Hospitals using their standardized protocol.

(vii) Liquid chromatography and mass spectroscopy

Extraction of samples, analysis and interpretation of results was performed by Dr Srinivasarao Ravipati and Prof D Barrett between 20/12/2012 to 22/12/2013. The method used was developed by Dr A Wong (Biochemical Sciences, University of Nottingham). The data were analysed in GraphPad prism using a paired non-parametric T-test (Wilcoxon).

3.3.4 Outcomes

The primary endpoint was the difference in change in galanin and/or galanin receptor 1 expression from 0 (baseline) to 12 weeks (Visit 5) or on withdrawal between mesalazine & placebo treated groups. This end point was chosen based on previous work suggesting increased galanin and galanin receptor 1 mRNA and protein in patients with symptomatic diverticular disease^{80, 491}. Secondary endpoints included differences between mesalazine and placebo groups with respect of changes from 0-12 weeks or withdrawal of: (1) mRNA of inflammatory cytokines, (2) cell counts of CD3, CD68, 5HT (serotonin) and KI67 positive cells, (3) Faecal calprotectin and (4) Abdominal pain, stool frequency and mean stool consistency.

Galanin and Tachykinin staining for protein

Multiple attempts at staining for galanin, its receptors and for tachykinin receptors were performed. Despite the use of several different commercially available antibodies, we were not successful and could not replicate the results of Simpson et al⁸⁰, Simpson et al performed their staining at a specialist laboratory in Lund, Sweden, with antibodies which were not commercially available. Other groups have reported similar problems in the reliability of antibodies^{87, 573-575}. Thus we will only report changes in gene expression.

3.3.5 Sample Size Calculation

The aim of this pilot study was to identify inflammatory markers which may be useful in distinguishing painful symptomatic diverticular disease patients with low grade inflammation in whom Mesalazine may be an effective treatment. No prior study has looked at markers of inflammation in diverticular disease and so no formal power calculation can be performed. A prior

study using just 21 ulcerative colitis patients found a significant effect of Mesalazine on the levels of mucosal interleukin 1 (IL-1)⁵⁷⁶. Therefore given a sample size of 40, it should be possible to detect a significant difference between two groups (i.e. mesalazine and placebo).

3.3.6 Randomization and Blinding

The mesalazine granules (Salofalk) and placebo were manufactured and packaged by Dr Falk Pharma and were identical in packaging, colour, size and taste. The participants' randomisation numbers were generated by Dr Falk Pharma prior to commencement of the study and consecutively allocated as participants were recruited into the study. All medication was stored in the Clinical Trials Pharmacy (Queen's Medical Centre University of Nottingham, UK) prior to dispensing to the participants at each study visit. The code was kept in a sealed envelope in case of adverse reactions, when it could be opened by the Clinical Trials Pharmacy. The code was not broken during the duration of the study and the envelopes were only opened after completion of laboratory sample assessment. This maintained the blinding of all participants and clinical trial staff in the NDDC BRU and Clinical Trials Pharmacy.

3.3.7 Statistical Analysis

As this study is a pilot study of only 40 participants, no interim analyses were performed during the study. All questionnaire, genetic, biochemical and histological data was stored in Microsoft Office Access 2007 (Microsoft USA) database and transferred to SPSS (version 15, IBM, Portsmouth UK) and GraphPad Prism (Version 5, California USA) for further analysis. To determine whether randomisation had been successful the baseline characteristics of the two groups and the withdrawals were compared using Mann-Whitney U. The outcome measures of principal interest assessed in the biopsy samples taken at the screening visit and at final visit (3 months) in a per protocol analysis. As the study was not powered to assess efficacy of mesalazine in pain relief, an intention to treat analysis was not performed. A paired non-parametric T-test (Wilcoxon signed-rank test) was used in per-protocol analysis to compare between the baseline and final measures in the placebo and mesalazine group. Statistical significance was $p < 0.05$ level.

3.4 Results

3.4.1 Participant Recruitment

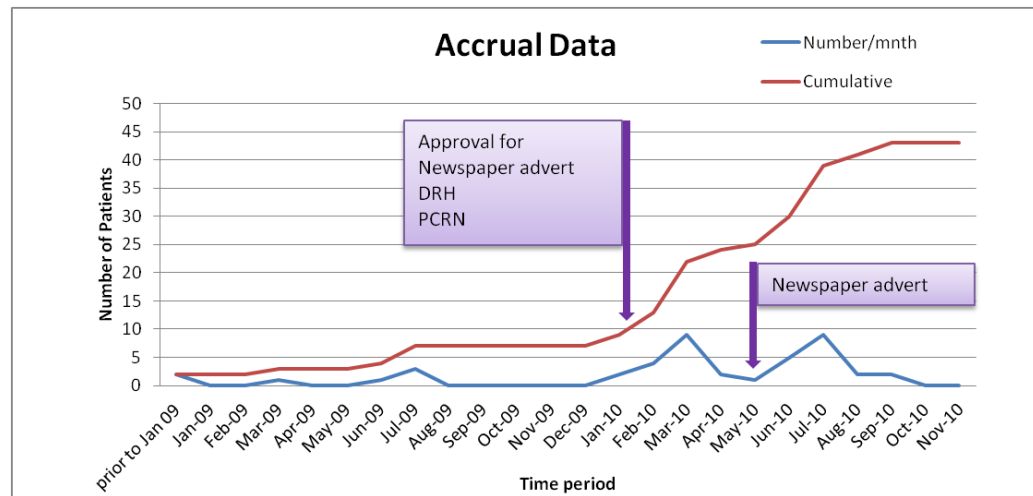
43 participants were recruited to the study between September 2008 and January 2011. Participants attended screening visits and follow up visits over a 3 month period as per protocol (Table M2.2). If a participant withdrew prior to commencing medication, the medication intended for them was re-allocated to the subsequent participant who completed the screening period. Ethical Approval was granted in August 2010 to recruit up to 50 participants to allow for the withdrawals prior to commencing medication. However once 40 participants had received medication the trial was closed to further recruitment, maintaining the study numbers as per the original protocol. One set of study medication was 'spoiled' during the study, resulting in 39 participants starting medication as part of the trial.

Recruitment for the study offered challenges, as many potential participants with painful SDD are treated by GPs in the community. Our initial poor recruitment rates were improved by targeting these potential participants through newspaper advertisements and via the Primary Care Research Network (PCRN). Figure R3.1 demonstrates the recruitment rate and the improvements obtained by using these alternative recruitment methods.

3.4.2 Participant Flow, Exclusions and Losses

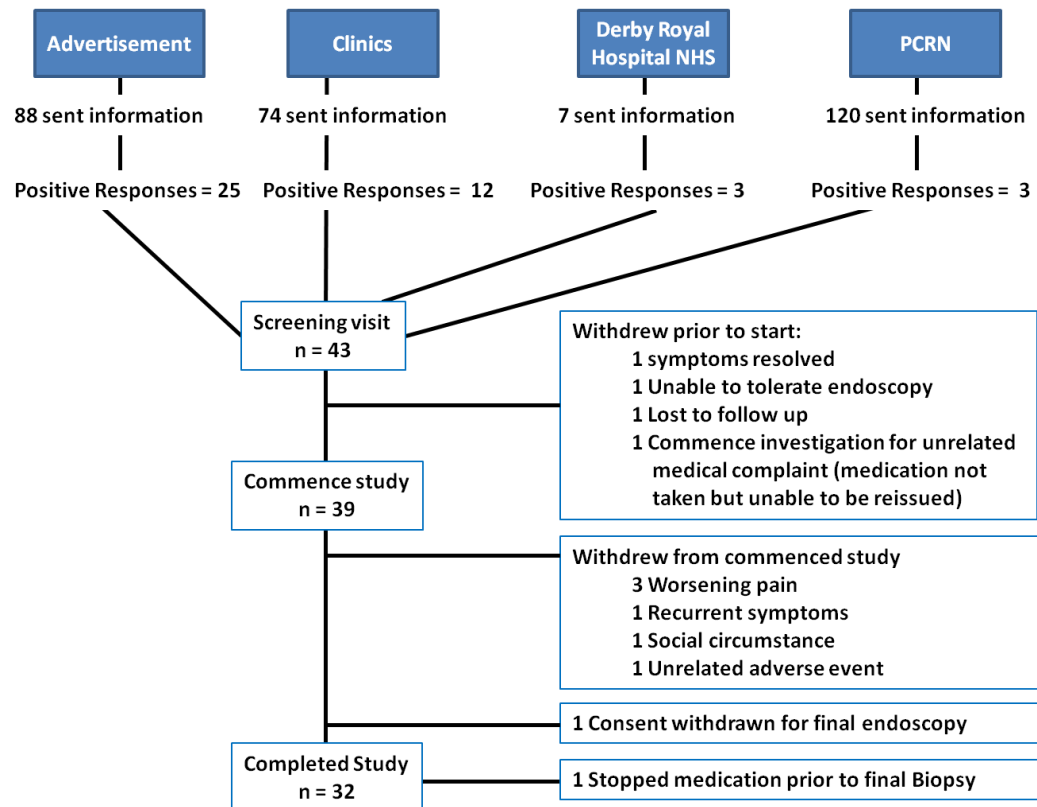
Two subjects withdrew during the 2 week baseline period due to 'resolution of their symptoms' or did not respond to further attempts to contact by the research team. Two more were withdrawn by the research team; 1 who was unable to tolerate an unprepared flexible sigmoidoscopy to obtain initial colorectal biopsy samples and 1 for an unrelated musculoskeletal problem, requiring further investigation by a NHS rheumatology team (Figure R3.2).

Figure R3.1 Annotated graph of recruitment rate for Mechanistic RCT.



Purple arrows indicate time at which recruitment interventions were initiated. All interventions were Ethical and Local Research and Development office approved. DRH = Derby Royal Hospital, PCRN = Primary Care Research Network.

Figure R3.2 Flow Diagram Overview of participants recruited to the Trial.



Advertisements include newspaper and hospital poster boards. Clinics are those based at Nottingham University Hospitals. PCRN = Primary Care Research Network.

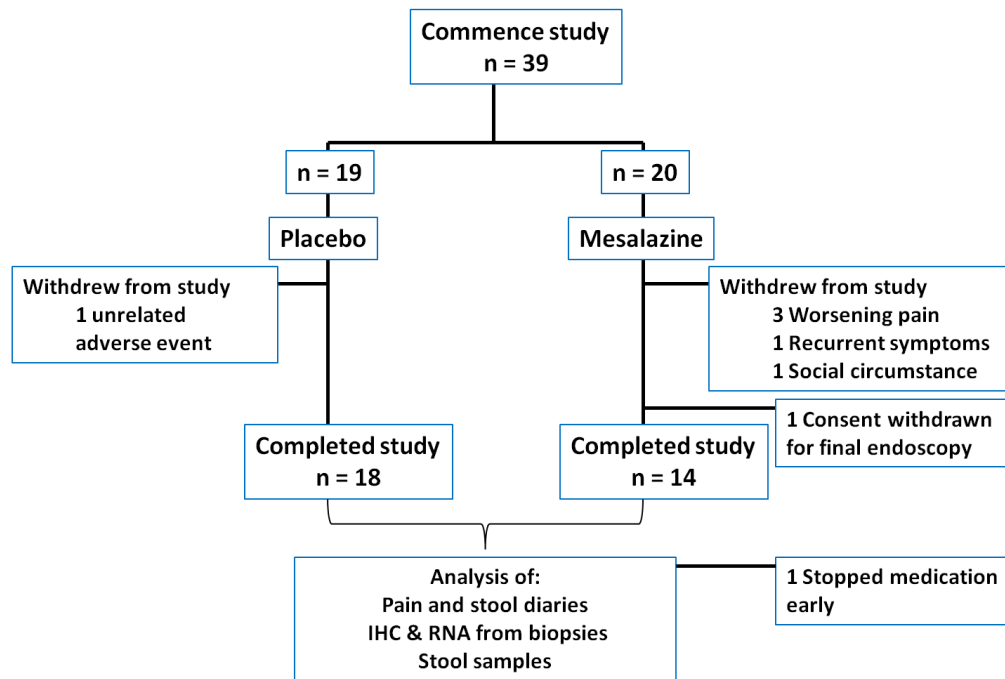
During the 12 week medication period of the study, 6 participants withdrew. Figure R3.3 outlines the withdrawals which occurred in each treatment arm. Four of these were for worsening abdominal pain or recurrence of original symptoms. The worsening abdominal pain resolved after stopping medication in each participant, but reoccurred with re-challenge. All these subjects were withdrawn with agreement between the participant and the research team. One participant had a change in family circumstances which prevented further travel to the NDDC BRU and asked to be withdrawn.

A further participant suffered a severe adverse event 2 weeks after commencing study medication (Visit 2). This was detected by the researcher after the participant mentioned symptoms of breathlessness on exertion, which had started before recruitment into the study but the participant

had not mentioned during screening. This symptom had subsequently become worse during the last 2 weeks before the visit. The participant had given a previous history of DVT, but investigations confirmed only a slight troponin rise of 0.070 ng/ml (upper limit normal 0.040 ng/ml), a normal ECG and a negative D-Dimer. The participant was admitted to the Nottingham University Hospitals where a cardiac echo, exercise tolerance test and CT Pulmonary Arteriogram (CTPA) were performed. These confirmed a diagnosis of a pulmonary embolism and the participant was commenced on warfarin. The participant ceased their medication on admission to hospital and was subsequently withdrawn from the study by the research team. Due to the onset and participant risk factors, the adverse event was classified as unrelated to the study. During the analysis phase the participant was found have been allocated to the placebo arm of the study.

During the final visit, 2 participants disclosed that they had not consistently taken or had prematurely stopped the medication. The reasons for this were not clear. One of these participants also withdrew consent for the final endoscopy and biopsies, although they did complete the questionnaires and provide blood and stool samples. The other informed the study team that she had not taken the medication for 5 days previously, which lead to some data loss during the analysis.

Figure R3.3 Participant Flow diagram and withdraws per treatment arm.



IHC = Immunohistochemistry, RNA = colorectal biopsy RNA analysis.

3.4.3 Baseline Data

The baseline characteristics of the mesalazine and placebo groups are demonstrated in table R3.1 A-E. As a considerable number of the withdrawals were from the mesalazine group, demographic data on the withdrawal group has also been included. However, 1 participant withdrew prior to collection of demographic data and before commencing medication, so only 10 out of the 11 withdrawal subjects have been reported.

Table R3.1A-B demonstrates the age range, sex, body mass index (BMI) and prevalence of a past medical history of diverticulitis, abdominal surgery, psychiatric history and smoking prevalence of the placebo, mesalazine groups as well as the withdrawals. The diagnosis of diverticulitis was based on hospital records, CT reports or on interview with the participant giving a history of >24hours of abdominal pain, fever and a prescription of antibiotics. The final column in table R3.1A and B includes p values for the continuous (Mann Whitney U, MWU) or categorical (Fisher Exact test) data. Participant method of diagnosis and recruitment into the study is shown in Table

R3.2 A-B. As pain experience can be influenced by social situation, demographic data on marital status was also collected Table R3.3.

Table R3.1 Demographics and Co-morbidities of participants per group.

	Placebo (A) N=18	Mesalazine (B) N=14	Withdrawn N=10	P Value A vs. B
A: Demographics				
Median Age (Yrs) (Range)	66 yrs (32-80)	64.5 yrs (45-77)	62.5 yrs (43-74)	P = 0.3417 MWU
Male (%)	9 (50%)	2 (14.3%)	7 (70%)	P = 0.0608 Fishers exact
BMI (Kg/M²) (Range)	28.94 (23.59-35.01)	32.98 (20.10-48.96)	26.82 (20.06-34.21)	P = 0.0800 MWU
Diverticulitis diagnosis	6 (33.3%)	6 (42.9%)	2 (20%)	n.s.
B: Co-morbidity				
Anxiety or Depression	2 (11.1%)	4 (28.5%)	3 (30.0%)	P=0.3649 Fisher Exact
Prev. Abdominal Surgery	13 (72.2%)	13 (92.9%)	6 (60.0%)	P=0.3589 Fisher Exact
Smoking				
None	7 (38.8%)	6 (42.9%)	5 (50.0%)	n.s.
Ex-smoker	7 (38.8%)	6 (42.9%)	4 (40.0%)	
Smoker	4 (22.2%)	2 (14.3%)	1 (10.0%)	

Operations include: appendectomy, total abdominal hysterectomies, surgery for an ectopic pregnancy, sterilisation, laparoscopy (+/- adhesiolysis), cholecystectomy, hernia repairs, lipoma removal, removal of un-descended testicle and TURP. n.s. = not significant

Table R3.2 Method of diagnosis and recruitment of participants in Placebo, Mesalazine groups and withdrawn groups.

A: Diagnosis Method	Placebo N=18	Mesalazine N=14	Withdrawn N=10
BE	7 (38.9%)	4 (28.6%)	3 (30%)
CT	0	4 (28.6%)	1 (10%)
Endoscopy	11 (61.1%)	5 (35.7%)	6 (60%)
Missing Data	0	1 (7.1%)	0
B: Recruitment Method			
Advert	11 (61.1%)	7 (50%)	8 (80%)
Clinic	3 (16.7%)	4 (28.6%)	2 (20%)
Database	1 (5.6%)	0	0
Royal Derby Hosp	1 (5.6%)	3 (21.4%)	0
PCRN	2 (11.1%)	0	0

BE = Barium Enema, Endoscopy = flexible sigmoidoscopy or colonoscopy,

Table R3.3 Marital status of participants in per group.

Social Support	Placebo N=18	Mesalazine N=14	Withdrawn N=10
Married or Living as married	12 (66.7%)	11 (78.6%)	7 (70%)
Living with Friend	0	1 (7.1%)	0
Divorced	2 (11.1%)	1 (7.1%)	1 (10%)
Widow	3 (16.7%)	1 (7.1%)	0
Single	1 (5.6%)	0	2 (20%)

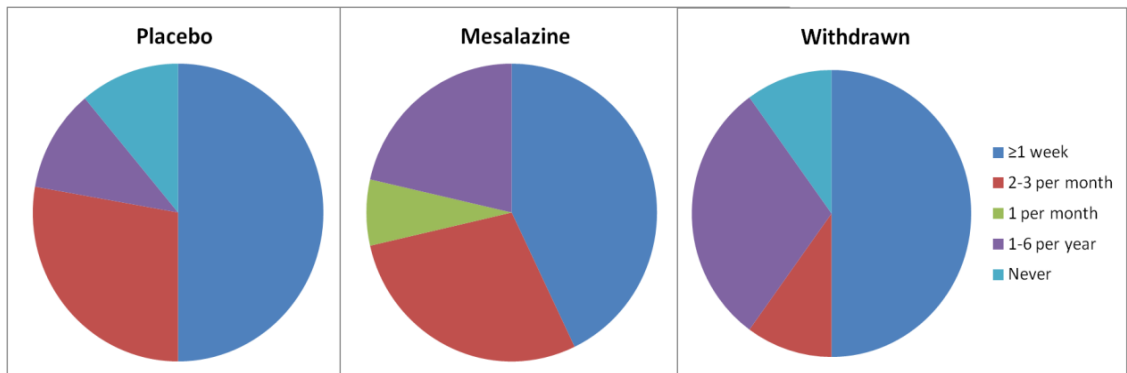
(a) Baseline Gastrointestinal symptoms

Both gastrointestinal pain and bowel habit were assessed at baseline. Gastrointestinal pain was divided into ‘attacks’ of pain lasting 1 day or longer and other episodes of pain or discomfort that occurred during their normal bowel habit.

(i) Pain > 24hrs duration

A pie chart demonstrating the incidence of pain lasting greater than 24hrs per treatment arm and withdrawals is shown in Figure R3.4.

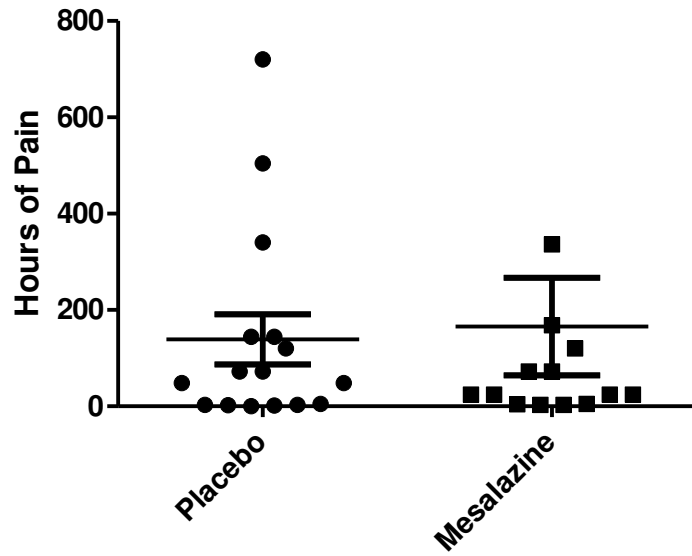
Figure R3.4 Incidence of Pain ≥ 24 hours duration per treatment group over the last 12 months prior to recruitment into study.



However although some of the participants answered that they did have pain lasting for >24 hours, they then answered further questions with estimates of their longest and typical attack of pain at values less than 24 hours. These results are shown in Table R3.4 and represented graphically in Figure R3.5. Figure 3.5B shows a biphasic distribution suggesting the while most patients with recurrent pain had short-lived pain some had bouts lasting several days suggesting a somewhat different mechanism.

Figure R3.5 Participants estimation of the duration of their (A) longest and (B) typical attacks of Pain. NB: In (A) 1 data point in the mesalazine group lies beyond the axis boundaries at 1440 hrs

(A) Duration of 'longest attacks' of pain



(B) Duration of 'typical attacks' of pain

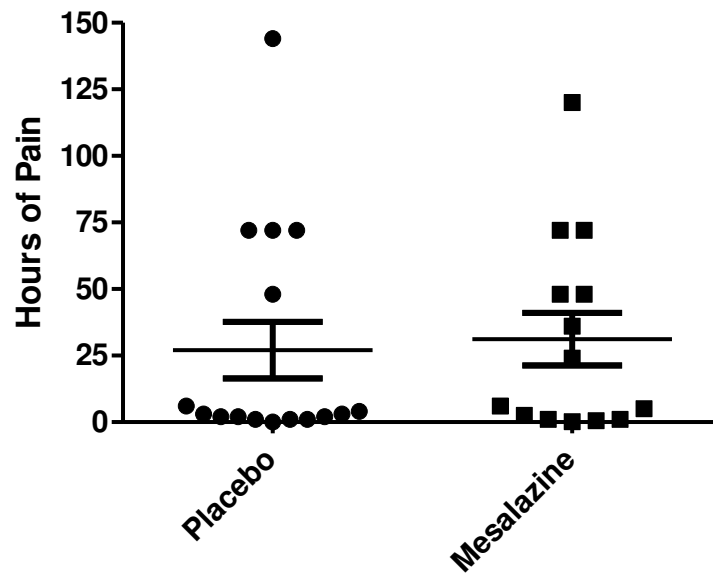


Table R3.4 Participant Answers to questions on pain greater than 24 hours in duration.

	Placebo (A)	Mesalazine (B)	Withdrawn	P Value A vs. B
Yes: Pain >24hrs: (%)	16 (88.9%)	14 (100%)	9 (90.0%)	
Est. pain >24hrs Longest Attack (%)	10 (55.6%)	10 (71.4%)	7 (70.0%)	
Est. pain >24hrs Typical (%)	5 (27.8%)	8 (57.1%)	6 (60.0%)	
All Longest Attack Median Duration (hrs) (Range)	60 (0.50-720)	24 (3.0-1440)	120 (3.0-8760)	P=0.9834
All Typical Attack Median Duration (hrs) (Range)	3 (0.08-144)	15 (0.17-120)	72 (2-8760)	P=0.7067

Est. = estimate

Two subjects in the placebo and 1 in the withdrawal group did not experience pain >24 hours but did report shorter lived episodes.

(ii) Pain with normal bowel habit

The number of days per month of pain that occurred without changes to the participants' normal bowel habits and the duration of that pain is shown in Table R3.5. Not all participants experienced pain as part of their normal bowel habit. However based on participants' answers to questions on pain lasting longer than 24 hours as well as pain during normal bowel habit, all fulfilled the pain inclusion criteria for the study. It is worth noting that the median duration of a typical attack in those withdrawing was 3 days suggesting that they may have had more severe symptoms.

Participants were also asked if they believed any dietary groups may influence their gastrointestinal symptoms, such as consuming fibre, fruit and vegetables or dairy foodstuffs. These are also shown in Table R3.5.

Table R3.5 Participants reporting and their estimation of pain duration during episodes of normal bowel habit and their beliefs on exacerbating factors.

	Placebo (A)	Mesalazine (B)	Withdrawn N=10	P Value A vs. B
Yes: Pain with normal bowel habit (%)	15	11	8	
Median Pain days/mnth (IQR)	16 (4.0-30.0)	7 (4.0-30.0)	18 (5.3-30.0)	n.s.
Median Pain duration (hrs) (IQR)	3 (1.0-24.0)	4.5 (2.0-24.0)	16 (4.1-24.0)	n.s.
Affected by Bran	3 (16.7%)	3 (21.4%)	4 (40.0%)	n.s.
Affected by Fruit	8 (44.4%)	9 (64.3%)	6 (60.0%)	n.s.
Affected by Dairy	3 (16.7%)	6 (42.9%)	2 (20.0%)	n.s.

The extent of which pain interfered with participants lives was indicated on the questionnaire by the number of participants who visited their GP or were admitted to hospital because of their gastrointestinal symptoms. Participants also gave an estimate how many days they had to stay at home in bed over the last year. This information is presented in Table R3.6.

(iii) Bowel Habit

Baseline bowel habit over the last year was assessed by questionnaire, with participants asked to estimate their bowel frequency and incidence of other symptoms per week (Table R3.7). This was used to confirm prospectively collected stool diaries.

Table R3.6 Hospital Admissions, GP Visits and Bed Days per study group.

	Placebo (A)	Mesalazine (B)	Withdrawn N=10	P Value A vs. B
GP visits: Yes (%)	10 (55.6%)	9 (64.3%)	6 (60%)	n.s.
Median Number of GP Visits (range)	1 (0-52)	1 (0-15)	1 (0-7)	n.s.
Hospital Admissions Yes (%)	4 (22.2%)	6 (42.9%)	1 (10%)	n.s.
Stay in bed (Home) (%)	6 (33.3%)	9 (64.3%)	4 (40%)	P=0.1527 Fisher exact

Table R3.7 Baseline bowel habits per study group over the last 12 months (retrospective)

% affected (Median days/week)[Range]	Placebo	Mesalazine	Withdrawn	P Value
Median BO/day (range)	1.5 (0.29-5.0)	2 (0.64-10.0)	1.5 (0.57-3.0)	n.s.
Loose Stools (number per week)	55.6% 3 (0-7)	71.4% 2.25 (0-7)	50% 0 (0-5)	n.s.
Hard Stools (number per week)	72.2% 1 (0-7)	57.1% 2 (0-7)	50% 1 (0-7)	n.s.
Strain (times per week)	55.6% 0.25 (0-5)	57.1% 2 (0-7)	40% 1 (0-7)	n.s.
Urgency (times per week)	44.4% 0 (0-7)	50% 0.5 (0-7)	20% 0 (0-5)	n.s.
Tenesmus (times per week)	66.7% 1.5 (0-7)	85.7% 2.25 (0-7)	70% 1 (0-7)	n.s.
Incontinence (times per week)	16.7% 0 (0-2)	21.4% 0 (0-1)	40% 0 (0-1)	n.s.
Mucus (times per week)	16.7% 0 (0-3)	42.9% 0 (0-7)	60% 0.25 (0-7)	n.s.
Bloating (times per week)	61.1% 3 (0-7)	71.4% 2.25 (0-7)	70% 4.5 (0-7)	n.s.
Blood (Number affected, %)	3 (16.7%)	6 (42.9%)	2 (20.0%)	n.s.

3.4.4 Analysis Groups

As mentioned previously, one participant in the mesalazine group stopped the medication 5 days prior to the end of the study. As it was only 5 days of continuous missed medication at the end of the study, we decided to include her data in the study up until visit 4. Her final biopsy sample was processed for RNA gene card but the data has not been included in the analysis as it is uncertain if 5 days of missed medication would potentially influence changes in gene expression. One further participant in the mesalazine group also failed to complete their visit 5 diary prior to completing the study. In this case participant diary information up to visit 4 was included in the analysis. As the participant had continued medication until visit 5 all biological samples were used in the per-protocol analysis.

Thus 18 participants in the placebo and 13 participants in the mesalazine group were used in analysis of mechanistic endpoints. For Baseline characteristics and end point symptom assessment, 18 participants in the placebo and 14 participants in the mesalazine group were included.

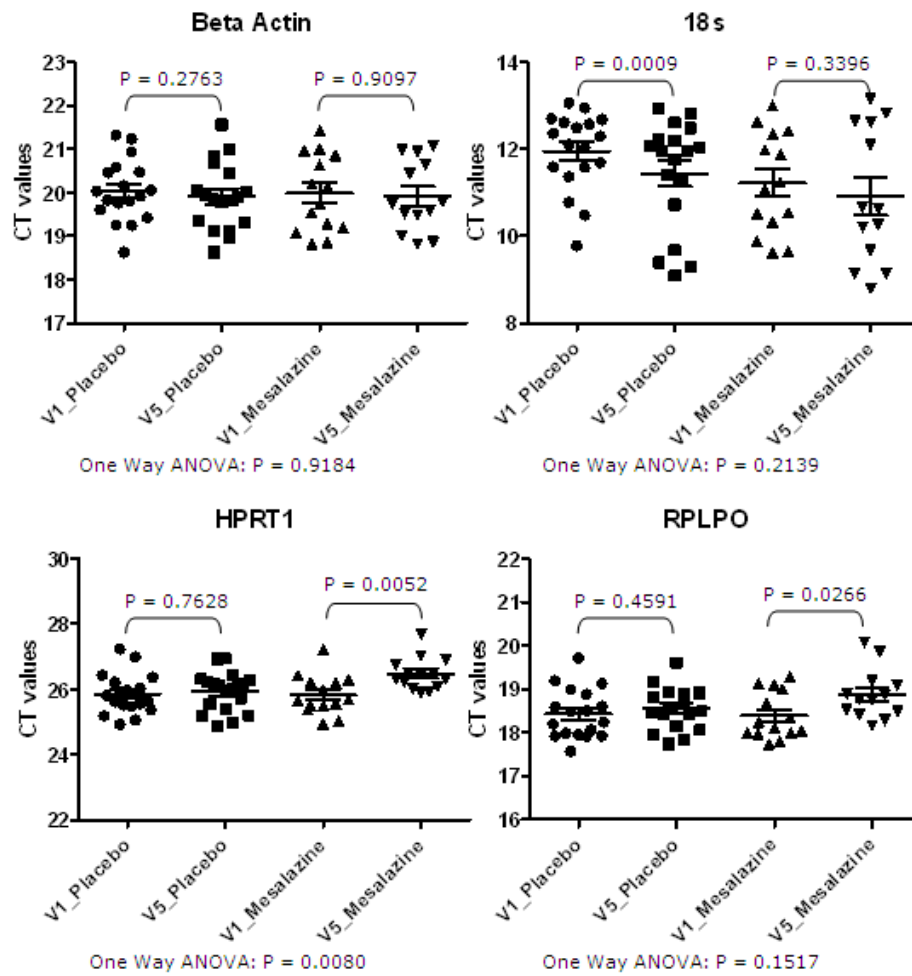
3.4.5 Endpoints and Outcomes

Mechanistic endpoints were achieved using the gene card to calculate the relative quantity. Four housekeeper genes were employed to be used in RQ calculation. These are genes to should remain unchanged by the interventions. However on statistical analysis of CT values for the house keepers, HPRT1 was significantly different between groups using one-way ANOVA (Kruskal-Wallis test) ($p=0.0080$). Paired t-test (Wilcoxon Signed-Rank Test) between baseline and final samples per treatment arm identified significant differences between the placebo (18S, $p = 0.0009$) or the mesalazine groups (RPLPO, $p = 0.0266$) in 2 further housekeeper genes which were excluded. Therefore all gene card RQ values were calculated using Beta-Actin (BA) as the housekeeper (Figure R3.6).

