UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Laboratory and Clinical Studies of Inflammatory Bowel Disease

by

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Thesis for the degree of Doctor of Medicine

March 2006

Errata

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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LABORATORY AND CLINICAL STUDIES OF INFLAMMATORY BOWEL DISEASE

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Inflammatory bowel disease (IBD) is the collective term for a group of chronic idiopathic conditions that affect the gastrointestinal tract, including Crohn's disease and ulcerative colitis, characterised by ongoing inflammation of the gut mucosa, in the absence of an obvious trigger. These cause significant long-term morbidity in both adults and children. The pathogenesis involves the gut mucosal immune system and genetic factors affect disease expression.

Previous studies have shown that Smad7, a negative regulator of transforming growth factor (TGF)-β, is expressed at high levels in IBD in gut mucosal biopsies and lamina propria mononuclear cells, preventing the anti-inflammatory effects of TGF-β. We confirm high Smad7 protein levels in IBD. This persists in lamina propria mononuclear cells *ex-vivo*. Tumour necrosis factor-α, but not interferon-γ or TGF-β, increases Smad7 in lamina propria mononuclear cells from normal gut mucosa. However, Smad7 mRNA levels are not significantly different in normal and IBD tissue.

Clinical studies of IBD patients examine the use of non-invasive methods to assess chronic gut symptoms and disease activity. Faecal calprotectin, a neutrophil protein, is a marker of gut inflammation. Using a highly sensitive assay, faecal calprotectin >50μg/g has 85% sensitivity for pathology in children with chronic gut symptoms, but is not specific for IBD. Calprotectin levels correlate with disease activity in ulcerative colitis, but not in Crohn's disease. Ultrasound imaging of the colon and terminal ileum shows that increased bowel wall thickness is also a marker of disease activity in IBD, but superior mesenteric artery blood flow, measured by Doppler analysis, does not correlate with disease activity. These data define new roles for both calprotectin and ultrasonography in the assessment of children with chronic gut symptoms and IBD.

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Acknowledgements

The research presented in this thesis was made possible by funding of a research training fellowship by the Crohn's Disease in Childhood Research Association (CiCRA), and their support is gratefully acknowledged.

My supervisors, Professor Tom MacDonald and Professor Derek Mann, have been unfailingly supportive and their guidance has been invaluable, particularly with experimental design and during thesis preparation.

I would like to thank all of the staff of the E-level labs in Southampton, who provided generous and open advice and expertise. Special thanks go to Dr Ute Günther, Dr Jo Prothero, Dr John Gordon and Dr Jude Holloway. Prof Giovanni Monteleone was a great teacher and was the perfect host when I visited him and his team both at St Bartholomew's Hospital, London, and at the University of Rome, Tor Vergata. Dr Jelena Mann provided expert advice and help with molecular biology experiments.

Dr Mark Beattie provided fantastic support, with collaboration in obtaining research biopsy samples, guidance for clinical studies and mentorship. His drive and enthusiasm have been inspirational.

Studies of calprotectin could not have been competed without Sohere Roked and Rebecca Robinson. Under supervision, they recruited patients and preformed assays as part of their 4th year undergraduate medical student projects, with the help of a grant from Nestlé UK for Calprest kits.

Radiology studies were in collaboration with the Paediatric Radiology Department at Southampton General Hospital, and supported by an Innovation grant from Hope UK. Dr Jo Fairhurst and Dr Mark Griffiths provided images for Figures 16, 17 and 22.

Thank you to Helen, my wife, for her encouragement and support throughout my fellowship.

Abbreviations

AIDS	acquired immunodeficiency syndrome
AIEC	adherent-invasive *E. coli*
APCs	antigen-presenting cells
ASCA	anti-*Saccharomyces cerevisiae* antibody
BaFT	barium follow-through
BMP	bone morphogenic protein
BWT	bowel wall thickness
CCR	chemokine (CC-motif) receptor
cDNA	complementary DNA
CMF-HBSS	calcium- and magnesium-free HBSS
CMV	cytomegalovirus
CrD	Crohn's disease
CRP	C-reactive protein
CSS	colitis symptom score
DCs	dendritic cells
DMEM	Dulbecco's modifed Eagle's medium
DNA	deoxyribonucleic acid
DSS	dextran sodium sulphate
DTT	dithiothreitol
EDTA	ethylene-diamine-tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immuno-sorbent assay
FAE	follicle-associated epithelium
FCS	foetal calf serum
GI	gastrointestinal
GSS	global severity scale
HBSS	Hank's buffered saline solution
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
ICAM	intracellular adhesion molecule
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-	interleukin
INFγ	interferon gamma
IκB	inhibitor of NFκB
KGF	keratinocyte growth factor
KLH	keyhole limpet haemocyanin

LPMC	lamina propria mononuclear cells
LPS	lipopolysaccharides
LREC	local and regional ethics committee
Mad-CAM	mucosal addressin cell adhesion molecule
MDR	multidrug resistance
Mϕ	macrophage
MHC	major histocompatibility
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NC	nitrocellulose
NCBI	National Centre for Biotechnology Information
NFκB	nuclear factor κB
OCTN	organic cation transporter genes
OMIM	online inheritance in man
PAGE	polyacrylamide gel electrophoresis
pANCA	perinuclear anti-neutrophil cytoplasmic antibody
PCDAI	paediatric Crohn's disease activity index
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PP(s)	Peyer's patch(es)
RI	resistive index
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase PCR
SARA	Smad-anchor for receptor activation
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
sIgA	secretory IgA
SMA	superior mesenteric artery
SNPs	single-nucleotide polymorphisms
STAT	signal transduction and activator of transcription factor
TBS	tris-buffered saline
TBS-T	TBS with Tween20
TCR	T-cell receptor
TGF-β	transforming growth factor
TE	tris-EDTA buffer
Th-cells	T-helper lymphocytes
TIMP	tissue inhibitor of metalloproteinases
TNFα	tumour necrosis factor
UC	ulcerative colitis
UK	United Kingdom
US	ultrasound
USA	United States of America
USS	ultrasound scan/scanning

1 INTRODUCTION

1.1 THE CLINICAL PROBLEM OF IBD

1.1.1 Clinical features

Inflammatory bowel disease (IBD) is the collective term for a group of chronic idiopathic conditions that affect the gastrointestinal tract. There are two main types of IBD, Crohn's disease and ulcerative colitis. Both cause long-term morbidity, and current treatment strategies have limited efficacy and cause significant side effects.

Crohn's disease (CrD) is an inflammatory disorder that can affect any part of the gut from the mouth to the anus. The associated pathological changes are chronic inflammation with a transmural inflammatory cell infiltrate and granuloma formation. Crohn's disease presents clinically with a history of abdominal pain, diarrhoea, with or without blood per rectum. There is often anorexia, weight loss and malnutrition, which can cause poor growth and delayed puberty in children. The natural history of the disease is one of periods of relapse and remission. CrD can also cause ongoing chronic ill health and/or fistula formation. Diagnosis is most commonly made in the second and third decades, with 10-15% of cases presenting under the age of 16 years. A systematic review of epidemiological studies in North American cohorts estimates the incidence at between 3 and 14 per 100 000 per year (Loftus, Jr. et al., 2002). There is evidence that the incidence of Crohn's disease in the UK is increasing (Ehlin et al., 2003).

Ulcerative colitis (UC) is limited to the colonic and rectal mucosa. The characteristic histology shows mucosal and submucosal acute and chronic inflammation with goblet cell

depletion, disrupted crypt architecture and crypt abscesses. Neutrophil and eosinophil infiltration of the mucosa is usually predominant. The inflammatory change is usually diffuse rather than patchy. The incidence is 7 per 100000 people per year, with a prevalence of 3 per 1000 at age 30 in the UK. The prevalence has not changed between the 1980s and 2000s (Ehlin et al., 2003).

The causes of these diseases are unknown. However, the pathogenesis is probably multifactorial, with inherited predisposition important in both CrD and UC, but environmental factors affectin disease expression. But, in both CrD and UC, chronic gut inflammation persists in the absence of an obvious trigger. In CrD there is evidence that the intestinal bacterial flora is the target of the T-lymphocyte responses (Shanahan, 2002; Weigmann and Neurath, 2002). UC may be an autoimmune disease, since circulating autoantibodies are often detected and it is associated with the development of other autoimmune diseases, such as sclerosing cholangitis and autoimmune liver disease. UC is "curable" by the surgical removal of the colon, but a similar inflammatory condition often occurs after reconstructive surgery with the ileoanal pouch that acts as a stool reservoir displaying inflammatory changes (pouchitis).

1.1.2 Intestinal bacteria

No single pathogen has been identified as the cause for either CrD or UC, but several bacteria and viral agents have been postulated as infectious causes of IBD. These have included *Mycobacterium paratuberculosis* and measles virus, although this work has been largely discredited (Shanahan and O'Mahony, 2005; Ghosh et al., 2001). *Yersinia enterocolitica* infection can mimic CrD in young children (Tuohy et al., 1999). The resident gut bacterial population that are normally present in the gut lumen, have also been implicated in the pathogenesis of IBD. A subgroup of patients with IBD responds well to treatment with broad-spectrum antibiotics, although clinical improvement is not often sustained (Turunen et al., 1998; Sandborn and Feagan, 2003).

There is also a large body of experimental data that supports a role for the intestinal bacterial flora in the pathogenesis of IBD. There are many natural and transgenic mouse strains, mostly with altered immunoregulatory genes, in which a spontaneous colitis develops under normal environmental conditions driven by bacterial antigens from the commensal flora (Rath et al., 2001; Elson et al., 2005).

Observational studies in humans have shown the presence of bacteria adherent to and invading the epithelial barrier of the mucosa in IBD, but no specific pathogen, or group of pathogens, seems to be responsible for these effects (Darfeuille-Michaud et al., 1998; Swidsinski et al., 2002). *Escherichia coli*, a species of bacteria with both pathogenic and non-pathogenic strains, is present in variable amounts in the normal bacterial flora, and is often present in increased numbers in CrD. Strains isolated from patients with CrD are able to adhere to and invade intestinal epithelial cells, known as adherent-invasive *E. coli*

(AIEC) (Darfeuille-Michaud et al., 1998). AIEC can invade epithelial cells *in vitro* (Boudeau et al., 2003).

Supplementing the gut flora with probiotics has a potential role in modulating IBD activity, but probiotics effective in inducing remission in active CrD have yet to be identified in randomised controlled clinical trials (Oliva-Hemker and Fiocchi, 2002; Shanahan, 2000). A combination of high dose probiotics (VSL#3) has shown benefit in mild or moderately active UC (Bibiloni et al., 2005). The same combination is effective in preventing pouchitis, an inflammatory condition affecting the constructed ileoanal reservoir after colectomy (Gionchetti et al., 2003). The role of probiotic strains of *Lactobacillus* in preventing relapse in CrD has been investigated recently, with no evidence of efficacy (Prantera et al., 2002; Bousvaros et al., 2005).

1.2 DISEASE DIAGNOSIS AND ASSESSMENT

The accurate diagnosis and assessment of IBD disease severity is useful in determining the requirements of any treatment/management plan. This is particularly important in children, in whom IBD disease activity affects normal growth and development. In idiopathic IBD, investigation aims to discriminate between CrD and UC, since the clinical course and therapeutic options are different. Multiple episodes of repeated investigations with radiological imaging or endoscopy are not desirable; thus tools for rapid, well-tolerated and non-invasive assessment would be useful to guide therapeutic choices and assess responses.

1.2.1 Clinical measures of disease activity

A specific paediatric CrD activity index (PCDAI) has been in use for over a decade (Hyams et al., 1991). This index scores symptom severity, physical examination findings and laboratory test results. It correlates well with the physician global score for disease severity and performs more accurately than the adult CrD activy index (CDAI) (Otley et al., 1999). PCDAI can detect short-term changes in disease activity (Kundhal et al., 2003). However, it has poor specificity for true disease remission, being unable to detect ongoing low-grade inflammation and cannot identify those likely to relapse after initial response to treatment (Hyams et al., 2005). Thus, a non-invasive investigation that can monitor sub-clinical inflammation could find a place in the routine assessment of disease activity and as a measure of effectiveness in clinical trials of new therapies.

1.2.2 Laboratory investigations used to diagnose and monitor IBD

Initial investigations of patients with colitic symptoms should include several stool examinations for bacterial and viral pathogens (including *Clostridium difficile*) and serological tests for *Yersinia entercolitica* and *Entamoeba histolytica*, since chronic intestinal infection can cause symptoms and clinical signs identical to idiopathic IBD. Routine blood investigations including full blood count, albumin, iron status and inflammatory markers (C-reactive protein or Erythrocyte Sedimentation Rate) will help discriminate between children with IBD and recurrent abdominal pain of childhood (Beattie et al., 1995; Cabrera-Abreu et al., 2004).

Auto-antibodies or antibodies against gut commensal flora and yeasts can be found in some patients with IBD. These include perinuclear anti-neutrophil cytoplasmic antibody (pANCA), which is more often found in patients with UC and anti-*Saccharomyces cerevisiae* antibody (ASCA) more common in CrD. However, these have little diagnostic value over standard laboratory tests for the diagnosis of IBD, because only a proportion of patients show positivity. A retrospective study found that 42 of 51 children (82%) with UC had positive pANCA, while 18 of 39 children (46%) with CrD had positive ASCA (Khan et al., 2002), giving an overall sensitivity of 68% and specificity of 92% for serology as a diagnostic test for IBD. Another series examined pANCA titres in 176 children with IBD and 78 controls with other bowel diseases. This study found that pANCA was 92% specific for UC and absent in all non-IBD controls, but sensitivity was only 57% (Ruemmele et al., 1998). A prospective cohort study of 97 adults with indeterminate colitis found that ASCA+/pANCA- predicts a final diagnosis of CrD in 80% and ASCA-/pANCA+ predicts UC in 63.6% (Joossens et al., 2002). When pANCA is positive in indeterminate colitis, the diagnosis is likely to be UC, but a significant proportion of patients with a final diagnosis

of UC have negative pANCA. The clinical importance of these serological markers is thus somewhat limited, and they are not widely used in the UK.

1.2.3 Endoscopy

Endoscopy is indicated in all cases for diagnosis and to assess disease extent. Histology is required to confirm the diagnosis of IBD, and will usually discriminate between Crohn's disease and UC. Inflammatory colitis of an indeterminate type occurs in 10-15% of cases, with histology consistent with IBD, but not characteristic of either CrD or UC, or having features of both.

Evaluation by both upper gastrointestinal endoscopy and ileocolonoscopy is recommended. Rectal biopsies taken at presentation in children later diagnosed with ulcerative colitis are less likely than adults to have signs of chronic mucosal damage (Washington et al., 2002). Distal colonic examination alone is therefore not sufficient. The addition of upper gastrointestinal endoscopy with biopsy improves diagnostic accuracy, since gastric granulomata and focal antral gastritis are more likely in Crohn's disease (Kundhal et al., 2003). One retrospective series reported that 28% of children with Crohn's disease had granulomata in the upper GI tract, compared with 39% having colonic granulomata in this group (Abdullah et al., 2002). Abnormal upper gastrointestinal histology can also be seen in UC, usually as a mild gastritis or mild oesophagitis.

Colonoscopy during follow-up can determine disease progression and assess response to treatment. It is particularly useful in children with difficult disease where repeat endoscopy can help to inform treatment choices. As colitis heals, less specific gastrointestinal

symptoms can occur including distension and constipation, presumably because these children are left with a functional "irritable bowel" syndrome.

1.2.4 Calprotectin

Calprotectin is a neutrophil cytosolic protein present at increased concentrations in the stool during bowel inflammation, correlating well with the results of radionulceotide-labelled neutrophil scans, endoscopic appearances and histology in inflammatory bowel disease (IBD) (Bunn et al., 2001). Reference values for normal children age 4-17 years have been established the using an improved ELISA-based assay technique (Calprest®, Eurospital, Trieste, Italy) which has greater sensitivity than previous assays (Fagerberg et al., 2003). Previous studies have demonstrated raised faecal calprotectin in children with IBD, but the role of faecal calprotectin in the diagnosis and assessment of IBD has not been fully defined (Olafsdottir et al., 2002; Berni Canani et al., 2004).

Studies in adults with Crohn's disease suggest there are higher levels present in those patients likely to relapse, although there is only a weak correlation between disease activity scores and faecal calprotectin (Tibble et al., 2000). In adults with ulcerative colitis (UC) there it is a stronger association with disease activity, probably refecting the predominance of neutrophils in the lesions (Roseth et al., 1997). There is a correlation between intestinal permeability and calprotectin levels in gut lavage fluid in adults with IBD (Berstad et al., 2000). Higher calprotectin levels have been associated with greater clinical disease activity in children with IBD, using a less sensitive test for calprotectin (Bunn et al., 2001).

1.2.5 Radiological imaging

Barium radiology, white cell scanning and ultrasound scanning have limitations in sensitivity and/or specificity for the diagnosis of IBD, but they can be helpful in defining disease extent and activity. Barium radiology of the small bowel can determine the presence of small bowel involvement in patients with an indeterminate colitis, thus making CrD more likely. Technetium-labelled white cell scanning is useful to assess disease activity in children with colitis, and can provide diagnostic information where there is clinical uncertainty between CrD (patchy involvement) and UC (continuous involvement) (Barabino et al., 1998; Bruno et al., 2002; Charron et al., 1999).

Transabdominal ultrasound scanning (USS) has been used as an adjunct in the assessment of paediatric inflammatory bowel disease (IBD), particularly with a view to identifying an inflammatory mass or abscess (Dinkel et al., 1986; Gasche et al., 1999; Ali and Carty, 2000; Hirche et al., 2002). It has advantages over other imaging procedures, being free of ionising radiation, and is non-invasive, well tolerated and widely available. The use of sonography as a primary imaging modality for the assessment of IBD has been described in adults and children, with one paediatric study showing sensitivity of 88% and a specificity of 93% in comparison to ileocolonoscopy (Faure et al., 1997; Haber et al., 2000; Haber et al., 2002; Baud et al., 2004). Sonographic changes have also been shown to follow clinical response in observational studies (Ruess et al., 2000).

1.3 IMMUNOPATHOGENESIS OF IBD

1.3.1 The immune system in the gut

Constantly exposed to bacterial and dietary antigens, the gut has several layers of defence against pathogens. These include structural and physiological adaptations, such as stomach acid and mucus secreted by goblet cells. The epithelial layer is constantly being shed and is repopulated with new cells every 2-3 days, yet the physical barrier between epithelial cells is maintained, preventing luminal contents from contact with the cells of the lamina propria. The cellular immune system is also constantly active in the gut. The immune cells and the cytokines they produce mediate pro- and anti-inflammatory effects through complex networks, orchestrating the local gut immune response.

The gut immune system contains organised lymphoid aggregates in the small bowel with a specialised overlying follicle-associated epithelium, the Peyer's patches (PP). PP are most prominent in the terminal ileum, especially during childhood. These contain active germinal centres that appear after birth with the introduction of antigen in the gut lumen (Bridges et al., 1959). PP are the inductive site for immune responses in the gut (Newberry and Lorenz, 2005). The follicle-associated epithelium (FAE) has specialised M-cells that transport luminal antigen by endocytosis (Neutra et al., 2001). In the sub-epithelial dome areas just beneath the FAE there are large numbers of immature dendritic cells (DCs) that process antigens and mature to become effective antigen-presenting cells (APCs) (Yamanaka et al., 2003). These DCs can present antigen locally or migrate to the PP T-cell zones, mesenteric lymph nodes or stimulate memory B-cells directly. Cytokines in the PP drives the production of T-helper (Th)-1 and Th2 cells in response to stimulation by

APCs, affecting the phenotype of immune responses in the gut (Figure 1). Human PP T-lymphocytes are predominantly Th1 cells, producing in high levels of INFγ and low levels of IL-4, IL-5 and IL-10 (Hauer et al., 1998). This is associated with high levels of IL-12 in human PPs, and activated STAT4, the transcription factor regulating INFγ production (Monteleone et al., 2003).

Naïve CD4+ T-helper lymphocytes (Th-cells) that are activated during the induction of immune response, depending on context, differentiate into effector (Th1- and Th2-cells) or regulatory subsets (Th3-cells) defined by their cytokine expression profile. The different effector subsets then direct the immune response as the architects of cellular defences (Figure 1). TGF-β is produced by the regulatory (CD4+CD25+) Th3-cells (Chen and Wahl, 2003). TGF-β and the immunosuppressive action of Th3-cells are thought to be central in preventing inappropriate ongoing immune activation in the gut. Regulatory and Th3-cells mediate tolerance and regulate immune responses in autoimmune diseases, transplantation, cancer and infection. As well as secreting TGF-β, active TGF-β is bound to the surface of regulatory T-cells, allowing contact-dependent immunosuppression (Chen and Wahl, 2003).

T- and B-lymphocytes activated in the PP leave and enter the lymphatic vessels that channel into regional lymph nodes (Brandtzaeg et al., 1999). From there, they enter the systemic circulation via the thoracic duct and migrate back to the gut. Lymphocytes from PP express the α4β7 integrin which binds specifically to the mucosal addressin Mad-CAM, expressed on endothelial cells in post-capillary high endothelial venues in the organised lymphoid tissues of the gut, but also on lamina propria endothelial cells (Briskin et al., 1997). This homing behaviour targets antigen-primed lymphocytes back to the anatomical

sites where they are likely to meet specific antigen, compartmentalising the immune response; but also directing effector cells from inductive sites to other regional areas. The expression of α4β7 integrin on lymphocytes is dependent on TGF-β (Bartolome et al., 2003).

Germ-free mice have poorly developed PPs, with no germinal centres, few IgA producing plasma cells or CD4+ T-lymphocytes in the lamina propria, and few CD8+ αβ IEL although the γδ IEL remain abundant (Bandeira et al., 1990; MacPherson et al., 2001). The introduction of a single bacterial strain results in a rapid increase in IgA production and IgA producing plasma cells in the lamina propria, resulting in a decrease in bacterial translocation across the epithelium (Shroff et al., 1995; Hooper and Gordon, 2001). These data suggest that, in mice, the gut immune system requires contact with luminal bacterial antigen in order to develop.

T-lymphocytes activated in the PP recirculate to the lamina propria and have a phenotype typical of primed effector T-lymphocytes and are efficient producers of Th1-cytokines. These cells are capable of activating local immune responses and inflammation, but in the normal gut, there is little evidence of active inflammation. The high prevalence of gut infection in patients with HIV/AIDS illustrates the important role of adaptive immunity in protecting against low-grade pathogens. Moreover, a high proportion of lamina propria T-lymphocytes are primed for apoptosis (Boirivant et al., 1996). Activating lamina propria T-lymphocytes can also lead to apoptosis, suggesting that in health, T-cell activation in the lamina propria is tightly controlled to prevent inappropriate responses (Boirivant et al., 1999).

There is also a massive production of secretory IgA (sIgA) in the gut by plasma cells derived form the PP, with adults making 3-5g per day. In healthy individuals, many of the bacterial flora are coated in sIgA and mucosal IgA can bind specifically to member of the flora (Macpherson et al., 1996; van der Waaij et al., 1996). Thus there is evidence of large-scale gut immune cell activity in the healthy state, perhaps a price that must be paid to allow the production of protective IgA in response to pathogens. Maturation of B-cells to IgA-preoducing plasma cells occurs is in response to TGF-β, although where this occurs in the gut is controversial. Previous data showed that lamina propria IgM-producing B-cells have the potential for class-switching to IgA (Fagarasan et al., 2001). However, recent data in humans suggests that class switching occurs *in vivo* in the PP only (Boursier et al., 2005).

There is also a large population of intra-epithelial lymphocytes (IEL) in normal gut, especially in the small bowel. They mostly express the αβ T-cell receptor (TCR) expression, with 5-10% γδ, somewhat higher than the proportion of γδTCR+ T-cells in peripheral blood. IEL TCR expression in oligoclonal, which suggests they originate from a limited number of progenitors (Blumberg et al., 1993). Freshly isolated human IEL are cytolytic and produce Th1-associated cytokines, especially IL-1β, IFNγ and TNFα, but have a low baseline rate of proliferation and reduced proliferation in response to mitogens (Lundqvist et al., 1996). However, their role in the gut immune system is not yet fully understood.

IEL are present throughout the gut, with variations in their frequency depending on the anatomical site. In the small bowel, there are 10-20 IEL per 100 enterocytes (Ferguson,

1977). Given the large surface area of the gut, the number of resident IEL in the normal gut is huge. CD8 expression can be used to classify IEL. Some express CD8αβ heterodimers, others CD8αα homodimers and others are $CD8^-$. IEL can be subdivided into "Type a" (conventional IEL) and "Type b" (unconventional IEL) on the basis of their functional characteristics and their TCR and CD8 expression profile. Type a IEL have TCRαβ+/CD8αβ+, and Type b have TCRαβ+/CD8αα+, TCRγδ+/CD8αα+ or TCRγδ+/CD8- (Hayday et al., 2001). The "Type a" IEL are MHC class I restricted and originate from PP, recirculate through the systemic circulation and home to the epithelium. There they may be stimulated *in situ* by antigen presented by enterocytes (Vezys et al., 2000). Recirculation is dependent on α4β7 integrin binding to Mad-CAM on the endothelium and CD103 (αEβ7 integrin) binding to E-selectin at the epithelium, as well as chemokine receptors CCR9 and CCR3 (Agace et al., 2000). "Type b" IEL are not MHC restricted and their development is less dependent on antigen-priming. They are present in athymic mice but their development is IL-7 dependent (Laky et al., 2000). Once in the gut, these cells respond poorly to stimulation (Guehler et al., 1998). However they can be stimulated to proliferate, for example in coeliac disease (gluten-sensitive enteropathy), where TCRγδ+ IEL are increased in number, then decline after the introduction of a gluten-free diet (Jarvinen et al., 2003). "Type b" IEL have TCR may be selected after gene rearrangement on the basis of reactivity to conserved antigens, or autoantigens, perhaps in response to either infection or cell damage (Boismenu and Havran, 1997).

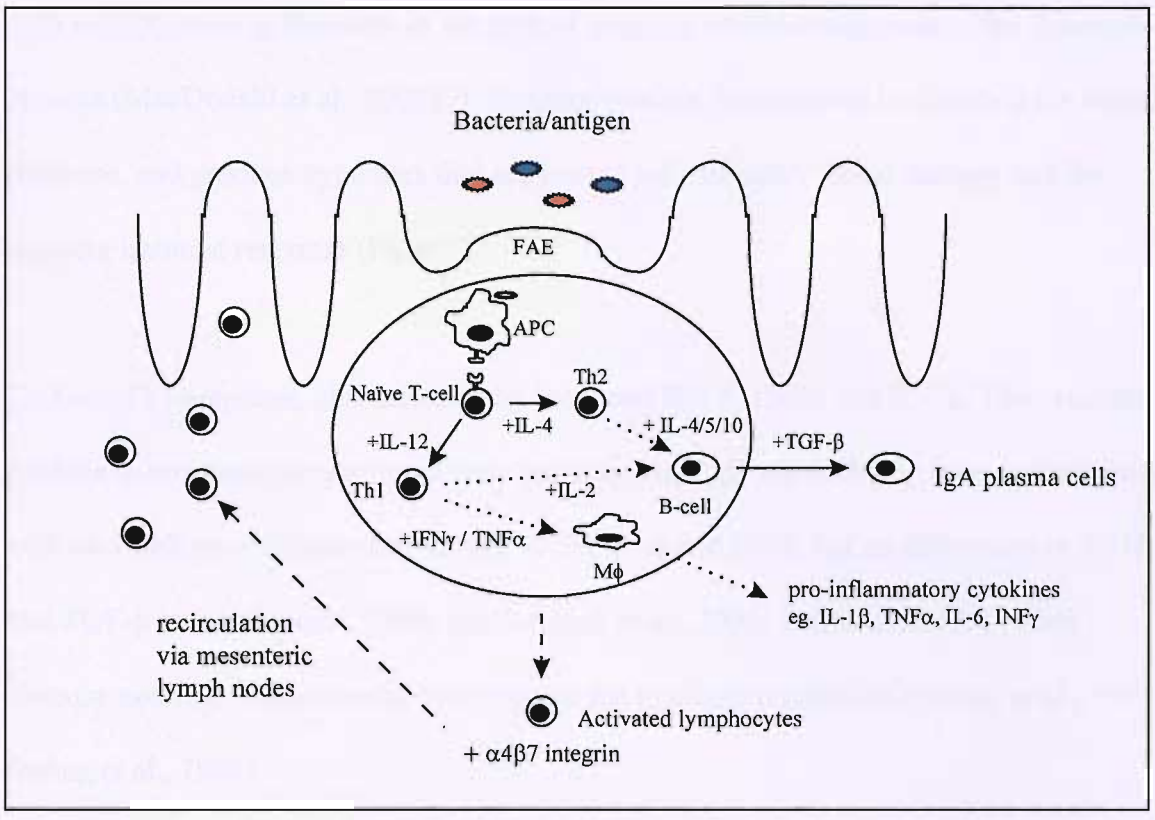


Figure 1: The organisation of the gut immune system.

