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Food intolerance testing and dietary manipulation in inflammatory bowel disease

A Thesis
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For Rosemary, Miguel & Arana

Declaration of authorship and originality

I, Stephen James Inns, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Stephen James Inns

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Abstract

The aetiology of inflammatory bowel disease (IBD) combines genetic predisposition and environmental factors. In both ulcerative colitis and Crohn disease, patients perceive that diet affects the course of their disease.

This thesis addresses the frequently observed compromise of the epithelial integrity of the gut in IBD and subsequent effect of the luminal content, which makes up the main part of the environmental stimulus, thus introducing the role of diet in IBD. Initially I conducted a survey, demonstrating the current practice of dietary manipulation and exclusion in IBD and irritable bowel syndrome, determining that advice given is generally empiric and that sensitivity testing is infrequently used in practice.

A subsequent observational study compared the occurrence of serum IgG antibodies to foods in IBD patients compared to controls. It showed that IBD is associated with increased serum IgG antibodies to a wide range of foods but that this does not correlate with patient reported food intolerance.

A further study investigated the colonic mucosal response to food antigen exposure, patient reported food intolerances, food specific serum IgG antibodies and intestinal permeability. The mucosal response did not correlate with patients' perception of food intolerance nor alterations in intestinal permeability.

This work reinforces the importance of food intolerance in IBD and attempts to correlate those intolerances to available tests. While gastroenterologists do give dietary advice to their patients with IBD, the available evidence does not allow unequivocal

advice. No objective relationship between patient-perceived food intolerance and hypersensitivity testing was demonstrated.

Future studies should seek to clearly define the association between intolerance tests and patient symptoms, investigate the mechanisms by which such tests might predict intolerance, and investigate the most promising strategies in carefully designed and controlled studies of dietary intervention.

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Abbreviations

51CrEDTA	51Chromium ethylenediamine-tetra-acetic acid
5ASA	5-aminosalicylic acid
AA	Ascorbic acid
AIEC	Adherent/invasive E. coli
ASCA	Anti-Saccharomyces cerevisiae antibodies
AU	Arbitrary unit
BSG	British Society of Gastroenterology
CD	Crohn disease
CDAI	Crohn's disease activity index
CGD	Chronic granulomatous disease
CL	Claudin
COLAP	Colonoscopic allergen provocation
COX	Cyclo-oxygenase
DAI	Disease activity index
DBPCFC	Double-blind placebo-controlled food challenge
DC	Dendritic cells
DSS	Dextran sodium sulfate
ECP	Eosinophil cationic protein
ELISA	Enzyme linked immunosorbent assay
EN	Enteral nutrition
EPO	Eosinophil peroxidase
EPX	Eosinophil protein X
ER	Endoplasmic reticulum
f	Phi
FA	Fatty acid
FODMAPs	Fermentable Oligo-Di-Mono-saccharides and Polyols
GALT	Gut associated lymphoid tissue
GBF	Germinated barley foodstuff
GI	Gastro-intestinal
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPs	General practitioners
GWA	Genome-wide association
H2	Hydrogen
HETE	Hydroxyeicosatetraenoic acid
HEV	High endothelial venules
HPETE	Hydroperoxyeicosatetraenoic acid
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IkB	Inhibitory subunit of NF-kB
IL	Interleukin
IL23R	Interleukin 23 receptor
IP	Intestinal permeability
ITT	Intention to treat analysis
L:M	Lactulose:mannitol
L:R	Lactulose:rhamnose
LAA	Leucocyte ascorbic acid
LCN-omega-3	Long-chain omega-3 fatty acids
LCT	Long chain triglycerides
LDF	Laser Doppler flowmetry

LM	Lactose malabsorption
LOX	Lipoxygenase
LPL	Lamina propria lymphocytes
LPS	Lipopolysaccharide
MAP	Mycobacterium avium subspecies paratuberculosis
MCT	Medium chain triglycerides
MDP	Muramyldipeptide
MHC	Major histocompatibility complex
MUFA	Monounsaturated fatty acids
NF-kB	Nuclear factor kappa B
NOD	Nucleotide-binding oligomerization domain
NS	Non-significant
NZ	New Zealand
NZSG	New Zealand Society of Gastroenterologists
OFER	Open food exclusion and rechallenge
OmpC	Outer membrane protein C
OR	Odds ratio
ORMH	Mantel-Haenszel odds ratio
PCR	Polymerase chain reaction
PCV	Post-capillary venules
PEG	Polyethylene glycol
PG	Prostaglandin
PN	Parenteral nutrition
PUFA	Polyunsaturated fatty acid
r	Pearson's correlation coefficient
RAST	Radioallergosorbent test
RBC	Red blood cell
RDBPCT	Randomised double blind placebo controlled trial
ROS	Reactive oxygen species
RR	Relative risk
SAA	Serum ascorbic acid or
SCFA	Short chain fatty acids
TiO2	Titanium dioxide
TIRAP	Toll–IL-1 receptor (TIR) domain-containing adapter protein
TLC	Thin layer chromatography
TLR	Toll-like receptors
TMG	2-(alpha-D-glucopyranosyl)methyl-2,5,7,8-tetra-methylchroman-6-ol
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumour necrosis factor
TPN	Total parenteral nutrition
TX	Thromboxane
UC	Ulcerative colitis
UCL	University College London
UK	United Kingdom

Chapter I Dietary factors in the aetiology of IBD

I.1 The pathogenesis of inflammatory bowel disease

The exact pathogenesis of inflammatory bowel disease (IBD) has proven elusive. What is clear is that susceptibility to IBD comes from the interaction of genetic predisposition and environmental factors. Mapping of the human genome has allowed genome wide scanning for abnormalities associated with IBD. Isolation of the environmental influences that modify those genetic influences has not been so complete. The interaction of genetic predisposition and environmental pressure has the net effect of causing epithelial barrier dysfunction and dysregulation of the mucosal immune system. Thus there are three essential, interactive cofactors in the pathogenesis of IBD: host susceptibility, largely determined by genetic factors; environmental factors, including the enteric microflora and the diet; and dysregulated mucosal immunity, which combines the mucosal immune system and epithelial barrier function (Fiocchi 1998; Xavier and Podolsky 2007).

This section serves as an overview of the information available regarding the aetiopathogenesis of IBD, prior to an in-depth discussion of the direct influences of diet as a specific environmental factor in the development of IBD. The intention is to place in context the relative importance of genetic, immune, environmental and dietary factors in the causation of Crohn disease and ulcerative colitis.

I.1.1 Dysregulated mucosal immunity

Immune dysregulation in the presence of active IBD is evidenced by changes in the inflammatory milieu within the mucosa of patients with IBD. Whether this represents a primary defect in the regulation of the immune system or a secondary consequence of immune activation is debated.

I.1.1.1 Disturbances in adaptive immunity

In IBD the balance of regulatory and effector cells is disturbed. The pattern of disruption differs somewhat between ulcerative colitis (UC) and Crohn disease (CD), the simplistic model being a greater activation of the Th1 effector T lymphocytes in CD and Th2 lymphocytes in UC, with decreased activity of the Th3 regulatory system in both diseases. The Th1/2 distinction was described initially in the mouse immune system (Mosmann, Cherwinski *et al.* 2005). Mosmann *et al.* characterised two distinct patterns of lymphokine activity in mouse helper T cells. One subset of lymphokines included interleukin-(IL)2, interferon-gamma, granulocyte monocyte-colony stimulating factor (GM-CSF), and IL3 and defined the type 1 T helper cell, the other clone produced distinct lymphokines, in particular IL4, and was designated Th2.

This distinction appears to have some applicability in CD (Niessner and Volk 1995). Neissner *et al.* used quantitative RT-PCR to determine the cytokine mRNA concentrations in the mucosa of patients with IBD. In CD there was greater expression of the Th1 cytokines: interferon (IFN)-γ and interleukin (IL)-2. The pattern of cytokine dysregulation in UC is less clear. There is greater expression of IL-5 and IL-13 present, cytokines more commonly associated with a Th2 response; however, the archetypal Th2 cytokine IL-4 is not upregulated, and greater levels of IFN-γ are seen (Brown and Mayer 2007).

Dysregulation of another immune effector system, the IL23/IL17 axis was recently described (Fujino, Andoh *et al.* 2003). Fujino *et al.* evaluated IL-17 expression in tissue samples and sera of patients with active and inactive IBD. IL-17 was not detected in the tissue or sera of normal individuals, infectious colitis, or ischaemic colitis patients. It was increased in the tissue samples and sera of active IBD patients. IL-17 is an inflammatory cytokine expressed by activated CD4+ T cells, referred to as Th17 cells (Harrington, Hatton *et al.* 2005). IL17 triggers the NF-kB signalling cascade and MAP kinase pathway, leading to T cell proliferation and up-regulation of inflammatory molecules (Hata, Andoh *et al.* 2002).

IL-23 is a cytokine which has been shown to play a strong role in the maintenance and expansion of Th17 cells (Bettelli, Carrier *et al.* 2006; Mangan, Harrington *et al.* 2006; Veldhoen, Hocking *et al.* 2006). The importance of IL23 in the production of intestinal inflammation was demonstrated by Yen *et al.* Using IL10 knockout mice as a model of T cell-mediated IBD, they showed that mice deficient for IL23 had marked suppression of colitis (Yen, Cheung *et al.* 2006). In addition treatment with anti IL-23p19 antibodies can cure established colitis in mice (Elson, Cong *et al.* 2007). Further evidence of the role of IL-23 in IBD comes from genome-wide association studies, which have linked CD to a number of IL-23 pathway genes, notably *IL23R* (interleukin 23 receptor). Similar associations in IL-23 pathway genes have been observed in UC (Abraham and Cho 2009).

I.1.1.2 Disturbed innate immunity

Recent investigation has centred on the role of the innate immune system in both CD and UC. The innate immune system depends on the immediate recognition of highly conserved signals, mostly derived from microbes (Janeway and Medzhitov 2002). The presence of both extracellular and intracellular microbial-associated molecular pattern is

detected by mammalian cells through pattern-recognition molecules, such as the cytosolic nucleotide-binding oligomerization domain containing (NOD)-like receptors and the membrane-bound Toll-like receptors (TLR) (Inohara, Chamaillard *et al.* 2005; Akira, Uematsu *et al.* 2006). The mucosal epithelium and the luminal microflora are in constant communication, resulting in fluxes in the expression of co-stimulatory molecules (Toy, Yio *et al.* 1997), components of the human major histocompatibility complex (MHC) (Hershberg, Framson *et al.* 1997), toll-like receptors (TLRs) (Hershberg 2002), NOD proteins (Kobayashi, Chamaillard *et al.* 2005), inflammatory cytokines (Yang, Eckmann *et al.* 1997), as well as antimicrobial peptides (Canny and Colgan 2005).

Disturbances of the innate immune mechanisms in IBD take the form of alterations in the pattern of TLR expression (Cario and Podolsky 2000), upregulation of NOD2 in intestinal epithelial cells (IECs) and disturbances in antigen recognition and processing by antigen-presenting cells (Berrebi, Maudinas *et al.* 2003).

I.1.1.2.1 TLR expression

Patients with IBD have disturbed innate immune mechanisms of the epithelial layer with alterations in the pattern of TLR expression in the mucosal epithelial cells (Cario and Podolsky 2000). Cario *et al.* characterised the expression pattern of TLR expression in biopsies from the small intestine and colon in patients with IBD as compared with controls. In the IECs of normal mucosa TLR3 and TLR5 were constitutively expressed, conversely TLR2 and TLR4 were only barely detectable. In active CD TLR3 was significantly down regulated in the IECs, but not in UC. In contrast, TLR4 was strongly upregulated in both UC and CD. TLR2 and TLR5 expression remained unchanged in IBD (Cario and Podolsky 2000).

The exact mechanism by which this alteration of expression might contribute to pathogenesis is not known. TLR signalling is crucial in maintaining intestinal homeostasis through regulation of the expression of cytokines, chemokines, and antimicrobial peptides. Mice deficient for MyD88, an essential mediator of TLR signalling, showed increased susceptibility to dextran sodium sulfate-induced colitis (Rakoff-Nahoum, Paglino *et al.* 2004).

Nenci *et al.* (Nenci, Becker *et al.* 2007) provided further elucidation of the underlying mechanism. They showed that deficiency of NF-kappaB, a master regulator of pro-inflammatory responses, led to a spontaneous, chronic inflammatory response in the mouse colon, initially dominated by innate immune cells but later also involving T lymphocytes. In mice deficient for the MyD88 adaptor protein the development of intestinal inflammation was prevented, demonstrating that Toll-like receptor activation by intestinal bacteria was essential for disease pathogenesis in their mouse model. NF-kappaB deficiency also increases susceptibility to infectious colitis in the mouse (Erdman, Fox *et al.* 2001).

In CD, but not UC, this is further supported by the finding that loss of function mutations of TLR2, TLR4, and their intracellular adaptor known as TIRAP [for Toll–IL-1 receptor (TIR) domain-containing adapter protein] confer increased predisposition to CD (Pierik, Joossens *et al.* 2006; De Jager, Franchimont *et al.* 2007).

I.1.1.2.2 Upregulation of NOD2

The NOD2 gene on chromosome 16q12 was the first susceptibility gene for CD to be successfully identified. NOD2 encodes an intracellular receptor predominantly expressed in monocytes and Paneth cells. It has been implicated in the innate immune response to muramyldipeptide (MDP), a component of peptidoglycan in bacterial cell

walls (Zhang, Massey *et al.* 2008). NOD2 activation leads to the activation of NF-kappaB and there is evidence of cross-talk between NOD2 and TLR pathways.

There is upregulation of NOD2 expression in the IECs of patients with IBD (Berrebi, Maudinas *et al.* 2003). However, the CD associated gene defects are associated with a significant diminution in responsiveness to MDP (Kelsall 2005). Thus, the exact mechanism by which defects in TLR and NOD2 expression and function might affect the pathogenesis of IBD remains unclear, but it may be that reduced ability to clear bacteria by innate immune mechanisms leads to immune dysregulation and the initiation of a chronic immune response.

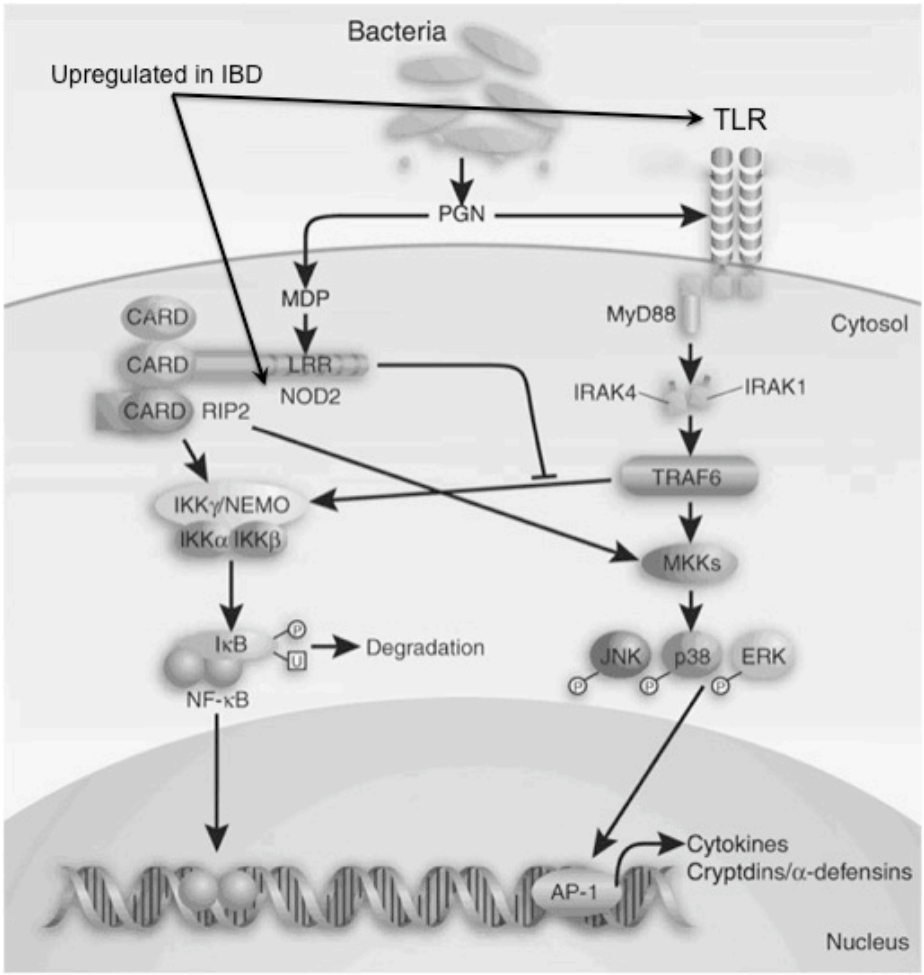


Figure 1. Models of activation and regulation of NOD and TLR pathways by peptidoglycan (adapted from Kelsall 2005)

I.1.1.2.3 Disturbances in antigen recognition

The dendritic cells (DCs) of the intestinal epithelium are the main antigen presenting cells in the gut (Hart, Al-Hassi *et al.* 2005). Their dendrites penetrate between epithelial cells, allowing constant sampling of luminal antigens without any effect on barrier function. Hart *et al.* investigated the properties of intestinal DCs in IBD compared with controls. They demonstrated that there was upregulation of TLR2 and TLR4 expression in DCs from patients with IBD compared with controls. Intestinal DCs in CD patients also expressed significantly higher levels of the maturation/activation marker CD40, and more DCs produced the pro-inflammatory cytokines IL-12 and IL-6 than controls, suggesting that intestinal DCs are activated in CD and produce pathologically relevant cytokines. They concluded that intestinal DCs were likely to be key initiators or perpetuators of the inflammatory response in IBD (Hart, Al-Hassi *et al.* 2005).

I.1.2 Autophagy

The autophagy process is a basic cellular process whereby cellular contents are encapsulated in a double membrane organelle, the autophagosome, and delivered to the lysosome for degradation. A role in the pathogenesis of IBD was not strongly suspected until genome wide association scans identified two genes, ATG16L1 and IRGM, known to be involved in autophagy, that were significantly associated with CD (Hampe, Franke *et al.* 2007; Parkes, Barrett *et al.* 2007; Rioux, Xavier *et al.* 2007; Wellcome Trust Case Control Consortium 2007; Barrett, Hansoul *et al.* 2008).

Functional studies have since shown that chimeric mice with Atg16L1- deficient haematopoietic cells have increased levels of the inflammatory cytokines IL-1b and IL-18 and are very susceptible to colitis induced by treatment with dextran sodium sulfate

(DSS). The DSS-induced colitis can be ameliorated by treatment with antibodies to IL-1b and IL-18 that block the activity of these cytokines (Saitoh, Fujita *et al.* 2008).

In turn these functional studies have drawn attention to the Paneth cell in the aetiology of CD. The role of Paneth cells in CD was previously suspected because NOD2 is highly expressed in these cells (Lala, Ogura *et al.* 2003) and the expression of antimicrobial peptides is reduced in NOD2 knockout mice (Kobayashi, Chamaillard *et al.* 2005) and patients with ileal CD (Wehkamp, Salzman *et al.* 2005). Mice that expressed reduced levels of ATG16L1 protein displayed defects in the Paneth cells of the small intestine (Cadwell, Liu *et al.* 2008). Homozygous deletion of another autophagy gene, Atg5, in the intestinal epithelium of mice also produced abnormal Paneth cells, indicating that these cells can be particularly sensitive to autophagy defects (Cadwell, Liu *et al.* 2008). Most exciting was the finding that CD patients homozygous for the ATG16L risk allele have abnormal Paneth cells in biopsies of uninvolved ileocolonic resection samples (Cadwell, Liu *et al.* 2008).

Less is known about IRGM, the other autophagy gene associated with CD and further functional studies are needed (Parkes, Barrett *et al.* 2007; Barrett, Hansoul *et al.* 2008). Common genetic variations in these and other autophagy genes are likely to have a major impact on the response of the innate immune system to intestinal microbiota and susceptibility to IBD.

I.1.3 Mucosal epithelial barrier function

The barrier function of the intestinal epithelium is crucial to the function of the mucosal immune system. The epithelium is made up of a single layer of IECs that are connected

by tight junctions. They interact with lamina propria DCs, lamina propria lymphocytes (LPL), intraepithelial lymphocytes (IEL) and mediators of the immune and the enteric nervous system (Neutra, Mantis *et al.* 2001).

The IECs are actively involved in immune regulation through expression of costimulatory molecules (Toy, Yio *et al.* 1997) and components of the human major histocompatibility complex (MHC) (Hershberg, Framson *et al.* 1997), toll-like receptors (TLRs) (Hershberg 2002), NOD proteins (Kobayashi, Chamaillard *et al.* 2005), inflammatory cytokines (Yang, Eckmann *et al.* 1997), as well as antimicrobial peptides (Canny and Colgan 2005). The role of antigen presentation and pattern recognition has been discussed above. This section focuses on epithelial barrier function, in particular intestinal permeability (IP), and defects in function that have been associated with IBD.

I.1.3.1 Intestinal permeability

Many methods are available for measuring IP. This section aims to deal with the published data regarding their use in IBD. Further comments as to the technical aspects of their use and their relative attributes are discussed in more detail in the methods section of Chapter V.

Medline was searched using the search terms “inflammatory bowel disease”, “ulcerative colitis”, “Crohn disease” and “intestinal permeability”. In addition the references of identified articles were checked for other appropriate publications. Publications were considered if they compared the results of IP testing in patients with UC or CD to that of a control group.

I.1.3.1.1 51Chromium EDTA intestinal permeability testing in CD

A total of eleven studies using 51Chromium ethylenediaminetetraacetic acid (51CrEDTA) testing in CD were identified. 9 were in adults and 2 in children. The total number of CD patients studied was 321 and the total number of controls 315 (see Table 1)

One study compared the IP of patients with CD depending on the site of disease (Bjarnason, O'Morain *et al.* 1983). This study showed that IP was highest in small bowel and ileocolonic disease, but was also increased in 5 of 11 patients with isolated colonic disease. Three studies demonstrated that permeability was greatest in those with active disease (Resnick, Royal *et al.* 1990; Adenis, Colombel *et al.* 1992; Berstad, Arslan *et al.* 2000). One further study used only radiolabelled 99mTc-diethylenetriaminopentaacetic acid in 16 adult CD patients and 10 controls and also demonstrated increased permeability (Casellas, Aguade *et al.* 1986). Resnick *et al.* used the same technique alongside 51CrEDTA, with similar results from both substrates (Resnick, Royal *et al.* 1990).

All but one study demonstrated increased IP to 51CrEDTA (Howden, Gillanders *et al.* 1994). This study enrolled 10 patients with CD remission who also had a relative who was willing to participate and had no gastrointestinal disease or past surgery. The median 24 hour urinary excretion of 51CR-EDTA was 1.7% in the CD patients compared with 1.35% in the unaffected relatives. This was compared to the study centre's own historical control value of 2% and active small intestinal CD value of 4.33%. Certainly the patient group studied in this report was very carefully selected for inactive disease. This is compared to the positive studies where generally patients with a range of disease activities were studied.

	Patient group	Number of patients	Findings
Bjarnason, O'Morain *et al.* 1983	Adults	28 controls, 10 small bowel CD, 11 ileocolonic CD, 11 CD colitis	Increased permeability in small bowel and ileocolonic CD and 5 of 11 CD colitis patients
Peled, Watz *et al.* 1985	Adults	27 controls, 6 CD	5 of 6 had increased permeability
Turck, Ythier *et al.* 1987	Children	7 control adults, 11 control children, 17 CD children	Increased permeability compared to controls
Ainsworth, Eriksen *et al.* 1989	Adults	28 controls, 15 CD	Increased permeability compared to controls
Resnick, Royal *et al.* 1990	Adults	11 controls, 35 CD	Increased permeability compared to controls
Adenis, Colombel *et al.* 1992	Adults	13 controls, 22 CD	Increased permeability compared to controls
Teahon, Smethurst *et al.* 1992	Adults	25 controls, 28 CD	Increased permeability compared to controls
Issenman, Jenkins *et al.* 1993	Children	26 paediatric controls, 51 active paediatric CD, 80 adult controls, 63 adults with active CD	Increased permeability compared to controls
Howden, Gillanders *et al.* 1994	Adults	10 CD with inactive disease (and 10 unaffected relatives)	No difference in permeability between patients and historical or related controls
Berstad, Arslan *et al.* 2000	Adults	18 IBS, 17 CD	Increased permeability compared to controls, permeability increased with increasing disease activity
Suenaert, Bulteel *et al.* 2005	Adults	31 controls, 25 active CD	Increased permeability compared to controls

Table 1. Studies of intestinal permeability in Crohn disease using 51Chromium EDTA

I.1.3.1.2 Polyethylene glycol intestinal permeability testing In CD

The results from studies that used polyethylene glycol (PEG) as a substrate for measuring IP are much more varied. A total of nine studies were identified. Eight were in adults and one in children.

Three studies showed a decrease in IP to PEG in CD, two studies showed an increase in IP and four studies showed no difference between CD patients and controls. One study that demonstrated a decrease in IP in CD was only able to do so for small bowel CD but not colonic CD (Teahon, Smethurst *et al.* 1992). In one study PEG was also instilled directly into the colon (Olaison, Sjodahl *et al.* 1989). This showed the greatest absorption of PEG across the colonic mucosa in those subjects with colonic CD.

	Patient group	**Number of patients**	**Findings**
Magnusson, Sundqvist *et al.* 1983	Adults	24 controls, 24 CD	Decreased permeability compared to controls
Olaison, Sjodahl *et al.* 1989	Adults	14 controls, 59 CD (15 colonic, 44 ileal)	Decreased permeability compared to controls
Teahon, Smethurst *et al.* 1992	Adults	25 controls, 28 CD	Decreased permeability in small bowel CD but not colonic CD compared to controls
Jenkins, Goodacre *et al.* 1986	Adults	40 controls, 15 CD	Increased permeability compared to controls
Hollander, Vadheim *et al.* 1986	Adults	17 controls, 11 CD	Increased permeability compared to controls
Zellweger, Freiburghaus *et al.* 1990	Adults	24 controls, 12 CD	No difference in permeability between patients and controls
Ruttenberg, Young *et al.* 1992	Adults	31 controls, 45 CD	No difference in permeability between patients and controls
Lindberg, Soderholm *et al.* 1995	Children	30 controls, CD 33	No difference in permeability between patients and controls
Munkholm, Langholz *et al.* 1994	Adults	31 controls, 47 CD	No difference in permeability between patients and controls

Table 2. Studies of intestinal permeability in Crohn disease using polyethylene glycol (ordered by result then year)

I.1.3.1.3 Lactulose:mannitol intestinal permeability testing In CD

The most extensively investigated technique for the assessment of IP is the lactulose:mannitol (L:M) absorption test. A total of 23 studies comparing the L:M ratio in CD patients to control subjects were identified. All but two showed an increase in the L:M excretion ratio (Munkholm, Langholz *et al.* 1994; Halme, Turunen *et al.* 2000).

The study by Munkholm *et al.* considered a relatively large sample for such studies (Munkholm, Langholz *et al.* 1994). There were 31 controls and 47 CD patients. The L:M analysis technique used was similar to a number of previous studies. Their samples were blindly analysed by the laboratory used in a previous, often quoted study that did find an increased L:M excretion ratio in CD (Hollander, Vadheim *et al.* 1986). However, the patients they tested had relatively mild disease with 75% having a Crohn's disease activity index (CDAI) of less than 150. In addition most patients were not receiving corticosteroids or immunosuppressive agents. Interestingly they did find a slight positive correlation between the activity of the disease and the L:M ratio. It is possible that the result of this study deviated from that of the majority of similar studies because of the clinical activity of the patient group selected.

The study by Halme *et al.* included a smaller sample of 22 patients with an exacerbation of CD and 10 healthy controls (Halme, Turunen *et al.* 2000). Patients on no medication or only on 5-ASA or sulphasalazine as maintenance treatment were included. The median L:M ratio was 0.037 (range 0.01 ± 0.260) in patients and 0.030 (range 0.004 ± 0.063) in controls (N.S.). In a comparison between activity indices and the permeation of test solutions, the L:M ratio showed significant correlation with endoscopic activity. In this series, 54% of the patients had an elevated L:M ratio, which is comparable with results from a previous study which found an increased overall L:M ration in CD

(Wyatt, Vogelsang *et al.* 1993). Thus, in this small study with similar proportions of patients with increased L:M ratios to previous positive studies but a relative increase in the L:M ratio in CD patients compared to controls which did not reach significance and a positive correlation between endoscopically assessed disease activity and L:M ratio, there is the possibility that type II error is responsible for the negative finding.

	Patient group	Number of patients	Findings
Pearson, Eastham *et al.* 1982	Children	31 controls, 8 CD	Increased permeability compared to controls
Ukabam, Clamp *et al.* 1983	Adults	16 controls, 13 ileal CD, 7 colonic CD	Increased permeability compared to controls
Andre, Andre *et al.* 1988	Adults	100 controls, 47 CD	Increased permeability compared to controls and permeability increased with disease activity
Katz, Hollander *et al.* 1989	Adults	29 controls, 25 CD	Increased permeability compared to controls
Murphy, Eastham *et al.* 1989	Children	31 controls, 17 CD	Increased permeability compared to controls and permeability increased with disease activity
May, Sutherland *et al.* 1993	Adults	31 controls, 36 CD	Increased permeability compared to controls
van Elburg, Kokke *et al.* 1993	Children	25 controls, 25 CD	Increased permeability compared to controls
Wyatt, Vogelsang *et al.* 1993	Adults	control numbers not given, 72 CD with quiescent disease	Increased permeability compared to controls
Peeters, Geypens *et al.* 1997	Adults	50 controls, 25 CD	Increased permeability compared to controls
Wyatt, Oberhuber *et al.* 1997	Adults	30 controls, 50 CD	Increased permeability compared to controls
Marsilio, D'Antiga *et al.* 1998	Children	30 controls, 10 CD	Increased permeability compared to controls
Puspok, Oberhuber *et al.* 1998	Adults	30 controls, 100 CD	Increased permeability compared to controls
Soderholm, Olaison *et al.* 1999	Adults	29 controls, 39 CD	Increased permeability compared to controls
Secondulfo, de Magistris *et al.* 2001	Adults	32 controls, 16 CD	Increased permeability compared to controls
Fries, Renda *et al.* 2005	Adults	controls 64, CD 29	Increased permeability compared to controls
Buhner, Buning *et al.* 2006	Adults	96 controls, 128 quiescent CD,	Increased permeability compared to controls
Buning, Geerdts *et al.* 2006	Adults	96 controls, 113 CD (CDAI <150)	Increased permeability compared to controls
D'Inca, Annese *et al.* 2006	Adults	35 controls, 115 CD	Increased permeability compared to controls
Benjamin, Makharia *et al.* 2008	Adults	controls 22, CD 125	Increased permeability compared to controls
Cuoco, Vescovo *et al.* 2008	Adults	controls 20, CD 13 active steroid free	Increased permeability compared to controls
Vilela, Torres *et al.* 2008	Adults	controls 15, CD 34	Increased permeability compared to controls
Dastych, Dastych *et al.* 2008	Adults	20 controls, 20 CD active	Increased permeability compared to controls
Halme, Turunen *et al.* 2000	Adults	10 controls, 22 exacerbation IBD	No difference in permeability between patients and controls
Munkholm, Langholz *et al.* 1994	Adults	31 controls, 47 CD	No difference in permeability between patients and controls

Table 3. Studies of intestinal permeability in Crohn disease using lactulose:mannitol (ordered by result then year)

I.1.3.1.4 Lactulose:rhamnose intestinal permeability testing in CD

A total of 6 studies comparing the lactulose:rhamnose (L:R) ratio in CD patients to control subjects were identified. All but one showed an increased in the L:R excretion ratio (Munkholm, Langholz *et al.* 1994), the limitations of this study that might have led to this discrepancy were outlined in section I.1.1.1.1.

	Patient group	**Number of patients**	**Findings**
Sanderson, Boulton *et al.* 1987	Children	6 control children, 14 CD	Increased permeability compared to controls
Katz, Hollander *et al.* 1989	Adults	29 controls, 25 CD	Increased permeability compared to controls
Teahon, Smethurst *et al.* 1992	Adults	25 controls, 28 CD	Increased permeability compared to controls
Miki, Moore *et al.* 1998	Children	36 controls, 12 CD	Increased permeability in active but not quiescent CD compared to controls
Iwata, Nakano *et al.* 2001	Adults	20 controls, 92 CD	Increased permeability compared to controls
Munkholm, Langholz *et al.* 1994	Adults	31 controls, 47 CD	No difference in permeability between patients and controls

Table 4. Studies of intestinal permeability in Crohn disease using lactulose:Rhamnose (ordered by result then year)

I.1.3.1.5 Iohexol intestinal permeability testing in CD

Two studies by the same authors used the radiological contrast agent iohexol as a probe for the determination of IP (Halme, Edgren *et al.* 1997; Halme, Turunen *et al.* 2000). They correlated the results of its use to the use of the L:M test and found the two to positively correlate. Both studies showed an increased excretion of iohexol by patients with CD compared to controls. They found that the urinary excretion of iohexol was significantly higher in active disease than in quiescent disease.

	Patient group	**Number of patients**	**Findings**
Halme, Edgren *et al.* 1997	Adults	16 controls, 40 CD	Increased permeability compared to controls and permeability increased with disease activity
Halme, Turunen *et al.* 2000	Adults	10 controls, 16 active CD	Increased permeability compared to controls

Table 5. Studies of intestinal permeability in Crohn disease using iohexol

I.1.3.1.6 Conclusions regarding intestinal permeability testing in CD

Thus the tests of intestinal permeability most studied in CD are those that use sugars as probes and the majority of these studies have shown increases in intestinal permeability, particularly in the setting of active disease. The same is true of those studies that used 51CrEDTA. Not all studies concur and this may reflect differences in levels of disease activity, disease location, the methodology used in testing and the test substrate. Certainly testing using PEG produces a very different pattern of absorption than the other probes used. It seems likely that PEG probes are more useful in demonstrating changes in permeability related to colonic disease.

While both sugar probes and 51CrEDTA give relatively consistent results in the setting of CD, in practice the requirements for the handling of the Chromium isotope make sugar probes a more pragmatic tool for the investigation of intestinal permeability in CD. It may be that a combination of sugar probes including lactulose, mannitol and rhamnose represents the best available approach to the measurement of intestinal permeability in CD.

I.1.3.1.7 Intestinal permeability testing in ulcerative colitis

Studies of IP in UC are much less numerous and those that exist generally investigated smaller numbers of patients than in CD. The evidence for a permeability defect using 51CrEDTA as the probe is greater than that for those using sugars or PEG as the probe (see table Table 6).

Four studies comparing 51CrEDTA excretion in UC and controls were identified. Two studies performed in the 1980's showed no difference between UC patients and controls (Bjarnason, O'Morain *et al.* 1983; Peled, Watz *et al.* 1985). In the 1985 study by Peled *et al.* the technique itself was called into question by the high rate of positive results from the control population (Peled, Watz *et al.* 1985). Nineteen of 27 patients with diseases thought not to affect the integrity of the small bowel had an abnormal test. All the patients with UC had normal excretion. In the 1983 study by Bjarnason *et al.* all the control patients had normal excretion of Cr51EDTA, as did the patients with UC, but not those with CD (Bjarnason, O'Morain *et al.* 1983).

Two later studies showed increased IP to 51CrEDTA in UC patients. Berstad *et al.* examined consecutive patients with abdominal pain or diarrhoea. They compared the 3 found to have UC to those with CD and those without any evidence of organic disease on endoscopy, abdominal ultrasonography, or barium X-ray examination of the small

bowel. The 3 patients with UC appeared to have higher IP than the patients without evidence of organic disease. Issenman *et al.* performed the largest study in UC to date using Cr51EDTA as the probe in children and adults. In their 1993 study this group examined 24 paediatric and 31 adult patients with active UC and compared them to 26 paediatric controls with recurrent abdominal pain or chronic non-specific diarrhoea and 80 adult controls (Issenman, Jenkins *et al.* 1993). Patients with UC demonstrated increased excretion of 51CrEDTA compared to controls.

Three further studies used non-sugar, non-PEG probes in UC patients. Casellas *et al.* used 99mTc labelled DTPA in 10 control adults and 12 adults with UC (Casellas, Aguade *et al.* 1986). They found that excretion was increased in UC patients compared to controls and was highest in those patients with active disease. Resnick *et al.* also compared 99mTc-DTPA excretion in 11 controls and 21 adult UC patients, showing a significantly greater mean percentage excretion of probe in UC (Resnick, Royal *et al.* 1990). Halme *et al.* used the radiological contrast agent Iohexol as a probe (Halme, Edgren *et al.* 1997). They compared 16 adult controls with 16 UC patients and demonstrated increased excretion of Iohexol in patients with UC.

One of three studies that used sugar probes showed a difference between UC patients and controls and then only in the subgroup of UC patients with active extensive UC (Miki, Moore *et al.* 1998). Miki *et al.* compared paediatric UC patients to historical paediatric and adult controls. In 6 of 7 patients with active extensive UC the L:R ratio was elevated. However, there was no difference between controls and 6 patients with inactive extensive UC. Only 1 of 5 patients with active left sided colitis demonstrated an increased L:R ratio.

None of the three identified studies that used PEG as a probe found a difference between controls and UC patients.

In summary, it appears that the IP changes associated with UC are much less impressive and less consistent than those seen in CD but that the available studies are small and heterogenous in terms of patient group and technique used. The radioisotope probes 99mTc-DTPA and 51CrEDTA may represent the most sensitive probes to changes in permeability and this may reflect their ability to detect changes in colonic permeability.

	Technique	Patient group	Number of patients	Findings in UC
Bjarnason, O'Morain *et al.* 1983	51 chromium EDTA	Adults	28 controls, 10 UC	No difference in permeability between patients and controls
Peled, Watz *et al.* 1985	51 chromium EDTA	Adults	27 control, 3 UC	No difference in permeability between patients and controls
Issenman, Jenkins *et al.* 1993	51 chromium EDTA	Children	26 paediatric controls, 80 adults controls, 24 paediatric UC, 31 adult UC	Increased permeability compared to controls
Berstad, Arslan *et al.* 2000	51 chromium EDTA	Adults	18 IBS, 3 UC	Increased permeability compared to controls
Casellas, Aguade *et al.* 1986	99mTc DTPA	Adults	10 controls, 12 UC	Increased permeability compared to controls and permeability increased with disease activity
Resnick, Royal *et al.* 1990	99mTc DTPA	Adults	11 controls, 21 UC	Increased permeability compared to controls
Halme, Edgren *et al.* 1997	Iohexol	Adults	16 controls, 16 UC	Increased permeability compared to controls
Ukabam, Clamp *et al.* 1983	Mannitol and Lactulose	Adults	16 controls, 7 UC	No difference in permeability between patients and controls
Miki, Moore *et al.* 1998	Lactulose and Rhamnose	Children	36 control, 18 UC	Permeability only increased in active extensive disease compared with controls
Munkholm, Langholz *et al.* 1994	PEG, Lactulose, Rhamnose and Mannitol	Adults	31 controls, 52 UC	No difference in permeability between patients and controls
Jenkins, Goodacre *et al.* 1986	PEG	Adults	40 control, 7 UC	No difference in permeability between patients and controls
Zellweger, Freiburghaus *et al.* 1990	PEG	Adults	24 controls, 8 UC	No difference in permeability between patients and controls

Table 6. Studies of intestinal permeability in ulcerative colitis (ordered by permeability technique used then result)

I.1.3.1.8 Pathogenesis of intestinal permeability defects in CD

Thus the balance of current evidence suggests the epithelial barrier exhibits increased permeability in CD and that permeability may be increased in, at least active, UC. The exact extent of the pathology underlying this disturbance of epithelial integrity is not fully elucidated by current methodology. All the permeability probes used in the studies described above measure predominantly the efficiency of the paracellular route. There are no clinical measures of transcellular permeability currently available (Gibson 2004). In addition molecular studies to date have focussed on the paracellular route. This evidence is summarised here.

The tight junction seals the space between adjacent epithelial cells. In intact gastrointestinal epithelia it is tight junction permeability that is the rate-limiting step that defines overall epithelial permeability (Weber and Turner 2007). Tight junctions are composed of multiple proteins that are involved in establishing the epithelial barrier, and they selectively determine which molecules are able to traverse the paracellular space. The claudin family of proteins has a critical role in selective ion permeability. Their involvement in the pathogenesis of the permeability defect of IBD has been recently demonstrated.

Prasad *et al.* used immunohistochemical techniques to examine the expression of claudins (CL) 2, 3 and 4 in tissue samples from UC and CD patients and control subjects (Prasad, Mingrino *et al.* 2005). They found that the pore-forming protein CL2 was strongly expressed along the inflamed crypt epithelium, whilst absent or barely detectable in normal colon. In contrast, CL 3 and 4 were present throughout normal colonic epithelium and were reduced or redistributed in the diseased surface epithelium. They went on to examine the effect of IL-13 treatment of an epithelial cell monolayer

model of the gut barrier. IL-13, a pro-inflammatory cytokine, produced no observed change in CL 3 and 4 but showed marked increases in CL 2 that correlated with reductions in trans-epithelial resistance.

Heller *et al.* in a study of colonic epithelial cells from UC patients, were also able to show increased expression of CL2 in response to increased IL-13 (Heller, Florian *et al.* 2005). This, in combination with increased numbers of apoptotic cells, led to a reduction in transepithelial resistance.

In another study of tight junction function in IBD Zeissig *et al.* used electron microscopy of epithelium affected by IBD, immunohistochemistry for claudin isoforms and in vitro studies of the effect of IBD related cytokines on intestinal cell barrier function (Zeissig, Burgel *et al.* 2007). Freeze-fracture electron microscopy of tissue from patients with active IBD showed morphological changes in the tight junctions particular to IBD and separate from the effects of gross epithelial damage such as ulceration, crypt abscesses or apoptosis. They also showed that CL2 expression was increased, particularly in the crypt epithelium, in patients with active disease. Additionally the observation that CL2 expression was normal in tissue from patients with inactive disease led the authors to conclude that the claudin expression patterns were likely to be a consequence rather than a cause of active disease. They were also able to demonstrate that CL2 expression was only subtly decreased by Interferon and modestly increased by tumour necrosis factor (TNF). Studies of cultured intestinal epithelial monolayers have demonstrated that TNF induces increases in permeability independent of apoptosis (Bruewer, Luegering *et al.* 2003).

Ultimately results such as those above have led to the conclusion that, while alterations in claudin expression might be important in IBD, TNF actually causes intestinal epithelial barrier dysfunction by mechanisms distinct from altered claudin isoform expression (Weber and Turner 2007). Instead the defect induced appears to relate to cytoskeletal mechanisms of permeability increase, in particular increases in epithelial cell myosin II regulatory light chain phosphorylation (Zolotarevsky, Hecht *et al.* 2002).

I.1.3.2 Defensins and mucus

The IECs produce a plethora of antimicrobial peptides. In themselves these peptides would be ineffective were they not retained in high concentration in the environment close to the epithelium by the action of mucus (McGuckin, Eri *et al.* 2009). The major macromolecular component of intestinal mucus is produced by goblet cells. Into this other compounds are secreted by the epithelium including phospholipids and antimicrobial compounds such as defensins, secreted in granules produced by Paneth cells (McGuckin, Eri *et al.* 2009).

It has long been considered that classical ileal CD results in goblet cell hypertrophy and increased, rather than decreased, mucus formation and so is not a result of decreased mucus layer function (Dvorak, Osage *et al.* 1980; Trabucchi, Mukenge *et al.* 1986). This dogma was challenged by the finding that defensin production by Paneth cells is reduced in CD (Wehkamp, Harder *et al.* 2004; Wehkamp, Salzman *et al.* 2005; Wehkamp, Schmid *et al.* 2005; Wehkamp, Schmid *et al.* 2007). In addition Paneth cell expression of NOD2 was reduced (Lala, Ogura *et al.* 2003; Ogura, Lala *et al.* 2003) and polymorphisms in the defensin gene were associated with CD (Fellermann, Stange *et al.* 2006). The methodology of many these studies relied on polymerase chain reaction (PCR) amplification of RNA from tissue biopsies from patients and controls. The relative abundance of epithelium decreases during intestinal inflammation. Therefore

such studies, based on whole tissue RNA, are fraught with the potential to wrongly attribute a decrease in the relative abundance of epithelial specific RNA to decreased production of molecules per remaining epithelial cell rather than to decrease in the relative abundance of epithelial cells. A recent study which used expression of another epithelial-specific gene, villin, as an indicator of relative defensin production demonstrated increased rather than decreased defensin expression in non-inflamed ileal CD (Simms, Doecke *et al.* 2008). The authors thus concluded that the evidence did not yet support a fundamental decrease in defensin production underlying ileal CD.

In colonic CD the evidence for a reduction in antimicrobial activity is stronger. Nuding *et al.* used a flow cytometric assay to test the antibacterial activity of cationic peptide extracts form colonic biopsies. In CD extracts there was decreased antimicrobial effect against E. coli and E. faecalis compared to UC. These differences were independent of the inflammation status or concurrent steroid treatment (Nuding, Fellermann *et al.* 2007). The same group also reported that there was a decrease in the antimicrobial protease inhibitors SLPI and elafin in inflamed CD tissue compared with inflamed UC tissue (Schmid, Fellermann *et al.* 2007).

Further, in UC there is a reduction in goblet cells, reduced size of goblet cell thecae, decreased MUC2 production (Tytgat, van der Wal *et al.* 1996; Van Klinken, Van der Wal *et al.* 1999), decreased mucin sulfation (Corfield, Myerscough *et al.* 1996; Hanski, Born *et al.* 1999; Van Klinken, Van der Wal *et al.* 1999) and a diminished mucus barrier. Whether these changes are primary or a response to inflammation is contentious. Similar changes are, however, observed in the unaffected proximal intestine of patients with distal UC (McGuckin, Eri *et al.* 2009) and vacuolisation of the endoplasmic reticulum (ER) and Golgi is seen in both inflamed and noninflamed

secretory cells in UC, suggesting that ER stress is occurring (Donnellan 1966; Gonzalez-Licea and Yardley 1966; Nagle and Kurtz 1967; Delpre, Avidor *et al.* 1989).

I.1.3.3 Regulation of reactive oxygen species

The intestinal mucosa is constantly exposed to reactive oxygen species (ROS) that are generated by the luminal contents, oxidized food debris, transition metals such as iron and copper, bacterial metabolites, bile acids and salivary oxidants (Rezaie, Parker *et al.* 2007). The intestinal mucosa is vulnerable to that oxidative stress and oxidant mediated injury plays an important part in the pathophysiology of IBD (Keshavarzian, Morgan *et al.* 1990). Studies in humans have demonstrated increased oxidative stress and decreased antioxidant defenses in IBD mucosa (Lih-Brody, Powell *et al.* 1996; Sido, Hack *et al.* 1998).

