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Paediatric Inflammatory Bowel disease - bench to bedside and nationwide

A detailed analysis of Scottish children with IBD

Dr Paul Henderson

A PhD thesis presented to the University of Edinburgh

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List of publications arising from this thesis

Permission from publishers and co-authors has been obtained for publications included in this thesis. (All papers are included on the attached CD)

Primary Data Articles

Henderson P, Anderson NH, Wilson DC. Fecal calprotectin for the diagnosis of pediatric inflammatory bowel disease: a systematic review and meta-analysis. Am J Gastroenterol. 14 May 2013; doi:10.1038/ajg.2013.131 [Epub ahead of print] (PMID: 23670113)

Stevens C, **Henderson P**, Nimmo ER, Soares DC, Dogan B, Simpson KW, et al. The intermediate filament protein vimentin is a regulator of NOD2 activity. Gut. 8 Jun 2012 [Epub ahead of print] (PMID: 22684479)

Henderson P, Casey A, Lawrence SJ, Kennedy NA, Kingstone K, Rogers P, et al. The diagnostic accuracy of fecal calprotectin during the investigation of suspected pediatric inflammatory bowel disease. Am J Gastroenterol. 2012;107(6):941-949 (PMID:22370604)

Henderson P, Hansen R, Cameron FL, Gerasimidis K, Rogers P, Bisset WM, et al. Rising incidence of pediatric inflammatory bowel disease in Scotland. Inflamm Bowel Dis. 2012;18(6):999-1005. (PMID: 21688352)

Henderson P, van Limbergen J, Anderson NH, Nimmo E, Russell RK, Satsangi J, Wilson DC. Letter: Variation in ICOSLG influences Crohn's disease susceptibility. Gut. 2011;60:1444. (PMID: 21335567)

Reviews

Henderson P, Stevens C. The role of autophagy in Crohn's disease. Cells. 2012;1(3):492-519 (doi:10.3390/cells1030492)

Henderson P, van Limbergen J, Schwarze J, Wilson DC. Function of the intestinal epithelium and its dysregulation in inflammatory bowel disease. Inflamm Bowel Dis. 2011;17(1):382-395. (PMID: 20645321)

Henderson P, van Limbergen J, Wilson DC, Satsangi J, Russell RK. Genetics of childhood-onset inflammatory bowel disease. Inflamm Bowel Dis. 2011;17(1):346-361. (PMID: 20839313)

Letters, Commentaries and Editorials

Henderson P, Wilson DC, Satsangi J, Stevens C. A role for vimentin in Crohn's disease. Autophagy. 2012;8(11) [Epub ahead of print] (doi: 10.4161/auto.21690) (PMID:22929019)

Henderson P, Wilson DC. Editorial: The rising incidence of paediatric-onset inflammatory bowel disease. Arch Dis Child. 2012;97(7):585-586. (PMID: 22745290)

Henderson P, Wilson DC, Satsangi J. Comment: Differences in phenotype and disease course in adult and paediatric inflammatory bowel disease - a population-based study. Aliment Pharmacol Ther. 2012;35(3):391-392. (PMID: 22221081)

Satsangi J, Kennedy NA, **Henderson P**, Wilson DC and Nimmo ER. Editorial: Exploring the hidden heritability of inflammatory bowel disease. Gut. 2011;60(11):1447-1448. (PMID: 21896637)

Henderson P, Russell RK, Satsangi J, Wilson DC. Comment: The changing epidemiology of paediatric inflammatory bowel disease. Aliment Pharmacol Ther. 2011(33):1380-1381. (PMID: 21569065)

Henderson P, Satsangi J. Editorial: Genes in IBD: lessons from complex disease. Clin Med. 2011;11(1):8-10. (PMID: 21404774)

Abstracts

(Where more than one version of an abstract has been published on several occasions, only one reference is given. Additionally, all abstracts have been presented as either an oral or poster presentation at national or international meetings).

Henderson P, Rogers P, Mitchell D, Devadason D, Gillett PM, Wilson DC. The epidemiology and natural history of paediatric inflammatory bowel disease in southeast Scotland: a prospective 13-year study. Gut. 2011;60(Suppl 3):A62.

Henderson P, Wilson DC. Faecal calprotectin for the diagnosis of paediatric inflammatory bowel disease: a meta-analysis. Gut. 2011;60(Suppl 3):A412.

Stevens C, **Henderson P**, Nimmo ER, Soares DC, Wilson, DC, Satsangi J. Vimentin is a regulator of NOD2 activity and responsiveness to adherent-invasive E.coli in mammalian cells. Gut. 2011;60(Suppl 3):A16.

Henderson P, Casey A, Lawrence SL, Kingstone K, Rogers P, et al. The value of faecal calprotectin in the investigation of suspected early-onset inflammatory bowel disease. Gastroenterology. 2011;140(5)(Suppl I):S-507.

Henderson P, van Limbergen J, Anderson NH, Cameron FL, Cameron E, Russell RK, et al. Variations in the gene encoding C-reactive protein suggest that CRP is a candidate susceptibility gene for inflammatory bowel disease in the Scottish paediatric population. Gut. 2011;60(Suppl I):A64

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Henderson P, Hansen R, Cameron F, Gerasimidis K, Rogers P, Bisset WM, et al . The rising incidence of early onset inflammatory bowel disease in Scotland. Gut. 2010 Mar:59(4)(Suppl I):A40.

Henderson P, Noble CL, Abbas AR, Schwarze J, Wilson DC, Satsangi J. Differences in costimulatory molecule gene expression on endoscopic biopsies from patients with ulcerative colitis. Gut. 2009;58(Suppl II):A313

Declaration of Originality

I declare that all the work in this thesis is entirely my own, unless otherwise indicated, performed in the Department of Child Life and Health, University of Edinburgh and the Gastrointestinal Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh between March 2009 and March 2012. This work has not been submitted for any other professional degree or qualification.

The data collection, experimental work and subsequent analysis was all carried out by Dr Paul Henderson with the exception of:

- Dr Richard Hansen, Dr Fiona Cameron, Dr Kostas Gerasimidis and Ms Pam Rogers provided clinical data pertaining to the Scottish-wide incidence data; Ms Pam Rogers also provided more detailed clinical data with regard to the South-East Scotland prospective cohort.
- A large proportion of clinical phenotyping of PIBD patients had previously been carried out by Dr Richard Russell, Dr Johan Van Limbergen and Mrs Hazel Drummond as part of previous PhD theses and ongoing as part of the Paediatric Inflammatory Bowel Disease Cohort and Treatment Study (PICTS).
- Dr Niall Anderson and Dr Johan Van Limbergen gave assistance with the transmission disequilibrium testing analysis during the *ICOSLG* and *CRP* genetic analysis, with Dr Anderson also providing the pooled sensitivity and specificity analysis of the faecal calprotectin meta-analysis data. Dr Nick Kennedy also performed statistical analyses during the faecal calprotectin case-control study analyses and gave general statistics advice.
- Dr Colin Noble collected and analysed the microarray data pertaining to the *ICOSLG* analysis in adult IBD patients.
- TaqMan genotyping was performed by the staff of the Wellcome Trust Clinical Research Facility, Western General Hospital (WGH), Edinburgh by William Hawkin under the supervision of Lee Murphy.
- Dr Sally Lawrence and Dr Aoife Casey helped collect data regarding patients in the faecal calprotectin study and Dr Elizabeth Freyer performed the flow cytometry analysis of the NOD2-variant cell lines at the Medical Research Council Facility, WGH.