Antigen presenting cells (APC) in the Peyer's patches receive antigen that has been transported through the follicle-associated epithelium (FAE) by M-cells and present the processed antigen to naïve T-lymphocytes. These can differentiate into effector Th1 cells under the influence IL-12 or into Th2 cells under the influence of IL-4. B-cells proliferate in response to cytokines (IL-2, IL-4, IL-5 or IL-10) from activated T cells and IgA class-switch is mediated by TGF-β. INFγ and TNFα activate mucosal macrophages (Mϕ), which produce pro-inflammatory cytokines (IL-1, TNFα, IL-6 and INFγ). Mature lymphocytes recycle through the mesenteric lymph nodes and the systemic circulation to colonise the gut lamina propria, targeted by the interaction of surface α4β7 integrin and MAdCAM on the gut endothelium.

1.3.2 Immune dysregulation in IBD

CrD and UC show differences in the type of ongoing immune responses in the diseased mucosa (MacDonald et al., 2000). T-lymphocytes are fundamental to directing the immune response, and produce cytokines that are lead to inflammatory tissue damage and the ongoing immune response (Figure 2).

CrD is a Th1-response, characterised by increased INFγ, TNFα and IL-12. The cytokine profiles in intestinal biopsy tissue from patients with CrD are different from normal tissue, with elevated pro-inflammatory IL-1β, TNFα, IL-6 and INFγ, but no difference in IL-10 and TGF-β levels (Fiocchi, 1993; McCormack et al., 2001; Fell et al., 2000). Both immune and non-immune cells contribute to the cytokine production (Breese et al., 1993; Strong et al., 1998).

Intestinal lamina propria T-lymphocytes isolated from patients with UC showed higher IL-10 mRNA than normal intestinal T-cells, lower levels of IL-2 and TNFα with no significant difference in TGF-β, INFγ and IL-4 (Melgar et al., 2003). TGF-β production by stimulated intestinal T-lymphocytes is lower in CrD than normal, but higher in UC. In CrD, the relatively low amount of TGF-β produced by stimulated T-lymphocytes is associated with a higher secretion of INFγ, and in UC higher IL-5 and IL-13 production (Del Zotto et al., 2003; Fuss et al., 2004).

The antigens driving T-lymphocyte activation and ongoing inflammation are likely to be derived from the gut lumen, but are still poorly characterised. Mouse models of IBD include gene knockouts (T-cell receptor, MHC class II, IL-2, IL-10, TGF-β, Smad3) and transgenic mice (IL-7, Gp39, STAT4) (Blumberg et al., 1999). The spontaneous colitis in

the C3H/HeJ Bir mouse strain can be transferred to littermates by T-lymphocyte transfer, suggesting T-lymphocyte activation in response to an environmental antigen can drive inflammation and cause significant pathology (Cong et al., 1998). Transfer of antigen-specific T-lymphocytes from normal mice to mice with a severe combined immunodeficiency (SCID) phenotype also causes colitis (Powrie et al., 1993; Rudolphi et al., 1994).

UC has high levels of mucosal pro-inflammatory cytokines, but also very high levels of IgG plasma cells and large numbers of neutrophils. The end result in both CrD and UC is the continued production of cytokines, chemokines and free radicals by infiltrating leukocytes, resident epithelial and mesenchymal cells, with upregulation of vascular adhesion molecules, attracting more inflammatory cells into the inflamed gut. NFκB is a transcription factor that mediates increased expression of pro-inflammatory genes, mostly in response to cytokines in IBD (Podolsky, 2002). The release of pro-inflammatory mediators, such as TNFα, affects tissue homeostasis by causing the production of growth factors by mesenchymal cells (eg. KGF) leading to epithelial hyperplasia and proteases that cause mucosal ulceration (eg. MMP3) (MacDonald et al., 2000). Costimulation of cells with bacterial products such as lipopolysaccharides (LPS) and peptidoglycan from the cell wall or bacterial DNA (CpG repeats) also contribute to immune cell activation (Stagg et al., 2003).

The role of the mesenchymal cell is relevant to both CrD, where transmural inflammation leads to fibrosis and stricture formation, and UC, where ulceration denudes the epithelium exposing the lamina propria beneath. In CrD, there is hyperplasia of muscle layers in fibrotic areas and smooth muscle cells become fibrogenic myofibroblasts (Matthes et al.,

1992). Increased matrix degradation mediated by MMP-3 without compensatory TIMP increases is seen in active IBD, contributing to mucosal ulceration (Heuschkel et al., 2000).

Treating IBD with immunosuppressive medication, such as corticosteroids or thiopurine derivatives (azathioprine, 6-mercaptopurine), improves symptoms and can allow mucosal healing (Hanauer and Present, 2003). Mucosal healing is also seen after immune reconstitution by bone marrow transplantation and in CD4+ T-lymphocyte depletion secondary to HIV infection (Lopez-Cubero et al., 1998; James, 1988). Biological therapies for IBD that target specific cells or activation pathways in the immune system offer a new therapeutic avenue. For CrD, the first of these to reach common clinical practice is a monoclonal human-mouse chimeric anti-TNFα antibody (Infliximab) that is effective in inducing remission in disease resistant to conventional immunosuppressive therapies (Hanauer et al., 2002). Many other targets for biological therapies are being investigated and include inhibitors of lymphocyte trafficking (anti-α4 integrin, ICAM-1 antisense oligonulceotide), Th1-cell polarisation (IL-10, anti-IL-12), NFκB, T-cell costimulation (anti-CD40) and CD4 cells (anti-CD4) (Sandborn and Targan, 2002). There have also been trials of topically applied epidermal growth factor (EGF) in UC limited to the distal colon (Sinha et al., 2003).

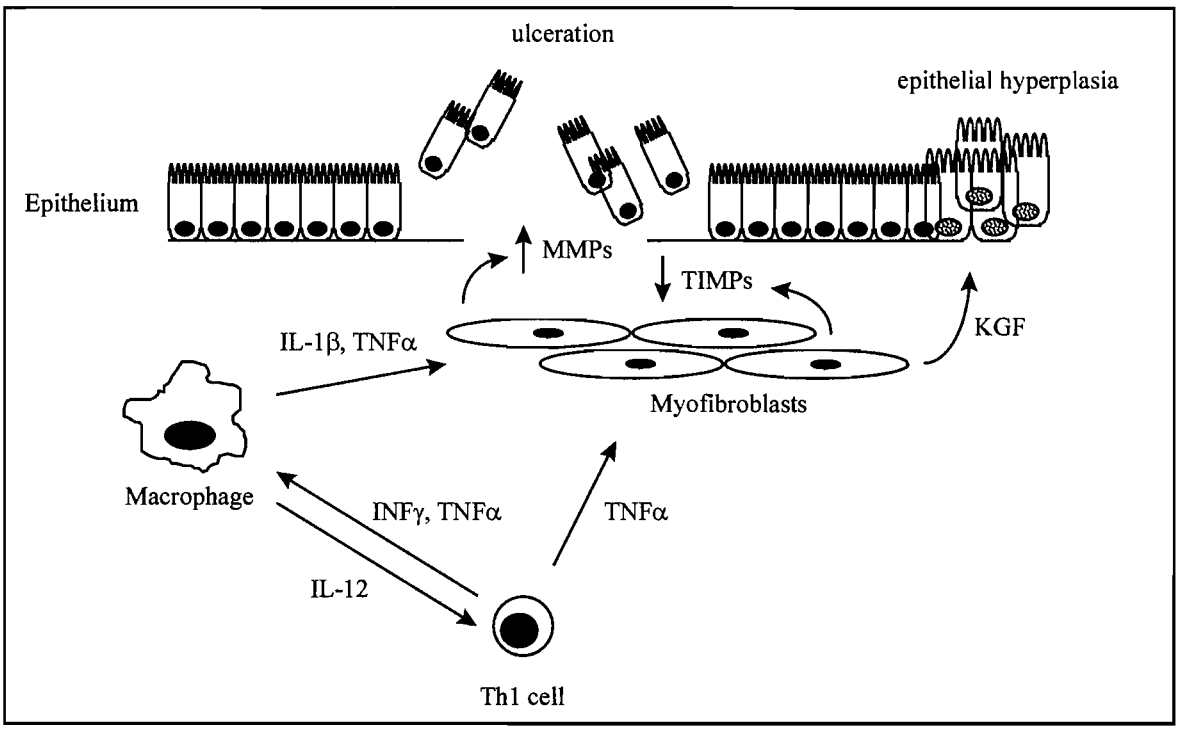


Figure 2: Mucosal mediators important in the pathogenesis of IBD.

Cytokine production by T-lymphocytes affects the release of mediators from resident cell populations in the gut. Tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) stimulate macrophages to produce inflammatory mediators. In response, mesenchymal myofibroblasts produce more matrix metalloproteinases (MMPs) less tissue inhibitors of MMPs (TIMPs), causing tissue damage and ulceration, and keratinocyte growth factor (KGF), which leads to epithelial hyperplasia.

1.3.3 Genetic predisposition to IBD

1.3.3.1 Clues from epidemiology

A positive family history for the disease is recognised clinically as a powerful risk factor for the development of the disease. Epidemiological studies have long suggested a genetic influence on the expression of IBD. Twin studies have shown concordance, and familial clustering is a feature of the diseases. The concordance of UC in dizygotic (fraternal) twins is 3% and in monozygotic (identical) twins is 10%. The relative risk to a sibling of a patient with CrD is between 13 and 36 fold and between 7 and 17 fold for UC.

CrD is rare in developing countries, and studies of immigrants show that the incidence changes towards that of the local population (Carr and Mayberry, 1999). Within a defined population, disease expression is influenced by socio-economic factors, with a higher incidence in more affluent areas (Blanchard et al., 2001). Thus environmental factors affect disease expression in genetically susceptible individuals.

1.3.3.2 Genetic linkage studies

The inflammatory bowel diseases are polygenic disorders, with a large environmental influence on disease expression. Despite this, the genetic component of disease expression is large, with familial clustering. Linkage studies have scanned the genome for areas that are associated with IBD. The initial results published in 1996 (Hugot et al., 1996; Satsangi et al., 1996), identified an area on chromosome 16, known now as IBD1. Since then, ten other disease-associated loci have been identified (Table 1). These loci with significant or suggestive linkage with IBD are variably associated with CrD and UC. There are many

candidate genes contained within these loci which could play important roles in the pathogenesis of IBD. The genes currently being investigated are involved in the immune response and include cytokines, the T-cell receptor and MHC loci. The most strongly associated regions of the genome for UC are at the IBD2 locus on chromosome 12 and the IBD3 locus on chromosome 6, mapping to the major histocompatibility complex (MHC).

Table 1: The genetic linkage studies in IBD.

Locus	Chromosomal location	Reference(s)	Associated diagnosis
IBD1	16q12	Hugot et al., 1996	CrD
IBD2	12q13	Satsangi et al., 1996	UC
IBD3	6p13	Hampe et al., 1999	CrD, UC
IBD4	14q11	Ma et al., 1999 Duerr et al., 2000	CrD
IBD5	5q31-33	Rioux et al., 2000	CrD
IBD6	19p13	Rioux et al., 2000	CrD, UC
IBD7	1p36	Cho et al., 1998	CrD, UC
IBD8	16p	Hampe et al., 2002	CrD, UC
IBD9	3p26	Satsangi et al., 1996	CrD, UC
Other loci	10q23	Stoll et al., 2004	CrD, UC
	7q	Satsangi et al. 1996	CrD, UC

1.3.3.3 NOD2

Abnormalities of a single gene, NOD2 (OMIM 60956), at the IBD1 locus has been identified as the cause of CrD in some families (Hugot et al., 2001). Homozygotes for NOD2 variants have a 20 fold increased risk of developing Crohn's disease, but fewer than 20% of patients with CrD have these disease-associated genotypes. NOD2 is not associated with UC, and in CrD is associated with mainly ileal disease (Cuthbert et al., 2002). In early-onset CrD, NOD2 is associated with severe disease (Russell et al., 2005). NOD2 encodes CARD15, a pattern-recognition receptor of the innate immune system that detects the bacterial product muramyl dipeptide (Inohara et al., 2003; Girardin et al., 2003). CARD15 is expressed in intestinal epithelial cells and macrophages and is regulated by TNFα and INFγ (Hisamatsu et al., 2003; Rosenstiel et al., 2003). NOD2/CARD15 expression is highest in the Paneth cells of the intestinal crypts, and NOD2 mutations are associated with reduced expression of defensins (Wehkamp et al., 2004). These antibacterial proteins are important in both the defence against bacterial pathogens and interactions with the normal flora (Eckmann, 2004). Mice deficient in NOD2 are susceptible to infection via the oral route, but not parenteral route, consistent with a role for NOD2 in protecting against bacterial invasion of the gut (Kobayashi et al., 2005).

1.3.3.4 OCTN

An association between genes within the IBD5 locus has been investigated by several groups (Rioux et al., 2000; Giallourakis et al., 2003; Negoro et al., 2003). Linkage disequilibrium within this region has hampered identification of risk-associated single

genes. Despite this, carriage of two specific mutations of the organic cation transporter genes (OCTN) is a risk haplotype, called OCTN-TC (Peltekova et al., 2004). These variants cause impaired function of the OCTN gene product. Also, OCTN variants interact with NOD2/CARD15 variants to increase risk (Mirza et al., 2003; McGovern et al., 2003). The OCTN-TC haplotype increases the risk of developing CrD 1.65 fold and is associated with earlier age at onset, colonic disease, and non-fistulising/non-fibrostenotic disease type (Torok et al., 2005).

1.3.3.5 DLG5

Another single gene implicated in the pathogenesis of CrD is DLG5, located on the pericentric region of chromosome 10 (Stoll et al., 2004). DLG5 is a protein involved in protein- protein interactions and is thought to act as a scaffolding protein. Single-nucleotide polymorphisms were associated with an increased risk of IBD with the risk increased 1.6 fold with the haplotype with the largest effect (DLG5 R30Q). There was also an association with this haplotype and disease-associated NOD2/CARD15 variants (Stoll et al., 2004). DLG5 polymorphisms have been examined in other studies of IBD with larger cohorts, and were not found to be associated with increased risk of CrD (Torok et al., 2005; Buning et al., 2006). Another study found that the DLG5 R30Q variant did confer some risk of CrD, but that the effect was seen in two of three cohorts they examined (Daly et al., 2005). The authors proposed that the previously reported association to other DLG5 haplotypes were due to linkage disequilibrium with the R30Q variant.

1.3.3.6 MDR1

Within the chromosome 7q risk-associated locus lies the multidrug resistance (MDR)1 gene. This gene codes a membrane-bound transport protein that confers chemotherapeutic drug resistance to tumour cells, and affects the pharmacokinetics of many drugs including corticosteroids and immunsuppressants. Mice deficient in MDR1 develop a spontaneous colitis (Schinkel et al., 1997). Consistent with this observation, inheriting a variant with lower activity (Ala893) is associated with IBD (Brant et al., 2003). Another study has found that SNPs of MDR1 are associated with refractory IBD (Potocnik et al., 2004).

1.4 THE ROLE OF TGF-β IN IMMUNE RESPONSES

TGF-β is a polypeptide growth factor with multiple roles in growth, development, wound healing, fibrosis and immune regulation (Wahl, 1994). This complex variety of effects, sometimes apparently contradictory, has led the functional role of TGF-β being described as: "not to have an intrinsic action, but to serve as a mechanism for coupling a cell to its environment or change in its own state" (Sporn and Roberts, 1990). TGF-β is present in most tissues of the body, including the gut, and is made by a variety of cell types. It has three isoforms, encoded by different genes that are differentially regulated in different cell types, with TGF-β1 mediating most of the important immunoregulatory functions in mammals (Letterio and Roberts, 1996). Cells respond to TGF-β in a contextual manner, allowing the receiving cell to respond differently depending on the other cytokine signals as well as its own lineage and developmental state.

Bone marrow-derived cells, which mainly secrete the TGF-β1 isoform, control TGF-β activity by regulating secretion and activation of latent TGF-β, not by altering mRNA expression. The conversion of the latent form is controlled by several tissue factors, including plasmin, thrombospondin and MMP-3 (Khalil, 1999).

TGF-β1 knockout mice are normal at birth, but die before 3 weeks of age with multiple organ inflammation, including colitis (Kulkarni et al., 1993). The degree of inflammation, but not the lethal phenotype, can be affected by exocrine production of active TGF-β1 by the liver, driven by the albumin promoter in double transgenic mice (Longenecker et al., 2002). Systemic inflammation does not occur in TGF-β1 null mice crossed with SCID mice, clearly showing that the adaptive immune system is needed for pathology to develop (Diebold et al., 1995). Also, bone marrow transplantation of lethally irradiated normal

mice with marrow taken from TGF-β null mice leads to systemic inflammation in the recipients. Thus the inflammation seen in TGF-β deficiency is mediated by leukocytes. Removing CD4+ lymphocytes improves survival in TGF-β1 deficient mice (Letterio et al., 1996).

TGF-β is also important in directing intestinal IEL and LPL trafficking, since it regulates the expression of αEβ7 integrin (Suzuki et al., 2002b).

TGF-β plays an important role in the isotype switching of B cells to make IgA (Zan et al., 1998). Both *in vitro* and *in vivo* studies have demonstrated that TGF-β1-acts as a potent negative regulator of mucosal inflammation, allowing healing (Wahl, 1994; Letterio and Roberts, 1998). In humans, T-lymphocyte proliferation in response to a gut bacterial antigen is inhibited by antigen-specific CD4+ lymphocytes secreting IL-10 and TGF-β (Khoo et al., 1997).

Tissue macrophages are responsive to TGF-β. Resting monocyte/macrophages express high levels of TGF-β receptors, which reduces as these cells mature and become activated by LPS or INFγ (McCartney-Francis and Wahl, 1994). TGF-β also increases phagocytic activity, both of apoptotic cells and of bound immunoglobulin, suggesting roles in both the prevention and resolution of inflammation (Rose et al., 1995; Welch et al., 1990). In the rat, exposing immature macrophages to TGF-β reduces their subsequent responses to TNFα or INFγ, an effect prevented by pre-incubating with INFγ(Erwig et al., 1998). This suggests that macrophage pro-inflammatory activity is affected by the cytokine context they developed in, and that once activated, respond less affectively to anti-inflammatory signals. In humans, intestinal macrophages from normal small bowel are relatively

unresponsive to pro-inflammatory stimuli, and do not produce TGF-β (Smythies et al., 2005). This relative anergy is dependent on stomal factors, including TGF-β itself.

TGF-β produced by antigen specific T-cells can induce reduced cytokine expression by all Th-lymphocyte subsets. In a model of IBD, the colitis induced in SCID mice by immune reconstitution with $CD45RB^{hi}$ (effector phenotype) T-lymphocytes, TGFβ appears to be important. In these mice, co-transfer with $CD45RB^{lo}$ (regulatory phenotype) T-lymphocytes that produce TGF-β prevents colitis (Powrie et al., 1996). In this colitis model, T-regs cannot suppress TGF-β-unresponsive effector T-cells that express a non-functional TGF-β receptor. However, functional T-regs develop normally in TGF-β-null mice, and these cells can suppress colitis, suggesting that although TGF-β is involved in this model, it may not be made by T-cells (Fahlen et al., 2005).

Intestinal myofibroblasts in the lamina propria synthesise the extracellular matrix, MMPs and TIMPs involved in tissue remodelling and repair of the gut. These have been implicated in the pathogenesis of CrD stricture formation and in mediating tissue damage in IBD. TGF-β decreases MMP expression and increases TIMP expression (Overall et al., 1991). Myofibroblasts isolated from patients with CrD produce higher levels of TIMP-1 than cells from normal or UC patients, with no differences in the amounts of MMP-1, MMP-2 and MMP-3 between disease groups (McKaig et al., 2003).

1.5 TGF-β1 SIGNALLING PATHWAYS

TGF-β1 signals through the ligand-dependent activation of a complex of heterodimeric transmembrane serine/threonine kinases, consisting of type I (TGF-βRI) and type II (TGF-βRII) receptors. The type III (TGF-βRIII) receptor is a co-receptor that captures extracellular TGF-β, allowing receptor ligation to occur. Upon TGF-β binding to the extracellular domain of TGF-βRIII, the type I and II receptors form heterodimers, with phosphorylation and activation of TGF-βRI by the constitutively active and auto-phosphorylated TGF-βRII (Piek et al., 1999) (Figure 3).

Once TGF-β binds to the receptor on the cell surface, the complexes are internalised by clathrin-coated endosomal vesicles. This is essential to facilitate signalling successfully (Itoh et al., 2002). The signalling pathway is facilitated by the Smad-Anchor for Receptor Activation (SARA), which binds the lipid membranes via phosphotidyl inositol 3-phosphate. SARA is present on the cell surface, so that internalisation does not have to occur before there is access to signal transduction proteins.

TGF-β signals from the receptor to the nucleus through Smad proteins, homologous to the *Drosophila sp.* Mad and the *Caenorhabditis elegans* Sma proteins. Ten different Smad proteins have been identified across all species which fall into three distinct functional sets: receptor-activated Smads (R-Smads), which include Smad2 and Smad3; a common mediator (Co-Smad), Smad4; and inhibitory Smads (I-Smads) 6 and 7 (Heldin et al., 1997; Derynck et al., 1998). Activated TGF-β1RI directly phosphorylates Smad2 and Smad3 at serine residues in the carboxy-terminal (Derynck et al., 1998; Abdollah et al., 1997). Once activated, Smad2 and Smad3 can associate with Smad4 in various combinations as hetero-trimers and then translocate to the nucleus where the complexes participate in

transcriptional control of a variety of target genes (Kawabata et al., 1998). Nucleo-cytoplasmic recycling is required to maintain active Smad complexes in the nucleus after TGF-β stimulation (Inman et al., 2002).

Targeted disruption of the Smad3 gene is associated with diminished T lymphocyte responsiveness to TGF-β1 (Yang et al., 1999). Smad3 mutant mice exhibit a massive infiltration of T lymphocytes and pyogenic abscess formation in the stomach and intestine, supporting the view that Smad3 is an essential mediator of the TGF-β-induced anti-inflammatory and suppressive activities *in vivo*. Activated R-Smad and Co-Smad complexes act as transcriptional regulator by co-activating or co-repressing gene transcription with other DNA transcription factors (eg AP-1) or independently by binding to DNA at a specific sequence.

Transgenic mice lacking intestinal epithelial TGF-β type II receptors have reduced ability to heal mucosal injury after induction of colitis (Hahm et al., 2001). They develop a severe spontaneous colitis if kept under normal conditions, but have no colitis in pathogen-free conditions. These mice had an increased susceptibility to dextran sodium sulphate (DSS)-induced colitis, which has histological features similar to ulcerative colitis in humans. Cytokine mRNA expression is higher in transgenic mice, both before and after induction of colitis, including mRNA for TGF-β. DSS also induced mucosal autoantibodies and increased expression of MCH class II markers on intestinal epithelial cells.

Smad proteins are found in many cell and tissue types and are necessary for normal embryonic development. There is cross-species homology and conserved functional roles in activin and bone morphogenic protein (BMP) signalling (Raftery and Sutherland, 1999).

In mammals, these cytokines and growth factors (which include TGF-β) have many roles, which are often cell-type specific and involved in growth and development in many organs systems. These effects, and the Smad signalling pathway, are crucial during embryonic development in mammals. In humans, the role of Smad proteins has been examined in several tissue and cell types. However, the regulation of Smad protein expression and function has yet to be fully examined in health and disease.

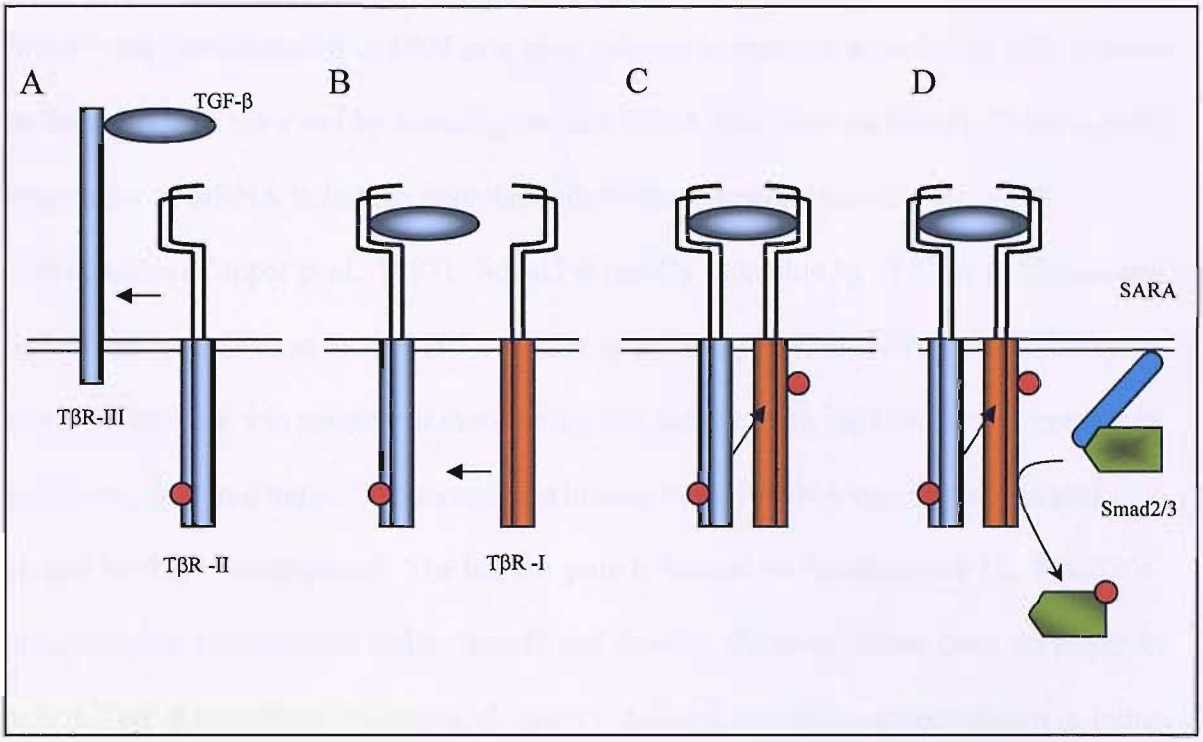


Figure 3: Mechanism of TGF-β receptor activation

(A) TGF-β1 initially binds the accessory TGF-β type III receptor (TβR-III), which is then presented to the type II receptor (TβR-II). (B) Subsequently, TGF-β bound to TβR-II, recruits TGF-β type I receptor (TβR-I) into the complex, the final receptor complex is a heteromer of two TβR-IIs and two TβR-Is. (C) TβR-II activates the TβR-I by phosphorylation (red circle). (D) TβR-I triggers downstream signals through phosphorylation of Smad2 or Smad3. Recruitment of these to the TGF-ß receptor complex is mediated by membrane associated SARA, capable of binding both Smad2/3 and the TGF-β receptor complex.