I.1.4 Genetic factors

A genetic influence on the pathogenesis of IBD has long been evidenced by twin studies in IBD. The combined concordance rate for IBD of 36% in monozygotic twins and only 4% in dizygotic twins was strong evidence for a genetic basis. This was supported by familial studies that showed an increased risk of both CD and UC in relatives of patients with either of these disorders; the greater risk being in siblings than in other family members (Gaya, Russell *et al.* 2006).

Recent large genome wide association (GWA) enterprises have led to the identification of numerous candidate genes. This is turn has focussed attention on hitherto unsuspected pathological mechanism in the aetiology of IBD. A particular example is that of the IL23/IL17 axis previously described in this chapter (see I.1.2). The techniques used in GWA studies and the findings from these studies have been

extensively reviewed elsewhere (Gaya, Russell *et al.* 2006; Kim and Misra 2007; Parkes, Barrett *et al.* 2007; Barrett, Hansoul *et al.* 2008; Zhang, Massey *et al.* 2008).

I.1.5 Environmental Factors

I.1.5.1 Microbial factors

There is a dynamic balance between microbes, particularly commensal flora, and the host defense response at the mucosal frontier (Xavier and Podolsky 2007). The fact that CD and UC occur in the areas of highest intestinal bacterial concentrations and faecal flow, and that diversion of that flow can reduce the inflammation of CD (Winslet, Allan *et al.* 1994), as well as the observation that CD and experimental colitis in rodent models respond to treatment with antibiotics (Kang, Bloom *et al.* 2008), suggest that this microbe-host interaction may play a pivotal role in the pathogenesis of IBD. How this interaction produces disease has not been clearly defined. There are three main hypotheses:

1. A single luminal pathogen, or functionally altered commensal bacteria, produces IBD.
2. An alteration of the microbial composition in the lumen causes disease.
3. That defective clearance and killing of bacteria leads to immune dysregulation and disease.

The evidence supporting each of these hypotheses is discussed below.

I.1.5.1.1 Pathogens as the cause for IBD

Studies of the microbiota in IBD have failed to demonstrate enrichment of an individual pathogenic species in IBD but theories regarding the presence of such an organism continue to attract attention because of the similarities between CD, UC and enteric infections (Packey and Sartor 2009). There are two main organisms that have attracted

attention with respect to this theory: Mycobacterium avium subspecies paratuberculosis (MAP) and adherent/invasive E. coli (AIEC).

I.1.5.1.1.1 Mycobacterium avium subspecies paratuberculosis

MAP causes a spontaneous granulomatous enterocolitis (Johne's disease) in ruminants, making it a credible causative agent for CD (Sartor 2005). However it has proven very difficult to either substantiate or invalidate a link between MAP and CD (Packey and Sartor 2009).

MAP was first cultured from resected CD tissue in 1984 (Chiodini, Van Kruiningen *et al.* 1984). A number of studies have replicated this finding but the reported detection rate has ranged from 0% to 100% (Autschbach, Eisold *et al.* 2005; Sartor 2005; Behr and Schurr 2006). Interest in MAP as a causative agent was regenerated with the identification of defective innate immune mechanisms, such as the NOD2 polymorphism (Sartor 2005; Behr and Schurr 2006). However, no association between NOD2 polymorphisms and MAP serology (Bernstein, Wang *et al.* 2007) or MAP culture was seen (Sechi, Gazouli *et al.* 2005). Credence was added to the theory by an uncontrolled report of long-lasting cure of CD by anti-mycobacterial antibiotic treatment (Gui, Thomas *et al.* 1997). However, a well-designed, 2-year prospective trial of clarithromycin, rifabutin, and ethambutol has failed to show sustained response (Selby, Pavli *et al.* 2007).

I.1.5.1.1.2 Adherent/invasive E. coli

E. coli comprise 99% of invasive bacterial isolates in mucosal biopsies of patients with CD as opposed to 42% in patients with UC and 2% in normal controls (Sasaki, Sitaraman *et al.* 2007). Serum antibodies directed against E. coli outer membrane protein C (OmpC) are present in 37-55% of patients with CD, in contrast to 5% or less

of patients with UC and without IBD. High serum reactivity to E. coli OmpC is associated with severe CD with longer disease duration, frequent disease progression, small bowel involvement and increased resections (Mow, Vasiliauskas *et al.* 2004).

AIEC are commensal E. coli that exhibit functional changes that allow them to persist and even replicate within macrophages, inducing the secretion of large amounts of TNF (Glasser, Boudeau *et al.* 2001). Three independent studies have demonstrated that AIEC selectively colonise the ileum of patients with CD (Sasaki, Sitaraman *et al.* 2007), (Darfeuille-Michaud, Boudeau *et al.* 2004), (Baumgart, Dogan *et al.* 2007). In addition the prototypic AIEC strain, LF82, induced in-vitro granulomas using blood-derived mononuclear cells (Meconi, Vercellone *et al.* 2007). The mechanisms by which AIEC achieve increased epithelial adherence and invasion are still being elucidated. That these virulence factors might combine with IBD associated deficits in innate immunity and barrier function to promote disease is evidenced by the observation that macrophages from NOD2-deficient mice display defective clearance of a murine AIEC strain, with prolonged secretion of IL-12/23 p40 and tumour necrosis factor (Packey and Sartor 2009).

Attempts to define the luminal microbiota in a quantitative fashion have been aided by the development of molecular techniques. For example Conte *et al.* used conventional culture techniques for aerobic and facultative-anaerobic microorganisms, and molecular analysis (16S rRNA-based amplification and real-time polymerase chain reaction assays) for the detection of anaerobic bacterial groups or species in biopsy specimens of the ileum, caecum and rectum obtained at colonoscopy. They studied 12 patients with Crohn's disease, 7 with ulcerative colitis, 6 with indeterminate colitis and 7 controls (Conte, Schippa *et al.* 2006). No single pathogen was linked to the presence of IBD.

However, there was an overall decrease in some bacterial species or groups belonging to the normal anaerobic intestinal flora; in particular, occurrence of Bacteroides vulgatus was low in Crohn's disease, ulcerative colitis and indeterminate colitis specimens.

Thus it may be that the intestinal microbiota contributes to disease not by harbouring a single pathogenic agent but by subtle alterations in the normal flora that somehow promote disease. This concept has been termed dysbiosis.

I.1.5.1.2 Dysbiosis

The luminal microbiota is altered in IBD. In both CD and UC there is decreased complexity of the commensal bacteria (Packey and Sartor 2009). The most notable changes are the reduction in the members of the phyla Bacteroidetes and Firmicutes (Backhed, Ley *et al.* 2005). Faecal and mucosa-associated microbial communities in patients with CD and UC are consistently less diverse with increased temporal instability (Frank, St Amand *et al.* 2007; Dicksved, Halfvarson *et al.* 2008; Martinez, Antolin *et al.* 2008; Ott, Plamondon *et al.* 2008; Nishikawa, Kudo *et al.* 2009). The abnormal microbiota also correlates with the occurrence of abscesses in patients with CD, and IBD patients with dysbiosis undergo surgery at a younger age than those with normal microbiota (Frank, St Amand *et al.* 2007).

Evidence that restitution of the luminal microbiota towards normal is beneficial to disease is provided by studies of probiotic preparations in IBD. There is evidence, albeit in small clinical trials, that VSL#3 can be efficacious in certain clinical situations (Packey and Sartor 2009). Recently particular attention has focussed on Faecalibacterium prausnitzii, a major member of the family Firmicutes. This bacteria was reduced in patients with CD and the reduction was associated with a higher risk of post-resection recurrence of ileal CD (Frank, St Amand *et al.* 2007; Swidsinski,

Loening-Baucke *et al.* 2008). In a supporting study oral administration of either live F. prausnitzii or its supernatant reduced the severity of trinitrobenzene sulfonic acid (TNBS) colitis and corrected the associated dysbiosis (Sokol, Pigneur *et al.* 2008).

Changes in the intestinal microbiota produce changes in the intestinal environment that may contribute to disease. The preferred energy substrate of the colonic epithelial cell is short chain fatty acids (SCFA) such as butyrate. Clostridia and Bacteroides species produce SCFA and decreased concentration of certain Clostridial groups could explain the observed decreased SCFA concentration in faecal extracts from IBD patients (Marchesi, Holmes *et al.* 2007). In addition overgrowth of sulfate-reducing bacterial species in UC and ileal pouches could enhance hydrogen sulfide production, which in turn blocks the utilisation of butyrate by colonocytes (Roediger, Duncan *et al.* 1993). Finally, some commensal bacteria produce chemicals, such as hydrogen sulfide, nitric oxide and serine proteases, capable of harming colonocytes and matrix components (Roediger, Duncan *et al.* 1993).

I.1.5.1.3 Defective clearance and killing of bacteria

Parallels between the pathophysiology of CD and the gastro-intestinal manifestations of chronic granulomatous disease (CGD), a condition caused by defective clearance of commensal, opportunistic or pathogenic bacteria, invite speculation that the aetiological mechanism in CD might involve an underlying defect in bacterial killing and clearance. As outlined in section I.1.1, multiple defects in the mucosal defences have been identified in IBD, many of them involving underlying genetic defects.

I.1.5.2 Dietary factors

Aside from the luminal microbiota, the other main luminal constituent is that which is ingested. The focus of the proceeding section is to outline the role that dietary factors might play in the aetiopathogenesis of IBD.

I.2 Dietary influences on the aetiology of IBD

Many methodologies have been employed to examine for a relationship between the development of IBD and specific dietary factors. With the obvious exception of breast-feeding practices, the initial identification of associations has largely been by observational studies regarding the pre-illness intake of multiple dietary constituents. The important associations discovered by this method are summarised in Table 7 and the literature regarding the further elucidation of each of those associations in turn is outlined in the section below. It should be noted that the main methodology employed has been case-control studies. These introduce a real risk of recall bias. Only one study to date has attempted to overcome this by using a prospective cohort methodology. In their study Hart *et al.* were able to examine a prospective cohort of 260,686 men and women resident in the UK, Sweden, Denmark, Germany and Italy (Hart, Luben *et al.* 2008). Prospectively collected data on diet was available. In total there were 139 subjects with incident UC in the cohort. No statistically significant association between diet and UC was found but there was a marginally significant positive association with increasing percentage intake of energy from total polyunsaturated fatty acids.

	Subjects	Metho-dology	Dietary Associations with UC	Dietary Associations with CD
Martini and Brandes 1976	63 CD, 63 controls	Case control	NA	Sweets and pastries
Thornton, Emmett *et al.* 1979	30 CD, 30 controls	Case control	NA	Refined sugar (negative association with dietary fibre, raw fruit and vegetables
Kasper and Sommer 1979	35 CD, 70 controls	Case control	Sugar, starch and total energy	NA
Persson, Ahlbom *et al.* 1992	152 CD, 145 UC, 305 controls	Case control	Fast foods	Sucrose and fast foods (negative association with fibre)
Japan 1994	101 UC, 143 controls	Case control	Western foods (bread for breakfast, butter, margarine, cheese, meats, and ham and sausage)	NA
Tragnone, Valpiani *et al.* 1995	104 UC and CD, 104 controls	Case control	Total protein, total carbohydrate, starch and refined sugar	Total carbohydrate, starch and refined sugar
Shoda, Matsueda *et al.* 1996	CD cohort	Population cohort	NA	Animal protein, increased ratio of n-6 to n-3 polyunsaturated fatty acids
Reif, Klein *et al.* 1997	33 CD, 54 UC, 144 controls	Case control	Sucrose, animal fat, cholesterol	Sucrose
Sakamoto, Kono *et al.* 2005	111 UC, 128 CD, 239 controls	Case control	Sweets	Sugars and sweeteners, sweets, fats and oils, fish and shellfish, total fat, monounsaturated fatty acids, and polyunsaturated fatty acids vitamin E and n-3 and n-6 fatty acids
Amre, D'Souza *et al.* 2007	130 CD, 220 control children (<=20)	Case control	NA	(Negative association with vegetables, fruits, fish and dietary fibre)
Hart, Luben *et al.* 2008	139 UC	Prospective cohort study	No statistically significant association	NA

(NA: not applicable)

Table 7. Studies of pre-illness diet In IBD

I.2.1 Infant feeding practices

Breastfeeding has been shown to protect against many immune-mediated diseases such as bronchial asthma and atopic dermatitis (Gdalevich, Mimouni *et al.* 2001), allergic rhinitis (Mimouni Bloch, Mimouni *et al.* 2002), and type 1 diabetes mellitus (Gerstein 1994). This, combined with the observation that breast feeding may protect infants from gastrointestinal infections, (Duffy, Byers *et al.* 1986; Howie, Forsyth *et al.* 1990; Beaudry, Dufour *et al.* 1995) suggests it is reasonable to postulate that breastfeeding might have an effect on the development of IBD.

The available epidemiological studies of the effect of breastfeeding on the development of IBD to 2003 was meta-analysed by Klement et al (Klement, Cohen *et al.* 2004). Together a total of 17 studies giving 2577 patients with UC and 3551 control subjects, and 3190 patients with CD and 4026 control subjects, were studied. They found that the protective effect of breastfeeding against both UC and CD remained statistically significant for all calculated pooled odds ratios (ORs) independent of the quality of the studies, with ORs of 0.67 (95% CI: 0.52, 0.86) for CD and 0.77 (0.61, 0.96) for UC in breastfed subjects. However, the results for both diseases appeared to be heterogeneous. In addition exploration for the possibility of publication bias, using funnel plots, indicated a possible publication bias in the studies for CD.

Soon after the completion of the above meta-analysis a further case-control study was performed by Baron *et al.* examining the environmental risk factors prior to the development of IBD in a paediatric population (Baron, Turck *et al.* 2005). They studied a total of 222 incident cases of CD and 60 incident cases of UC compared to the same number of control subjects matched by sex, age and geographical location. They recorded 140 study variables in a questionnaire that covered familial history of IBD,

events during the perinatal period, infant and child diet, vaccinations and childhood diseases, household amenities, and the family's socioeconomic status. This study showed that while breastfeeding had no significant impact on the development of UC, it was significantly associated with an increased risk of developing CD (OR 2.1 (95% confidence interval 1.3-3.4)).

Klement *et al.* went on to repeat their meta-analysis using the data from the study of Baron *et al.* They concluded that the study was conducted with the use of excellent methods and that it would diminish the significant results of protective breastfeeding on CD [Mantel-Haenszel odds ratio (OR_{MH}): 0.62; 95% CI: 0.27, 1.43] but would not affect significantly the summary estimate of the protective association between breastfeeding and UC (OR_{MH}: 0.62; 95% CI: 0.43, 0.91). In addition, inclusion of this study further increased the high heterogeneity in the CD studies. This finding again emphasized the need for further high-quality studies of other population types to fully understand the association between breastfeeding and IBD (Klement and Reif 2005).

I.2.2 Cow's milk proteins

In the earlier part of the 20th century a role for cows milk in the pathogenesis of IBD was widely propounded (Cashman and Shanahan 2003). In 1989 Koletzko *et al.* reported their controlled study of the association between infant feeding practices and IBD (Koletzko, Sherman *et al.* 1989). They compared 114 patients and their 180 unaffected siblings from 107 families. Univariate analysis showed that patients with CD were less likely to have been breast fed (relative risk 3.6, 95% confidence interval 1.4 to 9.0, $p<0.01$), more likely to have received formula food from birth (3.1, 1.3 to 7.4, $p<0.02$), and more likely to have had diarrhoeal illnesses during infancy (2.7, 1.5 to 5.8, $p<0.02$). Multivariate analysis, however, showed that only two factors, lack of breast-

feeding and episodes of diarrhoeal disease during infancy, were independently associated with later development of CD.

In 1990 Glassman *et al.* went on to investigate the association between cow's milk sensitivity in infancy and the development of IBD (Glassman, Newman *et al.* 1990). They surveyed 78 patients with IBD (35 with CD and 43 with UC) and compared them to a control population of 36 children without organic disease. They asked patients and controls to report symptoms compatible with cow's milk intolerance. The incidence of a history compatible with cow's milk intolerance was 8.5% (3/35) in patients with CD, similar to the 2.8% (1/36) incidence in controls. Patients with UC, however, had a significantly greater prevalence of cow's milk intolerance compared with the other patient groups (20.9%, 9/43; $p<0.03$). In addition, patients with a history of cow's milk allergy, who subsequently developed UC, did so at an earlier age (6.68 +/- 2.05 yr vs. 10.62 +/- 0.74 yr: $p<0.02$) than those without a history of cow's milk sensitivity. However, these results need to be interpreted with reservations because of the difficulty in assessing sensitivity to cow's milk in this retrospective study.

Further evidence for a role of cow's milk in the pathogenesis of IBD is limited to studies of cow's milk specific antibodies in IBD (see section I.3.2.8). The results from such studies vary widely. This may be due to different techniques used for measuring antibodies in those studies (Cashman and Shanahan 2003). One commentator has concluded that the only recognised value of studies showing elevated levels of serum antibodies to cow's milk proteins is that they provide evidence that the lack of breast feeding and increased prevalence of cow's milk antibodies may be risk factors for the later development of IBD (Mishkin 1997).

I.2.3 Dietary fat

The epidemiological support for a role for dietary fat in the aetiology of IBD stems largely from the striking association between the increasing intake of animal fat in Japanese society and a parallel increase in the incidence of CD. Shoda *et al.* used data that recorded the daily intake of each dietary component using annual prospective interviews for 5 consecutive days for between 16500 and 68000 people each year from 1966 through to 1985 (Shoda, Matsueda *et al.* 1996). They then compared this to incidence data for CD in Japan, obtained from a nationwide multicenter survey of the annual numbers of new patients with CD. This data suggests the number of patients with IBD in Japan has increased sharply during the past three decades. This increase may reflect improved diagnostic and recording practices as well as an increased awareness of the disease in recent years. Nonetheless, it does appear true that the actual incidence has increased as well (Yamamoto, Nakahigashi *et al.* 2009).

Univariate analysis showed that the increased incidence of CD was strongly ($p < 0.001$) correlated with increased dietary intake of total fat ($r = 0.919$), animal fat ($r = 0.880$), n-6 polyunsaturated fatty acids ($r = 0.883$), animal protein ($r = 0.908$), milk protein ($r = 0.924$), and the ratio of n-6 to n-3 fatty acid intake ($r = 0.792$). It was less correlated with intake of total protein ($r = 0.482$, $p < 0.05$), was not correlated with intake of fish protein ($r = 0.055$, $p > 0.1$), and was inversely correlated with intake of vegetable protein ($r = -0.941$, $p < 0.001$). The multivariate analysis showed that increased intake of animal protein was the strongest independent factor with a weaker second factor, an increased ratio of n-6 to n-3 polyunsaturated fatty acids (Shoda, Matsueda *et al.* 1996). The eicosanoid biosynthesis pathway and the association of n-3 fatty acids with the product of anti-inflammatory cytokines vs. that of n-6 fatty acids with the pro-inflammatory Arachidonic acid, provides a plausible biological mechanism for such an

association (see Figure 2). The role of the dietary ratio of n-3 to n-6 polyunsaturated fatty acids in IBD is considered in more detail in sections II.1.4.7 and II.2.5.

Figure 2. Eicosanoid biosynthesis pathway (adapted from Calder 2004)

A case control study, also conducted in Japan, found that fat intake, among other foods, was positively associated with CD (Sakamoto, Kono *et al.* 2005). They compared 239 IBD patients (111 UC, 128 CD) to the same number of control subjects matched for sex, age and hospital. Using a semi-quantitative food frequency questionnaire they retrospectively estimated pre-illness intakes of food groups and nutrients. The intake of total fat (OR, 2.86; 95% CI, 1.39 to 5.90), monounsaturated fatty acids (OR, 2.49; 95% CI, 1.23 to 5.03), polyunsaturated fatty acids (OR, 2.31; 95% CI, 1.12 to 4.79), n-3 (OR,

3.24; 95% CI, 1.52 to 6.88) and n-6 fatty acids (OR, 2.57; 95% CI, 1.24 to 5.32) were all positively associated with CD risk.

This data is supported by the 2007 study of Amre *et al.* (Amre, D'Souza *et al.* 2007). They examined the impact of diet on new onset CD in Canadian children, in a case-control study. Newly diagnosed patients with CD 20 years old and younger were compared to population or hospital controls who were matched for time of diagnosis (+/-6 months) and area of residence. Dietary consumption 1 yr prior to disease diagnosis was evaluated using a validated food frequency questionnaire, administered within 1 month of diagnosis. A total of 130 CD patients and 202 controls were studied. The consumption of long-chain omega-3 fatty acids (LCN-omega-3) was negatively associated with CD (OR 0.44, 95% CI 0.19-1.00, $p<0.001$). A higher ratio of LCN-omega-3/omega-6 fatty acids was significantly associated with lower risks for CD (OR 0.32, 95% CI 0.14-0.71, $p=0.02$).

Reif *et al.* observed such an association for UC in a study conducted in Israel (Reif, Klein *et al.* 1997). Quantified dietary histories were obtained from 87 patients with recent IBD (54 UC and 33 CD) and 144 controls. A high fat intake was associated with an increased risk for UC; this was particularly marked for animal fat (OR 4.09, $p=0.02$) and cholesterol (OR 4.57, $p=0.02$).

The observation of an association between fat intake and CD is not universal however. Tragnone *et al.* studied the dietary habits of 104 patients with IBD just prior to the onset of disease and compared this with the habits of a matched control population in Italy (Tragnone, Valpiani *et al.* 1995). They found no difference in fat consumption between IBD patients and controls. Other commentators have, however, pointed out that they

failed to correct for total energy intake, and this methodological limitation could have biased the results (Geerling, Stockbrugger *et al.* 1999).

I.2.4 Margarine consumption

A series of studies from Germany in the early 1980s suggested an association between margarine consumption and CD (Cashman and Shanahan 2003). This finding was also seen in UC patients in a multisite, hospital-based, case-control study conducted in Japan (EGotRCoIBDiJ 1994). 101 patients who had been diagnosed with UC within the previous 3 years were surveyed using self-administered patient questionnaires and compared to 143 control subjects. Margarine was positively associated with UC ($p=0.005$).

An international epidemiological study failed to confirm an association between margarine consumption and IBD (Sonnenberg 1988). In that study the per capita consumption of margarine was correlated with the incidence and mortality of CD from different countries and the time trends of mortality from CD. No significant correlation was found between margarine consumption and the incidence of CD. The time trends of CD in different countries were not matched by similar time trends of margarine consumption.

I.2.5 Refined carbohydrates

Interest in the association of sugar and refined carbohydrate and the development of CD was ignited by the findings of Martini and Brandes in 1976 (Martini and Brandes 1976). They compared the nutritional habits of 63 patients with CD using questionnaires and compared them to a 63 person control group matched for age, sex and social status. There was a significantly higher pre-diagnosis consumption of refined carbohydrates in the CD group compared to controls. They, however, found no significant difference in

the intake of other foodstuffs such as proteins, fats, vegetables or alcohol. Since then there have been numerous studies confirming the observation (Cashman and Shanahan 2003).

The repeatability of this observation has led some authors to conclude that the most consistent, distinct dietary association with IBD is the relationship between increased consumption of refined carbohydrates and CD (Cashman and Shanahan 2003). One study allowed the estimation of the relative risk of developing CD with a high sugar intake. Katschinski *et al.* used a postal questionnaire to examine the association between smoking, added sugar intake and CD (Katschinski, Logan *et al.* 1988). They questioned 104 CD patients and 153 community controls. They found that added sugar intake was associated with CD, independent of smoking and with a dose response pattern, with a relative risk for no added sugar vs. added sugar of less than 50g/day and greater than 50g/day of 1.0, 1.8 and 4.6 ($p<0.005$) respectively. However, they found that in smokers, the addition of sugar to the diet did not significantly increase the risk of CD and thus concluded that the influences of smoking and added sugar may be operating through a common mechanism.

Nonetheless, epidemiological studies have not shown a correlation between the rising incidence of IBD and any marked change in sugar consumption over the last 50 years (Riordan, Ruxton *et al.* 1998). In addition CD remains extremely rare in countries such as Saudi Arabia and Morocco despite a large indigenous intake of sugar (Kirsner and Shorter 1982). The differences in diet, therefore, could be interpreted as a consequence rather than a cause of the disease, or that other factors are required in combination for the development of disease (Cashman and Shanahan 2003). It also remains possible that increased refined carbohydrate intake may simply be an expression of the “modern

lifestyle" or "urban diet" and is confounded by other risk factors for the development of IBD (Russel, Engels *et al.* 1998; Mahmud and Weir 2001).

I.2.6 Dietary fibre

The role of breakfast foods, and by inference cereals, in the pathogenesis of CD was brought into the spotlight by the 1977 report of James (James 1977). In that report the breakfast habits in adult life of 34 patients with CD were compared with those of 68 matched controls. An association between the consumption of cornflakes, but not other cereals, and CD was reported. This generated a flurry of discussion and further descriptive studies, the results of which varied widely but did not coincide with that of James (Archer and Harvey 1978; Rawcliffe and Truelove 1978). Since then several other studies have failed to show an association between cereal intake and CD (Cashman and Shanahan 2003).

The consumption of dietary fibre, however, especially in the form of fruit, has been shown to be negatively associated with the risk of IBD (Cashman and Shanahan 2003). Reif *et al.* used quantified dietary histories from 87 patients with recent IBD (54 UC and 33 CD) and 144 controls to investigate the association between intake levels of various foods and IBD (Reif, Klein *et al.* 1997). They found a statistically non-significant trend toward a negative association between a high intake of fruits and IBD. Conversely, decreased consumption of fruit, fruit juice and vegetables has been reported among IBD patients. Kasper *et al.* obtained individual dietary histories from 35 patients with CD and 70 normal controls (Kasper and Sommer 1979). In the patients with CD the mean dietary fiber intake was established as 26.6 +/- 1.4 g/day, compared to 22.3 +/- 0.9 g/day in the controls ($p<0.05$). Mayberry *et al.* interviewed 48 men and 52 women with CD and compared them with 100 controls, matched for age and sex (Mayberry, Rhodes *et al.* 1978). They recorded breakfast food intake, but not total fruit and

vegetable intake. They found that significantly fewer patients than controls consumed fruit or fruit juice for breakfast.

Thornton *et al.*, in an attempt to reduce the recall bias inherent in the above studies, interviewed thirty newly diagnosed patients with CD and compared them with 30 healthy controls, matched for age, sex, social class, and marital status (Thornton, Emmett *et al.* 1979). The patients ate slightly less dietary fibre, and considerably less raw fruit and vegetables, than the controls. In a further study by Porro *et al.* the smoking habits, and intake of refined carbohydrates and vegetable fibre were evaluated in 124 patients suffering from UC, 109 patients with CD, and 250 controls matched by sex and age (Bianchi Porro and Panza 1985). A considerable intake of vegetables and fruit seemed to reduce the risk for IBD (relative risk (RR) for CD=0.36 and 0.66 respectively; RR for UC=0.30 and 0.38).

Each of these studies has inherent flaws. Many were subject to recall bias, with patients asked to remember their past intake of different food groups. In addition many asked specifically about breakfast intake, ignoring intake throughout the rest of the day. None were designed to be able to determine the causality of any association between intake and IBD.

Nonetheless an association between dietary fibre intake and IBD remains biologically plausible as dietary fibre actively contributes to the intestinal anti-inflammatory effect associated with an increased production of short-chain fatty acids, mainly acetate, propionate and butyrate in the colonic lumen (Galvez, Rodriguez-Cabezas *et al.* 2005). These substances may accelerate intestinal repair, preserving the integrity of the intestinal mucosa, and downregulate the exacerbated immune response associated with

IBD (Segain, Raingeard de la Bletiere *et al.* 2000). The production of short-chain fatty acids, can also contribute to the inhibition of the production and release of proinflammatory cytokines, including IL-6, IL-8, TNF-alpha, and other mediators of inflammation such as reactive oxygen and nitrogen metabolites (Rodriguez-Cabezas, Galvez *et al.* 2002).

I.2.7 Dietary protein

In their 1985 study Gee *et al.* compared the dietary intakes of two groups of gastrointestinal patients, one group with IBD and the other with functional disorders - irritable bowel syndrome, nonulcer dyspepsia, or gastro-oesophageal reflux disease (Gee, Grace *et al.* 1985). They assessed the patients' diet using 48-hour recalls and found an increased mean intake of protein among IBD patients compared with functional disorders subjects.

Tragnone *et al.* also used dietary recall questionnaires in 104 IBD patients in Italy to study dietary habits immediately prior to the onset of disease (Tragnone, Valpiani *et al.* 1995). They compared IBD patients to healthy subjects matched for age, sex and city of residence and found that total protein intake was significantly higher in UC, but not in CD patients, than in controls. In contrast, Shoda *et al.* used prospectively collected annual dietary information for a Japanese population between 1966 and 1985 (Shoda, Matsueda *et al.* 1996). On univariate analysis they found a weak correlation between the incidence of CD and total protein ($r = 0.482$, $P < 0.05$) but a strong correlation with increased dietary intake of total fat ($r = 0.919$). The multivariate analysis showed that increased intake of animal protein was the strongest independent factor associated with an increased risk of CD.

Finally Reif *et al.*, again using dietary recall histories, compared 87 IBD patients (54 UC and 33 CD) and 144 controls (Reif, Klein *et al.* 1997). They failed to find an association between total protein intake and risk of IBD.

Apart from the Japanese study, these studies were subject to recall bias and the dietary histories were based on dietary recall. None of the studies were designed to prove a causal relationship. It remains possible an increase in dietary protein might represent a response to the disease rather than an aetiological factor.

I.2.8 Dietary microparticles

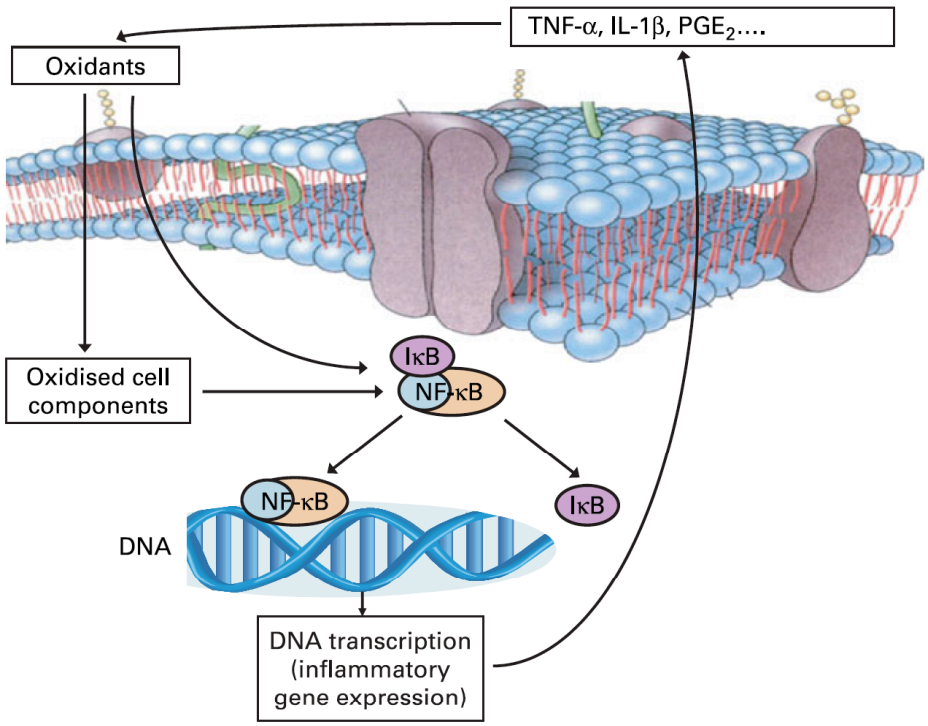
Powell *et al.* described microparticles resident in phagolysosomes of macrophages at the base of human gut associated lymphoid tissue (GALT) (Powell, Ainley *et al.* 1996). They identified three distinct types of microparticle: type I - spheres of titanium dioxide; type II - aluminosilicates; and type III - mixed environmental silicates without aluminium. The association of such microparticles with non-gastrointestinal disease raised the concern that such microparticles might be pathogenic in the gut.

The same group investigated whether one of these microparticles, titanium dioxide (TiO2), could alter intestinal cell responsiveness to lipopolysaccharide (LPS) (Powell, Harvey *et al.* 2000). They took colonic biopsy specimens from 28 patients with UC, 21 with CD, and 36 healthy controls. These samples were incubated either alone, with LPS, with TiO2, or with LPS adsorbed to TiO2. They saw no increase in IL-1 secretion when the intestinal organ cultures were challenged with TiO2 or LPS alone, but saw a two-to-three-fold increase in IL-1 production with the addition of the TiO2-LPS conjugate. They concluded the ultrafine particles were not immunologically inert and that they might be important adjuncts in the mediation of the response of the gut immune system to luminal antigens.

This group then went on to define the intake of such microparticles in the diets of 91 CD patients compared to 91 general practice-based controls (Lomer, Hutchinson *et al.* 2004). While there was wide variation in intakes of microparticles between individuals there was no significant difference between subjects with CD and controls.

I.2.9 Antioxidant vitamins (C, E and carotenoids)

In epidemiological studies a negative association was observed between the intake of fruits and vegetables, in particular raw fruits and vegetables, and the development of IBD (Thornton, Emmett *et al.* 1979) (Amre, D'Souza *et al.* 2007). The role fibre intake might play in this has been discussed (see section I.2.6). It is also possible that other micronutrients found predominately in those foods might play a role. The antioxidant vitamins (C, E and the carotenoids) are found in high relative concentrations in these foods. There is a strong interaction between oxidative stress and inflammation (Calder 2009). Oxidants and oxidised cell components, acting through transcription factors such as NF-kB, induce production of inflammatory eicosanoids and cytokines (see Figure 3). Many of these mechanisms are held in common with IBD. This section briefly outlines the evidence to link the antioxidant vitamins to a protective role in IBD.



(IkB: inhibitory subunit of NF-kB; IL: interleukin; NF-kB: nuclear factor kappa B; PG: prostaglandin; TNF: tumour necrosis factor)

Figure 3. The interaction between oxidant stress and inflammation (adapted from Calder, Albers *et al.* 2009)

I.2.9.1 Vitamin C

The term vitamin C includes ascorbic acid (AA) and dehydroascorbic acid. Vitamin C is essential for humans as we are unable to synthesise AA from glucose due to a lack of the enzyme gulonolactone oxidase. The main dietary sources of vitamin C in the Western diet are fruits and vegetables. The recommended five servings per day of fruits and vegetables should provide at least 200 mg of vitamin C, compared to the recommended daily intake of 75 to 90 mg/day. Vitamin C plays an important role in mechanisms involved in immune function and inflammatory processes, including free radical scavenging and protection against lipid peroxidation. In addition to its antioxidant action, vitamin C is a cofactor for enzymes involved in the biosynthesis of collagen, carnitine and neurotransmitters as well as corticosteroids, the microsomal drug-metabolising enzymes and cytochrome P-450 electron transport. It modulates iron absorption, transport and storage. AA has also been shown to modulate prostaglandin

synthesis. Vitamin C also affects antimicrobial and natural killer cell activities, lymphocyte proliferation, chemotaxis and delayed-type hypersensitivity (Calder, Albers *et al.* 2009).

Medline was searched using the terms “vitamin C”, “ascorbic acid”, “ulcerative colitis”, “Crohn” and “inflammatory bowel disease”. All articles that compared the intake of vitamin C, or the serum, tissue or other levels of ascorbic acid, to control values or reference parameters are summarised in Table 8.

All studies show either decreased serum ascorbic acid (SAA) or leucocyte ascorbic acid (LAA) in IBD patients. In those studies where vitamin C intake was measured it was generally reduced in IBD. There was, however, evidence of normal absorption of vitamin C into the mucosa of the small bowel in CD patients. Colonic samples from CD and UC patients demonstrated reduced levels of AA in inflamed compared to non-inflamed tissue. Only one study compared SAA in patients with active vs. inactive CD and no difference was seen (Geerling, v Houwelingen *et al.* 1999).

	Patient group	Method	Results in UC	Result in CD
Gerson 1975	20 CD, 20 CD on vit C supplement and 32 controls	SAA and LAA	NA	Decreased SAA and LAA in CD, higher with supplements but still low cf controls
Linaker 1979	10 CD and 10 controls	Vit C intake and LAA	NA	Normal intake but reduced LAA cf controls
Hodges, Gee *et al.* 1984	47 CD	Vit C intake	NA	Adequate intake cf. reference parameters
Imes, Dinwoodie *et al.* 1986	137 CD	Vit C intake and SAA and LAA	NA	Lower than reference range vit C intake and low SAA and LAA
Fernandez-Banares, Abad-Lacruz *et al.* 1989	15 UC and 8 CD with acute or subacute disease and 89 controls	SAA	Decreased SAA cf. controls	Decreased SAA cf. controls
Pettit, Shaffer *et al.* 1989	12 CD and 6 controls	Measured absorption of labelled AA into small bowel samples	NA	AA absorption normal in CD
Buffinton and Doe 1995	17 CD and 13 UC	Compared AA in inflamed and non-inflamed colonic biopsies	AA decreased by 73%	AA decreased by 35% in inflamed tissue
Hoffenberg, Deutsch *et al.* 1997	12 CD and 12 UC children and 23 controls	SAA	Decreased SAA cf. controls	Decreased SAA cf. controls
Geerling, Badart-Smook *et al.* 1998	32 CD and 32 matched controls	SAA	NA	Decreased cf. controls
Geerling, v Houwelingen *et al.* 1999	12 active CD, 50 inactive CD and 70 controls	SAA	NA	Decreased cf. controls, no signifcant difference between active and inactive CD
Wendland, Aghdassi *et al.* 2001	37 CD and 37 matched controls	SAA	NA	Decreased cf. controls
Filippi, Al-Jaouni *et al.* 2006	54 CD in remission and 25 controls	Vit C intake and SAA	NA	Decreased intake cf. control and decreased SAA
Hengstermann, Valentini *et al.* 2008	100 CD, 67 UC (majority in remission) and 45 matched controls	SAA	Decreased cf. controls (did not differentiate UC from CD)	Decreased cf. controls (did not differentiate UC from CD)

(AA: ascorbic acid; LAA: leucocyte AA; SAA: serum AA; cf: compared with; NA: not applicable; Vit C: vitamin C; UC: ulcerative colitis; CD: Crohn disease)

Table 8. Observational studies of vitamin C in IBD

I.2.9.2 Vitamin E

Vitamin E is a potent chain-breaking antioxidant that acts mainly in the lipid phase and interrupts the chain reaction of lipid peroxidation and, consequently, prevents the propagation of free radical-initiated reactions (Calder, Albers *et al.* 2009).

Medline was searched using the terms “vitamin E”, “tocopherol”, “ulcerative colitis”, “Crohn” and “inflammatory bowel disease”. All articles that compared the intake of vitamin E, or the serum, tissue or other levels of tocopherols, to control values or reference parameters are summarised in Table 9.

The reports of tocopherol levels in IBD are conflicting. Reports of no difference in levels in control compared with diseased subjects are balanced by reports of low levels in disease. Notably there is even one paper that reports increased levels of alpha-tocopherol in UC and CD (Hoffenberg, Deutsch *et al.* 1997).

	Patient group	Method	Results in UC	Result in CD
Kuroki, Iida *et al.* 1994	13 CD at diagnosis and 12 controls	Serum and RBC vit E	NA	Serum level decreased cf. control, RBC level no difference
Buffinton and Doe 1995	28 CD and 28 UC	Compared alpha-tocopherol in inflamed and non-inflamed colonic biopsies	No difference in inflamed vs. non-inflamed tissue	No difference in inflamed vs. non-inflamed tissue
Hoffenberg, Deutsch *et al.* 1997	12 CD and 12 UC children and 23 controls	Plasma alpha- and gamma-tocopherol	Increased alpha-tocopherol cf. controls, gamma-tocopherol unchanged	Increased alpha-tocopherol cf. controls, gamma-tocopherol unchanged
Ramakrishna, Varghese *et al.* 1997	19 active UC and 19 controls	Plasma vit E	No difference from controls	NA
Bousvaros, Zurakowski *et al.* 1998	61 CD, 36 UC and 23 controls (paediatric and young adult)	Serum alpha-tocopherol	Decreased cf. controls	Decreased cf. controls
Geerling, Badart-Smook *et al.* 1998	32 CD and 32 matched controls	Serum vit E	NA	Decreased cf. controls
Geerling, v Houwelingen *et al.* 1999	12 active CD, 50 inactive CD and 70 controls	Serum vit E	NA	No difference cf. controls
Genser, Kang *et al.* 1999	24 CD and 33 controls	Alpha- and gamma-tocopherols	NA	Gamma-tocopherol elevated and alpha-tocopherol no difference cf. controls
D'Odorico, Bortolan *et al.* 2001	46 UC, 37 CD and 386 controls	Plasma vit E	Decreased cf. controls	Decreased cf. controls
Sampietro, Cristaldi *et al.* 2002	20 CD listed for surgery and 134 controls	Plasma vit E	NA	Decreased cf. control, returned to control levels following surgery
Filippi, Al-Jaouni *et al.* 2006	54 CD in remission and 25 controls	Vit E intake and plasma concentration	NA	Decreased intake cf. control and decreased plasma concentration
Kawakami, Okada *et al.* 2007	27 UC and 27 controls	Serum alpha-tocopherol	Decrease cf. controls	NA
Hengstermann, Valentini *et al.* 2008	100 CD, 67 UC (majority in remission) and 45 matched controls	Plasma vit E	No difference cf. controls (did not differentiate UC from CD)	No difference cf. controls (did not differentiate UC from CD)

(NA: not applicable; vit: vitamin; RBC: red blood cell; cf: compared with)

Table 9. Observational studies of vitamin E in IBD

I.2.9.3 Carotenoids

Carotenoids are coloured pigments found in nature. They are responsible for the typical colour of fruits and vegetables as well as some animals. The most prevalent carotenoids in the Western diet are alpha-carotene, beta-carotene, lycopene, beta-cryptoxanthin, lutein and zeaxanthin. Carotenoids possess immunomodulatory activities in human subjects and animals including stimulation of the phagocytic and bacteria-killing ability of peripheral blood neutrophils and peritoneal macrophages and of lymphocyte blastogenesis, increasing the population of specific lymphocyte subsets and lymphocyte cytotoxic activity, as well as stimulation of the production of various cytokines. Beta-carotene inhibits inflammatory gene expression by suppressing the activation of the redox-sensitive transcription factor, NF-kB (Calder, Albers *et al.* 2009).

Medline was searched using the terms “carotene”, “ulcerative colitis”, “Crohn” and “inflammatory bowel disease”. All articles that compared the intake of carotenoids, or the serum, tissue or other levels of carotenoids, to control values or reference parameters are summarised in Table 10.

All available observational studies but one found that serum carotenoids were reduced in UC and CD. Most studies examined serum beta-carotene levels but those that investigated a wider range of carotenoids showed similar results among the other carotenoids. The carotenoid levels were lower in active disease. One study looked at intake and showed that CD patients had a lower intake of beta-carotene than controls (Filippi, Al-Jaouni *et al.* 2006).

	Patient group	Method	Results in UC	Result in CD
Sharman, Dick *et al.* 1979	107 UC and controls	Serum carotenoids	Decreased only in active UC	NA
Fernandez-Banares, Abad-Lacruz *et al.* 1989	15 UC and 8 CD with acute or subacute disease and 89 controls	Serum beta-carotene	Decreased beta-carotene cf. controls	Decreased beta-carotene cf. controls
Hoffenberg, Deutsch *et al.* 1997	12 CD and 12 UC children and 23 controls	Plasma beta-carotene	Beta-carotene not different from controls	Beta-carotene not different from controls
Geerling, Badart-Smook *et al.* 1998	32 CD and 32 matched controls	Serum beta-carotene	NA	Decreased cf. controls
Geerling, v Houwelingen *et al.* 1999	12 active CD, 50 inactive CD and 70 controls	Serum beta-carotene	NA	Decreased cf. controls and in active cf. inactive CD
Genser, Kang *et al.* 1999	24 CD and 33 controls	Plasma alpha- and beta-carotene, and cryptoxanthin	NA	All carotenoids decreased cf. controls
Rumi, Szabo *et al.* 2000	28 CD and 23 controls	Serum lutein, zeaxanthin, alpha- and beta-carotene, alpha- and beta-cryptoxanthin	NA	Decreased lutein, zeaxanthin, alpha- and beta-carotene
D'Odorico, Bortolan *et al.* 2001	46 UC, 37 CD and 386 controls	Plasma total carotenoids	Decreased cf. controls	Decreased cf. controls
Wendland, Aghdassi *et al.* 2001	37 CD and 37 matched controls	Plasma alpha- and beta-carotene, lycopene and beta-cryptoxanthin	NA	All decreased cf. controls
Filippi, Al-Jaouni *et al.* 2006	54 CD in remission and 25 controls	Beta-carotene intake and plasma concentration	NA	Decreased intake cf. control and decreased plasma concentration
Kawakami, Okada *et al.* 2007	27 UC and 27 controls	Serum beta-carotene	Decrease cf. controls	NA
Hengstermann, Valentini *et al.* 2008	100 CD, 67 UC (majority in remission) and 45 matched controls	Plasma beta-cryptoxanthin, lycopene, alpha- and beta-carotenes and sum of carotenoids	Decreased cf. controls (did not differentiate UC from CD). Beta-carotene lower in active than inactive disease	Decreased cf. controls (did not differentiate UC from CD). Beta-carotene lower in active than inactive disease
Maor, Rainis *et al.* 2008	16 active CD, 27 stable CD and 15 controls	Serum beta-carotene	NA	Decreased in active cf. stable and both cf. control
Drai, Borel *et al.* 2009	20 CD and 21 controls	Plasma carotenoids	NA	Decreased cf. controls

(NA: not applicable; cf: compared with)

Table 10. Observational studies of carotenoids in IBD

I.2.9.4 Studies of antioxidants in animal models of IBD

Medline was searched using the terms “carotene”, “vitamin E”, “tocopherol”, “vitamin C”, “ascorbic acid”, “ulcerative colitis”, “Crohn” and “inflammatory bowel disease”. All studies that examined the effect of antioxidants on an animal model of IBD are summarised in Table 11.

The reported studies show a universal benefit of antioxidant treatment in animal models of IBD. Two studies showed that antioxidants were particularly beneficial when chemically induced colitis was augmented by the administration of iron (Carrier, Aghdassi *et al.* 2002; Reifen, Nissenkorn *et al.* 2004).