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Abbreviations

AIEC:	adherent, invasive *E.coli*
AUC:	area under the curve
AZA:	azathioprine
CD:	Crohn's disease
CI:	confidence interval
CRP:	C-reactive protein
DGH:	district general hospital
DNA:	deoxyribonucleic acid
ESR:	erythrocyte sedimentation rate
FC:	faecal calprotectin
GFP:	green fluorescent-labelled protein
GI:	gastrointestinal
GWAS:	genome-wide association scan
Hb:	haemoglobin
IBD:	inflammatory bowel disease
IBDU:	colonic inflammatory bowel disease, type unclassified
ICOS:	inducible T cell co-stimulator
ICOSLG:	inducible T cell co-stimulator ligand
IEC:	intestinal epithelial cell
IQR:	interquartile range
LC3:	microtubule-associated protein light chain 3
LD:	linkage disequilibrium
LRR:	leucine-rich repeat
MDP:	muramyl dipeptide
6-MP:	6-mercaptopurine
MTX:	methotrexate
NCC:	non-Crohn's colitis
NOD2:	Nucleotide-binding oligomerisation domain-containing protein 2
OR:	odds ratio
PIBD:	paediatric inflammatory bowel disease
ROC:	receiver operating curve
SES:	south-east Scotland
SNP:	single nucleotide polymorphism
TDT:	transmission disequilibrium test

TNF-α:	tumour-necrosis factor-alpha
UC:	ulcerative colitis
UK:	United Kingdom
UTR:	untranslated region
WCC:	white cell count
Yr:	year

Abstract

The inflammatory bowel diseases (IBDs) are a group of chronic conditions affecting the gastrointestinal tract, often presenting with non-specific clinical features such as abdominal pain, weight loss and diarrhoea. Approximately 25% of patients are diagnosed with IBD in childhood.

For epidemiological studies, previously collected (1990-1995) and original (2003-2008) Scottish incidence data were used to determine national trends in newly diagnosed paediatric IBD (PIBD). A smaller, geographically defined, prospective 14-year cohort (1997-2011) in South-East Scotland (SES) was used to assess regional trends in incidence, point prevalence, disease extent, medication use and PIBD surgery rates in 326 children. For the detailed analysis of the role of ICOSLG and CRP in Scottish children with PIBD, haplotype-tagging of both genes in 448 children (and their parents) registered on the Paediatric Inflammatory bowel disease Cohort and Treatment Study (PICTS) database was performed. Further clinical information from this database and previously gathered adult mRNA microarray data were also used to inform the analysis. For the faecal calprotectin (FC) case-control study, all PIBD patients diagnosed in SES between 01.01.05 and 31.12.10 (aged 1-17yrs) with a FC performed during initial workup were identified; controls were matched non-IBD patients who had similarly undergone endoscopy with a referral FC level available. The systematic review and meta-analysis of FC case-control studies was performed with keywords relating to IBD and calprotectin in electronic resources from 1946 to May 2012. Inclusion criteria were studies that reported FC levels prior to the endoscopic investigation of IBD in children less than 18 years old. Laboratory work used newly derived HEK293 and HCT116 cell lines stably expressing wild-type NOD2 and the CD-associated NOD2 frameshift mutant, as well as utilising previously derived HEK293 and HCT116 cells stably expressing green fluorescent-labelled protein LC3 during the assessment of autophagy. Western blot, immunofluorescent microscopy and flow cytometry were used for analysis.

There was a significant rise in PIBD incidence in Scotland since the early 1990s, with 260 new cases between 1990-1995 (4.45/100,000/year) and 436 in the 2003-2008 epoch (7.82/100,000/year) ($p<0.001$). A five-fold increase in Crohn's disease (CD) in the last 40 years was also demonstrated. SES was shown to have the highest recorded PIBD incidence rate in the UK for the six-year epoch from 2006-2011 (9.50/100,000/year) with a significant rise in ulcerative colitis (UC) to 2.67/100,000/year ($p=0.010$). Point prevalence rates for PIBD in SES had also risen significantly to 41.2/100,000 between the 2000-2005 and 2006-2011

epochs ($p=0.016$). With a follow up of 1577 patient years, the severe phenotype in children with PIBD was confirmed; 34% of children with CD presented with pan-enteric disease (44% at follow up), and 76% of children with UC had pancolonic disease at diagnosis (81% at follow up). 26% of patients required methotrexate and 18% were exposed to infliximab/adalimumab, with the time to first exposure of both significantly lower in children diagnosed between 2006-2011 ($p=0.001$ and $p<0.001$ respectively). A total of 70% of children were exposed to azathioprine and 20% underwent IBD-related surgery.

Using a haplotype-tagging approach and transmission disequilibrium testing (TDT) in 230 PIBD case-parent trios there was significant overtransmission of the rs8126734-A single nucleotide polymorphism (SNP) in *ICOSLG* following correction ($p=0.0467$). In the CD TDT analysis the same SNP was overtransmitted ($p=0.0084$). The strongest susceptibility signal was evident across the two marker haplotype rs762421-A / rs8126734-G ($p=0.0072$), suggesting that the 3-prime untranslated region in *ICOSLG* may be targeted for deep sequencing. mRNA microarray data from adult patients showed downregulation of *ICOSLG* expression in the ascending colon ($p=0.023$) and upregulation in the descending colon ($p=0.0351$) in uninflamed biopsies from CD patients and non-IBD controls; no difference in gene expression was shown in UC patients. Using a similar approach, the A allele of two SNPs tagging *CRP* showed significant over-transmission to affected IBD patients after correction (rs1417938, $p=0.006$; rs1130864, $p=0.015$). The six-marker haplotype (ACACAC) showed significant distortion of transmission to affected individuals ($p=8x10^{-4}$). CD and UC patients demonstrated differences in rs1205 genotype ($p=0.0085$) and *CRP* haplotype ($p=0.0024$), with the influence of the rs1205 SNP on response to anti-tumour necrosis factor-alpha therapy also shown ($p=0.021$).

During the FC case-control study significantly elevated FC levels at diagnosis were demonstrated compared to controls (1265 µg/g vs 65 µg/g; $p<0.001$). FC also outperformed commonly used blood parameters (e.g. CRP, ESR, platelets), with an area under the curve of 0.93 (95% CI 0.89-0.97) and good sensitivity (0.93 [95% CI 0.86-0.98]) and specificity (0.74 [95% CI 0.64-0.82]) when values above 200µg/g were used. FC levels were not influenced by disease location in CD or UC. The systematic review and meta-analysis highlighted the often poor methodological quality of previous studies and concluded that across all studies FC had a pooled sensitivity of 0.98 (95% CI 0.95-1.00) and pooled specificity of 0.68 (95% CI 0.50-0.86) for PIBD at diagnosis.