(adapted from Piek et al, 1999)

1.6 SMAD7

Smad7 was first identified in 1997 as a gene induced in vascular endothelial cells exposed to shear stress *in vitro* and by screening mouse cDNA from various tissues. There is high expression of mRNA in human vascular endothelium, as demonstrated by *in situ* hybridisation (Topper et al., 1997). Smad7 is rapidly inducible by TGF-β1 in human and mouse cell lines (Nakao et al., 1997). Tissue specific expression of Smad7 mRNA by Northern blotting was greatest in mouse lung and kidney, with high levels also present in gut, heart, liver and brain. The mouse and human Smad7 cDNA was sequenced and cloned by these investigators. The human gene is located on chromosome 18. Smad7 is phosphorylated *in vivo* and, unlike Smad2 and Smad3, phosphorylation does not affect its role in TGF-β signalling (Pulaski et al., 2001). Several stimuli have been shown to induce Smad7. These include TGF-β1, TNFα, IL-7 and INF-γ (Mori et al., 2000; Ulloa et al., 1999); (Bitzer et al., 2000; Huang et al., 2002). However, the cell type used seems to be important in determining the response in Smad7 expression. Verruchia *et al* (2000) examined the role of TNFα in antagonizing TGF-β/Smad3 signalling in dermal fibroblasts. These data suggested that neither TGF-β nor TNFα induced Smad7. This is contrary to previous reports using mouse fibroblast cell lines, which have shown Smad7 induction by LPS, IL-1β and TNFα (Bitzer et al., 2000). There is more evidence supporting the induction of Smad7 by TGF-β1 in human cells (von Gersdorff et al., 2000; Mori et al., 2000; Nakao et al., 1997). Thus the control of Smad7 expression and TGF-β signalling is tissue and cell specific.

Smad7 stability in the nucleus is enhanced by acetylation mediated by the histone acetyltransferase and transcriptional co-activator p300 (Gronroos et al., 2002).

Acetylation reduces Smad7 degradation by preventing ubiquitination at the same site by Smurf1, the ubiquitin ligase that targets the protein for degradation. This mechanism protects nuclear Smad7-Smurf complexes from being removed before export to the cytoplasm where the inhibition of TGF-β signalling occurs.

The nuclear export of Smad7 is also mediated by E3 ubiquitin ligases (Smurf1 and Smurf2) and these are required to target activated receptor complexes for degradation (Ebisawa et al., 2001; Kavsak et al., 2000). Smad7-Smurf complexes bind to the TGF-β receptor through co-operation with STRAP, a protein containing WD40 repeats that facilitate protein-protein interactions (Datta and Moses, 2000). The mechanism of TGF-β signaling inhibition by Smad7 is by directing the activated TGF-β receptor complex via endocytosis into Calveolin positive vesicles and degradation in the proteosome (Di Guglielmo et al., 2003). A balance of signal transduction blockade and receptor complex degradation allows cells to regulate their responses to TGF-β (Figure 4) (Shi and Massague, 2003).

Smad7 activity is tightly regulated. Export from the nucleus is facilitated by Smurf, through a high affinity interaction with Smad7, allowing functional activity in the cytoplasm, but also mediating ubiquitination that leads to degradation (Suzuki et al., 2002a; Kavsak et al., 2000). Acetylation at lysine residues causes increased stability of Smad7, protecting from targeting to the proteosome (Gronroos et al., 2002). The balance of these regulatory mechanisms controls the stability of Smad7 (Simonsson et al., 2005).

A transgenic mouse with ectopic Smad7 expression driven by the epithelial-specific keratin K5 promoter, has been described (He et al., 2002). This promoter targets transgene

expression in the epidermis, hair follicle, stratified and pseudo-stratified squamous epithelial tissues and in the thymus. This mouse has epithelial hyperplasia, aberrant eye development and thymic atrophy, and dies within a few days of birth. This phenotype is consistent with an important role of TGF-β in inhibiting apoptosis in epithelial tissues and a crucial role in thymic development.

Another transgenic mouse had Smad7 overexpression driven by a distal *lck* promoter, targeting expression to mature T-lymphocytes (Nakao et al., 2000). These mice have a normal developmental phenotype, but show enhanced cytokine production in response to inhaled antigen. Thus, mature T-cells that over-express Smad7 have impaired negative regulation of immune activation. The pathological effect of chemical-induced colitis or intestinal infection has not been described in these animals.

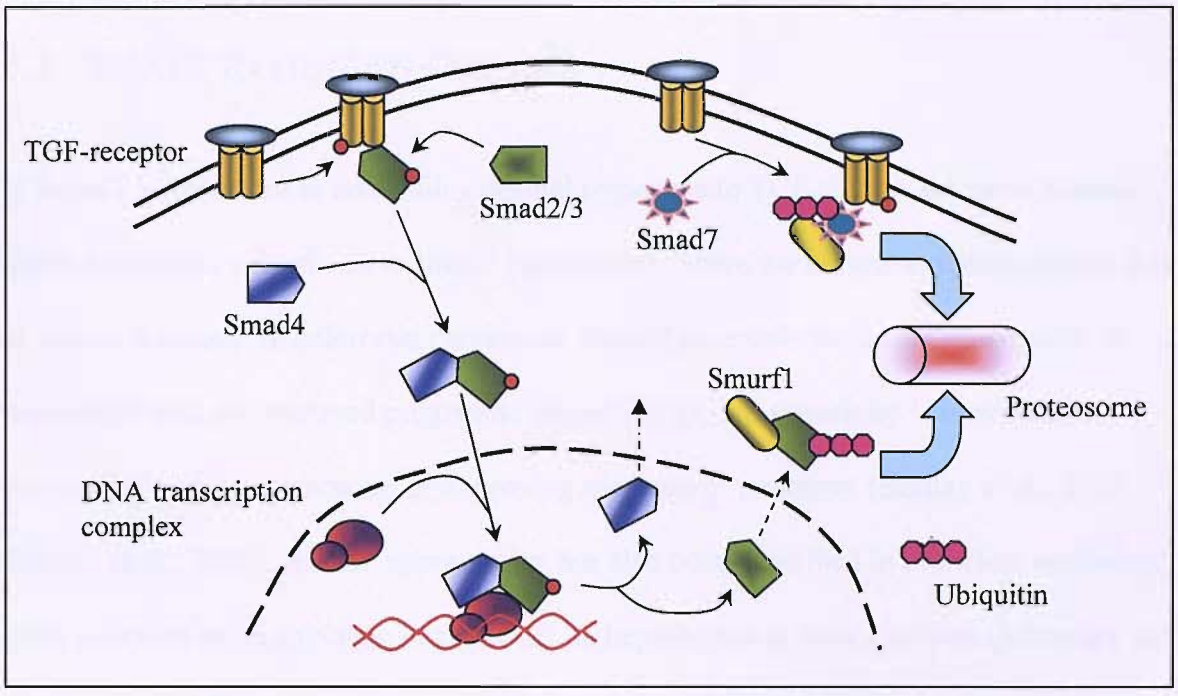


Figure 4: The Smad signalling pathway.

Activated TGF is captured by the TGF-β receptor and heterodimerisation of the type I and type II leads to phosphorylation of the type I receptors (red circles). The activated receptor complex on the cell membrane is internalised from the cell surface via clathrin-coated pits where activation of Smad2/3 and signal transduction occurs, in endosomes. The signal transduction process involves activation of Smad2/3 by phosphorylation, which forms complexes with Smad4. Smad4 allows the complex to enter the nucleus, where they affect gene transcription in co-operation with other co-activators, co-repressors and DNA binding co-factors, altering cell function. Smad4 and Smad2/3 shuttle to and from the nucleus while TGF-β receptors are active, but can be targeted for degradation in the proteosome through ubiquitination by Smurf1. Alternatively, in the presence of Smad7, the receptor complex is internalised via caveolin positive vesicles and targeted for degradation mediated by Smurf1.

(adapted from Shi and Massague, 2003).

1.7 SMAD7 IN HUMAN DISEASE

If Smad7 is important in controlling normal responses to TGF-β, then are some disease states associated with aberrant Smad7 expression? There are several examples where this is indeed the case. In colorectal carcinoma, Smad7 gene deletion in the cancer cells is associated with an improved prognosis, suggesting TGF-β sensitivity is important in preventing cancer progression or improving response to treatment (Boulay et al., 2003; Boulay et al., 2001). Smad7 upregulation has also been described in bronchial epithelial cells in severe asthma (Nakao et al., 2002), in hepatocytes in liver cirrhosis (Kitamura and Ninomiya, 2003) and in chronic renal disease (Schiffer et al., 2002). Smad7 deficiency is seen in scleroderma, a chronic inflammatory and fibrotic skin condition, with associated increased Smad3 activity and hyper-responsiveness to TGF-β (Dong et al., 2002).

1.8 SMAD7 AND GUT INFLAMMATION

TGF-β1 is present in abundance in the gut mucosa (Babyatsky et al., 1996). Monteleone *et al* (2001) observed that Smad7 protein expression is high in inflamed IBD mucosal biopsies and isolated lamina propria mononuclear cells (LPMC). In IBD LPMC, TGF-β1 was unable to induce Smad3 phosphorylation, a requirement for signal transduction. Blocking the production of Smad7 using a specific antisense oligonucleotide restored Smad3 phosphorylation in response to exogenous TGF-β1 in IBD LPMC. Thus the high levels of Smad7 expression in IBD cause unresponsiveness to TGF-β1, but the mechanisms leading to its increased expression are not known. Blocking the production of Smad7 in inflamed tissue could restore TGF-β signalling, and decrease pro-inflammatory cytokines. This suggests Smad7 as a potential target for new therapies for IBD and other inflammatory diseases. Further studies have shown that Smad7 over-expression prevents IκB-mediated inhibition of NFκB activation in LPMC isolated from IBD resection specimens (Monteleone et al., 2004a). This also suggests that Smad7 is central to the control of cellular responses to pro- and anti-inflammatory signals, tipping the balance in IBD towards pro-inflammatory responses (Figure 5). The factors that lead to the production or control the degradation of Smad7 in IBD could provide clues to the pathogenesis of the ongoing inflammation in IBD.

Smad7 is also overexpressed in mucosal biopsies from patients with *H. pylori* gastritis and Smad7 can be induced by IFNγ in normal gastric mucosal biopsies in vitro organ culture (Monteleone et al., 2004b). These data showed that Smad7 expression was associated with Th1-type inflammatory responses in the lamina propria, and that in gastritis not caused by *H pylori*, Smad7 expression was not increased.

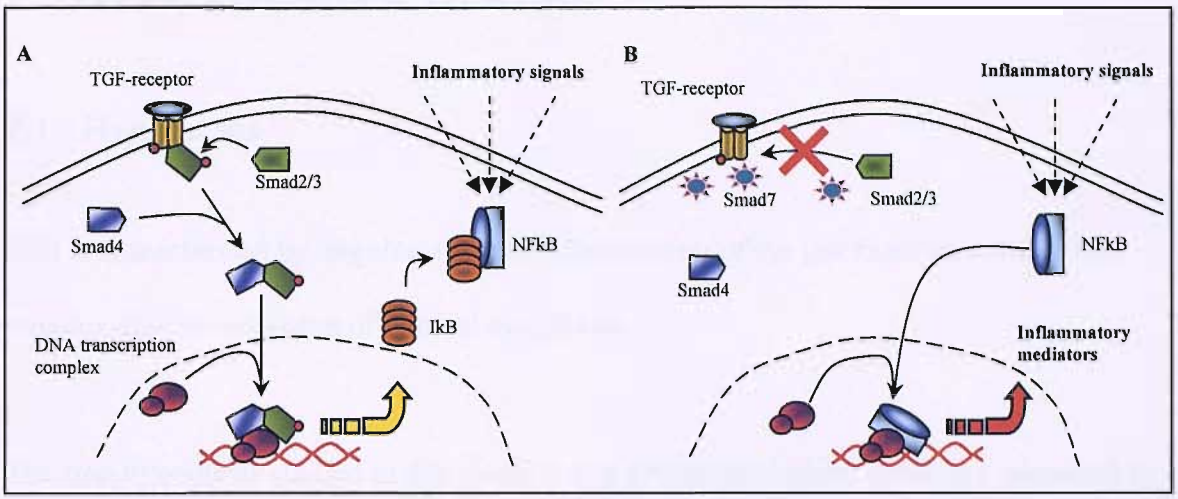


Figure 5: Cross-talk between Smad signalling and NFκB.

(A) In the absence of Smad7, TGF-β receptor activation leads to signal transduction by Smad2/3 and Smad4 to affect gene transcription, including IκB. The presence of IκB stabilises NFκB, preventing it entering the nucleus. (B) In the presence of Smad7, IκB production is reduced, permitting NFκB activation and translocation to the nucleus to induce the production of inflammatory mediators.

(adapted from Monteleone *et al*, 2004a).

2 HYPOTHESES AND AIMS

2.1 Hypotheses

IBD is characterised by ongoing chronic inflammation of the gut mucosa, which often remains despite remission of clinical symptoms.

The first hypothesis studied in this thesis is that pro-inflammatory cytokines produced by T-cells in the gut increases Smad7 expression, which makes cells unresponsive to the anti-inflammatory effects of TGF-β. Determining factors that determine increased Smad7 expression would suggest targets for new therapies.

The second hypothesis is that non-invasive assessment of ongoing gut inflammation using faecal calprotectin or ultrasound scanning would provide new measures of paediatric IBD activity in clinical practice and for assessment of therapies in clinical trials. This is particularly important in children with IBD, in whom normal growth and development is often affected when disease activity is poorly controlled.

2.2 Aims for basic science studies

1. to examine Smad7 expression in IBD
2. to establish methods to investigate Smad7 mRNA transcription in IBD
3. to assess the effects of T-cell cytokines on Smad7 protein expression

2.3 Aims for clinical studies

1. to assess the role of calprotectin as a diagnostic test for chronic gastrointestinal symptoms and for disease activity in IBD
2. to examine bowel wall thickness and mesenteric blood flow on USS in colon and terminal ileum in children with IBD

3 BASIC SCIENCE MATERIALS AND METHODS

3.1 *In vitro* culture of COS1 cells

COS1 cells (African green monkey kidney cell line transformed with SV40 virus) have fibroblast-like morphology. The cells were a gift from Dr Jelena Mann (Division of IIR, Southampton University School of Medicine). Cells are adherent to plastic *in vitro* and were cultured in 250ml flasks (Greiner) with 15ml of DMEM (Invitrogen) with 10% FCS (Invitrogen) with penicillin and gentamicin. Cells were subcultured when confluent, by treating the monolayers with typsin/EDTA (Invitrogen) at 37°C in 5% CO_2 for 1-2 minutes and splitting the cells 1:12.

3.2 Transformation of competent bacteria

Smad7 expression vector plasmid DNA was kindly provided by Prof C-H Heldin (The Ludwig Institute for Cancer Research, Uppsala, Sweden). This plasmid was based on the pcDNA3 expression vector (Invitrogen) with a cDNA insert coding for human Smad7. Dr J Mann (Division of IIR, University of Southampton School of Medicine) kindly provided empty pcDNA vector (Appendix A). These plasmid expression vectors allow transfected cells to produce proteins under the control of a CMV promoter and also codes for ampicillin resistance. 0.1-1μg of plasmid (5μl + 20μl of the resuspended plasmid DNA in TE buffer) was added to 50μl JM109 competent cells (in rubidium chloride buffer). The mix was allowed to incubate on ice for 30 minutes. The water bath was set at 42°C, and the cells subjected to heat shock for 45 seconds. After this treatment, 350μl of SOC medium (a rich bacterial culture medium that improves bacterial transformation) was added, and the tube placed in a shaking incubator at 37°C for 90 minutes. The cells were plated out at different dilutions on agar plates, with the surface smeared with 300μg ampicillin. Plates are sealed with Parafilm, and incubated at 37°C overnight (16 hours).

3.3 Miniprep of plasmid DNA

Individual colonies of bacteria selected by ampicillin resistance by ovenight culture on agar plates after JM109 cell transformation are selected for DNA extraction. Each colony was placed in 5ml of LB growth medium with 500μg ampicillin and incubated at 37°C for 6 hours with agitation. Plasmid DNA was purified using the Wizard Plus Miniprep system (Promega) per the manufacturer's protocol. Cells in LB medium (3ml) were pelleted by centrifugation at maximum speed and then in resuspended in 200μl of Cell Resuspension Solution. 200μl of Cell Lysis Solution was added and then the tube inverted to mix. Neutralization Solution (200μl) was added and again mixed by inverting. The lysate was centrifuged at maximum speed for 5 minutes. DNA binding resin (1ml) was prepared in a 3ml Luer-lock syringe barrel attached to the Minicolumn, then the lysate added. The resin/lysate mix was syringed through the Minicolumn. Wash Solution (2ml) containing ethanol was pushed through the Minicolumn, and then transferred to a 1.5ml Eppendorf tube for centrifugation at maximum speed for 2 minutes. DNA bound in the column was eluted with 50μl of nuclease-free water by centrifugation at maximum speed into a clean 1.5ml Eppendorf tube after incubation at room temperature for 1 minute. Plasmid DNA was stored at -20°C.

3.4 Maxiprep of plasmid DNA

Transformed JM109 cells with Smad7-pcDNA3 and empty-pcDNA (300µl) were prepared for DNA purification with Qiagen HiSpeed Maxiprep kit. First the cells were cultured overnight by incubation in 100ml of LB broth with 10mg ampicillin at 37°C with agitation. The resulting broth was centrifuged at 6000g and the cells resuspended in 10ml Buffer P1, lysed by the addition of 10ml Buffer P2 and mixing by inversion. The mixture was neutralised and protein precipitated by mixing with 10ml chilled Buffer P3 and transferred to the filter syringe for 5 minutes before filtration. The anion exchange resin was equilibrated with buffer for 5 minutes before the cell lysate was plunged through gently. The cleared filtrate was allowed to enter the resin under gravity. The resin was washed through with 60ml of Buffer QC. DNA was eluted with 15ml of Buffer QF. The resulting DNA suspension was precipitated with 10ml of isopropanol, and the mixture filtered through the QIAprecipitator under vacuum. The DNA was eluted from the column with 0.5ml TE buffer and measured by optical density at 260nm. Purified plasmid DNA was stored at -20°C.

3.5 Restriction digests of plasmid DNA

The expression vector for human Smad7 has a cDNA insert of 1.9 Kbp (Heldin, personal communication). The pcDNA-3 plasmid is 5.4Kbp in length. Restriction enzymes cut the DNA at specific sites, allowing the presence of the insert to be checked. Plasmid DNA was subject to restriction enzyme digestion by EcoR1, Xba1 or both enzymes. Both these enzymes have a single restriction site in the pcDNA3 plasmid (Appendix A). Plasmid DNA samples were incubated in 0.5ml PCR tubes at 37°C for 1 hour, in 20μl reaction volumes with 1μl of EcoR1, Xba1 or both. The resulting DNA fragments were separated by electophoresis on a 1% agarose gel with ethidium bromide in 0.5x TBE buffer and visualised by UV fluorescence.

The restriction enzymes cut the plasmid DNA, allowing it to linearise. The plasmid containing Smad7 cDNA is longer than the 5.4kbp long empty pcDNA3 vector, unless subject to digestion by both EcoR1 and Xba1, whose combined action excised a shorter DNA fragment, with the 1.9kBP length of the cDNA sequence for Smad7 (Figure 6).

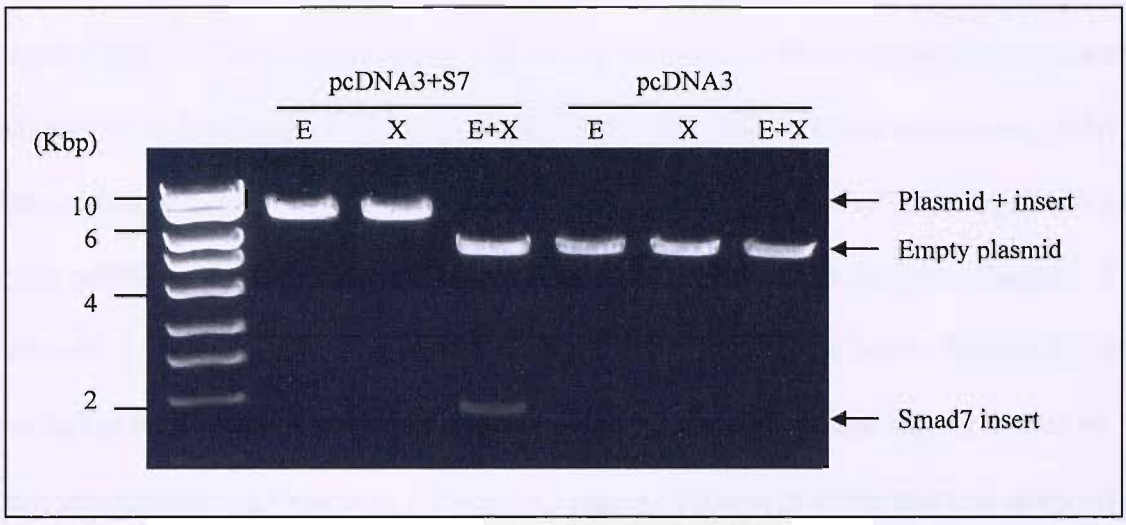


Figure 6: Restriction enzyme digests of pcDNA3 plasmid vectors.

Plasmid DNA was incubated with EcoR1 (E), Xba1 (X) or both enzymes (E+X). The first lane shows a standard ladder. The 1.9 Kbp Smad7 cDNA insert was excised by the combination EcoR1 and Xba1.

3.6 Transfection of COS1 cells

Transfections of COS1 cells was performed in 6-well tissue culture plates (Greiner), with cells seeded at $2x10^5$ cells/well with 1.6ml DMEM with 10%FCS and antibiotics. After overnight culture, the cells reached 70-80% confluent. Tranfection of Smad7-pcDNA and empty pcDNA plasmids was performed using liposomal Effectene Reagent (Qiagen). For each well, 1μg DNA was dissolved to 100μl in DNA condensation buffer (Buffer EC) and then 8μl of Enhancer added with brief mixing by vortex. The mixture was incubated at room temperature for 5 minutes. Effectene Reagent (10μl) was added and mixed by vortex for 10 seconds. While this was left to incubate at room temperature for 10 minutes, the cell culture medium removed and replaced with 1.6ml of DMEM with 10% FCS and antibiotics. 600μl of DMEM with 10% FCS and antibiotics was added to the DNA-Effectene mix and mixed gently by pipette, then added dropwise to the cell culture medium (700μl per well). The 6-well plates were incubated at 37°C in 5% CO_2 for 48 hours before harvesting the cells. First, the adherent cells were washed with 2ml PBS per well, and harvested by scraping in 200μl of PBS per well. The resulting cell suspensions were pelleted by centrifugation at 735x g in a 1.5ml Eppendorf tube, the supernatant removed and the cells snap frozen in liquid nitrogen. Cell pellets were stored at -80°C prior to protein extraction.

3.7 Colonoscopic mucosal biopsies

Fresh and snap frozen mucosal biopsies were obtained from children undergoing endoscopic investigations for chronic intestinal symptoms, or for assessment of disease activity, according to clinical need as determined by the attending physician. Specific informed and written consent was obtained prior to the procedure for research biopsies in addition to normal diagnostic biopsies. Local ethics committee approval was sought and granted prior to the study's commencement (LREC protocol number 130/01). Standard clinical and pathological criteria were applied for the diagnosis of IBD. Biopsies were defined as "Normal" where no histopathological changes consistent with a diagnosis if IBD were present in any of the diagnostic biopsies taken at the same attendance as the research biopsies. No subjects were receiving anti-TNFα therapies.

3.8 Isolation of cells from intestinal operative resection specimens

Normal mucosa was obtained from bowel resection material distant from resection margins and not within 2cm of any suspected pathological lesion, such as carcinoma or diverticular disease. Diseased mucosa was obtained from resected bowel from patients undergoing elective or emergency operative treatment of IBD. In the case of adults, patients gave specific informed consent for the use of tissue for research where surplus to diagnostic requirements (Southampton University Hospitals Trust patient consent for operative procedure form). For children under the age of 16 years undergoing operative resections, specific consent was obtained for research tissue in addition to the standard consent for the operative procedure from their parent/guardian. Research tissue specimens (surface area 50-100cm^2) were obtained fresh and transported to the laboratory in tissue culture medium (RPMI 1640 with 10% FCS, penicillin, streptomycin and gentamicin).

Isolation of LPMC was as described (Breese et al., 1993).The mucosa was dissected with scissors from the submucosa after washing the tissue with Calcium- and Magnesium-free Hank's Buffered Saline Solution (CMF-HBSS) (Invitrogen). The mucosal strips were washed in 100ml 0.1M DTT in CMF-HBSS with penicillin, streptomycin and gentamicin at room temperature for 15 minutes with stirring to remove mucous and debris. The clean mucosa was minced with crossed scalpel blades into 2-3mm diameter pieces on a sterile 100mm Petri dish and washed with 0.1M EDTA (pH 8.0) in CMF- HBSS with antibiotics at 37°C for 30 minutes with constant stirring. The supernatant, containing epithelial cells and IEL, was decanted from the second and third washes, after debris and bacteria has been washed from the epithelium. These washes were repeated at least twice more or until the supernatant was clear. The remaining tissue fragments were retrieved with forceps,

minced finely with crossed scalpel blades and transferred to a clean 100ml pot. The tissue fragments were incubated in 1mg/ml collagenase type I (Sigma) in RPMI with 10% FCS (Invitrogen) and antibiotics at 37°C with constant stirring for 3 hours or until digested. The resulting cell suspension was decanted, passed through a 100μm filter and the cells pelleted by centrifugation (300x g for 10 minutes). The cells were washed with CMF-HBSS with antibiotics twice before being prepared for separation by density.

Percoll (Sigma) mixtures were prepared at different densities (see table). The washed cells were resuspended in 4-6ml 40% Percoll, depending on the size of the cell pellet. Discontinuous Percoll gradients were prepared with 100%, 60%, 40% and 30% Percoll, using 2ml of each layered in a 15ml Falcon tube, taking care not to disrupt the interface between layers (see Table 2). The cells obtained after collagenase digestion were resuspended in 40% Percoll with CMF-HBSS. After centrifugation without brake at 450x g for 25 minutes, the 40%/60% interface holds low density LPMC (containing fibroblasts) and the 40%/60% interface holds the higher density LPMC (predominantly lymphocytes). The cells were harvested and washed with CMF-HBSS at 550x g. The cells were then resuspended after a further wash in serum-free RPMI with antibiotics. Viability was determined by Trypan blue exclusion. The viability of LPMC was rarely less than 90%. LPMC yields from resection specimens were 5-60 $x10^6$ cells, typically 15-20 $x10^6$ cells.