	Animal model	Intervention	Result
Sato, Kanazawa *et al.* 1998	TNBS induced colitis in rats	Alpha-tocopherol	Decreased colonic damage
Carrier, Aghdassi *et al.* 2002	DSS induced colitis in rats augmented by iron	dl-alpha-tocopherol acetate	Decreased colonic inflammation and rectal bleeding but did not affect oxidative stress
Lavy, Naveh *et al.* 2003	Acetic acid induced enteritis in rats	Beta-carotene	Prevented acid induced histological change and reduced mucosal myeloperoxidase activity
Ademoglu, Erbil *et al.* 2004	TNBS induced colitis in rats	Selenium and vitamin E	Combination improved histopathology but either alone did not
Reifen, Nissenkorn *et al.* 2004	Iodoacetamide induced colitis in rats, augmented by iron ingestion	Lycopene and beta-carotene	Weight of ulcerated area of colon was reduced by lycopene but not beta-carotene
Isozaki, Yoshida *et al.* 2006	TNBS induced colitis in rats	The water-soluble vitamin E derivative TMG injected intraperitoneally	TMG reduced the damage score, wet weight of colon, peroxidative activity
Bitiren, Karakilcik *et al.* 2010	Acetic acid induced colitis in rats	Vit E and selenium	Decreased microscopic and macroscopic colonic damage and improved oxidative status

(TNBS: trinitrobenzene sulfonic acid; TMG: 2-(alpha-D-glucopyranosyl)methyl-2,5,7,8-tetra-methylchroman-6-ol; DSS: dextran sulfate sodium)

Table 11. Studies of antioxidants in animal models of IBD

I.2.9.5 Human studies of antioxidants in IBD

Medline was searched using the terms “carotene”, “vitamin E”, “tocopherol”, “vitamin C”, “ascorbic acid”, “ulcerative colitis”, “Crohn” and “inflammatory bowel disease”. All studies that examined the effect of antioxidants on clinical activity of IBD, or on oxidative status in patients with IBD, are summarised in Table 12.

In patients with IBD, supplementation with antioxidant vitamins can improve the serum levels of antioxidants (Geerling, Badart-Smook *et al.* 2000; Akobeng, Richmond *et al.* 2007). To date studies examining the effect of antioxidant preparations on human

disease have been small in size and methodologically limited. One large randomised controlled trial is available and did show a benefit but a combination of fish oil, fibre and antioxidants was used, making it impossible to divine the benefit exhibited by the antioxidant part of the intervention (Seidner, Lashner *et al.* 2005). A recent pilot study of the effect of alpha-tocopherol enemas in UC is encouraging but further randomised controlled trials will be necessary before this treatment can be recommended (Mirbagheri, Nezami *et al.* 2008).

	Study type	**Subjects**	**Intervention**	**Results**
Hermanowicz, Sliwinski *et al.* 1985	Open label study	38 UC	Ascorbic acid 6 months	No clinical benefit
Bennet 1986	Case report	1 UC	Alpha-tocopherylquinone	Colonic and extra-intestinal manifestations improved with treatment and worsened with withdrawal
Aghdassi, Wendland *et al.* 2003	RDBPCT	57 CD	Vit E (800 iu) and vit C (1000mg) or placebo for 4 weeks	Reduced oxidative stress measures
Tsujikawa, Kanauchi *et al.* 2003	Open label study	11 CD	Chitosan (fibre) and ascorbic acid for 8 weeks	No effect on disease activity
Seidner, Lashner *et al.* 2005	RDBPCT	121 CD	Fish oil, soluble fiber, vit E, vit C and selenium cf. carbohydrate formula for 6 months	No difference in DAI but reduced prednisone requirement
Mirbagheri, Nezami *et al.* 2008	Open label study	15 mild to moderate UC	d-alpha tocopherol enema (8000 U/d) for 12 weeks and concomitant 5ASA or immunomodulator	Average DAI reduced after 12 weeks, 9/14 (64%) completing followup in remission

(RDBPCT: randomised double blind placebo controlled trial; DAI: disease activity index; 5ASA: 5-aminosalicylic acid)

Table 12. Studies of antioxidants in IBD

I.2.9.6 Conclusions regarding antioxidant vitamins in IBD

Levels of antioxidant vitamins, in particular ascorbic acid and carotenoids but also potentially tocopherols, are reduced in patients with IBD. Supplementation of patients with IBD does improve their antioxidant levels and can improve their oxidation status. Animal studies of the effect of antioxidant supplementation in animal models of IBD have invariably shown benefit. However, high quality studies of this sort in humans are lacking and those which are available have combined treatments, limiting our ability to draw conclusions as to the benefit of any individual component. Further high quality randomised controlled trials of antioxidant therapies in IBD are indicated by the positive results to date.

I.3 Dietary allergy in the aetiology of IBD

I.3.1 The potential for food allergy to act as a pathogenic agent in IBD

The lack of concordance for IBD in twin studies and the partial penetrance of genetic defects associated with IBD suggest an environmental influence in the aetiology of the disease. The two main triggers implicated are luminal microbiota and ingested substances.

The bowel mucosa acts as a physical barrier to dietary and microbial antigens. However, even under normal physiological conditions, intact food antigens can penetrate the mucosal barrier via transcellular or paracellular routes (Warshaw, Walker *et al.* 1974; Walker 1986; Sanderson and Walker 1993; Beier and Gebert 1998). The integrity of the GI mucosa is further compromised by inflammatory conditions such as IBD. Hence the immune components of the GI tract, principally resident in the lamina

propria and Peyer's patches, are continually exposed to the potentially antigenic components of the luminal contents.

Early work suggested an association between IBD and atopic and allergic disease (Hammer, Ashurst *et al.* 1968; Jewell and Truelove 1972; Roberts, Rhodes *et al.* 1978; Pugh, Rhodes *et al.* 1979). However, other investigaters were unable to substantiate those associations (Mee, Brown *et al.* 1979; Troncone, Merrett *et al.* 1988). Nonetheless the observation has fuelled further investigation into an allergic component in the pathogenesis of IBD. This section serves to outline potential allergic mechanisms in the pathogenesis of IBD, in particular IgE mediated or Type I allergy and also IgG mediated hypersensitivity, and the literature supporting and refuting these.

I.3.2 Hypersensitivity and IBD

Hypersensitivity can be described as taking two forms, immediate and delayed. The immediate types include type I – anaphylactic, type II – antibody dependent cytotoxic, and type III – immune complex mediated. The immediate hypersensitivity most relevant to gastro-intestinal disorders and food allergy is type I. In addition there is a fourth type, delayed or cell mediated hypersensitivity, often referred to as non-IgE allergy or non-atopic allergy (Roitt 1997; Kay 2001).

Although there is little direct evidence that IgE mediated hypersensitivity to food antigens plays a role in the pathogenesis of IBD, a role for the effector mechanisms of hypersensitivity in IBD would raise the possibility that hypersensitivity might be implicated in the pathogenesis of IBD. This section briefly discusses the pathogenesis of hypersensitivity and then goes on to discuss the evidence of a role for its different constituent parts in the aetiopathogenesis of IBD.

I.3.2.1 Nomenclature in GI hypersensitivity

A combined position statement from the European Academy of Allergology and Clinical Immunology, and the World Allergy Organization clearly defines the appropriate nomenclature to be used when discussing gastrointestinal allergy (Johansson, Bieber *et al.* 2004). The aim of this document was to present a globally acceptable nomenclature for allergic diseases, with the ultimate goal of improving communication in the field of allergy

In that document hypersensitivity is defined as "objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons". Such hypersensitivity to food is termed "food allergy" only when immunologic mechanisms have been demonstrated. All other reactions to foods should be referred to as "nonallergic food hypersensitivity".

These definitions were not designed to encompass those gastrointestinal symptoms that have not been objectively and repeatedly proven to relate to a defined ingested stimulus. In this section and the sections that follow I have used the term "food intolerance" to describe those instances where hypersensitivity to food, as evidence by repeated objective adverse response to a defined food, has not been proven but nonetheless, an adverse response to a food has been reported or observed.

I.3.2.2 Immediate hypersensitivity

In the type I reaction previously sensitised mast cells are induced to release inflammatory mediators by contact with cross-linked IgE. These mediators result in increased vascular permeability, smooth muscle contraction and the classical wheal and flare response.

The main preformed inflammatory mediators involved in the type I reaction are histamine, heparin, neutral protease, eosinophil and neutrophil chemotactic factors, and platelet activating factor. These are stored in cytoplasmic granules in the mucosal mast cell and are released following cross-linking of IgE antibodies, and more weakly of IgG4 antibodies, with IgE receptors on the surface of the mast cell. In addition to degranulation, the leukotrienes LTB4, LTC4 and LTD4, the prostaglandin PGD2 and thromboxanes are newly synthesized on activation, as well as IL-3, IL-4, IL-5 and IL-6 and GM-CSF (Roitt 1997).

Preferential accumulation of eosinophils occurs because of the actions of IL-3, IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF). This applies particularly to IL-5 which primes eosinophils for enhanced locomotor attractions towards other type I inflammatory mediators (Roitt 1997).

I.3.2.3 Delayed hypersensitivity

The immunomodulators and pro-inflammatory agents released by mast cell degranulation result in granulocyte, lymphocyte and monocyte/macrophage migration and activation. This results in a more protracted hypersensitivity response (Dvorak, Mihm *et al.* 1976; Charlesworth, Hood *et al.* 1989; Gauchat, Henchoz *et al.* 1993; Bischoff 1996). The role of this delayed phase reaction in asthma and atopic eczema is well demonstrated (Brostoff, Johns *et al.* 1977; Metzger, Hunninghake *et al.* 1985). It has also been suggested that a similar mechanism may play a role in food allergy in the gut (Crowe and Perdue 1992).

I.3.2.4 IgE in IBD

I.3.2.4.1 Total serum IgE

Total serum IgE is elevated in a number of conditions that are mediated by type I allergy (Grammatikos 2008). If IBD were mediated in a similar fashion it might be expected that total serum IgE levels might be elevated in IBD also. Levo *et al.* looked at the serum concentration of IgE in patients with IBD (Levo, Shalit *et al.* 1986). They found that the serum concentration of IgE, as well as the prevalence of patients with "high IgE", was significantly increased in IBD. Among patients with IBD, those with CD or those in relapse had the highest levels of IgE. They concluded that, based on these findings, allergy might play a pathogenic role in a subset of IBD patients.

Other investigators, however, have not replicated this finding. In their 1977 study Pepys *et al.* found that only 12 of 39 patients with CD and 5 of 20 with UC had elevated serum levels of IgE. Their patient group included patients with documented atopy (Pepys, Druguet *et al.* 1977). Mee *et al.* compared the serum IgE levels of 39 UC and 35 CD patients with control subjects (Mee, Brown *et al.* 1979). They found no difference in serum IgE levels between the groups. Troncone *et al.* found no difference between controls and IBD patients, or IBD subgroups, when they measured serum IgE levels in 122 patients with IBD and 103 age-matched controls (Troncone, Merrett *et al.* 1988). Finally, Brignola *et al.* studied 50 patients with UC, 50 patients with CD and 100 healthy controls matched for sex and age (Brignola, Miniero *et al.* 1986). There was no significant difference in the total serum IgE level between UC, CD and controls.

I.3.2.4.2 Serum food specific IgE antibodies

In addition to measuring the total serum IgE in IBD patients, Brignola *et al.* evaluated the presence of food specific IgE antibodies to 10 foods using the radioallergosorbent test (RAST). They found that a positive reaction to specific IgE was significantly less frequent in controls than in UC and CD. The result was also more often positive in CD patients with colonic or ileocolonic involvement than in isolated ileal disease.

Frieri *et al.* found an increase in total serum IgE in only 3 of 11 CD patients (Frieri, Claus *et al.* 1990). They in turn looked for specific IgE antibodies to foods, this time using enzyme linked immunosorbent assay (ELISA) for specific IgE antibodies to five foods (egg, milk, wheat, soy and corn). In their study all patients had low to negative serum IgE levels to all foods. Bartunkova *et al.* also used ELISA to detect specific IgE to nine food allergens (chicken egg white, cow's milk, peanut, soy bean, apple, walnut, gliadin, carrot, and fish) in children with CD, UC and coeliac disease. Only 2 of 23 UC and 3 of 21 CD patients tested positive for food specific IgE antibodies. There was no difference between any of the groups. Unfortunately both these studies lacked a disease free control group with which to make comparisons regarding the significance of these results.

Finally Huber *et al.* considered whether a lack of detectable IgE specific food antibodies in serum might relate to the formation of IgG anti-IgE immune complexes, which would effectively 'hide' the specific IgE present from RAST studies (Huber, Genser *et al.* 1998). They examined the serum of 107 patients with CD and 65 healthy controls, unmasking specific IgE from anti-IgE autoantibodies by treating the purified immune complexes in a low pH environment in order to detect 'real' levels of specific IgE in RAST immunoassay. They again found no increase in food specific IgE antibodies in CD compared to controls.

I.3.2.4.3 Luminal IgE

The main secreted immunoglobulin subtype of the intestinal mucosa is IgA. Normally very little IgE secretion occurs but variable levels are detectable in the stool of well people (Kolmannskog, Florholmen *et al.* 1986). The absence of serum IgE reactivity in IBD reduces the likelihood that specific IgE antibodies play a strong role in the

pathogenesis of IBD, but it remains possible that IgE antibodies might play a role at the luminal surface that is not reflected in serum studies. Very little information exists regarding this possibility, largely because of the difficulty in designing techniques capable of examining any involvement.

Total IgE levels in the intestinal lumen are elevated in childhood allergy and parasitic disease (Schwab, Raithel *et al.* 2001). In a study of the excretion of IgE in faeces Kolmannskog *et al.* found that less than 10% of 88 presumably healthy infants, children, and adults had detectable IgE in their feces. Conversely 21 of 40 (53%) of children with various kinds of allergy had measurable fecal IgE. That was compared with the 6 of 25 (24%) adult patients with UC or CD in clinical remission who had IgE-positive fecal extracts. No information exists regarding the production of specific IgE antibodies in the intestinal mucosa. However, there is evidence that IgE dependant mechanisms can play a role in altering human IP (Crowe and Perdue 1993) raising the possibility that the negative results of serum studies do not preclude a role for IgE mediated reactions at the mucosal level.

I.3.2.5 Mast cells

The mast cell is the effector cell of type I hypersensitivity. Its role in IBD, however, is less well understood. Reports of increased mast cell numbers in IBD date back to 1966 when Bercovitz and Sommers, using light microscopic techniques, found increased numbers of mast cells in the rectal tissue of patients with active UC (Bercovitz and Sommers 1966).

Not only are the number of mast cells increased in the mucosa of IBD patients but also the function of mast cells are altered in comparison with normal subjects. Lilja *et al.* were able to show that mast cells are an important source of TNF-alpha in all layers of

the ileal wall in CD (Lilja, Gustafson-Svard *et al.* 2000). They looked for TNF-alpha immunohistochemically in full thickness specimens of ileal wall from patients with CD (histologically normal, n = 9; inflamed, n = 6) and controls (patients with colonic cancer, n = 8). In all layers of the ileal wall, and in every specimen investigated, mast cells were the main cell type that expressed TNF-alpha immunoreactivity out of the TNF-alpha-labelled cells.

Mast cells may also play an important role in the signalling of CD4+ lymphocytes in CD. A study by Middel *et al.* demonstrated increased numbers of IL-16+ mast cells in active CD in comparison with UC and controls (Middel, Reich *et al.* 2001). This correlated positively with an increased number of CD4+ lymphocytes, suggesting a role for mast cells in the initiation and persistence of the inflammatory process in CD.

I.3.2.5.1 The Role for therapies directed at the mast cell in IBD

Many of the therapies effective against IBD do have actions on the mast cell. As discussed above mast cells are a source of TNF-alpha in IBD and anti-TNF-alpha agents are effective in these diseases (Carter, Lobo *et al.* 2004). In addition the 5ASA preparations, which form the mainstay of UC treatment, are able to inhibit anti-IgE induced histamine and PGD2 release from human intestinal mast cells (Fox, Moore *et al.* 1991).

I.3.2.6 Histamine

As well as its action in the immediate allergic response as a potent vasoactive agent, smooth muscle constrictor, and stimulant of nociceptive itch nerves (Repka-Ramirez and Baraniuk 2002), histamine participates in the delayed allergic reaction by activating and chemo-attracting neutrophils and eosinophils, and by increasing IL-8 and evoking

leukocyte rolling on endothelial cells (He, Peng *et al.* 1997). Using a variety of techniques histamine has been shown to be elevated in the involved tissues of IBD.

Knutson and colleagues used a segmental jejunal perfusions system with a two-balloon, six-channel small tube to measure the jejunal secretion rate of histamine in patients with CD (n = 15) of the terminal small bowel and in healthy controls (n = 24) (Knutson, Ahrenstedt *et al.* 1990). They found that histamine secretion was significantly increased in patients with CD compared with the secretion rate in controls. Moreover, the secretion of histamine was related to the disease activity. Similar results were found on examination of stool from 62 CD and 24 UC patients compared to 8 healthy controls (Bischoff, Grabowsky *et al.* 1997). Histamine levels in endoscopic gut mucosal biopsies and histamine release from those biopsies were also increased in both UC and CD (Baenkler, Lux *et al.* 1987; Horauf, Matek *et al.* 1989; Fox, Lichtenstein *et al.* 1993; Raithel, Matek *et al.* 1995).

Systemic production of histamine has also been measured in IBD using urinary levels of N-methylhistamine, a stable metabolite of histamine. Increased levels were seen in three reports provided by the same group. Initially they examined 41 patients with CD and, as controls, 27 persons being worked up for irritable bowel syndrome or food allergy (Weidenhiller, Raithel *et al.* 2000). They measured 24-hour urinary methylhistamine, correlating it with urinary creatinine to provide an internal comparator, expressing the urinary methylhistamine level as mg/mmol creatinine x m2 body surface area. The urinary methylhistamine level was significantly elevated in CD compared to controls. In addition there was a higher mean urinary methylhistamine level in active vs. inactive CD but this result did not reach statistical significance. The second report, in 2002, investigated 55 controls, 56 patients with CD, and in 36 patients with UC (Winterkamp,

Weidenhiller *et al.* 2002). Urinary excretion of methylhistamine was found to be significantly elevated in IBD. Patients with active CD and active UC had higher rates of methylhistamine excretion than patients in remission or controls. Methylhistamine excretion was also significantly correlated with disease activity. The latest paper, published in 2007, followed 8 CD patients in remission over a year, comparing them to controls (Kimpel *et al*. 2007). The urinary methylhistamine level remained in the normal range throughout follow-up, suggesting no accumulation and degranulation of mast cells during inactive phases of CD.

I.3.2.7 Eosinophils

As eosinophils are of particular relevance to the late phase allergic reaction, characterised by cellular infiltration and tissue destruction (Charlesworth, Hood *et al.* 1989), their role in the pathogenesis of IBD might suggest similar processes are involved.

Increased numbers of eosinophils are seen in inflamed and non-inflamed colonic tissues of IBD patients. Carvalho *et al.* used eosinophil specific histological staining techniques to examine colonic biopsies from 15 CD, 15 UC and 12 irritable bowel syndrome control patients (Carvalho, Elia *et al.* 2003). Increased proportions of eosinophils were found in the colon of patients with UC and in inflamed and non-inflamed colon of CD patients as compared with controls.

The quantity of eosinophils present in the mucosa may also correlate with the clinical activity of disease. In a study in 50 patients with ulcerative proctocolitis who were followed for a mean period of 70 months, Heatley and James found that the number of eosinophils in the rectal mucosa predicted the clinical course of disease (Heatley and

James 1979). They found that patients with a higher mucosal eosinophil count had disease that more readily responded to treatment.

In a study designed to examine the pathogenesis of eosinophil infiltration during the recurrence of CD in the post-resection neo-terminal ileum, samples were taken from nine patients with CD three months after ileocolectomy (Dubucquoi, Janin *et al.* 1995). Tissue eosinophils were studied by histochemical methods and electron microscopy. Mucosal expression of IL-5 was also studied using in situ hybridisation. These techniques were applied in normal and diseased areas of the neo-ileum. Eosinophil infiltration was more pronounced in diseased than in endoscopically normal areas and was associated with a high expression of IL-5 mRNA. Ultrastructural analysis showed features of eosinophil activation, but no cytotoxic lesions of surrounding inflammatory or epithelial cells. The authors concluded that local synthesis of IL-5 and associated eosinophil activation might participate early in the mucosal damage of CD.

In addition to increased infiltration of eosinophils in the mucosa of IBD patients there is evidence of increased mucosal eosinophil activity in IBD. Hallgren *et al.* measured the concentrations of eosinophil cationic protein (ECP), a specific eosinophil granule protein, in jejunal perfusion fluid from ten patients with CD and 14 controls (Hallgren, Colombel *et al.* 1989). While the jejunal segment perfused in patients with CD was endoscopically and histologically normal, the perfusion fluid concentration of ECP was double that in the control subjects. Another study used segmental perfusion of the sigmoid colon and rectum in 18 UC patients and 18 healthy controls to confirm a 10 to 20 fold increase in ECP, eosinophil protein X (EPX) and eosinophil peroxidase (EPO) in colitis patients compared with controls (Carlson, Raab *et al.* 1999). EPX and EPO, like ECP, are potent cytotoxic and neurotoxic mediators derived from eosinophilic

granules, which are involved in killing parasites and in tissue destruction during inflammatory processes. The same study found increased levels of GM-CSF and IL-8, and a correlation between all three eosinophil granule proteins and the levels of IL-8/GM-CSF in the sigmoid segments of patients with colitis. This correlation suggested a role for GM-CSF and IL-8 as eosinophil priming cytokines.

Berstad *et al.* were able to measure ECP in human stool samples (Berstad, Borkje *et al.* 1993). In a study of 10 patients with active CD, 19 with active UC, and 10 healthy controls they found elevated levels of ECP in both patient groups as compared to controls. A further study by Bischoff *et al.* provided evidence of increased eosinophil activation in IBD (Bischoff, Grabowsky *et al.* 1997). They collected stool samples from 62 CD patients, 24 UC patients and 8 healthy controls. They were able to measure ECP and EPX in the stool. Elevated levels of ECP and EPX were seen in patients compared with controls. The levels of ECP and EPX did not correlate with activity of disease and high levels of ECP and EPX were not specific for IBD, also occurring in other diseases associated with inflammation of the intestinal mucosa.

These studies confirm a role for the eosinophil in the pathology of IBD and suggest that the eosinophil may be an early player in the development of IBD related mucosal inflammation. They do not, however, allow conclusions to be drawn as to the exact role of the eosinophil in the aetiopathogenesis of these diseases.

I.3.2.8 IgG mediated food hypersensitivity

Whilst raised IgG levels are seen in patients with asthma, hayfever, eczema and atopic dermatitis, (Shakib, McLaughlan *et al.* 1977; Gwynn, Smith *et al.* 1978) the published data regarding IgG mediated immune reactions in food hypersensitivity is contradictory (Galant, Bullock *et al.* 1973; Wraith, Merrett *et al.* 1979). In fact it has been suggested

that IgG production may be a normal immunological response to dietary antigens (Barnes, Barton *et al.* 1983; Merrett, Burr *et al.* 1983; Johansson, Dannaeus *et al.* 1984; Husby, Oxelius *et al.* 1985). However, increased levels of food-specific IgG and IgG4 antibodies have been demonstrated in atopic eczema and respiratory allergy (Merrett, Barnetson *et al.* 1984; Shakib, Brown *et al.* 1986; Okahata, Nishi *et al.* 1990; Barnes, Lewis-Jones *et al.* 1993).

Raised levels of specific IgG antibodies to a limited range of foods have also been reported in IBD. In the first study of its kind, Davidson *et al.* were able to show that antibodies to maize were detectable, using an immunofluorescent technique, in 14% of controls, 33% of CD patients, and in 50% of UC patients (Davidson, Lloyd *et al.* 1979). They found similar levels in patients with coeliac disease and concluded, "The similar incidence of antibodies in the IBD and coeliac groups suggests absorption of dietary antigen secondary to an increased mucosal permeability".

Other authors have since promoted this conjecture, but it is interesting to note that a Medline search reveals no study that directly compares any measure of IP with food-specific IgG antibody production in IBD. The evidence that does exist regarding IgG reactivity to luminal antigens and IP relates to IgG antibodies to yeast. In their 2003 study Harrer *et al.* examined the relationship between serum levels of anti-Saccharomyces cerevisiae antibodies (ASCA) and IP at a given time and the probability of increased ASCA serum levels with increased IP in patients with CD (Harrer, Reinisch *et al.* 2003). They did not find a statistically significant association between ASCA IgG antibodies and IP and concluded, "elevated serum levels of anti-S. cerevisiae antibodies do not seem to result primarily from a defect of the gut barrier".

In a further study of food specific IgG antibodies in IBD Knoflach *et al.* measured serum IgG, IgM and IgA antibodies to five major proteins of cow's milk, casein, bovine serum albumin, alpha-lactalbumin, beta-lactoglobulin A, and beta-lactoglobulin B, using enzyme-linked immunosorbent assay in 51 patients with UC, 49 with CD, and 20 age-matched controls (Knoflach, Park *et al.* 1987). IgG and IgM antibodies to cow's milk proteins were significantly elevated in patients with IBD as compared to controls. These increased titres seemed to be specific and not due to a polyclonal immunoglobulin activation, as naturally occurring blood group antibodies were not elevated. In addition there was a correlation between disease activity and antibody titers.

Similar results were observed by Paganelli *et al.* on measuring the class-specific antibody response to the cow's milk antigen beta-lactoglobulin in sera from patients with UC and CD (Paganelli, Pallone *et al.* 1985). IgG antibodies were higher in active cases but antibody titres did not correlate with disease duration.

A slightly wider range of food-specific IgG antibodies was tested by Frieri *et al.* in their 1990 study (Frieri, Claus *et al.* 1990). They evaluated 11 CD patients by measuring serum IgG4 levels to five food proteins (egg, milk, wheat, soy, and corn) using a sensitive enzyme monoclonal antibody assay. They found an increased IgG4 humoral response to egg protein only.

Although little data exists regarding the prevalence of IgG antibodies to a wider range of foods in IBD, there is some information in the setting of IBS. Zar *et al.* examined the patterns of IgG4 food-specific antibody positivity in patients with IBS compared with controls (Zar, Benson *et al.* 2005). They measured IgG4 titers to 16 common foods (milk, eggs, cheese, wheat, rice, potatoes, chicken, beef, pork, lamb, fish, shrimps, soya

bean, yeast, tomatoes, and peanuts) in one hundred and eight IBS patients and 43 controls. They found that subjects with IBS had significantly higher titres of antibodies to wheat, beef, pork and lamb than controls.

The same group have reported an uncontrolled study of the effect of an exclusion diet based on IgG4 titres on IBS symptoms and rectal sensitivity and compliance (Zar, Mincher *et al.* 2005). They studied 25 patients with IBS, measuring IgG4 titres to the 16 foods mentioned above. Foods with titres >250 microg/l were excluded for 6 months. A statistically significant improvement was reported in pain severity, pain frequency, bloating severity, satisfaction with bowel habits and effect of IBS on life in general, at 3 months. This symptom improvement was maintained at 6 months.

Atkinson *et al.* were able to show similar results using non-subtype IgG food-specific antibodies in a randomised, sham controlled study of IBS patients (Atkinson, Sheldon *et al.* 2004). A total of 150 outpatients with IBS were randomised to receive, for three months, either a diet excluding all foods to which they had raised IgG antibodies or a sham diet excluding the same number of foods but not those to which they had antibodies. After 12 weeks, the true diet resulted in a 10% greater reduction in symptom score than the sham diet.

While the exclusion diets were open, patients and investigators were blinded as to the results of IgG testing. Potential confounding factors in this study include the fact that IgG antibodies to wheat, milk, whole egg and yeast were common amongst subjects with IBS and so were commonly excluded in the true exclusion diet compared with the sham diet. These foods are widely reported as causing food intolerance in IBS and may be associated with worsening of IBS symptoms by non-immunological mechanisms.

Critics of the study concluded, “regardless of IgG antibody status, the dietary restrictions in one group were not controlled for by the other group” and “it is therefore difficult to assess whether a diet excluding these foods would have led to symptomatic improvement in all patients regardless of their antibody status” (Mawdsley, Irving *et al.* 2005; Sewell 2005).

Following the above studies, and the experiments that make up this thesis, Bentz *et al.* published their experience with IgG antibody guided diets in CD (Bentz, Hausmann *et al.* 2010). In an initial observational pilot study they examined 20 healthy volunteers without a history of food intolerance and 79 CD patients with different disease status. Forty-seven of them had clinical and endoscopic signs of acute inflammation (i.e. diarrhea and mucosal ulcerations). Twenty-four CD patients had chronically active disease and 8 were in remission. They used the ImuPro300 test (Evomed, Darmstadt, Germany) to detect food specific IgG antibodies in serum. Disease activity was assessed by the patient’s medical record.

They found a significant difference in serum IgG antibodies between CD patients and healthy controls ($p < 0.0001$, t test). The ten most frequently measured IgG antibodies in CD patients were against processed cheese (84%), yeast (83%), agave syrup (78%), camembert cheese (76%), poppy seeds (74%), aloe vera (74%), bamboo sprouts (73%), kamut (durum wheat, 70%), unripe spelt grain (69%) and wheat (60%). More CD patients showed reactions against the evaluated food components than healthy controls, i.e. 35% of healthy controls had IgG antibodies against wheat in contrast to 60% of CD patients. Moreover, 39% of CD patients had IgG antibodies against hazelnut in contrast to only 15% of healthy controls. This was even more pronounced in IgG antibodies against linseed, where 70% of CD patients and only 10% of healthy controls showed

IgG antibodies. The same was seen with processed cheese (60% of healthy controls vs. 84% of CD patients). The most frequently detected IgG antibodies in healthy controls were against yeast (66%), Aspergillus niger (60%), whey (60%), processed cheese (60%), bamboo sprouts (55%), paprika spice (55%), crawfish meat (50%), cottage cheese (45%), yoghurt (45%) and zander (45%).

In the proceeding interventional study 40 CD patients were evaluated in a randomised, double blind, cross over, sham diet controlled fashion. Each diet was followed for 6 weeks. Similar to the Atkinson *et al.* study in IBS the specific and sham diets were based on similarity of the excluded food components. If, for example, IgG against hazelnut was detected, then almond was excluded in the sham diet; if cauliflower IgG was found, broccoli was excluded. Sixteen male and 24 female subjects were randomised but, in a non-intention to treat analysis, only 23 patients were included in the final analysis because of a high dropout rate (n=17) from the treatment first and sham first groups.

There was a significant reduction in the daily stool frequency by 11% in the active diet compared to the sham diet group ($p = 0.004$, 95% CI: 4%, 18%). However, the effect was confounded by a significant increase in stool frequency of 9% in the second intervention phase of the study, regardless of type of diet ($p = 0.025$, 95% CI: 1%, 18%; fig. 2). Only those patients who first followed the specific diet had a significant reduction in stool frequency. The group of subjects who first followed the sham diet had no significant change in their stool frequency.

This study demonstrated that IgG antibodies against a number of food antigens are elevated in patients with CD in contrast to healthy controls. An improvement in some

IBD symptoms was observed in patients eliminating foods to which they were found to exhibit elevated IgG antibodies. Forty-eight percent of patients in the intervention group had an improvement in stool frequency and general well-being (total score). Only 9% of patients described the opposite effect. However, the high drop out rate meant that an intention to treat analysis would have shown much less effect than that seen in the per-protocol analysis performed. In addition the 40 patients initially included in this study were on different medications, allowing no control for the confounding effect of different medication regimens on IgG food antibody results or course of disease. As with the Atkinson *et al.* study in IBS, it could also be argued that the sham diet was too similar to the specific diet, as the definition of specific and sham diet was based on the similarity of excluded food components. There may be some cross-reactivity of the respective antigens, which could explain some effects of the sham diet on IBD symptoms. Finally, the study did not include a washout phase at the cross-over point, which may have led to some transmission of effects into the sham arm of the study.

Although these studies exhibit methodological problems, their findings challenge the dogma that IgG antibodies to food are non-specific and of no relevance to GI disease. The possibility exists that IgG antibodies to food might be useful in guiding dietary management of those GI diseases that are responsive to dietary manipulation.

Chapter II Current evidence and recommendations for the use of dietary manipulation in the management of IBD

II.1 Crohn Disease

II.1.1 Attitudes

While there is no evidence that specific immune mediated reactions to food play a role in most patients with either CD or UC, (Bischoff, Mayer *et al.* 2000) it is commonplace for patients with GI disorders to believe that something in their diet has caused their condition (Crowe 2001; Joachim 1999). Some studies have claimed that food intolerances are common in CD and have found that when food intolerances are detected, patients on an exclusion diet maintain remission significantly longer than those on an unrestricted diet (Jones, Dickinson *et al.* 1985; Riordan, Hunter *et al.* 1993). However, when these patients are subjected to double-blind food challenges only 15% show a positive response (Pearson, Teahon *et al.* 1993).

That both newly diagnosed and chronically affected patients with CD have significant nutritional inadequacies is clear. Malnutrition is often reported, especially in CD patients with active disease (Harries and Heatley 1983; Fernandez-Banares, Abad-Lacruz *et al.* 1989; Fernandez-Banares, Mingorance *et al.* 1990; Janczewska, Bartnik *et al.* 1991; Stokes 1992; Kuroki, Iida *et al.* 1993; Royall, Greenberg *et al.* 1995; Teahon, Pearson *et al.* 1995; Zurita, Rawls *et al.* 1995; Azcue, Rashid *et al.* 1997; Capristo 1998). In addition several nutritional and functional deficiencies, especially of antioxidants, in patients with longstanding CD in remission, have been described (Geerling, Badart-Smook *et al.* 1998). Serum vitamin B12 concentrations are

significantly lower in CD than controls even at the time of diagnosis (Geerling, Badart-Smook *et al.* 2000).

The degree to which these nutritional deficiencies are related to malabsorption and inflammation versus dietary deficiencies is not clear. It does seem likely that dietary exclusions which do not positively influence disease activity might have a negative effect on nutritional status and thus a negative effect on wellbeing. Certainly patients with CD do avoid those foods to which they report intolerance (Joachim 1999). No consensus statements regarding the nutritional advice that should be given to CD patients exist, in particular with respect to exclusion of foods from the diet. In the following section the current evidence, where any exists, for those dietary manipulations commonly practised in CD are discussed.

II.1.2 Milk and dairy products

The possible mechanisms for dairy intolerance in CD are multiple and include lactose malabsorption (LM) due to lactase inactivity secondary to mucosal disease, the long-chain triacylglycerol content of milk, or allergy to milk proteins (see Table 13). Milk has always featured highly in the list of foods investigated for IBD related food intolerances, originating in the early part of last century with the theory that milk allergy might contribute to the pathogenesis of IBD (Truelove 1961; Binder, Gryboski *et al.* 1966). This was later replaced by the recognition that in large part the GI symptoms produced by milk relate to malabsorption of lactose (Saavedra and Perman 1989) and, specifically in CD, might relate to the presence of long-chain triacylglycerol content as evidenced by improved response to enteral diets excluding these fats in CD (Middleton, Rucker *et al.* 1995).

There is some data to show that dairy intolerance (as reported by patients) occurs in CD patients more commonly than controls. In a questionnaire study of 33 patients with CD and 27 patients with UC Joachim *et al.* determined those foods which patients reported as affecting them positively and compared them to those that affected them negatively. Dairy products were reported as affecting both disease groups negatively (Joachim 1999). Using similar methodology Triggs *et al.* examined a Caucasian CD population in New Zealand (Triggs, Munday *et al.* 2010). They reported similar rates of dairy intolerance. Interestingly, it seemed the greater the fat content of the dairy product, the higher the frequency of reports of intolerance. Von Tirpitz *et al.* asked 49 patients with CD specifically about dairy intolerance (von Tirpitz, Kohn *et al.* 2002). 46.9% of the disease group reported milk intolerance compared to 16.6% of controls. However, the H2 lactose breath test was positive in just 70% of the patients reporting milk intolerance. The mechanism and data surrounding LM in CD is discussed further below (see section II.1.2.1).

Very little data exists regarding the veracity of milk allergy as an aetiological mechanism in CD. The majority of data that is available concentrates on the use of classical allergy tests (including skin prick and RAST tests) and also on the incidence of non-IgG food-specific antibodies in detecting immune sensitisation to food antigens. Direct testing for allergy as a mechanism is fraught in the gastrointestinal tract. While double blind placebo controlled food challenge is perhaps the gold standard for detecting food hypersensitivity, it is not specific for the mechanism of hypersensitivity and in no way rules out non-immune mechanisms (Bischoff and Crowe 2005). Novel methods for detecting immune reactivity to foods in the gut have yielded results in CD (Bischoff, Herrmann *et al.* 1996; Van Den Bogaerde, Cahill *et al.* 2002). This topic is considered separately in detail in section V.1.1

Additionally it may be that any effect of milk ingestion on CD activity is not only mediated by sugar content in the form of lactose, or protein as a potential allergen, but also by the fat constituent. This is supported to an extent by studies manipulating the type of fat given to CD patients receiving therapy with enteral nutrition and is discussed in detail below (see section II.1.4.6).

Potential Mechanisms for Milk Intolerance in CD	Evidence for a role in CD
Long chain triacylglycerols	There is an improved response to enteral diets excluding these fats in CD (Middleton, Rucker *et al.* 1995) the greater the fat content of the dairy product, the higher the frequency of reports of intolerance (Triggs, Munday *et al.* 2010)
Allergy to milk protein	Despite the initial interest in a mechanism for milk allergy in IBD (Truelove 1961; Binder, Gryboski *et al.* 1966) there is little direct evidence of a role for milk allergy in CD
Lactase inactivity	The activity of duodenal lactase has been shown to be reduced only in active disease (von Tirpitz, Kohn *et al.* 2002)
Other mechanisms for Lactose Malabsorption	Disease location and activity, small bowel transit time and previous surgery are implicated (Pironi, Callegari *et al.* 1988; Mishkin, Yalovsky *et al.* 1997)

Table 13. Summary of the evidence for a role of each potential mechanism for dairy intolerance in CD

II.1.2.1 Lactose malabsorption in Crohn Disease

Disaccharide lactose, the principal carbohydrate of animal milks, requires the enzyme lactase to split it into glucose and galactose. Undigested lactose passes to the colon where fermentation produces hydrogen and short-chain fatty acids that can cause abdominal distension, pain, and sometimes diarrhoea (Scrimshaw and Murray 1988). The term LM is widely used for an arbitrarily defined "significant" increase in breath hydrogen (H2) (10-20 ppm) after an oral lactose challenge (King and Toskes 1983; Saavedra and Perman 1989). This is based on an equally arbitrary 50g lactose challenge that is only applicable to the hereditary form of LM, which is not associated with any

organic gastrointestinal disorder (Mishkin 1997). It also fails to include the variable fraction of the population (10-20%) who do not excrete appreciable H2 during colonic fermentation, which can lead to false-negative results in the breath test (Corazza, Strocchi *et al.* 1993). On the other hand, a positive hydrogen breath test may just be an indicator of bacterial overgrowth (Mishkin 1997).

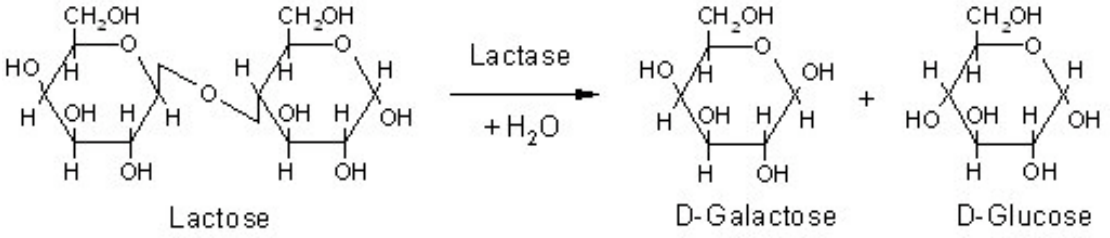


Figure 4. Lactase converts lactose to galactose and glucose in the small intestine

The frequency of LM in patients with CD, particularly those with active disease, appears to be higher than that in the normal population (Littman, Cady *et al.* 1968; Pironi, Callegari *et al.* 1988; Mishkin, Yalovsky *et al.* 1997; von Tirpitz, Kohn *et al.* 2002; Barrett, Irving *et al.* 2009). However, reports are contradictory (Gudmand-Hoyer and Jarnum 1970; Park, Duncan *et al.* 1990; Banos Madrid, Salama Benerroch *et al.* 2004). It is still only a small proportion (8% in one study) that experience symptoms of intolerance when challenged with milk (Pironi, Callegari *et al.* 1988) and most of these patients consume some dairy product without any significant discomfort (Mishkin 1997). The available evidence is summarised in Table 14.

Authors	Country	Crohn disease		Controls		Diagnostic method	
		Number of patients	Lactose Malab-sorption	Number	Lactose Malab-sorption	Lactose tolerance test	Determination of mucosal enzyme activity
Chalfin and Holt 1967	USA	5	3 (60%)			+	
Littman, Cady *et al.* 1968	USA	11	5 (45%)	93	18 (19%)	+	+
Gudmand-Hoyer and Jarnum 1970	Denmark	71	4 (6%)	700	3-7%	+	+
Kirschner, DeFavaro *et al.* 1981	USA	50	17(34%)	40	"similar to IBD"	+	
Pironi, Callegari *et al.* 1988	Italy	37	18 (49%)	67	11(16%)	+	
Park, Duncan *et al.* 1990	Scotland	62	2(3%)	13 (IBS patients)	1(8%)		+
Huppe, Tromm *et al.* 1992	Germany	124	21(17%)			+	
Mishkin, Yalovsky *et al.* 1997	Canada	121	48(40%)	158	46(29%)	+	
von Tirpitz, Kohn *et al.* 2002	Germany	49	23(47%)	24	4(17%)	+	+
Banos Madrid, Salama Benerroch *et al.* 2004	Spain	18	3(17%)	25	5(20%)	+	
Barrett, Irving *et al.* 2009	Australia	92	39(42%)	71	13(18%)	+	

Table 14. Studies of lactose malabsorption in Crohn disease

II.1.2.2 Mechanisms of lactose malabsorption

There are many mechanisms for LM. In the general population the two main risk factors for LM are related to reduced levels of duodenal lactase. They are ethnicity, with very high rates in Asians and Native Americans (DiPalma and Narvaez 1988); and age, with

decreasing levels of duodenal lactase occurring with older age (Welsh, Poley *et al.* 1978; Rao, Bello *et al.* 1994).

However, in CD other mechanisms may also be important and the activity of duodenal lactase has been shown to be reduced only in active disease (von Tirpitz, Kohn *et al.* 2002). There is the suggestion that disease location influences the frequency of LM in CD with a higher incidence in proximal small bowel disease than terminal ileal and terminal ileum than colonic disease in turn (Mishkin 1997). Disease activity and previous surgery have also been discussed as determinants of LM (Pironi, Callegari *et al.* 1988; Mishkin, Yalovsky *et al.* 1997). In addition it may be that many CD patients who are positive for LM have a small bowel transit time that is significantly shortened (Mishkin, Yalovsky *et al.* 1997).

II.1.2.3 Lactose or dairy restriction in the treatment of CD

Much of the evidence for dairy avoidance in CD is embedded in studies that examined diets with multiple food exclusions. The majority of positive results stem from studies from one centre that examined the ability of dietary exclusion to maintain a remission induced by therapy with an elemental diet. Once those CD patients were in remission, 30% managed to stay in remission with avoidance of dairy products and other offending foods identified using elimination diets (Jones, Dickinson *et al.* 1985; Jones 1987; Riordan, Hunter *et al.* 1993). Although two other studies have confirmed these results, the benefit was much less than that seen in the original studies (Giaffer, Cann *et al.* 1991; Pearson, Teahon *et al.* 1993). It is not possible to extrapolate from these studies the degree to which dairy avoidance contributed to the results.

The only available evidence for a diet excluding milk alone is a dated case series in which three CD patients, who were milk drinkers with lactose malabsorption proven on

enzyme testing and blood sugar response to lactose challenge, experienced a total cessation of diarrhoea with a lactose-free diet. In addition nine patients without evident lactose malabsorption were treated with a lactose free diet and the diet was beneficial in three patients, as judged from the number of bowel movements and their feeling of well being (Gudmand-Hoyer and Jarnum 1970)

Nanji *et al.* made an interesting observation in their epidemiological study of the association between geographic incidence of CD and LM (Nanji and Denardi 1986). They found a strong negative correlation (-0.93, $p<0.01$) between the incidence of CD and LM over 13 countries. The authors propose that the mechanisms by which such protection occurs include: a) production of short chain fatty acids and lactate; b) increase in intestinal transit time; and, c) reduction in production of noxious substances that may be partially responsible in the pathogenesis of IBD.

Very little evidence exists regarding physician practice in the use of dairy exclusion in IBD. Mishkin reported the results of a survey conducted in > 100 physicians and nutritionists attending a Canadian IBD conference in 1995 (Mishkin 1997). 40% indicated that they advised their IBD patients to avoid dairy products.

Dairy products are an important source of calcium and other nutrients and a reduction in their intake might have significant consequences for the patient with CD (Mishkin 1997). This possibility is supported by a study of 38 patients with CD who underwent bone densitometry. Bone mineral densities of the spine were significantly decreased in those with reported milk intolerance (z-score, -1.33 ± 0.92 vs. -0.19 ± 0.95; $p=0.002$). Bone densities of the femoral neck also tended to be decreased in patients with milk intolerance, although this result did not reach significance (z-score, -1.26 ± 0.67

vs. -0.76 ± 0.95; not significant) (von Tirpitz, Kohn *et al.* 2002).

The available evidence, therefore, suggests that there may be an increased rate of detectable LM in the CD population but that the clinical significance of this is not clear. Evidence to support the recommendation of empiric dairy exclusion to patients with CD is lacking.

II.1.3 Carbohydrates

II.1.3.1 Simple sugars

An interest in the role of sugar in the evolution of CD was fuelled by the observation of Thornton *et al.* that newly diagnosed CD patients ate more refined sugar than controls (Thornton, Emmett *et al.* 1979). Further case control studies have shown an increased relative risk of CD for patients with a high intake of sucrose and total intake of carbohydrate (see Table 15).

Persson *et al.* performed a postal questionnaire of 152 CD patients asking about their food intake 5 years previously (Persson, Ahlbom *et al.* 1992). They showed a relative risk of 2.6 for CD in patients who had a high intake of sucrose. Reif *et al.* obtained dietary histories in 33 patients with CD of recent onset. (Reif, Klein *et al.* 1997). Again a high sucrose consumption was associated with an increased risk for CD.