Characterisation of cells stably-expressing wild-type NOD2 or the CD-associated NOD2 frameshift mutation demonstrated increased cell proliferation compared to empty vector, and

an accentuated apoptotic response to serum starvation. The NOD2 frameshift protein had a shorter half-life (at 11 hours) than the wild-type protein, with degradation of the NOD2 protein shown to be mediated through a proteasome-dependent pathway, possibly through lysine residues on the CARD domain. Following the establishment of a robust method of assessing autophagy in a cell culture system, experimental work showed that muramyl dipeptide-induced autophagy is unlikely to signal through the mammalian target of rapamycin, with the intermediate filament vimentin shown to be intimately involved in this pathway; the vimentin gene (*Vim*) was also shown to be a candidate susceptibility gene for CD. Using a panel of PIBD drugs azathioprine was shown to induce autophagy in a dose-dependent manner through an mTOR-dependent, ERK-independent pathway.

It can be seen that with the increasing incidence and prevalence of PIBD in Scotland that a greater understanding of epidemiological trends, the role of genetic susceptibility, the optimal use of biomarkers and translational functional biology are all needed to understand further the aetiopathogenesis of PIBD. This future work will undoubtedly help to inform service design and the clinical care pathways utilised to provide the best care for children in addition to targeting pathways for potential drug development, with these measures helping to prepare for the increasing disease burden generated by PIBD.

1. The pathogenesis and clinical phenotype of paediatric inflammatory bowel disease

1.1 Introduction

The inflammatory bowel diseases (IBDs) are a group of chronic conditions affecting the gastrointestinal (GI) tract, often presenting with non-specific clinical features such as abdominal pain, weight loss and diarrhoea. To date the precise pathogenic mechanisms underlying IBD remain unclear, however it is likely that dysbiosis of the intestinal microbiota and other environmental influences contribute to disease in those with genetic susceptibility.[1] The IBDs are divided into three disease types: Crohn's disease (CD), ulcerative colitis (UC) and colonic inflammatory bowel disease, type unclassified (IBDU).[2] CD is typically characterised by transmural, granulomatous inflammation affecting any part of the GI tract from mouth to anus, often discontinuously. In contrast, UC is limited to the colon and consists of superficial ulceration of the bowel mucosa. The third type, IBDU, describes patients with chronic colitis within the spectrum of IBD but in the absence of distinguishing features of either CD or UC.[2]

1.2 Inflammatory bowel disease genetics

One of the first indications that a significant genetic component was important in IBD pathogenesis was the observation of increased twin concordance in the late 1980s.[3] It has since been shown that the risk of CD in monozygotic twins is 36% and for UC 16%, in contrast to 4% for both diseases in dizygotic twins.[4] Furthermore, the risk of IBD has also been shown to be increased in first-degree relatives (more than a 1 in 10 lifetime risk of developing disease[5,6]) and also in certain ethnic groups[7,8]. In addition, several IBD genetic risk loci have been identified in other inflammatory disorders.[9] For example single nucleotide polymorphisms (SNPs) associated with the gene encoding interleukin-23 receptor (IL23R) have been shown to be involved in psoriasis[10] and ankylosing spondylitis[11] as well as IBD[12,13].

Following the strong evidence for heritability in IBD in the 1990s the search began to uncover the genetic variations that led to this increased disease susceptibility. Using non-parametric linkage analysis, a number of genome-wide searches were conducted between 1996-2006 in order to identify genetic regions involved in IBD (**Figure 1.1**).[14] This method of gene identification has come under scrutiny in recent years, mainly due to newer techniques

failing to replicate findings in these early analyses. However, these studies uncovered a large number of both putative and confirmed linkage regions including the identification of *NOD2*/*CARD15* which remains the strongest individual signal using more modern genome-wide association scanning (GWAS) techniques.[15]

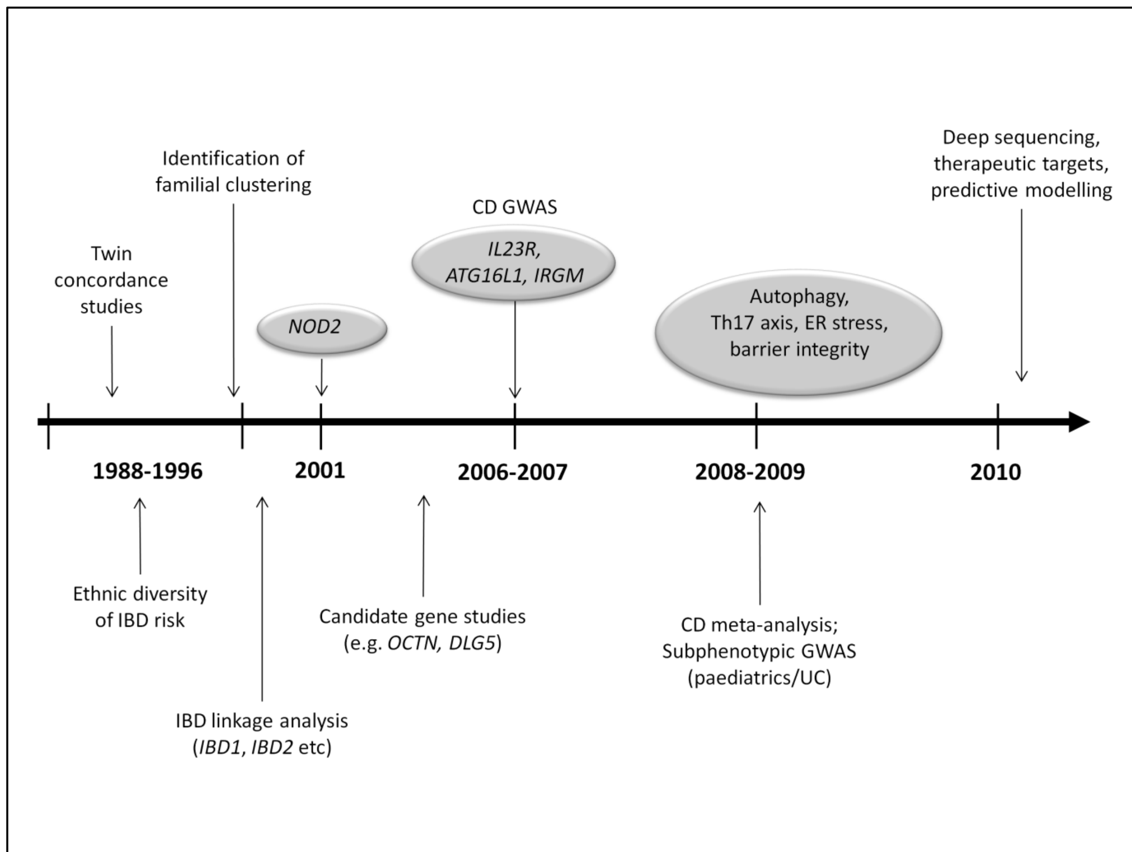


Figure 1.1. Timeline of epidemiological and genetic discoveries in inflammatory bowel disease. GWAS, genome-wide association scan; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; ER, endoplasmic reticulum.