Non-parametric test were used for all gene card statistical calculations due to the small sample size which reduced the confidence in assuming normal distribution of the data. Samples which were

greater than 3 standard deviations away from the mean were excluded. No genes became significant or not from these exclusions.

Figure R3.6 Graph of CT value distribution of Housekeeper genes between study treatments arms. ANOVA using Kruskal-Wallis test



(a) Primary Endpoint

The primary endpoint was the change in expression of galanin receptor 1 (GALR1) expression from baseline (pre) to final (post) visit due to mesalazine. No significant difference was identified (Table R3.8).

Table R3.8 Relative Quantification of GALR1 RNA from sigmoid biopsies per study treatment arm.

Gene	Placebo			Mesalazine		
	Pre N=17	Post N=18		Pre N=14	Post N=13	
	Median (IQR)	Median (IQR)	P value	Median (IQR)	Median (IQR)	P value
GALR1	2.253 (1.61-2.65)	1.916 (0.88-3.29)	0.4210	1.970 (1.19-3.18)	1.374 (0.85-2.57)	0.1465

(b) Secondary Endpoints

(i) RNA analysis

The results have been organized to reflect the pathways of interest or linked genes on which the gene card was originally designed. Significant results have been highlighted in bold.

a. Pain associated receptors and pathways

Several other neurochemical transmitters, receptors and pathways, apart from GALR1, were analysed and shown below in Table R3.9.

Table R3.9 Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Pain associated Receptors and Pathways. Wilcoxon Signed-Rank Test

Gene	Placebo			Mesalazine		
	Pre	Post		Pre	Post	
	Median (IQR)	Median (IQR)	P value	Median (IQR)	Median (IQR)	P value
GALR2	1.157 (0.82-2.76)	1.397 (0.61-2.19)	0.3165	1.375 (0.99-2.62)	1.522 (1.07-2.04)	0.7869
Bradykinin Receptor						
BDKRB2	1.419 (1.21-1.89)	1.394 (0.83-1.90)	0.2066	1.182 (0.83-2.01)	0.7459 (0.58-1.33)	0.0046
Endocannabinoid Signalling						
CNR2*	0.3523 (0.27-0.47)	0.3787 (0.18-1.03)	0.8498	0.5845 (0.18-1.06)	0.3249 (0.22-0.49)	0.1099
MGLL	1.880 (1.67-2.45)	1.863 (1.14-2.65)	0.8961	1.530 (1.13-3.64)	1.038 (0.75-1.94)	0.0171
NAPEPLD	1.805 (1.40-2.33)	1.624 (0.96-2.30)	0.0979	1.519 (0.99-3.22)	0.9221 (0.66-1.31)	0.0007
Serotonin (5HT) Signalling						
HTR4	0.9756 (0.83-1.51)	1.263 (0.79-1.76)	0.5713	1.197 (0.66-1.97)	0.8621 (0.69-1.57)	0.4973
SLC6A4	1.008 (0.49-1.53)	0.4318 (0.19-0.88)	0.0815	0.9134 (0.73-1.53)	0.5449 (0.28-0.87)	0.0681
TPH1	1.532 (0.94-2.59)	1.314 (1.02-1.97)	0.2959	1.419 (0.95-2.79)	0.9606 (0.74-1.71)	0.0803

* N is reduced to 17 for the placebo group.

IQR is interquartile range.

Table R3.9 Continued. Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Pain associated Receptors and Pathways. Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post	P value	Pre	Post	P value
	Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)	
Tachykinin Receptors						
TACR1	0.3457 (0.22-0.64)	0.4668 (0.26-0.63)	0.5713	0.6325 (0.37-1.22)	0.4254 (0.29-0.81)	0.3394
TACR2	1.461 (0.56-2.65)	1.042 (0.76-2.02)	0.8498	1.833 (0.96-2.17)	2.113 (1.17-2.69)	0.6848
Transient Receptor Potential Channels						
TRPA1	2.207 (1.30-3.04)	2.032 (1.26-2.74)	0.5700	2.108 (1.64-2.94)	1.402 (0.82-1.76)	0.0171
TRPV1	1.346 (1.09-1.94)	1.368 (0.94-1.90)	0.5136	1.432 (0.95-2.12)	0.7200 (0.58-1.60)	0.0574
TRPV4	0.8971 (0.57-1.57)	0.8188 (0.47-1.27)	0.3604	0.9204 (0.58-1.53)	0.7840 (0.49-1.14)	0.1677
Nerve Growth Factor (NGF) Signalling						
NGF	2.106 (0.77-3.41)	1.519 (0.80-3.21)	0.8961	1.821 (0.83-3.89)	2.173 (1.27-3.26)	0.6355
NGFR	0.5130 (0.33-0.93)	0.6019 (0.41-0.90)	1.0000	0.6593 (0.40-1.02)	0.4475 (0.38-0.53)	0.1677
NTRK1	0.7232 (0.31-1.21)	0.9197 (0.45-1.23)	0.5713	0.6162 (0.30-1.02)	0.5893 (0.30-0.97)	0.7869

* N is reduced to 17 for the placebo group.

b. Arachidonic acid Pathway (Table R3.10)

Table R3.10 Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Arachidonic acid Pathways. Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post	P value	Pre	Post	P value
	Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)	
Lipoxygenases						
ALOX 12	0.5187 (0.13-0.87)	0.3285 (0.13-0.99)	1.0000	0.4619 (0.06-0.96)	0.1682 (0.04-0.57)	0.0479
ALOX 5	0.7831 (0.62-1.22)	0.9174 (0.53-1.37)	0.9653	1.002 (0.67-2.65)	0.5747 (0.50-0.92)	0.0012
ALOX 15	1.055 (0.54-2.99)	1.475 (0.59-2.13)	0.8276	0.9200 (0.67-2.16)	0.6000 (0.50-1.08)	0.2439
ALOX15B	3.232 (1.55-5.31)	2.426 (1.41-4.74)	0.1169	4.002 (2.19-7.01)	2.757 (2.18-3.91)	0.0803
ALOX5AP	0.9717 (0.73-2.00)	1.102 (0.67-1.72)	1.0000	1.164 (0.84-1.71)	0.8888 (0.64-1.41)	0.1099
Leukotrienes						
LTA4H	1.816 (1.04-3.06)	2.289 (1.72-3.51)	0.6013	2.739 (1.47-3.48)	2.583 (2.00-3.26)	0.6355
LTB4R	0.9183 (0.78-1.50)	0.8554 (0.61-1.39)	0.1507	0.8711 (0.62-1.53)	0.6859 (0.50-1.37)	0.2439
LTC4S	1.297 (0.80-1.78)	1.340 (0.80-1.58)	0.7605	1.230 (0.91-2.17)	1.050 (0.78-1.62)	0.7869
Phosphodiesterases						
PDE4B	0.6052 (0.39-0.98)	0.6496 (0.39-0.74)	0.4859	0.7303 (0.50-1.05)	0.4073 (0.31-0.60)	0.0034
PDE4D	1.278 (1.05-1.94)	1.487 (1.06-1.78)	0.7605	1.402 (1.03-1.89)	1.452 (0.93-1.87)	0.7354

* N is reduced to 17 for the placebo group.

Table R3.10 Continued. Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Arachidonic acid Pathways. Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post	P value	Pre	Post	P value
	Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)	
Prostaglandins						
PTGES2	0.9289 (0.93-1.41)	1.041 (0.61-1.35)	0.5713	1.220 (0.73-2.05)	0.5498 (0.52-1.05)	0.0017
PTGES	0.8089 (0.54-1.32)	0.8915 (0.57-1.34)	0.4080	1.071 (0.89-1.92)	1.027 (0.83-1.24)	0.0803
PTGS1	1.121 (0.86-1.53)	1.063 (0.63-1.64)	0.3165	1.035 (0.86-1.97)	0.8106 (0.48-1.07)	0.0005
PTGS2	0.8578 (0.53-1.25)	0.7827 (0.50-1.04)	0.2763	0.8345 (0.61-1.53)	0.4170 (0.30-0.71)	0.0266
PTGER1	1.210 (0.71-1.74)	1.135 (0.67-2.06)	0.5136	1.988 (0.93-2.35)	0.9720 (0.72-1.35)	0.0215
PTGER3	1.158 (0.51-1.79)	0.9481 (0.53-1.14)	0.0674	1.129 (0.82-2.18)	1.107 (0.54-1.87)	0.7869
Thromboxanes						
TBXA2R	1.212 (0.88-1.92)	1.154 (0.80-1.69)	0.3838	1.842 (0.98-3.14)	0.9917 (0.74-2.00)	0.1677
TBXAS1	1.599 (1.03-2.51)	1.594 (0.86-2.21)	0.5136	1.643 (0.99-2.71)	0.9434 (0.68-1.35)	0.0002
Others						
PLA2G2A	0.9765 (0.42-1.52)	0.9838 (0.40-1.70)	0.9653	0.7582 (0.45-1.39)	0.4604 (0.31-0.98)	0.0574
EPHX2	1.711 (1.12-2.09)	1.708 (1.12-2.67)	0.8617	2.052 (1.12-2.77)	1.170 (0.85-1.53)	0.0574

* N is reduced to 17 for the placebo group.

c. Cytokines, inflammatory mediators and cell migration markers (Table R3.11)

Table R3.11 Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Inflammation and cell migration pathways. Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post	P value	Pre	Post	P value
	Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)	
Interleukin 1 family						
IL1B	0.7416 (0.47-1.40)	1.092 (0.37-1.49)	0.2575	0.9633 (0.37-1.96)	0.6220 (0.37-1.01)	0.0024
IL1RN	1.245 (0.50-2.14)	0.9245 (0.42-1.86)	0.2397	1.125 (0.77-1.59)	0.6921 (0.55-0.98)	0.0024
Tumour Necrosis Factor Family						
TNF*	0.6611 (0.47-1.05)	0.6341 (0.34-1.15)	0.2977	0.9170 (0.61-1.40)	0.4435 (0.36-0.69)	0.0034
TNFSF10A	1.266 (1.00-2.30)	1.650 (0.72-2.28)	0.7939	1.571 (0.96-2.74)	0.8645 (0.72-1.51)	0.0012
TNFSF10	1.166 (0.94-1.39)	1.315 (0.70-1.59)	0.6319	1.119 (0.70-1.61)	0.7356 (0.50-1.09)	0.0024
TNFSF15	1.258 (1.05-2.01)	1.195 (0.61-1.87)	0.4080	1.656 (1.03-2.73)	0.6662 (0.45-1.44)	0.0017
Transforming Growth Factor Beta Family						
TGFB1	1.086 (0.71-1.92)	1.277 (0.75-1.65)	0.8617	1.274 (0.78-2.09)	0.7495 (0.60-1.37)	0.0081
TGFBR1	1.364 (0.97-2.01)	1.308 (0.99-1.77)	0.2959	1.514 (0.91-2.43)	0.8380 (0.64-1.51)	0.0081
TGFBR2	1.237 (0.93-1.67)	1.444 (1.02-1.71)	0.9653	1.452 (0.77-2.06)	0.7351 (0.61-1.31)	0.0046

* N is reduced to 17 for the Placebo Group. ++ N is reduced to 11 in the Mesalazine Group

Table R3.11 Continued. Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Inflammation and cell migration pathways. Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post		Pre	Post	
	Median (IQR)	Median (IQR)	P value	Median (IQR)	Median (IQR)	P value
Interferon gamma						
INFG*	0.2105 (0.11-0.55)	0.1692 (0.07-0.33)	0.8129	0.2109 (0.12-0.37)	0.1638 (0.14-0.30)	0.2734
Interleukins						
IL2	1.223 (0.59-2.07)	1.620 (0.59-2.27)	0.5421	1.418 (0.65-2.18)	0.7911 (0.49-2.01)	0.1272
IL6	0.3339 (0.16-0.84)	0.3988 (0.16-1.01)	0.5136	0.3791 (0.19-0.87)	0.2646 (0.20-0.62)	0.4548
IL8*	0.4706 (0.33-0.77)	0.4880 (0.34-0.66)	0.4548	0.7753 (0.55-1.32)	0.4836 (0.23-1.11)	0.3757
IL10	1.114 (0.50-1.82)	1.124 (0.74-1.62)	0.8961	1.095 (0.72-1.60)	0.6988 (0.52-1.22)	0.4143
IL17A*	0.5148 (0.25-1.08)	0.3299 (0.12-0.65)	0.2763	0.6033 (0.05-0.99)	0.3640 (0.07-0.76)	0.8394
IL23A	0.3503 (0.28-0.46)	0.3600 (0.20-0.56)	0.7605	0.5219 (0.36-0.74)	0.3061 (0.21-0.75)	0.4143

* N is reduced to 17 for the Placebo Group. ++ N is reduced to 11 in the Mesalazine Group

Table R3.11 Continued. Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Inflammation and cell migration pathways. Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post	P value	Pre	Post	P value
	Median (IQR)	Median (IQR)		Median (IQR)	Median (IQR)	
Chemokines and receptors						
CMKLR1	1.531 (0.92-2.15)	1.521 (0.67-2.07)	0.4331	1.732 (0.99-2.10)	0.8651 (0.61-1.32)	0.0046
MCL1	1.749 (1.31-2.82)	2.100 (1.18-2.59)	0.7939	2.146 (1.09-2.93)	0.9454 (0.74-1.91)	0.0081
CCL11	3.490 (0.65-7.00)	2.614 (1.36-6.27)	0.8276	1.555 (1.28-5.16)	1.999 (1.45-3.55)	0.6848
CCL2	1.403 (0.73-2.35)	1.581 (1.14-1.99)	0.5136	1.415 (0.94-1.98)	1.026 (0.80-1.72)	0.9460
KITLG	1.289 (0.90-2.36)	1.541 (1.18-1.73)	0.9653	1.422 (0.95-2.15)	0.9693 (0.64-1.52)	0.1465
Cell migration Receptors and Ligands						
MADCAM1	0.9788 (0.69-1.45)	1.198 (0.57-2.09)	0.4859	1.103 (0.83-2.12)	0.8550 (0.59-1.64)	0.0215
VCAM1*	0.5868 (0.50-1.45)	0.8385 (0.52-1.09)	0.7764	0.9736 (0.55-1.35)	0.4717 (0.35-0.76)	0.0012
SELE*,++	0.8699 (0.45-1.41)	0.5690 (0.25-1.96)	0.8871	0.6249 (0.25-1.16)	0.4383 (0.17-1.30)	0.8984
ICAM1	0.4206 (0.34-0.64)	0.4536 (0.27-0.83)	0.5136	0.5373 (0.29-0.81)	0.5468 (0.30-0.65)	0.5879

* N is reduced to 17 for the Placebo Group. ++ N is reduced to 11 in the Mesalazine Group

d. Pattern recognition receptors Table R3.12)

Table R3.12 Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Toll-Like Receptor (TLR) Pathways. Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post		Pre	Post	
	Median (IQR)	Median (IQR)	P value	Median (IQR)	Median (IQR)	P value
TLR2	1.202 (0.62-1.66)	1.051 (0.63-1.94)	1.0000	1.170 (0.74-1.98)	0.7130 (0.60-1.14)	0.0327
TLR4	1.377 (0.94-2.49)	1.465 (1.05-1.94)	0.3604	1.537 (1.01-1.96)	0.8932 (0.70-1.52)	0.0479
TLR5	1.255 (0.78-2.11)	1.278 (0.80-1.70)	0.8961	1.413 (0.94-2.25)	0.9781 (0.69-1.56)	0.0574
TLR7	1.120 (0.70-1.73)	1.096 (0.73-1.31)	0.3604	1.306 (.99-1.78)	0.8202 (0.66-1.19)	0.0266
TLR8	1.064 (0.61-1.67)	0.9126 (0.48-1.30)	0.2227	1.803 (1.01-2.18)	0.7961 (0.46-1.76)	0.0398
TLR9*	0.4506 (0.34-0.73)	0.5264 (0.30-0.68)	0.4488	0.7991 (0.39-1.17)	0.4976 (0.32-0.64)	0.0215
MYD88	1.765 (1.51-2.66)	1.678 (1.22-2.44)	0.6013	1.780 (1.15-2.99)	1.134 (0.90-1.76)	0.0105
TOLLIP	1.754 (1.48-2.44)	2.100 (1.26-2.41)	0.9306	2.073 (1.08-3.15)	1.278 (0.93-1.88)	0.0803
NOD2	0.8938 (0.76-1.57)	1.021 (0.60-1.55)	0.6632	1.359 (0.79-1.96)	0.5925 (0.40-0.94)	0.0002

* N is reduced to 17 for the placebo group.

e. Tight junctions, Cytoskeleton and Extracellular matrix (Table R.2.13)

Table R3.13 Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm – Tight junctions, cytoskeleton and extracellular matrix pathways.

Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post		Pre	Post	
	Median (IQR)	Median (IQR)	P value	Median (IQR)	Median (IQR)	P value
CLDN2*	1.232 (1.04-2.26)	1.466 (0.85-2.09)	0.2366	1.589 (0.93-2.94)	0.7469 (0.55-1.52)	0.0266
MUC1	1.070 (0.90-1.75)	1.064 (0.60-1.43)	0.2763	1.043 (0.72-2.17)	0.7260 (0.35-1.23)	0.0105
MUC3A:3B	0.9571 (0.85-1.58)	1.182 (0.50-1.55)	0.6013	0.8763 (0.64-1.86)	0.6878 (0.20-1.29)	0.0327
Tight Junction Proteins						
TJP1	1.803 (1.26-2.62)	1.622 (1.04-2.15)	0.0210	1.427 (0.94-2.54)	0.9285 (0.61-1.64)	0.0017
TJP2	1.753 (1.62-2.49)	2.270 (1.28-2.69)	0.5421	1.958 (1.07-3.47)	1.310 (0.81-1.74)	0.0681
Matrix Metalloproteinases						
MMP2	1.303 (0.82-1.57)	1.367 (0.84-1.85)	0.7605	1.352 (0.71-1.90)	0.7690 (0.49-1.33)	0.8961
MMP9	0.3518 (0.29-0.52)	0.3347 (0.16-1.18)	0.9653	0.4848 (0.27-1.04)	0.5800 (0.33-0.73)	0.4973

* N is reduced to 17 for the placebo group.

f. Other Pain Related Genes (Table R3.14)

Table R3.14 Gene Card analysis of sigmoid colonic samples at baseline and final visit per treatment arm.. Wilcoxon Signed-Rank Test.

Gene	Placebo			Mesalazine		
	Pre	Post		Pre	Post	
	Median (IQR)	Median (IQR)	P value	Median (IQR)	Median (IQR)	P value
F2RL1(PAR2 receptor)	1.841 (1.40-2.34)	1.571 (1.20-2.08)	0.4080	1.944 (1.49-3.37)	1.056 (0.77-1.25)	0.0007
FPR2*	0.3096 (0.21-0.88)	0.4003 (0.21-0.54)	0.6701	0.6899 (0.38-0.86)	0.2195 (0.13-0.50)	0.0081
PPARG	2.130 (1.66-3.25)	1.946 (1.23-2.55)	0.0553	1.692 (1.17-3.24)	1.068 (0.83-1.86)	0.0061
SOD1	1.234 (0.83-1.68)	1.258 (0.82-1.80)	0.7939	1.238 (0.72-1.88)	0.8718 (0.70-1.38)	0.0171
NOS2*	1.068 (0.71-1.43)	0.8621 (0.58-1.10)	0.1183	0.9210 (0.48-1.69)	0.6364 (0.42-0.83)	0.0479
CYP2J2	1.650 (1.07-2.14)	1.436 (1.05-1.95)	0.8276	1.665 (1.00-2.38)	1.152 (0.74-1.63)	0.0942

* N is reduced to 17 for the placebo group.

(ii) **Histology**

Coefficients of variation were calculated per stain used prior to further histological assessment (Table R3.15).

Table R3.15 Variation of Coefficients and reproducibility per immunohistochemistry antibody.

Stain	Number of samples assessed	Number of Areas per sample	Mean Coefficient of variance	Reproducibility (%)
5HT	5	10	0.0223	97.8
CD3 Epith	3	10	0.027	97.3
CD3 LP	3	10	0.053	94.7
CD68	4	10	0.062	93.8
KI67 SUPF	3	10	0.078	92.2
KI67 DEEP	3	10	0.057	94.3

Changes within the epithelium (Epith.), lamina and superficial (S/crypt) and deep (D/crypt) crypt mucosa are shown in Table R3.16. One sample from the baseline placebo group was lost and not processed. Two samples from the Mesalazine baseline group contained minimal tissue present and could not be assessed except for CD68 and CD3. Other sample losses were due to artefacts such as folding of the sample.

Table R3.16 Histological results of sigmoid biopsies from each arm of the study at baseline (Pre) and Final visit (Post). Wilcoxon Signed-Rank Test.

Stain	Placebo (A)			Mesalazine (B)		
	Pre	Post		Pre	Post	
	Median (cells/mm ²) (IQR)	Median (cells/mm ²) (IQR)	P value	Median (cells/mm ²) (IQR)	Median (cells/mm ²) (IQR)	P value
CD3 Epith.	N=17 511.4 (363.0-610.9)	N=17 374.9 (224.2-576.6)	n.s.	N=14 351.8 (247.3-540.0)	N=13 397.9 (288.0-609.0)	n.s.
CD3 Lamina.	1275 (893.3-1465)	1030 (828.5-1444)	n.s.	1037 (843.4-1406)	1051 (753.6-1621)	n.s.
CD68	N=17 1665 (1484-1806)	N=17 1631 (1316-1854)	n.s.	N=14 1703 (1384-1942)	N=13 1744 (1463-2118)	n.s.
KI67 S/crypt	N=17 79.1 (56.2-246.1)	N=17 98.0 (0.0-202.5)	n.s.	N=12 60.3 (0-199.4)	N=13 65.3 (0-148.2)	n.s.
KI67 D/crypt	2297 (1757-3156)	1913 (1349-2910)	n.s.	1817 (1585-2427)	2091 (1409-2970)	n.s.
5HT S/crypt	N=17 104.2 (64.7-165.3)	N=16 92.2 (77.7-112.3)	n.s.	N=12 103.7 (61.4-139.2)	N=13 86.96 (61.4-139.2)	n.s.
5HT D/crypt	277.8 (220.6-367.2)	342.7 (212.0-397.4)	n.s.	346.8 (298.3-484.1)	392.2 (264.8-555.3)	n.s.

(iii) Faecal Calprotectin

One of the mesalazine final visit samples was inadequate and not processed. Results are shown in table R3.17.

(iv) Super sensitive CRP

1 sample from the mesalazine group was excluded from analysis as the value was greater than 20 at both baseline and follow up visits. It is not certain why the CRP was consistently raised, but may be due to upper respiratory tract infections (Baseline) and/or urinary problems (Visit 4) that the participant disclosed during the study. Results are shown in table R3.18

Table R3.17 Results of Faecal Calprotectin ELIZA analysis of samples from each arm of the study at baseline (Pre) and Final visit (Post). Wilcoxon Signed-Rank Test.

	Placebo (A)			Mesalazine (B)		
	Pre N=18	Post N=18		Pre N=14	Post N=13	
	Median (mg/kg) (IQR)	Median (mg/kg) (IQR)	P value	Median (mg/kg) (IQR)	Median (mg/kg) (IQR)	P value
Calprotectin	15.60 (15.60- 40.83)	15.60 (15.60- 21.80)	0.2324	30.67 (15.60- 62.80)	31.98 (15.60- 62.38)	0.3750

Table R3.18 Results of Super sensitive C reactive protein (SS-CRP) analysis of samples from each arm of the study at baseline (Pre) and Final visit (Post). Wilcoxon Signed-Rank Test.

	Placebo (A)			Mesalazine (B)		
	Pre N=18	Post N=18		Pre N=13	Post N=13	
	Median (mg/l) (IQR)	Median (mg/l) (IQR)	P value	Median (mg/l) (IQR)	Median (mg/l) (IQR)	P value
SS- CRP	1.13 (0.56-2.87)	1.78 (0.72-4.01)	0.7939	1.780 (0.50-3.69)	1.81 (0.89-5.40)	0.2036

(v) **Liquid Chromatography and Mass Spectroscopy (LCMS)**

The results for LCMS are shown in table R3.19. Due to small sample size, 2 rectal and 1 sigmoid samples were combined to maximise detection of different arachidonic acid pathway components. Only arachidonic acid (AA), prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), 12-hydroxyeicosatetraenoic acid (12-HETE), linoleic acid (LA), thromboxane-B₂ (TXB₂), arachidonoyl ethanolamide (AEA), 2-arachidonoyl glycerol (2-AG), N-palmitoyl ethanolamide (PEA), N-oleoyl ethanolamide (OEA) and leukotriene-E₄ (LTE₄) were detectable. As detection was difficult, the number of samples (N) that were detected and used in analysis is given for each result in table R3.19.

Table R3.19 Results of LCMS analysis of samples from each arm of the study at baseline (Pre) and Final visit (Post). Wilcoxon Signed-Rank Test.

	Placebo (A)			Mesalazine (B)		
	Pre	Post		Pre	Post	
	Median (pmol/ml) (IQR)	Median (pmol/ml) (IQR)	P value	Median (pmol/ml) (IQR)	Median (pmol/ml) (IQR)	P value
AA	N=12 9.50 (7.6-20.8)	N=12 15.60 (10.2-23.6)	0.7695	N=11 10.80 (9.3-26.8)	N=11 10.20 (9.5-36.4)	0.9658
PGE2	N=13 3.90 (2.4 – 12.4)	N=14 6.95 (3.5 – 9.3)	1.000	N=11 6.70 (3.4 – 9.0)	N=10 4.60 (2.5 - 8.8)	0.6250
PGD2	N=13 3.77 (2.4-12.2)	N=14 7.02 (3.9-9.2)	0.9097	N=11 7.01 (3.4-9.0)	N=11 4.37 (2.4-8.7)	0.4648
2AG	N=14 183.4 (120.8–419.0)	N=15 205.1 (165.8-331.5)	0.7148	N=11 270.9 (142.8-327.9)	N=10 204.4 (102.0-325.3)	0.6250

Table R3.19 continued. Results of LCMS analysis of samples from each arm of the study at baseline (Pre) and Final visit (Post). Wilcoxon Signed-Rank Test.

	Placebo (A)			Mesalazine (B)		
	Pre	Post	P value	Pre	Post	P value
	Median (pmol/ml) (IQR)	Median (pmol/ml) (IQR)		Median (pmol/ml) (IQR)	Median (pmol/ml) (IQR)	
TXB2	N=13 2.40 (1.20-3.8)	N=14 3.40 (2.1-4.7)	0.2661	N=11 2.7 (2.0-3.9)	N=10 2.8 (1.3-5.0)	0.7695
LTE4	N=13 78.00 (49.5-177.0)	N=14 148.5 (100.5-430.5)	0.1294	N=11 73.00 (66.0-156.0)	N=11 109.0 (55.0-207.0)	0.4648
AEA	N=14 28.50 (23.7 - 50.1)	N=14 40.55 (23.2 - 60.7)	0.2634	N=11 39.20 (24.3 - 53.6)	N=9 29.00 (25.2 - 46.1)	0.9102
OEA	N=14 9.85 (3.1 - 33.0)	N=14 13.40 (7.8 - 38.2)	0.1099	N=11 6.40 (3.8 - 19.8)	N=9 10.90 (3.4 - 34.8)	0.1289
PEA	N=15 5.00 (2.8 - 15.7)	N=14 5.55 (2.4 - 17.3)	0.6257	N=10 3.30 (1.3 - 7.0)	N=10 4.50 (1.7 - 11.6)	0.4961
LA	N=13 77.60 (61.0-318.5)	N=13 260.2 (123.1-347.5)	0.1016	N=11 126.7 (96.4-182.5)	N=11 135.1 (103.8-332.9)	0.5771
5-HETE	N=14 24.45 (19.0-56.7)	N=14 33.80 (8.5-57.8)	0.5879	N=11 32.20 (25.6-53.9)	N=10 24.65 (5.8-86.9)	0.7695

(vi) **Participant Symptoms**

Participant symptoms were calculated from diary sheet prospectively collected during the study period. Median values were calculated from scores given between 0 and 10. Two participants in Mesalazine Group did not complete visit 5 diary and values for visit 4 used instead (IXZ024 and RWP033). Some participants did not report bloating as a normal symptom. Therefore results on those who complained of bloating initially as well as bloating for all subjects have been reported. The difference between the baseline and final (Visit 5) scores are presented in table R3.20. The median pain duration per day (shown as VAS on 0-10 scale) per study group is shown graphical in figure R3.7.

Figure R3.7 Median pain duration per day per group occurring during the trial.

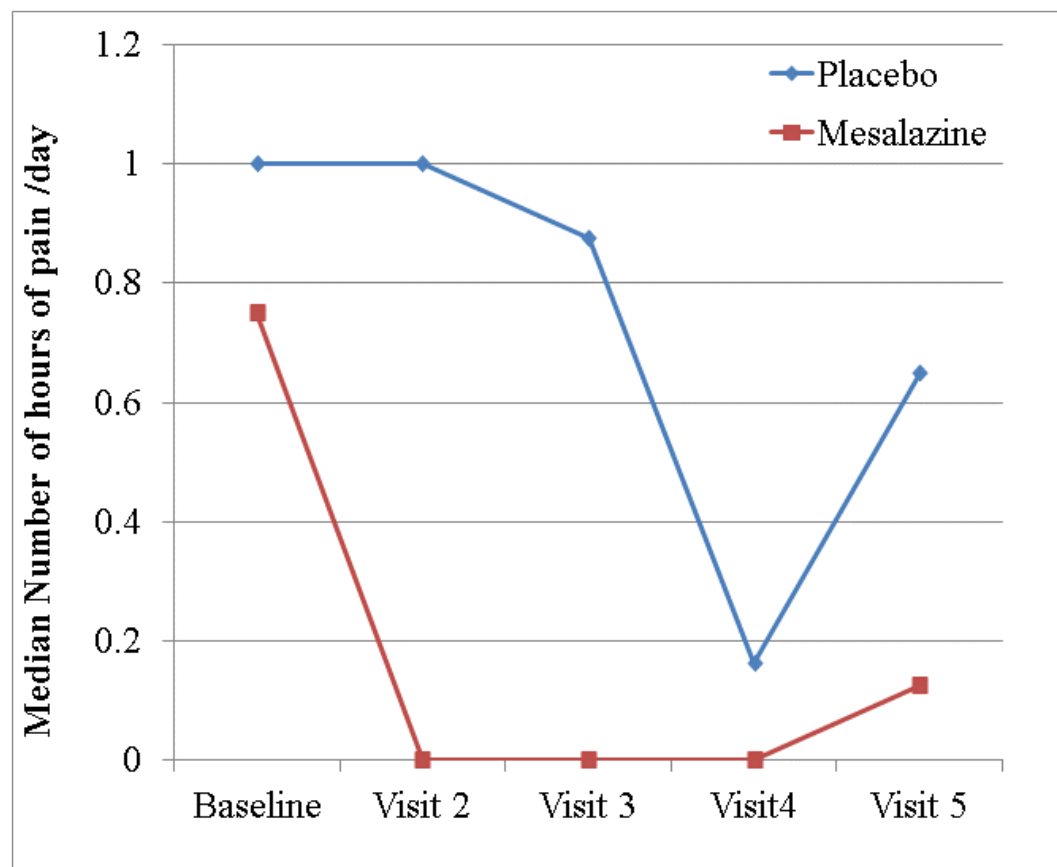


Table R3.20 Changes in participant gastrointestinal symptoms from diary sheets at baseline (Pre) and final visit (Post). Wilcoxon Signed-Rank Test.