104 UC and CD, 104 controls *et al.* studied the dietary habits of 104 IBD patients immediately prior to the onset of disease using a recall questionnaire (Tragnone, Valpiani *et al.* 1995). They found that patients with CD have a high intake of total carbohydrate, starch and refined sugar. This result was corroborated in a study by Geerling *et al.* who took a dietary history in 23 CD patients within 6 months of initial

diagnosis (Geerling, Badart-Smook *et al.* 2000). They also found a higher mean daily intake of carbohydrates in CD patients compared to controls.

	Study methodology	**Sample size**	**Result**
Thornton, Emmett *et al.* 1979.	Case-control, retrospective interview	30 CD and 30 controls	Median refined sugar intake CD 122 vs. controls 65 g/day ($p<0.002$)
Persson, Ahlbom *et al.* 1992	Case-control, retrospective postal questionnaire	152 CD and 305 controls	RR 2.6 for CD if >55g/day sucrose intake
Tragnone, Valpiani *et al.* 1995	Prospective cohort study	104 UC and CD, 104 controls	High intake of total carbohydrate, starch and refined sugar
Reif, Klein *et al.* 1997	Case-control, retrospective interview of patients with recent onset CD	33 CD and 144 controls	OR 2.85 for CD if high sucrose consumption
Geerling, Badart-Smook *et al.* 2000	Case-control, contemporary dietary interview	23 CD and 23 controls	Total carbohydrate (en%) CD 51% vs. controls 46% ($p<0.05$)

(RR: relative risk; OR: odds ratio; en%: percentage of energy intake)

Table 15. Studies of sugar consumption in Crohn disease

In the study by Geerling *et al.* the total carbohydrate intake expressed as a percentage of energy was significantly higher in patients with active disease compared to those in remission. It has been suggested that the above observations of an association between CD and carbohydrate intake might simply represent a consequence of active disease (Riordan, Ruxton *et al.* 1998)

A study by Heaton *et al.* retrospectively compared 32 CD patients treated with conventional therapy plus a fibre-rich, unrefined-carbohydrate diet to 32 matched patients who had received no dietary instruction. There were fewer hospital admissions and surgical interventions in the diet treated patients (Heaton, Thornton *et al.* 1979).

Ritchie *et al.* followed this up with a controlled, multicentre study of 162 patients given the above diet compared to 190 patients given a diet unrestricted in sugar and low in fibre (Ritchie, Wadsworth *et al.* 1987). They found no difference between the groups in terms of the relapse rate over 2 years.

Certainly it seems that patients with CD consume more refined sugar and have a higher total carbohydrate intake that controls. However, it is not clear that this association is causal and the available evidence does not suggest that restricting refined sugar intake in patients with CD results in an improvement in disease outcomes.

II.1.3.2 FODMAPs

The variable evidence of a role for lactose malabsorption in CD has led in recent years to interest in other fermentable carbohydrates. Incomplete absorption of fructose is relatively common in the normal population and has been identified as a possible precipitant of functional gastrointestinal symptoms (Simren and Stotzer 2006). In a study by Barret *et al.* 91 patients with CD were tested for fructose malabsorption using a hydrogen breath test (Barrett, Irving *et al.* 2009). They found that fructose malabsorption was more common in CD than in controls (61% vs. 34%, $p<0.05$).

Other rapidly fermented and osmotically active carbohydrates may be malabsorbed and contribute to gastro-intestinal symptomatology. These poorly absorbed short-chain carbohydrates have been termed the Fermentable Oligo-, Di-, Mono-saccharides and Polyols (FODMAPs) (Gibson and Shepherd 2005). They include oligosaccharides, disaccharises, and polyols (see Table 16).

Fructose	Fructans	Lactose	Galacto-Oligosaccharides	Polyols
Honey Apples Mango Pear Watermelon High fructose corn syrup Corn syrup solids	Artichokes (globe and jerusalem) Asparagus Beetroot Chicory Dandelion leaves Garlic (in large amounts) Leek Onion Raddicio lettuce Spring Onion (white part) Wheat (in large amounts) Rye (in large amounts) Inulin Fructo-oligosaccharides.	Milk Icecream Custard Dairy desserts Condensed and evaporated milk Milk powder Yoghurt Margarine Soft unripened cheeses (eg. Ricotta, Cottage, Cream, Marscarpone).	Legume beans (eg. baked beans, kidney beans, bortolotti beans) Lentils Chickpeas	Apples Apricots Avocado Cherries Longon Lychee Nectarines Pears Plums Prunes Mushrooms Sorbitol Mannitol Xylitol Maltitol Isomalt

Table 16. Common examples of the FODMAP foods (adapted from Gearry, Irving *et al.* 2009)

Gearry *et al.* conducted a retrospective phone interview survey of 52 CD patients who had been identified as having previously received dietary advice for probable functional gastro-intestinal symptoms in quiescent CD (Gearry, Irving *et al.* 2009). The dietary advice given included the exclusion of dietary FODMAPs. They found that up to 70% of patients were adherent to the diet. They found that more than half of the patients with abdominal pain, diarrhoea and bloating reported improvement following dietary advice.

The evidence to date is not sufficient to warrant empiric exclusion of FODMAPs from the diet of CD patients suspected of having functional symptoms. Large scale comparative studies do, however, seem indicated.

II.1.4 Enteral and parenteral nutrition

CD patients benefit from treatment with parenteral or enteral formula nutrition. Such treatment has not been shown to have a particular role for the management of UC (Dickinson, Ashton *et al.* 1980; Seidman 1989; Sitzmann, Converse *et al.* 1990). The mechanisms by which such treatment might benefit CD patients are not clear but possibilities include exclusion of deleterious dietary substances (eg. lactose, fat), resting the bowel, altering the microflora of the gut, altering the balance of beneficial to harmful precursors in foods (eg n3- vs. n6-polyunsaturated fatty acids), nutritional improvements, or perhaps reducing the antigenic load presented to the immune mechanisms of the bowel. These potential mechanisms, and the evidence to support them, are discussed in detail in the ensuing section.

Potential mechanism of effect for enteral nutritional	**Evidence for a role in CD**
Bowel rest and reducing the antigenic load	TPN is not superior to EN (Lochs, Meryn *et al.* 1983; Greenberg, Fleming *et al.* 1988), however total EN may be more effective than partial EN (Johnson, Macdonald *et al.* 2006) suggesting removal of something from the diet and resting the gut may play a role
Altering the enteric microflora	EN can change the intestinal microbiota (Pryce-Millar, Murch *et al.* 2004; Lionetti, Callegari *et al.* 2005; Schneider, Girard-Pipau *et al.* 2006), the mechanism by which this might then affect disease is not clear
Altering metabolic precursors	The main example of this is the alteration of the n-3 to n-6 fatty acid ratio (see II.1.4.7 n3 vs. n6 polyunsaturated fatty acids)
Nutritional improvements	Suboptimal levels of micronutrients impair mucosal healing and defence (Gassull 2004) and improvements in nutritional status is important in achieving and maintaining remission (Royall, Jeejeebhoy *et al.* 1994)
Direct anti-inflammatory effect	A decrease in the production of inflammatory cytokines is observed when inflamed mucosa is incubated with formula (Meister, Bode *et al.* 2002), the mechanism for this is unclear

Table 17. Summary of potential mechanisms for an effect of enteral nutrition in Crohn disease

Initial investigations into dietary intervention in CD centred on the use of enteral feeding using elemental diets consisting of amino acids rather than peptides as the nitrogen source, and varying amounts and types of fats. Polymeric (whole protein) drinks have the advantage of lesser cost as well as enhanced taste and palatability, thereby improving tolerance and compliance (Day, Whitten *et al.* 2008). They have been shown to be equally efficacious to elemental enteral nutrition (EN) (Gonzalez-Huix, de Leon *et al.* 1993; Royall, Jeejeebhoy *et al.* 1994; Verma, Brown *et al.* 2000; Zachos, Tondeur *et al.* 2001; Zachos, Tondeur *et al.* 2007). While 5 metaanalyses have concluded that steroid therapy is more efficacious than enteral feeding for attaining remission of CD (Fernandez-Banares, Cabre *et al.* 1995; Griffiths, Ohlsson *et al.* 1995; Messori, Trallori *et al.* 1996; Zachos, Tondeur *et al.* 2001; Zachos, Tondeur *et al.* 2007), it remains true that the remission rate obtained with enteral feeding is approximately 60%, and higher than the 20-30% placebo response (Singleton, Hanauer *et al.* 1993; Lochs 2007). It appears that the benefit might be greater in children than adults and meta-analysis of the paediatric studies (not including any adult studies) using EN for CD has ascertained that EN and steroids were of equal efficacy in the induction of remission in children with active CD (Heuschkel, Menache *et al.* 2000; Dziechciarz, Horvath *et al.* 2007).

Even if steroid is more effective than EN at attaining clinical remission, there is evidence it may not be so efficacious in healing the mucosa. Yamamoto *et al.* treated 28 patients with EN and saw endoscopic healing in 44% and improvements in 78% of patients (Yamamoto, Nakahigashi *et al.* 2005). This is in keeping with a study of 37 children randomised to polymeric EN vs. corticosteroids in which 74% of the children given EN had mucosal healing compared to only 33% in the steroid group ($p<0.05$) (Borrelli, Cordischi *et al.* 2006).

In addition to induction of remission, EN can be used to maintain remission. This has been achieved either by giving enteral formula as intermittent intensive periods of exclusive feeding via a NG tube (Belli, Seidman *et al.* 1988), or using supplements of formula in addition to an ongoing standard diet (Harries, Jones *et al.* 1983; Verma, Kirkwood *et al.* 2000; Esaki, Matsumoto *et al.* 2005; Yamamoto, Nakahigashi *et al.* 2005; Takagi, Utsunomiya *et al.* 2006). An additional benefit of EEN is the nutritional improvement seen during and following therapy in many clinical studies (Sanderson, Udeen *et al.* 1987; Azcue, Rashid *et al.* 1997; Gavin, Anderson *et al.* 2005). EEN also has specific benefits on bone nutrition (Day, Whitten *et al.* 2008). Additionally EN is associated with few side effects, principally loose, unformed motions and flatulence, but also nausea and constipation (Borrelli, Cordischi *et al.* 2006; Day, Whitten *et al.* 2006). Finally, it has been shown to improve QOL scores in children treated with EN for active CD (Afzal, Van Der Zaag-Loonen *et al.* 2004).

II.1.4.1 Direct effects on the epithelium

That EN itself may have a direct effect at the epithelial level is supported by data demonstrating that, although corticosteroids fail to induce epithelial healing (Modigliani, Mary *et al.* 1990; Landi, Anh *et al.* 1992), in the course of inducing clinical remission EN does result in epithelial healing (Afzal, Davies *et al.* 2005; Fell, Paintin *et al.* 2000; Yamamoto, Nakahigashi *et al.* 2005; Berni Canani, Terrin *et al.* 2006).

Suboptimal levels of micronutrients may participate in this process due to defects in tissue repair mechanisms or via impaired defence (Gassull 2004). In addition recent data suggest EN may have a direct effect on inflammatory processes in the epithelium

(Meister, Bode *et al.* 2002; Fell, Paintin *et al.* 2000; Yamamoto, Nakahigashi *et al.* 2005; de Jong, Leach *et al.* 2007).

II.1.4.2 Improvement in nutritional status

Improvement in nutritional status and correction of micronutrient deficiencies are considered key factors for the effect of enteral nutrition and may be associated with an effect on disease activity (Lochs 2006). Improvement in nutritional status appears to be important not only in achieving but also in maintaining remission. (Royall, Jeejeebhoy *et al.* 1994).

II.1.4.3 Resting the bowel from the mechanical effects of digestion

One theory for the beneficial effects of both TPN and EN in CD was that of "resting" the bowel, although exactly what the mechanism underlying this might be was never elucidated. Only two studies have been performed to investigate the validity of this hypothesis, and those in the 1980's. In those studies no advantage of total parenteral nutrition (TPN), or total enteral nutrition compared with partial parenteral nutrition (PN) in addition to standard food, was found (Lochs, Meryn *et al.* 1983; Greenberg, Fleming *et al.* 1988). Hence it was concluded that bowel rest is not necessary, enteral nutrition should be preferred, and patients might eat ad libitum in addition to enteral nutrition. It is interesting to note that despite this fact all later studies put patients on ''bowel rest'' whilst on enteral nutrition.

The waters are somewhat muddied by a recent randomised controlled study that showed that total EN was superior to partial EN, with 50% of calories provided as EN and 50% as normal diet (Johnson, Macdonald *et al.* 2006). While this study seems supports the bowel rest hypothesis, the study was not designed to test this hypothesis. It fails to control for the beneficial nutritional effects provided by total EN versus relying on oral

food intake to provide 50% of the daily caloric requirements. Thus the difference between the groups is as likely to be explained by a lack of nutritional repletion, or a reduced exposure to potential intrinsic anti-inflammatory effects of EN, in the partial EN group.

II.1.4.4 Altering the gut flora – A prebiotic effect for EN?

That the expression of CD is modulated to some degree by the intestinal flora is well established (Shanahan 2004). It seems reasonable therefore that the effects of EN might be mediated by alterations in that flora. It has been shown that EN, albeit a fibre enriched formula, given to healthy individuals is capable of altering the intestinal flora and the characteristics of the bacterial metabolic activity (Schneider, Girard-Pipau *et al.* 2006). In CD EN leads to alterations in the bacterial flora present on mucosal surfaces (Pryce-Millar, Murch *et al.* 2004) and produces large changes in the faecal flora patterns (Lionetti, Callegari *et al.* 2005). It remains unclear how treatment with EN modulates changes in faecal or mucosa-associated flora. It could be because of prebiotic properties of the formula used for EN or because EN alters the micro-environment in the colon, perhaps as a result of alterations in pH, short-chain fatty acids or changes in bacterial growth factors (Day, Whitten *et al.* 2008).

II.1.4.5 Reducing the antigenic load presented to the immune mechanisms of the bowel

One of the driving theories behind the emergence of enteral and parenteral therapy for CD was that they removed a potential source of antigenic load confronting the immune cells in the lamina propria of the gut (Stenson and Alpers 1997). No studies have directly addressed this hypothesis. However, studies attempting to define whether bowel rest is an important factor give some information. As described above, in these studies PN was compared to PN plus formula and food ad libitum (Lochs, Meryn *et al.* 1983) or

PN to EN alone to partial PN and oral food (Greenberg, Fleming *et al.* 1988) (see II.1.4.3 Resting the bowel from the mechanical effects of digestion). No significant differences were found, suggesting that introducing food to the lumen has no deleterious effect. This is further supported by studies showing equivalence between elemental and polymeric EN (Gonzalez-Huix, de Leon *et al.* 1993; Royall, Jeejeebhoy *et al.* 1994; Verma, Brown *et al.* 2000; Zachos, Tondeur *et al.* 2001; Zachos, Tondeur *et al.* 2007).

However, any immune response to food antigens might be expected to be dose dependent and there are likely to be significant differences in the antigenic load to the intestine between a normal diet and a polymeric diet or a PN regimen with additional oral food. This conjecture is supported by a recent study that compared patients on exclusive enteral EN to those receiving approximately 50% of their intake as formula with the remainder as a normal diet, ie. a greater proportion of calories as whole food than the previous studies. The exclusive EN group had a remission rate of 42%, which was superior to that of the partial EN group (remission in 15%: $P < 0.035$) (Johnson, Macdonald *et al.* 2006).

One must also consider that TPN might have deleterious effects on the GI immune system. TPN has been shown to induce atrophic changes in Peyer's patches, with a decrease in number of CD4+ T cell subsets and immunoglobulin A-containing cells in the intestinal mucosa of rats (Tanaka, Miura *et al.* 1991). Total parenteral nutrition also resulted in inhibition of lymphocyte transport through intestinal lymphatics, suggesting the importance of oral nutrition in maintaining immunological function in general, and GALT function in particular. This further confounds the ability to extrapolate the effects of oral food vs. TPN in terms of direct effects on the GI immune system.

II.1.4.6 The Role of the amount and type of fat

Although the protein source in EN does not appear to impact on effectiveness in the treatment of CD (Gonzalez-Huix, de Leon *et al.* 1993; Royall, Jeejeebhoy *et al.* 1994; Verma, Brown *et al.* 2000; Zachos, Tondeur *et al.* 2001; Zachos, Tondeur *et al.* 2007), the effects of the type and amount of fat used in the diet has proven important, and the optimal formulation for the induction of remission in CD has been elusive.

More than 90% of dietary fat is triglycerides, which are made up of fatty acids and glycerol. Short-chain fatty acids have four or six, medium-chain fatty acids have eight to 12, and long-chain fatty acids have 14 to 22 carbon atoms. Fatty acids with one double bond are monounsaturated, while those with more are polyunsaturated. The predominant monounsaturated fatty acid in the diet is oleic acid. The predominant dietary polyunsaturated fatty acid (PUFA) is linoleic acid, an essential fatty acid. The position of the first double bond in a PUFA is designated by the omega (n-) number. The two main families of PUFAs are n-6 and n-3 (Gorard 2003).

Middleton *et al.* in 1995 performed meta-analysis of the existing data that showed that the response rate to enteral feeding in CD is inversely correlated with the amount of fat in the feed, in particular long chain triglycerides (LCT) (Middleton, Rucker *et al.* 1995). Subsequently Bamba *et al.* showed that the efficacy of an elemental diet in inducing remission in Crohn disease is impaired by the addition of LCTs (Bamba, Shimoyama *et al.* 2003). In addition Middleton *et al.* went on to show that adding medium chain triglycerides (MCT) to the feed did not impair therapeutic efficacy (Middleton, Rucker *et al.* 1995). This is supported by the results of Sakurai *et al.* who obtained the same response from elemental feeds with low fat and polymeric feeds with 25% energy as fat, mainly in the form of MCTs (Sakurai, Matsui *et al.* 2002). Finally, Khoshoo *et al.*

obtained similar responses in adolescents with Crohn disease when using a peptide-based feed containing 9% energy as fat, of which 45% was MCT, and a peptide feed containing 33% energy as fat, of which 70% was MCT (Khoshoo, Reifen *et al.* 1996).

The mechanisms by which fat administration might affect disease expression in CD are multiple. Various saturated and unsaturated fatty acids can modulate the immunological role of lymphocytes both in vitro and in vivo (Erickson 1986; Palmblad and Gyllenhammar 1988). Theories as to the mechanisms for this include effects on lymphocyte migration, lymphocyte proliferation, and changes in receptor affinity and signalling of lymphocytes; as well as effects on other cell types of the GI immune system, oxidative mechanisms and GI immunoglobulin production. Most of the evidence regarding these mechanisms applies to animal models and how important each might be in the mediation of CD remains to be determined. The evidence to support each of these proposed mechanisms is summarised in the proceeding text. The possible pro- and anti-inflammatory effects of the two main PUFA types, n-3 and n-6, are discussed in detail in the following section (see section II.1.4.7).

Potential mechanisms for an effect by fats	Evidence for an effect
Lymphatic lymphocyte transportation	LCTs are transported with lymphocytes in the lymphatic system and so could affect lymphocyte transportation (Miura, Sekizuka *et al.* 1987)
Lymphocyte trafficking	Fat absorption can affect the expression of adhesion molecules on the lymphocyte (Tsuzuki, Miura *et al.* 1997; Fujiyama, Hokari *et al.* 2007)
Lymphocyte proliferation	Lipoproteins are required for lymphocyte proliferation and the type of fat available to lymphocytes can affect their proliferation (Cuthbert and Lipsky 1989; Miura, Imaeda *et al.* 1993)
Lymphocyte receptor affinity and signalling	Unsaturated fatty acids are incorporated into the lymphocyte cell membrane and can effect changes in membrane fluidity, changes in receptor affinity to cytokines and effects on signal transduction (Goppelt, Kohler *et al.* 1985; Shinomura, Asaoka *et al.* 1991; Zurier 1993; Suchner, Senftleben *et al.* 1995)
Effects on other immune cells	Oleic acid stimulates fibroblasts (Zugaza, Casabiell *et al.* 1995) and long chain fatty acids enhance the secretion of cytokines from intestinal epithelial cell lines (Hirokawa, Miura *et al.* 1997)
Effects on immunoglobulin production	Intestinal IgA secretion is stimulated by oleic acid in the rat (Imaeda, Miura *et al.* 1993)

Table 18. Potential mechanisms for an effect on disease of the amount and type of fat ingested

II.1.4.6.1 Lymphatic lymphocyte transportation

Both long-chain fatty acids and lymphocytes utilize intestinal lymphatics as the major pathway for transport from the intestinal mucosa to the systemic circulation, whereas medium chain fatty acid is mostly transported via the portal system. Thus associated changes in lymphocyte transport in the lymphatic system might be greater with administration of LCTs than MCTs. In one study this effect was demonstrated to cause a significant increase in lymphocyte flux in the mesenteric collecting lymphatics after administration of olive oil (Miura, Sekizuka *et al.* 1987).

II.1.4.6.2 Lymphocyte trafficking

Lymphocytes interact with endothelium and cross endothelial linings of post-capillary venules (PCV), especially in Peyer's patches, where morphologically distinct venules called high endothelial venules (HEV) are recognized as sites of lymphocyte traffic. Extravasation of lymphocytes through HEV involves multiple steps; primary interaction (rolling), activation-dependent adhesion (sticking) and transmigration. Adherence of lymphocytes to PCV of Peyer's patches is mediated by a variety of lymphocyte adhesion molecules, including L-selectin, CD44, LFA-1 and the a4b7 integrins (Miura, Tsuzuki *et al.* 1998).

That fatty acid exposure and the fatty acid composition of lymphocyte membranes can affect patterns of lymphocyte migration has been demonstrated in a number of studies. (Novo, Fonseca *et al.* 1987; Twisk, Detering *et al.* 1991; Twisk, Rutten *et al.* 1992; Tsuzuki, Miura *et al.* 1997). In one of those studies potential mechanisms were examined and it was found that olive oil absorption enhanced expression of both a4-integrin and L-selectin on lymphocytes. Nearly complete inhibition of the olive oil-stimulated lymphocyte–PCV interactions by a combination of a4-integrin- and L-selectin-specific antibodies suggested that dual upregulation of a4-integrin and L-selectin after fat absorption largely accounts for the associated lymphocyte adhesion in Peyer's patches (Tsuzuki, Miura *et al.* 1997).

A recent study into T-lymphocyte adherence to microvessels of the small intestinal mucosa showed that this was significantly enhanced after butter (long chain, saturated fatty acid) ingestion. This enhancement was due to an increase in expression levels of adhesion molecules of the intestinal mucosa (Fujiyama, Hokari *et al.* 2007).

II.1.4.6.3 Lymphocyte proliferation

Lipoproteins are needed for lymphocyte proliferation in vitro (Cuthbert and Lipsky 1989). Hence the amount and type of lipoprotein available to lymphocytes in vivo might affect their proliferation (Miura, Tsuzuki *et al.* 1998). This is supported by experiments showing that absorption of long-chain fatty acids, specifically oleic acids, stimulates mitogen-induced blast transformation of lymphocytes in intestinal lymphatics, whereas MCTs do not (Miura, Imaeda *et al.* 1993). Another group have shown that triacylglycerols containing PUFA inhibit lymphocyte proliferation, while triacylglycerols containing saturated fatty acids do not (Cuthbert and Lipsky 1989).

II.1.4.6.4 Changes in lymphocyte receptor affinity and signalling

Unsaturated fatty acids are readily taken up by lymphocytes and incorporated into phospholipids and triglycerides (Goppelt, Kohler *et al.* 1985; Cuthbert and Lipsky 1989). They then have direct effects on immunocompetent cells, some of which do not depend on the eicosanoid actions discussed further below. These include changes in membrane fluidity, changes in receptor affinity to cytokines, and also direct interactions of the PUFA with intracellular signal transduction and by acting as signal transducers themselves, affecting such cellular enzymes as cyclases or protein kinase C (Goppelt, Kohler *et al.* 1985; Shinomura, Asaoka *et al.* 1991; Zurier 1993; Suchner, Senftleben *et al.* 1995).

II.1.4.6.5 Effects on other cell types of the GI immune system

There is evidence that fat absorption might affect other GI cell types. In one series of experiments the monounsaturated fatty acid, oleic acid, stimulated fibroblasts exposed to endothelial growth factor to enter the replicative cycle more quickly and exhibit earlier cell division (Zugaza, Casabiell *et al.* 1995). In contrast long chain fatty acid has

been shown to enhance the secretion of cytokines during experiments on intestinal epithelial cell lines (Hirokawa, Miura *et al.* 1997).

II.1.4.6.6 Effects on oxidative mechanisms

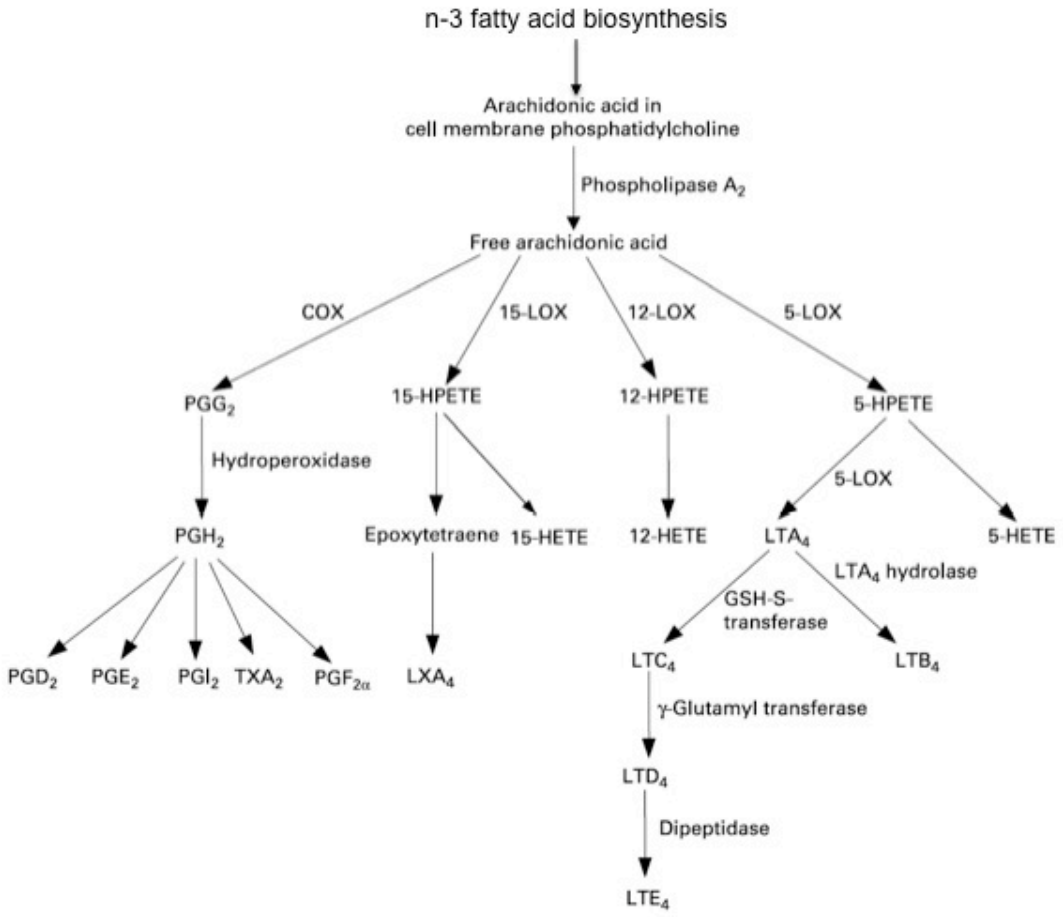
The potential effects of different fatty acids in inflammatory states are demonstrated by experiments on neutrophils pretreated with TNF. TNF augmented superoxide production in response to PUFA and mono-unsaturated fatty acids, such as oleic acid, but not to saturated fatty acids (Li, Ferrante *et al.* 1996).

II.1.4.6.7 Effects on gastrointestinal immunoglobulin production

Finally, there is a documented effect of fatty acids on intestinal IgA production in the rat small intestine. Release of IgA into the intestinal lumen is stimulated by absorption of oleic acid and transport of IgA into the lymphatics is decreased (Imaeda, Miura *et al.* 1993).

II.1.4.7 n3 vs. n6 polyunsaturated fatty acids

More recently attention has focussed on the individual fatty acids (FA) that make up the fat content of feeds. Polyunsaturated fats (PUFA) can be made up of two main subtypes of FA, n-3 and n-6. n-6 FAs are derived from Linoleic acid and are precursors for Arachidonic acid. Arachidonic acid forms an important part of the pro-inflammatory pathway via its conversion to Thromboxane A2 and Leukotrienes. In contrast n-3 FAs consist primarily of Linolenic acid, which has been shown to exert an anti-inflammatory effect (Caughey, Mantzioris *et al.* 1996). It has been speculated that increasing the amount of n-3 FA in feeds, relative to n-6, may exert a greater anti-inflammatory effect in CD.



(COX: cyclo-oxygenase; HETE: hydroxyeicosatetraenoic acid; HPETE: hydroperoxyeicosatetraenoic acid; LOX: lipoxygenase; LT: leukotriene; PG: prostaglandin; TX: thromboxane)

Figure 5. Eicosanoid biosynthesis (adapted from Calder, Albers *et al.* 2009)

Support for this concept comes from the link between increased intake of n-6 FAs and increasing incidence of CD in Japan (Shoda, Matsueda *et al.* 1996). In addition patients with CD have lower concentrations of n-3 PUFAs in plasma phospholipids and adipose tissue compared with controls (Geerling, Stockbrugger *et al.* 1999).

Whiting *et al.*, using a (SCID) mouse model of chronic colitis that resembles Crohn disease, compared standard diet or a diet enriched in n-3 PUFAs (Whiting, Bland *et al.* 2005). Animals fed n-3 FAs had similar immune cell infiltration, but significantly reduced disease scores, reduced neutrophil infiltration and lower mucosal levels of pro-inflammatory cytokines (TNF-a, IL-1b and IL-12). Expression of epithelial tight

junction protein ZO-1 – a marker of intestinal permeability – was also better preserved, suggesting improved epithelial barrier function. The authors interpreted their findings as demonstrating that n-3 PUFAs reduce inflammation by reducing myeloid cell infiltration and activation, which, in turn, means lower levels of cytokines and less damage to the barrier.

In a randomised controlled trial Bamba *et al.* showed that the addition of LCT with high n-6 FA levels did decrease the effectiveness of enteral feeding in CD (Bamba, Shimoyama *et al.* 2003). A multi-centre European study (Gassull, Fernandez-Banares *et al.* 2002), which was stopped early because of a low remission rate in one of the enterally fed groups, muddies the waters somewhat. It did not compare n-3 to n-6 enriched feeds but did compare a high n-6 feed to a feed composed almost solely of monounsaturated fatty acids (MUFA). Of compliant patients treated with a diet high in linoleate (n-6 PUFA) and low in oleate (MUFA), 67% achieved remission compared with only 27% of those compliant with a formula low in linoleate and high in oleate. This suggests that the magnitude of response to enteral nutrition is influenced by lipid composition, but the relative importance of specific fatty acids might not so clearly relate to n-3 to n-6 ratio alone.

Finally, in a recent study of nutritional supplementation in combination with steroids in CD, both the n-6 and n-3 enriched nutritional supplements were able to improve nutritional status, and clinical and biochemical markers (Nielsen, Nielsen *et al.* 2007).

II.1.4.8 Maintenance using exclusion diets

Following the induction of remission of CD by the use of an elemental diet or other enteral feeding regimen, remission can be maintained by the avoidance of foods to which intolerance has been demonstrated (Jones, Dickinson *et al.* 1985; Riordan,

Hunter *et al.* 1993). However the oral food challenges which inform dietary exclusion remains cumbersome and unreliable (Pearson, Teahon *et al.* 1993) and the regimens used need to be individualised.

II.1.4.9 Implications of a role for enteral nutrition in CD

The research outlined in the above section suggests that multiple mechanisms combine to produce a benefit for enteral nutrition in CD and that no one molecular mechanism is responsible for all the effects seen. That mechanical rest for the gut may play a role seems likely but this effect may be less than the role of the significant changes in the luminal content that occur with enteral nutrition. While enteral nutrition may well have direct anti-inflammatory effects on the mucosa and change the bowel flora in an advantageous manner, it remains plausible that a part of the effect of EN on active CD comes from the reduction or removal of antigenic substances from the lumen.

II.1.5 Short chain fatty acids

Studies of a role for SCFAs in the treatment of CD have been lower in number and have largely followed similar studies in UC (see Chapter III.2.4 Dietary fibre and short chain fatty acids). In their study of 17 CD patients and 6 healthy controls Segain *et al.* cultured intestinal biopsy specimens with and without butyrate (Segain, Raingeard de la Bletiere *et al.* 2000). They were able to show that butyrate decreased proinflammatory cytokine expression in inflamed tissue and that that change was mediated by the inhibition of NFkappaB activation and IkappaBalpha degradation.

Di Sabatino performed the first in vivo study of butyrate treatment in CD (Di Sabatino, Morera *et al.* 2005). Thirteen patients with mild-moderate ileocolonic CD were given enteric-coated butyrate tablets for a total of 8 weeks. Crohn disease activity index and endoscopic and histological scores were assessed at the beginning and end of treatment.

Seven (53%) patients entered clinical remission and a further two had a partial response. Endoscopic and histological scores were significantly improved at the end of treatment. One patient withdrew from the study and three did not experience clinical improvement.

While these results are encouraging further large scale controlled trials will be required before oral butyrate for CD could be recommended in clinical practice.

II.1.6 Dietary fibre

Concerns that dietary fibre might exacerbate IBD, and in particular cause obstruction in CD, was largely discounted by work done in the 1980s. Levenstein *et al.* in 1985 randomly assigned 79 patients with non-stenosing Crohn disease to normal or low residue diet and saw no difference between groups (Levenstein, Prantera *et al.* 1985). Following this Ritchie *et al.* randomised 352 patients with inactive or mildly active Crohn disease to a diet containing either little or no sugar and high unrefined carbohydrate or unrestricted in sugar and low in fibre (Ritchie, Wadsworth *et al.* 1987). Again there was no difference between the groups.

Thus it seems unlikely that fibre in the diet of CD patients is detrimental, but neither is there evidence to support the use of a high fibre diet in the treatment of CD.

II.1.7 Yeast

A role for yeast in the pathogenesis of CD was suggested by an early study that showed, in patients with CD of the ileum, decreased capacity of the leukocytes to phagocytose yeast (Krause, Michaelsson *et al.* 1978). Further indirect evidence comes from studies on occupational distribution of Crohn disease prevalence and mortality. Analyses of occupational mortality from Crohn disease in England and Wales (Sonnenberg 1990) have shown a proportional mortality ratio for bakers close to 3.5 times greater than that

for managers in retail (who had the second highest ratio of all professions). Another study from Germany showed that, among all professions in men, bakers have the highest odds ratio for Crohn disease (Sonnenberg 1990).

There is one clinical study of 19 patients looking at the effect of yeast exclusion and exposure on CD (Barclay, McKenzie *et al.* 1992). This involved patients continuing their usual diet during the 1st month (base-line period), but during the next 2 months dietary yeast was excluded except that during 1 month patients took baker's yeast capsules while for the other month they took placebo capsules. The mean of each patient's maximum CDAI during yeast exclusion was significantly lower than those during the base-line and baker's yeast inclusion periods. Patients with elevated yeast antibodies tended to develop a higher CDAI while receiving baker's yeast. These results suggest that dietary yeast may affect the activity of Crohn disease but larger studies are required to confirm this finding.

II.2 UC

II.2.1 Attitudes

The majority of patients with UC believe that certain foods affect the activity of their disease, and restrict their diets accordingly (Ballegaard, Bjergstrom *et al.* 1997; Green, Issenman *et al.* 1998; Joachim 1999; Jowett, Seal *et al.* 2004). The frequency and pattern of food intolerance does not differ between patients with CD and UC (Ballegaard, Bjergstrom *et al.* 1997). A wide range of foods are cited as causing symptoms. Most commonly these are vegetables, fruit, milk products, liver, diet drinks and artificial sweeteners, high fibre foods, spicy foods, corn and corn products, nuts, chocolate, cheese, wheat, and tomato sauces (Ballegaard, Bjergstrom *et al.* 1997; Green, Issenman *et al.* 1998; Joachim 1999).

Ballegaard *et al.* performed a postal survey of 53 CD, 69 UC patients and 70 healthy controls, asking whether the participants had problems with any particular food item and, if so, to describe the symptoms experienced from it (Ballegaard, Bjergstrom *et al.* 1997). Food intolerance was equally common in CD (66%) and UC (64%), compared to 14% of controls. The pattern of foods to which intolerance was reported and the symptoms reported were similar for UC and CD patients.

In a later study Joachim *et al.* questioned 33 CD and 27 UC patients about their consumption of 122 different foods (Joachim 1999). They asked subjects to rate their reaction to each food. In contrast to the study by Ballegaard *et al.* they found that there was a higher number of foods that caused problems for people with CD than UC.

As well as reporting intolerances to foods, patients with UC do alter their diet in response to those intolerances. In one study 80% of paediatric UC patients, who altered their diets to avoid foods that they felt worsened their condition, reported subjective symptomatic improvement as a result (Green, Issenman *et al.* 1998). Forty-nine UC patients responded to a questionnaire enquiring about changes they had made to their diets as a result of their disease and the effect this had had on the disease. Lactose-free, nonspicy, low acid, additive-free, caloric supplement and low fibre diets were used by more than 15% of UC patients. UC patients commonly avoided corn and corn products, nuts, milk and bran. More than 20% of UC patients also avoided tomato, chocolate, cheese, wheat, tomato sauces and fruit juice. The authors claimed, “of the 55 UC patients who had modified their diet, 44 (80%) reported a benefit”. The discrepancy between the denominator for this result and the total number of respondents may signify

that the there were actually a total of 55 dietary changes amongst the 49 UC patients surveyed.

Conversely, a prospective study using an objective measure of disease activity found dietary beliefs did not modify the risk of relapse, as much as adversely affect nutrient intake (Jowett, Seal *et al.* 2004). In this 1-year cohort study nutrient intake was assessed using a food frequency questionnaire and relapse defined using the simple clinical colitis activity index. Food beliefs, demographics and disease characteristics were also recorded. Of the one hundred and eighty-three patients studied 52% relapsed. 68% of patients held dietary beliefs and reported having altered their diet because of this, most commonly by avoiding milk and dairy products. No reported behaviour reduced the risk of relapse but patients who avoided dairy products did have a significantly lower intake of calcium. That dietary beliefs can affect nutritional status in UC is further evidenced by a study of patients with UC in whom the median calcium intake was significantly lower in those patients who reported avoiding milk and dairy products (Bernstein, Ament *et al.* 1994).

Jowett *et al.* also examined the types of dietary advice received by UC patients and their adherence to it. The minority of patients (33%) had received advice. It was more common for those who believed that food was important to their disease to have received dietary advice compared to those that did not think food was important (40% vs. 17%). The advice received most commonly came from dietitians, then hospital doctors and general practitioners. It consisted of a variety of recommendations, and the minority of patients were following the advice received (Jowett, Seal *et al.* 2004).

As with CD, analysis of the nutritional status of recently diagnosed UC patients shows that body weight and body mass index are significantly lower in UC patients than controls and that there are differences in intake of carbohydrate, protein, calcium, phosphorus, and riboflavin between patients and controls, and also major differences in the serum concentrations of several nutrients (beta-carotene, magnesium, selenium and zinc) in UC patients compared to controls (Geerling, Badart-Smook *et al.* 2000). However, the decrease in serum antioxidants of UC patients may be explained by increased consumption of antioxidants by inflamed tissue rather than by impaired digestion and absorption of nutrients. The effects of disease on body protein, body fat and hydration state were corrected within 12 months by curative colectomy in a separate study (Christie and Hill 1990). The degree to which these nutritional deficits might relate to the dietary manipulation effected by patients and their advisors is difficult to evaluate.

Thus it appears that patients with UC commonly experience food intolerances and commonly alter their diet based on these intolerances. In addition, patients who alter their diets do report improvement in their disease. However, the real efficacy of such practices has not yet been clearly defined, although there remains a real possibility of deleterious nutritional effects.

Non-dietary disease factors are likely to be statistically more important than dietary factors. In their study of dietary effect on risk of relapse, Jowett *et al.* demonstrated a greater magnitude of effect of previous disease activity on relapse rate than that of the greatest dietary contributor, red meat (odds ratio 9.30 vs. 5.19 respectively). However, nutrient intake cannot be ignored as a modifiable factor in disease activity. Patients

already commonly modify their diet in response to its perceived effect on their disease, and, when asked, see this as an area of great research interest (Jowett, Seal *et al.* 2004).

No consensus statement as to appropriate use of dietary modification in the treatment of UC has been published. The impact of food on the course of disease has been examined only for a small number of specific dietary factors, including milk, meat, sulphate containing foods, fish oils, fat and dietary fibre. The current evidence regarding dietary manipulation in UC is summarized below.

II.2.2 Milk

The perception that dairy products have a negative effect in UC dates back to studies from the 1960s, conducted by Truelove's group. They described a marked symptomatic and histological improvement for a small group of UC patients on a milk-free diet (Truelove 1961). Five patients were described who had a clear temporal relationship between the reintroduction of milk to the diet and the onset of clinical and histological exacerbation of disease.

In the controlled trial that followed from the same group, patients on a milk free diet were found to be less likely to relapse than controls (Wright and Truelove 1965). Patients were randomly assigned to either a low fibre, dairy free diet or a "dummy" diet in which they were instructed to exclude a variety of items, such as fried foods, condiments, and icecream. In addition all patients received "standard medical therapy" which included oral prednisone. Relapse was defined as diarrhoea with an average of four or more stools a day for at least a week and with macroscopic blood present, together with sigmoidoscopic evidence of diffuse inflammation. There were a total of 26 patients in the milk free diet group and 24 in the "dummy" diet group. The milk free diet group experienced fewer relapses than the "dummy" diet group. These results only

reached statistical significance if an one-tailed Chi-square test was used rather than a two-tailed test.

These studies, by today's standards, can be considered to have clear methodological defects (Jowett, Seal *et al.* 2004; Taxonera and Mendoza 2004). That said, the concept remains in force amongst patients with UC, who commonly adhere to a lactose free diet (Bernstein, Ament *et al.* 1994; Jowett, Seal *et al.* 2004). However, the evidence is that the prevalence of lactose malabsorption (as determined using hydrogen breath testing) is similar to (Gudmand-Hoyer and Jarnum 1970; Busk, Dahlerup *et al.* 1975; Bernstein, Ament *et al.* 1994; Ginard, Riera *et al.* 2003; Banos Madrid, Salama Benerroch *et al.* 2004) or even lower than controls (Huppe, Tromm *et al.* 1992; Mishkin, Yalovsky *et al.* 1997) when the risk is adjusted for ethnic group. Not even during flares in disease do patients with UC appear to have a higher rate of LM (Bernstein, Ament *et al.* 1994; Rosinach, Maurer-Pons *et al.* 2002)(see Table 19).

Authors	Country	Ulcerative colitis		Controls		Diagnostic method	
		Number of patients	Lactose Malab-sorption	Number	Lactose Malab-sorption	Lactose tolerance test	Determ-ination of mucosal enzyme activity
Binder, Gryboski *et al.* 1966	USA	39	19 (49%)	37	18 (49%)	+	
Cady, Rhodes *et al.* 1967	USA	32	15(47%)	122	38(31%)		+
Newcomer and McGill 1967	USA	24	2 (8%)	100	6 (6%)	+	+
Chalfin and Holt 1967	USA	9	4 (44%)			+	
Littman, Cady *et al.* 1968	USA	29	13 (45%)	93	18 (19%)	+	+
Kojecky and Matlocha 1968	Czechos-lovakia	18	9 (50%)			+	+
Montgomery, Frazer *et al.* 1968	England	11	2(18%)				+
Gudmand-Hoyer and Jarnum 1970	Denmark	85	8 (9%)	700	3-7%	+	+
Busk, Dahlerup *et al.* 1975	Denmark	120	11 (9%)			+	+
Kirschner, DeFavaro *et al.* 1981	USA	20	3(15%)	40	"similar to IBD"	+	
Huppe, Tromm *et al.* 1992	Germany	53	2 (3.8%)			+	
Bernstein, Ament *et al.* 1994	USA	29	13(44%)	14	5(36%)	+	
Mishkin, Yalovsky *et al.* 1997	Canada	139	18(13%)	158	46(29%)	+	
Ginard, Riera *et al.* 2003	Spain	52	13 (25%)	34	11(32%)	+	
Banos Madrid, Salama Benerroch *et al.* 2004	Spain	24	4(17%)	25	5(20%)	+	

Table 19. Studies of lactose malabsorption in ulcerative colitis

(Adapted from Gudmand-Hoyer *et al.*)

That mechanisms other than LM might play a role in UC has received less research attention. In a dated study of 21 patients with ulcerative colitis, none of whom had lactose malabsorption, a milk-free diet was beneficial in five patients, as judged from the numbers of movements and their feeling of well being (Gudmand-Hoyer and Jarnum 1970). In another study an increased number of basophils degranulated in the presence of cows' milk in UC patients, but normal responses occurred in patients with CD (Smart, Danis *et al.* 1986).

It seems therefore that a subset of patients with UC do believe intake of dairy products contributes to their disease. However, there is no data to suggest that lactose malabsorption plays a more important role in this than it does in the general population and no further mechanisms by which milk might contribute to symptoms in UC have been elucidated in the literature.

II.2.3 Meat and sulphates

Concerns that a diet high in red meat might predispose to UC are raised by epidemiological data from Japan, a country in which increasing rates of UC have been paralleled by an increased intake of red meat (Kitahora, Utsunomiya *et al.* 1995). Prospective data is limited, but in a study by Jowett *et al.* a high meat, protein, or alcohol intake was associated with an increased risk of UC relapse (Jowett, Seal *et al.* 2004). The biological plausibility of such observations are supported by animal experiments demonstrating that sulphated dextrans, but not dextrans without sulphur, are able to induce experimental colitis in rodents (Ohkusa 1985). The presence of faecal sulfides is directly related to the intake of red meat (Magee *et al.* 2000).