The discovery of *NOD2* in 1996 was a major breakthrough in not only IBD genetics but complex polygenic disease overall. Discovered by the French paediatric gastroenterologist JP Hugot following fine mapping of the IBD1 locus,[16] Hugot's group later identified variants in the leucine-rich repeat region (LRR) of the gene as conferring susceptibility to IBD,[17] something which has been widely replicated in both adult and paediatric disease[13,18]. The gene encoded by *NOD2* is intimately involved in the innate immune response and has gut expression limited to anti-microbial Paneth cells, with the LRR domain recognising muramyl dipeptide (MDP), a product of bacterial cell wall degradation.[19,20] Since these initial studies further work has demonstrated a diverse range of functions for this pattern recognition

receptor including the regulation of commensal bacteria,[21] the control of certain immune cell populations[22] and viral recognition[23].

The process of autophagy (i.e. the delivery of portions of the cytoplasm to the lysosome for degradation) is now receiving increasing attention, with three loci containing autophagy-related genes (*ATG16L1*, *IRGM* and *LRRK2*) reaching genome-wide significance in the recent CD GWAS meta-analysis[13] and two (*ATG16L1* and *IRGM*) in UC[24]. Recent work in this area has shown that, in the ileal epithelium, ATG16L1 is crucial for Paneth cell biology[25] with CD patients homozygous for the risk allele displaying Paneth cell granule abnormalities[26]. IRGM, a member of the GTPase family, has similarly been shown to have key roles in the protection against adherent-invasive *E.coli*,[27] the protection of effector CD4+ cells against interferon-gamma (IFN-γ)-induced autophagic cell death[28] and macrophage motility[29]. It should also be noted that a link between innate immune recognition molecules (NOD1 and NOD2) and autophagy has now been established,[30,31] with alterations in the ubiquitin-proteasome system (which accounts for the remaining 80% of intracellular component degradation) also demonstrated in IBD[32].

A number of genetic susceptibility loci containing genes involved in T cell biology have also been identified by recent analyses. Following innate immune system signalling, a coordinated T helper (Th) cell response by the adaptive immune system leads to tissue-specific inflammation.[33] One of these recently described subsets are the Th17 effector cells, classified on the basis of intracellular transcription factors such as signal transducer and activator of transcription 3 (STAT3), RAR-related orphan receptor gamma T (RORγT) and RAR-related orphan receptor alpha (RORα).[34] The association with CD of a germline variation of the *IL23R* gene in 2006 provided the initial stimulus to look more closely at the Th17 subset.[12] Specifically the Arg381Gln variant in *IL23R* has been shown to be protective for the development of IBD although other signals within the gene also confer disease susceptibility independently of this SNP.[35] IL23R is expressed on T cells with IL-23 promoting the stabilisation of the Th17 subset after differentiation through various intracellular transcription factors such as STAT3. Furthermore, germline variations in the genes encoding STAT3, the tyrosine kinase Janus kinase 2 (JAK2) and the G protein-coupled chemokine receptor C-C chemokine receptor type 6 (CCR6) (all intimately involved in Th17 biology) have also been implicated in CD pathogenesis.[13] Similarly, Barrett et al. demonstrated that a SNP at chromosome 21q22.3 conferred susceptibility to CD, with the recent paediatric GWAS showing significance in both CD and UC.[18] The gene encoding inducible T cell co-stimulator ligand (ICOSLG) was suggested as the candidate susceptibility gene at this locus, although no replication or expression data has yet confirmed this association.[13] Early in the differentiation of the Th17 subset ICOSLG binds with its T cell ligand (ICOS) leading to T cell activation.[36] The interaction of ICOSLG with its ligand ICOS

is not only essential in the early differentiation of naive T cells but is likely to be important in the careful balance of IL-10-producing regulatory cells and the Th17 population.[37] An overview of the genetic architecture of IBD is shown in **Figure 1.2**.

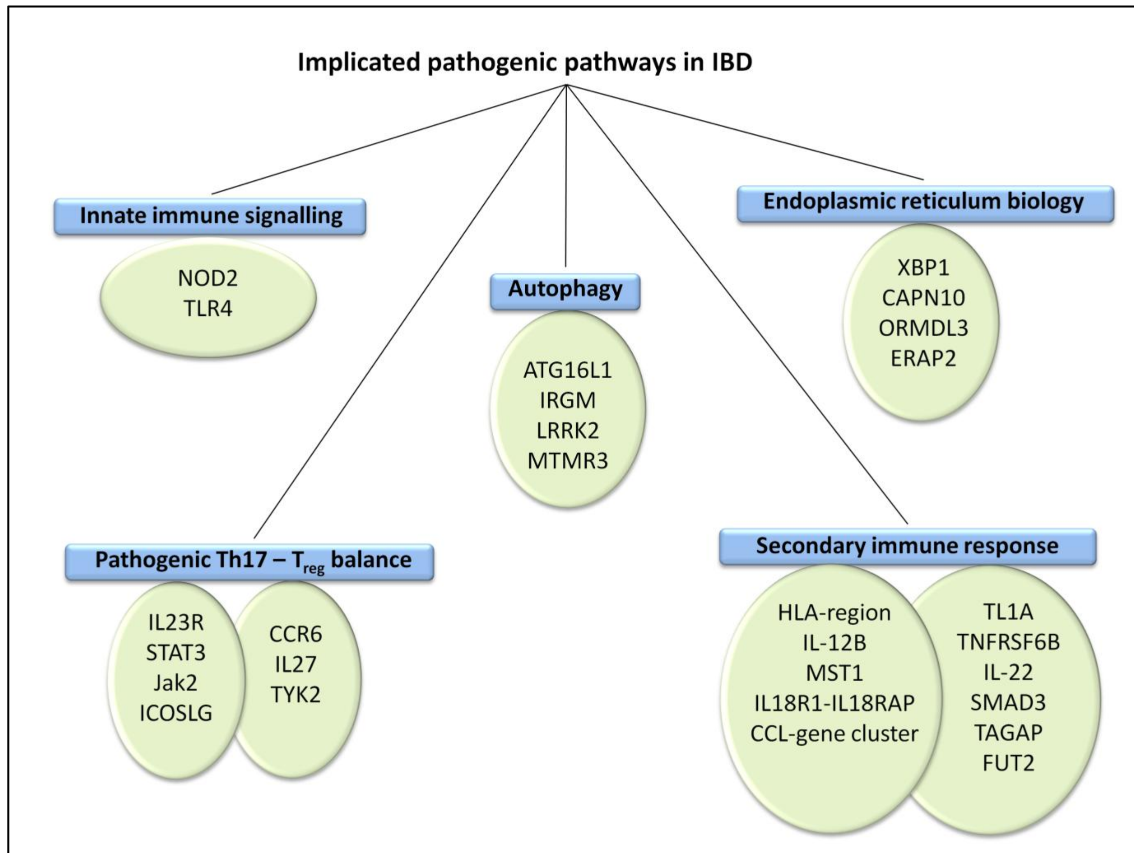


Figure 1.2. Broad overview of the genetic architecture of inflammatory bowel disease giving examples of confirmed and candidate genes implicated in each pathway. T_{reg}, regulatory T cell.