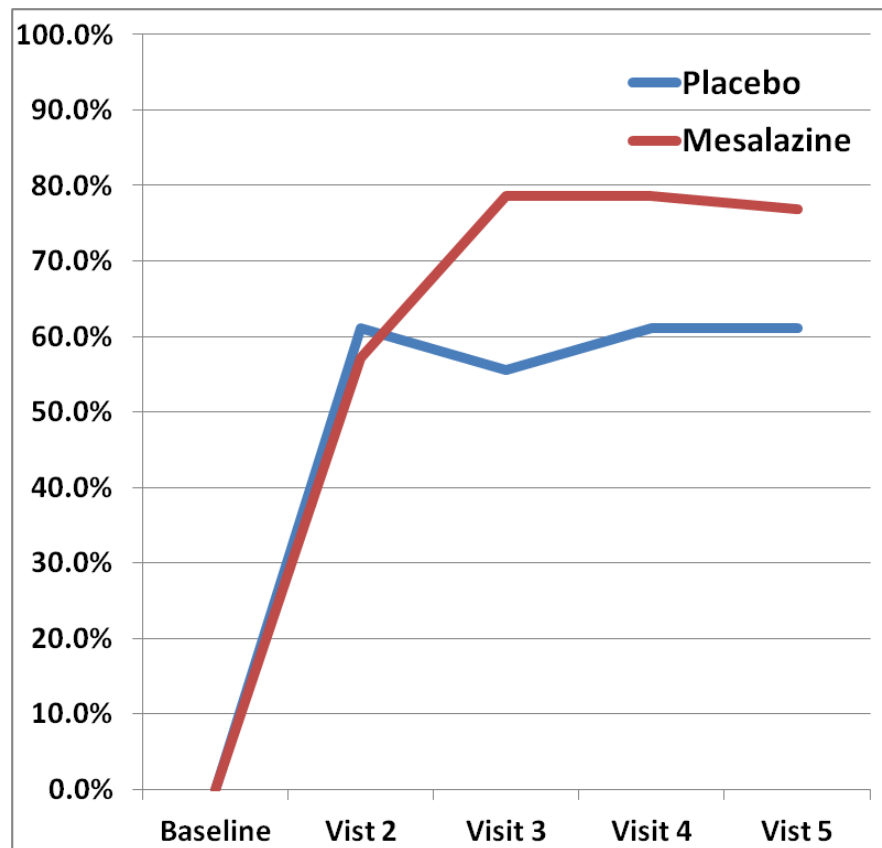
Gene	Placebo			Mesalazine		
	Pre N=18	Post N=18	Wilcoxon P value	Pre N=14	Post N=14	Wilcoxon P value
	Median Score (Range)	Median Score (Range)		Median Score (Range)	Median Score (Range)	
Pain Intensity	4.0 (0-6)	2.5 (0-6)	0.5979	2.0 (0-6)	0 (0-6)	0.1366
Pain Duration	1.0 (0-5)	0.65 (0-4)	0.1919	0.75 (0-20)	0.125 (0-5.5)	0.0413
Stool Frequency	2.0 (0-3.5)	2.0 (1-4)	0.7393	2.0 (1-8)	2.0 (1-8)	0.5236
Stool Consistency	4.0 (3-6)	4.0 (1-6)	0.4729	3.975 (2-6.5)	3.750 (2-5)	0.1452
All Bloating	4.0 (0-6)	2.5 (0-10)	0.6617	1.5 (0-8)	1.5 (0-8)	0.5065
Reported Bloating	N=11 5 (2-6)	N=12 4.5 (0-10)	0.4154	N=9 5 (0.5-8)	N=9 3 (0-8)	0.1604
General Wellbeing	7.0 (5-10)	7.0 (4-10)	1.00	6.75 (1.5-10)	8.0 (3-10)	0.5474

Pain etc. 0 = none 10 = severe; General well being 0 = unwell, 10 = excellent

(vii) **Participant Beliefs**

At each visit participants were asked if they had relief or not from their diverticular symptoms during the last 2 or 4 weeks. Figure 3.8 demonstrates the percentage of participants who indicated they did have relief per group.

Figure 3.8 Percentage of participants who had relief from symptoms per visit per study treatment arm.



NB: Placebo N= 18, Mesalazine N=14 except visit 5, where data from the participant who ceased medication early was not included (N=13).

At the end of the study, without breaking the blinding of participants or researchers, the participant was asked if they thought they were taking the Mesalazine or the placebo during the study. These results are presented graphically in figure R3.9. Fisher exact test (Taking Mesalazine vs. Taking placebo) demonstrated a p value 0.0914.

Participants were also asked whether they would continue to take the medication if offered. This data is represented in figure R3.10 and was not statistically significant.

Figure R3.9 Graphical representation of participants' beliefs about the study medication they were taking during the trial.

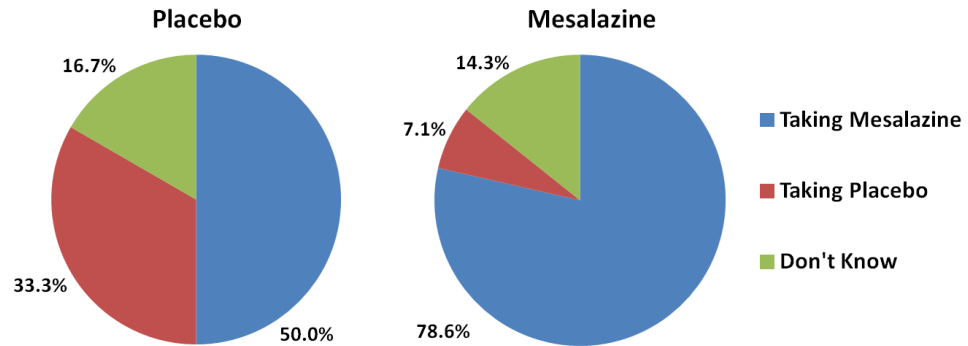
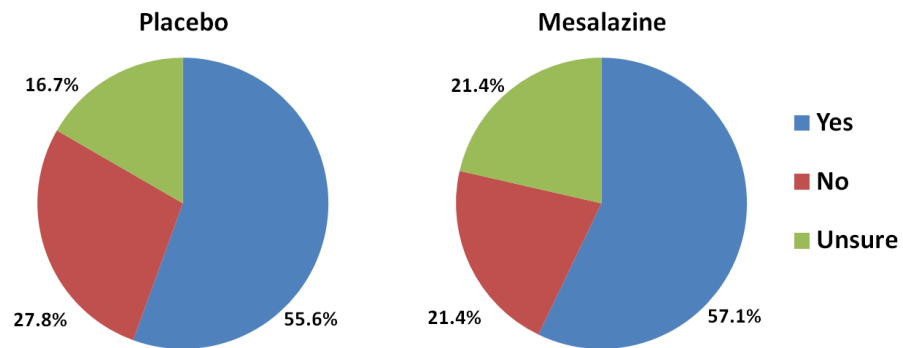


Figure R3.10 Graphical representation of the percentage of participants wanting to continue the study medication they were taking beyond the duration of the trial.



3.4.6 Complications and side effects

Out of all participants who entered the study 14 subjects (N=19, 73.7%) in the placebo and 16 subjects (N=20, 80%) in the mesalazine group reported new symptoms during the study. The majority of these were unrelated to the study. In the mesalazine group 5 subjects were thought to have side effects from the mesalazine medication (N=20, 25%). Four participants developed increasing abdominal pain which necessitated withdrawal from the study and were discussed above. A further participant developed diarrhoea with mesalazine. The participant had relief of other symptoms from the study and was keen to stay within the trial. Therefore the dose of the medication was reduced from 3g to 1g during the last 4 weeks of the study with good effect.

Important to note is 1 participant in the mesalazine arm of the study who reported abdominal pain and raised temperatures, consistent with an episode of acute diverticulitis. The participant consulted their own GP and was prescribed a 5 day course of metronidazole and cephalexin, which resolved the pain. The participant did not contact the research team during these events, and there was no imaging or biological samples to confirm the diagnosis. However the participant did continue her medication throughout this period. Other symptoms reported by participants in the study are shown in table R3.21 and are similar between groups.

Table R3.21 Reported new symptoms during the study period by group.

Symptoms attributed to mesalazine medication are shown in italics

Symptoms	Placebo Group	Mesalazine Group
Gastrointestinal	Diarrhoea and Vomiting illness*2 New Abdominal Pain *3 e.g. upper or right sided Diarrhoea Constipation *3 Nausea Perianal pain Reflux	Diarrhoea and Vomiting illness New Abdominal Pain *5 Diarrhoea
Respiratory tract	Infection e.g. 'Chest infection', Flu like Illness	Infection e.g. Sinusitis, Flu like Illness
Neurological and Musculoskeletal	Headaches *2 Leg Cramps Dizziness Myalgia	Headaches *2 Leg Cramps Dizziness 'Tingling' in legs
Genitourinary		Haematuria UTI
Other	Bruising Breast discomfort Dental abscess	Palpitations Hot Sweats

3.5 Discussion

3.5.1 Synopsis of the key findings

This pilot study was designed to look at the mechanistic effects of mesalazine in SDD. The study was not powered to identify efficacy of mesalazine in improving patient symptoms or for performing an intention to treat analysis. Although the sample size is small, this study has demonstrated some marked gene changes within the mesalazine but not the placebo groups. Interestingly these are all located within gene families or pathways which support the anti-inflammatory effects of mesalazine. These mechanisms of mesalazine have become of increasing interest recently, especially its anti-cancer activities⁵⁷⁷ and may contribute to its effects in visceral pain reported in other open labeled studies^{562, 566-569, 578}.

3.5.2 Comparison with relevant findings from other published

(A) Gene card results

(a) Pain associated receptors and pathways

There have not been any previous studies which have identified neurochemical and/or receptor changes that occur with mesalazine. Galanin and galanin receptor 1 (GALR1) expression have previously been shown to be increase in animal models of visceral pain and in patients with symptomatic diverticular disease^{80, 300}. It was from this work that we chose changes in Galanin receptor 1 as our primary end point for the study. However these studies were in patients with diverticulitis, and these findings have not reached significance in further studies of SDD by our group⁴⁹¹. This may be why no significant difference in GALR1 and GALR2 was found in the mesalazine group between the first and final visit (Table R3.8 and 3.9). GALR1 is expressed on smooth muscle and colonic epithelial cells as well as nerves, and is thought to be important in fluid secretion and motility⁵⁷⁹. It can also be up-regulated by NFkB activation and down-regulated by NFkB inhibitors, such as dexamethasone⁵⁷⁹. The GALRs detected in our study will be from epithelial cell expression rather than nerves, where the associated mRNA transcripts are found within the dorsal horn cell bodies and not the mucosa. As galanin and GALR1 anti-human

antibodies are unreliable, it is difficult to assess any nerve associated galanin changes in our study⁴⁹¹.

(i) Tachykinins

Tachykinin receptors are found in the enteric nervous system, smooth muscle, epithelium and immune cells⁵⁸⁰. Our previous studies showed Substance P⁸⁰ and its receptors, TACR1 and TACR2¹⁰⁴ are up-regulated in SDD and animal models of colitis^{80, 300, 491}. They are also thought to have a role in IBS, with TACR1 antagonists improving mood and pain ratings and emotional arousal circuits on fMRI imaging⁵⁸¹. However no studies have investigated the effect of mesalazine. In our study Mesalazine did not appear to alter their expression. This may be because mRNA from the enteric and afferent nervous system would not have been included in our biopsies and changes in neuronal expression would not have been identified. Unfortunately TACR1 antibodies are unreliable and it was not possible to examine changes in neuronal expression in our study⁴⁹¹.

(ii) Bradykinin Receptors

Bradykinin is an inflammatory mediator in the gastrointestinal system and Bradykinin receptor B2 (BDKRB2) has been identified on submucosal ganglia in the distal rat colon⁵⁸². These receptors are up-regulated in animal models of colitis⁵⁸³ but their expression in DD and IBS has not been assessed. In our study, BDKRB2 was significantly decreased compared to other nociceptive receptors (Table R3.8 and 3.9). No other studies have assessed the effect of 5-ASA on the Kallikrein-kinin system, but a decrease in BDKRB2 would support the anti-inflammatory effects associated with Mesalazine. BDKRB2 have also been shown to be up-regulated by pseudomonas aeruginosa inflammation via an NFkB pathway⁵⁸⁴ and act via NFkB to increase IL6 expression in synovial fibroblasts⁵⁸⁵. Also in cultured human coronary artery cells, BDKRB2 expression was down-regulated with decreased cell proliferation⁵⁸⁶. As Mesalazine has been shown to decrease cellular proliferation^{535, 544, 587} and inhibit NFkB activity⁵³⁰, these mechanisms could be involved in the decrease in BDKRB2 we observed.

(iii) Transient Receptor Potential receptors

Although TRPA1 is commonly associated with gastrointestinal motility and pain, it also influences anion secretion^{588, 589}. In our study TRPA1 was significantly decreased from visit 1 to 5 while the change in TRPV1 was of borderline significance (p0.0574, Table R3.9) in the mesalazine group. TRPV1 and TRPA1 on nerves fibres have been implicated in pain in human gastrointestinal conditions⁵⁹⁰⁻⁵⁹², and are up-regulated in experimental animal models of inflammation^{588, 589, 593, 594}. However it is unlikely that the change in mRNA in our study is due to changes in expression on gastrointestinal nerves as the mRNA that produces these receptors comes from the nerve cell body located in the dorsal root ganglion. As well as unmyelinated enteric nerves, TRPV1 and A1 are expressed as chemo receptors in epithelial and enterochromaffin cells and smooth muscle layers⁵⁹⁴. Therefore the mRNA changes must be due to alteration of TRPV1 and TRPA1 in immune and epithelial cells. This and any altered expression on nerve terminals would need to be confirmed with immune-fluorescence methods. Unfortunately reliable commercially available anti-human antibodies for TRPV1 and TRPA1 are limited.

The effects of mesalazine on TRPV1 and TRPA1 have not previously been reported but their decreased mRNA does support the known anti-inflammatory and potential anti-nociceptive activity of mesalazine. The reduction in TRPV1 and TRPA1 mRNA expression may be either as a direct action of mesalazine or through by downstream effects from other mesalazine affected genes. There are many genes which could be involved as TRPV1 and TRPA1 can be sensitised by bradykinin and tryptases via PAR2^{595, 596} and have been linked to endocannabinoids and serotonin pathway⁵⁹⁷.

(iv) Endocannabinoids

The endocannabinoid system is thought to be important in gastrointestinal inflammation and pain processing, by interaction with TRP, PPARalpha and other receptors⁵⁹⁸. Endocannabinoid receptors are found in epithelium, submucosa and muscle as well as the enteric nervous system^{598, 599}. There are many enzymes involved in the manufacture and elimination of endocannabinoids such as,

MGLL (monoglyceride lipase) which acts to hydrolyse 2-AG and NAPE-PLD which is important in the creation of anandamide⁵⁹⁸.

Endocannabinoid receptor agonists have previously been shown to inhibit contractions in colonic muscle strips from controls to a greater extent than strips from DD patients⁸⁸. A potential endogenous endocannabinoid, anandamide, has also been found at increased levels in DD compared to controls⁸⁸. Increased expression of CBR2 in SDD compared to ADD has also been identified⁴⁹¹. Endocannabinoids may also have a role in other gastrointestinal conditions as different alleles of FAAH enzyme (C385A), which is the rate limiting step in anandamide metabolism, is associated with D-IBS⁶⁰⁰. Treatment of diarrhoea in D-IBS by endocannabinoid receptor agonist dronabinol, have also shown to be influenced by different SNPs of endocannabinoid receptor 1 (CNR1)^{601, 602}. The endocannabinoid system has also been implicated in UC, with expression of key enzymes and receptors in the mucosa, submucosa, muscle and enteric plexus changing with severity of inflammation⁵⁹⁹.

In our study expression of CBR2 was not altered in the mesalazine group, but there was a significant decrease in MGLL and NAPE-PLD. This is in contrast to a study of treated UC patients which found an association between 5-ASA and increased expression of MGLL and CBR2⁵⁹⁹. However in this study most subjects included were treated with steroids or other immunomodulators as well as 5-ASA. This along with the different disease process underlying UC makes interpretation of their results and comparison with our study difficult. In other studies in UC, increased anandamide levels, but not 2-AG, have been found in untreated UC patients' mucosa and animal models of colitis treated with 5-ASA using LC-MS⁶⁰³. However gene changes were not assessed. The finding in 2-AG in this study supports our LC-MS analysis, where no difference in 2-AG was found between groups. However we did not see a change in anandamide (AEA). This may be again due to the different disease process and use of cell lines. Also, although we have demonstrated a reduction in MGLL and NAPE-PLD, which manufacture 2-AG and anandamide, we have not investigated the enzymes involved in their breakdown. It is hypothesised that increase

anandamide levels may be due to 5-ASA inhibition of COX-2, reduced oxidation and further processing^{603, 604}. This may explain the counterintuitive decrease in MGLL and NAPE-PLD in our study with the maintenance and/or increase in some endocannabinoids in other studies⁶⁰³. Thus, further assessment of the endocannabinoid system would be required in larger RCTs of mesalazine to confirm our findings and hypothesis.

(v) Serotonin pathway

Using immunocytochemistry, increase in 5HT producing cells in resected DD specimens has previously been reported¹³¹. In contrast decreased 5HT transporter SERT, but not tryptophan hydroxylase 1 (TPH1) mRNA, the rate limiting enzyme in 5HT manufacture, or numbers of 5HT positive cells have also been reported in mucosa of patients with a history of diverticulitis within the last 6 months⁶⁰⁵. In a recently published study from our group, no significant difference was found in the number of 5HT positive cells in SDD or ADD groups⁴⁹¹. However in IBS, there is evidence of increased mucosal serotonin availability in IBS with diarrhoea (IBS-D) and 5HT3 receptor antagonists have been shown to relieve symptoms⁶⁰⁶⁻⁶⁰⁸. No studies have identified alteration in components of the serotonin pathway with mesalazine. In our study a trend to decreased SERT (SLC6A4) and TPH1 was identified and may underlie some of the motility effects associated with mesalazine. However larger mechanistic RCT are needed to confirm these findings.

(b) Arachidonic acid pathway

Increased expression in prostaglandin E synthase has been recently identified in SDD compared to ADD⁴⁹¹. As Mesalazine and other 5-ASA are known to alter the Arachidonic acid pathway, its effect on these pathways may be important in treatment of pain in this group. In our study significant decreased mRNA expression was found for lipoxygenases (ALOX12 and ALOX5), prostaglandins (PTGES2 [prostaglandin E synthase 2], PTGS1 [COX-1], PTGS2 [COX-2] and PTGER1 [Prostaglandin E receptor 1]) and for thromboxane synthase 1 (TBXAS1). A trend for reduced expression was also found for prostaglandin E synthase (PTGES), 15-lipoxygenase type

II (ALOX15B), phospholipase A2 and epoxide 2 hydrolase (EPHX2). However no difference was found for enzymes involved in the leukotriene pathway (LTA4H, LTB4R, LTC4S).

The changes in prostaglandin pathways fit well with the literature. 5-ASA compounds have been found to inhibit COX-2 expression and prostaglandin E2 production in a colorectal cancer (CRC) cell line (HT-115), even when the cells are stimulated with TNFalpha and IL-1B⁵⁴⁴. Mesalazine has also been shown to inhibit COX-2 expression and production of PGE2 in TNBS treated colitic mice⁶⁰⁹. 5-ASA has also been shown to inhibit thromboxane A2 activity and 5-lipoxygenase⁶¹⁰⁻⁶¹², which agrees with our study findings.

However, when 5-ASA was added to a suspension of isolated colonic mucosal cells from healthy volunteers there was a reduction in LTB4 but not PGE2 synthesis⁶¹³. This is in contrast to our work. However it is supported by another study in an animal model of colitis, where 5-ASA compounds have been shown to reduce PGE2 and TBX2 production and a non significant decrease in LTB4 release⁶¹⁴.

Unfortunately the changes in gene expression were not supported by our liquid chromatography and mass spectroscopy results of AA pathway products which is discussed below.

(c) Cytokines and inflammatory mediators

Several cytokines have been linked to DD and other painful gastrointestinal conditions. In a recent study by Humes et al, who used genetic techniques and biopsies from unprepared colon, an increase in TNFalpha was found in symptomatic compared to asymptomatic DD individuals⁴⁹¹. This finding is supported by a small study which demonstrated increased TNFalpha mRNA in Symptomatic DD compared to healthy volunteers with and without diverticulosis⁶¹⁵. Polymorphisms in another member of the TNF superfamily, TNFSF15 have also been associated with both IBD and IBS (OR 1.37, especially constipation predominant OR 1.79) and with the higher risk allele resulting in increased TNFSF15 mRNA in rectal mucosa¹⁵³.

In our study, a reduction in mRNA was seen in TNF (TNFalpha, TNFSF10, TNFSF10A and TNFSF15), TGFbeta (TGFB1, TGFBR1 and TGFBR2) and IL-1beta (IL1B and IL1RN) gene families following 3 months of mesalazine. Our findings agree with the current literature of the effects of mesalazine with down regulation of IL-1B, TNFalpha and NFkB pathways reported by several studies in experimental animals^{529, 552, 553, 616, 617} and in vitro cells^{577, 618-620}. In one contrary study, IL-1B increased with 5-ASA in rheumatoid synovial fibroblasts in vitro⁶¹⁹ however other in vitro studies using colorectal cell lines HCT116 and colonic fibroblasts have shown that 5-ASA inhibits TGF-beta 1 downstream signalling, which is independent of PPAR-gamma⁶²¹.

Other cytokines (IFNgamma, IL2, IL6, IL8, IL10, IL17 and IL23A) were not altered by mesalazine in our study. Whether these cytokines play a part in SDD is unclear. A recent study examined endoscopic biopsies from 10 SDD (excluding those with a past history of or suspected diverticulitis) and 10 controls. Cytokines were measured using a chemiluminescent multiparametric assay for IL-2, 4, 5, 8, 10, 13, 12, IFNgamma and TNFalpha¹³², with no difference was found between the groups. This is contrary to our previous study which showed an increase in TNFalpha and IL6⁴⁹¹ in patients with symptomatic compared to asymptomatic diverticular disease. These studies are all small and underpowered to show clear differences given the inherent variability in patient groups.

Other studies have identified an effect of 5-ASA drugs on cytokine production. These include a study using cultured monocytes stimulated by endotoxin, where 5-ASA reduced IL-1 and TNF synthesis but not IL-6⁶²², and in peripheral blood mononucleocytes from patients with beryllium sensitisation or chronic beryllium disease, where 5-ASA inhibits production of IFNgamma and TNFalpha when the cells were stimulated with beryllium⁶¹⁸. In many of these studies the ability of 5-ASA to decrease cytokine release or production comes from isolated cell lines, which highly express these products, or by artificial stimulation of the cells. In our study, the biopsy samples were not cultured or stimulated and so the ability of 5-ASA to suppress up-regulation of genes or

release of pre-made cytokines through stimulation could not be assessed. This may account for the difference between our results and others which report cytokine changes.

(d) Pattern recognition receptors

Some of the most striking and consistent changes with mesalazine were seen in the expression of these receptors, which have not previously been studied in diverticulosis and SDD. Their ability to recognise bacterial and other 'alarm factors' and influence the immunological response suggests a possible role in the low grade chronic inflammation responsible for SDD symptoms^{13, 80}. They may also play a role in the maintenance of chronic inflammation in other conditions. In IBS, TLRs are thought to play a role in IBS symptoms and gut mucosal permeability⁶²³, with genetic variants in TLR9 being independent risk factor for developing IBS¹⁵² and PI-IBS¹²². Different TLR9 alleles may interact with other SNPs, such as PR domain zinc finger protein (PRDM1), an inflammation regulator protein, to alter mucosal barrier functions and transit through the colon¹⁵².

In our study, the mRNA expression of TLR 2, 4, 7, 8 and 9 and co-signalling factor MYD88 were reduced in the mesalazine group after treatment. This is in contrast to one small open labelled study of mesalazine with and without lactobacillus casei DG which showed no effect of TLR4 expression, except when the L Casei was administered rectally, which caused both TLR4 and IL-1 β to be reduced⁶²⁴. In another small study in UC patients treated with 5-ASA and steroids or 5-ASA and azathioprine, TLR4, MYD88 and NF κ B protein expression was increased in the 5-ASA and steroid treated patients but not the 5-ASA and azathioprine group compared to healthy controls⁶²⁵. However, these differences in results may be due to the different pathological processes underlying UC and diverticular disease, or a combination of drugs or confounding by indication (so that the increase in TLR4 reflected increased severity causing increased steroid use) and the small sample size (7 to 13 subjects per group⁶²⁵). These western blotting results were also not confirmed with other techniques such as IHC or RT-PCR and biopsies were taken from prepared colons. These solutions can be irritative and may have altered expression. The healthy controls selected were also had abdominal pain and had been diagnosed with IBS. Since TLR expression have been found to

be altered in this patient group⁶²³, they may also not have been the most ideal control sample to compare. In contrast Cell lines treated with 5-ASA have shown a down regulation of other TLR associated genes, such as TRAF3, which supports our findings⁵¹³.

Another pattern recognition receptor is NOD2. Identified in 2001, it is of increasing interest in as genetic variants have been linked to inflammatory bowel disease⁶²⁶ and malignancy, but so far it has not been found to be associated with IBS⁶²⁷. There are no previous studies examining the expression of NOD2 in diverticular disease. NOD2 appears to reduce bacterial translocation and production of TNF and IFN γ , suggesting it has a protective role in inflammation⁶²⁸. In our study, the expression of NOD2 was decreased in the mesalazine group, which would initially appear counter-intuitive. However 5-ASA alters bacterial profiles⁵⁶⁰ and invasiveness⁵¹⁶ and reduces mucosal permeability⁵⁵³. This may reduce the bacterial stimuli to the mucosa leading to a down regulation of NOD2. However further work is required to confirm this hypothesis.

(e) Tight junctions, cytoskeleton and extracellular matrix

Mucosal barrier is altered in IBS^{123, 157, 629-631}, with polymorphisms of molecules involved in control of tight junctions, being implicated in the risk of developing of PI-IBS¹²². Other groups have shown that IBS mucosal supernatants also alter barrier function in epithelial monolayers and disrupt tight junctions⁶³². However mucosal barrier function, permeability and tight junctions have not been assessed in diverticular disease. Several different components, such as mucin layers, tight junctions (such as Zona occludens, claudins and occludin), extracellular matrix proteins, immune and nerve cells are important in maintaining the intestinal barrier homeostasis^{633, 634}.

(i) Tight Junction Proteins

In IBS, mRNA levels of Zona occludens 1 (ZO-1) and occludin have been found to be reduced by some groups⁶²⁹ but not others⁶³⁰. PAR-2 has been implicated in barrier function disruption as the effect of IBS supernatants is lost in PAR2 knockout animals⁶³² and *Vibrio Cholerae* derived ZO1 toxin is also thought to cause tight junction disassembly through PAR-2⁶³⁵. Claudin 2 forms part of

the tight junction complex but, unlike other claudins, producing pores to allow paracellular diffusion and increased mucosal permeability⁶³⁶⁻⁶³⁸. It is up-regulated by IL6⁶³⁹ and TNFalpha⁶⁴⁰⁻⁶⁴² and has been shown to be up-regulated in active Crohn's and UC^{636, 642}. There are no studies which have examined tight junctions in diverticular disease.

In our study TJP1 (ZO-1) mRNA expression was reduced in the mesalazine and controls groups, while TJP2 (Zona occludens 2, ZO-2) was also reduced but did not reach significant in the mesalazine group. This may be a spurious result, especially as the decrease would appear counter intuitive, as 5-ASA compounds protect intestinal permeability in animal models of colitis. Both Mesalazine and balsalazide decreased mucosal injury to dextran sodium sulphate and mucosal permeability⁵⁵³. Balsalazine showed reduced disruption of the mucosal and tight junction on electron microscopy⁵⁵³, while Immunofluorescence of occludin, showed that mesalazine, attenuated its disruption and irregular distribution within the cells⁶⁴³. Thus it would be expected that with 5-ASA, ZO-1 and ZO-2 mRNA levels would increase. However, in a study of IBS, mRNA levels were shown to be unchanged but protein levels were reduced and the distribution of ZO-1 and occludin within the cell was altered in IBS patients compared to controls⁶³⁰. Thus the lack of protein data needs to be considered when comparing our results with others studies.

In our study Claudin-2, was significantly decreased in the mesalazine treated group. A decrease in Claudin-2 is associated with a decrease in mucosal permeability and would complement the decrease in TNFalpha mRNA seen in our study. Although the expression of claudin-2 with 5-ASAs has not been assessed, other anti-inflammatory agents, such as NSAIDS, inhibit the expression of Claudin 2 mRNA, while up-regulating other tight junction proteins associated with decreased mucosal permeability (Claudin 1, 4 and occludin)⁶⁴⁴. Our results are in keeping with a reduction in the mucosal permeability in diverticular disease which would fit with the known action of mesalazine. However further clinical and biomolecular work in assessing mucosal permeability in different subgroups of diverticular disease and with mesalazine is needed to confirm these findings and hypothesis.

(ii) Mucins and Matrix Metalloproteinases

Mucin is produced by goblet cells and acts as a protective barrier between the luminal contents and the epithelia⁶⁴⁵. There are 5 mucin genes (MUC 1, 2, 3, 4 and 5AC) expressed in the colon⁶⁴⁶, which can be further modified post transcription by glycosylation. MUC-2 is the major gel forming mucin produced. MUC-1 has also been implicated in barrier function in knock out animal models^{647, 648}. Although MUC-1 and MUC-3 mRNA expression have been found to be reduced and increased in erosive oesophageal reflux respectively⁶⁴⁹, there is little information about their role in IBS or diverticular disease. In contrast, the turn-over of extracellular matrix by matrix metalloproteinases (MMP) are thought to be important in the development of diverticular disease²³. Altered expression of MMPs have previously been identified in complicated DD^{21, 23, 491} and IBD⁶⁵⁰.

In our study both MUC-1 and MUC-3A and B were significantly decreased in the mesalazine treated group. Mucin-2 (MUC-2) has been shown to decrease in animal models of colitis, which is reversed by 5-ASA⁶⁵¹, but there have been no studies on MUC-1 or 3 and 5-ASAs. However an animal study using probiotic VSL#3 has shown increased mucus production and MUC2 gene expression but not MUC1 or 3⁶⁵². This was not replicated in an in-vitro cell line model⁶⁵², but suggests that bacterial components may stimulate the production of mucin genes. 5-ASA exerts an antibacterial effect⁶⁵³ and reduces invasiveness⁵¹⁶, which might result in a secondary decrease in mucin mRNA expression as seen in our study.