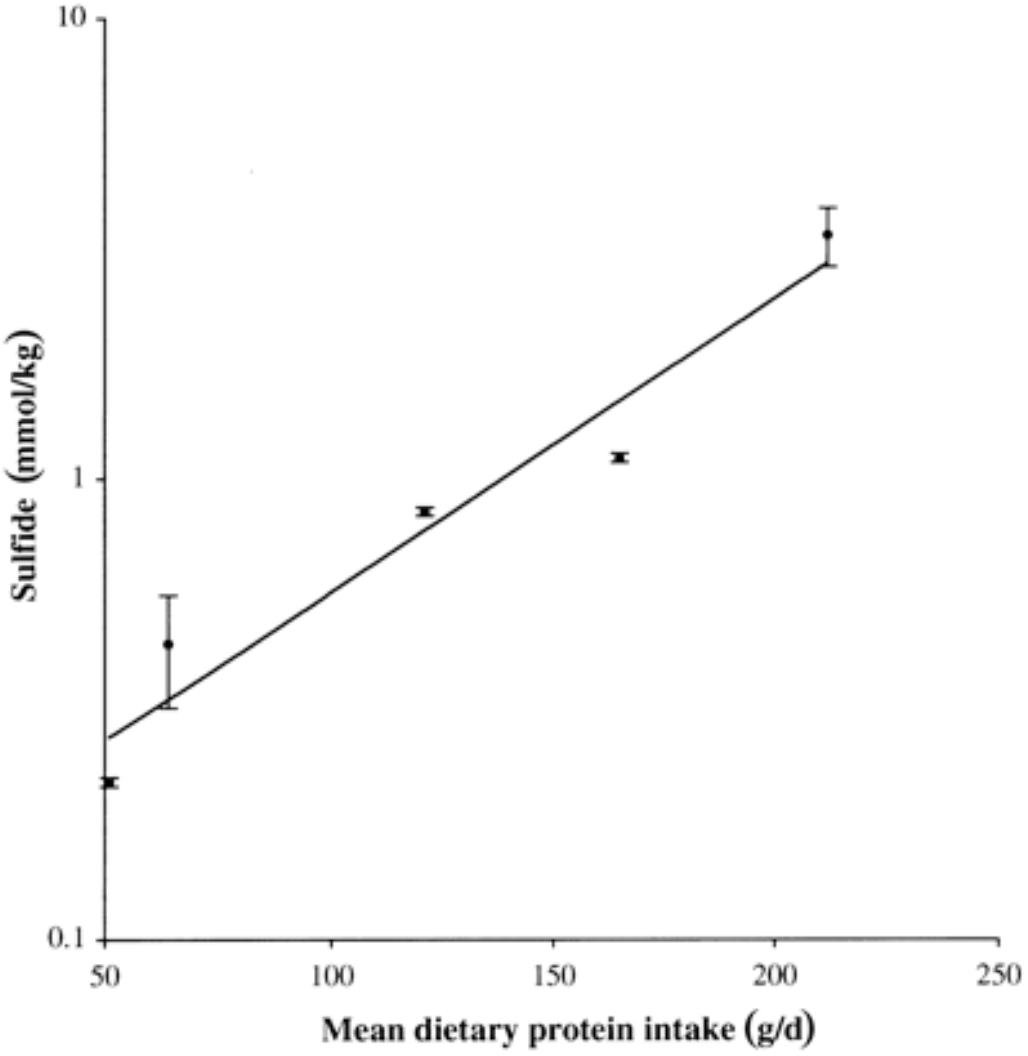


Figure 6. Faecal sulfide content as a function of dietary meat protein intake (from Magee *et al.* 2000)

Clinical data to support limiting the intake of sulphur amino acids is limited to a pilot study of 4 patients with chronic UC and 4 with acute UC, in which all patients experienced clinical improvement (Roediger, Duncan *et al.* 1993). Current evidence therefore suggests an association between the intake of red meat and other sulphate containing foods, and the incidence of ulcerative colitis. While there is experimental data to suggest a biological mechanism, there are no controlled clinical trials demonstrating the utility of dietary avoidance of sulphate containing foods.

II.2.4 Dietary fibre and short chain fatty acids

N-butyrate, a SCFA produced by the colonic fermentation of some forms of fibre, is the preferred respiratory fuel of the colonocyte (Roediger 1980). Butyrate has also been shown to inhibit both the production of some cytokines and the activation of the transcription factor NFkB (Segain, Boureille *et al.* 1997; Wu, Huang *et al.* 1997; Luhrs, Gerke *et al.* 2002; Sanderson 2004). These findings have fuelled investigation into the role of SCFAs in the management of UC. The results of clinical studies performed using SCFA preparations are summarised in Table 20.

	Preparation	Design	Patients	Results
Senagore, MacKeigan *et al.* 1992	SCFA enema vs. corticosteroid enema vs. mesalamine enema	Open label	Proctitis (SCFA enema n=14, corticosteroid n=12, 5ASA n=19)	Recovery in 12/14 SCFA, 10/12 corticosteroid and 17/19 5ASA treated patients
Scheppach, Sommer *et al.* 1992	Butyrate enema	RDBPCT cross-over (8 weeks)	Distal UC unresponsive to standard therapy (n=10)	Reduced stool frequency, bleeding and inflammation
Steinhart, Brzezinski *et al.* 1994	Butyrate enema	Open label	Distal UC unresponsive to standard therapy (n=10)	60% response
Vernia, Marcheggiano *et al.* 1995	SCFA enema	RDBPCT (6 weeks)	Mild to moderate distal UC (n=40)	Improvement in 14/20 treatment vs. 5/20 placebo patients
Patz, Jacobsohn *et al.* 1996	SCFA enema	Open label (6 weeks)	Distal UC unresponsive to standard therapy (n=10)	50% clinical and endoscopic response
Steinhart, Hiruki *et al.* 1996	Butyrate enema	RDBPCT (6 weeks)	Distal UC (n=38)	Clinical improvement in 37% butyrate treated and 47% placebo treated patients
Scheppach 1996	SCFA enema vs. butyrate enemas vs. saline placebo	RDBPCT (6 weeks)	Active distal UC (n=47)	Trend toward improvement with active treatment but no statistical differences
Breuer, Soergel *et al.* 1997	SCFA enema vs. placebo	RDBPCT (6 weeks)	Distal UC (n=103)	Improvement in 33% SCFA vs. 20% placebo treated patients (p=0.14)
Vernia, Monteleone *et al.* 2000	Oral sodium butyrate tablets plus oral mesalazine vs. mesalazine plus placebo	RDBPCT (6 weeks)	Mild to moderate UC (n=30)	Remission in 7 butyrate vs. 5 placebo treated patients and improvement in 4 butyrate vs. 5 placebo treated patients
Vernia, Annese *et al.* 2003	Butyrate and 5-ASA enema vs. 5-ASA enema alone	RDBPCT (6 weeks)	Distal UC refractory to topical 5-ASA and cortisone (n=51, 24 butyrate vs. 27 placebo)	Remission in 6 butyrate vs. 1 placebo treated patients and improvement in 12 butyrate vs. 13 placebo treated patients
Assisi 2008	Mesalazine, butyrate and inulin tablets	Open label	UC unresponsive to mesalazine (n=216)	110/216 (51%) clinical and endoscopic remission (ITT)

(RDBPCT: randomised, double-blind, placebo controlled trial; SCFA enema = sodium acetate, sodium propionate and sodium butyrate; ITT: intention to treat analysis)

Table 20. Studies of short chain fatty acids in the treatment of ulcerative colitis

The treatment of UC with SCFA preparations has given contradictory results, with early results being encouraging, but the results from more recent, larger studies disappointing. Subgroup analysis of the largest of the studies to date did suggest that despite there being no therapeutic value of SCFA enemas for the whole group studied, patients with a short period of disease activity prior to treatment, and those who used more of the treatment, were more likely to benefit (Breuer, Soergel *et al.* 1997).

Anecdotal reports of clinical improvement of UC with a high fibre diet fuelled a 1978 report from Davies and Rhodes who took patients with UC in remission and either continued them on Sulphasalazine (n=15) or withdrew Sulphasalazine after initiation of a high-fibre diet (n=24). Four patients could not tolerate the diet and were withdrawn. At the end of 6 months 15 of 20 patients who continued on the high fibre diet had relapsed as compared to 3 of 15 patients who continued Sulphasalazine.

In 1991 Hallert *et al.* described their placebo-controlled cross-over experience with Ispaghula husk in UC in remission (Hallert, Kaldma *et al.* 1991). Twenty-nine patients with UC in remission but with ongoing disturbance of bowel habit were studied. The trial analysis was not on an intention to treat basis and only the results of patients who completed treatment were considered. They found a statistically significant rate of symptomatic improvement in Ispaghula treated patients.

The hypothesis upon which the above studies were based stemmed from evidence that patients with quiescent UC were subject to irritable bowel syndrome-like symptoms. However, the advent of evidence for a role of SCFA in maintaining the health of the colonocyte, and initial data suggesting a role for topical SCFA in the treatment of UC,

led to the conjecture that oral ingestion of substrates promoting the production of SCFA in the colon might be beneficial in UC.

The first of these studies, by Fernandez-Banares et al, found no difference in the efficacy of Plantago Ovata vs. Mesalamine, or a combination of the two, for the management of UC in remission (Fernandez-Banares, Hinojosa *et al.* 1999). In an open label randomised clinical trial 105 patients with UC in remission were randomised to receive one of the above treatments. The primary outcome measure was maintenance of remission at 12 months. The rate of treatment failure was 40% in the Plantago ovata, 35% in the mesalamine and 30% in the combined treatment groups. A significant increase in faecal butyrate was observed with Plantago ovata administration.

Mitsuyama *et al.* used germinated barley foodstuff (GBF) to treat 10 patients with mild to moderate UC who had been unresponsive to or intolerant of standard treatment. In this open label study there was a clinical and endoscopic improvement in the patients treated at the end of 4 weeks (Mitsuyama, Saiki *et al.* 1998). This improvement was associated with an increase in stool butyrate concentrations and in luminal Bifidobacterium and Eubacterium (the bacteria responsible for converting fermentable fibre to SCFA) levels.

The follow up study was again open label and examined 18 patients with mild to moderate UC who were randomly allocated to receive either standard therapy alone (n=7) or standard therapy plus GBF (n=11) (Kanauchi, Suga *et al.* 2002). After 4 weeks of treatment, the GBF-treated group showed a decrease in clinical activity score compared with control ($p<0.05$). A group of 21 patients was further followed to 24 weeks whilst on open label treatment (Kanauchi, Mitsuyama *et al.* 2003). Again the

treatment group showed a decrease in clinical activity index compared to the control group ($p<0.05$).

Hanai *et al.* then went on to use GBF for the treatment of 59 patients with UC in remission (Hanai, Kanauchi *et al.* 2004). They compared patients on conventional therapy (n=37) with patients treated with conventional therapy plus GBF (n=22). At 12 months the clinical activity index was better in the GBF treatment group.

In a study examining the advice given to patients with UC, they most commonly recalled being told to take a high fibre diet, but the advice was not consistent and a minority were told to have a low fibre diet (Jowett, Seal *et al.* 2004). Interestingly, those patients who reported taking a high fibre diet because they felt it helped their disease did not have a significantly greater intake of dietary fibre compared to those who did not believe high fibre helped (Jowett, Seal *et al.* 2004). In another study patients with UC consumed significantly less fibre than control subjects (Rosman-Urbach, Niv *et al.* 2006).

Recently interest has focused on the anti-inflammatory properties of SCFAs and dietary fibre outside of luminal disease (North, Venter *et al.* 2009). These agents act on multiple inflammatory pathways, not all of which are particular to the gut (see Figure 7).

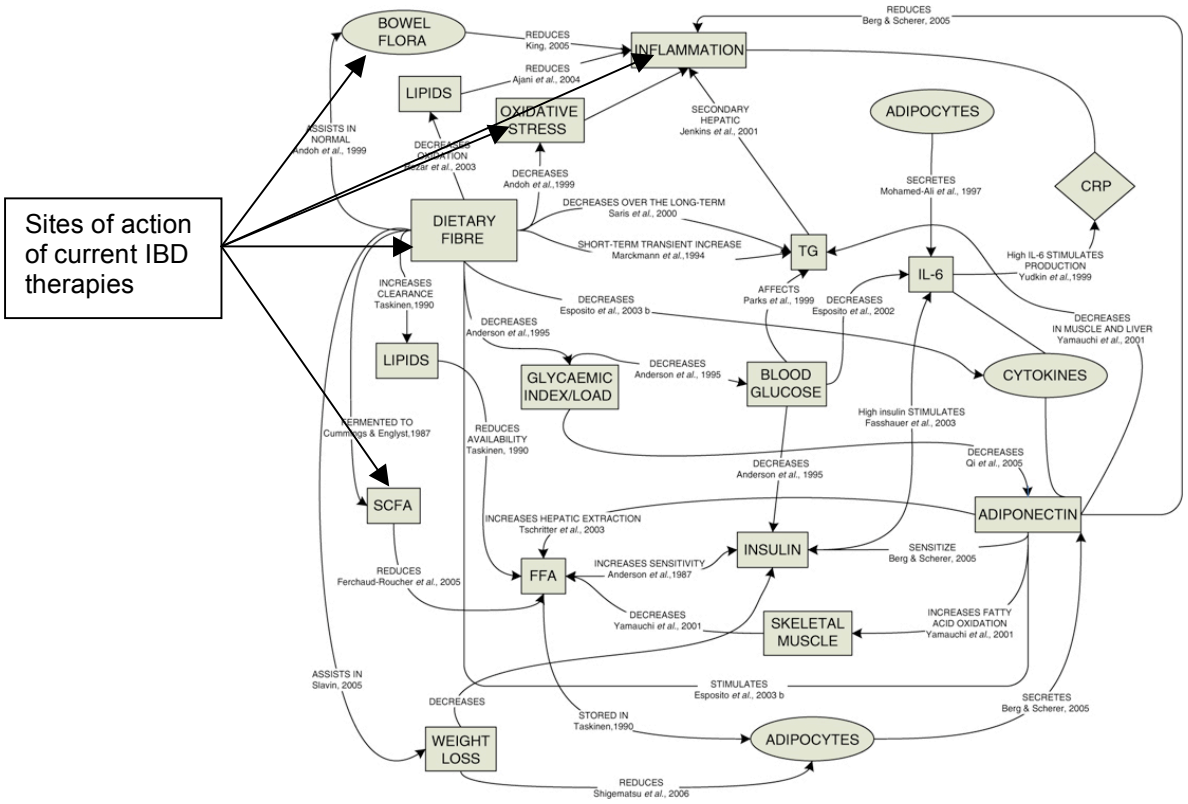


Figure 7. The mechanisms through which dietary fibre may influence inflammation (adapted from North, Venter *et al.* 2009)

A biological mechanism for benefit is provided by the demonstration that dietary fibre in UC can safely increase the amount of luminal butyrate (Fernandez-Banares, Hinojosa *et al.* 1999; Hallert, Bjorck *et al.* 2003). However, larger scale placebo controlled trials are required to ensure the finding that the addition of fermenting fibre to conventional remission maintenance strategies robustly improves outcome. In addition, placebo controlled trials of fibre for the treatment of active UC are required before this treatment can be recommended.

II.2.5 Fish oils

As discussed in section II.1.4.7 a dietary intake high in n-3 fatty acids, such as are found in fish oil preparations, may have anti-inflammatory effects. The evidence to support a role for the use of fish oil preparations in UC are summarised in Table 21.

	Study description	Subjects	Results
Lorenz, Weber *et al.* 1989	7 month, double-blind, placebo controlled cross-over study	Active UC (n=10)	Non-significant trend towards an improvement in clinical activity in the fish oil treated group
Aslan and Triadafilopoulos 1992	8-month, double-blind, placebo-controlled, crossover trial	Eleven patients with mild to moderately active UC	A reduction in the mean disease activity index of 56% for patients receiving fish oil compared to 4% for those on placebo
Stenson, Cort *et al.* 1992	4 month randomised, double-blind, placebo-controlled crossover trial	24 patients with active UC	Histological improvement and weight gain in treatment group, no improvement in placebo group
Hawthorne, Daneshmend *et al.* 1992	randomised, double blind, placebo-controlled study	96 patients with UC, 56 of whom were in relapse at enrolment and 40 in remission	For those patients who entered the trial with active disease there was a significant reduction in corticosteroid use but only a trend toward achieving remission
Almallah, Ewen *et al.* 2000	6 month randomised, double blind study of fish oil or sunflower oil placebo	18 patients with active proctitis	Significant reduction in the clinical activity score for the fish oil compared to the placebo group

Table 21. Randomised controlled studies of fish oil in active UC

Lorenz *et al.* were able to show that dietary n-3 fatty acids were incorporated into plasma and enteric mucosa phospholipids at the expense of n-6 fatty acids (Lorenz, Weber *et al.* 1989). Almallah *et al.* demonstrated a possible mechanistic effect in the form of a significant reduction in the circulating numbers and activity of natural killer and lymphokine-activated killer cell activities (Almallah, El-Tahir *et al.* 2000).

A Cochrane review of this subject found a level of inconsistency, in particular regarding reporting of outcome measures, between the above studies that obviated meta-analysis (De Ley, de Vos *et al.* 2007). Their conclusion was that the available information is insufficient to make recommendations for practice regarding the use of fish oils in active UC.

Studies that have used fish oil as maintenance therapy for UC in remission have, however, been largely negative. In the trial conducted by Hawthorne *et al.* described above, there was no significant difference in the rate of relapse of those in remission between the fish oil treated and placebo groups (Hawthorne, Daneshmend *et al.* 1992). In a study by Greenfield *et al.* 24 patients with stable UC were randomised to receive fish oil (n=16) or olive oil placebo (n=8) (Greenfield, Green *et al.* 1993). The only clinical effect of active treatment was an improvement in stool consistency. Stool frequency, rectal bleeding, disease relapse, sigmoidoscopic appearance and rectal histology were unchanged between the treatment groups.

A relapse prevention study by Loeschke *et al.* randomised 64 patients with UC in remission, both on and off steroids, to fish oil or placebo. After 3 months of treatment all 5-ASA preparations were stopped and clinical disease activity was monitored for two years. The relapse-free survival was improved in the fish oil group only during months 2 and 3. By 2 years the relapse rate was similar for both groups.

Mantzaris *et al.* allocated 40 patients with UC in remission to be treated either with fish oil (n=22) or olive oil placebo (n=18), in a randomised fashion (Mantzaris, Archavlis *et al.* 1996). There was no difference in relapse rates at one year between the two groups.

Recent meta-analysis from the Cochrane group of 3 of the above studies was unable to show a clear benefit for fish oils alone in the maintenance of UC (Turner, Steinhart *et al.* 2007).

Other studies have compared fish oil treatment to active controls. Dichi *et al.* compared fish oil head-to-head with sulphasalazine in a randomised, cross-over study of 10 patients with mild to moderate active UC. The fish oil group fared significantly worse than the sulphasalazine group in terms of clinical, endoscopic and histological activity of disease.

Barbosa *et al.* performed a randomised controlled cross-over study comparing sulphasalazine alone with the combination of sulphasalazine and fish oil in 9 patients with mild or moderate UC. Although they were able to show a reduction in plasma oxidative stress with fish oil treatment, this did not translate into an improvement in disease activity as measured by laboratory indicators, and sigmoidoscopy and histology scores.

A further open label study examined the use of seal oil, administered via nasoduodenal feeding tube, for the treatment of UC (Arslan, Brunborg *et al.* 2002). Five patients were treated for 10 days with a benefit in terms of disease activity.

Interest in the action of n-3 FAs and fibre as described above has logically led to the investigation of combinations of these supplements in the management of IBD, in particular in UC. Seidner *et al.* compared a combination of fish oil, soluble fibre and antioxidants in the management of mild to moderate UC. They were able to demonstrate

a reduction in the use of steroid therapy in the treatment group, clinical response was similar for treatment and placebo control (Seidner, Lashner *et al.* 2005).

Thus it seems unlikely that fish oil preparations constitute a beneficial approach to the maintenance of remission in UC. The possibility remains that larger, parallel group, double-blind, placebo controlled studies might find a benefit for fish oil over placebo in the treatment of active UC. However, the limited available evidence suggests it likely fish oil will remain inferior to standard 5-ASA therapy for the treatment of such patients.

II.3 Conclusions

The only conclusion the above Chapter allows is that there remains a lack of consensus on the dietary management of IBD. This is explicable by the uncertainty of the underlying science regarding the effect of dietary factors on disease development and the influence of dietary change over disease course. In the light of this I wanted to ascertain a baseline status of current practice. Much information exists regarding how patients view the interaction of diet with their disease, but there is very little descriptive data regarding the approach gastroenterologists take to the interaction between diet and disease. The proceeding Chapter describes a survey study, conducted in both the United Kingdom and New Zealand, designed to ascertain how gastroenterologists approach dietary management and manipulation in IBD and to compare that with their dietary management in irritable bowel syndrome, another common gastroenterological condition .

Chapter III Survey of United Kingdom and New Zealand gastroenterologists' practice regarding dietary advice and food exclusion in irritable bowel syndrome and inflammatory bowel disease

III.1 Introduction

In 1950 Loveless (Loveless 1950) and Graham (Graham, Wolf *et al.* 1950) synchronously demonstrated an association between food and gastro-intestinal symptoms in adults. Since then food intolerance has commonly been reported in the general population, and 20-45% of the adult population believe that they suffer from adverse reactions to food (Burr and Merrett 1983; Crowe and Perdue 1992; Shanahan 1993; Young, Stoneham *et al.* 1994). However, double-blind food elimination and challenge is positive in only a small proportion of these people.

It has long been thought that food intolerance plays at least some role in the production of symptoms in irritable bowel syndrome (IBS) (Crespo and Rodriguez 2003; Sicherer 2003). The perception of food intolerance is common amongst patients with IBS, 20-65% of patients attribute their symptoms to adverse food reactions (Nanda, James *et al.* 1989; Dainese, Galliani *et al.* 1999) and patients commonly experiment with elimination diets or alternative therapies before seeking medical help (Smart, Mayberry *et al.* 1986). Recent studies have shown the ability of an exclusion diet guided by the results of testing for IgG antibodies to foods in the serum to improve the symptoms of patients with IBS (Atkinson, Sheldon *et al.* 2004; Zar, Mincher *et al.* 2005).

The association between symptoms of IBD and diet has also received much attention (Jones, Dickinson *et al.* 1985; Riordan, Hunter *et al.* 1993; Crowe 2001). It is commonplace for patients with GI disorders to believe that something in their diet has caused their condition (Crowe 2001). Some studies have claimed that food sensitivities are common in CD and have found that when food intolerances are detected, patients on an exclusion diet maintain remission significantly longer than those on an unrestricted diet (Jones, Dickinson *et al.* 1985; Riordan, Hunter *et al.* 1993). However, when these patients are subjected to double-blind food challenges only 15% show a positive response (Pearson, Teahon *et al.* 1993).

Despite these associations the available evidence is insufficient for strong recommendations regarding the use of exclusion diets in these conditions to be made and current guidelines for the management of IBD and IBS give very little specific recommendation regarding testing for food intolerance/allergy nor the treatment of it (Carter, Lobo *et al.* 2004; Spiller, Aziz *et al.* 2007). No information exists as to how commonly exclusion diets are used in practice or what forms of advice are given. This study aimed to determine what current practice regarding dietary advice, in particular advice about food exclusion, is amongst gastroenterologists in the United Kingdom (UK) and New Zealand (NZ).

III.2 Collaborators

The author performed all data collection and analysis. Dr A Emmanuel and Dr S Bloom assisted with study and questionnaire design.

III.3 Ethical approval

Advice was sought from the chair of the Joint UCL/UCLH Committees On The Ethics

Of Human Research. It was their opinion that committee approval was not needed for the conduct of this study.

III.4 Subjects

This survey aimed to question the majority of adult gastroenterologists in NZ and the UK. Both countries have professional gastroenterological societies with high rates of membership by practising gastroenterologists [the British Society of Gastroenterologists (BSG) and the New Zealand Society of Gastroenterologists (NZSG)], although the exact proportion of gastroenterologists who are members is not known. Both societies maintain lists of active members, providing the only reliable route for identification of a large body of practising gastroenterologist in each country.

Both societies also contain many members who are not practising adult gastroenterologists. All respondents were questioned regarding the nature of their practice. All but those currently practising as gastroenterologists in adult medicine were excluded from the analysis. In addition the list of non-responders was examined for non-adult gastroenterologists by means of qualification, e.g. those holding FRCS and FRCPath qualifications were excluded, and location of practice, e.g. those practising only in a paediatric setting were excluded. This differentiation proved straightforward in NZ, where the number of practising gastroenterologists is lower. However, it remains probable that non-adult gastroenterologists are represented in the final numbers of non-responders in the UK audit, thus increasing the apparent non-response rate in that survey.

III.5 Methods

Gastroenterologists are no strangers to surveys of practice. A Medline search using the terms “gastroenterologist” and “survey” returned 10 published, large scale, practice surveys in the year 2008 alone. Mandal *et al.* and Eaden *et al.* have extensively reviewed the aspects that are essential to maintaining the quality of survey work in this area (Eaden, Mayberry *et al.* 1999; Mandal, Eaden *et al.* 2000). They are careful to point out that, while survey techniques can produce speedy results usually without significant capital investment, questionnaires cannot be easily constructed and used without training. Careful attention to reliability and validity is needed. The ideas from these review articles are heavily drawn upon in the proceeding discussion. Each aspect of the survey design process for this study is outlined below and the compromises and solutions decided upon are discussed.

III.5.1 Question type

Closed questions may unduly lead the respondent, yet open questions give responses that may be difficult to analyse in a quantitative fashion. Semi-closed questions can provide a compromise for both the investigators and the respondents; besides providing answer choices, they give the subject a freedom to include additional information. For this reason two main question types, dichotomous and multiple choice, were used in this survey but ample opportunities for additional open responses were provided.

III.5.2 Survey medium

The choice of survey medium depends on the type and the size of the population to be studied as well as its geographical distribution. In this survey the desire was that a large population over a wide geographical distribution be included. This precluded face-to-face or over-the-telephone interviews, leaving mail out and Internet based surveys as the applicable tools.

Internet based techniques allow researchers to conduct surveys through self-administered questionnaires and data can be collected using the World Wide Web or through electronic mail. The main advantages of this method are: 1. cheapness, as it needs no postage, printing, packaging, interviewer or telephone call; 2. it is quick – it takes only few seconds to send and return messages; 3. sending a repeat message, reminder or clarification is easy; 4. recipients can easily and quickly complete proformas and return the data with a keystroke; 5. easy access to respondents throughout the world which removes geographic barriers.

The disadvantages are: 1. there remains a concern that the internet community is not yet representative of the general population and this will lead to selection bias; 2. respondents have generally had a higher education and higher household income; 3. if the survey is done through e-mail, access to the e-mail addresses of potential respondents is necessary; 4. impersonation of patients or other subjects will be difficult to exclude.

At the time of their review Mandal *et al.* concluded that data collected through the Internet should be interpreted with caution because of these selection biases (Mandal, Eaden *et al.* 2000). However, this tool has received increasing popularity of use in healthcare related research (Braithwaite, Emery *et al.* 2003). Recently, Lusk *et al.* evaluated determinants of response to Internet-based surveys in a sample (n = 5600) of Texas healthcare professionals (Lusk, Delclos *et al.* 2007). Participants were given the option of responding by mail or over the Web. Overall, Web-based responses represented a consistent 9% to 10% of the total responses. Missing questionnaire items were significantly higher among Web responders. In the final multivariate logistic

regression, only male gender (OR = 2.09, 95% CI = 1.56-2.80) and younger age remained significantly associated with response over the Internet, suggesting that there is a significant and perhaps growing minority of health professionals who would prefer to respond over the internet and that the selection biases one could expect might be mostly gender and age based.

Mail surveys are also quick, cheap and free of interviewer influence. The major issues are of non-response bias, response quality and item non-response. In addition they allow only limited control over whether the intended respondent or someone else completes the form. Incorrect addresses and temporary absence of the respondent at the time of the survey (e.g. holiday) will also affect response rates.

Both the BSG and NZSG lists include email addresses for the majority of members, allowing a Web-based approach. However, it was recognised that the age range in both societies is wide and that, as evidenced by the results of Lusk *et al.* (Lusk, Delclos *et al.* 2007), sole use of a Web-based approach would have been likely to produce non-response bias, at least in terms of age of respondents vs. non-respondents. For this reason a two-pronged approach was chosen where all society members would first be emailed. Then, in subsequent rounds, those not responding and those without listed, functioning email addresses would additionally be contacted by conventional mail.

III.5.3 Questionnaire design

The main principles of questionnaire design, as set out by Stone, were adhered to (Stone 1993).

1. Clear and appropriate objectives for the study were set with a well-defined end-point.
2. Questions were designed to be unambiguous.

3. The questions were designed to be appropriate to the social and educational background of the respondent.
4. Consultant gastroenterologists were approached as it was felt they would be most willing and able to answer accurately.
5. The potential for external events to bias the answer was considered. Thus the decision to use two forms of survey medium. In addition the decision to use an incentive strategy was very carefully considered (discussed in section III.5.5) and only embarked upon when it was felt that overcoming non-response bias might outweigh the bias introduced. In addition the effect of the incentive on the pattern of response was analysed in a sensitivity analysis.
6. Approval from the Chair of the Joint UCL/UCLH Committees On The Ethics Of Human Research was sought.

III.5.3.1 Length of questionnaire

Available evidence suggests that the length of a survey directly contributes to non-response. Mandal *et al.* and Eaden *et al.* have suggested that one A4 page is an appropriate length of survey (Eaden, Mayberry *et al.* 1999; Mandal, Eaden *et al.* 2000). A study by Jepson *et al.* showed that there might be a threshold length for surveys, beyond which non-response rates increase (Jepson, Asch *et al.* 2005). In a pilot study they administered questionnaires of 30 different lengths (849 to 1,867 words), by mail, to 192 physicians in April 1999. This was followed by a study involving surveys of 16 different lengths (564 to 988 words) sent to 1,700 physicians between June 1999 and January 2000. They concluded there appeared to have been a threshold of approximately 1,000 words. Questionnaires above the threshold had lower response rates than those below it (38.0% vs. 59.4%). However there was no direct association, on logistic regression analysis, between word count and response.

Attempts to confine the questionnaire for this survey to one A4 page did not allow identical surveys to be administered for IBS and IBD with clear layout. Although the focus of this thesis is food intolerance testing in IBD, I was very interested to investigate how current practice compared between IBD and IBS, two diseases with very different evidence bases regarding food intolerance and its management. For this reason it was decided the questionnaire would be extended to two A4 sheets. However, at 833 words, the final word count was kept below 1000.

III.5.3.2 Layout of questionnaire

The level of compliance and quality of response depends on the initial questions, thus emotional content should be avoided at the beginning of the questionnaire and the first few questions should be simple, objective and interesting. More sensitive items are better placed later. This is known as a 'funnel approach' (Mandal, Eaden *et al.* 2000). In addition the overall questionnaire should move from topic to topic in a logical manner with all questions on one topic being completed before the respondent moves to the next.

These principles were carefully applied in the design of the questionnaire for this study. Initial questions were regarding the nature of the respondents' practice and the resources available to them, prior to asking direct questions about their personal management. The two parts to the questionnaire, practice in IBS and IBD, were carefully separated by a header statement in the Web-based and mail-out versions and by separate pages in the mail-out version.

III.5.4 Questionnaire pilot study

Mandal *et al.* summarise the characteristics of a pilot study (Mandal, Eaden *et al.* 2000). Pilot studies should address the following:

1. Validity of the data (extent to which an instrument measures what it is supposed to measure)
2. Reliability of the data (extent to which the questionnaire can give consistent results)
3. Whether the understanding was similar for all respondents
4. Non-response to one or more questions
5. Uninterpretable responses to any question
6. Researcher bias in question design
7. Whether the questionnaire and the covering letter adequately explain the purpose of the study.

Test–retest examines the ability of a questionnaire to produce identical responses when given to the same person on two separate occasions. However, if the test–retest is done too quickly the result may be confounded by memory.

Within this study a pilot study was conducted with 14 consultant gastroenterologists in the London region. 14 gastroenterologists were emailed and requested to complete an online survey. They were then requested to repeat the survey 4 weeks later. In the light of their comments the questionnaire was modified. The responses to both pilot questionnaire rounds were used to assess the test-retest reliability of the survey, by comparing each item and identifying items as unreliable if the correlation coefficient was below 0.5 using Pearson's correlation coefficient (r) for continuous variables and by calculating Phi (f) for dichotomous variables.

III.5.5 Avoiding non-response bias

Mandal *et al.* have summarised the factors associated with response rates greater than 90% (Mandal, Eaden *et al.* 2000):

1. Motivation and interest in the subject matter.
2. The nature of the sponsor, for example sponsorship by a professional body.
3. A relatively short and non-contentious questionnaire that includes a description of purpose and benefits of the study.
4. Notification by mail or telephone.
5. A handwritten note attached to the questionnaire.
6. A supporting letter from the patient's general practitioner.

Attention was paid to those aspects that could be addressed in this study. It was presumed that gastroenterologists, by and large, would exhibit interest in what is recognised as an aspect that is of great interest to their patients in the management of two of the commonest diseases that they treat. In addition the covering letter sought to promote that interest by clearly stating the objectives of the study and recapping the absence of similar data or authoritative recommendations regarding practice in this area. In the NZ study a hand-written note accompanied all second mail out letters from the author. Although sponsorship from the professional bodies was not available, the use of the mailing list from that body was mentioned.

One of the major advantages of Internet-based surveys is the ease of repeated reminders to potential respondents. In work by Braithwaite *et al.* looking at the response of UK general practitioners (GPs) to an electronic survey, this strategy was associated with a doubling of the initial response rate after a total of five reminders and response rates

very similar to those reported in studies of mail-out surveys of UK GPs (Braithwaite, Emery *et al.* 2003).

Monetary incentives can also increase response rates substantially, especially if they are prepaid (Mandal, Eaden *et al.* 2000). In this study it was decided that response rate would be monitored in the initial email rounds and if unexpectedly low response rates were occurring an incentive in the form of a prize would be offered. The effect of this on the responses would be monitored using sensitivity analysis of responses before and after the offering of a prize.

III.5.5.1 Sensitivity analysis

After each successive wave of contact with a group of potential respondents the researcher should run a sensitivity analysis. Its purpose is to ascertain how different non-respondents would need to be from respondents to alter the significance of the data supplied by current respondents. If the most extreme foreseeable answers by the non-respondents would not alter the decision no further efforts are needed. If the non-respondents could alter the decision then the researcher should examine the trend over the first, second and third mailings. The attributes of the non-respondents are assumed to be similar to a projection of the trend between early and late respondents.

In this study the responses from early vs. late respondents, which also made up the groups offered vs. not offered an incentive, and also email vs. conventional mail respondents, where analysed for significant differences in their replies to any question.

III.5.6 Statistical methods

Sensitivity analysis comparing mail rounds was performed by calculating the 95% confidence intervals for each response within each mail round. Confidence intervals

were compared between rounds to examine for statistically significant differences in response between mail rounds. Assessment of questionnaire test-retest reliability was performed in a pilot study using Pearson's correlation coefficient (*r*) for continuous variables and by calculating Phi (*f*) for dichotomous variables. Paired proportions were compared using the McNemar test.

Web-based survey data were collected using the tool provided by the UK based online market and research systems provider Problemfree Ltd. at their website www.Freesurveysonline.com. Data were collated in Microsoft® Excel 2003 (Microsoft Inc). Statistical analysis was carried out using SPSS™ 14.0 (SPSS Inc).

III.6 Results

III.6.1 Pilot study (UK)

In a pilot study to assess questionnaire design and reliability 14 gastroenterologists were emailed and requested to complete an online survey. Eight (57%) replied to the initial request, of whom six (43%) replied to a request to repeat the survey 4 weeks later. In the light of their comments the questionnaire was modified. The responses of the six respondents to both questionnaire rounds were used to assess the reliability of the survey, by comparing each item and identifying items as unreliable if the correlation coefficient was below 0.5 using Pearson's correlation coefficient (*r*) for continuous variables and by calculating Phi (*f*) for dichotomous variables. Correlation coefficients could not be calculated for some dichotomous variables as they generated identical responses from all participants in either mail round.

Four items considered in the final analysis were shown to be unreliable using these criteria. These questions were:

1. Physicians please indicate the percentage of time spent in gastroenterology versus medicine.
2. Please indicate which patients with IBS you are most likely to give or send for dietary advice: Difficult to control IBS?
3. If you do ask patients to exclude foods please indicate the types of foods: Yeast?
4. Please indicate which patients with IBS you are most likely to give or send for dietary advice: Constipation predominant?

All other items gave a correlation coefficient greater than 0.5 where one could be calculated (see Appendix 1. Correlation coefficients for two rounds of survey pilot study).

III.6.2 United Kingdom national survey

Altogether there were three email invitations at intervals of approximately six weeks. A single conventional mailout was made to all BSG members who had no email address listed or whose listed email address was non-functioning. In addition, following all email rounds, all BSG members who did not respond to email requests were sent a conventional mail request. Eighty-nine potential subjects were excluded because a response was received stating they were not eligible for the survey. This included non-adult gastroenterologists, retired gastroenterologists, deceased members, members with no clinical practice and members on maternity leave. Two members responded declining to participate and were excluded.

This gave a total of 983 potential respondents, 834 of whom appeared to have functioning email addresses, in that no reply from the email server was received stating that delivery was not possible. This, of course, does not mean that all the members emailed received the email or indeed read the email. Those members with non-functioning or no email address were sent a questionnaire by first class post. In addition, all members with an apparently functioning email address who did not reply to the email requests were sent a questionnaire by first class post. Following a response rate of only 17% to the first email round it was evident that response rates were likely to fall below predicted and an incentive, in the form of entry in a prize draw for all respondents, was offered at all subsequent mailings. In total there were 363 replies, constituting 37% of the 983 potential respondents identified (Figure 8).

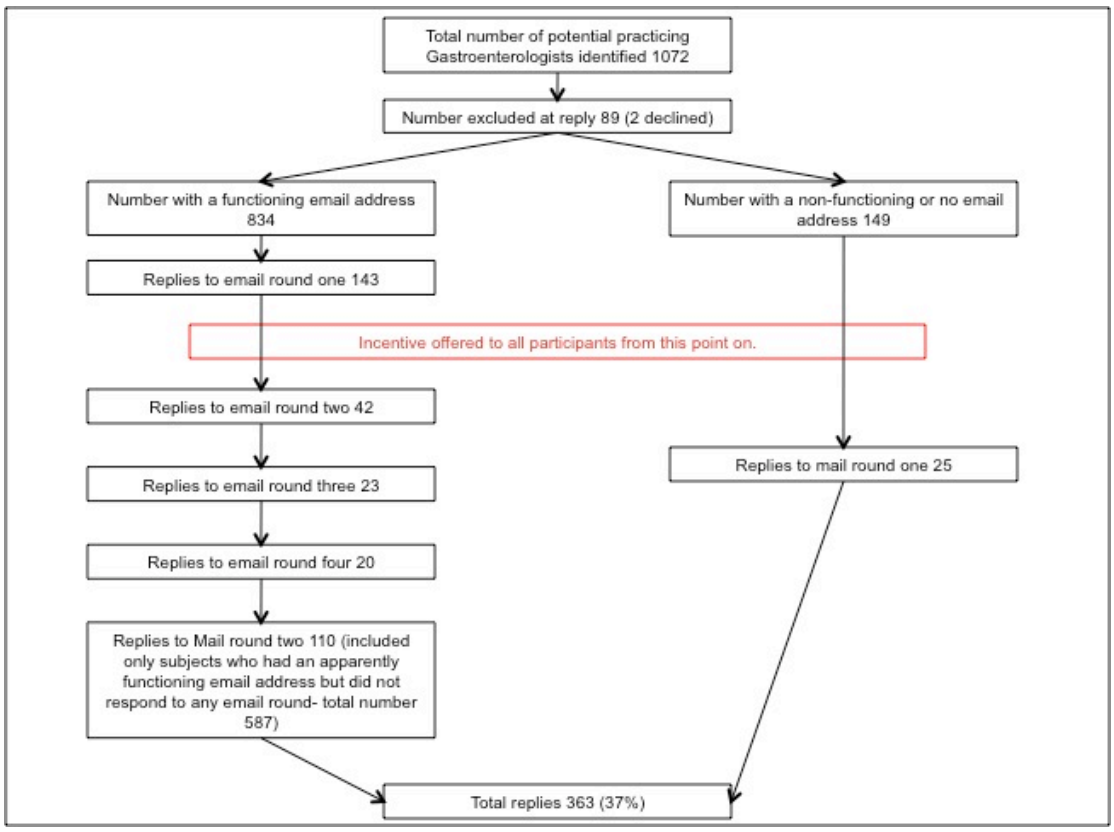


Figure 8. Number of responses received at each mail round

III.6.2.1 Sensitivity analysis

Early vs. late respondents, which also made up the groups offered vs. not offered an incentive, were not found to differ significantly in their replies to any question, nor were email vs. conventional mail respondents.

III.6.2.2 Respondent demographics

The median percentage of time spent in gastroenterological practice was 80% (range 5% to 95%) with 98.6% of respondents having access to dietetic services, equal numbers having access to general dietetic services and specialist gastroenterological dietetic services. Respondents were asked how much dietetic resource (in hours) was allocated to their service and the median was 6 hours (range 0 to 100) but 172 respondents were unable to or did not answer this question.

49% of respondents were involved in a specialist IBD clinic whereas only 8.5% were involved in a specialist IBS clinic. Overall, respondents reported seeing similar numbers of IBS and IBD patients in outpatient clinics with the majority of respondents seeing between 20 and 60 IBS and IBD patients in a month (

Figure 9).

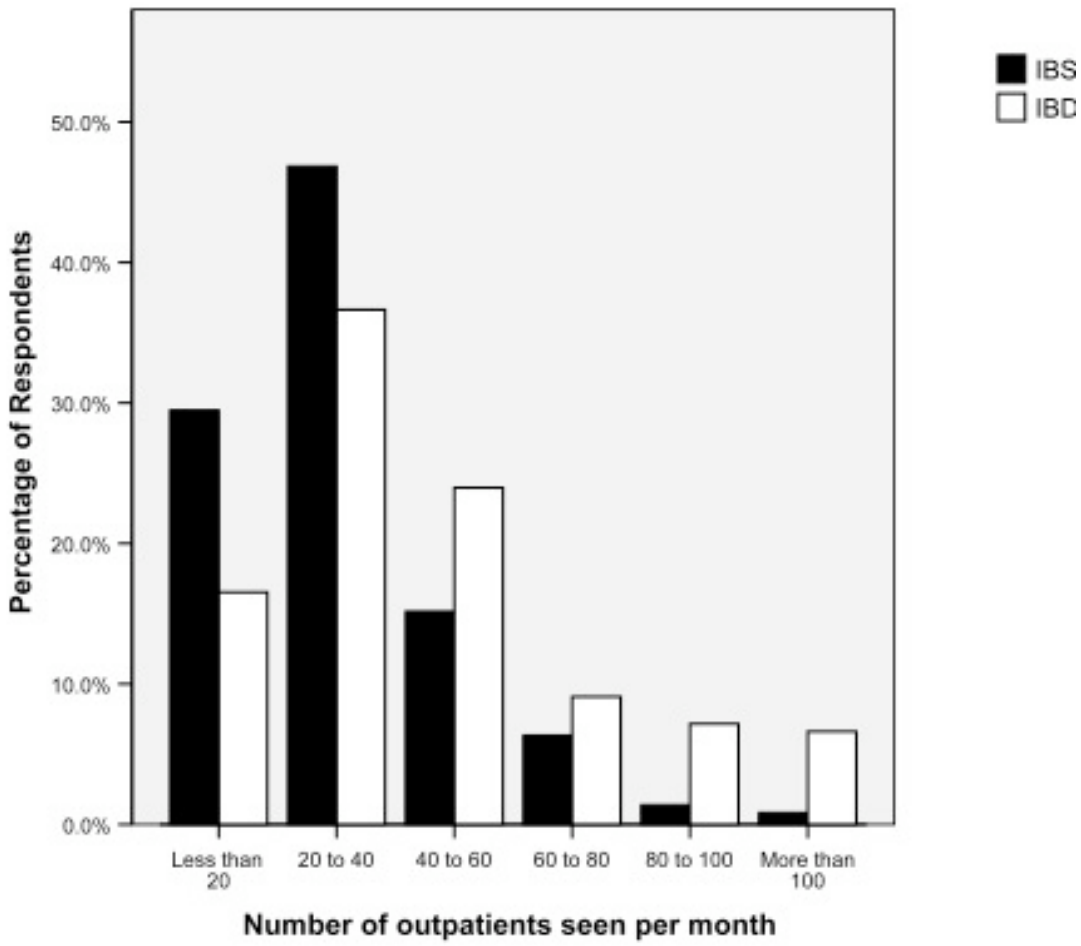


Figure 9. Number of IBD and IBS patients seen by gastroenterologists per month

III.6.2.3 Dietary advice

Clinicians reported giving specific dietary advice to patient with IBS more commonly than IBD. The majority of respondents (84%) reported giving advice to more than 25% of patients with IBS, whereas this was the minority (27%) in IBD (p=0.001) (Figure 10). The proportion of patients sent for dietetics referral was similar for both groups of

patients, the majority of respondents reporting that they refer less than 25% of IBD and IBS patients to see a dietitian.