Table 2: Preparation of Percoll (Sigma) dilutions for density separation

100% Percoll (50ml)	45ml Percoll + 4ml 10x PBS + 1ml CMF-HBSS
60% Percoll (10ml)	6ml 100% Percoll + 4ml CMF-HBSS
40% Percoll (10ml)	4ml 100% Percoll + 6ml 1x PBS
30% Percoll (10ml)	3ml 100% Percoll + 7ml CMF-HBSS

3.9 *Ex vivo* short-term culture of LPMC

LPMC from the 40%/60% interface were cultured in RPMI (Invitrogen) with antibiotics supplemented with 10% FCS (Invitrogen) or HL-1 (Sigma). HL-1 is a serum-free, low protein culture medium supplement containing growth-promoting substances including insulin, transferring, testosterone, fatty acids and minerals. It used as a serum replacement for lymphoid cells. For *ex-vivo* experiments, cells were resuspended at 1-2 $x10^6$ cells/ml in sterile cell culture plates. Cell cultures were maintained at 37°C in a 5% CO_2 atmosphere for up to 72 hours. Cultures were harvested and cooled on ice prior to pelleting cells by centrifugation. Cell pellets were stored at -70°C before protein extraction.

3.10 Extraction of total cellular protein

Hypotonic cell lysis buffer with protease and phosphatase inhibitors was prepared with HEPES 10mM (pH 8.0), EDTA 1mM (pH 8.0), potassium chloride 60mM, 0.2% Nonidet NP-40, DTT 1mM, PMSF 1mM, Aprotinin 10μg/ml, Leupetin 10μg/ml, activated sodium vanadate 1mM and sodium fluoride 1mM. Snap frozen cell pellets or biopsies were homogenised by serial passage through needles (19G to 26G) in lysis buffer on ice. Cell pellets were lysed in 100-150μl of lysis buffer, whereas biopsies were lysed in 200-300μl buffer. The resulting suspension was centrifuged at 1700x g for 30 minutes at 4°C. The supernatant was saved for protein measurement using 1ml BioRad solution (diluted 1:5) per sample with spectrophotometric analysis by optical density at 595nm. Protein standards for comparison were immunoglobulin (BioRad), using 7-35μg/ml for the standard curve. An equal volume of lysis buffer was added to the samples of standard protein before comparison to the protein lysates. Aliquots of protein lysate (1-20μl) were

used for protein measurement, in order that the test samples provide optical density readings within the linear range of the standard curve.

3.11 Western blotting by SDS-PAGE

Protein separation was by polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli, 1970). The reagents used are listed in Appendix B. Protein lysates were diluted with lysis buffer to equal concentrations in order for samples with equal protein content to have equal volumes for loading sample wells. The lysates were boiled for 5 minutes in an appropriate volume of sample buffer containing β2-mercaptoethanol to reduce disulphide bonds and denature proteins. SDS-PAGE gels were prepared with a 5% stacking gel and 10% running gel and electrophoreses was in Tris-Glycine buffer. The stacking gel was run at 50mA and the separating gel initiated at 100mA, with the voltage kept constant throughout. The protein was transferred from the gel to nitrocellulose (NC) membrane (BioRad) at 100V in chilled transfer buffer for 1 hour. Ponceau's stain (0.1% w/v Acid Red 150 (Sigma) in 5% v/v acetic acid) of the membrane was used to assess transfer efficiency and equal protein loading prior to immunoblotting. Non-specific antibody binding to the NC membrane was blocked with 5% w/v fat free milk in TBS with Tween20 0.05% v/v (TBS-T) at room temperature for 1 hour.

Rabbit anti-Smad7 (H-79) polyclonal (2mg/ml, Santa Cruz Biotechnology) was used at 1:400 dilution, as determined in previous work by Monteleone *et al* (personal communication). The optimal concentration of Smad7-specific antipeptide polyclonal rabbit serum (a gift from Prof C-H Heldin, Uppsala, Sweden) was determined by titration (see Section 4.1.2). Primary antibody was diluted in 5% milk in TBS-T and incubated for 12-14 hours at room temperature with constant agitation. The membrane was washed at

least 3 times with TBS-T for 20-30 minutes between primary and secondary antibody incubations. The secondary antibody was goat anti-rabbit HRP conjugate (Immunopure, Pierce) used at 1:20000 final dilution in 5% milk in TBS-T. Incubation with secondary antibody was for 2-3 hours at room temperature. Nitrocellulose membranes were again washed at least 3 times for 20-30 minutes with TBS-T before being prepared for chemiluminescent detection with ECL-Plus (Amersham) or DURA (Pierce) kits. Highly sensitive photographic film was used for visualisation (Hyperfilm-ECL, Amersham).

Actin was used as an internal loading control. Nitrocellulose membranes were stripped of primary and secondary antibody prior to re-probing. Membranes were sealed in plastic with 10ml stripping solution (2% SDS, 62.5mM Tris-HCl (pH6.8) and 100mM β2-mercaptoethanol). Membranes were then incubated in a waterbath at 60°C, for 30 minutes. After stripping, membranes were washed 3 times for 20-30 minutes with TBS-T. Blocking was with 5% milk-TBST for one hour then membranes were incubated with rabbit polyclonal anti-actin antibody H-196 (200μg/ml, Santa Cruz Biotechnology) at 1:500 dilution in 5% milk-TBST. Development was as previously described, with ECL Plus (Amersham).

3.12 Smad7 specific anti-peptide antibody

An immunogenic peptide sequence from Smad7 has been previously identified and used to produce a polyclonal anti-Smad7 rabbit serum by Prof Heldin's group at the Ludwig Institute in Uppsala, Sweden (personal communication). The 19 amino acid sequence is: KAFD YEKAYSLQRP NDHEF. This sequence maps near the c-terminus of the protein, within the conserved MH2 region, critical to the protein's function in negative regulation of TGF-β signalling (Hanyu et al., 2001).

This peptide was synthesised and conjugated to Keyhole Limpet Haemocyanin (KLH) for immunisation of 2 rabbits by Moravian Biotechnology Ltd (Czech Republic). Sera collected after 2nd and 3rd immunisations were tested using Western blotting against lysates of human gut tissue and transfected COS cells. Affinity purification against the peptide was performed after the 4th immunisation. The results obtained with these antibodies are shown in Appendix D.

3.13 Design of PCR primers for human Smad7

Oligonucleotide PCR primer sequences were chosen with the aid of a computer software model (Primer Designer 4, Sci Ed Software) using the nucleotide sequence for human Smad7 published in the NCBI RefSeq DNA sequence database (gi:2252821).
The primer sequences chosen were:

Forward primer: 5'-CATCACCTTAGCCGACTCTG-3'

Reverse primer: 5'-TGTACGCCTTCTCGTAGTCG-3'

These were selected because they met criteria for effective PCR reaction primer function. These criteria were the predicted melting point (70-75°C), GC content (50-60%) and the absence of hairpins or dimers at annealing temperatures. The forward primer sequence is located in exon 1 of the Smad7 gene and the reverse primer in exon 4. The predicted PCR product from cDNA is 540 base pairs long. The genomic DNA product is predicted to be over 2000 base pairs long. The primer sequences were checked for specificity against the published human consensus DNA sequences from the Human Genome Project (NCBI RefSeq Database).

3.14 Purifying and sequencing the Smad7 RT-PCR product

The PCR product obtained with an annealing temperature of 61°C was purified after separation on a 1% agarose gel with ethidium bromide (1µl per 100ml). The purification was using QIAquick DNA purification kit as per the manufacturer's protocol (Qiagen). The PCR product DNA was cut within a section of the agarose gel with a weight of 300mg. Buffer QC (900µl) was added to dissolve the agarose at 50°C for 10 minutes. Then 300 µl of isopropanol was added. The sample was transferred to the QIAquick spin column, centrifuged at high speed and the flow-through discarded. 0.5ml of Buffer QG was washed through the column to remove all agarose. Buffer PE (0.75ml) was added to the column, which was left to stand for 5 minutes to equilibrate. The column was centrifuged at high speed and the flow-though discarded. The dry column was then centrifuged again to remove all traces of ethanol. The column was then transferred to a clean 1.5ml Eppendorf tube and DNA eluted twice by centrifugation with 50µl nuclease-free water.

The purified DNA was sent to the Advanced Biotechnology Centre at Imperial College, London, for PCR-based direct sequencing (Appendix C). The product sequence was read with both primers and the resulting sequences submitted to NCBI Nucleotide Blast to compare specificity against the published human consensus DNA sequences from the Human Genome Project (NCBI RefSeq Database).

3.15 Total RNA extraction from biopsies

Whole mucosal biopsies (obtained with 2mm colonoscopic forceps) were lysed in 1ml Trizol reagent by serial passage through 19-26G needles. Chloroform (200µl) was added and the mixture shaken for 15 seconds then incubated at room temperature for 2 minutes. After separation of the phases by centrifugation at high speed for 15minutes at 4°C, the upper layer was removed carefully by pipette. Isopropanol (500µl) was added and mixed by vortex. This mixture was kept at -20°C overnight to precipitate RNA. After centrifugation at high speed for 10 minutes at 4°C, the RNA pellet was washed with 1ml 75% ethanol and RNA dissolved in 20-40µl nuclease-free water for measurement by optical density at 260nm. RNA quality was determined by 260:280nm absorbance (ratio 1.6-2.0) and by visualisation of 18S and 28S bands after separation by electrophoresis on a 1% agarose gel with ethidium bromide in 0.5x TBE buffer.

3.16 Semi-quantitative RT-PCR for Smad7 and β-actin

Reverse transcription of RNA into cDNA was performed using M-MLV reverse transcriptase (RT) per the manufacturer's protocol (Invitrogen). RNA preparations were mixed with 1µl of oligo-dT (mRNA specific primer), and nuclease-free water to 12 µl in a thin walled 0.5ml PCR tube and the RNA linearised by heating to 65°C for 15 minutes followed by chilling on ice. A 20µl RT reaction volume was completed with the addition of x5 RT-buffer (Invitrogen), 2µl 0.1M DTT (Invitrogen), 1µl of 0.1M dNTPs and 1unit of RT (Invitrogen). Reactions were incubation for 5 minutes at 25°C, then 60 minutes at 37°C and termination by heating to 72°C. Resulting cDNA samples were stored at -20°C.

Primer sequences (Sigma Genosys) specific for β-actin were:

Forward: 5'-CGAGGCCCAGAGCAAGAGA

Reverse: 5'-CACAGCTTCTCCTTAATGTCACG

PCR was in 50μl reaction volumes under mineral oil in thin walled PCR tubes. Nuclease-free water, 10x PCR buffer with magnesium chloride (Promega), primer pairs, dNTPs and Taq polymerase (Promega) were premixed and equal volume aliquots were used in experiments. PCR for Smad7 had an initial denaturation for 3 minutes at 96°C. Cycles were denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 1 minute, with a final extension step at 72°C for 5 minutes. Samples were stored at 4°C. For β-actin, initial denaturation at 96°C for 3 minutes was followed by cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds. A final extension at 72°C was performed for 5 minutes.

4 CLINICAL STUDIES: PATIENTS AND METHODS

4.1 Calprotectin

One hundred children aged 4-17 years (median 12 years) were recruited from children attending the paediatric gastroenterology clinic and ward referrals to the gastroenterology team between January 2002 and April 2003. Approval was granted by the local and regional ethics committee, and participants gave informed written consent. Samples supplied by out-patients were collected at home and sent the same day by post, and from in-patients transported at room temperature, to the laboratory for storage at -20°C until analysis. Faecal calprotectin was measured using the Calprest® ELISA-based testing kit (Eurospidal, Trieste, Italy). Samples were tested in duplicate using 80-120mg of stool according to the manufacturer's instructions. The lower limit of detection for the assay is 15.6μg/g. CrD activity was assessed using PCDAI (Hyams et al., 1991). UC disease activity was using Colitis Symptom Score (CSS) (Beattie et al., 1996). Patient recruitment, clinical data collection and laboratory analysis of calprotectin using Calprest® kits was undertaken by Ms Sohere Patel and Ms Rebecca Robinson, 4th Year Medical Students.

Forty three children with IBD, defined by standard clinicopathological criteria, provided stool samples (CrD n=29, age median 13 years, range 9-16, male=20; UC n=14, age median 12 years, range 8-16, male=5). There were 12 newly diagnosed patients, with 31/43 (72%) taking a 5-asa derivative and 14/43 (33%) taking azathioprine. None were being treated with corticosteroids when samples were obtained. CrD was confined to the ileum in 10, ileocolonic in 7, colonic in 10 and 1 had oral disease. Serum C-reactive protein (CRP) levels were measured in all children with IBD.

Comparative groups were: seven "normal" children without bowel pathology (age median 11, range 5-14, male=4), thirty one with longstanding functional constipation taking regular laxatives (age median 9, range 4-16, male=14) and nineteen children with other GI diagnoses (age median 10, range 4-15, male=5). Comparison between groups was using the Mann-Whitney U-test. Differences were considered statistically significant where $p<0.05$.

4.2 Ultrasound scanning

Subjects were recruited from the regional paediatric gastroenterology clinic when either barium meal and follow though or endoscopy was clinically indicated for diagnosis of suspected IBD or assessment of known IBD. Informed written consent was obtained from participants at recruitment. The Local and Regional Ethics Committee approved the study (protocol 436/02). All had an abdominal USS prior to their other investigations. The USS were performed and reported by the radiologist blind to the results of the other investigation. All subjects had study investigations during the same period of disease activity. Clinical disease activity was assessed depending on final diagnosis using PDCAI for Crohn's disease and colitis score for ulcerative colitis or indeterminate colitis (Beattie et al., 1996; Hyams et al., 1991).

A standard pre-procedure bowel preparation prior to endoscopy was using sodium picosulphate solution (2 sachets) 24 hours prior to procedure, followed by clear fluids only. Subjects were fasted for 6 hours prior to BaFT, without bowel cleansing.

USS were performed with standardised reporting issued by a radiologist (Dr J Fairhurst, Dr M Griffiths, or Dr J Argent, Southampton General Hospital) prior to either barium studies

or colonoscopy. Following conventional abdominal USS with particular reference to the hepatobiliary system using a 5-7.5 MHz transducer as appropriate for patient size, the bowel was examined using a 10 MHz linear probe (Toshiba Aplio). The right iliac fossa was examined and the terminal ileum identified. The entire colon was then reviewed systematically. Bowel wall thickness was measured from serosa to lumen, with separate measurements taken for each colonic segment and terminal ileum. Measurements were obtained from: caecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. Superior mesenteric artery (SMA) flow was measured using Doppler analysis, using the Resistive Index (RI). The result was not known by either the radiologist undertaking barium examination or the endoscopist prior to the procedure.

Colonoscopy was performed by Dr RM Beattie (Consultant Paediatric Gastroenterologist, Southampton General Hospital) and was reported during withdrawal of the endoscope by consensus between two clinicians (Dr RM Beattie and Dr AR Bremner) blind to the USS result by anatomical bowel segment for macroscopic disease severity, and overall using a 10-point analogue global severity scale (GSS). Mild disease was defined as mild erythema of the mucosa with loss of normal vascular pattern. Moderate disease was superficial ulceration or contact bleeding. Severe lesions were deep ulceration, spontaneous bleeding or stenosis.

Barium studies were performed with per-oral pneumocolon if required to provide double-contrast imaging of the terminal ileum. Imaging was performed in the Department of Paediatric Radiology, Southampton General Hospital. Studies were performed blind to USS results, supervised and reported by one of three paediatric radiologists (Dr J Fairhurst, Dr M Griffiths, Dr J Argent). Abnormalities considered consistent with IBD included

thickened bowel loops, ulceration and strictures. Abnormalities consistent with lymphoid nodular hyperplasia were not considered diagnostic of IBD.

Comparison between BWT and endoscopic severity was using one-way ANOVA, followed by Bonferroni's multiple comparison t-tests between groups. Comparison between BaFT abnormality and BWT was using t-tests. Pearson's test was used for correlations. Results were considered statistical significant where $p<0.05$.

5 RESULTS AND DISCUSSION: LABORATORY STUDIES

5.1 EVALUATION OF ANTI-SMAD7 ANTIBODIES

Preliminary experiments with a commercial rabbit polyclonal anti-Smad7 antibody (Santa Cruz H-79) showed that there were multiple protein bands detected on Western blotting, and the band at the expected apparent molecular weight (51kD) was indistinct. The specificity of this and other sera were evaluated against a standard protein prepared by tranfection of COS1 cells with a Smad7 cDNA expression vector (a gift from Prof C-H Heldin, Uppsala, Sweden). The plasmid was tested to ensure it contained the cDNA insert and that it could induce over-expression of Smad7 in COS1 cells using a specific rabbit antiserum kindly provided by Prof Heldin's group. The commercially available polyclonal antibody, also raised in rabbit (H-79), was also tested for comparison using the same secondary antibody for detection.

5.1.1 Titration of anti-Smad7 rabbit serum in COS cell lysate

A Smad7-specific anti-peptide rabbit serum was evaluated (serum supplied by Prof Heldin, Uppsala, Sweden). The transfected COS cell lysates with Smad7 (+S7) or with empty vector (-S7) were used to assess serum dilutions for the detection of Smad7 by Western blotting. Each lane was loaded with 20µg of protein.

The recombinant Smad7 protein was detected at all dilutions, with antiserum at 1:2000 detecting a single protein band at 43kD not present in the cells transfected with the empty pcDNA vector (Figure 7). Specificity was best at 1:2000, since at dilutions of 1:500 and 1:1000, the serum detects bands in both lysates.

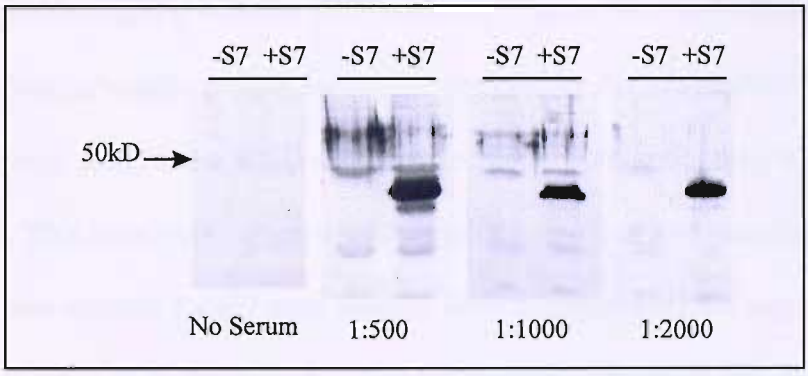


Figure 7: Antibody titrations for Smad7 in COS cell lysates.

Western blots of COS cell lysates with and without recombinant Smad7 protein, using Heldin's anti-Smad7 serum. There are no bands detected in the absence of primary antibody. At all dilutions, the serum detects a 43kD band in the Smad7-transfected cells (+S7) not present in the control lysate (-S7).

5.1.2 Comparison of Smad7 antibodies against recombinant protein

COS cell lysates after transfection with pcDNA3 plasmid with and without Smad7 cDNA were used as standards to compare the specificity of the rabbit polyclonal antibody (Santa Cruz) and the anti-peptide serum from Prof Heldin's group (Uppsala, Sweden).

Twenty μg of protein from COS cells transfected with Smad7-coding expression vector and empty vector, were tested using the affinity-purified rabbit polyclonal anti-Smad7 H-79 antibody (1:400) and Heldin's Smad7 anti-peptide rabbit serum (1:2000).

Both antibody reagents, the antiserum and the affinity-purified antibody, recognised a protein at 43kD in the Smad7-overexpressing cells, not detected in the cells with empty vector (Figure 8). There was detection of other bands with both reagents, with more apparent non-specific binding with the H-79 antibody (Figure 8a). H-79 detected several other higher molecular weight protein bands, not detected by the antipeptide serum (Figure 8b). There are bands close to the 50kD marker detected by both antibodies, though less clearly by H-79. This band may represent endogenous Smad7, or non-specific cross-reactivity. The unprocessed Smad7 gene product from consensus cDNA sequences has a molecular weight of 46.4kD. Known sites for post-translational modifications include (serine-249), acetylation (lysine-64 and lysine-70) and ubiquitination (Pulaski et al., 2001; Gronroos et al., 2002). These alterations are likely to affect its apparent molecular weight on Western blotting. In order to investigate the nature of this protein band, as Smad7 or another non-specific protein, a monoclonal antibody could be raised against recombinant Smad7 protein. These reagents could then be used to immunoprecipitate Smad7 from human tissue lysates and the resultant purified protein then subjected to Western blotting

with the alternative Smad7 reagents (monoclonal, polyclonal or affinity-purified). Blocking studies using recombinant protein or Smad7-specific peptides could confirm antibody specificity, but cannot exclude cross-reactivity to a different protein with the same epitope. To improve confidence in specificity, the immunoprecipitated proteins could be separated by 2-dimensional electrophoresis or analysed using mass spectrometry.

The small amount of anti-peptide serum provided by Prof Heldin's group was not sufficient to undertake further experiments. H-79 detects Smad7 in COS cell lysates, and was used to investigate Smad7 expression in human specimens. Antibodies were raised against the same peptide sequence used by Heldin's group for use in future experiments. Evaluation of these antibodies is presented in Appendix D.

(a) Heldin serum (1:2000)

(b) St Cruz H-79 (1:400)

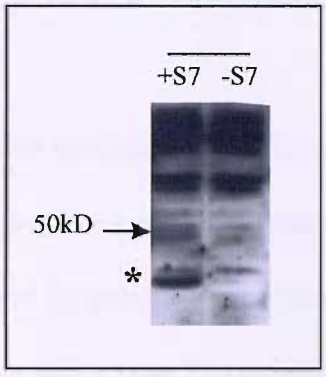


Figure 8: Comparison of Smad7 antibodies in COS cell lysates.

Western blots of COS cell lysates, transfected with Smad7-coding expression vector (+S7) and empty vector (-S7), using Heldin's Smad7 anti-peptide rabbit serum (a) and rabbit polyclonal anti-Smad7 H-79 antibody (b). Both antibodies recognised a protein at 43kD (*) from the Smad7-overexpressing cells, not detected in empty vector lysate. There are higher molecular weight non-specific bands detected by the H-79 antibody, not detected by Heldin's serum.

5.2 SMAD7 PROTEIN EXPRESSION IN IBD

5.2.1 Introduction

Monteleone *et al* found that Smad7 is overexpressed in IBD (Monteleone et al., 2001). The following experiments aim to repeat this observation using the commercially-available rabbit polyclonal anti-Smad7 peptide antibody H-79 (St Cruz Biotechnology, USA).

These experiments aim to examine whether that the Smad7 specific antibodies show in human tissue the same changes in Smad7 expression between normal and IBD. Snap frozen gut mucosal biopsies were homogenised in lysis buffer, and the protein lysate subjected to Western blotting for Smad7 using affinity-purifed polyclonal rabbit anti-peptide Smad7 antibody H-79. Band intensity was quantified with a computer software package (Quantity One, BioRad).

5.2.2 Results

Biopsies were obtained from 33 children aged 9-17years old (median 13). 19 subjects were male. Children were undergoing assessment for diagnosis (n=19) or known IBD (n=14). Details of medications were not recorded.

Snap frozen colonoscopic mucosal biopsies were homogenised in lysis buffer, and the protein lysate subjected to Western blotting. Each lane had 50μg of protein loaded. Three separate experiments were undertaken, with 9 normal biopsies, 14 with CrD and 10 with UC. Immunoblotting was with StCruz H-79 rabbit polyclonal anti-Smad7 (diluted 1:400). Each lane had 50μg of protein loaded, with equal transfer confirmed by Ponceau's staining prior to immunoblotting. Blots were stripped and re-probed for actin, as an internal loading control. Band intensity data is presented as a ratio of intensity to actin.

Smad7 is seen in all human gut biopsy specimens at a calculated molecular weight of 51kD, and there is generally higher expression in biopsies from patients with IBD, reflected by increased signal density from chemiluminescent detection reagents with Smad7 antibody stained Western blots (Figure 9). There were statistically significant differences between mean Smad7 expression in CrD (n=14, Student's t-test, p=0.01) and in UC (n=10, p=0.002), compared to normal gut (n=9). The H-79 antibody detects a series of bands between 49kD and 51kD, and these may represent the effects of post-translational modifications, including phosphorylation and acetylation. The effect of acetylation is to stabilise Smad7, preventing the poly-ubiquitination that targets it for degradation by the proteosome (Gronroos et al., 2002).

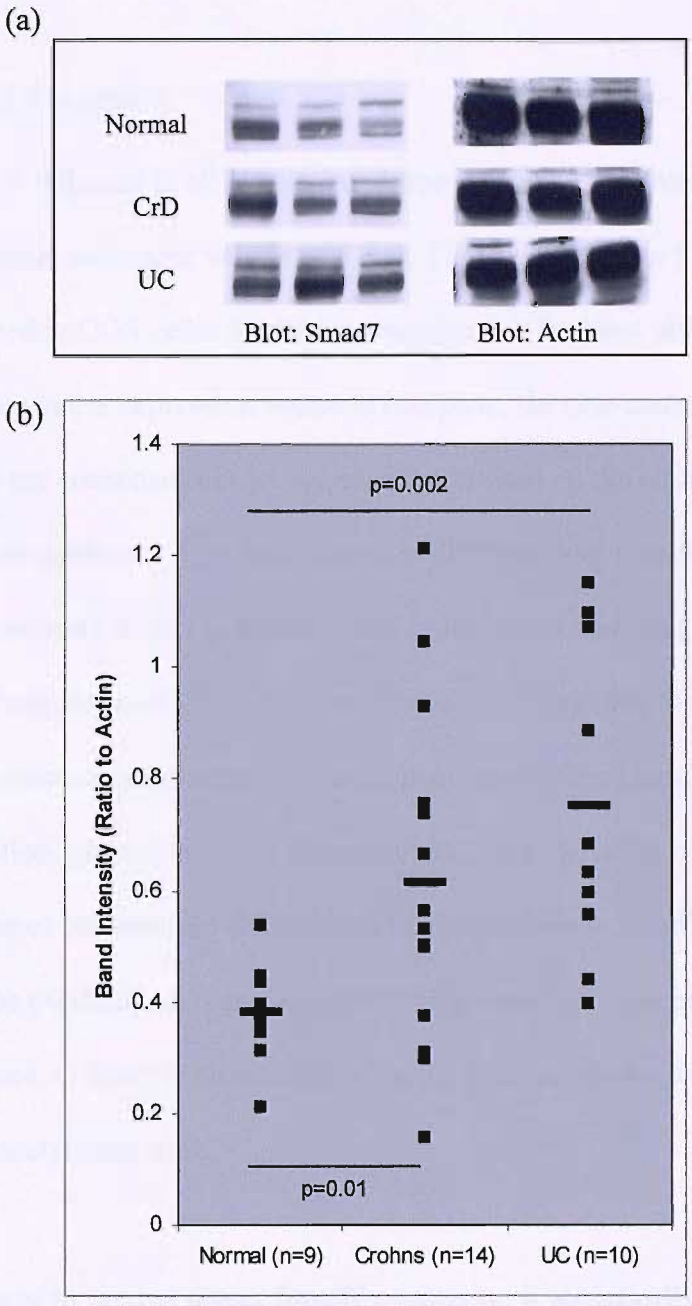


Figure 9: Smad7 expression in human gut biopsies.