1.3 The intestinal epithelium - barrier function, immune regulation and the microbiota

The intestinal mucosa is constantly exposed to a milieu of both commensal bacteria and dietary antigen. The gut is therefore uniquely characterised by a state of hyporesponsiveness through carefully balanced local responses.[38] This active process not only maintains mucosal integrity but also prevents the induction of an overwhelming immune response leading to intestinal inflammation. At the heart of this complex interface is a single monolayer of intestinal epithelium acting as a physical barrier to foreign antigen. However, in addition to this barrier function, the intestinal epithelial cell (IEC) also recognises, processes

and presents antigen, produces a myriad of signalling molecules and directly affects immune cell proliferation and differentiation; IBD is characterised by inflammation at this mucosal surface. It is now becoming apparent that abnormalities within the epithelial layer, such as increased permeability and abnormalities in IEC-immune cell interactions, play a key role in the disease process.[39,40]

The proximal and distal sections of the human intestinal tract (i.e. the oral cavity, oesophagus and anus) are lined with stratified squamous epithelium and are commonly affected in CD patients. The epithelium lining the remainder of the gut is composed of non-ciliated, columnar epithelium. This rapidly self-renewing tissue is primarily involved in the absorption of water and nutrients, but also has a vital role in acting as a barrier to luminal pathogens. The epithelial monolayer is carefully folded, producing crypts [of Lieberkühn] and villous protrusions. The crypts are an important site of IEC differentiation and it is here that the four IEC types are derived from pluripotent stem cells.[41] Three of the cell types (goblet, enteroendocrine and absorptive) migrate to the tip of the villus where they undergo spontaneous apoptosis several days after terminal differentiation.[42] On the contrary, Paneth cells remain within the crypt after differentiation and have a considerable role in antibacterial defence.[43] The secretory lineages (Paneth, goblet and enteroendocrine cells) are small in number within the epithelial layer with the absorptive lineage (the enterocyte) accounting for over 80% of the epithelial cells.[44] These enterocytes are highly polarised, with an apical brush border that is in constant contact with the luminal contents and a basolateral component adjacent to the stroma.

Interspersed between the single epithelial cells lining the intestine are intraepithelial lymphocytes (IELs). These cells, which express αβ T-cell receptors (TCRs) or γδTCRs, monitor for stressed or damaged IECs.[45] Several pathways involving IELs are of particular interest in IBD, particularly the so-called natural killer T (NKT) IELs,[46] the key chemokine receptor C-C chemokine receptor 9 (CCR9)[47] and IEL-expressed α4β7 integrin[48]. Additionally, individual epithelial cells effectively compartmentalise the underlying stroma through intercellular junctions, contributing to the epithelium's overall barrier function. These proteins help maintain cellular polarity by regulating the flux of water, electrolytes, lipids and proteins across the epithelium, in addition to providing a physical barrier to both commensal and pathogenic bacteria. Defects in these intercellular junctions have been shown to lead to increased epithelial permeability which has been reported in those suffering from IBD and their first–degree relatives.[49-52] Disordered intestinal permeability has also been shown to predict and possibly cause relapse[53,54] and is increased in those at high risk of CD at baseline[55]. Defects in E-cadherin have been of particular interest in IBD research with upregulation in areas of active inflammation[56] and downregulation by pathogens implicated in the development of colitis such as adherent-invasive *E.coli*[57]. Similarly, the tight junction

protein junctional adhesion molecule-A (JAM-A), occludin expression,[58] claudin dysregulation[59] and altered cytokine production[60,61] have also been implicated in the overall barrier disruption in IBD, however the mechanisms involved remain unclear and often controversial[62]. An overview of the intercellular junctions within the intestine is shown in **Figure 1.3**.

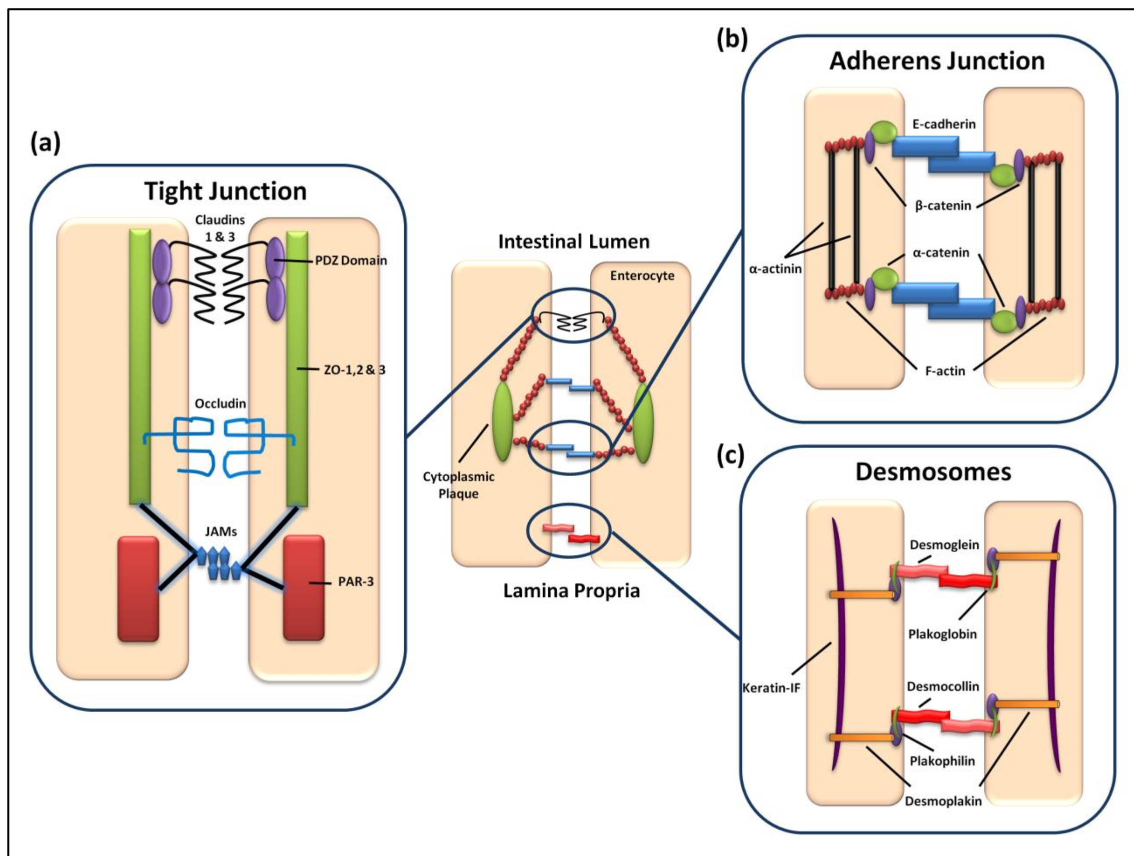


Figure 1.3. The intestinal epithelial junctional complexes. Each enterocyte is connected at the tight junction (TJ), adherens junction (AJ) and desmosomes (DMs) from the luminal to lamina propria side respectively. **(A)** The TJs are localised to the apical-lateral membrane junction and consist of three integral transmembrane proteins (claudins, occludin and JAMs) with the proteins connected to the actin cytoskeleton through PDZ-domain-containing proteins via the zonulins (ZO-1,2 & 3) or PAR adaptor proteins. **(B)** E-cadherin is the main transmembrane protein at the AJ and associates with the alpha and beta catenins, this complex is further maintained through interaction with the serine/threonine phosphatase PP2A. **(C)** The DMs lie at the basal end of the enterocyte and are in contrast to the TJ and AJ in that they associate with the keratin filament cytoskeleton of the cell. JAMs, junctional adhesional molecules; PAR-3, partitioning defective-3; ZO, zonulin; Keratin IF, keratin intermediate filament.