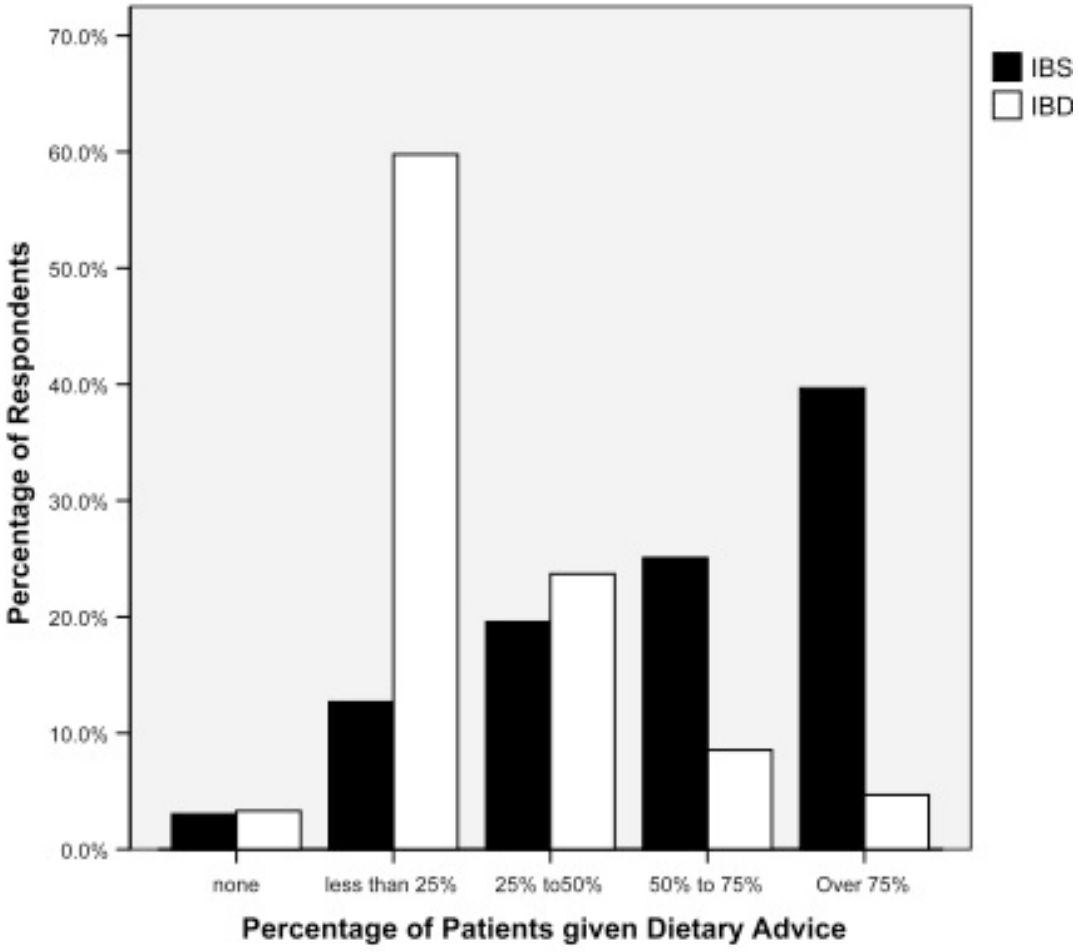


Figure 10. Percentage of IBS and IBD patients given dietary advice

Respondents were also more likely to give advice specifically about dietary exclusion to IBS than IBD patients. The majority of respondents reported giving advice to more than 25% of their IBS patients (87%) compared to the majority who reported giving advice about dietary exclusion to less than 25% of their IBD patients (61%, $p<0.001$). The foods respondents most commonly advised patients to avoid were similar for IBD and IBS, fibre being common in both. Wheat and dairy exclusion were also commonly recommended in both conditions, however these recommendations were more common

in IBS than IBD (66% vs 20% of respondents for wheat ($p<0.001$) and 70% vs. 45% for dairy ($p=0.001$).

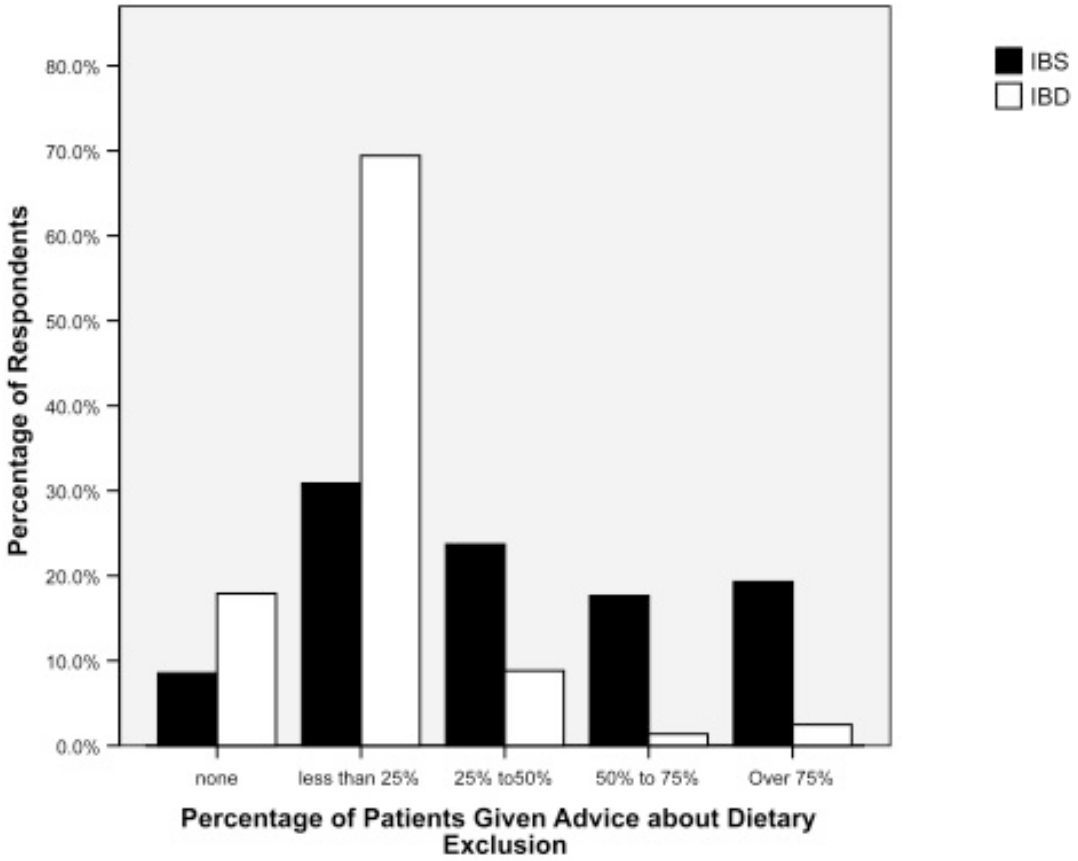


Figure 11. Percentage of IBD and IBS patients given dietary exclusion advice

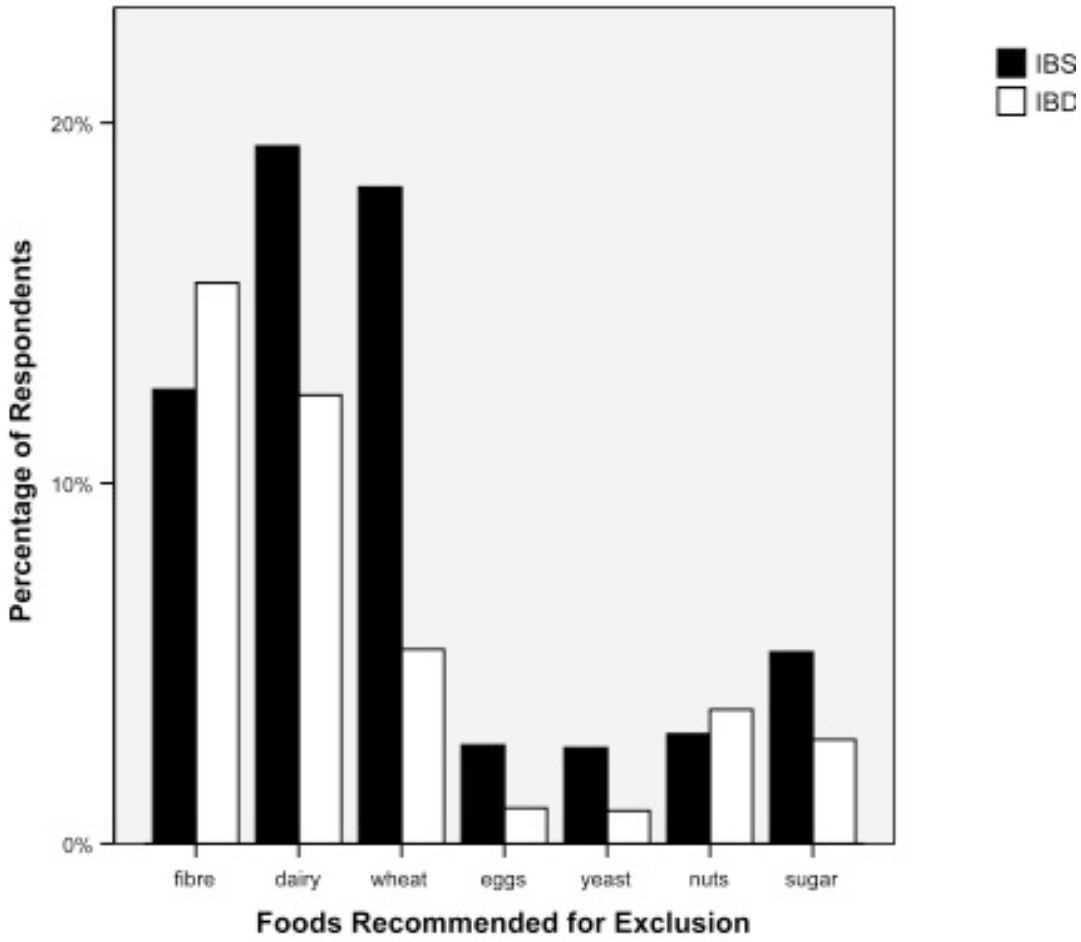


Figure 12. Foods recommended to IBD and IBS patients for exclusion

A low utilisation of allergy testing was reported in both IBD and IBS. In both conditions the majority of respondents reported no or very little (0-25% of patients) use of allergy testing (97% in IBD and 89% in IBS) (Figure 13). Where allergy tests were used they were most commonly "open food exclusion and rechallenge" (14% of respondents in IBD and 23% in IBS) and RAST (14% in IBD and 18% in IBS). There was also a small proportion who reported using skin prick testing (4% in IBD and 6% in IBS) and the Yorktest IgG antibody test (3% in IBD and 8% in IBS) (Figure 14). Respondents reported being most likely give dietary advice to, or send for dietary advice, patients with small bowel Crohns disease (84% of respondents), difficult to control IBD (46%), diarrhoea predominant IBS (59%) and difficult to control IBS (59%).

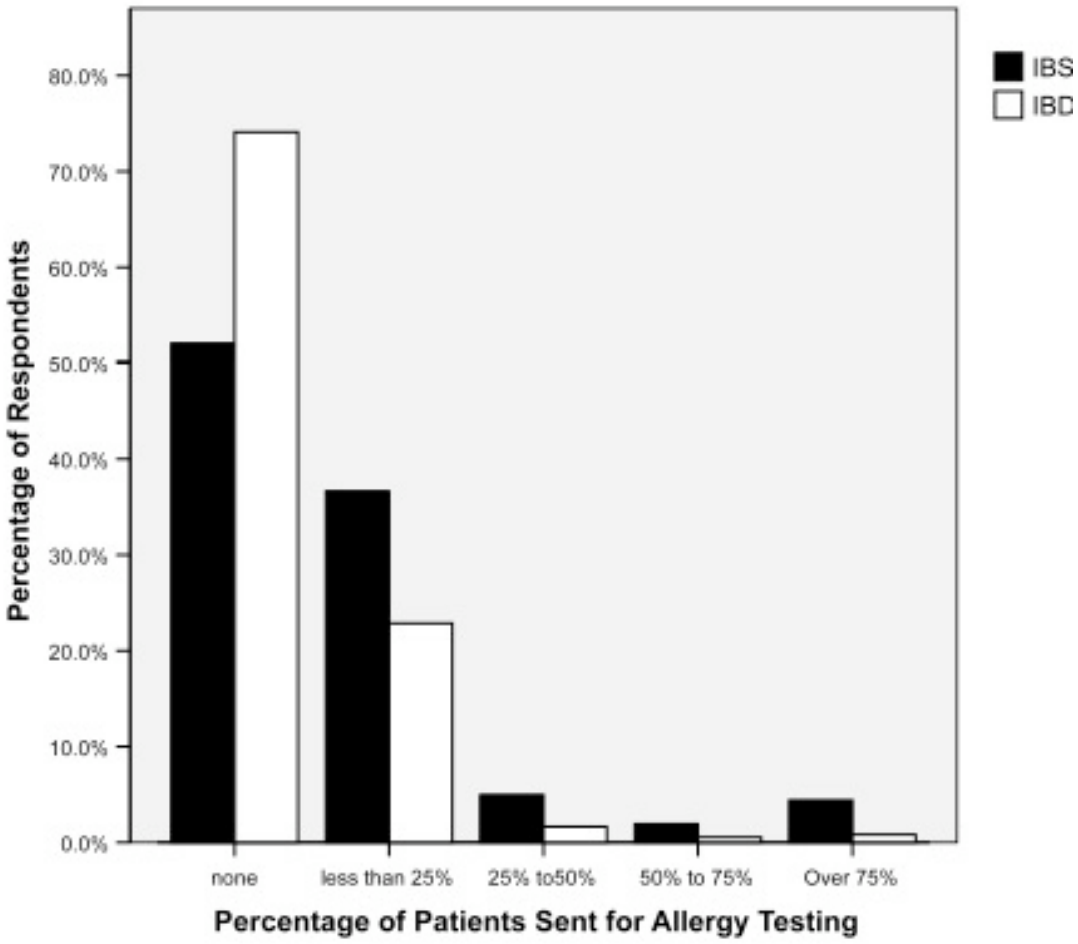
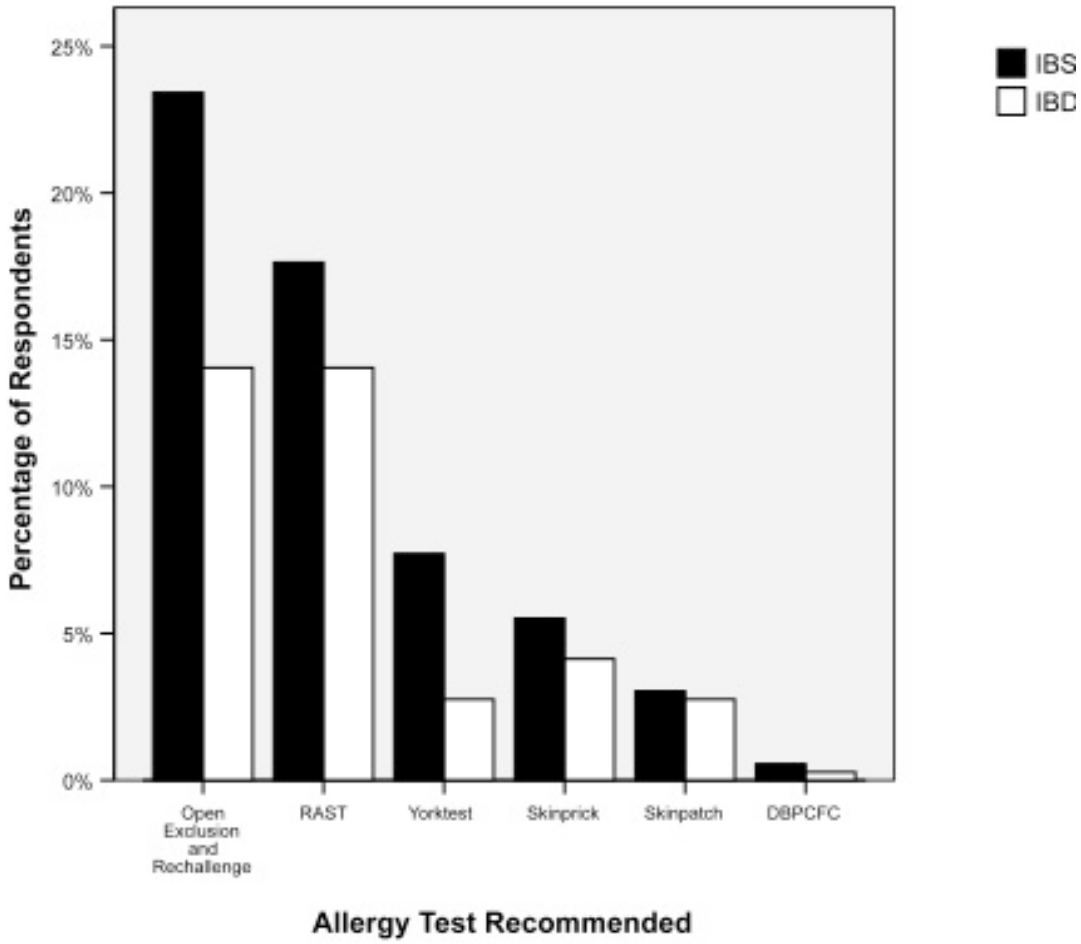


Figure 13. Percentage of IBD and IBS patients sent for allergy testing



(RAST: radioallergosorbent test; DBPCFC: double-blind placebo-controlled food challenge)

Figure 14. Allergy tests recommended to IBS and IBD patients

When asked whether participants agreed that dietary exclusion was an effective strategy in IBD, responses were mixed with only a small proportion agreeing strongly (7%) and the rest either agreeing a little (32%), neither agreeing or disagreeing (20%), disagreeing a little (20%) or disagreeing a lot (21%). When asked the same question in IBS the majority of respondents reported either agreeing strongly or agreeing a little (71%).

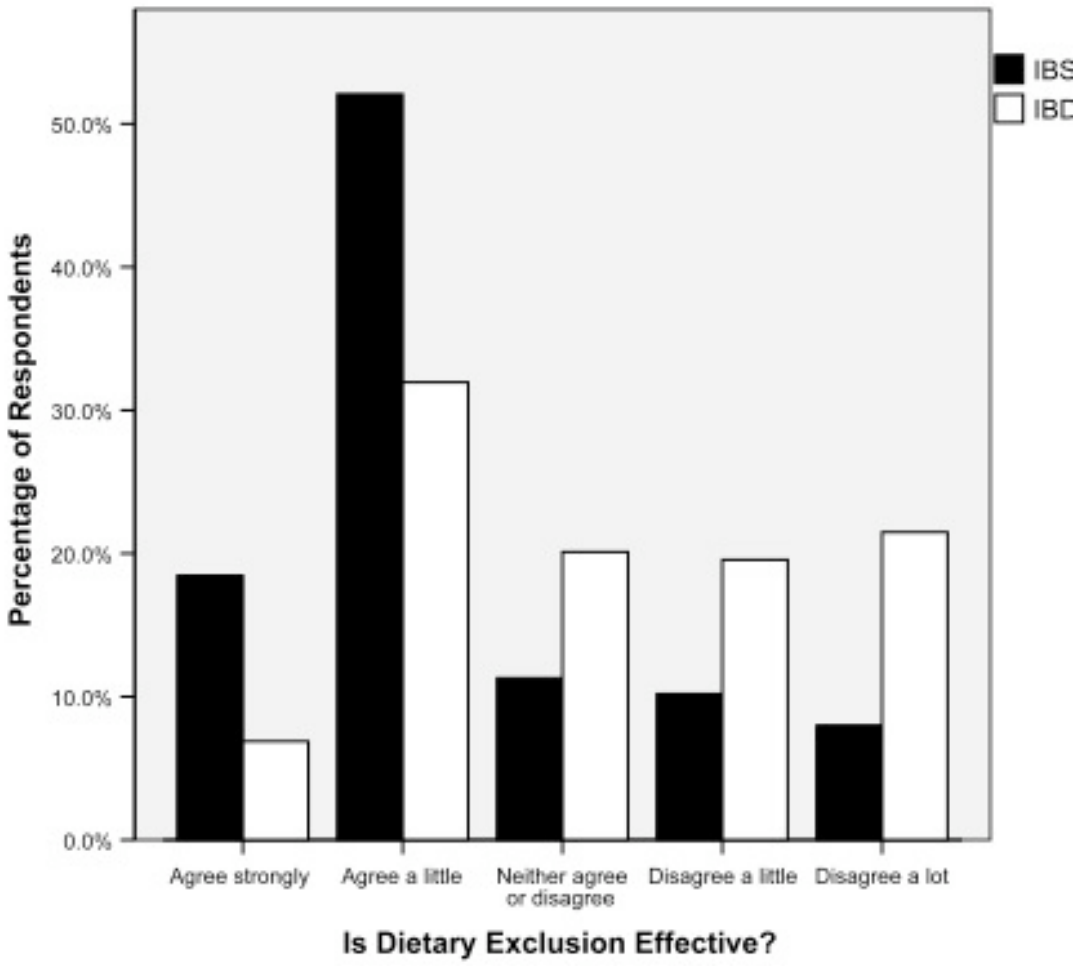


Figure 15. Response to the question "do you think dietary exclusion is an effective strategy" for IBD and IBS patients

III.6.3 New Zealand national survey

Altogether there were five email mailings at intervals of approximately two weeks. Two conventional mailouts were made to all NZSG members who had not responded two weeks after the final mail round. The two mail rounds were separated by approximately four weeks. Fifty-five potential subjects were excluded because a response was received stating they were not eligible for the survey. This included non-adult gastroenterologists, retired gastroenterologists, deceased members, members not currently practising or practising outside of NZ.

This gave a total of fifty-four potential respondents, all of whom appeared to have functioning email addresses, in that no reply from the email server was received stating that delivery was not possible. After five email rounds a total of forty-three members had responded. The remaining eleven were sent a mail-out reminder letter, to the first round of which six replied and to the second round, two.

Thus in total there were fifty-one replies, constituting 94% of the fifty-four potential respondents identified (Figure 16).

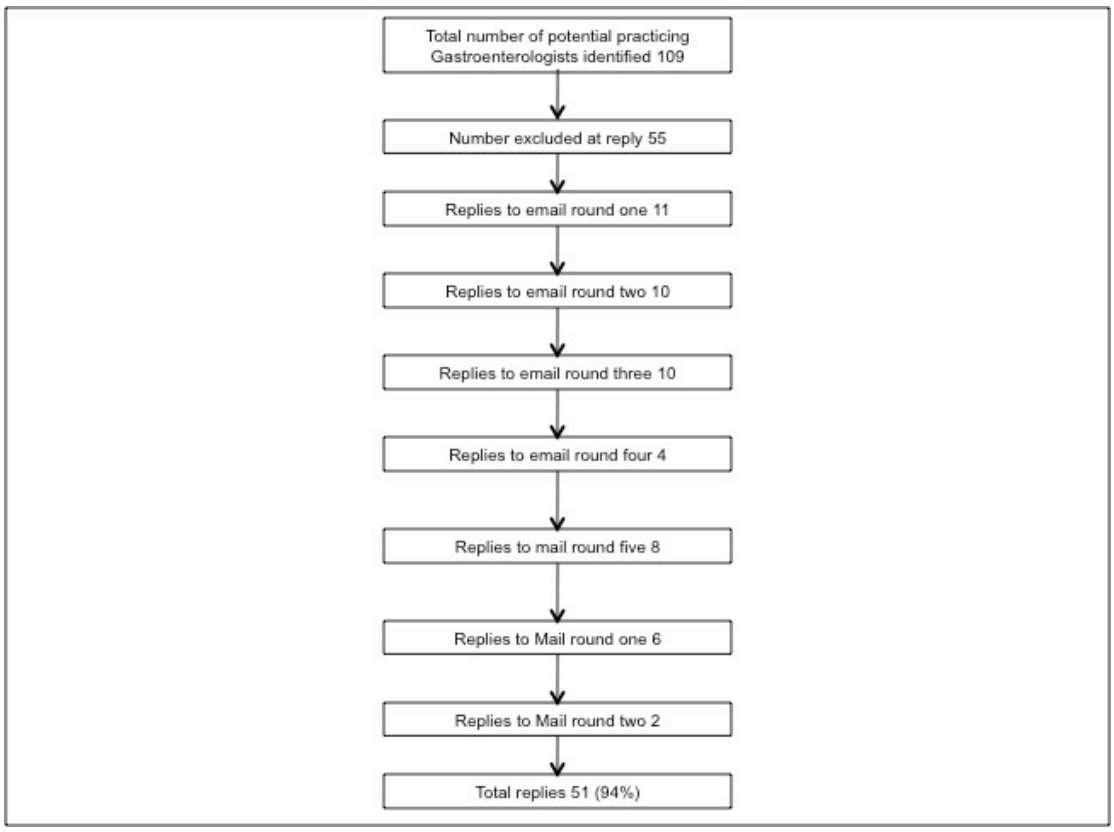


Figure 16. Number of responses received at each mail round

III.6.3.1 Sensitivity analysis

Because of the high response rate for the NZ survey non-response bias is very unlikely and sensitivity analysis was not required.

III.6.3.2 Respondent demographics

The median percentage of time spent in gastroenterological practice was 93% (range 10% to 100%) with 96% of respondents having access to dietetic services, the greater proportion (67%) having access to general dietetic services versus specialist gastroenterological dietetic services (29%). Respondents were asked how much dietetic resource (in hours) was allocated to their service and the median was four hours (range 0 to 20) but 30 respondents were unable to or did not answer this question.

31% of respondents were involved in a specialist IBD clinic whereas 18% were involved in a specialist IBS clinic. Respondents reported seeing greater numbers of IBS than IBD patients in outpatient clinics with the overwhelming majority of respondents seeing 20 to 40 IBS patients per month (Figure 17).

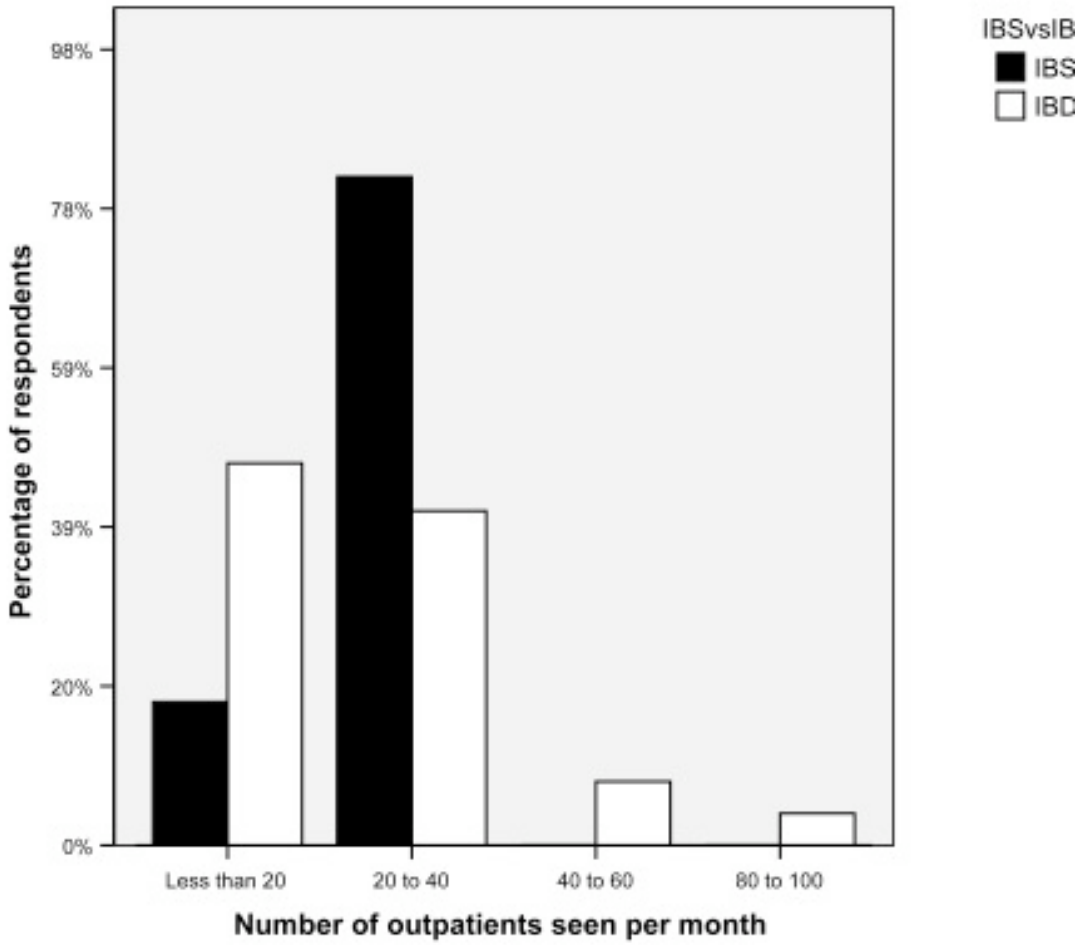


Figure 17. Number of IBD and IBS patients seen by gastroenterologists per month

III.6.3.3 Dietary advice

Clinicians reported giving specific dietary advice to patients with IBS more commonly than IBD. 90% of respondents reported giving advice to more than 25% of patients with IBS, whereas this was much lower (55%) in IBD ($p<0.001$) (Figure 18). The proportion of patients sent for dietetics referral was similar for both groups of patients, the overwhelming majority of respondents reporting that they refer less than 25% of IBD and IBS patients to see a dietitian.

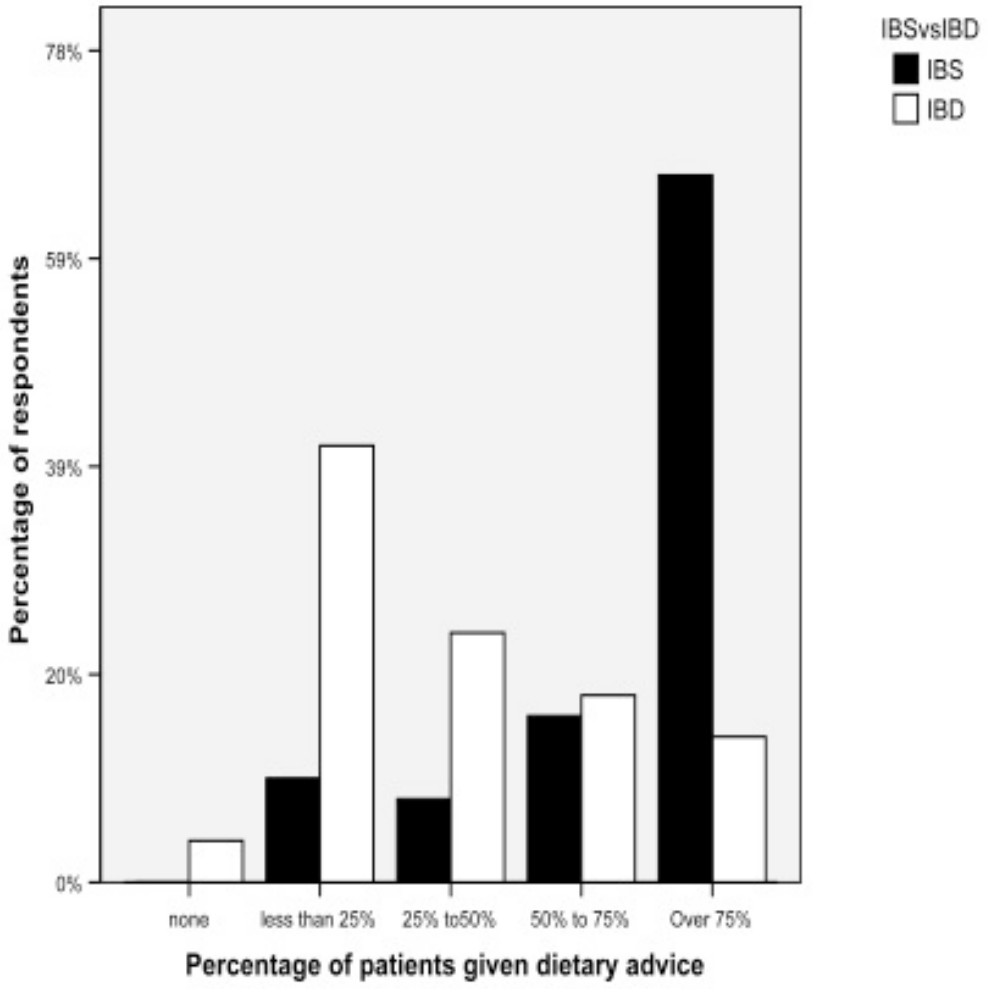


Figure 18. Percentage of IBS and IBD patients given dietary advice

Respondents were also more likely to give advice specifically about dietary exclusion to IBS than IBD patients. The majority of respondents reported giving advice to more than 25% of their IBS patients (77%) compared to the majority who reported giving advice about dietary exclusion to less than 25% of their IBD patients (86%, $p<0.001$). The foods respondents most commonly advised patients to avoid were similar for IBD and IBS, fibre being common in both. Wheat, sugar and dairy exclusion were also commonly recommended in both conditions, however these recommendations were more common in IBS than IBD (45% vs 14% of respondents for wheat ($p<0.001$), 47% vs 20% for sugar ($p=0.001$) and 55% vs. 26% for dairy ($p=0.001$) (Figure 19).

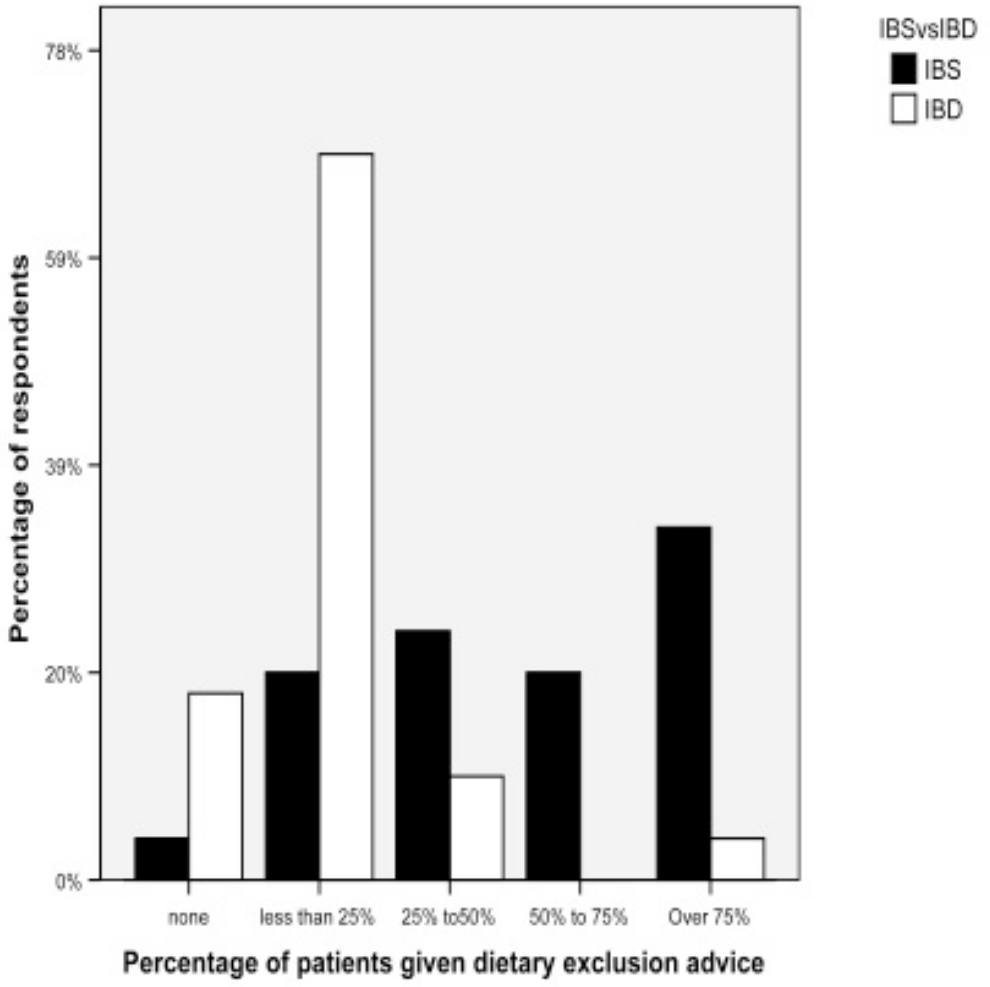


Figure 19. Percentage of IBD and IBS patients given dietary exclusion advice

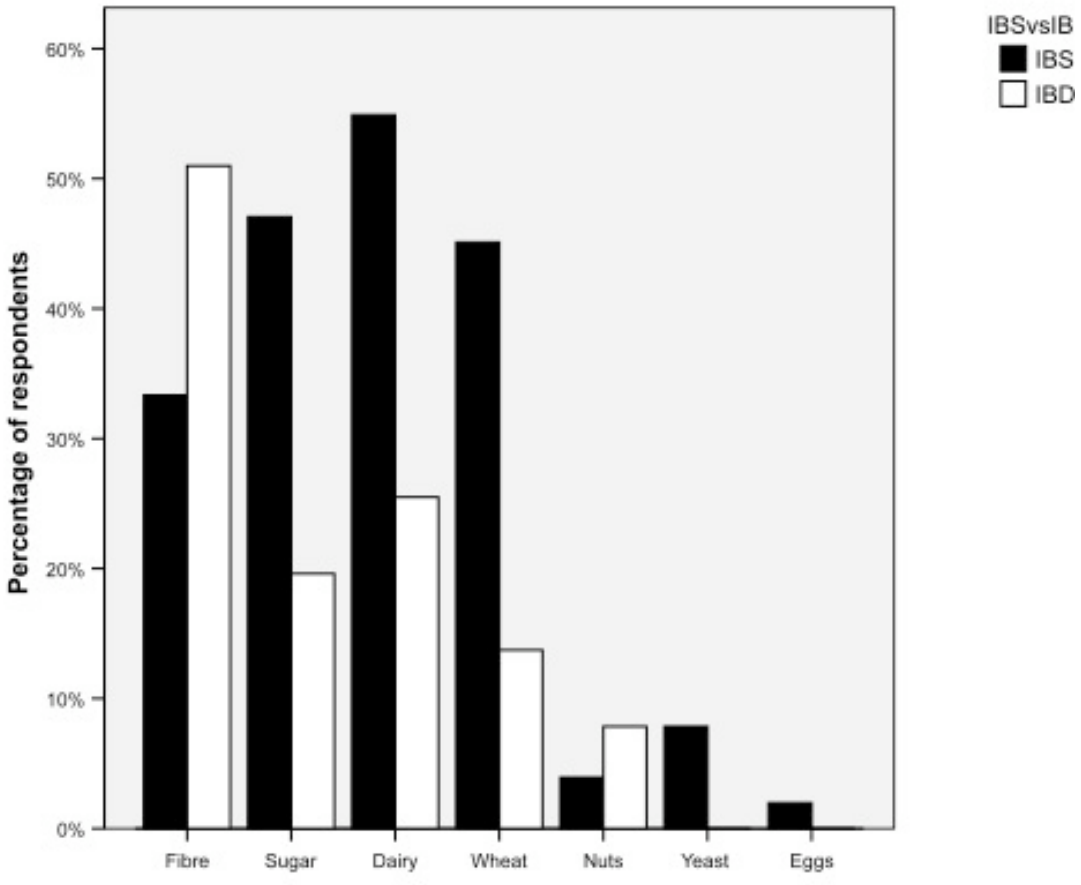


Figure 20. Foods recommended to IBD and IBS patients for exclusion

A low utilisation of allergy testing was reported in both IBD and IBS. In both conditions the majority of respondents reported no or very little (0-25% of patients) use of allergy testing (82% in IBD and 89% in IBS) (Figure 21). Where allergy tests were used they were most commonly "open food exclusion and rechallenge" (14% of respondents in IBD and 29% in IBS) and RAST (4% in IBD and 14% in IBS). There was also a small proportion that reported using skin prick testing (4% in IBD and 6% in IBS) and skin patch testing (2%in IBD and 4% in IBS) (Figure 22). Respondents reported being most likely to give dietary advice to, or send for dietary advice, patients with small bowel Crohn disease (71% of respondents), difficult to control IBD (59%), diarrhoea predominant IBS (49%) and difficult to control IBS (69%).

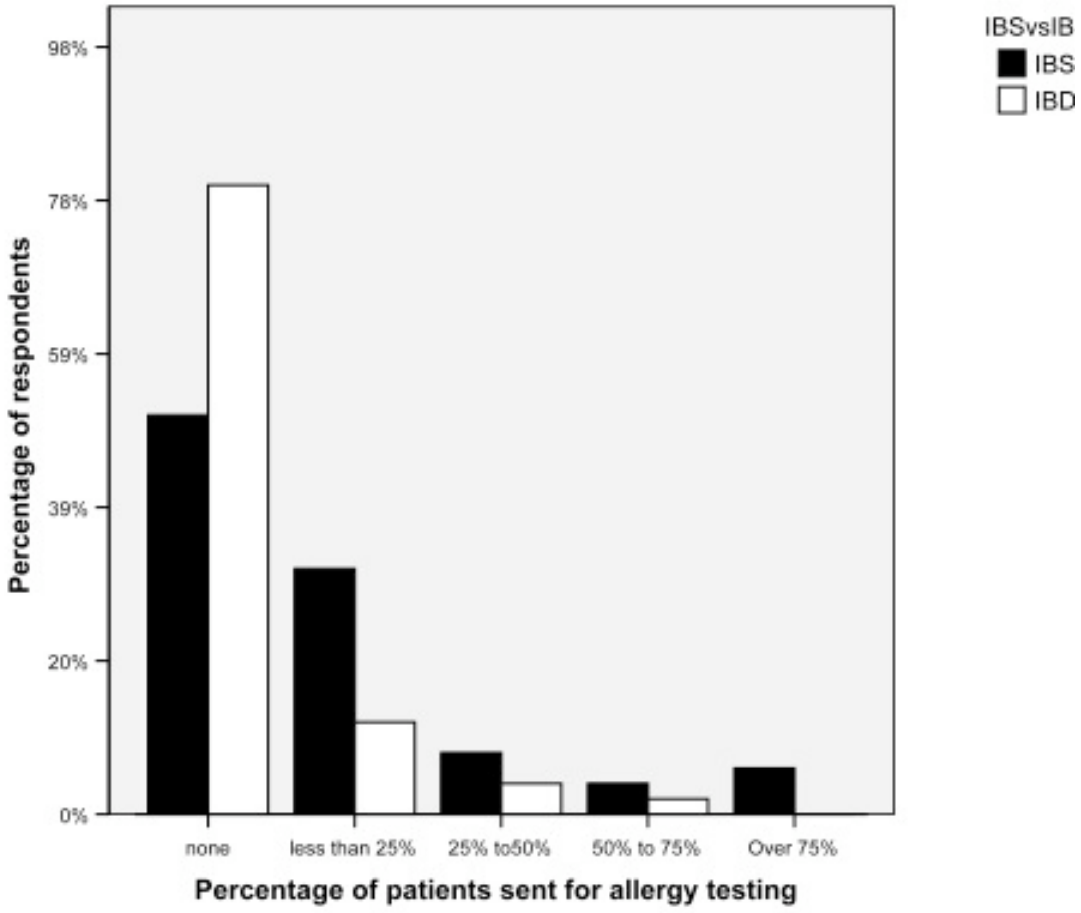
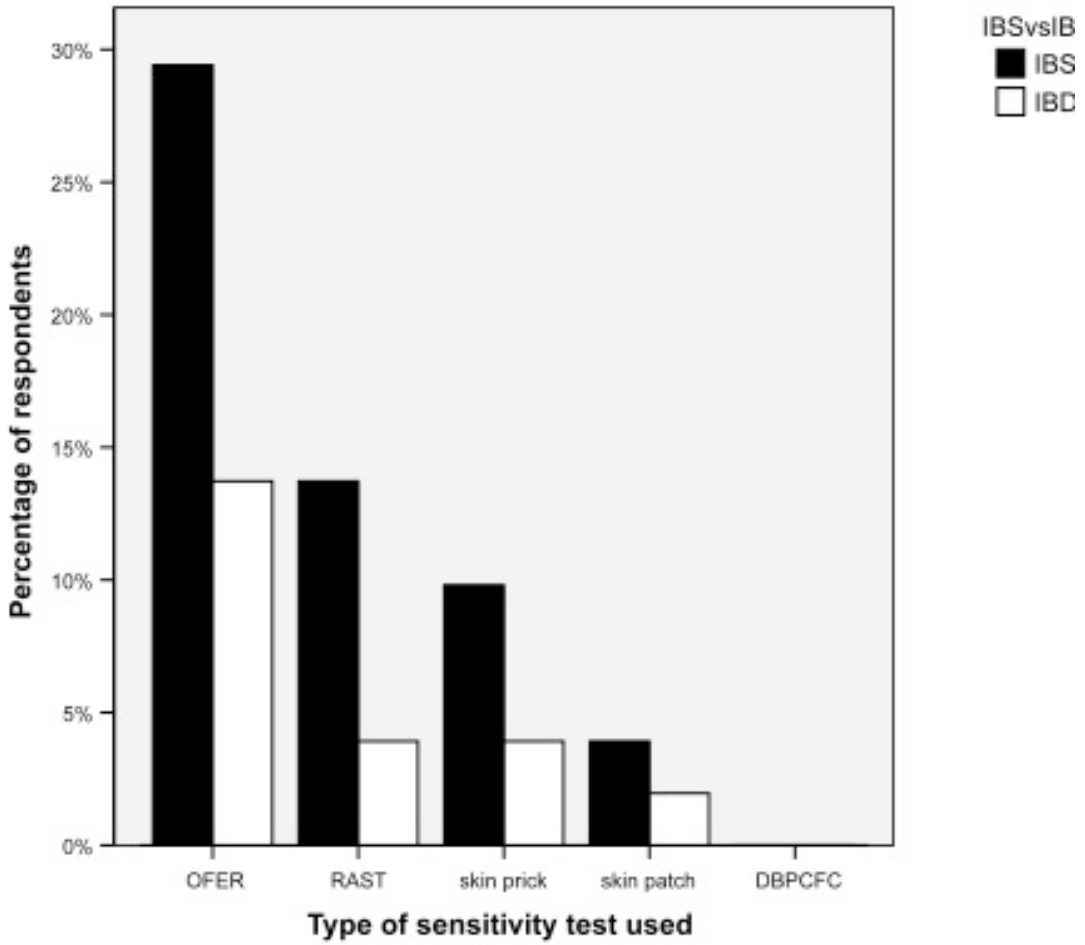


Figure 21. Percentage of IBD and IBS patients sent for allergy testing



(OFER: open food exclusion and rechallenge; RAST: radioallergosorbent test; DBPCFC: double-blind placebo-controlled food challenge)

Figure 22. Allergy tests recommended to IBS and IBD patients

When asked whether participants agreed that dietary exclusion was an effective strategy in IBD, responses were mixed with only a small proportion agreeing strongly (2%) and the rest either agreeing a little (41%), neither agreeing or disagreeing (16%), disagreeing a little (16%) or disagreeing a lot (26%). When asked the same question in IBS the majority of respondents reported either agreeing strongly or agreeing a little (84%) (Figure 23).