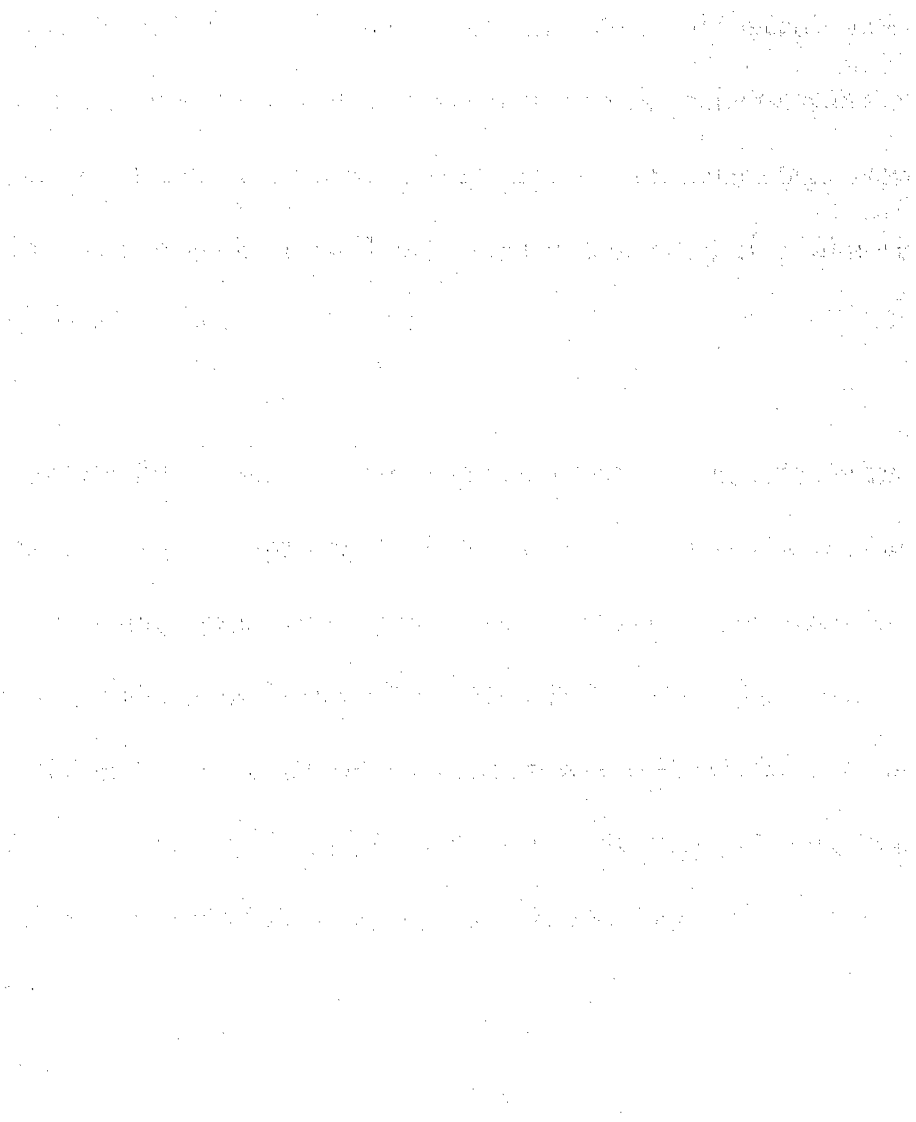
(a) Representative Western blots for Smad7 in normal, CrD and UC biopsies, with actin as loading control. (b) Band intensity is generally higher in biopsies from patients with IBD (boxes). There was statistically significant differences between mean Smad7 expression (bars) in normal gut (n=9) compared to CrD (p=0.01, Student's t-test, n=14) and to UC (p=0.002, n=10).

5.2.3 Discussion

Smad7 is detected in all human gut tissue samples. The Smad7 protein band detected is at an apparent molecular weight of 51kD. This is higher than the recombinant protein expressed in COS cells (43kD) (see Section 5.1.2). Also, although the cDNA coding sequence in the expression vector is complete, the non-coding sequences do not match exactly the consensus cDNA sequence published on the NCBI's consensus human genome sequence database. This could result in different post-translational modification in COS cells compared to that in human cells, or that the recombinant protein is truncated. Altered coding sequences in the cDNA could result in changes in the protein sequence, affecting length, secondary structure or site for post-translational modification (eg. phosphorylation, acetylation, glycosylation or ubiquitination sites). In order to investigate the potential differences between the endogenous and recombinant Smad7 proteins, the two proteins could be purified and then sequenced using mass spectrometry. The sequences could be compared, to identify characteristic motifs that are known to affect function, such as the lysine acetylation sites.

Compared to normal tissue, Smad7 expression is statistically significantly higher both in Crohn's disease (t-test, $p=0.01$) and UC (t-test, $p=0.002$). Overall, IBD specimens have a significantly higher expression of Smad7 than normal tissue (t-test, $p=0.00016$). There is a range of Smad7 expression in IBD, with more CrD biopsies falling within the range of that seen in normal biopsies. This is likely to reflect the degree of inflammation at the site the biopsies were taken from, since CrD causes patchy disease, and colitis is not always a prominent feature. UC affects the colon more evenly, and thus is any biopsy is likely to reflect the inflammatory changes over a larger area. In some UC biopsies the Smad7

expression falls within the normal range, and this reflects the spectrum of disease in UC, with some patients having more active inflammation than others. Some biopsies may have been taken from areas with no active inflammation, or during disease remission. Monteleone *et al* (2001) showed that Smad7 expression correlated with histological severity.



5.3 SMAD7 EXPRESSION IN LPMC CULTURED *EX VIVO*

5.3.1 Introduction

The factors which control expression of Smad7 in normal and inflamed bowel are not known. As a necessary step prior to investigating exogenous factors which could induce Smad7, preliminary experiments were carried out to determine whether Smad7 levels in cells remained stable *in vitro*, since for example, if growth factors in calf serum induced Smad7, this would have to be factored into the design of subsequent experiments. The literature on this topic is very limited. Monteleone *et al* (2001) extracted LPMC from patients with IBD and cultured these overnight with Smad7-specific antisense oligonucleotides and a control antisense. In the samples cultured with the control oligonucleotide overnight, Smad7 protein expression remained high. However the expression of Smad7 in normal LPMC was not examined during culture *ex- vivo* in this series of experiments.

Thus, the stability of Smad7 protein expression was investigated in order to interpret results of subsequent experiments with LPMC evaluating the effects of exogenous stimuli. Also, the cultures were continued for longer than has been previously reported, to determine if high Smad7 expression in IBD LPMC is maintained *ex-vivo*. LPMC were isolated from 6 patients and cultured in RPMI with 10% FCS at $2x10^6$ cells/ml in 37°C in 5%CO_2. Immunoblots were performed using the StCruz H-79 rabbit polyclonal anti-Smad7 antibody (1:400 dilution), with actin as a loading control (StCruz H-196, 1:500 dilution).

5.3.2 Results

The figure shown is representative of two separate experiments, using in total LPMC extracted from 6 separate gut resection specimens. Normal LPMC have a low expression of Smad7, which decreases after *in vitro* culture and is lower at 36 hours than at 12 hours. Like whole mucosal biopsies, the expression of Smad7 was high in IBD, with UC higher than CrD at the start of culture. Notably however, Smad7 expression remained high in IBD LPMC samples, persisting at 60 and 84 hours. Between 36 and 84 hours Smad7 levels were stable in all patients.

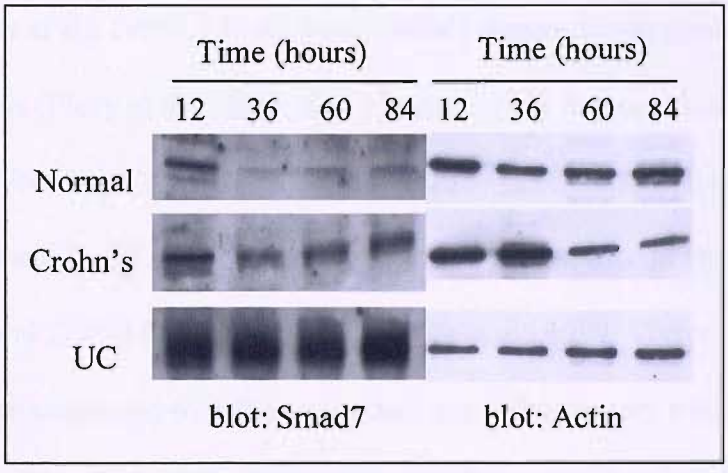


Figure 10: Smad7 expression in LPMC *ex-vivo*.

Smad7 is detected in all samples after 12 hours, higher in IBD samples. At 36 hours and thereafter, there is persistent high Smad7 expression in IBD samples, but lower expression in normal LPMC. Smad7 expression is stable up to 84 hours *in vitro*. Actin blots serve as loading controls.

5.3.3 Discussion

LPMC extracted from normal and IBD gut resection specimens have different Smad7 expression, in line with the data from gut biopsies. Importantly in terms of investigating which exogenous factors might increase Smad7, in normal LPMC, culture per se did not increase Smad 7 levels. The increased Smad7 expression in IBD LPMC persists *ex vivo*, it is unlikely therefore that epithelial cells which are largely lacking from LPMC are responsible for maintaining high Smad7 levels, however LPMC do contain some stromal cells which may have a role in Smad7 maintenance.

Previous data have shown that normal LPMC produce large amounts of IFNγ, and little IL-4 or IL-5 (Hauer et al., 1998). LPMC from Crohn's disease tissue produce higher levels of INFγ and TNF-α (Plevy et al., 1997). IL-12 is also highly expressed in CrD, increasing production of IFNγ in LPMC (Monteleone et al., 1997). UC tissue also contains very high levels of TNFα and IL-1β. Smad7 mRNA expression is induced *in vitro* in cell lines by TNF-α, IFNγ and TGF-β (Mori et al., 2000; Ulloa et al., 1999; Verrecchia et al., 2000). These results are consistent with the notion that pro-inflammatory cytokines can increase the expression of Smad7 in IBD, though this would appear to be a quantitative rather than a qualitative effect.

In normal human gut LPMC, Smad7 is ubiquitinated and degraded by the proteosome (Monteleone et al., 2005). This process does not occur in LPMC from patients with IBD and in IBD, but not in normal LPMC, Smad7 is acelylated. Acetylation prevents ubiqutination, stabilising the protein, an effect blocked by p300-specific short interfering RNA. These mechanisms explain the different patterns of Smad7 expression observed in normal and IBD LPMC cultured *ex-vivo*.

5.4 INDUCTION OF SMAD7 IN NORMAL LPMC

5.4.1 Introduction

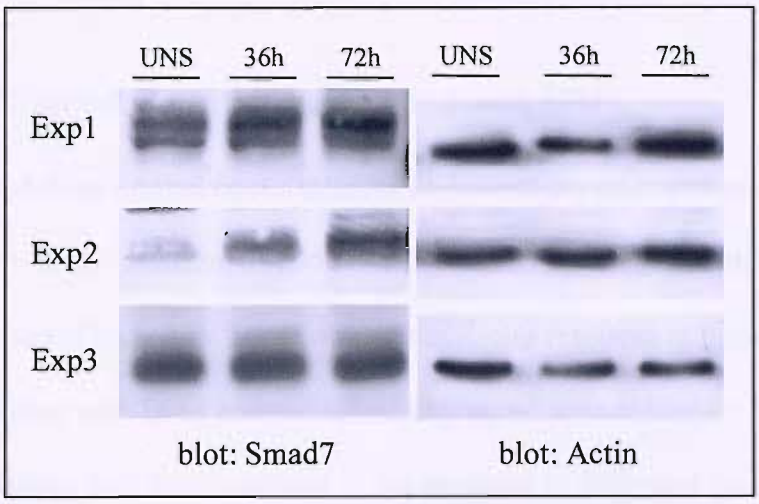
The control of Smad7 expression in LPMC is not known. LPMC in IBD produce large amounts of IFNγ and TNF-α and there is also increased TGFβ in IBD tissue (Plevy et al., 1997; Monteleone et al., 1997; Babyatsky et al., 1996). Studies of fibroblast cell lines *in vitro* have shown that several cytokines important in the pathogenesis of IBD increase Smad7 gene expression. These include TGF-β, TNF-α and IFNγ (Mori et al., 2000; Ulloa et al., 1999; Verrecchia et al., 2000). Thus these three cytokines were chosen for a series of experiments examining the effect on Smad7 expression in normal LPMC in short-term culture.

Cells were isolated from fresh gut resection specimens, cultured *ex-vivo* and the resulting cell pellets stored at -60°C prior to cellular protein extraction, as previously described (Sections 3.8, 3.9, 3.10). When using TGF-β, the LPMC were cultured in serum free medium. After Western blotting, equal protein transfer was confirmed using Ponceau's stain. Immunoblots were using StCruz antibody H-79, with 50μg of protein loaded in each lane, and using actin as a loading control (StCruz H-196, 1:500 dilution).

5.4.2 Smad7 protein in LPMC stimulated with TNFα

Lamina propria mononuclear cells from normal gut tissue from three separate control patients were cultured *in vitro* in the presence of 5ng/ml TNFα in RPMI supplemented with 10% FCS for 36 or 72 hours. Smad7 expression increased after 36 hours of culture in the presence of TNFα, an effect maintained at 72 hours in three experiments. The overall effect of TNFα is to significantly increase Smad7 expression, relative to actin, compared to that of unstimulated LPMC from the same individual (t-test, p=0.01) (Figure 11). The mean increase in Smad7 band intensity relative to actin after 36 and 72 hours in the presence of TNFα is 2.3 fold and 2.9 fold respectively.

(a)



(b)

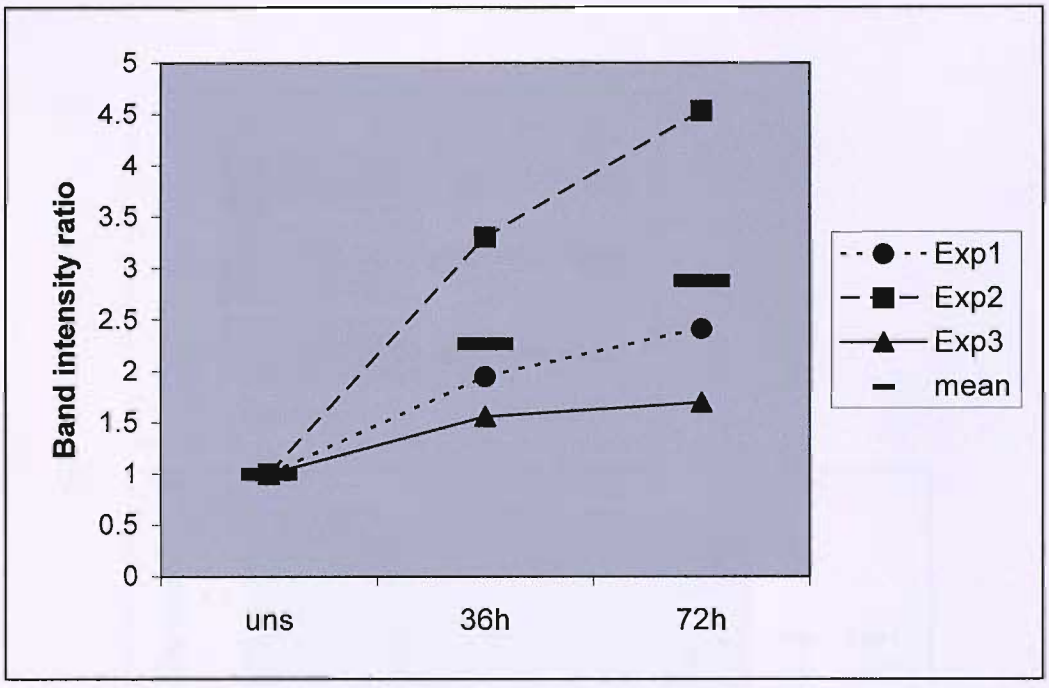


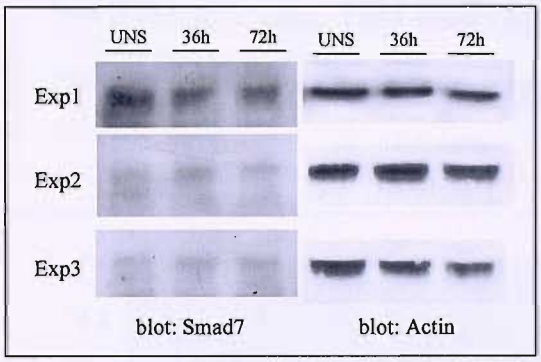
Figure 11: The effect of TNFα on Smad7 expression in LPMC.

(a) Western blots of LPMC show increased Smad7 expression with TNFα, with actin as a loading control. (b) Comparing Smad7 band intensity (relative to actin), normalised to the baseline level and after 36 or 72 hours stimulated by TNFα in three separate experiments and the mean value (bars). Smad7 expression is significantly increased in the presence of TNFα (t-test, p=0.01).

5.4.3 Smad7 protein in LPMC stimulated with IFNγ

LPMC extracted from normal bowel mucosa from three separate control patients were cultured *in vitro* in the presence of 100iu/ml INFγ in RPMI supplemented with 10% FCS for 36 or 72 hours (Figure 12). There was no consistent response in Smad7 expression after *ex-vivo* stimulation with INFγ and no statistically significant difference between the Smad7 expression between the LPMC cultured in the presence of INFγ and the unstimulated LPMC.

(a)



(b)

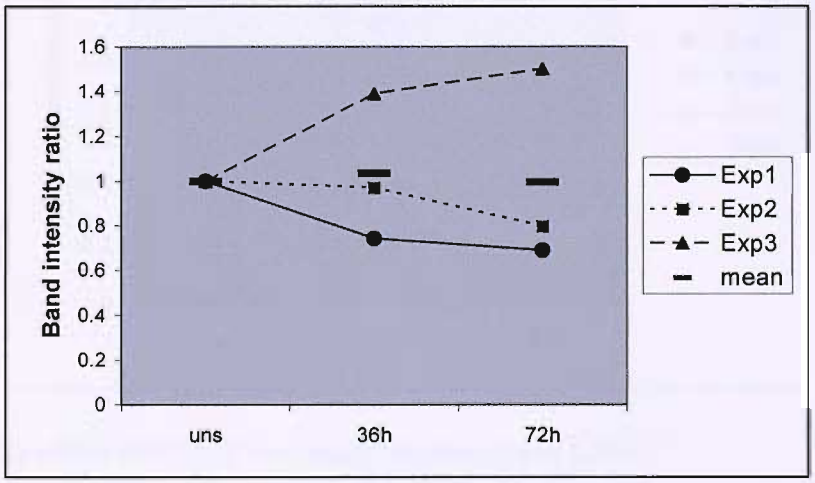


Figure 12: The effect of INFγ on Smad7 expression in LPMC

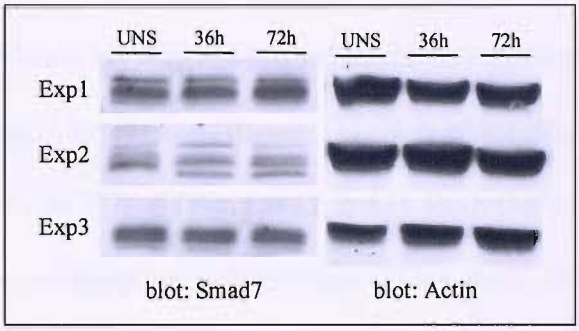
(a) Western blots of LPMC Smad7 expression with INFγ, with actin as a loading control.

(b) Comparing Smad7 band intensity, relative to actin, at baseline and after 36 or 72 hours stimulated by INFγ in three separate experiments and the mean value (bars). Smad7 expression is not significantly affected.

5.4.4 Smad7 protein in LPMC stimulated with TGF-β1

LPMC extracted from normal bowel mucosa from three separate contol patients were cultured *in vitro* in serum-free medium, RPMI supplemented with HL-1 (Sigma) and in the presence of TGF-β1 (10ng/ml) for 36 or 72 hours (Figure 13). There was no significant change in Smad7 expression in the presence of TGF-β1.

(a)



(b)

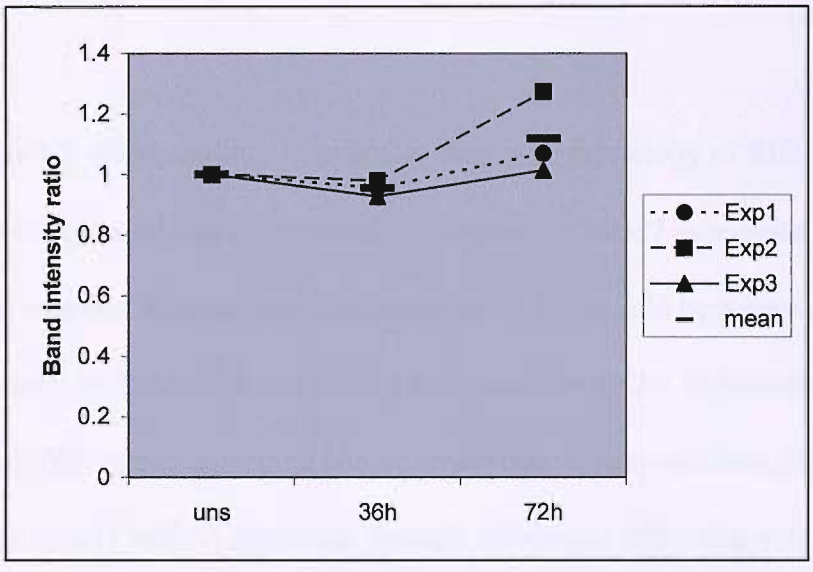


Figure 13: The effect of TGF-β on Smad7 expression in LPMC

(a) Western blots of LPMC Smad7 expression with TGF-β1, with actin as a loading control. (b) Comparing Smad7 band intensity, relative to actin, at baseline and after 36 or 72 hours stimulated by TGF-β1 in three separate experiments and the mean value (bars). Smad7 expression is not significantly different.

5.4.5 Discussion

The previous chapter showed that Smad7 expression remains higher in LPMC from IBD patients cultured *ex-vivo*, in the presence of calf serum, but in the absence of exogenous cytokines (Figure 10). Thus higher Smad7 expression in IBD LPMC is not dependent on tissue factors alone, and could be driven by endogenous factors. Stimulation of normal LPMC cultured *ex-vivo* with the pro-inflammatory cytokine TNFα results in a significant increase in Smad7 expression (Figure 11). This effect is not observed when LPMC are exposed to INFγ or TGF-β (Figures 12 & 13). Both INFγ and TGF-β are known to increase Smad7 mRNA transcription in fibroblasts (Ulloa et al., 1999; Brodin et al., 2000; Nakao et al., 1997).

TNFα, through NF-κB signalling, is central to the pathophysiology of IBD (Section 1.3.2), and the novel observation that TNFα results in increased Smad7 expression suggests that the altered the ongoing inflammation characteristic of IBD could be related to Smad7 over-expression caused by TNFα. Smad7 mRNA is induced by TNFα in fibrolast cell lines (Bitzer et al., 2000). In a transformed human embryonic kidney cell line (HEK-292), TNFα reduced Smad7 mRNA expresion through NF-κB, an effect that was reversed by overexpression of the transactivating histone acyltransferase p300 (Nagarajan et al., 2000). Taken together, these data suggest that TNFα can affect Smad7 mRNA expression in different ways depending on context. However, Smad7 protein levels are uncoupled from mRNA expression, being regulated by a balance of acetylation and ubiqutination (Gronroos et al., 2005). Thus increased Smad7 protein stability prevents cellular anti-inflammatory responses to TGF-β, supporting the pro-inflammatory state. In IBD LPMC,

Smad7 over-expression enhances NF-κB activity, an effect inhinited in normal LPMC by GF-β inducing IκB (Monteleone et al., 2004a). Also in IBD, p300 is over-expressed, and acetylates Smad7, reducing its degradation through ubiquin-mediated targeting to the proteosome (Monteleone et al., 2005). Therefore Smad7 and p300 interactions are likely to be central in mediating cross-talk between these major signalling pathways. The observed effect of p300 over-expression on Smad7 mRNA levels may be as a result of p300 affecting Smad7 protein stability (Nagarajan et al., 2000). In IBD, Smad7 acetylation by p300 explains the over-expression of Smad7 and TGF-β unresponsiveness in LPMC. However, the control of p300 expression and the cells types in which these effects occur is not clear.

Smad7 mRNA is induced by IFNγ in cell lines via the Jak/STAT signaling pathway (Ulloa et al., 1999). However, in normal LPMC, there was no consistent response in Smad7 levels with stimulation by IFNγ. This would suggest that the effect of INFγ on mRNA expression does not translate to increased protein expression. This is in contrast to findings in mouse lung tissue and skin, where INFγ increased Smad7 (Wen et al., 2004; Ishida et al., 2004). In human gastric mucosal organ cultures, INFγ induced Smad7 protein expression (Monteleone et al., 2004b). Normal intestinal LPMC produce IFNγ *in vitro*, and the finding that Smad7 expression is not affected by exogenous IFNγ may be because the unstimulated control cells produce sufficient to affect Smad7 (Hauer et al., 1998). This hypothesis could be addressed by the examining the effects of IFNγ-neutralising antibodies on the Smad7 levels in normal LPMC stimulated with IFNγ.

Smad7 mRNA is rapidly induced by TGF-β via Smad3 and Smad4 (Afrakhte et al., 1998; von Gersdorff et al., 2000; Nakao et al., 1997). However, efficient induction of Smad7

mRNA involves several transcription factors, including AP-1 that can be activated by TNFα (Brodin et al., 2000; Verrecchia et al., 2000). In normal LPMC, stimulation with TGF-β in serum-free medium resulted in no change in Smad7 protein levels. This suggests tight regulation of Smad7 protein levels in the presence of TGF-β. Small changes in Smad7 levels would result, maintaining the ability of LPMC to respond to changes in TGF-β levels in the lamina propria, coupling the cell to its environment. Thus, the cells maintain an anti-inflammatory state, unless there are mechanisms to stabilise Smad7, leading to TGF-β-unresponsiveness.

5.5 SMAD7 mRNA EXPRESSION IN IBD

5.5.1 Introduction

The control of Smad7 gene expression has not been investigated in human gut tissue. Differential regulation of Smad7 gene expression may be reflected in different mRNA levels in IBD, consistent with the observed changes in protein expression (section 5.2). The following experiments examine Smad7 mRNA expression in gut biopsies from paediatric patients, both normal and with IBD, using novel primers with a semi-quantitative RT-PCR method. The extraction of RNA was as previously described with the quality of extracted RNA confirmed by spectrometry and by visualisation of 18S and 28S ribosomal RNA bands on agarose gel electrophoresis (Section 3.15). Oligo-dT primers were used to prepare cDNA from mRNA, and the PCR primers were designed to cross exons, so than contaminating genomic DNA would not produce an amplicon of the same length. The expression of Smad7 in tissue was compared to β-actin expression, and results presented as the ratios of band intensity during the amplification phase of PCR.

5.5.2 Results

Total RNA was extracted from colonoscopic mucosal biopsies from 4 normal children, 5 with CrD and 3 with UC. RNA (1μg/reaction) underwent reverse transcription, with oligo-dT primers and 10% of the resulting cDNA amplified using PCR. The product obtained after 36 cycles of amplification with Smad7-specific primers were compared to the detection of β-actin after 28 cycles. A negative control sample of RNA/DNA-free water was used in each experiment.

Agarose gel electrophoresis of the Smad7 amplicon produced after 36 cycles of PCR, and actin amplicon at 28 cycles shows Smad7 is mRNA present in all samples, with no significant difference between normal and IBD (Figure 14). Amplification at these cycle numbers was not at plateau phase, with 40 cycles of PCR for Smad7 and 30 for actin gave more intense bands.

Although there was lower Smad7 mRNA expression in UC, there was no statistically significant difference in the ratio of Smad7 and β-actin signal intensity between normal and IBD samples. These data suggest that the observed Smad7 protein overexpression in IBD is not as a result of increased Smad7 gene transcription in gut tissue biopsies. These results are contrary to a model of Smad7 expression being directly affected by gene expression in IBD, and suggest that post-translational control is more likely to be important in affecting Smad7 protein levels. This could be by reducing Smad7 degradation, as has previously described in *in vitro* cell culture models (Simonsson et al., 2005).