Recently a renewed interest in the role of mucosal dendritic cells (DCs) in IBD pathogenesis has occurred.[63,64] This has mainly been as a result of work demonstrating that DCs are primed locally to undertake tissue-specific roles within the intestine.[65] Some of

these local functions include driving the differentiation of certain T cell populations,[66,67] the development of IgA-producing B cells[68] and the selective imprinting of gut-homing T cells[69]. There is considerable cross-talk between the IEC and lamina propria DCs, with IECs presenting antigen to DCs,[70] in addition to DCs playing an important role in IEC homeostasis[71]. The observation that DCs could express tight junction proteins and sample luminal antigens by intercalating their dendrites between IECs[72] has led to further work demonstrating the ability of the IEC to promote the differentiation of tolerogenic DCs, a capacity lost in CD[73].

In both murine and human models the ability of the IEC to alter T cell proliferation has been demonstrated, although many of the mechanisms involved remain unclear.[74-77] IECs do not express classical co-stimulatory molecules (such as CD80 and CD86) which are needed to interact with local T cells. However, they do express other co-stimulatory molecule ligands such as ICOSLG,[78] suggesting that co-stimulation may also be involved in IBD pathogenesis.[18,79] The area of T cell differentiation and its role in inflammatory diseases is also expanding rapidly, with multiple T cell lineages now recognised.[80] The aberrant differentiation of this immune cell population has been postulated to be involved in the pathogenesis of several chronic inflammatory conditions including IBD.[81] Researchers have demonstrated that T cell co-culture of IECs from IBD patients and normal controls produce significantly different T cell populations.[40,78,82] Similarly, it has been recognised that T regulatory (T_{reg}) cells play a key role in oral tolerance,[83] and that defects in the T_{reg} response may be important in IBD pathogenesis[84]. Although the precise role of T_{regs} in human IBD is not fully understood, patients do show a reduced number of T_{regs} in the blood and colon with the remaining cells functional in vitro.[85] Other regulatory cells are currently under investigation, most notably the CD28-CD101+CD103+ subset of CD8+ T-cells, with these having been shown to expand following interaction with healthy IECs and reduced in IBD.[86]

The gut microbiota is a complex anaerobic environment, with approximately 10^{14} bacteria composed of four major phyla: Firmicutes, Bacteriodetes, Actinomycetes and Proteobacteria.[87] Around 70-80% of intestinal bacteria have yet to be cultured, therefore bacterial diversity in both health and disease has only really been evaluated by culture-independent techniques.[88] These techniques have demonstrated that the gut microbiome contains hundreds of species,[89] which are stable over long periods of time[90] and are person-specific[91]. Much of this work has been based on 16S rDNA sequencing of faecal material, however the debate on the usefulness of this method over more specific 16S rRNA sequencing (i.e. metabolically active gut microbial diversity) or the use of mucosal samples is still ongoing.[92] Additionally, large scale metagenomic analyses of the intestinal microflora are now underway which will undoubtedly begin to tease out the complexities of microbial diversity, especially in relation to IBD.[93]

Since the first description of CD in the early 20th Century[94,95] there have been several lines of evidence implicating bacteria in the pathogenesis of IBD. Firstly, faecal stream diversion (via a diverting ileostomy) after ileal resection and ileocolonic anastamosis prevented microscopic recurrence of CD in a small group of adult patients, suggesting that faecal material, carrying a high bacterial load, may be involved.[96] Further work by the same group demonstrated that deliberate infusion of intestinal luminal contents in three patients following resection led to mononuclear infiltration and lymphocyte recruitment in the lamina propria.[97] Secondly, in several murine models of IBD (such as interleukin-10-deficient[98] and interleukin-2-deficient[99] mice) housing of animals a pathogen-free environment limited the development of chronic enterocolitis, with antibiotic therapy also shown to prevent the development of disease and treat active inflammation in another model[100]. Thirdly, it has been shown that peptide extracts from biopsies of IBD patients have reduced antimicrobial activity compared to healthy controls,[101] supporting the work demonstrating abnormal Paneth cell biology in CD[102,103]. All of this work is greatly enhanced by the plethora of genetic susceptibility genes involved in the innate immune response, bacterial handling and adaptive immunity.[104]

Although a large volume of research has now been performed looking specifically at the role of the intestinal flora in IBD, no specific pathogen has yet been identified as a disease-causing microorganism. However, a number of bacteria have been implicated including *Mycobacterium avium paratuberculosis*, *Helicobacter* species and *E.coli*.[105] Of particular interest are adherent-invasive *E.coli* strains (AIEC) that have been consistently isolated by independent investigators from CD patients with ileal disease, and shown capable of inducing epithelial injury and subsequent inflammation.[106] A direct role for AIEC in CD is supported by the relationship of colonisation density to mucosal inflammation,[107] their ability to exploit host defects in bacterial killing and autophagy conferred by CD-related polymorphisms in *ATG16L1*, *IRGM* and *NOD2*,[27,108] their binding of inflammation-induced proteins such as CEACAM6[109] and the ability to induce granulomas *in vitro*[110].

1.4 Environmental aspects of IBD pathogenesis

Although the genetic susceptibility of patients with IBD is now being dissected, the predisposing or triggering environmental events have yet to be firmly established. Over the decades there have been a multitude of factors suggested as possible influences on IBD pathogenesis. Several perinatal factors have been suggested to influence the risk of PIBD development, such as breast-feeding[111] and the protective effect of delivery by caesarean section (on a background of genetic predisposition[112]). However the studies drawing these

conclusions have often been small or of poor methodological quality and not adequately powered to determine effect size.

A paradoxical relationship has been demonstrated between smoking and IBD pathogenesis. A meta-analysis concluded that active smokers were less likely to develop UC compared to individuals who were never smokers or ex-smokers; in contrast, active smokers, followed by ex-smokers, were at an increased risk for acquiring CD.[113] However, it should be noted that the highest incidence of smoking is found in countries with the lowest incidence of IBD.[114] A similar relationship has also been proposed with passive smoke exposure, however, a meta-analysis did not demonstrate an association for childhood passive smoke exposure or prenatal smoke exposure.[115] Appendicectomy has been consistently shown to be negatively associated with UC, especially in children with appendicitis under the age of 10 years. This was confirmed by meta-analysis, although no causal mechanism has been suggested.[116] The relationship between appendicectomy and CD is less clear, with positive[117] and negative[118] associations published. However any attributable risk for CD is consistently strongest in the first year after appendicectomy[119] suggesting that this may be part of the clinical spectrum of disease, rather than a cause in itself.