The mRNA expression of MMP9 or MMP2 was not significantly different between the groups in our study. However 5-ASA decreases changes in MMP2 and 9 in animal models of UC, which was induced by iodoacetamide⁵⁵². In-vitro cell lines treated with 5-ASA⁵¹³ and COX-2 inhibitors⁶⁵⁴ have also shown a decrease in MMP2 and 9 enzyme activity. TNFalpha has also been shown to increase expression of MMP9 in vitro⁶⁵⁵ and in Crohn's disease, the use of TNFalpha inhibitors has been also demonstrated to decrease MMP9, while increasing MMP2⁶⁵⁶. PPARgamma agonists also reduced MMP9s⁶⁵⁷, suggesting that 5-ASA can act through PPARgamma and TNFalpha to

influence MMP9 expression⁵⁵². With the above studies, a change in MMP9 may have been expected but this lack of change in our study may again be due to the small numbers. Larger studies may be identified in significant change.

The control of barrier function in the intestine is complex^{633, 634}, but involves several pathways which are known to be influenced by 5-ASAs such as TNFalpha, IL-1B, TLRs, PAR-2 and NFkB (see above). Thus one of the actions of mesalazine in alleviating pain in SDD may be through altering intestinal barrier function, by influencing mucin and tight junction protein levels and distribution. But further assessment is required to confirm the effect of mesalazine on ZO-1 and other tight junction and mucin components. The small but significant decrease in the control group for ZO-1 may have occurred by chance or due to changes in stress or other psychological factors not specifically measured during the study but which have been shown to influence intestinal permeability⁶³⁴. This and the potential effects of 5-ASAs on mucosal permeability require further study.

(f) Other genes

(i) Cell migration and apoptosis

The understanding of the role of chemokines and cell adhesion molecules in diverticular disease and IBS is limited, although E-selectin has recently been shown to be up-regulated in SDD compared to ADD patients⁴⁹¹. 5-ASAs influence of cell migration molecule expression is also not well understood. Our study has shown a significant decrease in the expression of VCAM-1 and MAdCAM-1. This is supported by one study which suggests 5-ASA can decrease the up-regulation of P and E Selectin and VCAM-1 in LPS stimulated mouse intestinal tissues⁶⁵⁸. In dental pulp cells, PPARgamma, which is thought to interact with 5-ASA, can also decreased production of MMPs, ICAM1 and VCAM1⁶⁵⁷. However another study has shown that 5-ASA have no effect on the expression MAdCAM1 in epithelial monolayers, which was induced by TNFalpha⁶⁵⁹. These differences may be due to the type of stimulation and/or cell lines used compared to our in-vivo work.

Our study also showed down-regulation of CMKLR1 (also known as ChemR23) and MCL-1. CMKLR1 has been associated with migration of macrophages and dendritic cells in vitro and with pro and anti-inflammatory effects^{660, 661}. Its expression can be regulated by TNFalpha, IFNgamma, IL-1B, IL-6 and TGF-B1⁹⁸. MCL-1 (myeloid leukaemia cell differentiation protein), part of the BCL-2 (B Cell lymphoma 2) family of genes, is important in protecting cells from apoptosis and is of interest in cancer therapies⁶⁶². Sulphasalazine but not Mesalazine has been found to down regulate BCL-2 and induce apoptosis in T lymphocytes from Crohn's patients⁶⁶³. However the effect of mesalazine on CMKLR1 and MCL-1 has not been previously investigated, but its decrease may be due to down regulation of it or other genes known to be effected by 5-ASA or due to alteration in cell populations within the GI tract.

(ii) Protease-activated receptor 2 (PAR2, gene F2RL1)

Protease-activated receptor 2 (PAR2, gene F2RL1) has been of increasing interest in IBS as it can induce mechanical hypersensitivity and alter gut permeability¹⁴⁷ by re-organization of tight junction proteins⁶⁶⁴. Another protease-activated receptor, PAR4, has been found to modulate pain by inhibiting the actions of PAR2 and TRPV4 in nerves⁶⁶⁵, but can be pro-inflammatory⁶⁶⁶. In the GI tract, mRNA levels of PAR2 appear to be similar in IBS colonic tissue as in controls^{147, 664}, but PAR4 mRNA is decreased and expression of tryptase and trypsin, activators of PAR2, are increased. Immunofluorescence studies have identified PAR4 on mast cells, with decreased expression found in PI-IBS⁶⁶⁷. Meanwhile, PAR2 has been linked to increased neuronal excitability in culture¹⁴⁸ and in chronically stressed mice infected with citrobacter rodentium⁶⁶⁸. However PAR2 and PAR4 roles in diverticular diseases have not been published. In our study PAR2 (F2RL1) mRNA expression was significantly decreased in the mesalazine group. PAR4 (F2RL3) was so poorly expressed that results were excluded from further analysis. No studies using 5-ASA compounds have reported changes in expression of PARs, but the decrease in PAR2 is in keeping with the anti-inflammatory properties of mesalazine and might contribute to the reduction in pain we observed.

(iii) PPARgamma

PPARgamma has many functions in the GI tract. It is highly expressed in many cells including activated macrophages⁶⁶⁹. Disruption of PPARgamma in macrophages increases susceptibility of colitis in animal model of IBD⁵²⁸. PPARgamma can also be found on epithelial cells, which are important in IBD models⁶⁷⁰. Activated PPARgamma suppresses NFkB activity, a key inflammatory nuclear transcription factor, and reduced inflammatory mediators⁶⁷¹. There is evidence that PPARgamma is down-regulated in experimental inflammatory bowel animal models, which can be restored by PPARgamma agonists and probiotics. Previous studies have suggested that in colonic epithelial cell lines, 5-ASA increases the expression of PPARgamma at mRNA and protein levels⁵²⁷. This was identified only after a short incubation of 12 hours. 5-ASA has also been shown to result in translocation of PPARgamma from the cytoplasm to the nucleus after 24hours of incubation with colonic epithelial cells⁵²⁷. In an animal experiment of PPARgamma and 5-ASA in radiation colitis, rats were treated with or without 5-ASA for 7 days, prior to irradiation exposure. The 6 control animals, who did not have irradiation and where treated with 5-ASA, showed a slight not non-significant rise in PPARgamma mRNA⁵⁴⁹. Interestingly, STAT3, which is part of a signalling pathway involved in inflammation^{672, 673}, was significantly elevated in this study⁵⁴⁹.

In contrast in our in vivo study, the mRNA expression of PPARgamma was decreased (Table R3.14). However, our in-vivo study was of 3 months duration and it is not known if 5-ASA induced increases in PPARgamma expression described above are maintained long term or if these changes are cell specific.

Although we didn't identify any significant changes in IHC slides, our sample size was small and the antibodies used for macrophages (CD68) and T lymphocytes (CD3) would not have distinguished between the sub-classifications of cells or their activation states. Therefore it is possible that mesalazine increases PPARgamma mRNA and/or activity in some cells, as suggested by other studies^{527, 538, 674}, but it may also alter the immune cell populations within the colonic mucosa resulting in an overall decrease in the total PPARgamma mRNA of the biopsy. This

hypothesis is supported by a study of mesalazine in IBS, where decreased mast cell numbers were identified in the mesalazine treated group⁵⁴³. PPARgamma is expressed in mast cells and thought to effect their maturation, function and release of mediators^{675, 676}. Mast cells have been linked to bloating and 'dysmotility-like dyspepsia'⁶⁷⁷ and proximity to nerve fibres¹³⁹ in IBS, supporting their involvement in patient symptoms. 5-ASA compounds have also been found to decrease mast cell mediator release as well^{678, 679}, offering a potential mechanism of action. Mast cell tryptase was not stained for in our study, but future work should include this histological assessment.

(iv) INOS

INOS (NOS2) was found to be significantly decreased in the mesalazine group in our study. This finding is supported by animal studies^{609, 680} and in human epithelial cells⁶⁸¹. In the human study, cultured epithelial cell lines were stimulated with IL-1B and IFNgamma and 5-ASA compounds. 5-ASA was found to inhibit iNOS production and the expression of mRNA and protein⁶⁸¹. This enzyme can be induced by inflammation and its reduction supports the anti-inflammatory properties of mesalazine.

(v) Superoxide dismutase (SOD1)

SOD1 is a cytoplasmic copper/zinc superoxide dismutase and is part of an antioxidant defence system. SOD1 has also been shown to be up-regulated and correlates with disease activity in active Crohn's Disease, but is down-regulated in UC with anaemia⁶⁸². It is decreased by corticosteroids treatment⁶⁸². In contrast, Balsalazine, another 5-ASA, has been found to increase the activity of SOD in mice with DSS-induced colitis⁵⁵³. Mesalamine has also been found to increase manganese-SOD in rat non-transformed small intestine cell lines⁶⁸³. However in both these studies 5-ASA was only given for a short time prior to analysis for SOD. Thus in the long term, 5-ASAs may act through anti-inflammatory and antioxidant activities to reduce SOD1 activity. However this finding would need to be confirmed.

(vi) Phosphodiesterase 4 (PDE4B and PDE4D)

We found a significant decrease in PDE4B but not PDE4D. Phosphodiesterase 4 (PDE4B and PDE4D) is important in cAMP breakdown and is found in many inflammatory cells. A recent study suggests that PDE4B is increased in SDD compared to ADD⁴⁹¹. PDE4 inhibitors are already used in other chronic inflammatory conditions, such as chronic obstructive pulmonary disease (COPD), and have been linked to decreased TNF α expression in peripheral blood mononuclear cells⁶⁸⁴. A small animal model of colitis also suggests that PDE4 inhibitors can reduce inflammation and mediators such as TNF α and TGF- β 1⁶⁸⁵ and there has been recent interest in selective PDE4 inhibitors in IBD⁶⁸⁶. However, although a decrease in PDE4 links with the anti-inflammatory effects of mesalazine, no previous studies have examined the effects of 5-ASA on PDE4 but our data suggest that this might contribute to its anti-inflammatory effect.

(vii) Formyl peptide receptor 2 (FPR2)

FPR2 acts as a G protein coupled receptor for, among 30 others, lipoxin A4 and annexin, which have been implicated in the promotion and resolution of inflammation^{687, 688}. FPR2 (which are also known as FPRL-1) are located on immune cells, such as neutrophils and macrophages, and are involved in cellular adhesion, migration and diapedesis. Some FPR2 ligands, e.g. lipoxin A4 and annexin, have shown anti-migration influences⁶⁸⁸. Mast cells also have FPR2 receptors, with annexin 1 inhibiting their activity⁶⁸⁸. Pro-inflammatory signals from this receptor are thought to act through NF κ B, while anti-inflammatory activities by SOCS-2, TRAF2 and TRAF6, desensitising the cell to classical stimuli from TLR receptors.

FPR2 has been implicated in several inflammatory diseases such as asthma, rheumatoid arthritis, Alzheimer's, coronary artery and Crohn's disease. Its expression has been shown to be increased in the mucosa of Crohn's disease and in THP-1 cells treated with LPS or IFN γ , which fits with an up-regulation of lipoxin signalling in inflammation⁶⁸⁹. In our study, FPR2 was significantly down regulated in the mesalazine group. However there have been no other studies of 5-ASA and FPR2. The reduction in FPR2 expression may be related to the reduction in TNF α and TLRs

which are known to up-regulate its expression⁶⁸⁸. This would correspond to the anti-inflammatory mechanism of mesalazine, leading to a down-regulation of natural resolution of inflammation (or anti-inflammatory) mechanisms which are up-regulated in an inflammatory event. However further studies would need to confirm these findings.

(B) Cell Counts

In Humes et al, no difference was found between SDD and ADD 5HT and CD3 cell counts⁴⁹¹. This agrees with our work where no difference was found between V1 and V5 and stained cell numbers/area (Lymphocytes CD3, Macrophages CD68, Enterochromaffin cells 5HT or Cellular proliferation KI67) in the mesalazine and placebo groups. Interestingly no significant difference was found in CD3 and CD68 in the Corinaldesi et al's study of mesalazine in IBS as well, although they did report a marked reduction in Mast cells⁵⁴³.

Tursi et al reported a significant decrease in number of KI67 stained cells in the whole crypt in 20 patients with symptomatic diverticular disease who were treated with mesalazine for 1 year⁵³⁵. This is supported by studies of colorectal carcinoma and the reduction in proliferation seen on mesalazine treatment^{513, 538, 544, 690}. A reduction in KI67 staining was not seen in our study and may be due to several factors. Firstly our study only has 13 subjects in the mesalazine group which may have been too small to identify a significant change. Secondly our study was of shorter duration than Tursi et al's and did not include an initial treatment with rifaximin. Thirdly, our cell counts were derived using computer assistance and expressed as an area rather than a percentage of positive stained cells. All of these may have contributed to our lack of significant results.

(C) Calprotectin and SS-CRP

Faecal calprotectin (FC) is released from inflammatory cells, mainly neutrophils, during cell activation or death. It is stable in faeces over several days and has been found to correlate with inflammation and disease activity in a variety of gastrointestinal conditions, such as IBD and colonic polyps⁶⁹¹⁻⁶⁹⁷.

In 2009 Tursi et al⁶⁹⁸ found increased FC levels in patients with diverticulitis and SDD when compared to HV and IBS patients. They also showed a decrease in FC in patients treated with mesalazine and rifaximin for 10 days followed by mesalazine alone for 8 weeks. This is in contrast to our study where no difference was found in the treatment of placebo group for faecal calprotectin. However it is important to note that, the FC in Tursi's study was detected using CAL Detect (Sofar SpA Milan Italy). This is a semi-quantitative method that gives 1 to 4 bands of colour to indicate FC concentration rather than the gold standard quantitative ELIZA method. In our study, we used an ELIZA (Calprest) technique which gives a more accurate quantitative measurement. Also in our study, patients did not require to have a prior proven episode of diverticulitis. Thus it is possible that in some of our patients central pain processing changes may be important in their pain experience rather than peripheral low level inflammation. Thus in some subjects their initial and final calprotectin levels may have been low and no change would have been identified in their FC. This and the use of rifaximin in Tursi's study may explain the difference in our results. In both studies only a small number of patients were assessed. Thus FC should be assessed in larger studies to confirm its usefulness in SDD and in identifying and predicting which patients may benefit from and/or are responding to treatment with mesalazine.

CRP is an acute phase reactive protein which can be increased by a wide range of inflammation or trauma related stimuli. SS-CRP, which allows detection of CRP below the standard reference range and is a marker of micro-inflammation, has been shown to increase in a study of IBS and HV⁶⁹⁹. It has been shown to be decreased in an open study of 20 patients with Ankylosing spondylitis who were treated with mesalazine for 24 weeks, but this reduction did not reach significance⁷⁰⁰. Although CRP may have uses in diagnosing and monitoring treatment of acute diverticulitis, its role in the monitoring of SDD treatment has not been demonstrated. Experience in Crohn's disease suggests that it is likely to be less sensitive than fecal calprotectin⁷⁰¹. In our study no change was found in SS-CRP between time points or treatment groups, but larger studies are required to confirm this finding.

(D) Liquid Chromatography and mass spectroscopy

In our study, LCMS results showed no difference between the time points within or between the treatment groups despite the fact that many of the enzymes involved in the manufacture and destruction of these products were altered in the mesalazine group. This may be because the rectal and sigmoid biopsy samples used in the analysis were small and difficult to assess. Also many of the studies, which demonstrated a change in prostaglandins and leukotrienes, used single cell cultures, such as leucocytes, and/or stimulated the production of arachidonic acid pathway products e.g. ionophore A23187^{611, 612, 702, 703}. Some studies also suggest that different 5-ASA compounds may inhibit different enzymes within the AA pathway to different extent, which makes comparison of mesalazine with other 5-ASA difficult^{612, 704, 705}. This may explain our inability to detect a significant change in these important inflammatory compounds and that future studies may require other techniques such as cell culture and stimulation of patient samples to demonstrate the changes inflammatory products resulting from the altered gene expression.

(E) Patient Symptoms

Our study was not powered to detect significant changes in reported patient symptoms, but we did find a significant decrease in the median duration of pain in the mesalazine group (Figure R3.7). No other significant change in symptoms, including general overall wellbeing, bloating, stool frequency or consistence or bloating was identified. There was evidence of a significant placebo effect, with reported improvement in pain relief in both the placebo and mesalazine groups (Figure 3.8). The placebo effect was also identified in the fact that 50% of patients taking placebo believed they were taking mesalazine, compared to 78.6% in the mesalazine group (Fig R3.9).

This is the first double blinded, placebo controlled RCT of mesalazine in SDD. Few other studies of mesalazine in SDD have been randomised. In 2010 Gatta et al published a meta-analysis of 3 studies of mesalazine in SDD, which showed symptomatic benefit. However these were open labelled studies and not placebo controlled. Humes et al published a systematic review in 2011⁵⁰². In this only 2 RCT of mesalazine and rifaximin were identified, both of which were open labelled,

with little detail on the method of randomisation or power calculations used⁵⁰². Both used a non-validated global symptom score to show significant benefits for the mesalazine, which was given as a pulsed rather than continuous medication^{566, 569}. Other open labelled or non-blinded studies (table I3.1) have suggested benefit of mesalazine with varying length of follow up.

In Corinaldesi et al's⁵⁴³ RCT of mesalazine in 20 IBS patients a significant improvement in general well being but not abdominal pain, bloating or bowel habits was reported. This agrees with a prospective study by Andrews et al⁶⁵³ of 12 women with PI-IBS, who had improved number of days of discomfort, increased bowel movement satisfaction on a global relief questionnaire. In a larger RCT of mesalazine in 360 IBS patients, which included all IBS types, significant changes in pain intensity and duration were identified⁷⁰⁶. Both these studies support our findings of an improvement in pain and general well being with mesalazine.

3.5.3 Limitations of the present study

There are several limitations to our study which include its small size and limited 3 month duration. This was because the study was powered to assess biochemical changes to mesalazine and not symptom improvement. Thus there was significant placebo effect, with many subjects in the placebo group reporting improvement in their symptoms (Figure R3.7-3.9), although these did not reach significance (Table R3.19).

There was also a female predominance in the mesalazine group ($p=0.0608$). This primarily occurred as male subjects developed adverse events and withdrew from the study. All female withdrawals were due to protocol violations or social circumstances. Poor tolerance of daily mesalazine has been highlighted in other gastrointestinal conditions, such as IBS⁷⁰⁷, and inflammatory conditions, such as Ankylosing spondilitis⁷⁰⁰. In a recent RCT meta-analysis of 5-ASA medications in UC, the frequency of abdominal pain or dyspepsia with mesalazine ranged from 1-27% (median 4%) and diarrhoea from 1-9% (median 2%)⁷⁰⁸. In a 5 year observational open labelled study from Gatta et al⁵⁶⁸, 16.8% of patients in the mesalazine group withdrew. This is

similar to a meta-analysis of several small open labelled studies of mesalazine in SDD and recurrent diverticulitis, where the incidence of abdominal pain was reported as 13 (n= 81, 16%) and 2 (n=20, 10%) mesalazine patients⁵⁷⁸. In our study 15% of participants (3/20) withdrew due to exacerbation of pain and 5% (1/20) reported diarrhoea in the mesalazine group, which is within the reported incidence of these complications⁷⁰⁸. However the gender difference between the groups is unlikely to have altered our biochemical results as at baseline both final analysis groups had no significant difference in their gene, stool or blood marker expressions.

The inclusion criteria for our study included patients diagnosed with at least 1 diverticulum in their left colon and abdominal pain which was thought to be related to it after investigation. Participants did not require a confirmed episode of diverticulitis and in our study only 6 participants in each of the final analysis groups had a history of diverticulitis. As the pain in diverticular disease may be similar to that in IBS, having a mix of peripheral and central processing changes^{434, 709, 710}, it is likely that our study population included participants with predominantly central and well as those with predominantly peripheral 'pain sensitivity'. This does not necessarily prevent mesalazine having beneficial effect in DD since mesalazine appears to be effective in IBS⁷⁰⁶ where peripheral factors are likely to be less obvious than central ones. There is undoubtedly an interaction between central psychological and peripheral mucosal factors in IBS and this may also be true in symptomatic DD where a peripherally acting drug like mesalazine may still be effective. It is still unknown how peripheral and central factors contribute to the sensation of pain and if selective treatments of one will affect the other. Mesalazine is poorly absorbed and is thought to act locally within the gastrointestinal tract. Thus Mesalazine may only have effect on those patients who have a predominantly peripheral component to their pain and thus our results may be diluted by inclusion of patients with a predominant central pain component. However this hypothesis and the potential to identify subjects though biopsy or questionnaires needs further investigation by larger randomised control trials of longer duration.

It will also be interesting to look at the results of trials of mesalazine in IBS, where larger numbers of participants with PI-IBS and other IBS types have been included. As PI-IBS may have similar underlying pain mechanisms to post-diverticulitis pain, this may support the use of mesalazine for patients with a peripheral pain component, with alternative such as amitriptyline for those with central pain^{486, 711, 712}. Alternatively alteration of peripheral inputs in all patients may help to reduce central pain processing changes^{434, 709}.

3.5.4 Summary of the clinical and research implications of the work,

This study has implications for future research and clinical practice. It has increased our understanding of the actions of mesalazine, suggesting alteration in key genes within the arachidonic acid pathway, cytokines and inflammatory pathways. Importantly we have shown previously unknown actions on pattern recognition receptors, mucosal barrier function genes and other key genes such as PAR2 and FPR2 which have become of increasing interest in gastrointestinal disorders such as IBS and IBD^{150, 713, 714}. Although larger studies are needed to confirm these results in other DD patients and diseases, these findings will aid inform future studies on the action of mesalazine and will help the development of future research and design of medications in the future.

This is the first RCT of mesalazine in diverticular disease, and although not powered to assess symptomatic improvement, has shown a reduction in the median numbers of hours of pain experienced by patients with SDD. This agrees with other open labelled studies and will support the design and powering of much larger multicentre RCTs into the symptomatic improvement of SDD. As SDD patients are a heterogeneous group and probably have both peripheral and/or central pain mechanisms, it is unlikely that mesalazine will provide effective pain relief for all sufferers. However by assessing larger group of SDD patients with validated questionnaires and focused biomedical investigations, it may become possible to select which patients will respond to different medicinal approaches based on their 'biomarkers'. By clarifying pain mechanisms further, future

treatments can be devised to help selected groups with different pain mechanisms allowing more personalised medical care.

However future work on the effectiveness of symptomatic relief and cost benefit need to be undertaken before mesalazine can be offered as a standard treatment to patients with SDD.

Chapter 4: Conclusions

This study was designed to identify the processes which underlie pain in SDD. Our hypothesis was that a spectrum of both peripheral and central pathologies were involved, with those that had a more peripheral problem having abdominal symptoms only while those with multiple symptoms throughout the body, having an altered central pain processing. The first study has supported this hypothesis. Although a statistically significant difference in sensory pain threshold was not demonstrated between the groups, fMRI imaging has shown greater emotional processing during pain and reduced anticipatory inhibitory responses in the HSDD groups. However this is not as clear cut as we had anticipated which may be due to subject selection and demonstrate a spectrum of mixed peripheral and central changes as well as those with only peripheral or central components.

In the second study, mesalazine showed interesting effects on reducing genes expression associated with inflammation in SDD patients. A reduction in the median number of hours of pain per week was seen. The study was not designed to allow intention to treat analysis but has shown promising results which will need to be consolidated with future large scale studies.

Both these studies also have implications for future research and suggest tailored approach to SDD patient treatment. The means of identifying each patients underlying pain process remains a challenge. Our studies have suggested that the PHQ12 may be one simple measure of doing this, but again needs to be confirmed with further larger studies. A rectal biopsy to identify biomarkers is another potential means and may assist in identifying the type of medication required for patients with a predominant peripheral pain component.

The identification of possible peripheral and central treatments is a challenge and requires further understanding of the underlying pathogenesis of pain in SDD beyond the scope of the work presented here. The mesalazine study has been useful in identifying potential targets for treatment

in SDD by highlighting several gene pathways which were down regulated. These have not previously been identified in SDD, but are becoming of increasing interest in IBS^{715, 716} and IBD.

Firstly it would be important to compare the inflammatory, pattern recognition receptor pathways and cell membrane permeability in SDD as well as healthy controls to confirm our findings. Mucosal permeability and pattern recognition of gut microbiota is of increasing interest in IBS⁷¹⁷⁻⁷¹⁹ and work to assess gut permeability with biological and imaging techniques in SDD would be a potential avenue to explore. Currently, stool samples from the mesalazine and fMRI study are being processed using gene cards to assess the different types of microflora in ADD and SDD patients and changes which can occur with mesalazine. Whether other treatments combined with or separate from mesalazine, such as probiotics, can also help in treatment of SDD patients could also be suggested from further work in this area.

One area we did not examine in the study was changes to peripheral nerves and whether mesalazine can alter these. The mucosal biopsies would not have contained any RNA from the sensory peripheral nerves supplying the mucosa or other layers of the bowel. Unfortunately we were not able to establish a reliable staining method to identify nerve fibres or receptors in the tissue. It may be that, by altering the ongoing inflammation in the bowel, any nerve changes would have reversed. Again further work needs to be carried out. We still have remaining samples from the study in storage under our studies original ethical approval. It may be possible, if a reliable technique is established, to perform this assessment at a future date, give appropriate ethical approvals. The gene changes identified in the mesalazine study as well as work in other conditions such as IBS would help design this work further. Further studies to see if mesalazine or other central or peripheral medications would be possible, if peripheral nerve assessment in biopsy samples or fMRI imaging techniques of spine cord are reliably established.

The fMRI study also suggested central pain processing changes. These techniques in identifying altered pain processing are important as it may allow us to assess different medications or CBT

techniques to see if they can produce prolonged reduction in pain processing^{486, 489, 499}. Work by others has also suggested that prolonged pain can also alter the structure of the brain^{274, 279, 282}. The long term effects of pain related brain changes are not currently clear. Work is currently being performed to identify any structural brain changes in our study group using the T1 weighted images. Brain pain changes have also been shown to be reversed once the cause of pain is treated^{499, 500}. If brain related changes are identified in SDD, the effect of mesalazine or other treatments in reversing this could be assessed using MRI.

Other mechanism of pain can also be investigated. With recent epidemiological studies suggesting that obesity may play a role in the development of symptoms in SDD⁷²⁰⁻⁷²², we have started a project using MRI imaging of the abdomen to quantify visceral and subcutaneous fat and peripheral blood adipokines. This will allow correlation with patient symptoms and may suggest new potential mechanisms and targets for treatment.

Understanding the mechanisms of pain in SDD is still behind that of other gastrointestinal conditions such as IBD and IBS. However with the increasing aging population and obesity, an increase in SDD is anticipated. With a greater appreciation of chronic symptoms related to SDD⁷, further work in this area and cross fertilisation of ideas between chronic gastroenterology and other chronic pain condition would be beneficial. This work has continued to progress our understanding of the condition but much further work is needed to understand the mechanisms. However this work does suggest that both peripheral and central pain processes are important and that treatment for patients with SDD will probably involve a tailored approach.

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Chapter 6: Appendices

6.1 Inclusion and exclusion criteria for fMRI study participants

List 6.1.1 Inclusion (a) and exclusion (b) criteria

(a) Inclusion Criteria:

Participants must have either:

- Symptomatic diverticular disease with short lived recurrent abdominal pain on 3 or more days a month and the condition confirmed on endoscopy/ telescope test, barium enema or CT scan.
- Asymptomatic diverticular disease, with no abdominal pain but the condition has been confirmed on endoscopy/ telescope test, barium enema or CT scan.
- Irritable bowel syndrome, which has been diagnosed by a gastroenterologist at the hospital using ROME II or III criteria.
- No abdominal problems e.g. a healthy participant.
- 18 – 85 years of age.
- Right handed – define as writing or drawing with the right hand.
- Signed informed consent.

(b) Exclusion Criteria:

General exclusions

- Pregnant or lactating women.
- Severe co-morbidity; e.g. heart failure, respiratory failure, alcoholism or drug dependence.
- Inability to give informed consent.
- Participation in any other study on Nottingham University campus in the last 3 months.