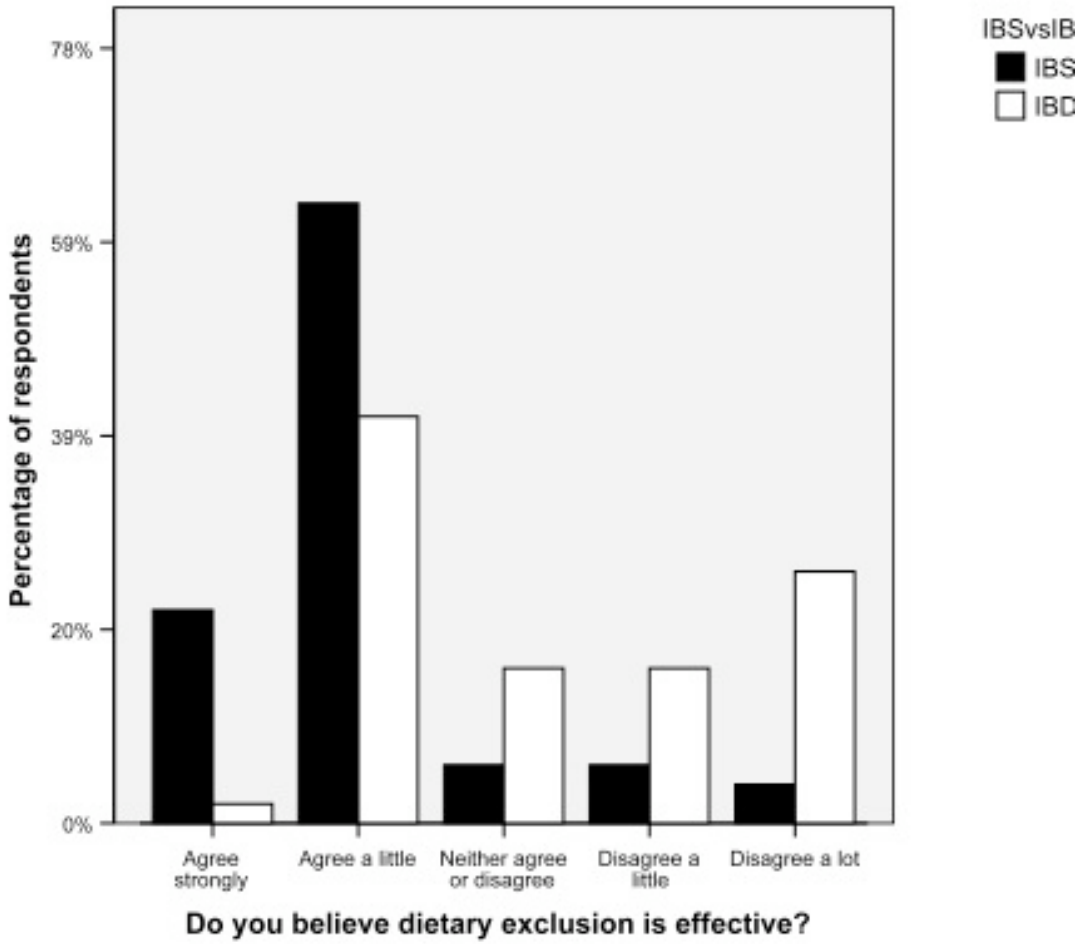


Figure 23. Response to the question "do you think dietary exclusion is an effective strategy" for IBD and IBS patients

III.7 Discussion

This is the first study to examine the attitudes of gastroenterologists to dietary manipulation in IBD and IBS. The use of the BSG membership list as a source for UK gastroenterologists is likely to have included the majority of UK gastroenterologists. However it will have also included a number of non-practicing and non-gastroenterologist members, who are unlikely to have replied, thus contributing to the high non-response rate. This was not the case for the NZ survey.

Although the response rate for the UK survey was low at 37% it is still a large survey of UK gastroenterologists, including 363 British gastroenterologists. This sample size is comparable to the sample size of previous such surveys including that of Eaden *et al.* in 2000 who reported a response rate of 83% of UK Gastroenterologists with a sample size of 341 (Eaden, Ward *et al.* 2000). We believe our sample is likely to be representative of gastroenterological practice in the UK because of the size of the sample and the fact that sensitivity analysis showed no significant difference in demographics or responses between early and late email responders, email and conventional mail responders, nor responders offered an incentive and those not. The internal validity of the questionnaire used was not directly tested during the pilot study. The questionnaire was, however, shown to be reliable using test-retest methods. The questionnaire's validity is supported by the comparability of responses between the two countries.

This is the first such survey of gastroenterologists conducted using email as the communication method. It could, therefore, be argued that this contributed to the poor response rate in the UK. However, the first round response rate to a conventional mail questionnaire sent to UK gastroenterologists without email addresses listed gave an identical response rate, suggesting this was not the case.

Access to dietetic support was almost universal. However, utilisation was low in both groups. This is in keeping with the recent UK national audit of IBD services (UK IBD Audit Committee 2008). In that study 204 of 207 sites reported access to GI dietetic support with a median number of dietetic hours per week of eight (range 0-24). In our study the median reported hours of GI dietetics service in the UK was six hours (range 0 to 100) and in NZ four hours (range 0 to 20).

This survey clearly demonstrates that practice regarding dietary manipulation in the UK and NZ differs between IBD and IBS, and that practice in NZ and the UK is very similar. Patients with IBS are more likely to be given dietary advice by the gastroenterologist and are more likely to be given advice regarding dietary exclusion than IBD patients. Both groups of patients are equally likely to be sent for dietetic consultation and receive allergy testing, although the rates of utilisation of both are low. Where dietary exclusion was recommended, in both conditions, the commonest exclusions recommended were fibre, dairy and wheat, as well as sugar in NZ but not the UK.

Where allergy testing was used this was most commonly "open exclusion and rechallenge" and RAST. It must be noted that both in the UK and NZ such testing services are not routinely available in the public healthcare setting. In addition the NICE guidelines on the management of IBS in primary care state, "There are no objective tests available to identify food intolerance and few to confirm food allergy" (NICE 2008). Those patients most likely to receive dietary advice are those with small bowel Crohn disease, difficult to control IBD, diarrhoea predominant IBS and difficult to control IBS.

Finally, overall most respondents agreed strongly or a little that dietary exclusion was effective in IBS. When asked the same question for IBD the level of agreement was much lower and very few respondents agreed strongly.

This data suggests that there is a role for dietary manipulation and exclusion in the modern care of IBD and IBS, particularly IBS, but that the advice given is largely empiric and mostly comprises the exclusion of fibre, dairy and wheat. Particularly in

IBS the level of confidence in this approach is high. These results would suggest that further research in this area is likely to be supported and utilised by the gastroenterological community.

Chapter IV Food specific IgG antibodies and patient perceived food intolerance in inflammatory bowel disease

IV.1 Introduction

As the previous Chapter demonstrated, gastroenterologists are generally uncertain, and frequently contradictory, in their approach to dietary intervention in IBD. By contrast, several lines of work have shown that patients frequently have a strong belief in the role of dietary factors in their disease (Ballegaard, Bjergstrom *et al.* 1997). When patients with IBD were surveyed regarding the frequency and pattern of food intolerance there existed no significant difference in findings between UC and CD (Ballegaard, Bjergstrom *et al.* 1997). Food intolerance was reported at a significantly higher rate in patients with both UC and CD compared with normal controls. Very little information exists comparing intolerance to individual foods in patients with IBD compared to controls.

While food intolerance appears to play a role in the symptomatology of IBD, testing for specific allergic or other mechanisms of intolerance has proven very difficult. As discussed previously classical allergy testing is not reliable for the detection of GI food hypersensitivity (see section I.3.2.4). One reason for this may be that hypersensitivity in the gut is mediated by local, rather than systemic, processes (Shanahan 1993; Bruijnzeel-Koomen, Ortolani *et al.* 1995; Crowe 2001). For example IgE levels in stool and intestinal juices do not correlate with IgE levels in blood (Belut, Moneret-Vautrin *et al.* 1980; Kolmannskog and Haneberg 1985; Andre, Andre *et al.* 1995). The other possible explanation for this discrepancy is that an IgE-independent mechanism, such as IgG mediated reactions, immune complex disease or lymphocyte-triggered reactions are involved (Bischoff, Mayer *et al.* 2000).

IgG antibodies to a limited number of foods have been shown to be more prevalent in IBD than in controls and there is evidence to support a role for exclusion diets guided by IgG levels in other GI disorders. However, IgG antibodies to foods are also found in people without GI disease and authors have dismissed IgG antibodies to foods as being a non-specific phenomenon, perhaps related to changes in intestinal permeability (see section I.3.2.8).

The primary aim of this study was to describe the self reported sensitivity to specific foods in IBD patients compared to controls. The secondary aim was to investigate the pattern of positivity of food specific IgG antibodies in IBD compared to controls, and to describe the association between food specific IgG antibodies and patient perceived food intolerance.

IV.2 Collaborators

The author completed the study design and analysis and performed the patient recruitment and sample collection. Dr Anton Emmanuel and Stuart Bloom, of University College London, assisted with trial design and analysis. Yorktest Laboratories Ltd completed the food specific IgG antibody testing.

IV.3 Ethical approval

This study was carried out in accordance with the declaration of Helsinki. It was approved by the University College London (UCL) / UCL Hospitals Joint Research Ethics Committee and written informed consent was obtained from all participants.

IV.4 Subjects

Patients diagnosed with ulcerative colitis or Crohn disease and controls aged 18 years and over were eligible to enter the study. As a significant proportion of patients with IBD receive some form of immunosuppressive therapy at some point in the course of their illness, patients on stable doses of immunosuppressant medication were not excluded from this study. Patients with other autoimmune disease or documented immune deficiency were excluded. Control subjects with diagnosed GI disease or symptoms suggesting significant GI disease were also excluded. The cases for this case-control study were identified from the UCLH IBD clinic register. All had confirmed diagnoses of UC or CD based on clinical, endoscopic and histological observations.

In order to avoid bias generated by advertising for control subjects, or selecting them from hospital clinic populations that might exhibit undiagnosed gastrointestinal or other immune conditions, the control group chosen consisted of patients presenting for assessment of thyroid lumps. All patients were biochemically euthyroid. They were approached for participation in this study when they attend for assessment in the UCLH thyroid nodule clinic.

Sequential patients with quiescent UC or CD were enrolled from the UCLH IBD clinic. Quiesence was determined by physician assessment of current symptomatology. Control patients were enrolled from the UCLH thyroid nodule clinic and had normal thyroid biochemistry and no history of GI disease.

IV.5 Methods

IV.5.1 Blinding

From the point of initial clinic assessment patient samples and questionnaires were identified only by linked anonymised labelling, in order to provide blinding and protect subject data. Laboratory staff performing antibody testing were unaware of subject details.

IV.5.2 Self-reported food intolerance

No validated questionnaire for the determination of food intolerances in IBD exists. Locke et al (Locke, Zinsmeister *et al.* 2000) asked IBS patients about food sensitivity and allergy as a part of a validated questionnaire for the identification of risk factors for IBS. They first asked the subjects if they were allergic or sensitive to any foods. If yes, the subject was asked to indicate the types of foods to which they thought they were allergic or sensitive, and whether they had a rash or swelling of the lips and throat. The reliability of the questionnaire as a whole was reported but not that for the food sensitivity questions particularly.

Eggesbo et al (Eggesbo, Halvorsen *et al.* 1999) administered a validated questionnaire to parents asking whether they perceived their children to have intolerances to foods including vomiting, abdominal pain or diarrhoea as well as extra gastrointestinal symptoms.

In the absence of other validated tools we based our questionnaire on these techniques (see Appendix 3. Questionnaire regarding food sensitivities). For each of the 92 foods to be tested for IgG, each subject was asked to report gastro-intestinal (GI) intolerance in the form of bloating, abdominal pain, diarrhoea, constipation, heartburn or other

symptoms using a tick-box questionnaire. They were considered to be positive for perceived GI intolerance to that food if they reported any one of these symptoms.

IV.5.3 Blood testing for food-specific IgG

Blood was taken and sent, with only a numerical identifier, to YorkTest Laboratories Ltd (York, UK) where an ELISA test was performed to detect the presence of IgG antibodies specific to a panel of 92 different food extracts. This test has been described previously elsewhere using a smaller panel of 29 food antigens (Foster, Knowles *et al.* 2003; Atkinson, Sheldon *et al.* 2004).

Plates were coated with food antigens (Antigen Laboratories, Inc., Missouri, USA) diluted in carbonate/bicarbonate buffer. Plates were incubated at 4 ºC overnight with 100 µl of extract per well. Plates were subsequently blocked with 400 µl per well of 0.1% phosphate buffered saline/0.5% sucrose/1% fish gelatin, at room temperature for 1 hour. The blocking buffer was then decanted and the plates were left at 37 ºC overnight.

Serum samples were diluted in PBST/3% polyvinyl pyrolidone 10 kD to 1/50, 1/150, and 1/450 with each dilution applied to an allergen panel. After washing, anti-human IgG horse radish peroxidase conjugated was applied to each well for 10 min. 3,3',5,5'-Tetramethylbenzidine substrate was applied to each well for 10 min. The reaction was stopped using 50 µl per well of 0.5 M sulphuric acid and the plates were read using a Dynex ELISA plate reader at 450 nm. The mean absorbance of each test specimen was compared to the absorbance of a positive control serum using 0 arbitrary unit (AU) and 25 AU standards prepared from a serum with a high IgG titre to a cow's milk allergen extract. A 50 AU positive control was used to confirm that the slope formed by the 0 and 25AU standards was sufficiently precise and accurate. The test results were obtained from the 1/150 dilution of the specimen. Where a high specimen background

was observed, the test results were obtained from the 1/450 dilution. The threshold for a positive (reactive) result was arbitrarily set as 10 AU. Test results were scored as positive or negative only, relative to this cut off.

IV.5.4 Statistical considerations

Insufficient information exists on the prevalence of IgG antibodies to foods in differing populations to allow calculations of sample size required to yield adequate statistical power. A minimum sample size of 75 patients: 25 with UC, 25 CD, 25 controls was chosen. Analysis was performed on the SPSS statistical package. Results are reported using proportion of patients positive for the primary and secondary outcome measures. Comparisons between groups were made using Fisher's exact (2-sided) tests. Correlations were analysed using Spearman's rank correlation test.

IV.6 Results

Over a six month period 77 patients were enrolled (25 with UC, 28 with CD and 24 with thyroid nodules). Of the patients with CD 10 had disease isolated to the colon, 2 had disease isolated to the small bowel, 15 had ileocolonic disease and 1 had perianal disease. Of the patients with UC 12 had distal disease (up to and including the splenic flexure), 11 had pan-colonic disease and in 2 patients the exact extent was unknown. Because of recruitment problems with control patients with thyroid nodules one less patient than planned could be recruited in the trial period. Cases were not matched with controls but the distribution of age and sex between the three groups were well matched: the proportion of females in the UC, CD and control groups were 52%, 54% and 58% respectively (p=NS chi-squared). The mean age (and range) in these three groups were also well matched, respectively 36 (19-59), 35 (22-56) and 36 (20-61).

All patients were assessed by the referring physician to have quiescent disease. Fifty-one out of 53 patients with inflammatory bowel disease had contemporary serum C-reactive protein measurements available, all of which were normal (less than 10mg/dL). Serology for coeliac disease using anti-gliadin and anti-endomysial antibodies was available in all patients, no patient demonstrated positive antibodies. A total of 4 (14%) patients with CD and 16 (64%) patients with UC were receiving therapy with 5-ASAs, and 3 (11%) with CD and 3 (12%) with UC were receiving either azathioprine or 6-mercaptopurine. None were taking corticosteroids.

All control patients had been assessed with thyroid ultrasound, and where appropriate fine needle aspiration, no patient had abnormal thyroid function tests and no patient was found to have thyroid carcinoma.

IV.6.1 Patient reported food sensitivity

CD and UC patients reported gastro-intestinal sensitivity to a greater number of foods than controls (median (range)): 3(0,33), 4(0,29), 0(0,20) respectively, $p=0.05$ CD vs control, $p<0.01$ UC vs control) (Figure 24).

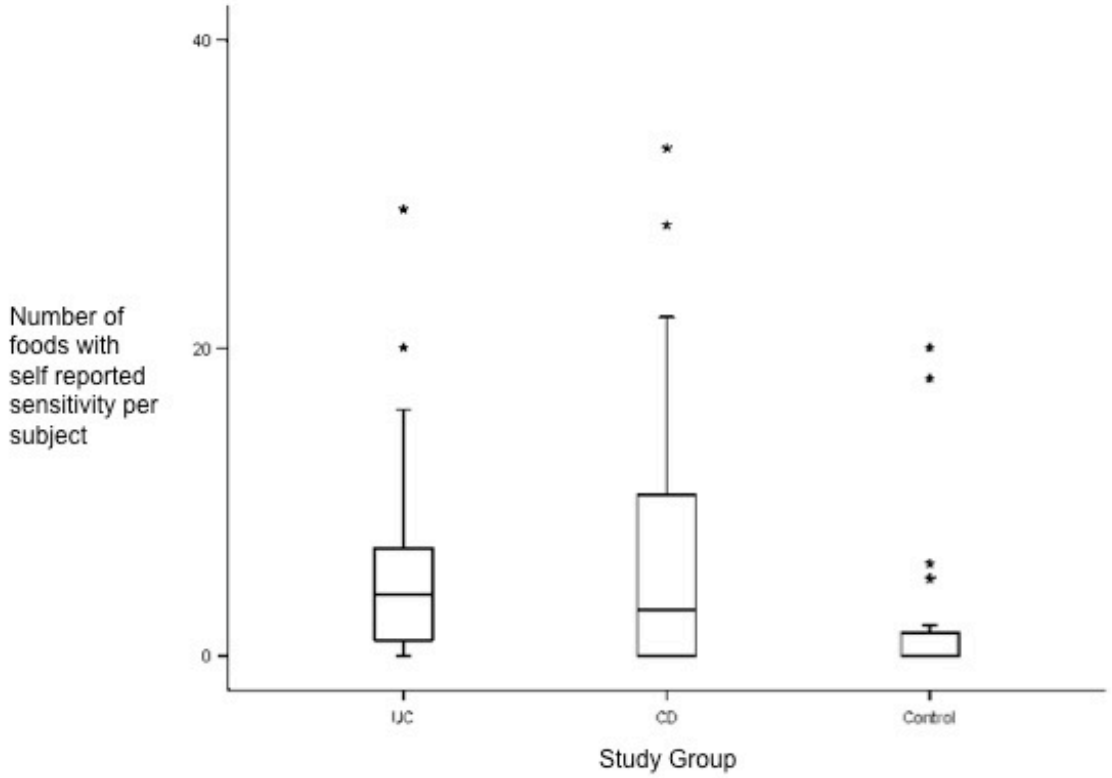


Figure 24. Box and whisker plots of the number of foods with self-reported sensitivity in patients with UC, CD and control, showing that inflammatory bowel disease patients reported significantly more foods they were "sensitive" to (* depicts outliers)

In CD subjects the most commonly reported sensitivities were to peanut (29% of CD subjects vs 13% of controls), cashew (25% vs 13%), lentils and broccoli (19% vs 4%), hazelnut and brazil nuts (19% vs 13%), and chilli (19% vs 8%). UC subjects also commonly reported sensitivity to chilli (44% vs 8%), but otherwise reported sensitivities to different foods than CD patients including wheat (40% vs 8%), milk (36% vs 8%), kidney and haricot beans (both 24% vs 0%), coffee and onions (20% vs 4%) and oranges (20% vs 0%) (Figure 25).

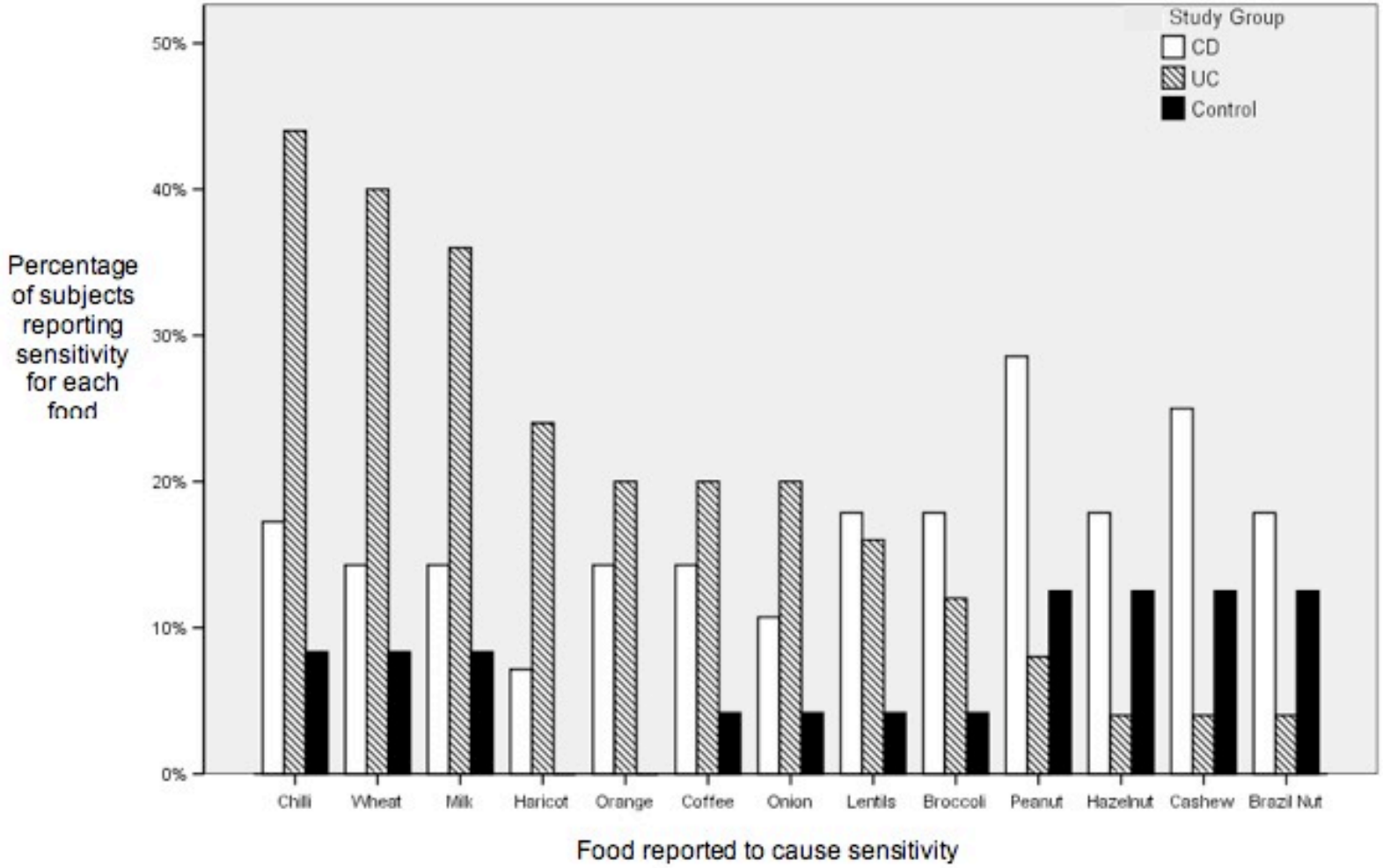


Figure 25. Histogram showing the wide range of specific foodstuffs that UC, CD and control patients reported subjective sensitivity to

IV.6.2 IgG food antibody positivity

The greatest difference in frequency of IgG positivity in CD patients vs. controls were seen for yeast (CD 82.1% positive vs. controls 54.2%, p=0.04), wheat 42.9% vs 12.5%, p=0.03), chilli (37.9% vs 8.3%, p=0.02), kiwi (35.7% vs 8.3%, p=0.02), corn (28.6% vs 0%, p=0.005), millet (28.6% vs 0%. p=0.005) and peanut (28.6% vs 0%, p=0.005) There was no correlation between patient reported food sensitivity and IgG antibody positivity to foods for CD subjects.

For UC vs control the greatest difference in frequency of IgG positivity was seen for corn (24% vs 0%, p=0.02), millet (20% vs 0%, p=0.05) and oat (20% vs 0%, p=0.05). A very modest correlation between patient reported food sensitivity and IgG positivity was seen for UC and control subjects (Spearman's rank correlation coefficients 0.23 and 0.26 respectively, $p<0.05$). No foods showed a significantly greater frequency of antibody positivity in controls compared to either IBD group (Figure 26).

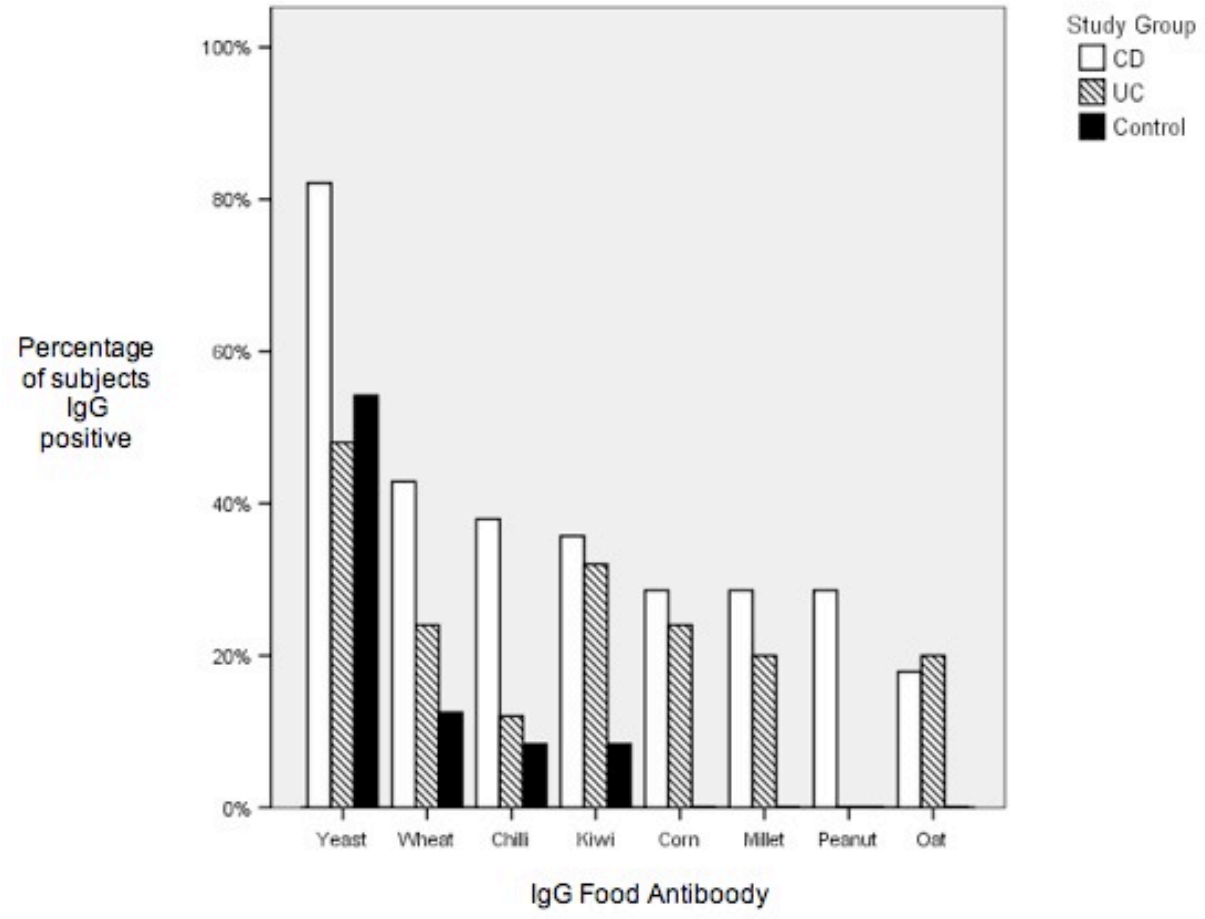


Figure 26. Histogram showing the range of specific foodstuffs that UC, CD and control patients demonstrated IgG positivity to

IV.7 Discussion

The rates of subjective food sensitivity in our study sample were high in both CD and UC compared to controls. This is similar to previous reports (Ballegaard, Bjergstrom *et al.* 1997).

Demonstration of a pathogenic association between such food intolerances and conventional markers of allergy has not proven possible. Skin tests and IgE measurements are known to be of limited value for the diagnosis of intestinal food allergy in general (Crowe and Perdue 1992; Shanahan 1993; Bruijnzeel-Koomen, Ortolani *et al.* 1995). Data regarding the association of food specific IgE antibodies and conventional allergy tests with IBD has been contradictory. In studies looking at the

presence of IgE antibodies to yeast, corn, celeriac, wheat, egg, milk and soy in CD sera, no or very low levels of food-specific IgE were detected (Frieri, Claus *et al.* 1990; Huber, Genser *et al.* 1998). A study of antibody response to the cow's milk antigen beta-lactoglobulin in ulcerative colitis and Crohn disease compared to non-atopic controls demonstrated no specific IgE response in any group (Paganelli, Pallone *et al.* 1985). However, in another study an increased number of basophils degranulated in the presence of cows' milk in UC patients, but normal responses occurred in patients with CD (Smart, Danis *et al.* 1986). Conversely a further study showed a considerable increase in positive results to RAST for dietary antigens in IBD (Brignola, Miniero *et al.* 1986).

The major immunoglobulin present in human secretions is a dimeric IgA; pentameric IgM is also actively enriched in most exocrine fluids (Brandtzaeg, Bjerke *et al.* 1987). It would seem logical that, in the absence of a convincing association with conventional markers of allergy, research should focus on these immunoglobulin isotypes. However, data in this area is sparse and inconclusive. One study using 26 monozygotic twin pairs with inflammatory bowel disease and 52 healthy controls to examine the presence of IgA, IgG and IgM antibodies against ovalbumin, betalactoglobulin, gliadin, whole yeast (Saccharomyces cerevisiae) and yeast cell wall mannan showed that individuals with ulcerative colitis were indistinguishable from healthy twins and controls, except for higher IgA levels to gliadin. Twins with Crohn disease displayed higher antibody titres towards yeast cell wall mannan and whole yeast (Saccharomyces cerevisiae) of all antibody types (IgA, IgG, and IgM). In all IBD patients the responses to gliadin, ovalbumin, and betalactoglobulin were even lower than in the controls (Lindberg, Magnusson *et al.* 1992).

While elevation of cow's milk specific IgG antibodies in IBD has been recognised in one study (Knoflach, Park *et al.* 1987) other studies have not confirmed this finding (Jewell and Truelove 1972; Hoier-Madsen, Holm *et al.* 1989). Elevated levels of IgG antibodies to foods in other conditions have been widely considered a phenomenon of no pathological significance and IgG antibodies to various food components are detectable in healthy individuals although usually at rather low levels. Thus the role of this class of antibody in the induction of symptoms remains highly controversial (Barnes 1995; Zar, Kumar *et al.* 2001; Teuber and Porch-Curren 2003). However, interest in this Ig isotype has been generated by the finding of apparent clinical utility in randomised controlled studies of the use of IgG food antibodies in determining an exclusion diet for IBS (Atkinson, Sheldon *et al.* 2004), and more recently IBD (Bentz, Hausmann *et al.* 2010). We chose to examine IgG food specific antibodies in IBD because of the support for an association with GI sensitivity in IBS by the above study and because of the lack of data regarding it's prevalence, except for cow's milk antibodies.

There is no accepted methodology for testing IgG antibody to food antigens. The ELISA test used by York Laboratories is commercially available and widely used, but is limited by the lack of calibration over the whole response range of the test, thus allowing its use only in a qualitative, rather than quantitative manner, requiring the selection of an arbitrary cutoff value for positivity. The chosen cutoff of 10 attempts to make the test as sensitive as possible. Sensitivity analysis using a higher cutoff of 20 (which still lies within 0 to 25 AU calibration curve) gives very similar results, in terms of the foods that are positive and the relative differences between IBD and control patients, but with lower proportions of subjects positive. The comparison of the presence of all food specific IgG antibodies to a single reference standard composed of

cow's milk raises the concern that antibodies to other foods might have different response characteristics, making the use of a single cutoff for all foods invalid.

Our study is the first report of the prevalence of IgG antibodies to a wide range of food antigens in IBD. It shows that patients with IBD do exhibit greater levels of positivity for certain IgG food specific antibodies than controls. The weakness of the correlation between food antibodies and patient reported sensitivity might be a result of the small number of patients in this study. In addition it is recognised that the varying temporal and dose-response relationship between foods and GI symptoms means that symptom reporting is a poor method for identifying food intolerance, as evidenced by the lack of correlation between reported sensitivities and double blind placebo controlled food challenge (Pearson, Teahon *et al.* 1993).

Support for IgG antibody testing as an indicator of GI sensitivity in IBD would require both the demonstration of a correlation between disease and IgG levels as well as the demonstration of a biologically plausible mechanism for that association. As symptom reporting in itself lacks reliability, it might be that correlation of IgG antibody production with other tests of GI hypersensitivity could strengthen the association between IgG antibody production and hypersensitivity.

As mentioned above it remains possible that IgG antibodies are physiological phenomenon not associated with disease. In considering why they might therefore be elevated in IBD, if not because of a direct association between the aetiopathogenesis of the disease and IgG antibody formation, an obvious conjecture is that changes in epithelial permeability, and thus exposure of the GI immune system to the luminal environment, might be the common mechanism.

For these reasons I decided, in the proceeding study, to investigate whether IgG antibodies can predict the foods associated with hypersensitivity using another measure of GI sensitivity, the recently described technique of colonic antigen provocation (Bischoff, Herrmann *et al.* 1996; Van Den Bogaerde, Cahill *et al.* 2002). This approach also allowed validation of the observation of elevated IgG antibodies to foods in IBD and allowed the theory that IgG antibody positivity relates to intestinal permeability to be tested.

Chapter V Comparison of gut mucosal response to food antigen injection with serum IgG food antibodies in Crohn disease

V.1 Introduction

The association between symptoms of IBD and diet has received much attention (see Chapter II). Some studies have claimed that food sensitivities are common in CD and have found that when food intolerances are detected, patients on an exclusion diet maintain remission significantly longer than those on an unrestricted diet (see section II.1.4.8).

However, the tools available for measuring and predicting GI food intolerances remain suboptimal. Traditional allergy testing has concentrated on the measurement of IgE mediated responses, but this approach has not proven useful in GI food sensitivity (Shanahan 1993; Bruijnzeel-Koomen, Ortolani *et al.* 1995; Crowe 2001). IgG antibodies to food components are detectable in healthy individuals, usually at rather low levels (Barnes 1995; Zar, Kumar *et al.* 2001; Teuber and Porch-Curren 2003). However, our own observation has been that these levels are significantly increased in UC and CD (see Chapter IV).

It has been suggested that the double blind, placebo controlled oral challenge should be the gold standard in testing for food hypersensitivity. However, it is a difficult and protracted procedure and has not proven practical in the clinical setting (Bischoff, Mayer *et al.* 2000).

V.1.1 The development of gastrointestinal mucosal provocation tests

Mucosal provocation tests involve localised testing of the mucosa within the organ system of interest. This approach has been successful in the bronchial, nasal and conjunctival mucosa (Bischoff, Mayer *et al.* 1997). They involve local application of a test substance, in the case of food allergy a food antigen, and measurement of the local response either: visually; by the detection of substances secreted in response to the provocation; or by the examination of tissue samples taken from the exposed site.

Compared to oral challenges, mucosal provocation tests have theoretical advantages. Because the amount of allergen is small, the risk of anaphylactic reaction present with oral food challenge is lessened (Sampson 1988). In addition the results can be interpreted immediately and directly, reducing the subjectivity inherent in interpreting the patient's symptoms (Bischoff, Herrmann *et al.* 1996).

Initial attempts to apply these techniques to the GI tract were made with the mucosal application of allergen intragastrically (Reimann and Lewin 1988; Bagnato, Di Cesare *et al.* 1995). In their 1988 study Reimann *et al.* included 30 patients whose food-allergic history had been proven through double-blind challenge tests; 20 healthy volunteers were also included as controls. They applied allergens endoscopically to the gastric mucosa. Two blinded, independent physicians observed the macroscopic reaction elicited. Biopsies were taken from the challenged areas for histological and histochemical analysis. The 30 patients with document food allergies exhibited swelling, erosions, and bleeding at the contact site (Reimann and Lewin 1988). A further study by Bagnato *et al.* elicited a gastric mucosal reaction to the plant Parietaria,

a common cause of pollen allergy, in those known to exhibit atopy to the plant (Bagnato, Di Cesare *et al.* 1995).

Jejunal perfusion with food allergen and measurement of inflammatory mediator release using a double balloon jejunal tube has also been carried out (Bengtsson, Knutson *et al.* 1997). Bengtsson *et al.* examined five patients with milk-related gastrointestinal symptoms diagnosed by double-blind placebo-controlled milk challenges, but with negative responses to skin prick tests and RASTs with milk. They compared these patients with eight healthy control subjects. They performed repeated perfusion studies with a two-balloon, six-channel tube by using milk, casein, and whey as antigens. Cow's milk induced a pronounced increase in intestinal secretion of histamine and eosinophil cationic protein in patients but not control subjects, during the first 20 minutes after challenge. This suggested that mast cells and eosinophils are effector cells not only in patients with allergic disease but also in patients intolerant to foods and lacking circulating antibodies. This strategy has been tested only in dairy allergy to date and is limited by the fact that local reactions in the mucosa cannot be observed visually or histologically.

Further attempts have centred around allergen injection into the colonic mucosa. Bischoff *et al.* described the development of this technique in their 1997 paper (Bischoff, Mayer *et al.* 1997). They studied 70 adult patients with abdominal symptoms suspected to be related to food allergy, and five healthy volunteers. Using their technique the caecal mucosa was challenged endoscopically with three food antigen extracts, a buffer control, and a positive control (histamine). The mucosal weal and flare reaction was registered semiquantitatively 20 minutes after challenge, and tissue biopsy specimens were examined for mast cell and eosinophil activation.

The provocation test was positive to at least one food antigen in 54 of 70 patients (77%), whereas no reaction in response to antigen was found in healthy volunteers. Antigen induced weal and flare reactions were correlated with intestinal mast cell and eosinophil activation, as well as with patients' history of adverse reactions to food, but not with serum concentrations of total or specific IgE or skin test results.

The challenge technique involved the injection of antigen extracts into the mucosa using a long plastic tube attached to a fine needle, similar to that used for endoscopic sclerotherapy of oesophageal varices. The plastic tube was filled with the test solution before introducing it into the working channel of the endoscope, the tube was drawn out of the endoscope after each application, and depleted of the test solution by washing with 4 ml 0·9% sodium chloride and pressing 8 ml air through the tube before it was filled again with the next test solution.

The food allergen extracts used were lyophilised and did not contain additives such as glycerine or preservatives. The concentrations used for intestinal challenge were assessed by previous dose response experiments. A 1:10 dilution of the stock solutions containing 3 mg/ml protein was used in all experiments.

As performed in skin prick tests, the mucosal weal and flare reaction was classified semiquantitatively 20 minutes after challenge using a scale of 0 to 4: 0=no reaction, 1=questionable reaction, 2=moderate reaction (<1 cm diameter), 3=strong reaction (1–2 cm), and 4=maximal reaction (>2 cm).

The caecum was chosen because its peristaltic movements proved to be less pronounced than that of other segments of the large intestine. The same group previously reported

studies performed in rectosigmoid colon of five subjects, where they obtained, compared with the caecum, similar but weaker mucosal responses (Bischoff, Wedemeyer *et al.* 1996).

In the 1997 paper Bischoff *et al.* also reported unpublished data where provocation was performed during gastroduodenoscopy on six patients(Bischoff, Mayer *et al.* 1997). The mucosal reactions in the stomach were inconsistent, whereas in the duodenum the test could be performed with good results. However, gastroduodenoscopy over 20 minutes was not well tolerated by the patients. Furthermore, the provocation test was more troublesome to carry out in the duodenum for the endoscopist because its peristaltic movements interfered with the test procedure.

The technique developed by Bischoff *et al.* was named the colonoscopic allergen provocation (COLAP) test. Detailed data comparing the COLAP test to elimination diet and food rechallenge are not available. The above results suggested that it might be a useful diagnostic measure in patients with suspected intestinal food allergy and may provide a new tool for the study of underlying mechanisms.

V.1.2 Gastrointestinal provocation tests in IBD

The COLAP technique was applied in CD by Van Den Bogaerde et al (Van Den Bogaerde, Cahill *et al.* 2002). Their case control study compared 10 patients with CD to 10 controls using rectal mucosal exposure to six food antigens (cereal, cabbage, citrus, milk, yeast and peanut) and control saline. As well as taking biopsies from exposed areas they used laser Doppler blood flowmetry to assess mucosal response to antigen as well as measuring in-vitro peripheral blood lymphocyte proliferation in response to the same antigens.

The Crohn disease group demonstrated higher rectal blood flow than controls in response to all food antigens, and this was significantly different for the responses to yeast ($P = 0.036$) and citrus fruits ($P = 0.038$). Lymphocyte proliferation occurred in 32 of 60 tests in Crohn disease patients and eight of 60 tests in controls ($P < 0.0001$).

The authors concluded that the findings support the concept that CD patients demonstrate gut specific sensitisation to food antigens and support the use of laser Doppler flowmetry as a measure of mucosal reaction to antigen challenge. None of the patients in the study had reactions when the antigens were tested by subdermal injection.

Why patients with CD might demonstrate higher titres to food specific IgG antigens and show greater changes in rectal blood flow in response to food antigen injection has not been investigated. CD is known to be associated with increased intestinal permeability (see I.1.3.1). Additionally, increased intestinal permeability has been found in patients with adverse reactions to foods (Ventura, Polimeno *et al.* 2006). Whether this increase in intestinal permeability, another factor associated with inflammation in the GI tract, or an unrelated immune phenomenon, might be the common factor causing raised food antibodies and/or provocation reactivity in CD remains to be determined.

The aim of this study was to evaluate a technique of direct colonic mucosal provocation in the setting of CD and to correlate this with the presence of serum food-specific IgG antibodies, patient perceived food intolerances and intestinal permeability.

V.2 Collaborators

The author completed the study design and analysis and performed the majority of the patient recruitment and provocation testing. Dr Anton Emmanuel and Stuart Bloom, of

University College London, assisted with trial design and analysis. Dr Farooq Rahman assisted with patient recruitment. Drs Anton Emmanuel, Farooq Rahman and Nora Thoua, of University College London, assisted with antigen provocation testing. Ms Audrey Duffy, of Kings College London, completed intestinal permeability testing.

V.3 Ethical approval

This study was carried out in accordance with the declaration of Helsinki. It was approved by the UCL / UCL Hospitals Joint Research Ethics Committee and written informed consent was obtained from all participants.

V.4 Subjects

Between August 2007 and February 2008 12 patients were enrolled in the study.
All patients had Crohn disease as assessed radiologically, colonoscopically and histologically. There was no history of previous rectal disease and no macroscopic rectal inflammation at the time of the study. Thus all measurements were undertaken in macroscopically uninvolved mucosal sites. At the time of the study all patients had quiescent disease as determined by the referring physician. No patients were receiving steroids, immunomodulatory therapy or biological therapy. Their demographics and distribution of disease are summarized in Table 22.

Patient	Age (years)	Disease distribution	Treatment
1	27	colonic	Pentasa
2	41	colonic	Pentasa
3	54	colonic	Nil
4	32	colonic	Pentasa
5	42	ileal	Nil
6	45	ileocolonic	Pentasa
7	33	ileal	Pentasa
8	37	ileal	Pentasa
9	29	ileocolonic	Pentasa
10	30	colonic	Asacol
11	39	colonic	Pentasa
12	55	ileocolonic	Nil

Table 22. Patient demographics

V.5 Methods

V.5.1.1 Colonic antigen provocation testing

Following warm water enema participants underwent flexible endoscopic examination of the rectum. During this procedure mucosal injection of the 5 food antigens being considered, as well as a control mixture of glycerine and saline, was performed. Antigen solutions were used at a 1:10 weight per volume solution. The allergens were pre-solubilised in 0.5% saline and preserved in 50% glycerine.

The location of injection sites were marked with three small tattoos, using a carbon particle based dye (Spot, GI Supply, Camp Hill, Penn.), placed at the points of an equilateral triangle, the points being at least 6cm apart. Antigen and control injections were then made, two along each side, allowing later differentiation of individual injection sites.

The antigen extracts were deposited onto the mucosa using an endoscopic injection needle (Variject, Boston Scientific, Tokyo, Japan). The needle was then passed through the bleb of antigen solution into the mucosa to a depth of 1 to 2mm. The needle was

filled with the test solution by aspirating the solution into the needle before introducing the needle into the working channel of the endoscope. The tube was drawn out of the endoscope after each application, and depleted of the test solution by washing liberally with 0·9% sodium chloride before it was filled again with the next test solution.

The presence of mucosal hyperaemia was measured using laser Doppler flowmetry immediately following antigen injection and again at repeat flexible sigmoidoscopy 3.5 hours following injection.

V.5.1.2 Laser Doppler flowmetry

Laser Doppler flowmetry (LDF) is a technique that utilises the frequency shift in light reflected from a moving object to estimate blood flow within tissue. A low intensity beam, almost exclusively consisting of monochromatic coherent 780 nm light, is generated by a near infrared laser diode source and delivered by a fibre optic probe to the tissue of interest. In tissue, red blood cells account for most of the moving structures, and the speed of their movement determines the frequency of light which is reflected. The light reflected is detected by a photocell and the signal processed to determine the frequency shift. The volume flow measured with this technique (expressed as "flux units") approximates to ml of blood per minute per 100 g tissue. The approximate area of measurement is 1 mm^2 at up to 1 mm depth from the tip of the probe. Movement artefact is eliminated by the built in software which averages recorded values over 0.1 second time intervals.

Laser Doppler flowmetry has found clinical application in a variety of conditions such as skin grafting, Raynaud's phenomenon, and cerebral hypoperfusion. Validation of this technique in the colon was undertaken by Emmanuel and Kamm in 1999 (Emmanuel and Kamm 1999). This study, in 26 healthy volunteers, of the use of LDF in the rectal

mucosa established the reproducibility of this technique and determined the physiological variables which affect mucosal flow.

The study showed excellent coefficients of variation for subjects studied under identical conditions on two, three, and four days (0.06, 0.05, and 0.06, respectively).
While mean mucosal blood flow increased after a standard meal, fasted measurements at 0900, 1200, 1600, and 2200 were similar.

However, blood flow was significantly affected by smoking with flow decreasing for 15 minutes after smoking and returning to baseline at 30 minutes. The menstrual cycle also affected flow with follicular phase mucosal flow lower and more reproducible than luteal. Ipratropium, metoprolol and sacral nerve stimulation increased flow but inhaled salbutamol did not change blood flow.

In our study patients were not permitted to smoke the morning of or during the study, patients taking medicines known to affect the mucosal blood flow were excluded and women were studied in the follicular phase of the menstrual cycle. At each site of injection a measurement was made, taken over at least thirty seconds. An arithmetic mean of blood flux (i.e. flow in ml/sec/mm^3 of tissue scanned) expressed as flux units, was used in analysis.

V.5.1.3 Intestinal permeability testing

The research regarding the use of permeability testing in CD was detailed in section I.1.3.1. The two methods most employed in IBD have used either sugars, namely lactulose, mannitol or rhamnose, or a radioactive moiety, usually Cr51EDTA, as the permeability probe. The decision regarding which probe to use in this study was limited largely by the difficulties, both practical and regulatory, in handling Cr51, the facilities

for which were not available in our unit or with any of our collaborators. Conversely, sugar probe permeability testing using lactulose, mannitol and rhamnose was freely available and in frequent use at Kings College Hospital in London. For these reasons that test was used in this study.