The role of diet and the cold-chain/hygiene hypothesis have been put forward as aetiological factors in IBD, however results have often been inconclusive. With regard to diet, insulin resistance, butyrate and polyunsaturated fatty acids have all been implicated as well as high sugar intake and dietary fibre.[120] The hygiene hypothesis suggests that reduced exposure to microbial antigen in early life increases the risk of IBD in later life. Although this has been shown in well designed case-control studies,[121,122] these studies rely heavily on patient questionnaires and assumptions based on geographical location and social class. Conversely the cold-chain hypothesis suggests that bacteria such as *Yersinia* and *Listeria*, which can survive the refrigeration process, contribute to disease.[123] Although demonstrated in a small case-control study,[124] this hypothesis is now difficult to evaluate, as people living in the most research-active countries now almost universally consume refrigerated food. The use of antibiotics in the first year of life has also been postulated as a possible factor,[125] in addition to the risk attributable to episodes of gastroenteritis,[126,127] however these factors, like those above, have yet to be confirmed.

1.5 Paediatric inflammatory bowel disease (PIBD)

Approximately 25-30% of patients are diagnosed with IBD in childhood, and in addition to the risk of morbidity and mortality from acute exacerbations of disease, the burden of chronic

illness can often lead to significant problems with linear growth,[128] education and future employment[129,130] as well as psychosocial effects[131]. Along with other complex immune diseases such as childhood asthma[132] and early-onset type 1 diabetes,[133] there has been consistent reporting of a rising incidence of paediatric IBD (PIBD) both in Scotland[134] and worldwide.[135,136] It is not yet clear if these observed rises are purely an essential increase prior to a plateau, or whether a sustained rise will be evident over time. A number of hypotheses have been put forward to explain this apparent rise, including westernisation of lifestyle,[137] the rising rates of obesity,[138] and the cold chain hypothesis[123] and the hygiene hypothesis as discussed above,[121] however to date none of these have been confirmed. Unlike incidence data, there has been a notable paucity in prevalence figures with regard to PIBD, with many data nested within larger adult cohorts.[139,140]

1.5.1 PIBD Diagnosis and Classification

Although a uniform definition of PIBD has not yet been universally agreed, extensive intestinal involvement and rapid early progression,[141,142] differences in baseline mucosal responses,[143,144] and the early requirement for second-line immunomodulatory drugs[145,146] suggest that early-onset disease has a distinct clinical phenotype from adult-onset disease, although the precise reasons for these differences are still under investigation. Due to these important differences, in addition to variations in symptomatology, a working group of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) was formed to recommend criteria for the diagnostic work-up of children with suspected PIBD. This work culminated in the publication of the Porto criteria in 2005 which presented consensus-based guidelines with regard to history taking, physical examination, laboratory investigation, endoscopic and histological assessment and radiological evaluation.[147] These criteria suggested that clinical suspicion of PIBD should be raised in children with persistent (≥4 weeks) or recurrent (≥2 episodes in 6 months) symptoms such as abdominal pain, diarrhoea, rectal bleeding and weight loss. They highlighted the importance of growth failure and pubertal development, as well as extra-intestinal manifestations and anal inspection. Suggested laboratory investigations included C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), with stool cultures for common enteric pathogens, notably *Clostridium difficile*. However, it was the promotion of both upper and lower endoscopy with a determined effort to perform terminal intubation which set apart these paediatric-based criteria from adult practice, with distinct histological findings also helping to standardise diagnosis.

Since the mid-1970s efforts have been made to comprehensively classify CD to allow meaningful multi-centre research to be performed. Following the first endeavour by Farmer et al.,[148] several systems were adopted including the Rome classification in 1992[149,150]. The

Rome classification included location, behaviour, extent of disease and operative history therefore theoretically resulting in as many as 756 subgroups of CD. A working party was therefore established to simplify this classification, resulting in the publication of the Vienna classification in 2000.[151]

Table 1.1. Comparison of Montreal and Paris IBD classification systems

	Montreal	Paris
Crohn's disease		
Age at diagnosis	A1: below 17 years A2: 17 to 40 years A3: above 40 years	A1a: 0 to <10 years A1b: 10 to <17 years A2: 17 to 40 years A3: above 40 years
Location	L1: terminal ileum ± limited caecal disease L2: colonic L3: ileocolonic L4: isolated upper disease	L1: distal 1/3 ileum ± limited caecal disease L2: colonic L3: ileocolonic L4a: upper disease proximal to the ligament of Treitz L4b: upper disease distal to the ligament of Treitz and proximal to the distal 1/3 ileum
Behaviour	B1: non-stricturing, non-penetrating B2: stricturing B3: penetrating P: perianal disease modifier	B1: non-stricturing, non-penetrating B2: stricturing B3: penetrating B2B3: both penetrating and stricturing disease, either concurrently or at different times P: perianal disease modifier
Growth	N/A	G_0: no evidence of growth delay G_1: growth delay
Ulcerative colitis		
Extent	E1: ulcerative proctitis E2: left-sided UC (distal to splenic flexure) E3: extensive (proximal to splenic flexure)	E1: ulcerative proctitis E2: left-sided UC (distal to splenic flexure) E3: extensive (hepatic flexure distally) E4: Pancolitis (proximal to hepatic flexure
Severity	S0: clinical remission S1: mild UC S2: moderate UC S3: severe UC	S0: never severe* S1: ever severe*

*Severe defined by paediatric ulcerative colitis activity index (PUCAI) ≥ 65. N/A, not applicable.

Subsequent to this, and in light of the growing volume of research being generated from genetic studies at the turn of the millennium, a further revision of the criteria were published to address the limitations in the Vienna system, notably the absence of UC and indeterminate colitis nomenclature; the Montreal classification was therefore published in 2005.[2] With regard to indeterminate colitis, the Montreal classification suggested that this pathological diagnosis should be reserved for patients following colectomy, based on the original description by Price in 1978.[152] However with the introduction of endoscopy since Price's report it was recognised that biopsies with histological assessment was commonplace, therefore patients with chronic colitis within the clinical spectrum of IBD but in the absence of distinguishing features of either CD or UC were given the diagnosis of colonic IBD, type unclassified (IBDU). Although many patients with IBDU are later reassessed and reclassified as either CD or UC, there are those who remain in this sub-group well into adult life.[153]

Following the eager adoption of the Montreal system paediatric gastroenterologists felt the need to further refine the system to account for the nuances of PIBD, especially with regard to disease extension, age at disease onset and linear growth parameters. Therefore in November 2010 a group of international experts published the Paris classification which introduced two subgroups of the Montreal CD A1 and L4 categories, the ability of stricturing and penetrating disease to co-exist (as B2B3 disease) as well as a growth parameter.[154] Other subtle adjustments were made to Montreal, especially with regard to UC extent; a comparison of the Montreal and Paris systems for CD and UC are outlined in **Table 1.1**.