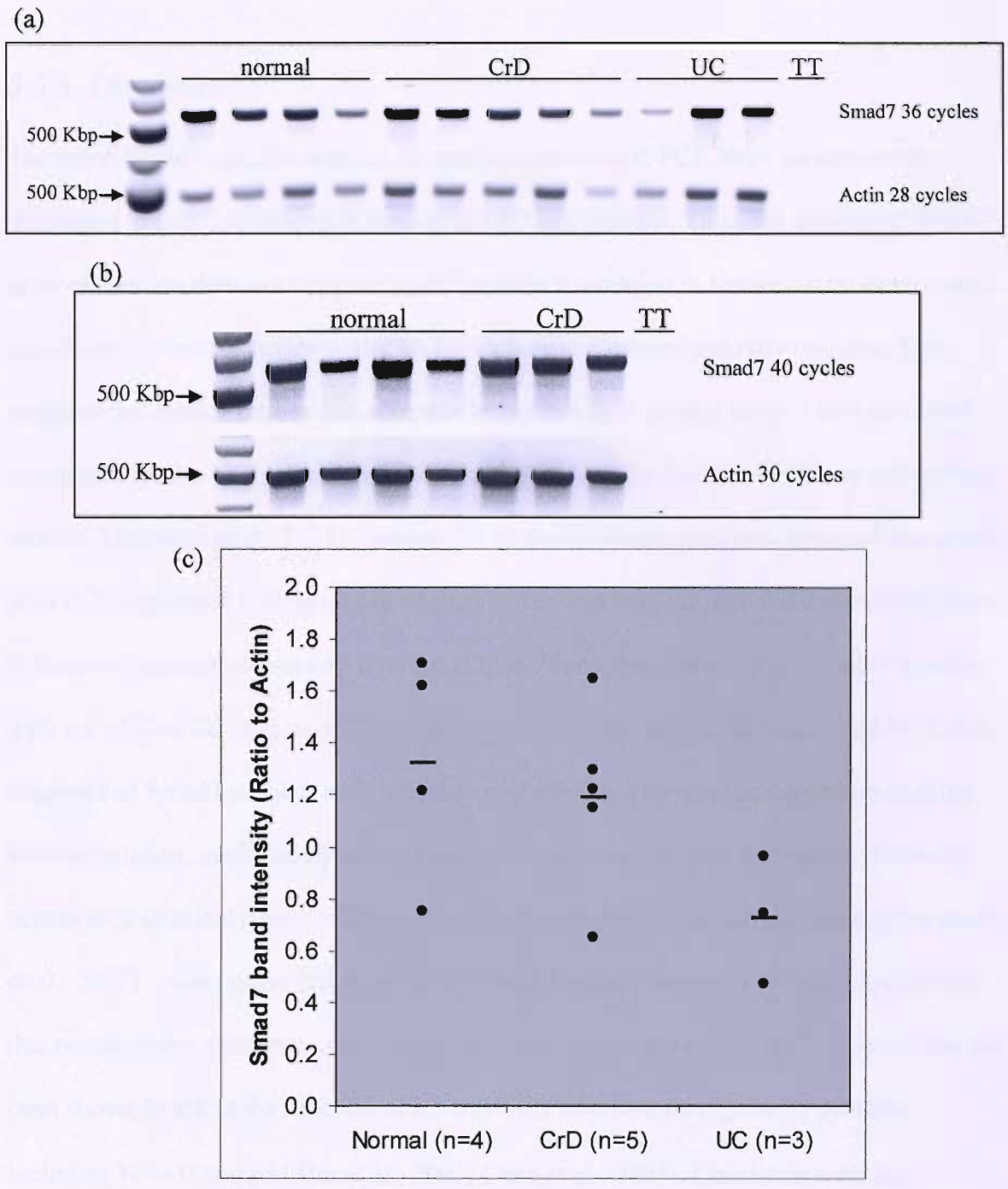


Figure 14: Smad7 mRNA expression in gut biopsies

(a) Smad7 mRNA is detected by rt-PCR in all gut biopsies at 36 cycles and actin at 28 cyles, with a negative control (TT). (b) The PCR reaction after 40 cycles for Smad7 and 30 cycles for actin gives more intense bands, with a negative control (TT). (c) Semi-quantitative analysis of Smad7 mRNA relative to actin (dots) and mean (bars) showing no significant difference in Smad7 expression in normal and IBD biopsies.

5.5.3 Discussion

The novel Smad7-specific primers for semi-quantitative rt-PCR show no detectable difference in gene expression in normal or IBD gut biopsies. Thus, the amount of Smad7 gene expression does not relate to Smad7 protein expression in tissues, since there is no significant difference between mRNA levels between normal and IBD biopsies. This suggests that Smad7 expression is controlled primarily at protein level. These are novel findings in human tissue, but are consistent with molecular biology studies in cell culture models (Gronroos et al., 2002). Subsequent to these experiments, data obtained using real-time PCR replicated this finding in adult subjects, and suggests that the observation of no difference between normal and IBD gut is true (Monteleone et al., 2005). Taken together with the *in vitro* findings in cell lines and the protein expression findings, these PCR data suggest that Smad7 stability and degradation is mediated by a balance between stability and degradation, mediated by acetylation and ubiquitination. The acetylation of Smad7 occurs at N-terminal lysine residues, regulated by the histone acyltransferases (Simonsson et al., 2005). Acetylation affects protein-protein interactions and prevents ubiquitination that occurs at the same residues (Chen et al., 2002; Gronroos et al., 2002). Acetylation has been shown to affect the function of several other non-histone regulatory proteins including NF-κB and p53 (Ito et al., 2001; Chen et al., 2001). Consistent with the hypothesis that histone acyltransferases act to stabilise Smad7, using short interfering RNA to inhibit p300 reduces Smad7 expression in IBD LPMC *ex vivo* (Monteleone et al., 2005). Thus, Smad7 protein levels are uncoupled from mRNA expression by differential post-translational modification. In the inflammatory state, Smad7 is stabilised, inhibiting TGF-β signalling.

5.6 CONCLUSIONS: LABORATORY STUDIES

Methods to detect Smad7 by Western blotting and RT-PCR in human gut tissue and LPMC have been evaluated. These data show that Smad7 protein expression is higher in IBD, consistent with previous data (Monteleone et al., 2001). Comparing Smad7 expression to β-actin expression may affect sensitivity when comparing different samples, since β-actin expression is high, causing high intensity signals when using chemiluminescent detection reagents. Where signals are intense, exposure times need to be short, to avoid over-exposure on light-sensitive film. With short exposure times, small differences in the signal could reflect relatively large absolute differences in the amount of protein present. Thus, differences in levels of β-actin expression may not be apparent between samples, since the resultant difference in signal intensity could be small. Consequently, when comparing Smad7/actin ratios to compare expression levels between samples, differences would appear less significant. These undetectable differences may have biologically important effects on cellular function. Therefore, interpretation of negative results in protein expression comparisons after stimulation *ex-vivo* is difficult. However, the reproducible observation that Smad7 expression is higher with exposure to TNFα is likely to be reliable. Future experiments could address the functional consequences of these differences, by measuring TGF-β responsiveness in LPMC, such as the phosphorylation of Smad2/3 (Monteleone et al., 2001).

Smad7 RT-PCR has been established, and the sequenced amplicon confirms specificity for Smad7 (Appendix C). The PCR product matches the published Smad7 cDNA sequence (NCBI GenBank Accession number: AH011391). The primers are in separate exons, so that detection of genomic DNA contamination cannot produce a band of the same length. Total RNA extracted from normal and IBD mucosal biopsies have similar levels of Smad7

mRNA expression. These data have been replicated using real-time PCR techniques, suggesting that the control of Smad7 expression in IBD is mediated by post-translational mechanisms (Monteleone et al., 2005).

A reproducible positive control for Smad7 protein was required to evaluate Smad7 antibodies. This has been established transfecting COS cells with a pcDNA3 expression vector encoding human Smad7. The recombinant protein is detected at 43kD. However, in human samples Smad7 is detected at 51kD. Blocking-peptide studies reveal that this band is likely to be a specific target (Appendix D). The difference in apparent molecular weight between the native Smad7 in LPMC or biopsies and recombinant protein could represent different post-translational modifications, glycosylation, phosphorylation, acetylation or covalent bonding with another peptide (eg. ubiquitin). In LPMC, Smad7 can be both acetylated and ubiquinated *in vivo* (Monteleone et al., 2005). Also, although the cDNA coding sequence in the expression vector is complete, the non-coding sequences do not match exactly the consensus cDNA sequence published on the NCBI's consensus human genome sequence database. Consequently, the COS cell expression system may not produce a full coding mRNA or a protein that is not processed identically to native Smad7.

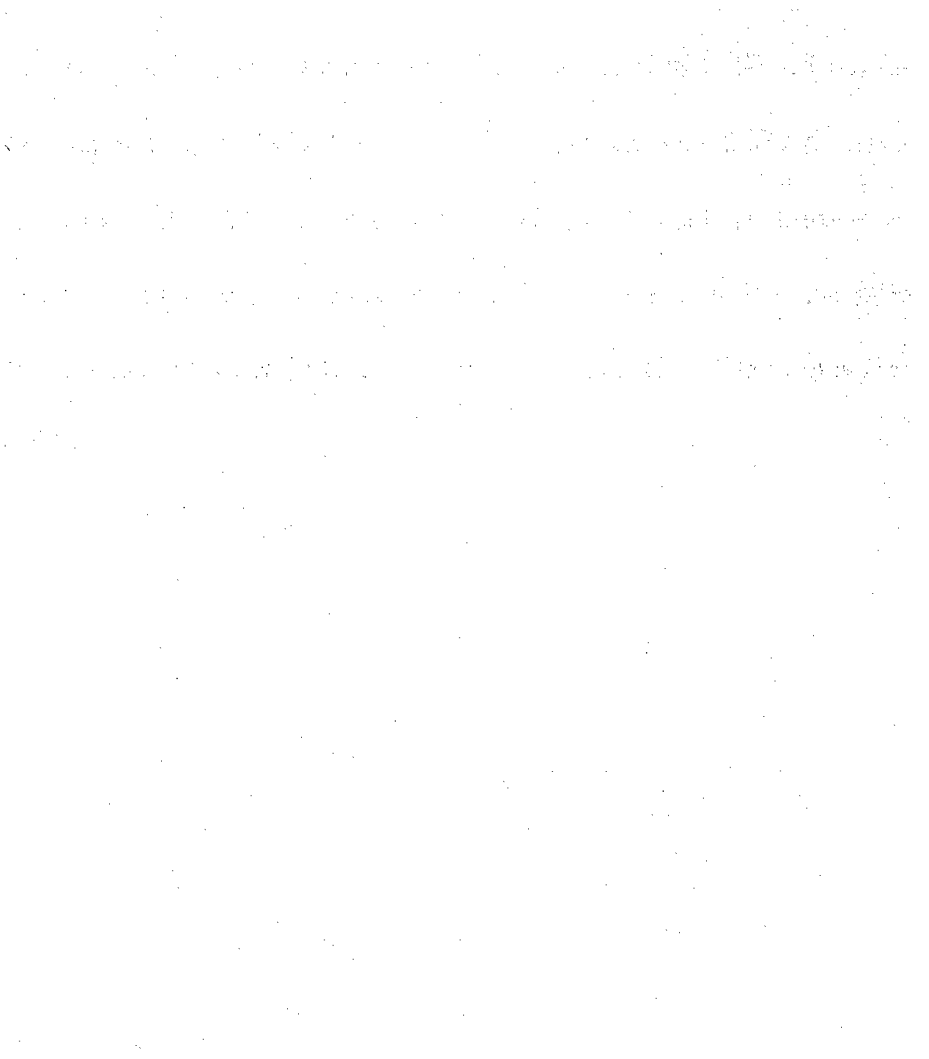
The regulation of cellular Smad7 protein levels has not previously been investigated in normal gut LPMC. These experiments found no changes in Smad7 expression in normal LPMC stimulated with TGF-β or IFNγ. Smad7 protein expression may be very transient in response to these cytokines in normal LPMC, with tightly regulated negative feedback returning the expression to baseline levels. TNFα increased Smad7 expression, at 36 and 72 hours. This may represent the effect seen in IBD and is consistent with the hypothesis that TNFα is a major contributor to the ongoing inflammation typically seen in IBD. This

is the first report of this in an *ex-vivo* study of normal human cells finding that TNFα results in increased Smad7 protein expression, and is consistent with the *in vitro* functional studies on cell lines (Bitzer et al., 2000).

However, Smad7 expression in IBD may not be induced directly by cytokines' effects on gene transcription, since Smad7 levels are affected by altered degradation or increased stability. Acetylation and ubiquitination affects levels of Smad7 in IBD LPMC, and these findings suggest new targets for therapy in IBD. For example, inhibiting the histone deacetylase p300 can reduce Smad7 expression in IBD LPMC (Monteleone et al., 2005). Moreover, p300 mediates acetylation of RelA, a central component of the TNFα/NF-κB signalling cascade, increasing gene transcription activity (Chen et al., 2005). Thus the effect of TNFα on Smad7 levels may not be a direct result of increased Smad7 production, but reflect an effect mediated by p300.

This apparent cross-talk between TNFα and TGF-β signalling cascades with counter-regulation would act to polarise a cell towards a pro- or anti-inflammatory state, depending on both the external signal context and its internal state. In LPMC from normal gut mucosa, the cell's Smad7 expression is sensitive to regulation by TNFα, promoting a pro-inflammatory state, and preventing the anti-inflammatory effects of TGF-β. Also, Smad7 expression in LPMC is relatively insensitive to TGF-β. This supports the observation that normal LPMC *ex vivo* maintain TGF-β responsiveness (Monteleone et al., 2001). Thus, Smad7 levels in LPMC are maintained at levels that allow TGF-β signalling to continue in the gut mucosa, where TGF-β is abundant (Babyatsky et al., 1996). This continued TGF-β responsiveness would tend to promote an anti-inflammatory state *in vivo*.

Previous data have shown that LPMC from normal mucosa produce IFNγ (Hauer et al., 1998). The finding that Smad7 expression is not significantly altered by the addition of exogenous IFNγ would suggest that the LPMC may maintain TGF-β responsiveness in the presence of pro-inflammatory IFNγ. The implications of this observation on gut physiology or its immune response are not clear, but this may represent a mechanism through which the balance of "physiological inflammation" in the gut is regulated.



6 RESULTS AND DISCUSSION: CLINICAL STUDIES

6.1 CALPROTECTIN IN CHILDREN WITH CHRONIC GASTROINTESTINAL SYMPTOMS

6.1.1 Introduction

Calprotectin is a stable neutrophil cytosolic protein detectable in the stool of patients with gut inflammation (Bunn et al., 2001). It has potential to be a reliable non-invasive test for bowel inflammation, and as a measure of disease activity in IBD. Data for calprotectin levels in a population of children aged 5-17 years has been defined, suggesting 50 μg/g as the upper limit of normal (Fagerberg et al., 2003). This cohort study examines the specificity of calprotectin as a test for organic bowel disease in children in this age group, and to compare calprotectin levels in children with active IBD to those in clinical remission.

6.1.2 Results

Paediatric gastroenterology clinic and ward referrals to the gastroenterology team provided 100 children aged 4-17 years (median 12 years) who provided a stool sample for examination. Comparison between groups was using the Mann-Whitney U-test. The numerical ELISA data from each subject is presented in Appendix E, and summarised by box-and-whiskers plots in Figure 15.

Overall, children with IBD had higher faecal calprotectin levels (median 336.6, range 22.4-1596, n=43) compared to normal children (median <15.6, range <15.6-39, n=7, $p<0.0001$) and children with functional constipation (median <15.6, range <15.6-63.1, n=31, $p<0.0001$). There were 2 children (4.7%) with IBD with calprotectin levels <50μg/g, one with UC in remission and one with Crohn's colitis in remission. Of the children with constipation, 3/31 (9.7%) had faecal calprotectin levels ≥50μg/g. Calprotectin levels were ≥50μg/g in 13/19 (68.4%) of the children with other (non-IBD) gastrointestinal diagnoses.

Those non-IBD children with calprotectin levels 50-200μg/g included: three with untreated coeliac disease, two with recurrent abdominal pain, one with viral gastroenteritis, one with Asperger's syndrome and food allergy, one with protein losing enteropathy secondary to complex congenital heart disease and one with an idiopathic enteropathy.

Levels ≥200μg/g were detected in a child with bacterial dysentery (716μg/g), one with recurrent abdominal pain with normal investigations (242μg/g), one with untreated coeliac disease and previous cerebrovascular stoke who was taking low-dose aspirin (220μg/g), and in one with enteropathy in association with an immunodeficiency syndrome (245μg/g).

In the children with IBD, CRP correlated with calprotectin (Spearman's correlation co-efficient 0.482, p=0.001, n=43). There was no statistically significant difference in calprotectin levels between CrD (n=29) and UC (n=14). In UC, children with clinically active disease (CSS >1) had significantly higher calprotectin (median 1250, range 156.3-1596, n=7) than with inactive disease (250, 45.6-573.5, n=7) (p=0.017). CSS correlated with calprotectin (Spearman's rho 0.757, p=0.002, n=14). For children with CrD, calprotectin levels in active disease (PCDAI ≥15) (median 410, range 77.8-1524, n=15) were not significantly different to inactive disease (263, 22.6-952.7, n=14).

PCDAI did not correlate significantly with calprotectin (Spearman's rho 0.09, p=0.642, n=29). Examined disease location in those CrD, patients with only the small bowel affected (median 269.4, range 62.0-799.8, n=11) did not have statistically different faecal calprotectin levels to those with colonic or ileocolonic disease (369.4, 22.6-1524, n=17), although the variance of these data and the small number of patients in each group makes interpretation difficult.

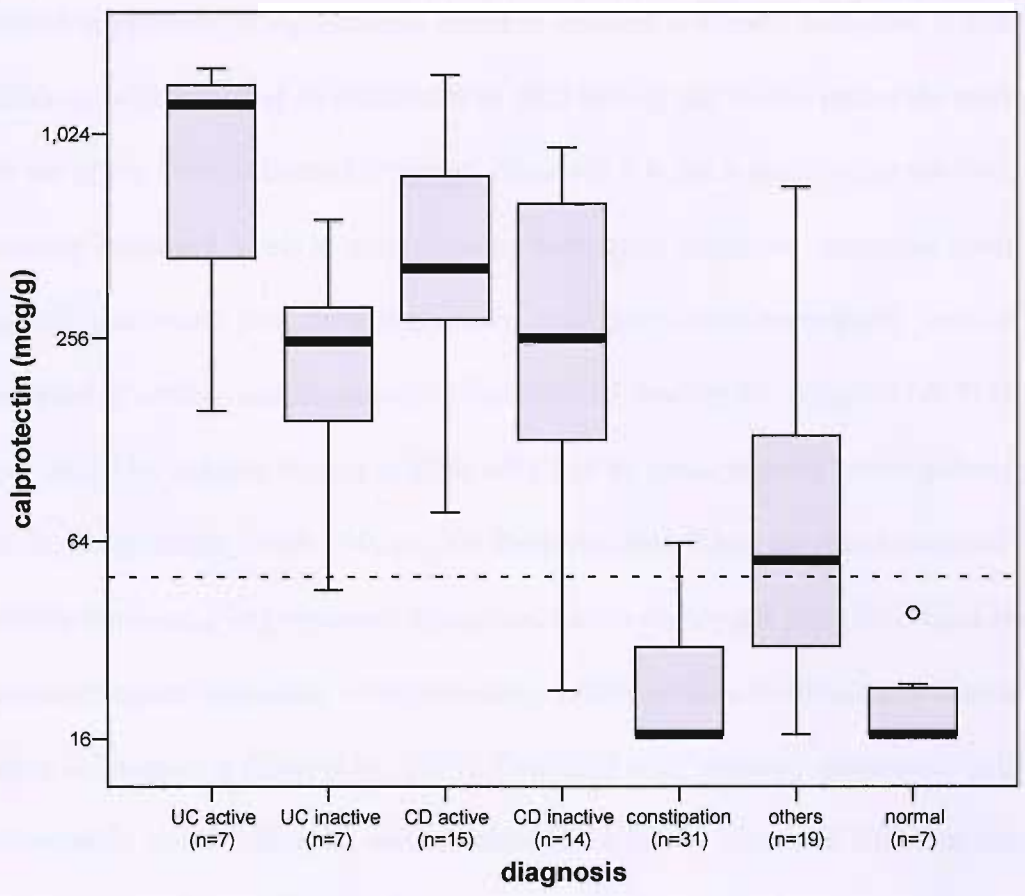


Figure 15: Faecal calprotectin results

Boxes represent the interquartile range and whiskers represent the maximum and minimum values, with outliers (values outside 1.5 box lengths) represented by open circles. Bold bars define the median value. The dotted line corresponds to 50μg/g, the reference value for the upper limit of normal in children age 4-17yrs. Calprotectin is significantly higher in those with IBD than normal ($p<0.0001$) and those with constipation ($p<0.0001$). Ulcerative colitis (UC) and Crohn's disease (CD) groups are separated according to disease activity scores. In ulcerative colitis, subjects with active disease (CSS >1) had significantly higher calprotectin than those with inactive disease ($p=0.017$), whereas there is no significant difference between active and inactive Crohn's disease.

6.1.3 Discussion

Faecal calprotectin is significantly raised in children with IBD compared to normal children, with only 2 of 43 children with IBD having test results below the reference value for the upper limit of normal (50μg/g). However it is not a specific test for IBD, with variably increased levels in some children with other diagnoses, including cases of non-specific abdominal pain, food intolerance, NSAID-induced enteropathy, coeliac disease, bacterial dysentery and immunodeficiency (n=19, median 56, range <15.6-716). The specificity for organic disease is 85%, with 6 of 41 cases without bowel inflammation having calprotectin levels >50μg/g. Of these children, 3 had chronic functional constipation and 3 had recurrent abdominal pain with normal other investigations. These results compare favourably with previously published data from radionucleotide-labelled white cell scanning (Shah et al., 1997). One child with recurrent abdominal pain had a calprotectin level >200 μg/g, and this child had a family history of IBD, but had normal endoscopic and histological findings. A raised calprotectin in this clinical context has been previously reported in adults (Thjodleifsson et al., 2003). Although IBD is unlikely where faecal calprotectin is less than 50μg/g, it cannot be excluded. The two individuals in this cohort with IBD and calproctectin levels <50μg/g. Both were in clinical remission with CRP <2mg/l. One was a boy age 8 years with CrD (PCDAI = 5). The other was a girl age 13 years with UC in complete clinical remission.

These data show that the calprotectin level correlates with CRP in IBD, consistent with the findings of other studies (Tibble et al., 2000). This suggests that calprotectin could be used as a marker of disease activity. Consistent with this hypothesis, faecal calprotectin levels in children with UC in remission are significantly lower than during relapse, but in most patients remain above the upper reference level for normal children (Figure 15). Similar

data have been reported in studies of adult patients. A persistently raised calprotectin may identify children that require second-line therapy and thus reduce cumulative steroid exposure. In Crohn's disease, however, previous data is conflicting on the relationship between calprotectin level and clinical disease activity. Another study of children with Crohn's disease found that calprotectin levels are lower in remission (Berni Canani et al., 2004). Our study has found a lower median value in those children in clinical remission (PCDAI <15), but this did not reach statistical significance (Figure 15). This may be because of a lack of statistical power, since the number of patients in these groups is small, or may represent disease severity in our cohort.

These data are similar to those described in adults with IBD, although calprotectin levels were generally higher in our cohort (Costa et al., 2005). A resolution of mucosal inflammation on histology does not always accompany clinical response to treatment in IBD, although the implications of this finding on long-term outcomes are not yet clear (Beattie et al., 1996). Thus, currently, faecal calprotectin has a limited potential role in the ongoing management of paediatric IBD because, in most cases, treatment decisions are based on the results of studies that have used measures of clinical activity rather than measures of intestinal inflammation as outcome data. Faecal calprotectin could however be used as a surrogate measure of mucosal healing during clinical trials of new therapies for IBD, and thus help to guide therapeutic decisions in new treatment regimes in the future.

Longitudinal follow-up of studies of faecal calprotectin levels in children with IBD undergoing treatment with comparison to follow-up endoscopy results would be crucial to test its usefulness as a research tool and in routine clinical practice. The likelihood of IBD in a child with faecal calprotectin levels <50μg/g is low in this cohort, in line with

previously published data. However, it has not yet been established whether there is an advantage in using faecal calprotectin levels as a screening test over clinical assessment and basic blood tests. Thus, faecal calprotectin >50μg/g in a child over the age of 4 years should prompt review, with decisions about further investigations determined in the light of a full clinical evaluation to avoid performing unnecessary investigations.

The data from this cohort study of calprotectin levels in children with chronic gastrointestinal symptoms support the association between high faecal calprotectin and IBD and support the use of faecal calprotectin to guide a diagnostic journey in a child with chronic GI symptoms. IBD is likely where faecal calprotectin is >200μg/g in the absence of intestinal infection. However, these levels are found in children with non-IBD enteropathy. One child who had a first-degree relative with IBD, but normal investigations, had a level >200μg/g. Levels 50-200μg/g are less specific for IBD, but unlikely in children without organic GI disease. In UC, patients in clinical remission faecal calprotectin levels are lower, but often remain above the upper limit of that found in the normal population.

6.2 A PROSPECTIVE COMPARISON OF COLONOSCOPY, BARIUM RADIOLOGY AND ULTRASOUND SCANNING

6.2.1 Introduction

The assessment of a child who presents with possible inflammatory bowel disease also includes imaging of the small bowel to determine disease extent and assess activity, often with barium radiology of the small bowel (Figure 16). The use of ultrasound scanning in abdominal pathology is well established for both inflammatory and ischaemic conditions, both in adults and children. The use of ultrasound scanning in children with CrD was first recognised as useful in the late 1980's, mainly to detect complications (Dinkel et al., 1986). Now, high frequency linear probes provide higher definition images of the bowel wall and enable bowel wall thickness to be measured (Figure 17). This measurement correlates well with clinical disease activity (Haber et al., 2000) and histological evidence of inflammation (Faure et al., 1997). Bowel wall thickening is not specific for CrD, but its presence is almost always pathological (Siegel et al., 1997).

Literature searches through PubMed and OVID online databases revealed no study comparing BaFT and USS in children with CrD. Studies of adults have shown USS to have sensitivity of 84-97% and specificity of 90%-97% (Hata et al., 1992; Solvig et al., 1995; Hollerbach et al., 1998; Parente et al., 2002).

A review of the imaging results of all the children seen in the paediatric gastroenterology clinic was undertaken. This revealed 24 subjects in whom both USS and BaFT were performed within one month. This case series illustrates a role for USS to reduce

cumulative radiation exposure in these children with an often chronic and relapsing disease.

An abnormal small bowel segment was detected by imaging in 20 of 24 children (83.3%). There were 8 (33%) in whom there was a discrepancy in the finding of USS and BaFT in the small bowel. Abnormality was detected at the distal ileum by both investigations in one case of CrD, but USS missed jejunal disease in this patient. Isolated proximal small bowel disease was noted in two (8%) cases, and not detected by USS in either of these. In the 16 remaining cases, the USS and BaFT results agreed for both the presence and extent of small bowel disease (Figure 18).

Comparing the results from USS and BaFT directly for terminal ileal disease, 18 (75%) cases had an abnormality detected at this site by either or both modalities. USS detected 13 cases with bowel wall thickening in the region of the terminal ileum. All 13 also had abnormality on their BaFT. In other words, increased bowel wall thickness on USS predicted abnormality on BaFT for terminal ileum disease. Terminal ileum histology was abnormal in 2 cases where imaging showed no abnormality at this site, illustrating that both imaging investigations are not sensitive when there is mild mucosal inflammation.