MRI exclusions

- Have a metallic implant e.g.
 - Cardiac pacemaker
 - Implanted cardiac defibrillator
 - Metallic heart valves
 - Aneurysm clips
 - Carotid artery vascular clamp
 - Neurostimulator
 - Insulin or infusion pump or implanted drug infusion device
 - Non-removable cochlear, otologic, or ear implant
- Ever been shot or have shrapnel inside the body
- Ever had metallic fragments in the eye
- Claustrophobia

List 6.1.1 Inclusion (a) and exclusion (b) criteria

(b) Exclusion Criteria:

Inflammatory exclusions

- Inability to stop NSAIDs (non-steroidal anti-inflammatory agents), antibiotics or immunosuppressant drugs
- Presence of other gastrointestinal inflammatory conditions such as ulcerative colitis, Crohn's disease and Coeliac disease.
- Previous abdominal surgery (other than appendectomy, hysterectomy, cholecystectomy and sterilisation, hernia repair)

Somatic exclusion

- Peripheral neuropathy (e.g. diabetic, alcohol)
- Broken skin

6.2 Timings and durations of paradigm stimuli

Figure 6.2.1 Durations from Start of Paradigm

	45°C	VAS	CUE
Paradigm 1	72	23	15
	197	150	60
	284	242	141
	407	331	186
	451	495	234
			276
			321
			402
			444
			483
Paradigm 2	113.4	24.4	12.4
	240.4	70.4	60.4
	282.4	155.4	108.4
	368.4	320.4	144.4
	452.4	495.4	228.4
			276.4
			315.4
			357.4
			444.4
			486.4

6.3 MRI Peltier participant subset selection

Central pain processing analysis to cutaneous heat stimulus applied consecutively to the hand and foot in:

IBS patients (IBS)

Asymptomatic Diverticular disease patients (ADD)

Symptomatic Diverticular disease patients

Low somatisation on PHQ12 score <6 (LSDD)

High somatisation on PHQ12 score >7 (HSDD)

All participants recruited

Number	IBS		ADD		LSDD		HSDD	
	MRI No	Data-base ID	MRI No	Data-base ID	MRI No	Data-base ID	MRI No	Data-base ID
1	4134	61	7352	62	7503	64	7499	65
2	6829	41	7574	67	7601	63	7631	68
3	7509	58	7769	59	7630	70	7632	66
4	7623	69	7787	50	7738	57	7788	56
5	7838	46	7801	53	7827	54	7845	51
6	7883	43	8255	33	7882	48	7918	28
7	7904	38	8346	8	7935	31	8155	35
8	7992	40	8354	6	8031	29	8196	30
9	7993	39	8418	10	8372	3	8235	32
10	8003	42	8429	11	8420	14	8253	36
11	8021	15	8522	25	8428	13	8258	34
12	8173	5	8523	23	8552	24	8373	7
13	8205	45	8595	20	8590	18	8680	75
14	8250	4	8685	73	8593	16	8682	71
15	8075	44	8598	1	8594	22	7903	26
16	7846	47	7799	55	8596	21	8032	27
17	7873	49	8276	37	8600	74	8592	17
18	6536	60	8647	19	8681	2		
19			8389	9	7820	52		
20			8599	72				

(**Bold** = patients excluded due to; abnormal scan, medication e.g. lorazepam, insufficient data on questionnaires to confirm group status)

Analysis

All patient images have been processed to correct for movement artefact, echos combined in matlab and normalised and smoothed to 8mm. All images were checked to look for additional movement artefact that was not identified in the processing graphs and the scans removed from further analysis. Participants included in subset analysis:

6.3.1 IBS

IBS		Foot 45oc?	Hand 45oC?	Foot p1	Footp 2	Handp1	Hand p2	Notes
No	MRI No							
1	4134	✓	✓	✓	✓	✓	✓	
2	6829	✓	✓	✓	✓	✓	✓	
3	7509	✓	✓	✓	✓	✓	✓	
4	7623	✓	✓	✓	✓	✓	✓	
5	7838	✓	✓	✓	✓	✓	✓	
6	7883		no	✓	✓	✓	✓	
7	7904	✓	✓	✓	✓	✓	✓	Vas scores 4 but consistent (F1 score missing)
8	7992	✓	✓	✓	✓	✓	✓	
9	7993	no	no	✓	✓	✓	✓	
10	8003	✓	✓	✓	✓	✓	✓	
11	8173	✓	✓	✓	✓	✓	✓	
12	8205	✓	✓	✓	✓	✓	✓	
13	8250	no	no	✓	✓	✓	✓	
14	8075	no	✓	✓	✓	✓	✓	
	7846	N/A	N/A	N/A	N/A	N/A	N/A	
	7873	N/A	N/A	N/A	N/A	N/A	N/A	
	6536	N/A	N/A	N/A	N/A	N/A	N/A	
	8021	✓	✓	✓	✓	✓	no	H2 vas 0

6.3.2 ADD

ADD		Foot 45oc?	Hand 45oC?	Foot p1	Footp 2	Handp1	Hand p2	Notes
No	MRI No							
1	7352	✓	✓	✓	✓	✓	✓	
2	7574	✓	✓	✓	✓	✓	✓	
3	7769	✓	✓	✓	✓	✓	✓	VAS scores consistently 1 or 2 for all
4	7787	✓	✓	✓	✓	✓	✓	
5	7801	✓	✓	✓	✓	✓	✓	
6	8255	no	✓	✓	✓	✓	✓	
7	8346	no	✓	✓	✓	✓	✓	
8	8354	✓	✓	✓	✓	✓	✓	
9	8418	no	no	✓	✓	✓	✓	
10	8522	✓	✓	✓	✓	✓	✓	
11	8523	no	no	✓	✓	✓	✓	
12	8595	✓	✓	✓	✓	✓	✓	
13	7799	✓	✓	✓	✓	✓	✓	
14	8276	✓	✓	✓	✓	✓	✓	
	8647	no	✓	no	✓	✓	✓	F1 vas score 0 rest 5-7
	8389	N/A	N/A	N/A	N/A	N/A	N/A	
	8599	N/A	N/A	N/A	N/A	N/A	N/A	
	8429	no	no	✓	no	✓	no	
	8685	no	no	no	✓	no	no	
	8598	no	✓	no	no	✓	✓	

6.3.3 LSDD

LSDD		Foot 45oc?	Hand 45oC?	Foot p1	Footp 2	Handp1	Hand p2	Notes
No	MRI No							
1	7601	✓	✓	✓	✓	✓	✓	
2	7630	✓	✓	✓	✓	✓	✓	
3	7738	✓	✓	✓	✓	✓	✓	
4	7827	✓	✓	✓	✓	✓	✓	
5	7882	✓	✓	✓	✓	✓	✓	
6	8031	✓	✓	✓	✓	✓	✓	
7	8372	no	no	✓	✓	✓	✓	
8	8428	no	✓	✓	✓	✓	✓	
9	8552	no	no	✓	✓	✓	✓	
10	8590	no	no	✓	✓	✓	✓	
11	8593	no	no	✓	✓	✓	✓	
12	8596	no	no	✓	✓	✓	✓	
13	8600	no	no	✓	✓	✓	✓	
14	8681	no	no	no	✓	✓	✓	
	7820	N/A	N/A	N/A	N/A	N/A	N/A	withdrew
	7503	✓	✓	No - ? missing	✓	✓	✓	1ST Subject F1 scan lost due to peltier fault.
	7935	no	no	✓	✓	No - bad	✓	H2 vas score 1 and H1 3
	8594	no	no	✓	No-missing	✓	No-missing	withdrew
	8420	no	no	✓	no	✓	✓	? H1 peltier fault not f2

6.3.4 HSDD

HSDD		Foot 45oc?	Hand 45oC?	Foot p1	Footp 2	Handp1	Hand p2	Notes
No	MRI No							
1	7499	✓	✓	✓	✓	✓	✓	
2	7631	✓	✓	✓	✓	✓	✓	VAS 3-4 but consistent
3	7632	✓	✓	✓	✓	✓	✓	
4	7788	✓	✓	✓	✓	✓	✓	VAS 3-4 but consistent
5	7845	✓	✓	✓	✓	✓	✓	
6	7918	✓	✓	✓	✓	✓	✓	
7	8155	no	no	✓	✓	✓	✓	
8	8196	no	✓	✓	✓	✓	✓	
9	8235	✓	✓	✓	✓	✓	✓	
10	8253	no	no	✓	✓	✓	✓	
11	8258	✓	✓	✓	✓	✓	✓	
12	8373	no	no	✓	✓	✓	✓	
13	8680	no	no	✓	✓	✓	✓	VAS 4-5 but consistent
14	8682	no	no	✓	✓	✓	✓	
	7903	NA	NA	NA	NA	NA	NA	
	8032	NA	NA	NA	NA	NA	NA	
	8592	NA	NA	NA	NA	NA	NA	

6.4 Notes for processing double echo data

Written July 2010 by Kay Head using first version of spm8, 4D datasets and using the MR Centre cluster computer (logged into modred) and updated by Jan Smith in December 2011. Knowledge of processing in spm8 is assumed – so only details of the double echo combination are given here.

Summary of process...

Convert PAR/REC data to IMG/HDR floats (ptoa -f)
spm8 realign first echo
Give echo2 the same realignment parameters
spm8 reslice echo1, reslice echo2
Run double echo matlab script
Spm8 normalise and smooth
Create 1st level model / estimate model / create contrasts
Create 2nd level model / estimate model / create contrasts
Making Masks
2-Sample T test Comparisons
Using Covariates

Details of method ...

Convert PAR/REC data to IMG/HDR floats (ptoa -f)

With only one set of PAR/REC data in any particular directory you can type...

ptoa -f * -this turns data to IMG/HDR data in float format

In window1 type **spm8 (choose fMRI option)**

spm8 realign first echo

Click Realign (**estimate**)

In Data

Session

Select the first echo only)

RUN

Give echo2 the same realignment parameters

In window2 copy the .hdr and .mat files from the first echo to the second

i.e. cp *echo01.hdr *echo02.hdr

cp *cdecho01.mat echo02.mat

This is a trick to pass the realignment parameters to the second echo

In spm8, run

spm8 reslice echo1, reslice echo2

Realign (**reslice**)

In Data

Session 1– select first echo data

Session 2 – select second echo data

RUN

Run double echo matlab script

In window2 type

Type **fslview**

Load in one of the echo02 files. Look for an intensity value that indicates the maximum background noise level – this should be around 10000-50000 and note this down.

Exit

type **matlab** - then choose option L

type **addpath ('/home/francis/matlab/DE/de/')**

type **de** (this should run the script)

select your first echo, second echo, type in the echo times and the noise level value

Output files

ss_map simple summation

ws_map summation of echos using t2*map create from the dataset

? 4D dataset of t2* values

? average t2*map

It is recommended to use the weighted summation for the rest of your processing

Spm8 normalise and smooth

Normalise the all images to a set template so they can be compared:

Click on Normalise in SPM8 – select estimate and write

For each subject/ scan paradigm create a separate module in the batch editor.

In each module:

- (1) Select the mean...img file for the Source Image
- (2) Select all corresponding rpelt...ws_map.img files for the Image to Write (the mean.img and ws files need to be for the same subject and paradigm)
- (3) For the template images navigate to the opt/magres/spm8/templates/ and select EPI.nii file

Save file and press Run

This will produce wr files

Smoothing

For fMRI, an 8mm³ smoothing is applied is allow some overlap between activation areas to occur and reduce the chance of missing a significant activation.

In SPM8 click on smooth

In the batch editor create modules for each subject and paradigm

For the Images to Smoothed select all the wr.pelt...ws_map.img

Change the FWHM to [8 8 8]

Save file and press Run

This will produce swr files

Quit SPM8 and go into each subject and paradigm file to create separate smooth_8mm folders:

mkdir smooth_8mm

Copy the swr files into the new directory: cp swr* smooth_8mm

Now it is time to create the 1st level random effects (RFX) Model

Make files for the results of the model which will be spm.mat files

Go into each subject file to create a results folder: mkdir combined_results

Create model / estimate model / create contrasts

1st level random effects (RFX) Model

SPM8 and select specify 1st level

In the batch editor create a module for each subject

In each module:

- Directory (where the model will be saved): select the combined_results file that you have just created
- Timing parameters:
 - Units for design: seconds
 - Interscan interval: 3 (this is the TR interval)
 - Microtime resolution: 16
 - Microtime onset: 1
- Data and design
 - Click on data and design header and create a subject/session for each paradigm e.g. footp1 footp2 handp1 handp2
- Subject/session: in each of these you need to enter the timings for the events e.g. cues or heat pulses and select the corresponding paradigm files and movement covariates which are not of interest but need to be taken into account when the model is created
 - Scans: select the smoothed images from the Smooth_8mm
 - Click on the X next to scans
 - A new window will appear
 - Navigate in to the appropriate subject and paradigm folders using the left hand box
 - The directory and folder you are in is shown in the box next to DIR at the top of the window
 - Click on the smooth_8mm file
 - Under the right hand box is a box with .* .
 - Type swr.* so that only the swr files are selected
 - The box immediately underneath this contains a 1.
 - Type 1:199 in this box or any number which is greater than the number of files in that folder. In the peltier study there are 177 files per paradigm used per site
 - Press return
 - The right hand box will be populated with all the swr files in the folder
 - Press REC under the left hand box and these selected swr files will be moved to the bottom box.
 - Press done and the window will disappear and the number of files selected will appear in the Scan row.
 - Click on Conditions
 - In the small grey box below click on new condition until you have created 3 conditions this is for the 45oC, VAS and cue events
 - Go back to the current module box above and below the condition header click on;
 - Name: enter e.g. 45, VAS or cue
 - Onsets: click on this and then edit value below
 - A new window should appear. Enter the times in seconds for the condition events for the appropriate paradigm:

	45	VAS	CUE
Paradigm 1	72 197 284 407 451	23 150 242 331 495	15 60 141 186 234 276 321 402 444 483
Paradigm 2	113.4 240.4 282.4 368.4 452.4	24.4 70.4 155.4 320.4 495.4	12.4 60.4 108.4 144.4 228.4 276.4 315.4 357.4 444.4 486.4

- Duration: 5
- Time modulation: No Time modulation
- Repeat for each condition
- Click on multiple regressors (to exclude movement artefact)
- Navigate to appropriate subject and paradigm folder and select the rp_pelt.....echo01.txt file
- Repeat for each paradigm for each subject

NB it is essential to select the correct files and corresponding rp_pelt...echo01.txt files and to make sure these match the paradigm condition event times that have been entered or the model will be wrong.

Once all the subject and paradigms have been completed save the model file and then press run.

Estimate the model

In SPM8 click on estimate
 Create a new module for each subject
 Load to spm.mat file created from the model above
 Method: Classical
 Click run to estimate the model

Defining contrasts

The model is designed as below

Paradigm 1	
Contrasts of interest	Movement artefact
45 VAS CUE	X Y Z A B C

In the peltier study the order of the paradigms in the model was foot paradigm 2, foot paradigm 1, hand paradigm 2 and hand paradigm 1

When creating contrasts click on the results tab in SPM8 and in the new window navigate to the spm.amt model file. Click on the file and select done.

A new window will appear.
 Click on define contrasts

To look at the maps for combined feet VAS events you need to name the contrast in the top box and enter in the box below
 0 1 0 0 0 0 0 0 0 0 1 (the highlighted 0 are for the movement artefact)

For the combined Hand vas events enter:
 0 1 0 0 0 0 0 0 0 1

Alternatively you can create a model with the model estimation and a standard list of contrasts built in. In the model file go to the header under batch editor and click on spm.
 Go down the list until stats.
 Click on **model estimate** and the estimation module will appear in the module list.
 In this model highlight the files selected and click dependency.
 A new window will open with all the model modules in order.
 Click on the appropriate factorial design module from the list.

Return to the spm button below the batch editor header and use the drop down box to select stats again.
 This time select **contrast manager**.
 A new contrast manager module will appear in the module list.
 Click on this
 Highlight the select spm.mat file and click on the dependency button
 Select the appropriate model estimate module from the list which appears in the new window.
 Click on contrast session and create new contrasts for all the events you are interested in. For the paradigm study 10 contrasts were created. Negative contrasts will identify areas which deactivated, while positive contrasts identify activations to the event.

Name	F1F2VAS	Con 1
T contrast Vector	0 1 0 0 0 0 0 0 0 1	
Name	F1F2VASNEG	Con 2
T contrast Vector	0 -1 0 0 0 0 0 0 0 -1	
Name	F1F2CUE	Con 3
T contrast Vector	0 0 1 0 0 0 0 0 0 0 1	
Name	F1F2CUENEG	Con 4
T contrast Vector	0 0 -1 0 0 0 0 0 0 0 -1	
Name	H1H2VAS	Con 5
T contrast Vector	0 1 0 0 0 0 0 0 0 1	
Name	H1H2VASNEG	Con 6
T contrast Vector	0 -1 0 0 0 0 0 0 0 0 -1	
Name	H1H2CUE	Con 7
T contrast Vector	0 1 0 0 0 0 0 0 0 1	
Name	H1H2CUENEG	Con 8
T contrast Vector	0 -1 0 0 0 0 0 0 0 0 -1	
Name	F1F2H1H2CUE	Con 9
T contrast Vector	0 0 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1	
Name	F1F2H1H2CUENEG	Con 10
T contrast Vector	0 0 -1 0 0 0 0 0 0 0 0 -1 0 0 0 0 0 0 0 0 -1 0 0 0 0 0 0 0 0 -1	

Make sure you select the 'don't replicate' for the 'replicate over session' for each contrast.

Viewing the model

View each model click on results in spm8

A new window will appear.

Navigate to the model file spm.mat you are interested in

Select file and click on done

A new window will appear with the contrasts listed

Click on the contrast of interest

In the window below the spm window click on options as follows

Mask : no mask

Stats: you can choose between corrections for multiple comparisons such as family wise error (FWE), FDR and or uncorrected

P value: keep the recommended value or alter this depending on the strength of the map blobs

Voxel threshold: keep as recommended to change to 3 to reduce small 'activation' dots and to sharpen the map

The map will appear in the graphics window

All model images for the subjects should be visualised to make sure movement artefact has been corrected satisfactorily and the images are of good quality.

2nd level model for the group activation maps

Create folders for the combined model in the main directory:

```
mkdir combined_results
```

```
cd combined_results
```

```
mkdir add high_sdd low_sdd ibs
```

Go into each file in turn and create folders for each contrast

```
mkdir f1f2cueneg f1f2cuepos f1f2vasneg f1f2vaspos h1h2cueneg h1h2cuepos h1h2vasneg h1h2vaspos f1f2h1h2cueneg f1f2h1h2cuepos
```

Type spm8 and select fMRI

Create a 2nd level effect model for all the contrasts as above – click on specify 2nd-level

Directory – select appropriate directory created above e.g. /combined_results/add/f1f2vaspos

Click on scans and then select files – in the new window go to each patient folder and the 1st level model results file and pick up the con files for that condition in all subjects in the study e.g. f1f2vas positive (con_1) or h1h2vas positive (con_5) etc

Do not add covariates

Add a model estimation after each factorial design

Go to spm on the top of the Batch editor window – go to stats and select model estimate

In the module list click on model estimation

Click on select spm.mat and then on dependency on the bottom right of the window

A New window will appear. Click on the appropriate factorial design specification file and then o.k.

You need a new model estimate for each factorial design specification

Add a contrast for the group maps

Go to spm on the top of the Batch editor window – go to stats and select contrast manager

In the module list click on contrast manager

Click on select spm.mat and then on dependency on the bottom right of the window

A New window will appear. Click on the appropriate model estimate file and then o.k.

Make contrast as before-

Name: grpmap

Contrast: 1

Save the model in the main or combined_results directory

Click on the green arrow to run the model

Once model completed

Click on results

In results window

No mask

P value FDR p0.05 or uncorrected p0.001 as strength of blobs allows

Voxel threshold 3

Check to make sure pictures o.k.

To create a list of the active brain regions use the stats window in which you selected the statistic test and p value etc and click on the 'whole brain' tab

A list will appear below the brain maps

Hover the mouse cursor over the list on the screen and press the right hand button on the mouse – several options will appear. Select the print list option and the list will appear in the modred window.

Click on the modred window on the header bar and then right click in the same area. A window and list will appear. Select 'copy all to clipboard'

Open notepad and then paste the copied clipboard. The list should appear with other text which can be edited to just leave the list.

Save as a text file.

Open Microsoft excel and import the saved text file. Make sure you select 'Delimited' and then hit next. In the next window select 'tab' and 'space' options and then 'finish'. The list should appear in the excel window.

The x y z co-ordinates are always at the right hand side. However in active areas, where there are several peaks, a list of minor peaks will appear under the major peak. These values and co-ordinates can be shifted to the left. Therefore drag this row to the right so that all the x y z co-ordinates line up in the same columns on the right hand side.

To determine what brain region the co-ordinate refers to you need to use the pick atlas.

To load the pick atlas go to the main named window of spm8 and click on 'wfupickatlas' tab under the spm for functional MRI.

A new window will load.

At the bottom of the window is several boxes to enter co-ordinates and in the middle a list of the 2 option boxes which determine how the brain areas are expressed. Select one to be the TA Brodmann's areas + and the other to be AAL.

Enter the x, y, and z co-ordinates for each activate into the middle row of boxes on the left marked as MNI. Press go at the end of the row and the brain regions will appear. You can check the location by looking for the pale blue spot that appears over the brain image above.

Write the brain region adjacent to the co-ordinates in the excel file.

To do further comparisons such as inter and intra group, a mask is sometimes needed so only the brain areas of interest are selected and the power of the maps is improved.

Making a mask:

Create folders for the masked data:

```
mkdir masks
```

```
cd masks
```

```
mkdir cueneg cuepos vasneg vaspos
```

in each of these folders make directories for the foot and hand – mkdir feet hand

Type spm8 and select fMRI

Create a 2nd level effect model for all the subjects for the e.g. cue or negative vas for the hand and foot – click on specify 2nd-level

Directory – select appropriate directory created above e.g. /masks/vaspos/feet

Click on scans and then select files – in the new window go to each patient folder and the 1st level model results file and pick up the con files for that condition in all subjects in the study e.g. flf2vas positive (con_1) or h1h2vas positive (con_5) etc

Do not add covariates

Add a model estimation after each factorial design

Go to spm on the top of the Batch editor window – go to stats and select model estimate

In the module list click on model estimation

Click on select spm.mat and then on dependency on the bottom right of the window

A New window will appear. Click on the appropriate factorial design specification file and then o.k.

You need a new model estimate for each factorial design specification

Save the model in the masks directory

Click on the green arrow to run the model

Once model completed

Click on results

Make contrast as before- grpmap, 1

In results window

No mask

P value none p 0.001

Voxel 3threshold 3

Check to make sure pictures o.k.

Click on imCalc to make into binary files

Input images – select spmT_0001 for 1 of masks e.g. vasneg feet

Output file e.g. vasnegfeet_308

Output directory e.g. /masks/vasneg/feet/

Expression = i1>3.08 (to get a p0.001)

Do not change the other settings

Save and run model

Repeat steps above but change the output file name and the expressions to

Output file e.g. vasnegfeet_258

Expression = $i1 > 2.58$ (to get a p0.005)

Output file e.g. vasnegfeet_235

Expression = $i1 > 2.35$ (to get a p0.01)

Check binary images by hit display and select output file(s) from above

When combining 2 binary models e.g. hand and foot vas positive to make a combined model select imcalc again

In the input file select both images of the same p value to be combined:

Input images – select vasposfeet_258.img and vasposhand_258.img

Output file e.g. sumf1f2h1h2vaspos_258

Output directory e.g. /masks/vaspos/

Expression = $i1 + i2$

By adding the binary models some of areas will become 2 (e.g. $1+1$)

To convert them all back to 0 or 1 again o back into imCalc

Input images – sumf1f2h1h2vaspos_308

Output file e.g. finalf1f2vaspos_binary_308

Output directory e.g. /masks/vaspos/

Expression = $i1 > 1$

NB In the peltier study - f1f2h1h2cuepos mask called vaspos_... by mistake! But are in the masks/cuepos/feet/ directory.

2-sample-t-tests

Using a mask in inter-group and intra-groups comparisons

For the comparisons between the groups and for the hand and foot comparisons within a group a mask is needed to exclude brain areas with no activations and to increase the power in those areas which activate or deactivate.

Foot_Hand Comparison

In modred create folders for the new comparisons

```
mkdir comp_handfeet
```

```
cd comp_handfeet
```

```
mkdir add low_sdd high_sdd ibs
```

In each of these folders make folders for the comparisons e.g.

```
cd add
```

```
mkdir vaspos vasneg cuepos cue neg
```

Once folders are made:

Type spm8 and select fMRI

Select 2nd level effects

In the batch editor create modules for all the comparisons e.g. vaspos cuepos vasneg.....

In each module

- Directory: Navigate and select the folder for the comparison e.g. comp_handfett/add/vaspos
- Click on design and in the box below click on 2-sample-t-test
- A list of group 1 and group 2 scans will appear
 - In group 1 select all the contrast files for the subjects in the group as previously that correspond to the e.g. handvaspos – con5

- In the group 2 select all the contrast files for the subjects in the group as previously that correspond to the e.g. feetvaspos – con1
- Do not add a covariate
- Under the masking heading click on explicit masks
- Navigate to the mask folders and select the combined hand and foot vas mask created earlier. Select the 308 mask first. If it doesn't work you can go back and try the looser 258 etc masks instead.
- Do not change the other parameters
- Add a model estimate and contrast manager as previous
- In the contrast manager you will need 2 contrasts
 - Group 1 vs. Group 2 i.e. areas where the hand has greater activations or deactivations compared to the foot.
 - Name the contrast e.g. hand_foot
 - Contrast [1 -1]
 - Group 2 vs. Group 1 i.e. areas where the foot has greater activations or deactivations compared to the hand.
 - Name the contrast e.g. foot_hand
 - Contrast [-1 1]
 - Repeat with the other modules
 - Save the model and press run
 - Check the images as before selecting the hand_foot contrast initially and then repeat the processing by selecting the results tab again but selecting the foot_hand contrast instead. The maps should be different. The blobs will be weaker than for the group maps made above and you may have to use *fdr* with a reduced *p* value to $p=0.1$ or even uncorrected and reduce the *p* value to $p=0.01$ or $p=0.05$ to see blobs.
 - You can also create lists of active areas and identify the using the pick atlas as above.

The mask is designed so that only the positive differences are seen on the maps and not negative contrasts from the other group. This will allow you to be confident that what you are seeing is just the areas where there is greater activation or deactivation for the group with the main contrast (i.e. 1)

Intergroup contrasts

To look at differences between groups repeat the same steps for the hand and foot comparisons above but creating new folders for the contrast.

Mkdir comp_groups

Cd comp_groups

Mkdir add low_sdd high_sdd ibs

Go into each of these e.g.

Cd add

Make folders for each contrast e.g.

Mkdir f1f2vaspos f1f2vasneg h1h2vaspos h1h2vasneg f1f2h1h2cuepos f1f2h1h2cueneg

In the module you will need to compare many contrasts e.g. f1f2vaspos, f1f2vasneg, h1h2vaspos, h1h2vasneg, f1f2h1h2cuepos, f1f2h1h2cueneg,

Make modules for all of these

Select the 2-sample-t-test in the design heading as before

- In group 1 select all the contrast files for the subjects in the group e.g. ADD as previously that correspond to the e.g. f1f2vaspos – con1
- In the group 2 select all the contrast files for the subjects in the group e.g. IBS as previously that correspond to the e.g. f1f2vaspos – con1

Add the mask to the module and model estimate as above

In the contrast manager make sure you name the contrasts as per the group e.g. if grp 1=ADD and Grp 2 =IBS so:

Contrast 1

- Name the contrast e.g. ADD_IBS
- Contrast [1 -1]

Contrast 2

- Name the contrast e.g. IBS_ADD
- Contrast [-1 1]

Save the model and press run.

Look at the maps, activation list and identify the areas with the pick atlas as above.

Using covariates

These allow you to see if there is any significant correlation between the e.g. anxiety score on questionnaires to the brain map activations.

start by creating new folders for the covariate of interest e.g. anxiety

```
mkdir anxiety
```

```
cd anxiety
```

```
mkdir add low_sdd high_sdd ibs
```

In each of these folders make folders for the comparisons e.g.

```
cd add
```

```
mkdir f1f2vaspos f1f2vasneg h1h2vaspos h1h2vasneg f1f2h1h2cuepos f1f2h1h2cueneg
```

Go into spm8 and select fMRI

Select modules for all the contrasts as above

In each module select the appropriate folder for the contrast in the 'directory'

In the design select the 1-sample -t -test

This will create only 'group 1' in which to select the con files

Add the con files for the group and contrast of interest e.g. all the subjects in ADD for the f1f2vaspos (con1)

Click on covariate

In the box below click on new covariate

Under the covariate a list of vector, name, interactions and centering will appear

Click on name and then edit at the bottom of the window

In the new window that appear label the covariate e.g. anxiety

Click on vector and then edit

In the new window enter the list of anxiety scores from the questionnaire

```
e.g. 7  
4  
8  
0
```

The order of the anxiety scores must correspond to the order in which the con files from each subject where selected in the 'group 1' files above e.g. if subject 08789 was loaded first the first number in the covariate anxiety list must be subjects 08789 score and so on.

Leave the interactions and centering as per recommendations

NB. Do not add an explicit mask for this model

Set up the other modules, add model estimation files and contrast manager
In the Contrast manager set up 2 contrasts

Contrast 1

- Name the contrast e.g. grp_map
- Contrast [1 0]

Contrast 2

- Name the contrast e.g. anxiety
- Contrast [0 1]

Save and run the model

To look at the maps for the covariates you need to increase the power by masking using the grp_map contrast.

Select results and navigate to the covariate and event of interest e.g. f1f2vaspos

Select and load the file as previously

In the contrast window select the anxiety contrast (e.g. contrast 2)

In the stats window it will ask for mask

Previously you should have selected no for this. This time click on yes and the contrast window will reappear again.

Select the grp_map as the contrast.

For the statistical power you may have to use *fdr* or *uncorrected* with a reduced *p* value as with the other comparisons above.

Select voxel threshold to 3 as previously

You can make list of the active areas and brain locations using the pick atlas as above.

For each covariate you are interested in make a new model repeating the steps above and creating new folders for each.