Intestinal permeability was tested in each subject on the day of COLAP testing using a previously validated technique (Papadia, Sherwood *et al.* 2007). Forty-eight hours before the test subjects abstained from taking any agents known to affect small bowel permeability including nonsteroidal anti-inflammatory drugs, alcohol, and antibiotics. After an overnight fast subjects then emptied their bladders, and drank the sugar mix test solution, commencing a 5-h study period during which a urine collection was maintained. The solution comprised 5 g lactulose, 1 g L-rhamnose, and 500 mg D-xylose in 100 mL tap water. A light breakfast with a drink was allowed after 2 h. The urine collections were preserved by the addition of 1 mL of thymol (5% in propanol) to the collecting vessel, the total volume was recorded.

Multiple 5-µL urinary aliquots from the 5-h collection, plus a range of primary sugar standards, were applied to a thin layer chromatography (TLC) plate and allowed to dry. The TLC plates used were 20 x 20 cm plastic sheets coated with silica gel. A plate application chart was used to assign positions on the plates; separate plates were used for monosaccharides and for disaccharides. The TLC plates were run using the "inverted beaker" technique. The edge of the plate, where the samples were located, was dipped into a mobile phase. The solvent mixture for monosaccharide sugars consisted of ethyl acetate, pyridine, acetic acid, and water (75 mL, 15 mL, 10 mL, 10 mL). The main solvent system for disaccharides was composed of butan-1-ol, ethyl acetate, ethanol, acetic acid, and water (35 mL, 10 mL, 45 mL, 7 mL, 7 mL). The sugars were located by

the colorigenic chemical reaction (a 4-aminobenzoic acid/sugar complex) after heating to 130°C for 10 min. The method provides quantification of the sugars in the urine samples by direct densitometry measurements of the chemically located zones on the TLC plate. The absorption of reflected blue light from equal zone areas of primary standards was used to provide a standard curve from which the study unknowns could be read. The differential five hour urinary excretion ratio of lactulose and L-rhamnose (percentage ingested dose) provides an index of small bowel permeability.

V.5.1.4 Serum food-specific IgG antibody testing

The technique for collection and testing of serum for food-specific IgG antibodies was identical to that described in Chapter IV. Serum IgG antibody levels to a food were considered positive when they were above 10 AU.

V.5.1.5 Questionnaire of patient perceived food sensitivity

Participants were asked to complete the same questionnaire used in the study described in Chapter IV, determining the frequency and severity of GI symptoms and the association of perceived food intolerances with these symptoms (see Appendix 3. Questionnaire regarding food sensitivities).

V.5.1.6 Blinding and avoiding bias

The endoscopist performing mucosal antigen injection and laser Doppler flowmetry was blinded as to the antigen and control mixtures being injected. They were also blinded as to the patient's pattern of serum IgG antibody reactivity. Patients remained blinded as to their serum IgG antibody reactivity and antigen injection results.

V.5.1.7 Inclusion criteria

Patients with small bowel or colonic CD and no history of perianal and rectal CD were eligible for inclusion.

V.5.1.8 Exclusion criteria

Patients with active rectal inflammation at the time of endoscopy were excluded. Patients taking topical rectal 5-ASA preparations, oral or topical steroid preparations or biological agents were excluded, as it is not possible to predict the effect these agents will have on local reactivity. Smokers and patients taking Ipratropium or Beta blockers were excluded because of the effects of these factors on laser Doppler flowmetry. For safety reasons patients with a history of systemic allergic reactions and anaphylaxis were excluded.

V.5.1.9 Statistical analysis

The main outcome measure for this study was the mean change in mucosal hyperaemia (immediately following and 3.5 hours following food antigen injection) between patients who had serum IgG positivity for that antigen compared to patients who did not.

The secondary outcome measures were correlation of changes in mucosal hyperaemia, serum food specific IgG, patient reported food sensitivity, and intestinal permeability as measured by lactulose/rhamnose absorption.

The study sample size was based on the primary outcome measure, mean change in laser Doppler flow in response to antigen injection in patients with positive IgG for that antigen compared to the mean change in patients negative for IgG to that antigen. The mean reaction in CD from the previous study (Van Den Bogaerde, Cahill *et al.* 2002) (unpublished data) was 112.5 flux units (sd 24.1) and a clinically significant difference would be a 50% higher flux reading in IgG positive vs. negative patients. Using a sample size calculation based on a 2-sided T-test with Bonferroni correction for 5 comparisons this gives a minimum total sample size of 12 patients.

T-tests were used to compare the means of normally distributed variables. Correlations were examined using Pearson's correlation coefficient.

V.6 Results

12 patients were enrolled in the study. Food specific IgG antibody tests were available for 11 of 12 subjects. All patients received rectal mucosal injections with the 5 food antigens and control solution and had the mucosal blood flow measured by LDF immediately after injection and at a mean of 212 minutes following. The absolute change in the LDF measurement (in flux units) between time point zero, immediately following injection, and three and a half hours later is summarized in Table 23.

Patient	Control	Yeast	Wheat	Milk	Egg	Kiwi
1	-15	63	-7	60	50	-22
2	52	47	-12	54	66	-18
3	-2	12	19	47	23	139
4	-32	90	-25	72	71	-26
5	33	6	26	33	-2	49
6	2	-11	21	25	17	29
7	-9	91	52	38	-22	48
8	-12	14	61	108	-11	-4
9	-14	35	8	-9	19	16
10	-4	29	-5	41	4	7
11	-3	57	-12	19	46	6
12	-5	50	54	43	24	-13
Mean change in LDF	-0.8	40.3	15.0	44.3	23.8	17.6
p value**		0.001	0.099	0.003	0.018	0.211

(**statistical significance set at p<0.01, *mucosal blood flow expressed in flux units derived by laser Doppler flowmetry)

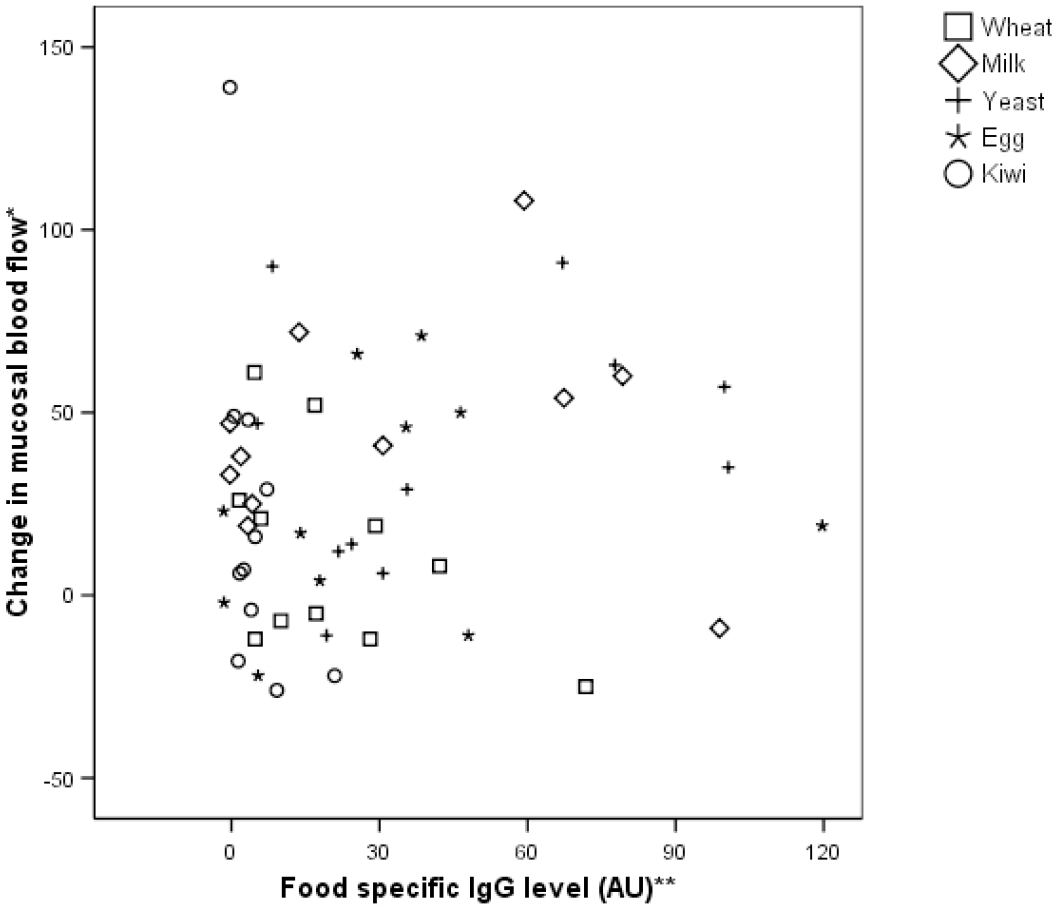
Table 23. Change in mucosal blood flow* at antigen injection sites between time zero and three and half hours after injection

There was no change in mean LDF for the control site injection in the study group. There was however an increase in LDF for each of the food antigen injection sites at 0 versus 3.5 hours with statistically significant increases for yeast and milk at a significance level of 0.01, using a Bonferonni correction to adjust for multiple comparisons across the five antigen injection sites.

V.6.1 Primary outcome measure

No difference was seen in mean change in LDF following antigen injection in patients with positive IgG for that antigen compared to the mean reaction in patients negative for IgG to that antigen (32 vs. 24.5 flux units, p=0.4).

This observation was also made when the absolute value of the IgG test as measured in AU was compared to the mucosal reactivity to antigen injection as measured by laser Doppler flowmetry (Figure 27).



(*mucosal blood flow measured in flux units; **AU=arbitrary units)

Figure 27. Scatterplot of change in mucosal blood flow vs. food specific IgG antibodies

V.6.2 Secondary outcome measures

No difference was seen in the mean change in LDF in patients who reported sensitivity for that food compared to the mean change in patients who did not report sensitivity to that food (28.9 vs. 27.6, p=0.9). Nor was there any association between the IgG result for each food and patient's reports of sensitivity to that food (Chi square p=1).

Comparison of intestinal permeability, according to lactulose:rhamnose excretion ratios, with IgG values and LDF values following antigen injection showed no correlation between permeability and IgG reactivity or mucosal reactivity to any food.

V.7 Discussion

This study confirms the practicality of performing antigen provocation testing in the rectum of patients with Crohn disease. It confirms a previous study showing that patients with Crohn disease exhibit greater mucosal hyperaemia in response to food antigen injection than in response to control injections (Van Den Bogaerde, Cahill *et al.* 2002). That study further demonstrated that this difference is greater in Crohn disease patients than in controls

Importantly, our study has failed to show an association of this phenomenon with the presence of IgG antibodies in the serum or patient's reports of sensitivity to the foods tested. In addition the mucosal response to antigen injection does not seem to be correlated with intestinal permeability as measured by lactulose:rhamnose permeability testing. This indicates that the mucosal reactivity to antigen injection does not appear to be a function of the activity of disease, as indicated by intestinal permeability. All subjects in this study were felt clinically to be in disease remission. In addition our study demonstrates that patient reported sensitivity to a food does not correlate with the presence of serum IgG antibodies to that food.

In determining the presence of subjective sensitivity to a food several methodological difficulties arise. No validated technique for the detection of food sensitivities in Crohn disease has been reported. Many authors recommend double-blind placebo controlled food challenge as the gold standard test for detecting food sensitivity. However, this test is cumbersome to administer and is unlikely to be widely applicable in the clinical setting. The use of food and symptom diaries has also been examined. However, this technique is also difficult to administer and has not been validated in this setting. Our technique of direct questioning about a range of sensitivity symptoms to a wide range of

foodstuffs may be subject to inaccuracy in the form of recall bias, over-reporting and, conversely, its ability to detect food sensitivities to particular foods commonly consumed in association with those foods the patient strongly feels to be associated with sensitivity. It does, however, provide a practical strategy for the detection of patient perceived food sensitivities and does allow direct comparison of patient perceived sensitivity with provocation or serological testing using specific food antigens.

The fact that patients with Crohn disease do react to food antigen injection in the rectum differently from control substance injection and differently from control patients cannot be ignored and the mechanism for this requires further investigation. Whether this phenomenon could be used to predict food sensitivities in Crohn disease patients, to which they are unaware, could best be tested by comparison with double blind placebo controlled food challenge.

Chapter VI Conclusions

VI.1 Dietary factors in the aetiology of IBD

The aetiology of IBD is a multifactorial process that combines a genetic predisposition and luminal environmental factors. The role of diet in this process was discussed in the introduction to this thesis. A number of specific dietary nutrients have been associated with the development of IBD in epidemiological studies. In IBD there is frequent compromise of the epithelial integrity of the gut, which leads to increased exposure of the GI immune system to luminal factors. Thus dietary products have the opportunity to provide antigenic exposure to the GI immune system.

The signalling mechanism for any such interaction does not appear to be via the classical type I allergy mechanism. However, the effector mechanisms for the type I response are altered in IBD, in particular mast cells are strongly implicated in the mediation of inflammation in IBD and therapy directed at the mast cell has proven effective in IBD. Additionally there is evidence for alteration in the effector mechanisms for other immunological processes particularly IgG mediated processes. Alterations of the levels of IgG antibodies directed against foods in IBD patients compared with controls has been demonstrated and there is evidence for the effectiveness of an exclusionary diet informed by the results of serum IgG antibodies to foods.

VI.2 Evidence for dietary manipulation in IBD

The available evidence regarding manipulation of dietary factors in IBD was discussed in chapter II. In UC and CD, disease is produced by an interaction between environment and genetically programmed predisposition. The exact mechanisms by which

environmental factors influence disease have yet to be fully elucidated but certainly the luminal content, as made up by microbiota and dietary products, contains the main part of this environmental stimulus.

In both UC and CD the patient perception that dietary components can affect the course of their disease is high. The association between the individual foods to which patients perceive intolerance and objective tests of hypersensitivity, including double blind placebo controlled food challenge, is poor however. In the studies that make up this thesis I was unable to demonstrate any association between patient perceived food intolerance and two emerging tests of food hypersensitivity, namely food specific IgG antibodies and the colonic antigen provocation test. That aside, patients with IBD do have increased IgG antibody positivity and increased epithelial reactivity to foods, and these changes are not explicable by changes in epithelial permeability, at least when measured at the time the hypersensitivity testing is undertaken.

The challenge faced is to resolve the patient perception that diet affects disease with the existing evidence and prioritise future work to areas with a probability of clinical success that might outweigh the deleterious effects of any dietary changes recommended.

VI.3 Survey of gastroenterologists' practice

Chapter III described a survey study conducted in New Zealand and the United Kingdom, which showed that dietary management differs greatly between IBS and IBD. IBS is a common intestinal condition to which the average gastroenterologist has frequent exposure. There is a strong environmental influence on the production and maintenance of disease in this condition. However, robust advice regarding how diet might best be manipulated to benefit disease is largely lacking. Thus, while the

pathophysiology of IBD and IBS are quite separate, some of the therapeutic challenges are shared. For this reason I was interested to test the differences in current practice between the two conditions.

Patients with IBS are more likely to be given dietary advice by a gastroenterologist and are more likely to be given advice about dietary exclusions than IBD patients. The two groups are equally likely to be sent for dietetic advice and receive allergy testing, although use of both is low. Where dietary exclusion was recommended both groups were given advice about fibre, dairy and wheat as well as sugar intake in New Zealand. Sensitivity testing, when used, was most commonly "open exclusion and rechallenge" and radio-immunosorbent assays.

Those patients with small bowel CD, difficult to control IBD, diarrhoea predominant IBS and difficult to control IBS were most likely to receive dietary advice. Respondents tended to agree that dietary manipulation was effective in IBS but were not so confident of its effect in IBD.

These results suggest that there is a current role for dietary manipulation and exclusion in IBD and IBS but that the advice given is generally empiric and that sensitivity testing is infrequently used in practice. The fact that practitioners do employ these techniques and have some confidence in their benefit, at least in IBS, encourages further research in this area.

VI.4 Food specific IgG antibodies in IBD

Chapter IV described an observational study of the occurrence of serum IgG antibodies to foods in IBD patients compared to controls. In this study CD and UC patients reported gastrointestinal intolerances to a greater number of foods than control subjects.

There was also a greater frequency of IgG food antibody positivity in UC and CD patients compared with controls. However, this was for a different range of foods in each disease and there was no correlation between patient reported intolerance and IgG food antibody positivity.

A study published following the completion of the investigations that make up this thesis generated similar results, albeit with positivity for a different range of foods (Bentz, Hausmann *et al.* 2010). Those authors went on to test the theory that a diet guided by the results of IgG food antibodies would be beneficial to gastrointestinal symptoms in patients with IBD. An improvement in stool frequency and general well-being was seen. However, methodological difficulties were observed including a high drop out rate, lack of control for concomitant medications and a sham diet that was potentially too similar to the specific diet.

Studies such as those above, and those that make up this thesis, challenge the dogma that IgG antibodies are a physiological phenomena that is not directly associated with the pathophysiology of disease. However, they fail to provide a robust biological mechanism for effect. Conversely, an alternative explanation for the presence of increased levels of IgG antibodies in IBD has not been forthcoming. In the first study that examined IgG food antibodies in IBD and other gastrointestinal disease Davidson *et al.* concluded, "The similar incidence of antibodies in the IBD and coeliac groups suggests absorption of dietary antigen secondary to an increased mucosal permeability" (Davidson, Lloyd *et al.* 1979). Other authors have since promoted this conjecture, but without any direct evidence to support it and, in fact, one study in the setting of IgG antibodies to yeast which demonstrated no association between IgG antibodies and changes in permeability (Harrer, Reinisch *et al.* 2003). The authors of that study

concluded, “Elevated serum levels of anti-S. cerevisiae antibodies do not seem to result primarily from a defect of the gut barrier”.

Thus, the evidence is that IBD is associated with increased serum IgG antibodies to a wide range of foods but that this does not correlate strongly with patient reported food intolerance. It is, however, well understood that patient perception of intolerance does not correlate well with more robust measures of intolerance such as double blind placebo controlled food challenge. The theory that the association between IBD and IgG food antibodies might merely reflect the intestinal permeability state had not been well tested. In addition the association between the colonic antigen provocation test, patient perceived food intolerance, and IgG food antibodies had not been examined. For these reasons I went on to compare IgG food antibodies, colonic provocation testing, intestinal permeability and patient perceived food intolerance in Chapter V of this thesis.

VI.5 Colonic provocation with food antigens in CD

The technique of direct mucosal antigen exposure has undergone much iteration. Many of the technical difficulties of the technique have been overcome by using the colonic mucosa for exposure. These techniques have been tested in CD and the responses found to be increased compared with controls. Chapter V described a study correlating colonic mucosal response to food antigen with patient reported food intolerances, food specific serum IgG antibody responses and intestinal permeability. Quantification of the mucosal response to antigen exposure was achieved using laser Doppler flowmetry.

A reaction to colonic mucosal exposure to food antigens was seen with all the foods tested, but not with control. Previous studies have shown that these responses are greater in CD than controls. The most significant reactions occurred with yeast, milk

and egg. However, this response did not correlate with IgG antibody reactivity, patient reported food intolerance or intestinal permeability.

Thus, colonic antigen provocation is practically applicable in CD. However, this reaction does not correlate with patients' perception of food intolerance. Nor does it appear to be an immediate product of alterations in intestinal permeability.

In conclusion, the studies that make up this thesis have been unable to demonstrate an objective relationship between patient perceived food intolerance and hypersensitivity testing in the form of serum IgG food antibodies or colonic antigen provocation. What has been demonstrated is that gastroenterologists do give dietary advice to at least some of their patients with IBD but they are not currently able to provide this information unequivocally based on the evidence available. The following future directions are therefore suggested.

VI.6 Future directions

That patients with IBD experience intolerances to foods is clear. Clinician response to this is currently guided by a very limited scientific literature. The result is largely generic advice and a lack of confidence in dietary techniques for the management of IBD. The research that makes up this thesis suggests that the response of the IBD patient to foods in terms of serum IgG antibody production and colonic mucosal reactivity is altered, but is unable to elucidate the mechanisms of these changes or correlate these changes with the clinical course of the patients' disease. Future studies of these techniques will need to demonstrate whether any association between the tests and the symptoms patients experience really exists and whether diets guided by tests such as these can be used with effect in the treatment of the symptoms of disease.

Finally they will need to clearly define the mechanisms by which such tests predict intolerance in patients.

VI.6.1 Studies comparing IgG food antibodies and COLAP to double blind placebo controlled food challenge

DBPCFC is considered the gold standard for the detection of food hypersensitivity. DBPCFC could be compared to a limited range of IgG food antibodies, and to colonic provocation, in an observational study. It would be important to ensure that the placebo preparation and the active food preparation were indistinguishable, which in practice can be very difficult. In addition the dose of active food preparation administered would need to be physiologically appropriate. With foods like wheat in a western diet this means a large volume of active preparation or, conversely, a large volume of placebo.

Outcome measures would also need to be carefully constructed. In the setting of Crohn disease the CDAI could be considered the outcome measure of choice in clinical trials. However, it would seem unlikely that the avoidance of a single food type would be likely to produce clinically significant changes in this index in the timeframes over which such comparisons could be run. Rather, the utilisation of a simple symptom diary may be more appropriate.

These considerations aside, a study design such as this would be the design most able to affirm or refute the hypothesis that these tests are able to detect food hypersensitivity in IBD patients.

VI.6.2 Studies comparing an IgG antibody or COLAP determined diet to a control diet

Strategies such as those described above might allow a diagnostic test for hypersensitivity to be validated or refuted but are unlikely to allow evaluation of the hypothesis that diets guided by such tests can have a clinically significant impact on disease status. One study in IBD and 2 studies in IBS have been able to show a clinical benefit of dietary manipulation guided by IgG food antibody testing when compared to a sham control diet. However, the complexity and diversity of the diets prescribed, and the fact that a few key constituent foods formed the main part of the "active" diets, has led to the concern that the IgG testing itself does little to inform the diets.

There would certainly be a role for the validation of these results in IBD. Design of the sham control presents some difficulties, however. From Chapter IV it seems that the rate of positivity to IgG food antibodies is much higher in CD than in controls. This makes it very likely that the rates of positivity are also higher in CD than IBS.

Criticisms of the past studies in IBS were that they put too many treatment group patients on a wheat, milk and yeast free diet compared to controls and that exclusion of those foods is commonly considered beneficial in IBS, hence IgG food antibody testing is not needed to design such a diet. This argument does not necessarily hold in CD where the range of positive responses to IgG food antibodies is greater. However, based on our data, it seems even more likely that patients on the true diet will be required to avoid specific foods (such as yeast, milk, wheat, kiwi, chilli and egg, all of which have greater than 30% positivity), more so even than in the previous IBS studies.

This raises an additional concern, in that it is likely that CD patients on the true diet will be instructed to avoid a very wide range of foods based on their IgG food antibody result. Hence design of sham diets that balance this significant dietary restriction will be particularly difficult.

In the face of this there are four options:

1. Exclude those six most frequently positive foods in all patients in both groups. This will mean a large number of treatment group patients are asked to exclude a diet to which they are IgG food antibody negative and vice versa, making any difference between the groups very difficult to detect.

2. Repeat the sham approach used in the previous IBS studies. The risk is that this will engender the same criticisms as the previous IBS papers and so be difficult to publish and unlikely to change clinical practice. However, as above, there is not the same level of concern that certain foods are important in generating symptoms for all patients with CD and the range of foods implicated is much wider. Nonetheless authors who criticised the IBS study are likely to have similar problems with milk and yeast in an IBD study.

3. Develop a sham approach that randomises control patients to an "IgG food antibody like diet" that is not their own IgG food antibody directed diet. This will mean a proportion of controls will be advised to avoid foods they would be predicted to be sensitive to by the YT diet. This will increase the apparent placebo effect, but inflation of the sample size would decrease the risk of no difference being seen (a type II error) and allow subgroup analysis of only those who do not have "crossover" in the diet. I have calculated this will affect about two thirds of subjects but will mostly apply only to

the foods yeast, wheat and milk and so in the worst case scenario would perform in a similar way to the study design of option 1.

4. Do away with a sham diet altogether. Some equivalent form of intervention would need to be instituted in order to control for the placebo effect of trial involvement and dietary advice. The most appealing would be to apply "standard dietary advice for IBD" as the control. Unfortunately no such consensus exists. The British Society of Gastroenterology's latest guidance on IBD has no such consensus on dietary intervention in IBD. However the best investigated dietary approach to CD could be considered a low fat, low fibre diet (Woolner, Parker *et al.* 1998). I believe that to place both groups on a low fat, low fibre diet, and additionally to give only the treatment group dietary advice based on the IgG food antibody result, is the most practical approach to design of the study that is most likely to demonstrate any difference in outcome. It has the additional benefit of allowing sample size calculation based on the effect size of past studies of such low fat, low fibre diets.

Essentially the same principles could be applied in the design of studies aiming to test the hypothesis that dietary modifications based on the results of colonic antigen provocation can be beneficial in IBD.

VI.6.3 Studies to elucidate the mechanisms by which IgG food antibodies and COLAP might detect food hypersensitivity

Studies investigating the link between antigen provocation or IgG food antibodies and food hypersensitivity in CD, if positive, will require the demonstration of a biological mechanism before widespread acceptance is likely. Therefore, any future study of the techniques applied in this thesis should attempt to define the mechanisms by which these tests might predict food hypersensitivity. The detection of IgG food antibodies in

subjects without disease has fuelled the concern that these antibodies might merely be physiological. The altered pattern and prevalence of these antibodies in disease states such as Crohn disease has been attributed to changes in the permeability of the gut in these conditions. However, in Chapter V we showed that alterations in these antibodies do not correlate with intestinal permeability at a single point in time. Longitudinal studies are necessary to correlate changes in IgG food antibody reactivity with changes in intestinal permeability and disease activity over time.

In vitro lymphocyte proliferation studies were utilised by Van den Boegarde *et al.* (Van Den Bogaerde, Cahill *et al.* 2002). In their study they tested 10 CD patients' blood lymphocytes with 6 different food antigens each. Proliferation was observed in a total of 32 of the 60 allergen/lymphocyte combinations. They compared this to colonic antigen provocation and in 15 of these 32, increased rectal reaction was observed in the same patient after exposure to the same antigen. This was compared to a total of 24 out of 60 positive rectal provocation responses. Thus, 15 of 24 (63%) rectal provocation responses were associated with a positive lymphocyte proliferation test. Similar techniques could be applied in a study of IgG food antibodies or revalidated in future studies of antigen provocation. If a correlation existed it might allow in vitro investigation to determine the mediators of the reaction including blocking of IgG mediated responses to investigate the hypothesis that IgG food antibodies directly mediate the immune reaction.

Histological studies should be undertaken in future studies of antigen provocation. Van den Boegarde *et al.* examined biopsy samples from antigen exposure sites using haemotoxylin and eosin staining and demonstrated submucosal oedema but no increase in lymphocyte numbers (Van Den Bogaerde, Cahill *et al.* 2002). Degranulation of mast

cells could not be accurately assessed using this technique. In the original study of the colonic antigen provocation technique in patients with food allergy, examination of biopsy specimens from the sites of antigen exposure showed no change in the number of eosinophils or mast cells present but did show evidence of mast cell degranulation and eosinophil activation in response to antigen provocation (Bischoff, Mayer *et al.* 1997). Application of these techniques in any future study of colonic antigen provocation in CD would determine whether the mucosal reactions seen in CD were mediated in a similar fashion to those in food allergy.

It is evident that patients with CD experience food intolerances. Those patients will continue to ask questions of the gastroenterological community regarding the mechanism and management of these intolerances. A likely consequence of not seeking answers to these questions is that our patients will take advice from other sources, with possible negative nutritional consequences. A strategy that allowed the detection of clinically significant food intolerances and the design of diets which were associated with improved disease outcomes without deleterious nutritional consequences would be of great benefit to our patients. This work reinforces the importance of food intolerance to patients with IBD and attempts to correlate those intolerances to available tests. Future studies should seek to clearly define the association between intolerance tests and patient symptoms, investigate the mechanisms by which such tests might predict intolerance, and investigate the most promising strategies in carefully designed and controlled studies of dietary intervention.

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Appendices

Appendix 1. Correlation coefficients for two rounds of survey pilot study

	Question	Correlation coefficient (*p* or *r*)
Q1.	How would you best describe your practice?	
	Gastroenterology time ____%	-0.06
	General medicine time ____%	0.73
Q2.	Are you able to refer to a dietician?	0.71
	Dietician time allocated to your service? _____hours	1.00
Q3.	Are you involved in a special IBD clinic	1.00
Q4.	Please indicate below how many new and follow-up patients with IBD would you see in outpatient clinics in an average month?	0.71
Q5.	What percentage of the patients with IBD that you see would you give specific dietary advice to?	NC
Q6.	What percentage of the patients with IBD that you see would you refer for dietetics advice?	1.00
Q7.	What percentage of the patients with IBD that you see would you ask to exclude specific foods from their diet?	0.53
Q8.	If you do ask patients to exclude foods please indicate the types of foods?	
	Fibre containing foods	0.71
	Refined sugars	NC
	Dairy products	NC
	Wheat	1.00
	Nuts	1.00
	Yeast	1.00
	Eggs	1.00
Q9.	What percentage of the patients with IBD that you see would you perform food allergy or intolerance testing on?	0.77
Q10.	If you do request intolerance testing which tests do you use?	
	Skin prick testing	1.00
	Skin patch testing	NC
	RAST	1.00
	Open food exclusion and rechallenge	1.00
	Double blind placebo controlled challenge	1.00
	Yorktest IgG test	1.00
Q11.	Please indicate which patients with IBD you are most likely to give or send for dietary advice?	
	Ulcerative colitis	1.00
	Small bowel Crohn's	1.00
	Large bowel Crohn's	1.00
	Perianal Crohn's	1.00
	Very difficult to control IBD	NC
Q12.	Do you agree that exclusion diets are effective in the treatment of IBD?	0.94

(NC: correlation coefficient not able to be calculated; *p*: Phi; *r*: Spearman's correlation coefficient, rho)

Appendix 1. Correlation coefficients for two rounds of survey pilot study (continued)

	Question	Correlation coefficient (*p* or *r*)
Q13.	Please indicate below how many new and follow-up patients with IBS would you see in outpatient clinics in an average month?	0.63
Q14.	Are you involved in a special IBS clinic	1.00
Q15.	What percentage of the patients with IBS that you see would your give specific dietary advice to?	0.93
Q16.	What percentage of the patients with IBS that you see would you refer for dietetics advice?	1.00
Q17.	What percentage of the patients with IBS that you see would you ask to exclude specific foods from their diet?	0.96
Q18.	If you do ask patients to exclude foods please indicate the types of foods?	
	Fibre containing foods	0.63
	Refined sugars	1.00
	Dairy products	0.50
	Wheat	1.00
	Nuts	1.00
	Yeast	0.25
	Eggs	NC
Q19.	What percentage of the patients with IBS that you see would you perform food allergy or intolerance testing on?	0.89
Q20.	If you do request intolerance testing which tests do you use?	
	Skin prick testing	1.00
	Skin patch testing	NC
	RAST	NC
	Open food exclusion and rechallenge	1.00
	Double blind placebo controlled challenge	1.00
	Yorktest IgG test	1.00
Q21.	Please indicate which patients with IBS you are most likely to give or send for dietary advice?	
	Diarrhoea predominant	0.71
	Constipation predominant	0.25
	Pain predominant	NC
	Difficult to control IBS	0.00
Q22.	Do you agree that exclusion diets are effective in the treatment of IBS?	0.59

(NC: correlation coefficient not able to be calculated; *p*: Phi; *r*: Spearman's correlation coefficient, rho)

Appendix 2. Covering letter and questionnaire for survey

Dear <<mail merge to follow>>,

Please find enclosed a short survey regarding dietary advice in inflammatory bowel disease and irritable bowel syndrome. This should take **no longer than 3 minutes** to fill out. This is a national survey that is being sent to all members of the British Society of Gastroenterologists. Currently there are no position statements regarding dietary advice in either condition. Information about current practice will greatly aid research in this area in the future.

Thank you very much for taking the time to fill in this short survey and returning it in the stamped, addressed envelope also enclosed.

Kind regards,

Anton Emmanuel

Can we please start by asking about the type of practice you are involved in?

Q1. How would you best describe your practice? (Physicians please indicate the percentage of time spent in gastroenterology versus medicine)

Colorectal surgeon ..... ☐
Other surgeon ..... ☐
Physician ..... ☐
Gastroenterology time ____%
General medicine time ____%
Other (PLEASE WRITE IN)_____

Q2. Are you able to refer to a dietician? (If yes please tell us how much dietetic sessional time is allocated to GI patients in your service?)

Yes, a specialist GI dietician ☐
Yes, a general dietician ..... ☐
No ..... ☐
Dietician time allocated to your service? _____hours

The first part of this questionnaire applies only to IBD.

Q3. Are you involved in a special IBD clinic

Yes ..... ☐
No ..... ☐

Q4. Please indicate below how many new and follow-up patients with IBD would you see in outpatient clinics in an average month?

Less than 20 ..... ☐
20 to 40 ..... ☐
40 to 60 ..... ☐
60 to 80 ..... ☐
80 to 100 ..... ☐
More than 100 ..... ☐

Q5. What percentage of the patients with IBD that you see would you give specific dietary advice to?

none ..... ☐
less than 25% ..... ☐
25% to 50% ..... ☐
50% to 75% ..... ☐
Over 75% ..... ☐

Q6. What percentage of the patients with IBD that you see would you refer for dietetics advice?

none ..... ☐
less than 25% ..... ☐
25% to 50% ..... ☐
50% to 75% ..... ☐
Over 75% ..... ☐

Q7. What percentage of the patients with IBD that you see would you ask to exclude specific foods from their diet?

none ..... ☐
less than 25% ..... ☐
25% to 50% ..... ☐
50% to 75% ..... ☐
Over 75% ..... ☐

Q8. If you do ask patients to exclude foods please indicate the types of foods?

Fibre containing foods ..... ☐
Refined sugars ..... ☐
Dairy products ..... ☐
Wheat ..... ☐
Nuts ..... ☐
Yeast ..... ☐
Eggs ..... ☐
*Other (PLEASE WRITE IN)*_____
_____
_____
_____

Q9. What percentage of the patients with IBD that you see would you perform food allergy or intolerance testing on?

none ..... ☐
less than 25% ..... ☐
25% to 50% ..... ☐
50% to 75% ..... ☐
Over 75% ..... ☐

Q10. If you do request intolerance testing which tests do you use?

Skin prick testing ..... ☐
Skin patch testing ..... ☐
RAST ..... ☐
Open food exclusion and rechallenge ..... ☐
Double blind placebo controlled challenge ..... ☐
Yorktest IgG test ..... ☐
*Other (PLEASE WRITE IN)*_____
_____
_____
_____

Q11. Please indicate which patients with IBD you are most likely to give or send for dietary advice?

Ulcerative colitis ..... ☐
Small bowel Crohn's ..... ☐
Large bowel Crohn's ..... ☐
Perianal Crohn's ..... ☐
Very difficult to control IBD ..... ☐

Q12. Do you agree that exclusion diets are effective in the treatment of IBD?

Agree strongly ☐
Agree a little ☐
Neither Agree nor Disagree ☐
Disagree a little ☐
Disagree a lot ☐

The second part of this questionnaire repeats the above questions but applies only to IBS.

Q13. Please indicate below how many new and follow-up patients with IBS would you see in outpatient clinics in an average month?

Less than 20 ☐
20 to 40 ☐
40 to 60 ☐
60 to 80 ☐
80 to 100 ☐
More than 100 ☐

Q14. Are you involved in a special IBS clinic

Yes ☐
No ☐

Q15. What percentage of the patients with IBS that you see would your give specific dietary advice to?

none ☐
less than 25% ☐
25% to 50% ☐
50% to 75% ☐
Over 75% ☐

Q16. What percentage of the patients with IBS that you see would you refer for dietetics advice?

none ☐
less than 25% ☐
25% to 50% ☐
50% to 75% ☐
Over 75% ☐

Q17. What percentage of the patients with IBS that you see would you ask to exclude specific foods from their diet?

none ☐
less than 25% ☐
25% to 50% ☐
50% to 75% ☐
Over 75% ☐

Q18. If you do ask patients to exclude foods please indicate the types of foods?

Fibre containing foods ☐
Refined sugars ☐
Dairy products ☐
Wheat ☐
Nuts ☐
Yeast ☐
Eggs ☐
Other (PLEASE WRITE IN)_____
_____
_____
_____

Q19. What percentage of the patients with IBS that you see would you perform food allergy or intolerance testing on?

none ☐
less than 25% ☐
25% to 50% ☐
50% to 75% ☐
Over 75% ☐

Q20. If you do request intolerance testing which tests do you use?

Skin prick testing ☐
Skin patch testing ☐
RAST ☐
Open food exclusion and rechallenge ☐
Double blind placebo controlled challenge ☐
Yorktest IgG test ☐
Other (PLEASE WRITE IN)_____
_____
_____
_____

Q21. Please indicate which patients with IBS you are most likely to give or send for dietary advice?

Diarrhoea predominant ☐
Constipation predominant ☐
Pain predominant ☐
Difficult to control IBS ☐

Q22. Do you agree that exclusion diets are effective in the treatment of IBS?

Agree strongly ☐
Agree a little ☐
Neither Agree nor Disagree ☐
Disagree a little ☐
Disagree a lot ☐

Appendix 3. Questionnaire regarding food sensitivities

Dear Participant,

Thank you for agreeing to take part in this study. Please take the time to complete the questions below. The questionnaire usually takes 20 to 30 minutes to complete.

Q1. Over the last month how many times per day on average have you opened your bowels? _____ times per day.

Q2. Over the last month what has the consistency of your bowel motions mostly been?

☐Hard pellets ☐Formed stool ☐Loose stool ☐Watery stool

☐Alternating between soft and loose stool

Q4. Do you have problems with persistent nausea or vomiting? ☐Yes ☐No

Q3. Are you allergic or sensitive to any food? ☐Yes ☐No

Q4. For each of the foods below please tick the box(es) that best describe the type of allergy/sensitivity you experience to that food, or write in the type of allergy/sensitivity you experience. If you do not experience allergy/sensitivity to the food please leave blank.

	Rash or swelling of the lips and throat	Eczema /derm-atitis	Asthma	Diarr-hoea	Constip-ation	Abdom-inal Pain	Bloat-ing	Heart-burn	Other (please describe)
Barley	☐	☐	☐	☐	☐	☐	☐	☐	
Buckwheat	☐	☐	☐	☐	☐	☐	☐	☐	
Corn (maize)	☐	☐	☐	☐	☐	☐	☐	☐	
Millet	☐	☐	☐	☐	☐	☐	☐	☐	
Oat	☐	☐	☐	☐	☐	☐	☐	☐	
Rice	☐	☐	☐	☐	☐	☐	☐	☐	
Rye	☐	☐	☐	☐	☐	☐	☐	☐	
Wheat	☐	☐	☐	☐	☐	☐	☐	☐	
Cows milk	☐	☐	☐	☐	☐	☐	☐	☐	
Egg - white	☐	☐	☐	☐	☐	☐	☐	☐	
Egg - yolk	☐	☐	☐	☐	☐	☐	☐	☐	

	Rash or swelling of the lips and throat	Eczema /derm-atitis	Asthma	Diarr-hoea	Constip -ation	Abdom-inal Pain	Bloat-ing	Heart-burn	Other (please describe)
Beef	☐	☐	☐	☐	☐	☐	☐	☐	
Chicken	☐	☐	☐	☐	☐	☐	☐	☐	
Duck	☐	☐	☐	☐	☐	☐	☐	☐	
Lamb	☐	☐	☐	☐	☐	☐	☐	☐	
Pork	☐	☐	☐	☐	☐	☐	☐	☐	
Turkey	☐	☐	☐	☐	☐	☐	☐	☐	
Crab, Lobster, Prawn / Shrimp	☐	☐	☐	☐	☐	☐	☐	☐	
Mussell, Oyster, Scallop	☐	☐	☐	☐	☐	☐	☐	☐	
Herring, Mackerel	☐	☐	☐	☐	☐	☐	☐	☐	
Plaice / Sole	☐	☐	☐	☐	☐	☐	☐	☐	
Salmon / Trout	☐	☐	☐	☐	☐	☐	☐	☐	
Tuna	☐	☐	☐	☐	☐	☐	☐	☐	
Cod, Haddock	☐	☐	☐	☐	☐	☐	☐	☐	
Almond	☐	☐	☐	☐	☐	☐	☐	☐	
Brazil	☐	☐	☐	☐	☐	☐	☐	☐	
Cashew	☐	☐	☐	☐	☐	☐	☐	☐	
Coconut	☐	☐	☐	☐	☐	☐	☐	☐	
Hazelnut	☐	☐	☐	☐	☐	☐	☐	☐	
Peanut	☐	☐	☐	☐	☐	☐	☐	☐	
Walnut	☐	☐	☐	☐	☐	☐	☐	☐	

	Rash or swelling of the lips and throat	Eczema /derm-atitis	Asthma	Diarr-hoea	Constip -ation	Abdom-inal Pain	Bloat-ing	Heart-burn	Other (please describe)
Asparagus	☐	☐	☐	☐	☐	☐	☐	☐	
Aubergine	☐	☐	☐	☐	☐	☐	☐	☐	
Avocado	☐	☐	☐	☐	☐	☐	☐	☐	
Carrot	☐	☐	☐	☐	☐	☐	☐	☐	
Celery	☐	☐	☐	☐	☐	☐	☐	☐	
Cucumber	☐	☐	☐	☐	☐	☐	☐	☐	
Haricot bean	☐	☐	☐	☐	☐	☐	☐	☐	
Kidney bean	☐	☐	☐	☐	☐	☐	☐	☐	
Spinach	☐	☐	☐	☐	☐	☐	☐	☐	
Lentils	☐	☐	☐	☐	☐	☐	☐	☐	
Lettuce	☐	☐	☐	☐	☐	☐	☐	☐	
Mushroom	☐	☐	☐	☐	☐	☐	☐	☐	
Broccoli, Brussell Sprouts, Cabbage, Cauliflower	☐	☐	☐	☐	☐	☐	☐	☐	
Onion	☐	☐	☐	☐	☐	☐	☐	☐	
Pea	☐	☐	☐	☐	☐	☐	☐	☐	
Peppers (Capsicum) / Paprika	☐	☐	☐	☐	☐	☐	☐	☐	
Potato	☐	☐	☐	☐	☐	☐	☐	☐	
Soya bean	☐	☐	☐	☐	☐	☐	☐	☐	
String bean	☐	☐	☐	☐	☐	☐	☐	☐	

	Rash or swelling of the lips and throat	Eczema /derm-atitis	Asthma	Diarr-hoea	Constip -ation	Abdom-inal Pain	Bloat-ing	Heart-burn	Other (please describe)
Apricot	☐	☐	☐	☐	☐	☐	☐	☐	
Banana	☐	☐	☐	☐	☐	☐	☐	☐	
Blackberry	☐	☐	☐	☐	☐	☐	☐	☐	
Black-currant	☐	☐	☐	☐	☐	☐	☐	☐	
Cherry	☐	☐	☐	☐	☐	☐	☐	☐	
Cranberry	☐	☐	☐	☐	☐	☐	☐	☐	
Grape	☐	☐	☐	☐	☐	☐	☐	☐	
Grapefruit	☐	☐	☐	☐	☐	☐	☐	☐	
Kiwi	☐	☐	☐	☐	☐	☐	☐	☐	
Lemon	☐	☐	☐	☐	☐	☐	☐	☐	
Lime	☐	☐	☐	☐	☐	☐	☐	☐	
Cantaloup, Honeydew, Water-melon	☐	☐	☐	☐	☐	☐	☐	☐	
Olive	☐	☐	☐	☐	☐	☐	☐	☐	
Orange	☐	☐	☐	☐	☐	☐	☐	☐	
Peach	☐	☐	☐	☐	☐	☐	☐	☐	
Pear	☐	☐	☐	☐	☐	☐	☐	☐	
Pineapple	☐	☐	☐	☐	☐	☐	☐	☐	
Plum	☐	☐	☐	☐	☐	☐	☐	☐	
Raspberry	☐	☐	☐	☐	☐	☐	☐	☐	
Strawberry	☐	☐	☐	☐	☐	☐	☐	☐	
Tomato	☐	☐	☐	☐	☐	☐	☐	☐	

	Rash or swelling of the lips and throat	Eczema /derm-atitis	Asthma	Diarr-hoea	Constip -ation	Abdom-inal Pain	Bloat-ing	Heart-burn	Other (please describe)
Chilli pepper	☐	☐	☐	☐	☐	☐	☐	☐	
Cinnamon / Clove	☐	☐	☐	☐	☐	☐	☐	☐	
Coriander / Cumin / Dill	☐	☐	☐	☐	☐	☐	☐	☐	
Garlic	☐	☐	☐	☐	☐	☐	☐	☐	
Ginger	☐	☐	☐	☐	☐	☐	☐	☐	
Basil, Mint, Sage, Thyme	☐	☐	☐	☐	☐	☐	☐	☐	
Mustard seed	☐	☐	☐	☐	☐	☐	☐	☐	
Nutmeg / Peppercorn	☐	☐	☐	☐	☐	☐	☐	☐	
Parsley	☐	☐	☐	☐	☐	☐	☐	☐	
Sesame seed	☐	☐	☐	☐	☐	☐	☐	☐	
Vanilla	☐	☐	☐	☐	☐	☐	☐	☐	
Carob	☐	☐	☐	☐	☐	☐	☐	☐	
Cocoa bean	☐	☐	☐	☐	☐	☐	☐	☐	
Coffee	☐	☐	☐	☐	☐	☐	☐	☐	
Cola nut	☐	☐	☐	☐	☐	☐	☐	☐	
Hops	☐	☐	☐	☐	☐	☐	☐	☐	
Sunflower seed	☐	☐	☐	☐	☐	☐	☐	☐	
Tea	☐	☐	☐	☐	☐	☐	☐	☐	
Yeast (brewers and bakers)	☐	☐	☐	☐	☐	☐	☐	☐	

If there are other foods, not listed above, that cause you significant symptoms, please list them using the blank table below									
Name of Food	Rash or swelling of the lips and throat	Eczema /derm-atitis	Asthma	Diarr-hoea	Constip -ation	Abdom -inal Pain	Bloat-ing	Heart-burn	Other (please describe)
	☐	☐	☐	☐	☐	☐	☐	☐	
	☐	☐	☐	☐	☐	☐	☐	☐	
	☐	☐	☐	☐	☐	☐	☐	☐	
	☐	☐	☐	☐	☐	☐	☐	☐	
	☐	☐	☐	☐	☐	☐	☐	☐	
	☐	☐	☐	☐	☐	☐	☐	☐	