Overall, USS and BaFT agreed in only 16/24 cases (67%), but at the terminal ileum, agreement was 19/24 cases (79%). Where USS identified affected small bowel segments, BaFT contributed further information in only one case. If no child with a positive USS had received a BaFT, then only this one affected segment would have been misdiagnosed, and this in a child with proximal ileal disease, in whom the treatment strategy would have been

the same (Bremner and Beattie, 2002), suggesting that USS could be a clinically useful non-invasive tool to assess ileal disease activity.

In order to quantify the accuracy of USS in evaluating inflammatory bowel disease further, we performed a double blind prospective study of a cohort undergoing endoscopy or barium radiology for suspected IBD or for disease activity assessment of known IBD. This study aimed to examine the diagnostic ability of USS in childhood IBD using bowel wall thickness (BWT) and Doppler analysis of superior mesenteric artery (SMA) flow. These children often undergo repeated radiological or invasive endoscopic investigations, and USS has potential to reduce this burden.

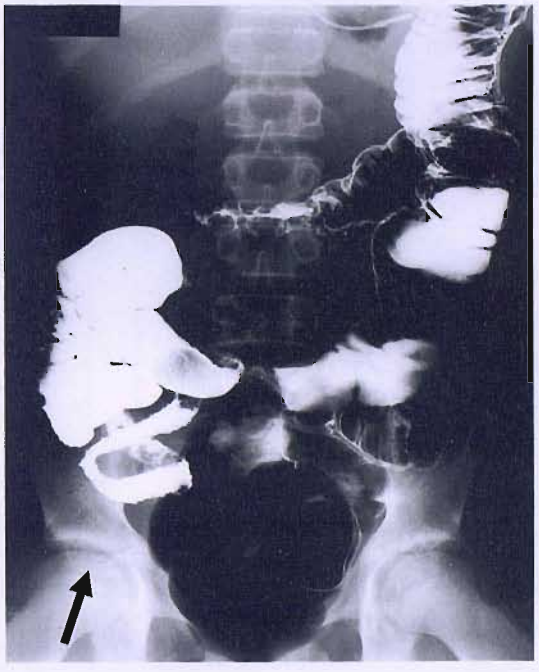


Figure 16: Barium radiology of Crohn's disease.

Barium follow-through with per-oral pneumocolon showing a narrowed and fissured terminal ileum with thickened walls, resulting in wide separation of bowel loops (arrrowed). There is a nasogastric feeding tube *in situ*.

(image supplied by Dr J Fairhurst, Southampton General Hospital)

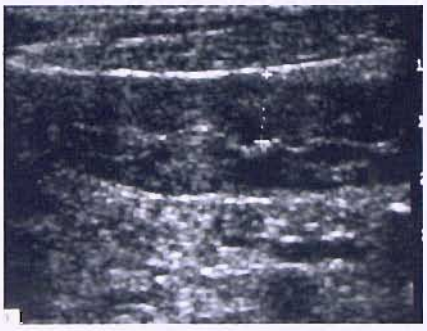


Figure 17: Ultrasound image of the terminal ileum in Crohn's disease.

A longitudinal image of the right iliac fossa using a high frequency linear probe, demonstrating abnormal terminal ileum measuring 6mm in wall thickness (crosshairs). The bowel wall is highlighted by echo-bright fat.

(image supplied by Dr J Fairhurst, Southampton General Hospital)

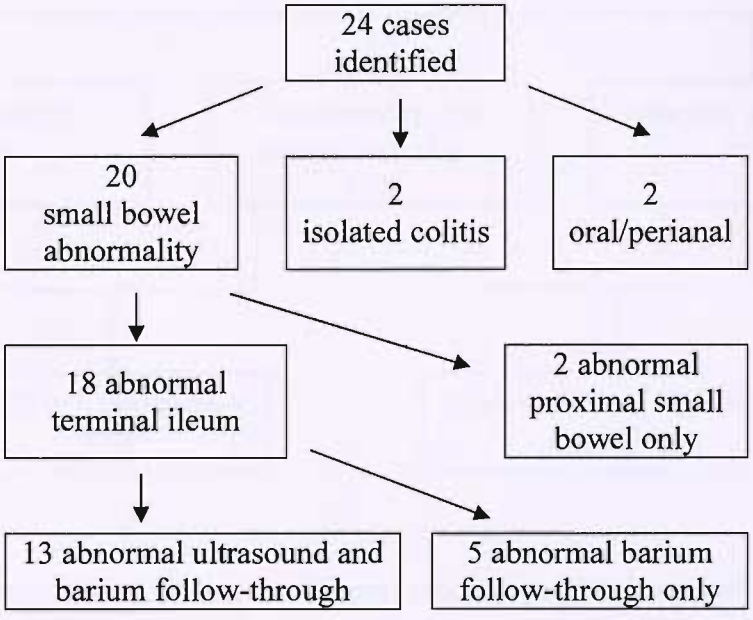


Figure 18: Retropective Barium radiology and ultrasound scan results.

A flow diagram of the results of imaging investigations in a retrospective comparison of ultrasound scanning and Barium radiology at the terminal ileum.

6.2.2 Results

In the prospective cohort study, 44 children (male= 25, median age 12yr, range 3.5yr-16.5yr) were recruited with chronic gut symptoms (n=29) or known IBD (n=15)). Diagnoses using standard clinicopathological criteria were CrD in 25, UC is 12, indeterminate colitis in 1, with the remaining 7 having normal investigations. Blinded ultrasound scans were performed in comparison to colonoscopy (n=19), BaFT (n=10) or both investigations (n=15). This provided data for comparison to colonoscopy in 34 subjects, and for BaFT in 25 subjects (Figure 19).

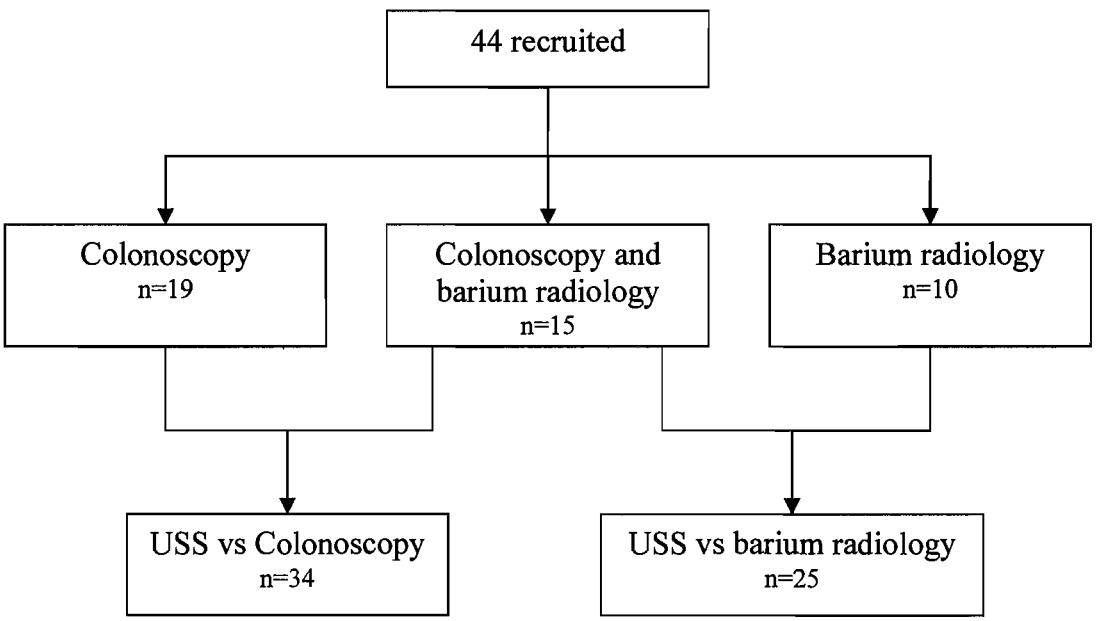


Figure 19: Prospective comparison of bowel imaging patient flow chart.

The 44 subjects had ultrasound scans prior to colonoscopy, barium radiology or both investigations. This resulted in comparative data for 34 colonscopies and 25 barium studies.

6.2.2.1 Colonoscopy v USS

Colonoscopy was performed in 34 subjects, providing data for six colonic segments in each. The rectum could not be imaged by USS in 16 subjects (52%). In order to reduce potential confounding, rectal BWT data were excluded from further analysis.

There were 3 patients in whom BWT could not be measured in one colonic segment, and one patient in whom obesity meant that no accurate BWT could be obtained. Colonoscopy was incomplete in one patient, in whom there was severe inflammation in the descending colon that could not be traversed. Thus BWT and endoscopic severity could be compared in 156 of a possible 170 colonic segments proximal to the rectum (Figure 20).

The mean BWT (with 95% confidence intervals) for normal segments was 1.5mm (1.3-1.6). For mildly affected segments (n=29) the BWT mean (and 95% CI) was 1.8mm (1.5-2.1). In moderately (n=46) and severely (n=20) affected segments the mean (and 95% CI) was 2.6mm (2.3-2.9) and 3.2mm (2.7-3.7) respectively. The mean colonic BWT in segments with severe endoscopic disease was 3.2mm in both CrD and UC.

There was no statistically significant difference between the BWT of mildly inflamed and normal segments, but there was between normal segments and moderately (t-test, $p<0.001$) or severely inflamed segments (t-test, $p<0.001$).

In endoscopically normal or mildly inflamed colonic segments (n=87) the mean BWT was 1.6mm and the 95th percentile 3.0mm. At endoscopy, 71 segments (43%) had moderate/severe disease. Using <3mm as the upper limit of normal for BWT, the

sensitivity of USS was 48% and specificity 93%, giving a positive predictive value of 84% (positive likelihood ratio = 7).

Sensitivity and specificity varied in each colonic segment. Specificity was 88% in the ascending and sigmoid, 90% in the transverse, and 100% in the caecum and descending colon. Sensitivity was 33% in the caecum, 46% in the ascending, 67% in the transverse, 54% in the descending and 50% in the sigmoid.

Although children with CrD had more endoscopically normal colonic segments than those with UC, there was no statistically significant difference between mean BWT in CrD compared to UC, between either the subgroups as a whole or when compared by the severity of endoscopic lesions. In both CrD and UC, the segments with endoscopically active disease (moderate/severe) had higher BWT than normal or mildly affected segments (t-test, $p<0.001$) (figure 21). Thus increased BWT does not differentiate between the lesions of CrD or UC.

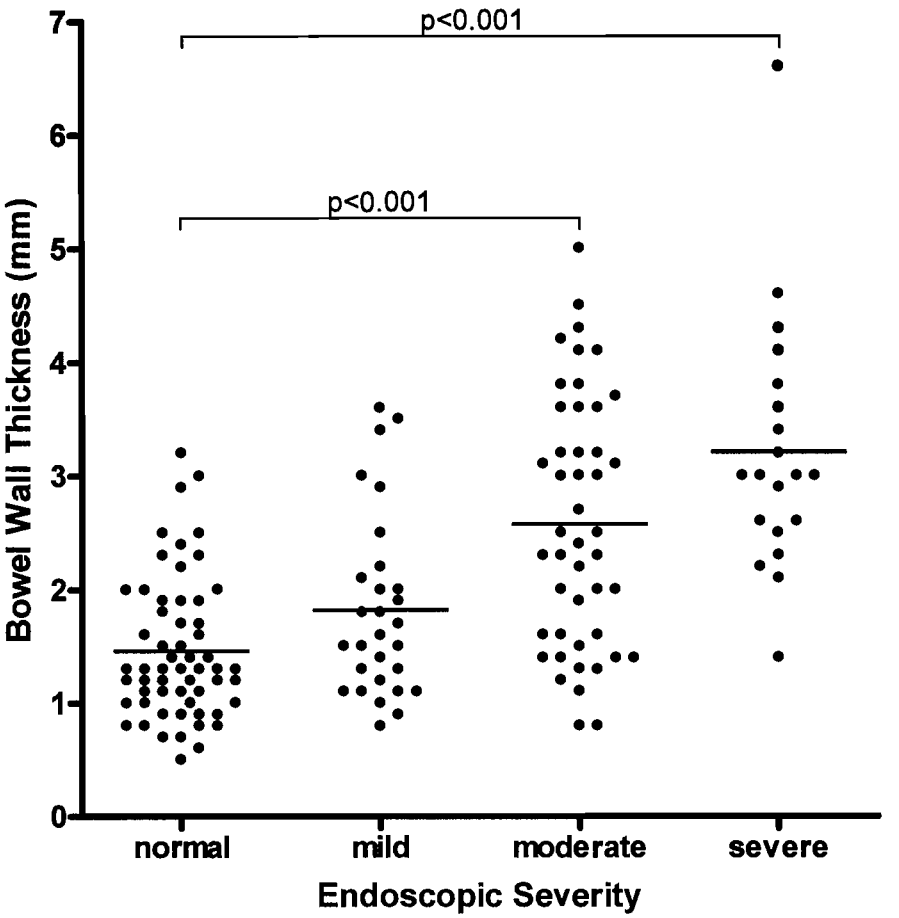


Figure 20: Bowel wall thickness compared to colonoscopic findings.

Colonic segment BWT measurements (dots) and the mean BWT (bars) for each group of patients. The mean BWT (with 95% confidence intervals) for normal segments was 1.5mm (1.3-1.6). For mildly, moderately and severely inflamed segments the BWT mean (and 95% CI) was 1.8mm (1.5-2.1), 2.6mm (2.3-2.9) and 3.2mm (2.7-3.7) respectively. There was a statistically significant difference between the BWT of normal segments and moderately ($p<0.001$) or severely inflamed segments ($p<0.001$).

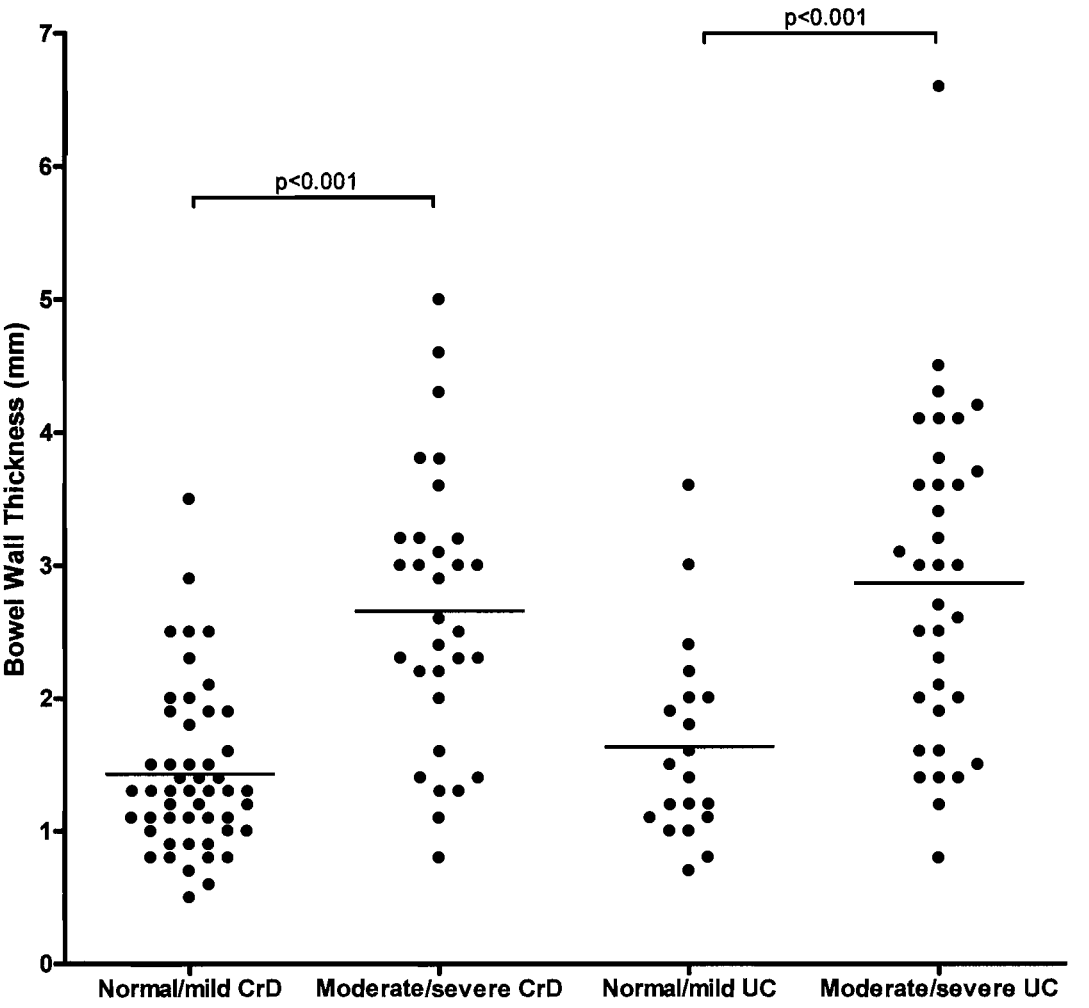


Figure 21: Bowel wall thickness compared to colonoscopic findings in CrD and UC

Differences in colonic BWT between segments with moderate/severe lesions are significantly higher than normal or mildly inflamed segments in both CrD and UC, with no significant difference between UC and CrD overall or by endoscopic severity.

6.2.2.2 Barium radiology v ultrasound scanning

Terminal ileum BWT was measured in 24 of 25 subjects who underwent BaFT. In one subject, USS identified the caecum, but the terminal ileum could not be well imaged. Subjects' final diagnoses were CrD (n=18), UC (n=3) and normal (n=4).

The terminal ileum was noted to be abnormal in 10/25 (40%) at BaFT. USS demonstrated BWT over 2.5mm in 8 subjects, of whom one had no abnormality detected on barium studies (Figure 22). One of the three subjects with UC had abnormality at the terminal ileum at BaFT, with USS measurement of BWT of 1.9mm.

Using 2.5mm as the upper limit of normal of ileal BWT, USS had a comparative sensitivity of 75% and specificity 92%, giving a positive predictive value of 88% (positive likelihood ratio = 9). There was a statistically significant difference between the mean BWT on USS in those with BaFT abnormality compared to those with no abnormality (t-test, $p<0.001$).

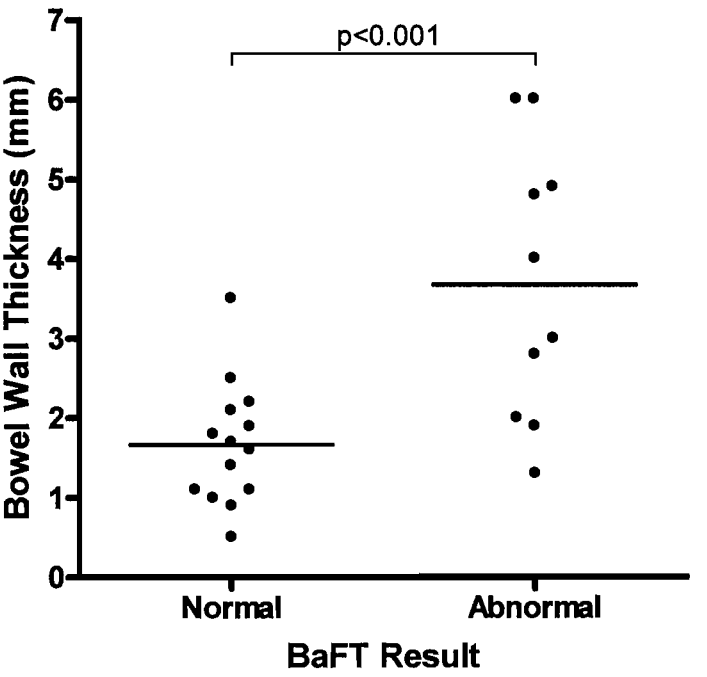


Figure 22: Ultrasound scanning compared to barium studies.

The graph illustrates measured bowel wall thickness (BWT) and barium follow-though results (dots), with the mean value for each group (bar). Mean BWT is significantly higher in those with abnormal barium studies (t-test, $p<0.001$).

6.2.2.3 SMA flow and disease severity

Doppler analysis of SMA flow (RI) was measured in 28 cases (85%) and did not correlate significantly with clinical disease activity, endoscopic disease severity or BaFT abnormality, in the whole cohort, in those with CrD (n=14) or those with UC (n=10).

Subjective increases blood flow on colour Doppler analysis of segments with increased BWT was often demonstrated, in line with previously published data (Quillin and Siegel, 1994) (Figure 23).

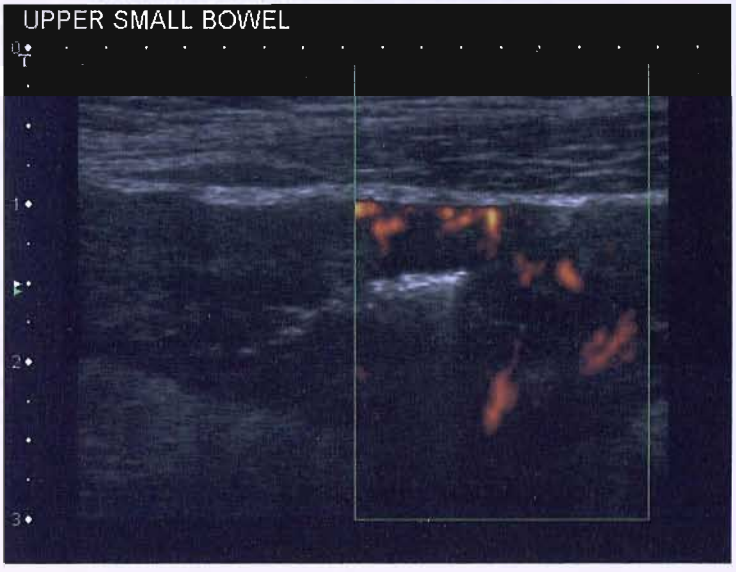


Figure 23: Colour Doppler studies of inflamed bowel wall.

An ultrasound scan still image showing Doppler signal (red) within the bowel wall, suggesting increased blood flow in the presence of active inflammation.

(image supplied by Dr M Griffiths, Southampton General Hospital)

6.2.3 Discussion

These data show that USS can provide a non-invasive measure of IBD activity in children in a blinded comparison against endoscopy or barium radiology. BWT measurements >2.9mm in the colon or >2.5mm in the terminal ileum reliably indicate moderate or severe inflammation in children with IBD.

Previous studies have suggested that USS can play an important role in the assessment of IBD, but it has yet to become part of the standard investigation of IBD. Interpreting USS images is operator-dependant and can be subjective, and thus we chose the quantitative measurements of BWT and SMA flow as parameters to study.

Combining the results from all patients increases the statistical power to assess the diagnostic accuracy of increased BWT. However, comparing the results from the UC and CrD subgroups shows that the difference between normal/mild and moderate/severe lesions persists (figure 21). This supports the hypothesis that USS can detect increased BWT in both conditions and that BWT on USS cannot discriminate reliably between CrD and UC.

This study was not designed to compare USS and histology as measures of intestinal inflammation, nor to examine the ability of USS to discriminate between different types of inflammatory lesion within the bowel. This acknowledges that histology is a requirement in the diagnostic assessment of any case of strongly suspected IBD and that increased BWT is not specific for IBD (Siegel et al., 1997; Hollerbach et al., 1998; Ledermann et al., 2000).

The investigation of the small bowel in IBD remains more difficult than that of the colon. Comparison of USS and BaFT to ileoscopy was not a primary objective of this study, and our cohort had few patients who underwent all studies during the same period of disease actvity (n=7, UC=3, CrD=3, normal=1). Therefore, it is not possible to make conclusions on the relative accuracy of BaFT and ileoscopy.

Barium radiology is the most widely used investigation to image the terminal ileum, but it has limitations in sensitivity for subtle mucosal lesions. Radionucleotide-labelled leukocyte scanning is useful for investigating IBD (Rispo et al., 2005). However, it is time-consuming and has limited specificity in the upper bowel (Grahnquist et al., 2003). Computerised tomography, magnetic resonance imaging (MRI) and capsule endoscopy can also be used (Hyer et al., 1997; Madsen et al., 2002; Magnano et al., 2003; Potthast et al., 2002). These investigations are more invasive and/or not easily available in some centres providing care for children with IBD. USS is attractive as an alternative, as it is non-invasive, well tolerated and easily available. Data in adult subjects suggest that USS is more accurate than MRI scanning, particularly in UC (Pascu et al., 2004). In this cohort, patients with CrD are predominant, and as such the comparative accuracy of USS in patients with UC cannot be evaluated.

Doppler analysis of SMA flow did not correlate with endoscopic or clinical disease severity, or measures of BWT. These data are at variance from some published data from adults with IBD (van Oostayen et al., 1994; Ludwig et al., 1999; Rapaccini et al., 2004; Yekeler et al., 2005). Other studies have found no correlation with clinical activity (Tarjan et al., 2000; Byrne et al., 2001). This may represent a different spectrum of disease severity

in study cohorts, or methodological flaws. The subjects in our cohort were being fasted in preparation for their other investigation, potentially reducing splanchnic blood flow.

Published data from studies of adults with CrD show that USS can identify strictures, fistulae and obstruction (Parente et al., 2002; Kohn et al., 1999; Maconi et al., 2002; Maconi et al., 2003). None of these complications was detected in this cohort with either USS or barium studies. One child had a caecal stricture at endoscopy, with increased BWT on USS (4.6mm) and abnormal barium studies of ileum and caecum, but no evidence of obstruction clinically or radiologically.

Lying deep in the pelvis, the rectum is not easily imaged by transabdominal USS. Our data show that USS imaging of the rectum is unreliable, with no measurement of BWT possible in 52% of subjects.

Overall, these data support the use of USS in the assessment of paediatric IBD, of the terminal ileum and colon, excluding the rectum. However, a normal BWT on USS does not exclude active inflammation or stricture, and cannot reliably determine disease extent. However, where increased bowel wall thickness has been identified on USS, it can direct medical management decisions without additional investigations, since these will be similar in most cases with moderate or severe inflammation, irrespective of the exact extent of mucosal lesions. If clinical improvement is not followed, then reassessment with consideration of alternative imaging modalities would be appropriate.

Longitudinal studies of patients with increased BWT during periods of disease activity would be useful to examine the role of serial USS in assessing disease progression.

Thus, increased BWT on USS has clinically useful diagnostic value for moderate and severe IBD lesions in the terminal ileum (>2.5mm) and colon proximal to the rectum (>2.9mm). BWT below these values does not exclude moderate or severe gut mucosal inflammation. These data support the use of USS a first investigation in a child with IBD to assess disease severity and extent and thereby obviate other imaging investigations. An increase in the use of USS will reduce the amount of ionised radiation that children with IBD are exposed to. Endoscopy remains the gold standard for diagnosis and disease assessment.

6.3 CONCLUSIONS: CLINICAL STUDIES

Reliable methods of non-invasive assessment of gastrointestinal inflammation would alter the diagnostic journey in children with chronic gastrointestinal symptoms and the assessment of IBD, reducing the reliance on invasive tests and reducing cumulative exposure to ionizing radiation from radiological procedures. Present clinical practice is to base assessment on clinical symptoms and examination findings, together with blood tests such as blood count and inflammatory markers (e.g. Erythrocyte Sedimentation Rate, CRP). The studies presented show potential roles of faecal calprotectin and transabdominal ultrasonography as methods to assess chronic abdominal symptoms and IBD activity in children.