6.5 Inter-Group Comparison functional MRI tables

ADD VS LSDD activations during VAS temperature stimulus left Foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Insula	9	0.013	2.35	2.22	34	-28	6	13	right insula (post)
Frontal	33	0.007	2.62	2.45	36	26	26		right inferior tri frontal
	5	0.019	2.18	2.07	56	-10	18		right rolandic operculum (s2)
Cerebellum	51	0.014	2.34	2.21	-20	-30	-28		left cerebellum (4,5) + pedicle
	5	0.041	1.8	1.74	0	-48	-26		vermis 10
Temporal	7	0.037	1.85	1.78	56	-8	-2	22	right superior temporal

LSDD VS ADD activations during VAS temperature stimulus left foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Insula	1081	0.001	3.66	3.26	32	0	-2		right putamen, insula (ant), amygdala
	17	0.028	2	1.91	-40	20	2	13	left insula (ant)
Cingulate	32	0.013	2.37	2.24	4	-22	40		right mid cingulum
	84	0.019	2.19	2.09	4	28	22	24	right anterior cingulum
Frontal	101	0.002	3.22	2.93	-48	6	18		left inferior operculo-frontal (?s2)
	208	0.002	3.09	2.83	8	18	52	6	right supplemental motor area, superior medial frontal, mid cingulum
	427	0.002	3.08	2.82	52	18	12	45	right frontal (inferior operculo-frontal, mid and inferior tri) (s2)
	93	0.005	2.74	2.54	46	12	48		right frontal (mid and inferior operculo-)
	61	0.016	2.26	2.14	40	2	36		right precentral
	5	0.035	1.88	1.81	50	10	20		right inferior operculo-frontal
	424	0.003	3	2.76	-16	6	0	putamen & med. globus pallidus	left pallidum and putamen
Subthalamic & Brainstem	141	0.007	2.66	2.48	-2	-24	-6		left upper brainstem/thalamus and vermis (3)
Temporal	13	0.018	2.2	2.09	-38	16	-20		left superior temporal pole
Amygdala & HippoC	30	0.019	2.17	2.07	-20	0	-16	34	left amygdala
Parietal	301	0.005	2.74	2.55	42	-56	46	40	right inferior parietal
	121	0.006	2.73	2.54	64	-22	32		right supra marginal
	19	0.007	2.63	2.45	12	-72	40	7	right precuneus
S1 & S2	133	0.002	3.12	2.85	42	-30	58		right post and precentral

ADD VS HSDD activations during VAS temperature stimulus left Foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Cingulate	76	0.006	2.73	2.53	0	-22	34		left mid cingulum
Frontal	23	0.012	2.38	2.24	-2	14	50	6	left supplemental motor area
	6	0.025	2.05	1.96	22	-18	68	6	right precentral
Thalamus and caudate	59	0.01	2.49	2.34	2	-18	6	pulvinar	right and left thalamus
Subthalamic & Brainstem	110	0.001	3.45	3.1	-20	-28	-32		left brainstem
	36	0.008	2.6	2.43	-14	-22	-12		left brainstem
	16	0.017	2.25	2.13	8	-24	-32		right brainstem
Cerebellum	62	0.013	2.37	2.23	-32	-44	-30		left cerebellum (6)
Temporal	10	0.04	1.82	1.75	56	-6	-2		right superior temporal (s2)
Amygdala & HippoC	13	0.022	2.1	2.01	52	-22	24		right supra marginal
Parietal	6	0.029	1.98	1.9	-52	-46	50	40	left inferior parietal
	5	0.037	1.85	1.78	-62	-46	36	40	left supra marginal
	8	0.012	2.38	2.25	10	-52	68	7	right precuneus

HSDD VS ADD activations during VAS temperature stimulus left foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Insula	106	0.011	2.43	2.29	36	18	-2	47	right insula (ant)
Cingulate	11	0.032	1.93	1.85	6	32	20	24	right ant cingulum
	12	0.034	1.89	1.82	-6	2	30		left ant cingulum
	6	0.036	1.87	1.8	16	16	36	32	right mid cingulum
								Post. corpus callosum & cingulum	
	11	0.026	2.04	1.95	-6	-28	24		
Frontal	263	0.003	3.04	2.79	32	44	4	10	left inferior tri frontal and right mid frontal
	59	0.008	2.57	2.41	52	32	22		right inferior tri frontal
	26	0.012	2.41	2.27	-48	8	18		left inferior operculo-frontal (s2)
	54	0.013	2.36	2.23	36	-2	42	6	right precentral
	8	0.013	2.35	2.22	-38	-2	44		left precentral
	22	0.022	2.11	2.01	34	-18	60		right precentral
Thalamus and caudate								putamen & lat. globus pallidus	
	160	0.004	2.82	2.61	-26	10	2		left putamen and pallidum
	307	0.009	2.54	2.38	32	0	-2	putamen	right putamen
Temporal	147	0.002	3.12	2.85	-50	-4	-6	38	left superior temporal + pole (s2)
	6	0.044	1.76	1.71	66	-40	22		right superior temporal
Amygdala & HippoC	121	0.013	2.34	2.21	-36	12	-20	amygdala	left superior temporal pole and amygdala
Parietal	32	0.017	2.22	2.11	42	-46	48		right inferior parietal and supramarginal
	6	0.03	1.97	1.89	-44	-40	26		left supra marginal
	6	0.04	1.81	1.75	66	-22	34	2	right supra marginal
S1 & S2	28	0.01	2.49	2.33	60	-12	22		right postcentral (s2)
	19	0.019	2.19	2.08	-60	-16	28	3	left postcentral

ADD VS IBS activations during VAS temperature stimulus left Foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Cingulate	8	0.033	1.91	1.84	2	38	12	24	left ant cingulum
Frontal	75	0.004	2.92	2.69	-8	28	34	9	left superior medial frontal
	15	0.014	2.32	2.2	-28	52	16		left mid frontal
	30	0.027	2.02	1.93	-2	14	48	6	left supplemental motor area
	3	0.031	1.94	1.87	-46	22	-8		left inferior orbito-frontal
	10	0.031	1.94	1.86	54	-4	40		right precentral
	3	0.034	1.9	1.83	-50	20	-10	47	left inferior orbito-frontal
	5	0.038	1.83	1.77	52	-14	22		right rolandic operculum (s2)
Temporal	56	0.002	3.27	2.96	66	-18	2		right superior temporal (s2)
	13	0.024	2.08	1.98	40	22	-26	38	right superior temporal pole
Parietal	8	0.024	2.07	1.98	-54	-40	50	40	left inferior parietal

IBS VS ADD activations during VAS temperature stimulus left foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Insula	1744	0	3.74	3.32	52	8	20	13	right (insula mid/post) inferior operculo-frontal, (s2)
	109	0.005	2.75	2.55	-38	18	4	13	left insula (ant)
	57	0.024	2.06	1.97	-50	2	22		left precentral, insula (mid) and rolandic operculum (S2)
Cingulate	30	0.008	2.59	2.42	-6	4	30		left ant cingulum
	49	0.018	2.2	2.09	8	-30	26	Post corpus callosum	right mid cingulum
Frontal	22	0.024	2.07	1.98	8	26	28	32	right ant cingulum
	325	0	3.75	3.32	48	38	-10		right inferior orbito-frontal
	64	0.004	2.85	2.63	52	34	26		right inferior tri frontal
	120	0.006	2.73	2.53	10	0	50		right supplemental motor area
	59	0.007	2.62	2.45	10	-10	66	6	right supplemental motor area
	48	0.008	2.56	2.4	36	-2	40	6	right precentral
	39	0.009	2.5	2.35	34	-20	60		right precentral
	8	0.016	2.26	2.14	-46	40	16	46	left mid frontal
	50	0.02	2.16	2.06	34	40	14		right mid frontal
	20	0.021	2.13	2.03	8	28	54		right superior medial frontal
	5	0.038	1.84	1.78	12	-24	48		right supplemental motor area
Thalamus and caudate	10	0.029	1.98	1.9	18	4	0	putamen	right pallidum
	24	0.015	2.29	2.17	16	-12	2		right thalamus
Cerebellum	7	0.026	2.04	1.95	-40	-48	-38		left crus (1) cerebellum
	179	0.002	3.21	2.92	-2	-20	-4	red nucleus	vermis (3)
	40	0.011	2.45	2.3	4	-60	-32		vermis (8)
	52	0.006	2.71	2.52	-20	-48	-30		left cerebellar pedicle and cerebellum (6)
Temporal	3	0.018	2.2	2.09	30	-28	16	13	right heschl
	13	0.006	2.67	2.49	56	-56	0		right mid temporal

IBS VS ADD Activations during VAS temperature stimulus left foot continued

Parietal	6	0.036	1.87	1.8	60	-42	24		right supra marginal
	9	0.027	2.01	1.93	12	-70	42		right precuneus
	430	0.001	3.53	3.16	66	-22	26	40	right supra marginal
	301	0.001	3.34	3.02	-52	-42	34	13	left supra marginal and superior temporal
	217	0.003	2.98	2.74	-46	-6	-6	22	left superior marginal
Occipital									Post corpus callosum left)/ post cingulum
	5	0.042	1.79	1.73	-6	-28	24		

ADD VS LSDD Deactivations during vas stimulus left Foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Temporal	18	0.024	2.08	1.98	-40	-38	8		left superior temporal and rolandic operculum (s2)
	8	0.013	2.35	2.22	54	-54	-18	20	right inferior temporal
Parietal	23	0.006	2.7	2.51	-28	-24	62		left precentral
S1 & S2	281	0.002	3.27	2.97	44	-28	58	2	right post and precentral
	329	0.004	2.9	2.67	-38	-32	58	3+4	left post and precentral
Occipital	232	0.004	2.91	2.69	-36	-82	16		left mid occipital
	97	0.005	2.77	2.57	40	-72	-12		right inferior occipital
	121	0.015	2.3	2.17	-46	-70	-8		left inferior occipital
	92	0.023	2.08	1.99	38	-76	18	19	right mid occipital
	6	0.021	2.13	2.03	-30	-70	-4	19	left lingual

LSDD VS ADD Deactivations during VAS temperature stimulus left foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Frontal	90	0.001	3.55	3.18	-20	56	16		left superior frontal
	757	0.001	3.42	3.08	6	54	12	10+9	left and right superior medial frontal
	22	0.014	2.33	2.21	-16	40	50		left frontal (superior and superior medial)
	16	0.019	2.19	2.09	-42	20	32		left inferior operculo-frontal
Cerebellum	16	0.011	2.45	2.3	-22	-28	-26		left cerebellum (4,5)
	33	0.014	2.31	2.19	30	-38	-28		right cerebellum (4,5)
Temporal	403	0.004	2.89	2.67	-48	-56	22		left mid temporal, angular and mid occipital
	11	0.007	2.66	2.48	66	-12	-12	21	right mid temporal
	51	0.01	2.47	2.32	48	0	-36	20	right inferior temporal
Parietal	24	0.017	2.24	2.12	-32	-62	60		left superior parietal
	55	0.026	2.04	1.95	-8	-60	42		left precuneus
	20	0.035	1.88	1.81	-8	-56	10		left precuneus
Occipital	34	0.022	2.11	2.01	12	-82	26		right cuneus

ADD VS HSDD Deactivations during VAS temperature stimulus left Foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Frontal	30	0.001	3.56	3.18	-36	20	24		left inferior tri frontal
	10	0.011	2.44	2.3	-50	22	30		left inferior tri frontal
	8	0.024	2.08	1.99	10	48	24		right superior medial frontal
	10	0.038	1.84	1.77	-26	14	54		left mid frontal
	22	0.025	2.05	1.96	34	-18	60		right precentral
	16	0.021	2.12	2.03	30	-22	44		right precentral
	5	0.04	1.81	1.75	-28	6	46		left mid frontal
	5	0.042	1.79	1.73	28	16	48	8	right mid frontal
	7	0.044	1.76	1.71	-14	50	0		left superior medial frontal
	Temporal	28	0.019	2.17	2.07	-54	-40	-14	
15		0.023	2.08	1.99	52	-54	-18	20	right inferior temporal
5		0.025	2.06	1.96	-26	-14	-34		left fusiform
39		0.03	1.95	1.88	24	-38	-14		right fusiform
334		0.004	2.87	2.65	-48	-66	-10		left inferior temporal
34		0.007	2.66	2.48	-30	-58	-12		left fusiform
50		0.007	2.65	2.47	-54	2	-18		left mid temporal
14		0.008	2.56	2.4	34	-10	-40	20	right fusiform
10		0.039	1.83	1.77	-32	-16	-26		left fusiform
5		0.042	1.79	1.73	-62	-14	-12	21	left mid temporal
Amygdala & HippoC	65	0.022	2.11	2.01	-32	-36	-12		left parahippocampus
	15	0.03	1.97	1.89	26	-12	-22	hippoC	right hippocampus
	71	0.016	2.27	2.15	-36	-36	12		left parahippocampus
	6	0.042	1.79	1.73	-24	-8	-20	amygdala	left hippocampus
Parietal	35	0.024	2.07	1.98	-18	-64	48	7	left superior parietal
S1 & S2	93	0.001	3.43	3.08	62	-8	22	43	right postcentral
	362	0.001	3.41	3.07	-32	-18	50		left pre and postcentral
	24	0.006	2.68	2.5	-60	-10	30		left postcentral

ADD VS HSDD Deactivations during VAS temperature stimulus left Foot continued

Occipital	383	0.003	3.06	2.8	18	-70	-12		right lingual, fusiform and inferior occipital
	90	0.009	2.54	2.38	38	-76	8		right mid occipital
	83	0.013	2.38	2.24	22	-86	28	19	right superior occipital
	20	0.029	1.97	1.89	-34	-82	14		left mid occipital

HSDD VS ADD Deactivations during VAS temperature stimulus left foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Cingulate	27	0.02	2.16	2.06	-8	-42	48		left mid cingulum
Frontal	6	0.015	2.3	2.17	-28	28	34		left mid frontal
	15	0.019	2.19	2.08	0	54	38		left superior medial frontal
	17	0.026	2.03	1.95	0	-38	66		paracentral lobule
	30	0.001	3.36	3.04	-20	54	18		left superior frontal
Thalamus and caudate	6	0.035	1.88	1.81	-12	-30	4	pulvinar	left thalamus
Subthalamic & Brainstem	6	0.032	1.92	1.85	-8	-30	-36		left brainstem
Cerebellum	9	0.008	2.58	2.41	-24	-28	-28		left cerebellum (4,5)
	33	0.014	2.33	2.2	24	-38	-30		right cerebellum (4,5)
	5	0.021	2.12	2.02	-34	-36	-30		left cerebellum (6)
	5	0.025	2.04	1.96	4	-48	4		vermis (4,5)
	53	0.014	2.32	2.2	42	4	-34		right mid temporal and pole
Temporal	5	0.021	2.14	2.04	-20	-46	-16		left fusiform
	11	0.023	2.09	2	-28	-4	-34		left fusiform
	35	0.02	2.15	2.05	-40	-72	48		left angular
Parietal	5	0.023	2.09	2	-52	-66	40	39	left angular
	9	0.031	1.94	1.87	-34	-60	58	7	left superior parietal
	21	0.008	2.57	2.41	10	-54	66	7	right precuneus
	14	0.025	2.05	1.96	10	-48	48		right precuneus

ADD VS IBS Deactivations during VAS temperature stimulus left Foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Insula	104	0.003	2.93	2.7	-38	-38	16	13	left insula (post) and superior temporal
Frontal	124	0.007	2.65	2.47	-36	-20	54	4	left precentral
	18	0.008	2.59	2.42	-36	20	22		left inferior tri frontal
	38	0.01	2.49	2.34	34	-20	60		right precentral
Thalamus and caudate	7	0.039	1.82	1.76	20	28	-6		right caudate
Cerebellum	17	0.017	2.23	2.12	24	-52	-34		left cerebellar pedicle
Temporal	6	0.038	1.84	1.78	-4	-46	64		left precuneus
	116	0.006	2.73	2.53	-50	-54	-20	19+20	left inferior temporal
	55	0.006	2.7	2.51	28	-30	16	13	right heschl
	20	0.014	2.33	2.2	50	-46	-6		right inferior temporal
	8	0.016	2.27	2.15	54	-54	-18	20	right inferior temporal
	235	0.008	2.59	2.42	32	-60	-12		right fusiform, putamen and lingual
	13	0.021	2.12	2.03	34	-10	-40	20	right fusiform
	38	0.023	2.09	2	-32	-20	-28		left fusiform
	8	0.027	2.01	1.92	-30	-60	-6		left fusiform
	14	0.029	1.97	1.89	-46	-24	-12		left mid temporal
Amygdala & HippoC	120	0.007	2.66	2.48	22	-34	-2		right hippocampus
	65	0.009	2.54	2.38	-20	-38	-6		left parahippocampal
	15	0.018	2.21	2.1	38	-18	-20		right hippocampus
Parietal	6	0.029	1.97	1.89	-26	-68	42		left inferior parietal
S1 & S2	65	0.006	2.68	2.5	-40	-32	58		left postcentral
Occipital	236	0.005	2.74	2.55	-34	-82	14	13	left occipital (mid and superior)
	236	0.01	2.47	2.32	24	-84	20		right occipital (superior and mid) and mid temporal
	162	0.005	2.77	2.57	-18	-84	-4		left lingual
	25	0.031	1.94	1.87	-40	-72	0		left mid occipital

IBS VSADD Deactivations during VAS temperature stimulus left foot

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Cingulate	6	0.036	1.87	1.8	-4	-42	48		left mid cingulum
Frontal	470	0.002	3.25	2.95	8	54	14	10	right frontal (superior medial and superior)
	37	0.006	2.67	2.49	-16	40	50		left superior frontal
	41	0.012	2.41	2.27	-28	28	34	8+9	left mid frontal
	10	0.017	2.23	2.12	-44	28	-12		left inferior orbito-frontal
	6	0.029	1.97	1.89	-42	22	34	9	left mid frontal
Cerebellum	197	0.011	2.45	2.3	4	-52	2	corpus callosum + 30	vermis (4,5) and right precuneus and calcarine
Temporal	6	0.023	2.08	1.99	-66	-30	-10	21	left mid temporal
	4	0.028	1.99	1.9	44	16	-34	38	right mid temporal pole
	10	0.03	1.97	1.89	64	-10	-12		right mid temporal
Amygdala & HippoC	24	0.024	2.07	1.98	-24	-24	-18		left parahippocampus
Parietal	95	0.006	2.7	2.51	-32	-60	60		left superior parietal
	47	0.019	2.18	2.07	-52	-70	34	19+39	left angular
	150	0.011	2.42	2.28	-10	-56	10		left precuneus
	70	0.016	2.26	2.14	4	-70	54		right precuneus
	26	0.018	2.2	2.09	-8	-58	48	7	left precuneus
S1 & S2	3	0.036	1.86	1.8	52	-12	36		right postcentral
Occipital	5	0.037	1.85	1.79	-14	-88	40	19	left superior occipital

ADD VS LSDD activations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Insula	5	0.038	1.84	1.78	-26	20	12		left insula (ant)
Cingulate	15	0.029	1.98	1.9	-6	-32	26		left post cingulum
Frontal	103	0	3.86	3.4	38	26	30		right mid frontal
	16	0.008	2.58	2.41	-44	46	16	10	left mid frontal
	17	0.01	2.49	2.33	-28	56	22	10	left superior frontal
	65	0.01	2.47	2.32	54	-12	20		right rolandic operculum (S2)
	15	0.011	2.44	2.3	-2	16	56	8	left supplemental motor area
	38	0.013	2.35	2.22	40	-30	22		right rolandic operculum
	5	0.019	2.18	2.07	14	32	36		right superior frontal
	9	0.019	2.18	2.07	-52	-4	46	6	left precentral
Subthalamic & Brainstem	15	0.012	2.38	2.24	2	-20	-22		right brainstem
Cerebellum	14	0.036	1.87	1.8	2	-38	-28		vermis
Temporal	6	0.024	2.07	1.98	62	-52	22		right superior temporal
Amygdala & HippoC	30	0.013	2.36	2.23	-40	-8	14		left amygdala/left mid temporal

LSDD VS ADD activations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Insula	9	0.041	1.8	1.74	34	24	-2		right insula (ant)
Cingulate	243	0.001	3.43	3.09	4	30	18	24	right ant cingulum
	219	0.005	2.8	2.59	16	16	38		right mid cingulum
Frontal	216	0.005	2.81	2.6	36	42	16	10	right frontal (mid and inferior tri)
	37	0.011	2.42	2.28	-12	8	48	32	left supplemental motor area
	44	0.02	2.17	2.06	-48	0	16		left rolandic operculum (s2)
	11	0.038	1.84	1.78	46	42	-12	11	right inferior orbito-frontal
	6	0.045	1.75	1.7	8	2	54	6	right supplemental motor area
Thalamus and caudate								red nucleus, ventral lateral nucleus, medial globus pallidus	
	734	0.001	3.64	3.24	-2	-18	-8		right thalamus
	10	0.02	2.17	2.06	12	8	-4	putamen	right pallidum
	6	0.041	1.8	1.74	10	-16	10	medial dorsal nucleus	right thalamus
Cerebellum	439	0	3.99	3.49	-32	-60	-26		left cerebellum (6)
	179	0.004	2.84	2.62	-4	-80	-20		left crus (1) cerebellum
	8	0.018	2.21	2.1	24	-72	-26		right cerebellum (6)
	42	0.025	2.05	1.96	0	-52	-16		vermis (4,5)
	6	0.039	1.83	1.76	0	-60	-26		vermis (8)
Temporal	5	0.041	1.8	1.74	-54	-36	14		left superior temporal
	7	0.034	1.89	1.82	50	4	-18		right mid temporal pole
	265	0.003	3.04	2.79	54	-20	4		right superior temporal
	12	0.029	1.98	1.9	-32	-26	4		left heschl
Amygdala & HippoC	1418	0.001	3.55	3.18	34	2	-18	28	right amygdala, insula (post), superior temporal pole
	709	0.003	2.97	2.73	-36	12	-18	47/12 and putamen	left putamen and amygdala

LSDD VS ADD activations during VAS temperature stimulus left hand continued

Parietal	220	0	3.93	3.45	62	-22	32		right supra marginal
	84	0.012	2.39	2.25	-58	-28	36		left supra marginal
	255	0.014	2.34	2.21	46	-44	44	40	right supra marginal and inferior parietal
	22	0.031	1.95	1.87	44	-38	56	40	right inferior parietal

ADD VS HSDD activations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Cingulate	116	0.004	2.89	2.66	-4	-24	30	23	left and right mid cingulum
	250	0.005	2.74	2.55	-6	22	32		left mid cingulum and left and right supplemental motor areas
	79	0.007	2.66	2.48	12	28	34	9	right mid cingulum
	12	0.019	2.17	2.06	-2	24	16	Corpus callosum	left anterior cingulum
Frontal	8	0.038	1.84	1.77	6	-26	48		right mid cingulum
	690	0	4.25	3.67	-44	46	16	10	left frontal (mid + inferior tri)
	311	0.003	2.98	2.74	34	34	28		right frontal (mid + inferior tri)
	24	0.01	2.47	2.32	-14	2	62		left supplemental motor area
	44	0.015	2.28	2.16	-42	-4	14	13	left rolandic operculum(s2)
	29	0.018	2.22	2.1	-60	4	18	44 + 45	left precentral and inferior operculo frontal
	46	0.022	2.12	2.02	36	22	10	13	right inferior tri frontal
	6	0.022	2.12	2.02	-38	40	34		left mid frontal
	6	0.028	2	1.92	44	-2	18		right rolandic operculum (s2)
	10	0.034	1.9	1.83	48	48	8	46	right mid frontal
Thalamus and caudate	8	0.031	1.94	1.86	-10	-4	4	ventral ant. nucleus	left thalamus
Subthalamic & Brainstem	94	0.001	3.37	3.04	0	-22	-24		brainstem
	277	0.001	3.29	2.98	6	-38	-30		brainstem + left and right cerebellar pedicle
Cerebellum	107	0.012	2.38	2.25	0	-22	4	optic tract	vermis (4,5)
	7	0.027	2.02	1.93	-46	-58	-30		left crus (1) cerebellum
Temporal	53	0.003	3.05	2.79	30	14	-28	38	right superior temporal pole
	116	0.007	2.65	2.47	62	-4	0	22	right superior temporal + rolandic operculum
	167	0.008	2.57	2.4	58	-48	16	13+21	right temporal (superior and mid)
Parietal	170	0.002	3.1	2.83	52	-20	24		right supra marginal
	45	0.007	2.65	2.47	-50	-26	22	22	left supra marginal
	27	0.013	2.35	2.22	-64	-36	30	40	left supra marginal

HSDD VS ADD activations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Frontal	18	0.006	2.68	2.49	-12	-20	70	6	left paracentral lobule
	9	0.024	2.06	1.97	30	48	6		right mid frontal
	23	0.033	1.91	1.84	34	24	-12		right inferior orbito-frontal
Thalamus and caudate	155	0.002	3.07	2.81	-24	-6	0	putamen + lateral globus pallidus	left pallidum
	50	0.004	2.87	2.65	30	-10	-2	putamen	right putamen
	70	0.009	2.53	2.37	14	-18	0		right thalamus
Cerebellum	13	0.029	1.98	1.9	-4	-82	-16		left cerebellum (6)
Temporal	84	0.002	3.25	2.95	52	4	-16	21	right mid temporal pole
	202	0.002	3.16	2.88	-50	-8	-6	21	left superior temporal + pole
	10	0.03	1.96	1.88	-32	-26	4		left superior temporal
Amygdala & HippoC	16	0.02	2.16	2.05	22	-12	-14		right hippocampus
Parietal	215	0.007	2.64	2.46	40	-50	50	40	right inferior parietal and supra marginal
S1 & S2	29	0.028	1.99	1.91	62	-18	36		right postcentral

ADD VS IBS activations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Insula	154	0.008	2.59	2.42	-46	22	-6		left insula (ant) and inferior orbito-frontal
	9	0.029	1.97	1.89	-36	4	-2		left insula (mid)/putamen
Cingulate	67	0.006	2.67	2.49	0	-12	34		left mid cingulum
	28	0.012	2.39	2.25	0	-36	26		left post cingulum
Frontal	249	0.001	3.34	3.02	-2	14	52	6	left supplemental motor area and frontal (superior medial)
	140	0.004	2.85	2.64	-44	46	16	10	left frontal (mid and inferior tri)
	109	0.004	2.83	2.62	60	4	34	6	right precentral
	69	0.008	2.6	2.43	48	24	-4		right inferior orbito-frontal
	12	0.01	2.48	2.33	-56	12	24	45	left inferior operculo-frontal
	15	0.032	1.93	1.86	52	-14	22		right rolandic operculum (s2)
	41	0.014	2.34	2.21	-26	50	22	10	left superior frontal
	43	0.015	2.29	2.17	48	16	40	9	right mid frontal
	17	0.025	2.06	1.97	-48	18	6	45	left frontal (inferior tri and inferior operculo-) (s2)
	11	0.026	2.03	1.94	-2	32	46	8	left superior medial frontal
Subthalamic & Brainstem	47	0.005	2.79	2.58	2	-20	-22		right brainstem
Cerebellum	17	0.033	1.92	1.85	38	-76	-24		right crus (1) cerebellum
Temporal	136	0.004	2.92	2.69	66	-16	2	21+ 22	right superior temporal
	50	0.004	2.86	2.65	58	12	-4	22	right superior temporal pole
	14	0.029	1.98	1.9	-62	-12	8		left superior temporal and heschl

IBS VS ADD activations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL	
Insula	421	0.001	3.45	3.1	48	6	-18	21+22	right insula (ant) and superior temporal pole (s2)	
	69	0.005	2.79	2.59	28	-26	12	13	right insula (post) and heschl	
	10	0.025	2.06	1.97	30	32	-4		right insula (ant)	
Cingulate	16	0.02	2.16	2.05	-10	28	18	24	left ant cingulum	
	9	0.02	2.15	2.05	10	-24	32		right mid cingulum	
	44	0.014	2.31	2.19	6	22	18	corpus callosum	right ant cingulum	
	14	0.021	2.14	2.04	8	40	24	9	right ant cingulum	
	9	0.022	2.11	2.02	14	20	34		right mid cingulum	
	14	0.025	2.06	1.97	-14	-24	36		left mid cingulum	
	Frontal	7	0.038	1.84	1.78	42	20	12		right inferior tri frontal
528		0	5.11	4.21	34	40	14		right frontal (mid and inferior orbito-)	
100		0.001	3.45	3.1	-14	-22	70	6	left paracentral lobule	
23		0.003	2.95	2.72	-14	6	46	32	left supplemental motor area	
84		0.004	2.87	2.65	48	6	16		right inferior operculo-frontal	
89		0.009	2.51	2.35	14	4	52		right supplemental motor area	
16		0.017	2.24	2.13	38	-4	44	6	right precentral	
10		0.018	2.21	2.1	10	30	52		right superior medial frontal	
Thalamus and caudate		48	0	4.35	3.74	20	-8	10	putamen	right putamen
		53	0.014	2.32	2.19	30	-4	12		right putamen
	6	0.017	2.22	2.11	-24	12	12		left putamen	
Cerebellum	9	0.021	2.14	2.04	-14	-6	-8	medial globus pallidus	left pallidum	
	9	0.013	2.37	2.24	-40	-52	-38		left crus (1) cerebellum	
	173	0.001	3.3	2.99	-8	-50	-28		left cerebellar pedicle and vermis (4,5,8)	
Temporal	195	0.006	2.7	2.51	-50	-4	-8	38	left superior temporal	
	17	0.028	1.99	1.91	-38	8	-24		left superior temporal pole	
	6	0.021	2.14	2.04	60	-54	-2		right mid temporal	

IBS VS ADD activations during VAS temperature stimulus left hand continued

Parietal	386	0.001	3.32	3.01	-48	-40	32		left supra marginal and temporal (superior)
	131	0.002	3.08	2.82	62	-24	30	40	right supra marginal
	180	0.003	2.96	2.72	50	-42	36		right supra marginal
	136	0.004	2.86	2.64	-16	-42	64	4	left precuneus
	15	0.035	1.88	1.81	58	-44	24	40	right supra marginal
S1 & S2	61	0.023	2.09	1.99	22	-42	66		right postcentral

ADD VS LSDD De activations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Frontal	11	0.019	2.19	2.08	-16	8	50	32	left superior frontal
	77	0.002	3.11	2.84	2	20	-4		right olfactory/ant cingulum/caudate
Cerebellum	5	0.038	1.83	1.77	16	-72	-16		right cerebellum (6)
Temporal	681	0.002	3.09	2.82	24	-44	-2	37	right lingual and fusiform
	66	0.013	2.36	2.23	-62	-58	-8	37	left temporal (inferior and mid)
	9	0.022	2.1	2.01	50	-44	-8		right mid temporal
	7	0.03	1.95	1.88	44	-46	0		right mid temporal
Amygdala & HippoC	235	0.01	2.46	2.31	-34	-36	-8		left hippocampus
	10	0.019	2.19	2.08	32	-6	-22		right hippocampus
	8	0.04	1.82	1.75	42	-32	-6		right hippocampus/right mid temporal
S1 & S2	18	0.028	1.99	1.91	44	-30	46	40	right postcentral
Occipital	758	0	3.73	3.3	-36	-88	14	31	left mid occipital (mid + superior) and calcarine
	328	0.007	2.67	2.48	18	-88	24	18+30	right superior occipital and calcarine
	51	0.007	2.61	2.44	-50	-70	-12	19	left inferior occipital
	198	0.01	2.5	2.35	-26	-80	0	18	left mid occipital and lingual
	100	0.014	2.33	2.2	36	-76	16		right mid occipital
	15	0.024	2.08	1.99	-14	-90	14	18	left superior occipital
	35	0.025	2.05	1.96	6	-76	-2		right lingual
	8	0.031	1.93	1.86	22	-74	32		right superior occipital
34	0.032	1.93	1.85	44	-76	-6		right inferior occipital	

LSDD VS ADD Deactivations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Cingulate	7	0.038	1.84	1.78	-4	-44	26		left post cingulum
	6	0.039	1.83	1.76	6	-40	30		right post cingulum
Frontal	244	0	3.73	3.31	-30	22	42		left frontal (mid and inferior operculo-frontal)
	499	0.001	3.58	3.2	-4	62	28	10	left superior medial frontal
	311	0.002	3.07	2.81	-10	56	6		left superior medial frontal
	10	0.008	2.55	2.39	18	32	38		right superior frontal
	25	0.014	2.33	2.2	-48	26	4	45	left inferior tri frontal
	13	0.019	2.18	2.07	-28	38	42		left mid frontal
	64	0.015	2.3	2.18	30	-38	-30		right cerebellum (4,5)
Temporal	117	0.001	3.41	3.08	56	2	-34		right temporal (inferior and mid pole)
	45	0.005	2.78	2.57	-46	2	-34	21	left temporal (inferior and mid)
	6	0.032	1.92	1.85	-26	-4	-38	36	left fusiform
	16	0.006	2.72	2.53	-16	-14	-28		left parahippocampus
	43	0.025	2.04	1.95	26	-4	-32	20	right parahippocampus and fusiform
Parietal	966	0.001	3.51	3.14	-12	-56	40		left precuneus and superior parietal
	131	0.004	2.9	2.68	-10	-58	12		left precuneus
	7	0.037	1.86	1.79	18	-70	60		right superior parietal
S1 & S2	8	0.012	2.38	2.25	-56	-12	46	3	left postcentral
	5	0.014	2.32	2.19	62	-4	36		right postcentral
Occipital	589	0.005	2.78	2.57	-38	-70	30	39	left mid occipital and angular
	91	0.007	2.6	2.43	50	-68	26		right mid occipital

ADD VS HSDD Deactivations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Cingulate	11	0.03	1.96	1.88	-14	46	16	10	left anterior cingulum
Frontal	22	0.005	2.78	2.57	30	14	54	8	right frontal (mid + sup)
	34	0.029	1.98	1.9	-22	20	54		left superior frontal
	22	0.005	2.78	2.57	30	14	54	8	right frontal (mid + sup)
	34	0.029	1.98	1.9	-22	20	54		left superior frontal
	8	0.032	1.92	1.85	-8	-26	68		left paracentral lobule
Thalamus and caudate	6	0.007	2.62	2.44	-28	-14	-2	putamen	left putamen
	6	0.035	1.88	1.81	8	20	-4		right caudate
	5	0.03	1.96	1.89	-10	18	4	caudate head	left caudate
Subthalamic & Brainstem	24	0.031	1.94	1.87	2	0	-10		right brainstem
Temporal	28	0.014	2.32	2.19	-64	-18	-10	21	left mid temporal
	36	0.02	2.16	2.05	50	10	-26	38	right mid temporal pole
	17	0.024	2.07	1.98	28	-66	52	7	right superior temporal
	57	0.008	2.58	2.41	-56	0	-16	38	left mid temporal and superior pole
	84	0.01	2.49	2.34	56	-12	-14		right mid temporal
Amygdala & HippoC	748	0.004	2.9	2.67	38	-22	-18		right hippocampus, parahippocampus and lingual
Parietal	70	0.014	2.33	2.2	-18	-60	56		left superior parietal and inferior temporal
S1 & S2	134	0.003	2.96	2.72	-38	-36	64		left postcentral
	40	0.004	2.91	2.69	42	-32	44	40	right postcentral
	20	0.009	2.55	2.38	-62	-10	30	4	left postcentral
	47	0.01	2.46	2.31	62	-10	32		right postcentral

ADD VS HSDD Deactivations during VAS temperature stimulus left hand continued

Occipital	389	0.002	3.23	2.94	-24	-82	36	19	left occipital (sup + mid)
	305	0.003	2.97	2.73	20	-88	36	19	right superior occipital
	100	0.006	2.7	2.52	40	-84	16	19	right mid occipital
	138	0.008	2.56	2.4	-50	-70	-12	19 + 37	left inferior occipital and temporal
	17	0.032	1.93	1.85	-36	-88	14		left mid occipital
	8	0.035	1.88	1.81	-28	-74	-4		left lingual
	45	0.036	1.87	1.8	0	-64	12		left calcarine

HSDD VS ADD Deactivations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Frontal	169	0.008	2.58	2.41	-30	22	40	9	left mid frontal
	127	0.013	2.37	2.24	0	58	32	10	left frontal (superior medial and sup)
	12	0.039	1.82	1.76	28	32	34	9	right mid frontal
Temporal	7	0.009	2.5	2.35	-44	-16	-22		left inferior temporal
Amygdala & HippoC	31	0.005	2.74	2.55	-16	-14	-28		left parahippocampus
	15	0.031	1.94	1.87	26	-4	-30		right parahippocampus
Parietal	12	0.008	2.59	2.43	58	-60	28		right angular
	33	0.016	2.26	2.14	-32	-62	26	39	left angular
	60	0.022	2.1	2.01	-52	-58	28		left angular
	39	0.024	2.08	1.98	-14	-54	40		left precuneus
	7	0.039	1.83	1.77	14	-38	4	corpus callosum	right precuneus
Occipital	8	0.026	2.03	1.94	-48	-78	28	39	left mid occipital

ADD VS IBS Deactivations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Frontal	37	0.002	3.11	2.84	-18	6	50	32	left superior frontal
	109	0.003	2.96	2.72	-8	-28	68	6	left paracentral lobule
	8	0.031	1.94	1.86	-16	40	20	9	left superior medial frontal
Thalamus and caudate	678	0	4.08	3.55	-12	20	4	caudate head	left and right caudate and frontal
Temporal	210	0.002	3.08	2.82	52	10	-24	38	right mid temporal pole
	14	0.019	2.17	2.07	-52	0	-18		left mid temporal
	10	0.022	2.11	2.01	56	-6	-14		right superior temporal
Amygdala & HippoC	3937	0	4.31	3.71	26	-40	4		right hippocampus, cingulum (post) and heschl
S1 & S2	43	0.012	2.38	2.25	-30	-34	64		left post and precentral
	18	0.022	2.12	2.02	-34	-28	40		left postcentral
	17	0.03	1.96	1.88	-28	-44	56		left postcentral
Occipital	2678	0	4.31	3.71	-22	-84	32	7+18	left occipital (superior and mid)

IBS VS ADD Deactivations during VAS temperature stimulus left hand

Brain regions	Cluster p(FDR)	Peak punc	Peak p(FDR)	Peak T equiv Z	X	Y	Z	Brodmann's area	AAL
Frontal	587	0.002	3.21	2.92	-2	58	32	10	left frontal (superior medial and superior)
	45	0.016	2.27	2.15	-30	36	42	9	left mid frontal
	23	0.017	2.25	2.13	-48	28	2		left inferior tri frontal
	29	0.021	2.14	2.04	-44	22	44		left frontal (mid and inferior tri)
	6	0.024	2.07	1.97	-34	14	34		left mid frontal
	6	0.031	1.95	1.87	24	46	42	9	right superior frontal
Cerebellum	34	0.015	2.29	2.17	6	-48	0	29	vermis (4,5) and right lingual
Temporal	35	0.003	3.02	2.77	-62	-20	-14		left mid temporal
Amygdala & HippoC	43	0.01	2.48	2.33	-26	-26	-16		left parahippocampus
Parietal	115	0.002	3.2	2.91	-30	-70	56	7	left superior parietal
	494	0.011	2.45	2.3	-8	-52	42	7	left precuneus
	5	0.03	1.96	1.88	6	-56	66		right precuneus
S1 & S2	7	0.014	2.33	2.2	-56	-16	48	3	left postcentral
Occipital	326	0.001	3.49	3.13	-18	-64	8		left calcarine and precuneus
	72	0.017	2.24	2.13	-48	-78	28		left mid occipital and angular

6.6 Covariate analysis of the brain activity during the VAS temperature stimulus

6.6.1 VAS score and actual VAS temperature °C analysis

Table A6.61 Covariates analysis of the brain activity during the painful VAS stimulus using the post-scanning VAS pain score out of 10 and the actual ‘VAS’ temperature, which was applied to the left foot (A) or left hand (B). ↑ = significant activation, ↓ significant deactivation, ↑ ↓ both significant activation and deactivations within the same brain region.