Raised faecal calprotectin is highly specific for organic bowel diseases, but cannot discriminate between causes. Thus, in a child with chronic abdominal symptoms and a normal faecal calprotectin (<50mcg/g), inflammatory bowel disease is very unlikely. There is potential confounding by use of non-steroidal anti-inflammatory drugs that cause a subclinical enteropathy. In children known to have IBD, calprotectin levels in stool often remain elevated, despite clinical improvement. In adults, raised calprotectin is associated with higher risk of later relapse (Tibble et al., 2000). Calprotectin has potential as a marker of mucosal inflammation in trials of therapies.

Data from the blinded comparison of endoscopic appearances and gut BWT measured with high frequency linear USS probes in children show that USS has low sensitivity as a tool to assess IBD activity. However, increased BWT in the colon proximal to the rectum is highly specific for moderate or severe mucosal lesions. Thus, USS can be used as an

adjunct to clinical assessment in the preliminary investigation of suspected relapse of both CrD and UC, potentially reducing the requirement for repeated endoscopy. In clinical practice increased BWT suggests moderate or severe disease activity is likely, and thus decisions on altering treatment would be made with improved confidence. Where relapse is suspected and USS shows BWT is not increased, disease activity is not excluded. Also, the accuracy of USS in detecting all affected segments is low, and therefore it cannot be used reliably to determine full disease extent or to discriminate between CrD and UC.

Taken together, these studies offer new data to support the use of non-invasive methods in both the diagnosis and assessment of IBD. Both calprotectin and USS deserve further evaluation during the course of disease in individual patients through their follow-up, to determine their role in predicting or assessing responses to therapies. Calprotectin may identify a sub-group in whom relapse is more likely, and thereby direct the early introduction of immunomodulatory therapies. Increased BWT on USS may resolve during treatment, and further studies would be necessary to determine if recurrence could predict later relapse.

7 SUMMARY DISCUSSION AND CONCLUSIONS

In laboratory studies, raised Smad7 expression in mucosal biopsies and in LPMC from patients with IBD has been demonstrated with a polyclonal antibody. Smad7 mRNA expression is not significantly different between normal and IBD mucosal biopsies. These data from children and adolescents mirror those previously described in adults (Monteleone et al., 2001). The observation that Smad7 protein expression can be induced *ex vivo* LPMC isolated from normal gut resection specimens with TNFα, but not with IFNγ or TGF-β is a novel finding.

Smad7-specific semi-quantitative RT-PCR shows no detectable difference in Smad7 gene transcription in IBD tissue, supporting the hypothesis that post-transcriptional control of Smad7 is important in regulating TGF-β signaling. This mechanism has been shown to be important in IBD LPMC, and suggest a critical role for p300, an acetyltransferase highly expressed in IBD tissue and capable of stabilising Smad7 (Monteleone et al., 2005). Further studies examining the expression and regulation of this protein would define the role of p300 in regulating gut immune responses and its role in IBD pathogenesis.

The data in this thesis taken together with other findings in human gut inflammation suggest that TNFα is an important cause of Smad7 over-expression (Monteleone et al., 2004a; Monteleone et al., 2005). Using TNFα-neutralising antibodies or inhibitors of the NFκB pathway in studies of normal and IBD LPMC could confirm these data. Further studies could examine the effects on the expression of Smad7 of other factors known to be

important to IBD pathogenesis, such as IL-12, IL-8 and bacterial products, such as LPS and peptidoglycan.

The mechanism of this apparent cross-talk regulating these pro-inflammatory and anti-inflammatory cytokines deserves further investigation. Smad7 inhibition represents a new target for therapy, and may be achieved by inhibiting p300 or by interfering with Smad7 RNA transcription.

In clinical studies, both calprotectin and USS offer non-invasive methods that contribute to the diagnosis and assessment of IBD in children. Raised faecal calprotectin has clinically useful specificity for organic bowel disorders, but cannot discriminate between CrD and UC. If used as a screening test in children with chronic abdominal symptoms, significantly raised calprotectin should prompt further investigations. Increased BWT in the colon suggests moderate or severe endoscopic lesions in children with IBD, but BWT is not useful to exclude active disease, as sensitivity is low (Section 6.2.2).

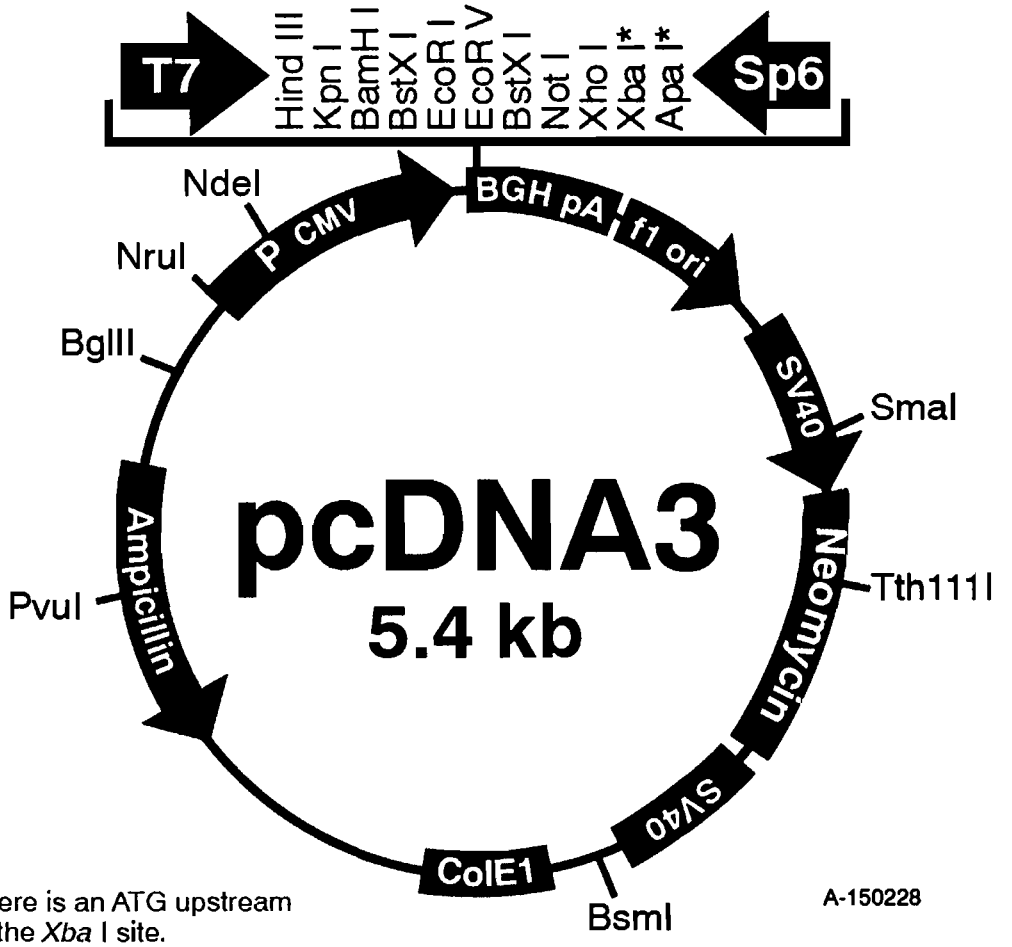
For the child at first presentation with bowel symptoms, raised calprotectin suggests an organic gut disorder, and thus prompt further investigations. Conversely, normal calprotectin makes IBD very unlikely, and in this case, invasive testing could be avoided. Increased BWT on USS identifies a group in whom endoscopy would be warranted, but BWT in the normal range does reliably exclude significant lesions in IBD.

Longitudinal studies would be necessary to assess the potential utility of USS and calprotectin to measure the response to treatment interventions or to identify a subgroup

likely to relapse. Both these investigations have a potential role in the assessment of new treatments for IBD, as proxy measures of mucosal healing.

Both the clinical evidence from faecal calprotectin data and laboratory studies of Smad7 protein expression add to the understanding of inflammation in IBD that can be ongoing despite clinical remission. The future therapies of IBD should be directed to promote "immunological" remission that could result in preventing relapse of disease in both UC and CrD or the stricturing consequences of chronic inflammation in CrD. In order to achieve this, methods of monitoring disease activity would have to be refined, to complement clinical evaluation and to avoid the requirement for repeated invasive tests, such as endoscopy. Currently, USS is not sufficiently sensitive to detect mild inflammation reliably in children with IBD. However, where BWT is increased, USS complements clinical assessment in children with symptoms suggestive of active disease, since moderate or severe lesions are likely. Thus, increased BWT helps the clinician, by providing evidence of the presence of active disease, supporting a decision to increase therapy.

Appendix A: pcDNA3 plasmid map



Plasmid information for pcDNA3 (Invitogen):

Length: 5446 nucleotides

f1 ori: filamentous bacteriophage origin of replication
CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
Neomycin resistance open reading frame: bases 2151-2945
SV40 poly A: bases 3000-3372
ColE1 origin: bases 3632-4305
Ampicillin resistance (β-lactamase) open reading frame: bases 4450-5310

Appendix B: Reagents for Western Blotting

SDS-PAGE gel preparation	10% Separating Gel	5% Stacking Gel
Deionised water	15.9ml	5.6ml
Tris (1.5M pH 8.8)	10.0ml	
Tris (0.5M pH 6.8)		2.5ml
30% acrylamide (Protogel)	13.3ml	1.7ml
10% SDS	400µl	100µl
10% Ammonium persulphate	100µl	100µl
Tetramethylethylenediamine	25µl	10µl
Total volume	40ml	10ml

Electrode (Running) Buffer		Transfer Buffer	
Tris base	3g	Tris base	7.6g
Glycine	14.4g	Glycine	36g
SDS	1g	Methanol	500ml
Deionised water	Make up to 1 litre	Deionised water	Make up to 2.5 litres

4x SAMPLE BUFFER	
Deionised water	19ml
Tris 0.5M pH 6.8	5ml
Glycerol	4ml
10% SDS	8ml
Bromophenol Blue 0.5%	2ml
Total volume	38ml
β2-mercaptoethanol (added just prior to use)	50µl per ml

Appendix C: Smad7 rt-PCR amplicon sequence

The upper sequence is from the cDNA sequence (in bold) from the Smad7 expression vector supplied by Pof Heldin (Uppsala, Sweden), starting and ending at the primer sites (in capitals). The lower is the PCR product, sequenced using the forward primer. Matching bases are linked with a vertical line.

```
              635  ATCACCTTAGCCGACTCTGCgaactagagtctccccccctccttac 682
                                          |      | ||||||||||||
                                        0  cantaangctccccctccttac 22

683  tccagatacccgatggattttctcaaaccaactgcagactgtccagatgctgtgccttcc 742
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
23   tccagatacccgatggattttctcaaaccaactgcagactgtccagatgctgtgccttcc 82

743  tccgctgaaacaggggggaacgaattatctggcccctgggggggctttcagattcccaactt 802
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
83   tccgctgaaacaggggggaacgaattatctggcccctgggggggctttcagattcccaactt 142

803  cttctggagcctggggatcggtcacactggtgcgtggtggcatactgggaggagaagacg 862
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
143  cttctggagcctggggatcggtcacactggtgcgtggtggcatactgggaggagaagacg 202

863  agagtggggaggctctactgtgtccaggagccctctctggatatcttctatgatctacct 922
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
203  agagtggggaggctctactgtgtccaggagccctctctggatatcttctatgatctacct 262

923  caggggaatggcttttgcctcggacagctcaattcggacaacaagagtcagctggtgcag 982
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
263  caggggaatggcttttgcctcggacagctcaattcggacaacaagagtcagctggtgcag 322

983  aaggtgcggagcaaaatcggctgcggcatccagctgacgcgggaggtggatggtgtgtgg 1042
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
323  aaggtgcggagcaaaatcggctgcggcatccagctgacgcgggaggtggatggtgtgtgg 382

1043 gtgtacaaccgtagcagttaccccatcttcatcaagtccgccacactggacaacccggac 1102
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
383  gtgtacaaccgcagcagttaccccatcttcatcaagtccgccacactggacaacccggac 442

1103 tccaggacgctgttggtacacaaggtgttccccggtttctccatcaaggctttCGACTAC 1162
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
443  tccaggacgctgttggtacacaaggtgttccccggtttctccatcttggctttcgactac 502

1163 GAGAAGGCGTACA 1175
     |||||||||||||
503  gagaaggcgtacaaannn..... 520
```

Appendix D: Preparation of Smad7 anti-peptide antibodies

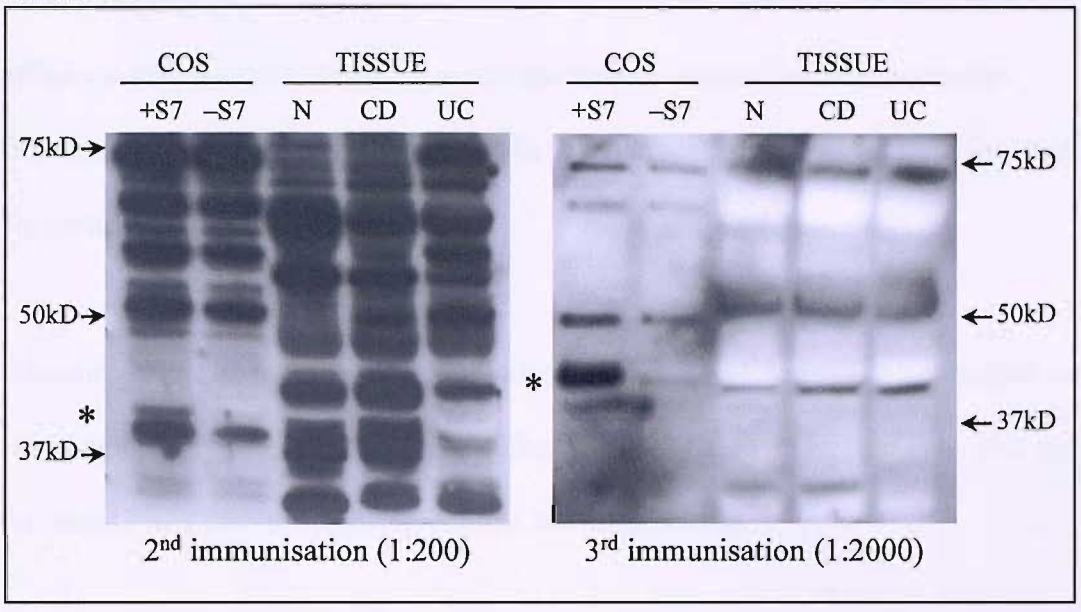
The small amount of anti-peptide serum provided by Prof Heldin's group (Uppsala, Sweden) was not sufficient to undertake future experiments. Taken together with the expense of purchasing antibodies from commercial sources, we decided to make our own antibodies to Smad7 in which we had confidence and which could be used for further studies.

Preparation of antibodies

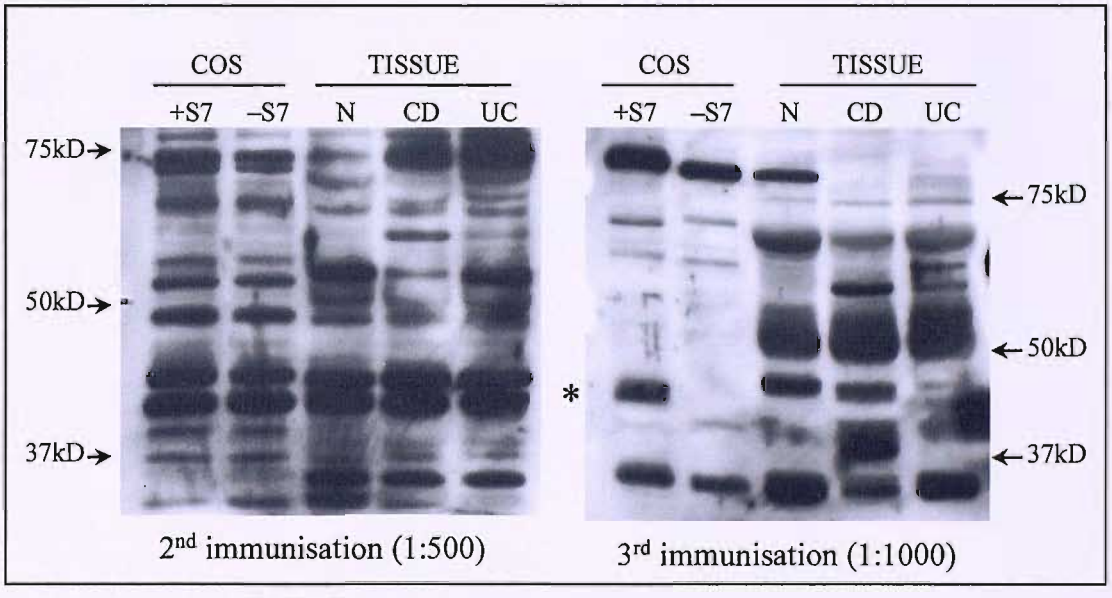
The peptide sequence used by Prof Heldin's group used to raise this antibody was synthesised and conjugated to Keyhole Limpet Haemocyanin (KLH) for immunisation of 2 rabbits (Moravian Biotechnology Ltd, Czech Republic). Sera obtained after the 2^{nd}, 3^{rd} and 4^{th} immunisations of KLH-conjugated Smad7-derived peptide were analysed for Smad7 specificity by Western blotting. Protein lysates were from COS cells transfected with Smad7-pcDNA3 or empty vector (20μg/lane) and whole tissue lysates from normal, CrD and UC (50μg/lane).

Immunoblots using the serum from both rabbits after 2 immunisations detected multiple non-specific protein bands. However, serum rabbit k26 recognises a protein band at 43kD in the Smad7 transfected COS cell lysate not present in cells transfected with empty vector (43kD marked: *). After the third immunisation, both rabbits' sera can detect this band in the Smad7-transfected COS cell lysates. In the human tissue lysates, bands remain at 51kD.

Rabbit #36:



Rabbit k26:



Evaluation of affinity purified antibodies

After a fourth immunisation, the rabbits were sacrificed and the sera were obtained for affinity purification of antibody against the Smad7-derived peptide (Moravian Biotechnology Ltd, Czech Republic). The resultant antibodies were tested for specificity for Smad7 for Western blotting.

Initial experiments tested antibody titrations in COS cell lysates. The antibodies were then tested against human gut tissue lysates from normal, CrD and UC patients. The specificity for Smad7 was then tested using peptide blocking studies.

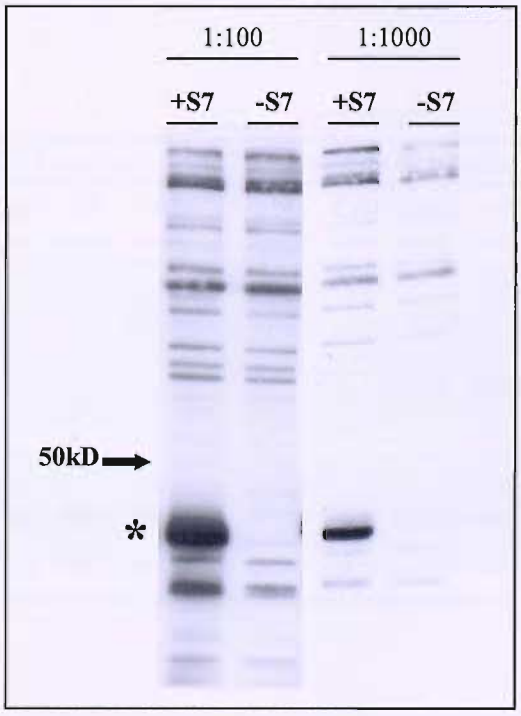
Antibody titrations

Antibodies were tested against COS cell lysates, transfected with Smad7 expression vector or empty vector. Each antibody was compared by immunoblots against 20 μg of COS cell lysate, using dilutions ranging from 1:100 to 1:2000. Blots were developed using ECL Plus chemiluminescent reagent.

Using 1:100 dilutions, there is detection of multiple protein bands with both antibodies. The results from 1:1000 dilutions show that antibody k26 has detects a protein band at 50kD in both COS cell lysates, and a band at 43kD in the Smad7-transfected cells only (*). Antibody #36 detects a protein band at 43kD in the Smad7-transfected cells, and weak detection of non-specific bands at higher and lower apparent molecular weights.

Antibody k26:

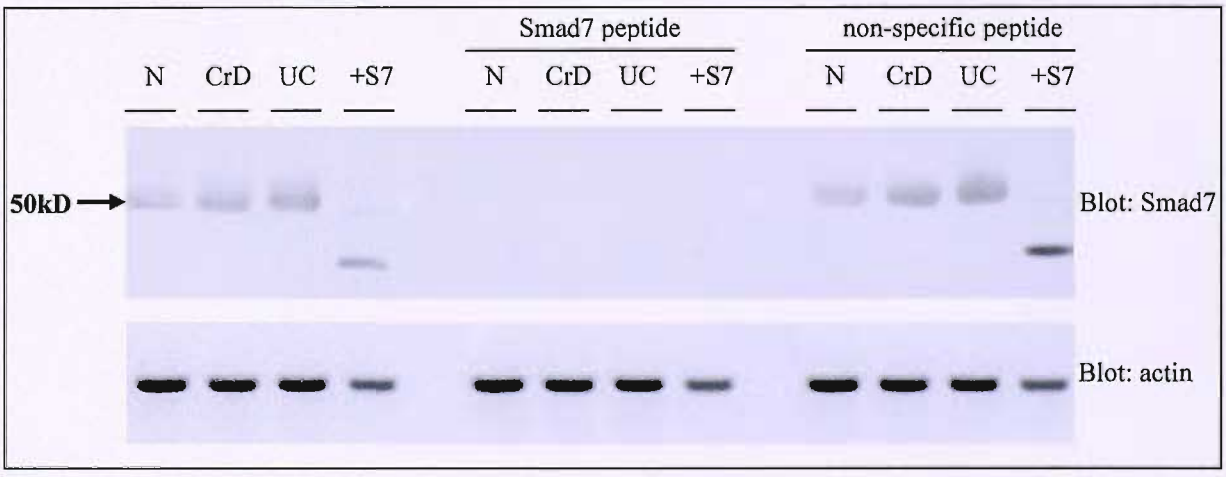
Antibody #36:



Peptide blocking studies

In order to examine antibody specificity, the affinity purified anti-peptide Smad7 antibody #36 was tested against transfected COS cell lysate (20μg) and human tissue lysate (50μg), from normal and IBD biopsy samples using. Antibody #36 (1:1000) was pre-incubated in TBST with Smad7-derived or non-specific peptides at 10μg/ml. Protein lysates were from COS cells transfected with Smad7-pcDNA3 or empty vector (20μg/lane) and whole tissue lysates from normal, CrD and UC (50μg/lane).

The Smad7-derived peptide prevents any binding of the primary antibody to both the tissue samples and the tranfected COS cell lysate. The actin blot confirms the presence of protein on the membrane after transfer in all lanes. This result demonstrates that the Smad7 antibody binds specifically to both the 43kD band in the transfected cells and the 51kD band in the human tissue samples.



Discussion

Affinity purified antibodies raised against the Smad7-derived peptide sequence show good specificity for recombinant Smad7 protein in COS cell lysate. The #36 antibody has better specificity, detecting a clear band at 43kD. In human tissue samples, there is a single band detected at 51kD, consistent with the results obtained with the commercial antibody H-79 (St Cruz). Detection of this 51kD band in human samples is affected by pre-incubating the antibody Smad7-specific peptide. This suggests that the 51kD band represents Smad7. The different apparent molecular weight in human tissue may reflect protein alteration by post-translational modification.

These data suggest that antibody #36 can be used for future experiments examining Smad7 expression in human gut tissue and extracted cells.

Appendix E: Calprotectin results table

SEX	AGE (yrs)	GROUP	DETAILS	CALPROTECTIN (µg/g)	PCDAI/CSS
F	12	CrD	ileocolonic	1001	15
F	15	CrD	ileocolonic	932.7	0
M	16	CrD	ileocolonic	894.6	20
M	13	CrD	ileocolonic	834.08	45
F	12	CrD	ileocolonic	560.5	45
M	14	CrD	ileocolonic	470.46	0
F	15	CrD	ileocolonic	77.8	65
M	16	CrD	colitis	1524.3	60
M	14	CrD	colitis	637.44	10
F	13	CrD	colitis	396.4	30
F	13	CrD	colitis	374.6	10
M	13	CrD	colitis	320.6	15
M	14	CrD	colitis	311.06	20
F	17	CrD	colitis	142.0	0
M	12	CrD	colitis	86.4	25
M	16	CrD	colitis	84.06	20
M	11	CrD	colitis	22.6	5
F	15	CrD	ileitis	799.8	0
F	14	CrD	ileitis	755.92	0
M	14	CrD	ileitis	708.0	20
M	16	CrD	ileitis	410.34	45
M	13	CrD	ileitis	327	0
M	12	CrD	ileitis	269.4	25
M	11	CrD	ileitis	199.9	5
M	13	CrD	ileitis	156.0	0
M	11	CrD	ileitis	128.0	10
M	11	CrD	ileitis	113.5	5
M	13	CrD	ileitis	62.0	5
M	9	CrD	oral/rectal	448.72	45
F	16	UC		1596.0	5
M	9	UC		1584.7	5
F	12	UC		1281.9	3
F	13	UC		1250	5
M	14	UC		575.6	4
F	13	UC		573.5	1
F	10	UC		336.6	4
M	12	UC		327.1	0
F	12	UC		304.1	0
M	11	UC		250.5	1
F	8	UC		224.8	0
F	8	UC		156.3	2
M	8	UC		94.2	1
F	13	UC		45.6	0

SEX	AGE (yrs)	GROUP	DETAILS	CALPROTECTIN (μg/g)
F	4	coeliac	on aspirin	220.1
F	12	coeliac		99.2
F	10	coeliac		38.0
M	4	coeliac		55.0
F	6	coeliac		50.0
F	4	other	bacterial dysentery	715.84
M	10	other	immunodeficiency	245.0
F	13	other	Relative with CrD	242.36
F	15	other	Asperger's synd. & food intolerance	173.0
F	11	other	protein-losing enteropathy	100.0
F	13	other	N/S abdo pain	68.8
M	8	other	N/S abdo pain	66.0
F	14	other	enteropathy	56.0
F	5	other	viral gastroenteritis	53.0
F	14	other	Familial Polyposis Syndrome	25.0
M	5	other	N/S abdo pain	15.6
F	6	other	food intolerance	15.6
F	5	other	Post-enteritis Syndrome	15.6
M	11	other	apthous ulcers	15.6

SEX	AGE (yrs)	GROUP	DETAILS	CALPROTECTIN (μg/g)
M	16	constipation		63.1
M	14	constipation		58.0
M	4	constipation		52.0
M	10	constipation		47.7
M	13	constipation		46.8
M	10	constipation		40.3
F	12	constipation		38.8
M	10	constipation		32.7
F	5	constipation		28.7
F	7	constipation		28.2
F	15	constipation		26.0
M	8	constipation		20.6
M	7	constipation		19.8
F	14	constipation		15.6
F	10	constipation		15.6
F	6	constipation		15.6
F	10	constipation		15.6
F	6	constipation		15.6
F	8	constipation		15.6
M	7	constipation		15.6
F	16	constipation		15.6
F	7	constipation		15.6
F	14	constipation		15.6
M	7	constipation		15.6
F	9	constipation		15.6
F	9	constipation		15.6
M	13	constipation		15.6
M	11	constipation		15.6
M	10	constipation		15.6
F	5	constipation		15.6
F	6	constipation		15.6
M	12	normal		39.0
M	13	normal		23.6
F	5	normal		22.3
F	14	normal		15.6
M	11	normal		15.6
M	11	normal		15.6
F	10	normal		15.6

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