(A) Foot stimulus

		VAS SCORE				Temperature (°C)			
Area	Side	ADD	LSDD	HSDD	IBS	ADD	LSDD	HSDD	IBS
SS	S1	L							
		R							
	S2	L							
		R		↑	↑				
SS	Post-Ins	L				↑	↑	↑	
		R				↑	↑		↑
Aff.	Mid-Ins	L						↑	
		R							↑
Aff.	Ant-Ins	L							↑
		R			↑			↓	↑
Aff.	ACC	L			↓				
Aff.	MCC	L						↑	
		R		↓	↑				↑
Aff.	PCC	L							↑
		R					↓		
Aff.	Medial PFC	L		↓	↓	↓			
		R		↓	↑	↑	↓		
DNIC	Lateral PFC	L		↓			↓	↑	
		R		↑	↑		↓	↑	↑
DNIC	Orbito-FC	L		↓				↑	↑
		R			↑	↓			↑
SS	Lentiform Nuclei and Thalamus	L						↑ ↓	
		R			↑				
Aff.	Amygdala (Hippocampus)	L			↑				
		R					↑	↑	
	Cerebellum	L		↑	↑ ↓			↑	
		R							↓
	Inferior Parietal	L		↓					
		R			↑		↓		
	Temporal	L	↑ ↓	↑	↑	↑ ↓	↑	↑	↑
		R	↑		↑ ↓	↑	↑	↑	
	Motor SMA	L							
		R							
		L							
		R	↑	↑				↑	↑
SS	Post-central Gyrus	L		↓	↓				
		R		↑			↓		
DNIC	Subthalamic/Brainstem		↑	↑		↓		↑	

Table A6.6.1 Covariates analysis of the brain activity during the painful VAS stimulus using the post-scanning VAS pain score out of 10 and the actual ‘VAS’ temperature, which was applied to the left foot (A) or left hand (B).

(B) Hand stimulus

		VAS SCORE				Temperature (oC)			
Area	Side	ADD	LSDD	HSDD	IBS	ADD	LSDD	HSDD	IBS
SS	S1	L							
		R							
SS	S2	L							↓
		R		↑			↑		↓
SS	Post-Ins	L		↓					
		R		↑	↑			↑	
SS	Mid-Ins	L							
		R		↑	↑				
Aff.	Ant-Ins	L	↑				↑	↑	↓
		R	↑		↑				
Aff.	ACC	L			↑				↓
		R				↓			↑
Aff.	MCC	L			↓		↓	↑↓	↑
		R		↑		↑		↑	↑
Aff.	PCC	L							
		R							
Aff.	Medial PFC	L		↓		↓			↓
		R		↑↓		↓			↑
DNIC	Lateral PFC	L	↑		↓	↓	↑↓		↓
		R	↑	↑	↑	↑		↓	↑
DNIC	Orbito-FC	L							↑↓
		R		↓			↑		
SS	Lentiform Nuclei and Thalamus	L	↑					↑	↑
		R	↑↓		↑	↓			↑
Aff.	Amygdala (Hippocampus)	L	↓			↓		↓	
		R	↓			↓			
Cerebellum		L				↑			↑
		R	↑↓		↑↓	↓			↑
Inferior Parietal		L	↓		↓		↑		
		R							
Temporal		L	↑↓	↓	↓	↓		↑↓	↓
		R	↓	↑↓	↑↓	↓	↑	↓	↓
Motor		L							
		R							
SMA		L	↑	↑					
		R		↑	↓				↑
SS	Post-central Gyrus	L		↑		↓	↑	↓	
		R		↑		↑		↑	
DNIC	Subthalamic/Brainstem		↑↓			↓		↓	

6.6.2 Hospital anxiety and depression score covariate analysis

Below are simplified fMRI results comparing activations (↑) and deactivations (↓) correlating with participant's HAD anxiety and depression scores used as the stimulus on the foot (A) and hand (B) during the stimulus (uncorrected $p < 0.01$) (Table A6.5.2). All effects were identified using group maps as a mask for the data.

Table A6.6.2 Covariates analysis of the brain activity during the pain heat VAS stimulus in the left foot (A) and left hand (B) using HAD questionnaire scores

(A) Foot stimulus

Area	Side	Anxiety				Depression			
		ADD	LSDD	HSDD	IBS	ADD	LSDD	HSDD	IBS
SS	S1	L							
		R							
SS	S2	L							
		R							
SS	Post-Ins	L							
		R							
SS	Mid-Ins	L							
		R							
Aff.	Ant-Ins	L			↑				↑
		R			↑				
Aff.	ACC	L							
		R			↓				
Aff.	MCC	L		↑					↓
		R	↓		↓				
Aff.	PCC	L							
		R							
Aff.	Medial PFC	L	↑		↑				↓
		R			↑				↑
DNIC	Lateral PFC	L			↑				↑
		R						↑	↑
DNIC	Orbito-FC	L						↑	↑
		R	↑						↑
SS	Lentiform Nuclei and Thalamus	L			↑				
		R	↑			↑			
Aff.	Amygdala (Hippocampus)	L							
		R			↓				
Cerebellum		L			↓			↓	↑
		R	↓	↓	↓			↓	↑
Inferior Parietal		L			↑				↑
		R							↑
Temporal		L	↓						
		R			↑			↑	↓
Motor	SMA	L							
		R							
		L							
		R							
SS	Post-central Gyrus	L		↓					
		R						↓	↑
DNIC	Subthalamic/Brainstem								

Table A6.6.2 Covariates analysis of the brain activity during the pain heat VAS stimulus in the left foot (A) and left hand (B) using HAD questionnaire scores

(B) Hand stimulus

		Anxiety				Depression			
Area	Side	ADD	LSDD	HSDD	IBS	ADD	LSDD	HSDD	IBS
SS	S1	L							
		R							
SS	S2	L							
		R							
SS	Post-Ins	L		↑					
		R			↑				
SS	Mid-Ins	L			↑	↑			
		R							
Aff.	Ant-Ins	L							
		R		↑	↑	↑			
Aff.	ACC	L							
Aff.	MCC	R	↓	↑	↑		↑		
		L	↓	↑	↑	↓		↑	
Aff.	PCC	R	↑	↓	↑↓	↓	↑		
		L							
Aff.	Medial PFC	R	↑					↓	↓
		L		↑				↓	↓
DNIC	Lateral PFC	L	↑	↓	↑		↑	↓	
		R	↑	↑↓	↑↓		↑	↑	↓
DNIC	Orbito-FC	L					↑	↑	↓
		R	↑				↑	↑↓	
SS	Lentiform Nuclei and Thalamus	L		↑	↓	↑		↑	↓
		R		↑	↓	↑		↑	↓
Aff.	Amygdala (Hippocampus)	L			↑				↓
		R				↑			↓
	Cerebellum	L	↑	↑		↑		↑	↓
		R		↑				↑	↓
	Inferior Parietal	L	↑	↓					
		R	↑	↑			↑		
	Temporal	L			↑↓				↓
		R		↓	↑↓	↓			↑↓
	Motor	L							
		R							
	SMA	L	↑				↑		
		R							
SS	Post-central Gyrus	L	↓	↑↓	↓	↓	↓	↑	↓
		R			↓	↓			↓
DNIC	Subthalamic/Brainstem		↑		↑↓				

6.6.3 Pain catastrophizing and Physiological health questionnaire 12 score covariate analysis

Below are simplified fMRI results comparing activations (↑) and deactivations (↓) correlating with participant’s PHQ12 and PC scores used as the stimulus on the foot (A) and hand (B) during the stimulus (uncorrected $p < 0.01$) (Table A6.5.3). All effects were identified using overall group maps as a mask for the data.

Table A6.6.3 Covariates analysis of the brain activity during the pain heat VAS stimulus in the left foot (A) and left hand (B) using PCS and PHQ12 questionnaire scores. (A) Foot stimulus

Area	Side	PCS				PHQ12			
		ADD	LSDD	HSDD	IBS	ADD	LSDD	HSDD	IBS
SS	S1	L							
	R								
SS	S2	L							
	R	↑							
SS	Post-Ins	L							
	R								
Mid-Ins	L								
	R								
Aff.	Ant-Ins	L		↑					
	R				↑			↑	↑
Aff.	ACC	L		↑	↓			↑	
R				↑	↓		↑	↑	
Aff.	MCC	L					↑	↑	↓
	R	↑		↑↓	↑		↑	↑↓	↓
PCC	L							↑	
	R	↓						↑	
Aff.	Medial PFC	L	↓	↑	↑↓	↓		↓	
	R			↑				↑	↑
DNIC	Lateral PFC	L			↑↓		↓	↑↓	
	R	↑							↑
DNIC	Orbito-FC	L				↓			
	R			↑	↓			↑	↑
SS	Lentiform Nuclei and Thalamus	L		↑		↑		↑	↑
	R	↓		↑	↑		↑	↑	
Aff.	Amygdala (Hippocampus)	L		↑	↑		↓	↑	
	R						↓		↑
Cerebellum	L	↓	↓	↑	↑		↑		
	R	↑		↓				↓	
Inferior Parietal	L			↓				↓	
	R								
Temporal	L	↑	↑↓	↑↓				↓	
	R	↑		↑↓	↓	↓		↓	↑
Motor	L								
	R								
SMA	L								
	R	↑						↑	
SS	Post-central Gyrus	L	↓		↑↓		↑↓	↑↓	
	R		↓	↓	↓			↓	↓
DNIC	Subthalamic/Brainstem		↑	↑	↑		↑	↑	↓

Table A6.6.3 Covariates analysis of the brain activity during the pain heat VAS stimulus in the left foot (A) and left hand (B) using PCS and PHQ12 questionnaire scores. (B) Hand stimulus

		PCS				PHQ12			
Area	Side	ADD	LSDD	HSDD	IBS	ADD	LSDD	HSDD	IBS
SS	S1	L							
		R							
	S2	L							
		R							
SS	Post-Ins	L						↓	
		R						↑	
Aff.	Mid-Ins	L		↑				↑↓	↑
		R		↑			↑	↑	
Aff.	Ant-Ins	L		↑		↑		↑	
		R	↑	↑		↑	↑	↑	↑
Aff.	ACC	L		↓				↑↓	
		R	↑	↑				↑↓	
Aff.	MCC	L				↑	↑		↑↓
		R	↑	↑↓		↑	↑↓	↑	
Aff.	PCC	L							
		R	↑						
Aff.	Medial PFC	L	↑	↓	↓		↑	↓	
		R		↑			↑		
DNIC	Lateral PFC	L		↑			↓	↑↓	
		R	↓	↑↓	↑		↑	↑	↑
DNIC	Orbito-FC	L				↑			
		R		↑			↑		
SS	Lentiform Nuclei and Thalamus	L	↑	↑↓	↑		↑	↓	↑
		R	↑	↓			↑	↓	↑
Aff.	Amygdala (Hippocampus)	L		↓				↑↓	
		R		↑			↑	↑↓	↑
	Cerebellum	L	↑	↑	↑		↑	↑	↑
		R			↓	↓		↑	↓
	Inferior Parietal	L						↑	
		R		↑				↑	
	Temporal	L	↑↓		↓	↑		↓	↓
		R	↑↓	↑↓	↓	↓		↑↓	↓
	Motor	L							
		R							
	SMA	L					↑		↑
		R	↑		↓	↑		↑	
SS	Post-central Gyrus	L		↓	↑↓			↑	↓
		R	↑			↓	↑	↑↓	↓
DNIC	Subthalamic/Brainstem		↑		↑↓			↑	

6.7 Intergroup analysis of the brain activity between the IBS and SDD groups during the Cue stimulus

6.7.1 Inter-Group Analysis: Differences between cue stimuli for IBS and SDD groups.

Table A6.6.1 are simplified significant results of 2 sample t test comparing activations (↑) and deactivations (↓) between the SDD groups for the visual cue (Uncorrected $p < 0.05$, voxel threshold 5) (Table R2.15). The first two columns on the left are areas where there is a significant probability that activations and deactivations are greater in the IBS group compared to the SDD groups (IBS>SDD). In the two right columns are areas where there is a significant probability that activations and deactivations are less in the IBS group compared to the SDD groups (SDD>IBS).

Table A6.7.1 Inter-Group Analysis: Differences between cue stimuli for IBS and SDD groups.

Area		Side	IBS> LSDD	IBS> HSDD	LSDD> IBS	HSDD> IBS
SS	S1	L				
		R				
	S2	L				
		R				↑
SS	Post-Ins	L		↓		
		R		↓	↓	
	Mid-Ins	L				
		R			↑	
Aff.	Ant-Ins	L				
		R	↑		↑	
Aff.	ACC	L			↑	
Aff.	MCC	R	↑	↑↓		
		L			↓	
	PCC	R			↑↓	↓
		L				
Aff.	Medial PFC	L			↓	↓
		R		↓	↓	↓
DNIC	Lateral PFC	L			↑	↓
		R	↓	↓		
DNIC	Orbito-FC	L				
		R	↑			
SS	Lentiform Nuclei and Thalamus	L	↑	↓	↑↓	↑
		R	↑		↑	↑
Aff.	Amygdala (Hippocampus)	L			↓	
		R		↓	↓	
	Cerebellum	L		↑	↓	
		R		↑	↑↓	
	Inferior Parietal	L				
		R				
	Temporal	L	↓	↑	↓	
		R	↑	↑↓	↓	↑
	Motor	L				
		R				
	SMA	L	↑	↑	↑	
		R			↑	
SS	Post-central Gyrus	L				↑
		R		↓		
DNIC	Subthalamic/ Brainstem					↓

6.8 Covariate analysis of the brain activity during the Cue stimulus

6.8.1 Hospital anxiety and depression score covariate analysis

Below are simplified fMRI results comparing activations (↑) and deactivations (↓) correlating with participant's HAD anxiety and depression scores during the cue stimulus (uncorrected $p < 0.01$) (Table A6.7.1). All effects were identified using group maps as a mask for the data.

Table A6.8.1 Cue stimulus: HAD questionnaire scores

		Anxiety				Depression			
Area	Side	ADD	LSDD	HSDD	IBS	ADD	LSDD	HSDD	IBS
SS	S1	L							
		R							
	S2	L							
		R				↓			↑
SS	Post-Ins	L			↓				
	R				↓			↑	
Mid-Ins	L							↑	
	R							↑	
Aff.	Ant-Ins	L		↑	↓			↑	↑
	R							↑	
Aff.	ACC	L		↑			↑	↑	
	R			↑			↑		
Aff.	MCC	L						↑	↓
	R			↓	↑			↑	
PCC	L						↑		
	R								
Aff.	Medial PFC	L		↑	↓				
	R			↓				↓	
DNIC	Lateral PFC	L	↑	↓			↓		
	R	↓		↓	↓			↑	
DNIC	Orbito-FC	L		↓			↑		
	R			↑			↑		
SS	Lentiform Nuclei and Thalamus	L		↓				↑	↑
	R							↑	
Aff.	Amygdala (Hippocampus)	L						↑	
	R	↓		↑	↓				
Cerebellum	L				↓				
	R	↑						↑	
Inferior Parietal	L		↓	↑			↓		
	R						↓	↑	
Temporal	L		↓	↑	↓				
	R		↓		↑	↓		↑	↑
Motor	SMA	L							
		R							
	SMA	L							
		R							
SS	Post-central Gyrus	L	↓				↓		
	R	↓					↓		↓
DNIC	Subthalamic/Brainstem			↑	↓			↑	↓

6.8.2 Pain catastrophizing and Physiological health questionnaire 12 score covariate analysis

Below are simplified fMRI results comparing activations (↑) and deactivations (↓) correlating with participant’s PCS and PHQ12 scores during the cue stimulus (uncorrected p<0.01) (Table A6.7.2). All effects were identified using group maps as a mask for the data.

Table A6.8.2 Cue stimulus: PCS and PhQ12 scores

		PCS				PHQ12			
Area	Side	ADD	LSDD	HSDD	IBS	ADD	LSDD	HSDD	IBS
SS	S1	L							
		R							
	S2	L							
		R							
SS	Post-Ins	L							
		R				↓			↓
Aff.	Mid-Ins	L							
		R							
Aff.	Ant-Ins	L					↓		
		R					↑		
Aff.	ACC	L		↑					
		R		↑					
Aff.	MCC	L		↑			↑	↑	
		R		↑	↑			↑	
Aff.	PCC	L							
		R							
Aff.	Medial PFC	L		↓	↓				↑
		R		↓	↓		↑		↓
DNIC	Lateral PFC	L			↓			↓	
		R				↑↓	↑		↑
DNIC	Orbito-FC	L		↑			↑		↑
		R		↑			↑	↑	
SS	Lentiform N. and Thalamus	L		↓	↓		↑	↓	↓
		R	↑	↓	↓	↑	↑		↑
Aff.	Amygdala (Hippocampus)	L			↓				↓
		R					↓	↓	↑↓
	Cerebellum	L			↓				
		R			↓			↑	
	Inferior Parietal	L					↑		
		R							
	Temporal	L			↓		↓	↓	↑↓
		R			↓	↑	↑↓	↓	↑
	Motor	L							
		R							
	SMA	L							↑
		R							↑
SS	Post-central Gyrus	L							
		R			↓			↓	
DNIC	Subthalamic/Brainstem		↓						

6.9 Patient diary sheets and Bristol Stool Chart

6.9.1 Front sheet

Nottingham University Hospitals 
NHS Trust

Version 2, 25th of June 2007

Name: Study Number:

Wolfson
Digestive
Diseases
Centre

Mesalazine in symptomatic
diverticular disease

PATIENT DIARY

Please record

1. Any unusual symptoms during the study period.
 2. Any use of non-prescription or newly prescribed medication
 3. Your symptoms and stool frequency for the week prior to your next visit or the two-weeks prior to the first visit.
-

6.9.2 Instructions

Version 2, 23rd of June 2007

Name: Study Number:

Have you used any other medications apart from your regular medication during the last week?

Please list below with dates and reason

Medication Name	Date of Use	Indication

The next page is for you to record your daily symptoms and stool frequency and form. Your symptoms can be graded using the following scale.

Scoring Instructions

Pain: Score 0-10 (0= none, 5=medium, 10= severe)

Bloating: Score 0-10 (0= none, 5=medium, 10= severe)

Global Well Being (how you feel overall): _____

Score, 0-10(0=very bad, 5=medium, 10=very good)

Your stool form can be graded using the scale given below.

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

6.9.3 Bristol Stool Chart

Bristol Stool Chart

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on the surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. Entirely Liquid

6.9.4 Diary Sheet

Patient ID

Bristol Stool Diary - Objective Assessment

Day	Hours of Pain	Abdominal Pain Severity 0-10	Straining Yes/No	Sense of Incomplete Evacuation Yes/No	Bloating 0-10	Global Well Being 0-10	STOOL TIME AND FORM											
							1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	8 th				
1							Form											
2							Time											
3							Form											
4							Time											
5							Form											
6							Time											
7							Form											
							Time											

6.9.5 Other symptoms

Version 2, 23rd of June 2007

Name: Study Number:

Please record any unusual symptoms other than those listed in the daily diary (for example; headache, dizziness, aches and pains etc). Please discuss any new or worsening medical problems with your study doctor.

Symptom	Start Date	Stop Date	Symptom Severity (tick one)
			<input type="checkbox"/> Mild <input type="checkbox"/> Moderate <input type="checkbox"/> Severe
			<input type="checkbox"/> Mild <input type="checkbox"/> Moderate <input type="checkbox"/> Severe
			<input type="checkbox"/> Mild <input type="checkbox"/> Moderate <input type="checkbox"/> Severe
			<input type="checkbox"/> Mild <input type="checkbox"/> Moderate <input type="checkbox"/> Severe
			<input type="checkbox"/> Mild <input type="checkbox"/> Moderate <input type="checkbox"/> Severe

6.10 FRAME Laboratory Standardized protocols

6.10.1 Method for the simultaneous preparation of RNA from cells and tissues

Preparation of reagents

- Bromo-3-Chloropropane (Sigma-Aldrich USA Pcode 1000840974 B9673)
- Sodium acetate (2M pH4) (Made in house from stocks (Sigma-Aldrich USA Pcode 1000564120) and treated with DEPC)
- Isopropanolol (HPLC Grade; Fisher Scientific P/7507/PB17)
- DEPC- treated water (Diethyl pyrocarbonate Sigma-Aldrich USA D5758)

Preparation of RNA from tissue by phenol-chloroform extraction

1. 50mg of frozen tissue was transferred to a 5ml polypropylene snap-cap tube (Falcon N.J. USA 352063) containing 2ml of ice-cold TRI reagent®(Sigma Aldrich USA Pcode101078497 T9424).
2. The tissue was homogenised (polytron homogeniser Janke and Kunkel Ultra Turrax T25) for 15-30 seconds at room temperature.
3. The homogenate was incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes.
4. 0.4ml of 1-Bromo-3-Chloropropane was added to the lysate and mixed by vigorous shaking.
5. The sample was centrifuged at 10,000g for 15 minutes at 4°C.(Beckman Coulter Allegra X-226 centrifuge)
6. The aqueous phase of the sample was then transferred to two fresh 1.5ml polypropylene snap cap eppendorf tubes.
7. The RNA was precipitated from the aqueous phase by the addition of 0.125ml of Sodium acetate (2M pH4) and 0.35ml of isopropanolol. After thorough mixing the final solution was stored for at least 30 minutes at -20°C.
8. The precipitated RNA was collected by centrifugation at maximum speed in an IEC (international equipment company) microfuge (model 3593 MA USA) at 4°C.

9. The RNA pellet was then washed twice with 70% ethanol, centrifuged as in step 8 each time. After washing the ethanol was allowed to evaporate, but not to dry completely by leaving on the bench uncovered for 5 minutes.
10. 50µl of DEPC- treated water was added to the washed RNA pellet and the then heated to 65°C for 5 minutes before being stored at -80°C.

6.10.2 RNA cleanup

Preparation of reagents

This method was performed using the RNeasy kit (Qiagen USA Cat No 74106) as per manufactures instructions. All buffers are part of the kit

Ethanol absolute (Sigma-Aldrich USA UN 1170)

1. Buffer RLT

10µl of β-Mercaptoethanol was added to each 1ml of Buffer RLT required in a fume hood.

2. Buffer RPE

The supplied concentrate was diluted in 4 volumes of 96-100% of ethanol.

3. DNAase I

10µl of DNAase I stock solution was diluted in 70µl of Buffer RDD and mixed gently by inverting the tube and briefly centrifuged to collect residual liquid from the sides of the tube before storing on ice until use.

1. 250µl of 100% ethanol was added to the RNA preparation obtained in step 10 of the preceding method and mixed thoroughly by pipetting.
2. The sample was then applied to an RNeasy mini column contained in a 2ml collection tube. The tube was closed gently and centrifuged at 10,000g at 20-35°C for 15s. The flow through and collection tube were then discarded.
3. 350µl buffer RW1 was added to the RNA easy spin column and centrifuged as in step 2. The flow through was discarded.
4. 80µl of the DNAase I incubation mix was added to the RNeasy column and incubated at room temperature for 15 minutes.

5. 350µl of buffer RW1 was then added to the RNAeasy spin column. And centrifuged as in step 2. The flow through was discarded.
6. The RNAeasy column was transferred to a fresh 2ml collection tube where 500µl of buffer RPE was added before centrifuging as in step 2.
7. A further 500µl of buffer RPE was added to the RNA easy column and centrifuged for 2 minutes at 10,000g.
8. The RNAeasy column was transferred to a fresh 2ml collection tube and centrifuged at 10,000g for 1 minute.
9. The column was transferred to a 1.5ml collection tube for the final elution step. 30µl of RNase-free water was added to the column and centrifuged for 1 minute at 10,000g.
10. The concentration of the RNA was estimated by measuring the absorbance at 260nm of an aliquot of the final preparation.

6.10.3 Quantitative RT-PCR Protocol

Preliminary Steps

Primers and probes for the target gene and for a reference gene (usually a housekeeping gene) can be ordered as a kit or designed using Primer Express 2. Extensive explanation on how to design primers and probes is given both in “TaqMan Universal Master Mix” protocol by ABI and in “Primer Express 2” user manual.

The amplicon (PCR product) should span an intron-exon boundary in order to avoid the amplification of a false positive product. Primers and probes should be blasted (BLAST N) in order to ensure that the chosen sequence is specific for the gene of interest.

Dilute primers and probes to 10 µM and store in smaller aliquots. Wrap probes' tubes with foil as they are light sensitive.

Always test primers with a DNA template before ordering probes. Primers should be tested with PCR reaction, using cycle parameters similar to the ones that will be used in the quantitative PCR:

- 95°C 10 mins
 - 95°C 15 sec
 - 60°C 1 min
 - 72°C 30 sec
 - 72°C 5 mins
- } x40

Run a gel with PCR products along with an appropriate DNA ladder to check the amplicon's correct size.

6.10.4 Reverse Transcription PCR

RNA can be prepared with the Trizol method (Invitrogen), with the solution D method or with the mRNA extraction kit (Invitrogen). Always use RNAase free tips while handling RNA.

Purified total/mRNA concentration is detected with the NanoDrop machine. Same amount of RNA (usually 1µg total RNA or 100ng mRNA) from different samples will be used as a template for RT-PCR synthesis of first strand cDNA.

Use either M-MLV reverse transcriptase or Superscript:

(a) M-MLV Reaction

- 1µg total RNA /100ng mRNA + 1µl Random Primers (as they come) + DEPC water to 15 µl. *Incubate at 70 °C for 5 mins then put on ice.*

- Add to the reaction mix:

RT Buffer 5X 5µl

dNTP (10mM) 1.25µl

RNAase inhibitor 0.5µl

M-MLV 1µl

DEPC water 2.25µl

FINAL REACTION VOL: 25µl

Incubate at 37 ° for 60 mins.

- Dilute 4X by adding 75µl HPLC water (This will be the **NEAT**).

(b) Superscript

Preparation of reagents

1. Random primers (Promega W.I. USA C118A)
2. Superscript III Reverse Transcriptase (200 unit/ μ l) (Invitrogen USA Cat No 18080-093)
 - 5* First-Strand Buffer
 - 0.1 M DTT
3. RNaseOUT™ (Invitrogen USA Cat No 10777-019)
4. dNTPs

1. 500ng of RNA was added to 1.5ul random primers and 1.5ul of dNTPs in a nuclease-free 0.5ml eppendorf and made up to a total volume of 19.5ul with DEPC treated water.
2. The tube was vortexed and pulsed in a centrifuge (IEC) for 5 seconds at 4°C to collect the contents.
3. Samples were heated to 65°C for 5 minutes to allow RNA dissociation and binding of random primers
4. 6ul 5X First-Strand Buffer, 1.5ul 0.1 M DTT, 1.5ul RNaseOUT™ Recombinant RNase Inhibitor were added to each tube.
5. 1.5ul of Superscript III RT (200 unit/ μ l) and the contents mixed by gentle pipetting.
6. The reaction was then incubated at 25°C for 5 minutes, at 50°C for 60 minutes before the reaction was stopped by heating to 70°C for 15 minutes on a Biometra TRIO-Thermobloc (No 9402208)
7. cDNA was stored at -20°C

6.10.5 cDNA standards

Serial dilutions of a standard cDNA are required in order to quantify relative concentrations of the target and reference gene in the samples. It is possible to use two different relative measurements: the relative standard curve method or the comparative Ct method. As a reference read: “Guide to performing relative quantitation of gene expression using real-time quantitative PCR” by ABI.

A mix of cDNAs from different samples or a cDNA from a sample believed to express the gene of interest can be used as a standard.

Dilutions of the standard and the samples need to be determined empirically. As a starting point use a 4-fold serial dilution of the NEAT for the standard, and dilute 5 or 10 times the NEAT for the samples.

(a) Setting up a TAQMAN 96-well plate

Serial dilutions of the standard cDNA and a non-template control (NTC) must be run for both the reference and the target gene in order to construct two standard curves. Run each standard dilution and each dilution of the sample in triplicates. When testing primers and probes for the first time, samples can be run in two dilutions in order to have a better possibility of using the correct one. This is a typical plate for a TaqMan reaction:

Neat	Neat	Neat	1:4	1:4	1:4	1:16	1:16	1:16	1:64	1:64	1:64
1:256	1:256	1:256	NTC	NTC	NTC						
Neat	Neat	Neat	1:4	1:4	1:4	1:16	1:16	1:16	1:64	1:64	1:64
1:256	1:256	1:256	NTC	NTC	NTC						
5X	5X	5X	5X	5X	5X						
Sample 1	Sample 1	Sample 1	Sample 2	Sample 2	Sample 2						
10X	10X	10X	10X	10X	10X						
Sample 1	Sample 1	Sample 1	Sample 2	Sample 2	Sample 2						
5X	5X	5X	5X	5X	5X						
Sample 1	Sample 1	Sample 1	Sample 2	Sample 2	Sample 2						
10X	10X	10X	10X	10X	10X						
Sample 1	Sample 1	Sample 1	Sample 2	Sample 2	Sample 2						

The reaction mixture for the reference gene is in red, the one for the target gene is in yellow.

- Prepare one master mix for the reference gene and one for the target gene for the numbers of wells required:

TAQMAN Rox-UDG Mix		13	μl	
FW Primer	(10 μM)	0.75	μl	
REV Primer	(10 μM)	0.75	μl	
PROBE	(10 μM)	0.5	μl	
HPLC Water		5	μl	
	Total Volume	20	μl	(per each well)

- Add 20 μl of the correct master mix in each well keeping the plate on ice
- Add 5 μl of the cDNA standards and samples
- Seal the plate with transparent film and place the rubber cover on the top. Keep the plate on ice and put the lid on the box in order to protect from the light
- Turn the TaqMan machine on and set up the plate document with ABI software. A “rough guide on how to use TaqMan” is in D53 in the Protocols’ Book.
- The reaction volume is usually set to 50 μl and must be changed to 25 μl
- The reaction will approximately take between 1 and 2 hours depending on the machine
- Refer to “Guide to performing relative quantitation of gene expression using real-time quantitative PCR” by ABI in order to analyze the results. Data can be exported from the ABI software as an excel file

6.11 Gene cards

6.11.1 Genes selected for gene card

IPA = Ingenuity Pathway analysis (Applied Biosystems, California, USA)

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
ACTB	Beta-Actin	Hs99999903_m1	Housekeeper			
ALOX12	12-Lipoxygenase	Hs00167524_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
ALOX15	15-Lipoxygenase	Hs00609608_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
ALOX15B	15-Lipoxygenase B	Hs00153988_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
ALOX5	5-Lipoxygenase	Hs01095330_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Literature, Planned LCMS	⁶¹⁰	
ALOX5AP	Arachidonate 5-Lipoxygenase activating protein	Hs00233463_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
BDKRB2	Bradykinin receptor 2	Hs00176121_m1	Neuropeptides: Inflammation	IPA linked, Literature	^{583, 723, 724}	

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
CALCA	Calcitonin-related polypeptide alpha	Hs01100741_m1	Neuropeptides: Inflammation cytokines	IPA linked		
CALCB	Calcitonin-related polypeptide beta	Hs00265194_m1	Neuropeptides: Inflammation cytokines	IPA linked		
CCL13	Chemokine (C-C motif) ligand 13 or MCP-4	Hs00237013_m1	Inflammation: chemokines	Literature	725	
CCL11	Chemokine (C-C motif) ligand 13 or eotaxin-1	Hs00234646_m1	Inflammation: chemokines	Literature	726	
CCL2	Chemokine (C-C motif) ligand 2 or Monocyte Chemotactic protein 1 (MCP-1)	Hs00234140_m1	Inflammation: chemokines	Literature	727	
CMKLR1	Chemokine-like receptor 1 or ChemR23	Hs01386064_m1	Inflammation: chemokines	Literature	728	
CNR2	Cannabinoid receptor 2	Hs00361490_m1	Inflammation: Endocannabinoids	Literature	729-731	
CRHR	Corticotrophin receptor	Hs00366363_m1	Neuropeptides: Inflammation cytokines	Literature	256, 732	
CYP2J2	Cytochrome P450, family 2, subfamily J	Hs00356035_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Literature	733	

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
EPHX2	Epoxide hydrolase 2 (SEH)	Hs00157403_m1	Arachidonic acid: Eicosanoid signaling	IPA linked		
F2RL1	Protease activated receptor 2 PAR2	Hs00173741_m1	Inflammation: PAR signaling	Literature	665, 734	
F2RL3	Protease activated receptor 4 PAR4	Hs00559732_m1	Inflammation: PAR signaling	Literature	665	
FPR2	Formyl peptide receptor 2,	Hs02759175_s1	Inflammation and Arachidonic acid signaling	Literature	735, 736	Primers not cross exon boundary
GALR1	Galanin receptor 1	Hs00175668_m1	Neuropeptides: Inflammation	Prev. Work	80, 104	
GALR2	Galanin receptor 2	Hs00605839_m1	Neuropeptides: Inflammation	IPA linked		
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Hs02800695_m1	Housekeeper			
HTR3A	5HT 3A receptor	Hs00356082_m1	Serotonin pathway	Prev. Work	605, 606, 608	
HTR3B	5HT 3B receptor	Hs00175775_m1	Serotonin pathway	Prev. Work	606, 608	
HTR4	5HT 4 receptor	Hs00410577_m1	Serotonin pathway	Prev. Work	608	
ICAM1	Intercellular adhesion molecule 1	Hs00164932_m1	Cell migration pathway	Literature	737	
IFNG	Interferon, gamma	Hs00989291_m1	Inflammation: Cytokine and interferon pathway	IPA linked		
IL10	Interleukin 10	Hs00961622_m1	Inflammation: Cytokine	IPA Linked		

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
IL13	Interleukin 13	Hs00174379_m1	Inflammation: Cytokine	Literature	738, 739, 740, 741	
IL17A	Interleukin 17A	Hs00174383_m1	Inflammation: Cytokine	Literature	725, 741, 742	
IL1B	Interleukin 1b	Hs00174097_m1	Inflammation: Cytokine	Literature	543, 743-745	
IL1RN	Interleukin 1 receptor antagonist	Hs00893625_m1	Inflammation: Cytokine	IPA linked		
IL6	Interleukin 6	Hs00174114_m1	Inflammation: Cytokine	Prev. Work	104, 740	
IL8	Interleukin 8	Hs00174103_m1	Inflammation: Cytokine	Literature	739, 740, 742, 743, 746	
KITLG	KIT ligand	Hs00241497_m1	Inflammation: chemokines	IPA linked		
LTA4H	Leukotriene A4 synthase	Hs00168505_m1	Arachidonic acid: Eicosanoid signaling	IPA linked		
LTB4R	Leukotriene B4 receptor	Hs00609525_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Literature	728	
LTC4S	Leukotriene C4 synthase	Hs00168529_m1	Arachidonic acid: Eicosanoid signaling	IPA linked		
MAdCAM1	Mucosal addressin cell adhesion marker-1	Hs00175533_m1	Cell migration pathway	Literature	747	

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
MCL-1	Myeloid cell leukaemia sequence 1	Hs01050896_m1	Inflammation: chemokines	IPA linked		
MGLL	Monoglyceride lipase	Hs00200752_m1	Inflammation: endocannabinoids	IPA linked		
MMP2	Matrix metalloproteinase 2	Hs00968305_m1	Cell migration pathway	Literature	748	
MMP9	Matrix metalloproteinase 9	Hs00957562_m1	Cell migration pathway	Literature	552, 748	
MUC1	Mucin 1, cell surface associated	Hs00159357_m1	Inflammation: barrier function	Literature	749, 750	
MUC3A	Mucin 3, cell surface associated	Hs03649367_mH	Inflammation: barrier function	Literature	750	
MYD88	Myeloid differentiation primary response protein 88	Hs00182082_m1	Inflammation: Toll receptor pathway	Literature	745, 751, 752	
NAPEPLD	N-acyl phosphatidylethanolamine phospholipase D	Hs00419593_m1	Inflammation: Endocannabinoids	Literature	599	
NGF	Nerve growth factor (beta polypeptide)	Hs00171458_m1	Inflammation: multiple	Literature	591, 730, 731, 753-756	
NGFR	Nerve growth factor receptor	Hs00609976_m1	Inflammation: multiple	IPA linked		
NOD2	Nucleotide-binding oligomerization domain containing 2	Hs00223394_m1	Inflammation: Toll receptor pathway and PPAR pathway	Literature	757-760	
NOS2	Nitric oxide synthase 2, inducible	Hs01075529_m1	Inflammation: Cytokine	Literature	609, 761, 762	
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1	Hs00176787_m1	Inflammation: multiple	IPA linked		

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
PDE4B	Phosphodiesterase 4B,	Hs00387320_m1	Arachidonic acid: Eicosanoid signaling	IPA linked		
PDE4D	Phosphodiesterase 4D,	Hs00174810_m1	Arachidonic acid: Eicosanoid signaling	IPA linked		
PLA2	Phosphatidolipase	Hs00179898_m1	Arachidonic acid: Eicosanoid signaling	IPA linked		
PPARG	Peroxisome proliferator-activated receptor gamma	Hs01115513_m1	PPAR signaling	Literature	⁷⁴⁹	
PTGER1	Prostaglandin E receptor 1	Hs00168752_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
PTGER3	Prostaglandin E receptor 3	Hs00168755_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
PTGES	Prostaglandin E synthase	Hs01115610_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
PTGES2	Prostaglandin E synthase 2	Hs00228159_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
PTGS1	Prostaglandin-endoperoxide synthase 1	Hs00377726_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
PTGS2	Prostaglandin-endoperoxide synthase 2	Hs00153133_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
RPLP0	Ribosomal protein, large, P0,Gene	Hs99999902_m1	Housekeeper			
SELE	E-selectin	Hs00174057_m1	Cell migration pathway	Literature	747 763	
SLC6A4	Serotonin transporter	Hs00169010_m1	Serotonin signaling	Literature	607, 608, 764, 765	
SOD1	Superoxide dismutase 1	Hs00533490_m1	Inflammation: oxidative stress	Literature	766, 767	
TACR1	Tachykinin receptor 1	Hs00185530_m1	Neuropeptides: Inflammation	Prev. Work, Literature	591 575, 768	
TACR2	Tachykinin receptor 2	Hs00169052_m1	Neuropeptides: Inflammation	Prev. Work, Literature	768	
TBXA2R	Thromboxane A2 receptor	Hs00169054_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
TBXAS1	Thromboxane A synthase 1 (platelet)	Hs01022706_m1	Arachidonic acid: Eicosanoid signaling	IPA linked, Planned LCMS		
TGFB1	Transforming growth factor beta	Hs00998130_m1	Inflammation: Cytokine - Growth factor	Literature	739	
TGFBR1	Transforming growth factor, beta receptor 1	Hs00610318_m1	Inflammation: Cytokine - Growth factor	IPA linked		

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	Hs00559660_m1	Inflammation: Cytokine - Growth factor	IPA linked		
TJP1	Tight junction protein 1 (zona occludens 1)	Hs01551876_m1	Inflammation: barrier function	Literature	629, 769	
TJP2	Tight junction protein 2 (zona occludens 2)	Hs00910541_m1	Inflammation: barrier function	IPA linked		
TLR2	Toll like receptor 2	Hs00152932_m1	Inflammation: Toll receptor pathway	Literature	739, 770	
TLR4	Toll like receptor 4	Hs00152939_m1	Inflammation: Toll receptor pathway	Literature	739, 745, 751, 752, 770, 771	
TLR5	Toll like receptor 5	Hs00152825_m1	Inflammation: Toll receptor pathway	Literature	739, 745, 771	
TLR7	Toll like receptor 7	Hs00152971_m1	Inflammation: Toll receptor pathway	Literature	739, 745	
TLR8	Toll like receptor 8	Hs00607866_mH	Inflammation: Toll receptor pathway	Literature	739	
TLR9	Toll like receptor 9	Hs00152973_m1	Inflammation: Toll receptor pathway	Literature	122, 739	

Name	Other names	Assay ID	Pathway	Evidence	References	Notes
TNF	Tumor necrosis factor	Hs99999043_m1	Inflammation: Cytokine	Prev. Work Literature	¹⁰⁴	
TNFSF10A	Tumor necrosis factor (ligand) superfamily, member 10A	Hs00269492_m1	Inflammation: Cytokine	IPA linked		
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	Hs00921974_m1	Inflammation: Cytokine	IPA linked		
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	Hs00353710_s1	Inflammation: Cytokine	Literature	^{772, 773}	No primers that cross exons
TOLLIP	Toll interacting protein	Hs00184085_m1	Inflammation: Toll receptor pathway	IPA linked		
TPH1	Tryptophan hydroxylase 1	Hs00188220_m1	Serotonin pathway	Prev. Work	^{605, 608}	
TRPA1	Transient receptor potential ankyrin 1	Hs00175798_m1	Inflammation: TRVP pathway	Literature	^{774, 775}	
TRPV1	Transient receptor potential vanilloid 1	Hs00218912_m1	Inflammation: TRVP pathway	Literature	^{590, 591}	
TRPV4	Transient receptor potential vanilloid 4	Hs01099348_m1	Inflammation: TRVP pathway	Literature	^{665, 734, 776}	
VCAM1	Vascular cell adhesion marker-1	Hs01003372_m1	Cell migration pathway	Literature	⁷⁴⁷	

6.12 Coefficients of variation for histology assessment

6.12.1 5HT

SD= standard deviation

Slide	Count	Area (mm2)	Cells	Perimeter (um)	Cells/area	
SR67-10-5HT	1.00	0.01	1.00		68.03	
	2.00	0.01	1.00		69.44	
	3.00	0.01	1.00		70.92	
	4.00	0.01	1.00		71.43	
	5.00	0.01	1.00		68.49	
	6.00	0.01	1.00	555.00	72.99	
	7.00	0.01	1.00	564.00	71.94	
	8.00	0.01	1.00	585.00	68.03	
	9.00	0.01	1.00	561.00	70.92	
	10.00	0.01	1.00	560.00	71.94	
	mean		0.01		565.00	70.41
	SD		0.00		11.64	1.79
	Coefficient of variation		0.03		0.02	0.03
	Reproducibility		97.44		97.94	97.46
SR81-10-5HT	1.00	0.03	2.00		66.89	
	2.00	0.03	2.00		66.45	
	3.00	0.03	2.00		67.34	
	4.00	0.03	2.00		67.57	
	5.00	0.03	2.00		65.36	
	6.00	0.03	2.00	876.00	66.89	
	7.00	0.03	2.00	877.00	66.01	
	8.00	0.03	2.00	879.00	68.73	
	9.00	0.03	2.00	884.00	67.80	
	10.00	0.03	2.00	890.00	66.01	
	mean		0.03		881.20	66.90
	SD		0.00		5.81	1.00
	Coefficient of variation		0.01		0.01	0.01
	Reproducibility		98.51		99.34	98.51

6.12.1 5HT continued

Slide	Count	Area (mm ²)	Cells	Perimeter (um)	Cells/area	
SR257-10-5HT	1.00	0.01	5.00		357.14	
	2.00	0.01	5.00		344.83	
	3.00	0.01	5.00		349.65	
	4.00	0.01	5.00		375.94	
	5.00	0.01	5.00		354.61	
	6.00	0.01	5.00	604.00	342.47	
	7.00	0.01	5.00	608.00	352.11	
	8.00	0.01	5.00	609.00	354.61	
	9.00	0.01	5.00	609.00	347.22	
	10.00	0.01	5.00	604.00	352.11	
	mean		0.01		606.80	353.07
	SD		0.00		2.59	9.26
	Coefficient of variation		0.03		0.00	0.03
	Reproducibility		97.47		99.57	97.38
SR173-10-5HT	1.00	0.03	3.00		106.76	
	2.00	0.03	3.00		99.67	
	3.00	0.03	3.00		102.04	
	4.00	0.03	3.00		101.35	
	5.00	0.03	3.00		99.34	
	6.00	0.03	3.00	978.00	103.09	
	7.00	0.03	3.00	986.00	102.39	
	8.00	0.03	3.00	1030.00	102.04	
	9.00	0.03	3.00	982.00	101.35	
	10.00	0.03	3.00	973.00	102.04	
	mean		0.03		989.80	102.01
	SD		0.00		22.98	2.04
	Coefficient of variation		0.02		0.02	0.02
	Reproducibility		98.04		97.68	98.00
SR201-10-5HT	1.00	0.02	3.00		126.05	
	2.00	0.02	3.00		120.97	
	3.00	0.02	3.00		122.95	
	4.00	0.02	3.00		132.74	
	5.00	0.02	3.00	790.00	125.52	
	6.00	0.02	3.00	781.00	128.21	
	7.00	0.02	3.00	790.00	127.66	
	8.00	0.02	3.00	793.00	125.00	
	9.00	0.02	3.00	776.00	127.12	
	10.00	0.02	3.00	780.00	127.12	
	mean		0.02		785.00	126.33
	SD		0.00		6.87	3.17
	Coefficient of variation		0.02		0.01	0.03
	Reproducibility		97.50		99.12	97.49
Overall coefficient of variance Mean		0.02		0.01	0.02	
Reproducibility		97.79		98.73	97.77	

6.12.2 CD68

Slide	CD68		Lamina Propria			
	Count	LP Area (mm2)	Cells	Perimeter (um)	cells/area	
SR162-10-CD3	1.00	0.02	21.00	971.00	1390.73	
	2.00	0.01	18.00	858.00	1267.61	
	3.00	0.01	18.00	873.00	1267.61	
	4.00	0.01	19.00	893.00	1366.91	
	5.00	0.01	20.00	907.00	1398.60	
	6.00	0.02	20.00	892.00	1333.33	
	7.00	0.01	20.00	921.00	1418.44	
	8.00	0.01	21.00	901.00	1438.36	
	9.00	0.02	20.00	962.00	1315.79	
	10.00	0.01	20.00	1000.00	1369.86	
	mean		0.01	19.70	917.80	1356.72
	SD		0.00	1.06	45.72	59.41
	Coefficient of variation		0.03	0.05	0.05	0.04
	Reproducibility		96.87	94.62	95.02	95.62
SR198-10-CD3	1.00	0.02	20.00	697.00	1315.79	
	2.00	0.02	19.00	698.00	1158.54	
	3.00	0.02	20.00	694.00	1298.70	
	4.00	0.02	19.00	672.00	1117.65	
	5.00	0.02	21.00	678.00	1320.75	
	6.00	0.02	20.00	690.00	1250.00	
	7.00	0.02	20.00	669.00	1219.51	
	8.00	0.02	21.00	686.00	1272.73	
	9.00	0.02	20.00	676.00	1204.82	
	10.00	0.02	21.00	668.00	1346.15	
	mean		0.02	20.10	682.80	1250.46
	SD		0.00	0.74	11.62	74.77
	Coefficient of variation		0.04	0.04	0.02	0.06
	Reproducibility		96.41	96.33	98.30	94.02
SR194-10-CD3	1.00	0.02	29.00	862.00	1502.59	
	2.00	0.02	25.00	729.00	1404.49	
	3.00	0.02	25.00	771.00	1428.57	
	4.00	0.02	28.00	830.00	1609.20	
	5.00	0.02	25.00	861.00	1213.59	
	6.00	0.02	31.00	889.00	1483.25	
	7.00	0.02	29.00	896.00	1450.00	
	8.00	0.02	31.00	861.00	1550.00	
	9.00	0.02	30.00	877.00	1304.35	
	10.00	0.02	31.00	870.00	1527.09	
	mean		0.02	28.40	844.60	1447.31
	SD		0.00	2.55	53.90	117.72
	Coefficient of variation		0.09	0.09	0.06	0.08
	Reproducibility		91.11	91.03	93.62	91.87

6.12.2 CD68 continued

Slide	Count	LP Area (mm ²)	Cells	Perimeter (mm)	Cells/area	
SR175-10-CD3	1.00	0.02	31.00	1.12	1371.68	
	2.00	0.02	32.00	1.11	1516.59	
	3.00	0.02	33.00	1.21	1617.65	
	4.00	0.02	38.00	1.28	1759.26	
	5.00	0.02	36.00	1.22	1565.22	
	6.00	0.02	32.00	0.87	1600.00	
	7.00	0.02	36.00	1.27	1531.91	
	8.00	0.02	36.00	1.25	1565.22	
	9.00	0.02	38.00	1.24	1652.17	
	10.00	0.02	37.00	1.20	1644.44	
	mean		0.02	34.90	1.18	1582.41
	SD		0.00	2.64	0.12	101.88
	Coefficient of variation		0.06	0.08	0.10	0.06
	Reproducibility		94.48	92.43	89.71	93.56
Overall coefficient of variance Mean		0.05	0.06	0.06	0.06	
Reproducibility		94.72	93.60	94.16	93.77	

6.12.3 CD3

CD3		Epithelium				Lamina Propria				
Slide	Count	Epi Area (mm2)	Cells	Perimeter (um)	Cells/area	LP Area (mm2)	Cells	Perimeter (um)	Cells/area	
SR70-10-CD3	1.00	0.02	12.00	797.00	677.97	0.02	23.00	714.00	1314.29	
	2.00	0.02	12.00	809.00	677.97	0.02	21.00	715.00	1280.49	
	3.00	0.02	12.00	813.00	670.39	0.02	22.00	739.00	1301.78	
	4.00	0.02	12.00	827.00	655.74	0.02	21.00	723.00	1265.06	
	5.00	0.02	12.00	823.00	648.65	0.02	21.00	747.00	1242.60	
	6.00	0.02	12.00	829.00	634.92	0.02	19.00	723.00	1187.50	
	7.00	0.02	12.00	818.00	662.98	0.02	20.00	731.00	1142.86	
	8.00	0.02	12.00	825.00	659.34	0.02	21.00	751.00	1200.00	
	9.00	0.02	12.00	827.00	655.74	0.02	21.00	722.00	1213.87	
	10.00	0.02	12.00	805.00	670.39	0.02	22.00	745.00	1264.37	
	mean		0.02	12.00	817.30	661.41	0.02	21.10	731.00	1241.28
	SD		0.00	0.00	10.89	13.52	0.00	1.10	13.62	54.43
	Coefficient of variation		0.02	0.00	0.01	0.02	0.03	0.05	0.02	0.04
Reproducibility		97.93	100.00	98.67	97.96	96.88	94.78	98.14	95.62	

6.12.3 CD3 Continued

Slide	Count	Epi Area (mm2)	Cells	Perimeter (um)	Cells/area	LP Area (mm2)	Cells	Perimeter (um)	Cells/area	
SR174-10-CD3	1.00	0.02	10.00	752.00	526.32	0.02	17.00	970.00	809.52	
	2.00	0.02	10.00	758.00	523.56	0.02	19.00	928.00	859.73	
	3.00	0.02	10.00	765.00	510.20	0.02	24.00	906.00	1100.92	
	4.00	0.02	10.00	766.00	518.13	0.02	22.00	908.00	973.45	
	5.00	0.02	10.00	766.00	518.13	0.02	22.00	931.00	1013.82	
	6.00	0.02	10.00	762.00	510.20	0.02	22.00	894.00	964.91	
	7.00	0.02	10.00	766.00	520.83	0.02	22.00	898.00	1023.26	
	8.00	0.02	10.00	733.00	526.32	0.02	21.00	925.00	985.92	
	9.00	0.02	10.00	769.00	500.00	0.02	22.00	902.00	995.48	
	10.00	0.02	10.00	759.00	510.20	0.02	22.00	905.00	986.55	
	mean		0.02	10.00	759.60	516.39	0.02	21.30	916.70	971.36
	SD		0.00	0.00	10.62	8.55	0.00	1.95	22.73	82.33
	Coefficient of variation		0.02	0.00	0.01	0.02	0.03	0.09	0.02	0.08
Reproducibility		98.33	100.00	98.60	98.34	97.40	90.86	97.52	91.52	

6.12.3 CD3 Continued

Slide	Count	Epi Area (mm2)	Cells	Perimeter (um)	Cells/area	LP Area (mm2)	Cells	Perimeter (um)	Cells/area	
SR270-10-CD3	1.00	0.02	20.00	882.00	1169.59	0.08	19.00	633.00	246.11	
	2.00	0.02	21.00	844.00	1354.84	0.07	19.00	641.00	255.38	
	3.00	0.02	20.00	857.00	1250.00	0.07	19.00	642.00	260.27	
	4.00	0.02	20.00	851.00	1307.19	0.08	18.00	639.00	237.15	
	5.00	0.02	20.00	860.00	1298.70	0.08	19.00	647.00	250.66	
	6.00	0.02	20.00	853.00	1290.32	0.08	19.00	634.00	250.00	
	7.00	0.02	20.00	839.00	1273.89	0.07	19.00	639.00	258.15	
	8.00	0.02	20.00	846.00	1183.43	0.07	19.00	658.00	256.76	
	9.00	0.02	20.00	836.00	1257.86	0.08	19.00	654.00	243.28	
	10.00	0.02	20.00	847.00	1257.86	0.07	19.00	642.00	254.35	
	mean		0.02	20.10	851.50	1264.37	0.08	18.90	642.90	251.21
	SD		0.00	0.32	13.07	55.56	0.00	0.32	8.03	7.28
	Coefficient of variation		0.04	0.02	0.02	0.04	0.02	0.02	0.01	0.03
Reproducibility		96.13	98.43	98.46	95.61	97.85	98.33	98.75	97.10	
Overall coefficient of variance Mean		0.03	0.01	0.01	0.03	0.03	0.05	0.02	0.05	
Reproducibility		97.47	99.48	98.58	97.30	97.38	94.66	98.14	94.75	

6.12.4 KI67

Ki67		Epithelium superficial				Epithelium deep						
Slide	Count	Area (mm2)	Cells	Perimeter (um)	cells/area	Count	Area (mm2)	Cells	Perimeter (um)	cells/area		
SR88-10-CD3	1.00	0.01	5.00	531.00	400.00	1.00	0.02	33.00	505.00	2037.04		
	2.00	0.01	5.00	556.00	354.61	2.00	0.02	31.00	506.00	2000.00		
	3.00	0.01	5.00	529.00	378.79	3.00	0.02	34.00	508.00	2098.77		
	4.00	0.01	5.00	578.00	335.57	4.00	0.02	35.00	511.00	2258.06		
	5.00	0.01	5.00		347.22	5.00	0.02	33.00	518.00	2037.04		
	6.00	0.01	5.00	550.00	362.32	6.00	0.02	32.00	522.00	1987.58		
	7.00	0.01	5.00	563.00	335.57	7.00	0.02	32.00	526.00	1963.19		
	8.00	0.01	5.00	557.00	340.14	8.00	0.02	35.00	509.00	2215.19		
	9.00	0.01	5.00	540.00	375.94	9.00	0.02	34.00	521.00	2060.61		
	10.00	0.01	5.00	538.00	364.96	10.00	0.02	34.00	521.00	2060.61		
	mean		0.01	5.00	549.11	359.51	mean		0.02	33.30	514.70	2071.81
	SD		0.00	0.00	16.11	21.12	SD		0.00	1.34	7.69	95.86
	Coefficient of variation		0.06	0.00	0.03	0.06	Coefficient of variation		0.02	0.04	0.01	0.05
Reproducibility		94.26	100.00	97.07	94.12	Reproducibility		97.73	95.98	98.51	95.37	

6.12.4 KI57 Continued

Slide	Count	Area (mm2)	Cells	Perimeter (um)	cells/area	Count	Area (mm2)	Cells	Perimeter (um)	cells/area	
SR196-10-CD3	1.00	0.01	0.00	490.00	0.00	1.00	0.01	22.00	474.00	1560.28	
	2.00	0.01	0.00	484.00	0.00	2.00	0.01	24.00	467.00	1690.14	
	3.00	0.01	0.00	506.00	0.00	3.00	0.01	25.00	466.00	1785.71	
	4.00	0.01	0.00	502.00	0.00	4.00	0.01	25.00	471.00	1851.85	
	5.00	0.01	0.00	506.00	0.00	5.00	0.01	24.00	472.00	1678.32	
	6.00	0.01	0.00	507.00	0.00	6.00	0.01	25.00	472.00	1773.05	
	7.00	0.01	0.00		0.00	7.00	0.01	24.00	471.00	1678.32	
	8.00	0.01	0.00	513.00	0.00	8.00	0.01	26.00	477.00	1805.56	
	9.00	0.01	0.00	507.00	0.00	9.00	0.01	26.00	466.00	1857.14	
	10.00	0.01	0.00	536.00	0.00	10.00	0.01	26.00	479.00	1793.10	
	mean		0.01	0.00	505.67	0.00	mean	0.01	24.70	471.50	1747.35
	SD		0.00	0.00	14.60	0.00	SD	0.00	1.25	4.40	93.32
	Coefficient of variation		0.03		0.03		Coefficient of variation	0.02	0.05	0.01	0.05
Reproducibility		96.65		97.11		Reproducibility	98.02	94.93	99.07	94.66	

6.12.4 KI57 Continued

Slide	Count	Area (um ²)	Cells	Perimeter (um)	cells/area	Count	Area (mm ²)	Cells	Perimeter (um)	cells/area	
SR391-10-CD3	1.00	0.01	7.00	577.00	800.00	1.00	0.01	29.00	555.00	2843.14	
	2.00	0.01	6.00	606.00	650.05	2.00	0.01	29.00	601.00	2929.29	
	3.00	0.01	6.00	615.00	652.88	3.00	0.01	27.00	593.00	3040.54	
	4.00	0.01	7.00	628.00	760.87	4.00	0.01	29.00	605.00	3251.12	
	5.00	0.01	6.00	600.00	653.59	5.00	0.01	28.00	648.00	3001.07	
	6.00	0.01	5.00	589.00	558.04	6.00	0.01	29.00	618.00	3065.54	
	7.00	0.01	5.00	592.00	568.83	7.00	0.01	26.00	629.00	2699.90	
	8.00	0.01	5.00	604.00	535.91	8.00	0.01	25.00	613.00	2564.10	
	9.00	0.01	4.00		459.77	9.00	0.01	30.00		3141.36	
	10.00	0.01	5.00	605.00	518.67	10.00	0.01	29.00	611.00	3059.07	
	mean		0.01	5.60	601.78	615.86	mean	0.01	28.10	608.11	2959.51
	SD		0.00	0.97	14.88	107.84	SD	0.00	1.60	25.66	207.03
	Coefficient of variation		0.03	0.17	0.02	0.18	Coefficient of variation	0.04	0.06	0.04	0.07
	Reproducibility		96.76	82.75	97.53	82.49	Reproducibility	95.73	94.32	95.78	93.00
Overall coefficient of variance	Mean	0.04	0.06	0.03	0.08		0.03	0.05	0.02	0.06	
Reproducibility		95.89	94.25	97.23	92.21		97.16	95.08	97.78	94.35	