1.5.2 Diagnostic biomarkers

Due to the variable and often vague symptoms in children presenting with suspected PIBD, a number of both serum and faecal biomarkers have been evaluated to effectively 'screen' patients prior to undergoing upper and lower endoscopy (the diagnostic gold standard). Until relatively recently only four common blood parameters have been in routine clinical use, namely haemoglobin, platelet count, albumin and ESR. Although anecdotally felt to be useful during the diagnostic work-up, a comprehensive evaluation of these parameters was carried out by Mack et al. through prospective enrolment of 526 PIBD patients at diagnosis in the United States and Canada.[155] These data demonstrated that 21% of patients with mild CD and 54% of patients with mild UC had values in the normal range for all four tests. Although the number of abnormal tests rose with increasing disease severity (more so for UC), it was clear that these parameters were of limited use when used in isolation. Within the United Kingdom the use of CRP during the diagnosis of inflammatory or infectious conditions has become routine, and although there is some evidence that CRP is useful when assessing IBD severity in the paediatric population,[156] results have been inconclusive[157]. To date

there have been only a small number of studies evaluating CRP at PIBD diagnosis. One of these studies by Quail et al. assessed the role of faecal calprotectin (FC), a well-characterised calcium-binding protein found in neutrophilic granulocytes,[158] in combination with the blood parameters outlined above, including CRP[159]. Although unable to provide sensitivity and specificity due to the lack of a control group, this study highlighted the previously reported poor performance of the isolated blood parameters. Furthermore, this study brought to light the role of FC, with 100% of the PIBD patients having at least one abnormal blood test and/or raised FC, and 96% of patients having a raised FC at diagnosis. The use of FC at PIBD diagnosis had been evaluated by a number of groups prior to this study, however many of these had small patient numbers or a non-representative PIBD cohort.[160,161] A recent meta-analysis looking at the role of FC during the diagnosis of both adult-onset and PIBD highlighted the poor methodological quality of the paediatric studies, and concluded that the discriminative power to safely exclude IBD was significantly better in adults than in children.[162]

As well as the routine blood tests available a number of serum antibodies specific to IBD have been investigated, although more often in the adult population. These markers have frequently been used within a research setting and include perinuclear anti-neutrophil antibody (pANCA), anti-*Saccharomyces cerevisiae* antibody (ASCA), and antibodies to *E.coli* outer membrane porin C (OmpC), anti-CD-related bacterial sequence I2 and CBir1 flagellin.[163] Many of these biomarkers, such as the anti-glycan antibody ASCA, OmpC and I2 are prevalent in approximately 55-60% of CD patients, with others more prevalent in UC.[163] Approximately 85% of CD patients are positive for at least one antibody, although only 4% are positive for all four;[164] this does not account for often highly variable quantitative responses. A recent study comparing these serological markers with routine blood investigations demonstrated that a combination of three abnormal blood indices had higher predictive values that a commercially available panel of seven antibodies, highlighting the difficulties in interpreting these biomarkers.[165] Other serum and faecal markers have been looked at with regard to IBD diagnosis, such as lactoferrin,[166] antilaminaribioside carbohydrate IgG (ALCA) and antichitobioside carbohydrate IgA (ACCA),[167] however these have only been assessed in the context of research and have yet to be fully investigated in routine clinical practice.

1.5.3 PIBD treatment summary

Following a diagnosis of PIBD the aims of management are to induce and maintain remission, prevent relapse, normalised growth and development and provide each child with a good quality of life.[168] Due to the complexities of PIBD management, especially with regard to disease severity and growth, it is important that the multidisciplinary team involved

is led by a paediatric gastroenterologist with an interest in PIBD. This was highlighted by the inclusion of IBD as a special condition in the UK National Specialised Services definition set.[169] Within the multidisciplinary team patients should have access to a paediatric gastroenterologist, paediatric surgeon, IBD nurse specialist, dietician, pharmacist and psychologist, in addition to support from local IBD-related charities.[170]

To date, there have been very few therapeutic trials in PIBD, with guidelines often extrapolated from adult data. In a comprehensive review of the literature as recently as 2003 only one placebo-controlled randomised controlled trial in PIBD was reported.[171] The IBD working group of the British Society of Paediatric Gastroenterology, Hepatology and Nutrition (BSPGHAN) recently carried out a systematic review of the evidence base for medical treatment in PIBD.[168] They highlighted the paucity of trials of high methodological quality with regard to PIBD treatment. They concluded that the development of clinical guidelines for managing PIBD should be consensus based, and informed by the best-available evidence from the paediatric literature and high-quality data from the adult IBD literature, together with the clinical expertise and multidisciplinary experience of PIBD experts. At present a number of therapeutic modalities are used to induce and maintain remission, treat exacerbations of disease and 'rescue' those with severe disease.

1.5.3.1 Exclusive Enteral Nutrition

A nutritional approach to managing gut inflammation, especially with regard to the induction of disease remission in paediatric CD, has been validated in recent years.[172,173] The main advantages of exclusive enteral nutrition (EEN) are the avoidance of drug side-effects (such as those encountered with corticosteroids) with the benefit of nutritional support and supplementation.[174] Although there is not yet universal consensus on the optimal regimen when introducing EEN, a typical course of therapy for a child newly diagnosed with paediatric CD would consist of 6-8 weeks of EEN using either an amino-acid based or whole protein (polymeric) formula;[175] it should be noted that EEN currently has no clear role in the treatment of UC. During this time adherence can be difficult and children may require naso-gastric feeding, with a sizeable proportion of children not completing their full course and instead opting for corticosteroids.[176] Although remission rates of up to 88% have been reported in some case series,[177] a recent Cochrane review yielded a pooled odds ratio of 0.33 (95% CI 0.21-0.53) favouring corticosteroid therapy during induction[178]. As well as induction of remission, EEN is often used during disease relapse and has been shown to result in mucosal healing,[179] improved linear growth[180] and better bone health[181]. The mechanism of action of EEN currently remains elusive and is almost certainly multifactorial, with potential effects on the bacterial microflora,[182] circulating antioxidants[183] and through direct anti-inflammatory pathways[184].

1.5.3.2 Corticosteroids

Corticosteroids have long been the mainstay of treatment for IBD in both adults and children, and often the first-line treatment of choice in UC. Although the anti-inflammatory properties of corticosteroids have been demonstrated to induce remission in approximately 85% of children with either CD or UC,[185] their potential side effects and detrimental effects on growth have led to their more cautious use in PIBD. Initial doses of approximately 1-2mg/kg/day, weaning slowly over 8-10 weeks, may be used in both CD and UC following diagnosis, although the evidence for this dosing regimen is lacking.[168] The majority of studies evaluating corticosteroids in PIBD have used either prednisolone or methyl prednisolone,[168] with one small randomised controlled trial evaluating the different rectal formulations available.[186] Corticosteroids are commonly used during PIBD relapses and constitute the basis of pharmacological management of acute severe ulcerative colitis.[187] Steroid dependence is widespread with between 30-45% remaining on steroids at one year,[168] with steroid-related side effects such as increased ocular pressure,[188] cataracts,[189] intracranial hypertension[190] and osteoporosis[191] all reported in PIBD studies.

1.5.3.3 5-aminosalicylate (5-ASA)

5-aminosalicylate (5-ASA) is a derivative of salicylic acid and is used as local therapy most often in the treatment of UC. In mild to moderate UC 5-ASA is commonly used to induce and maintain remission, however evidence is lacking with regard to both paediatric UC and CD.[168] 5-ASA rectal preparations are either administered as foam enemas or suppositories, with oral preparations releasing active 5-ASA in the distal small bowel and colon. To prevent absorption of the oral formulations, utilisation of different carrier molecules or pH- and time-dependent mechanisms are employed. Side effects are seldom serious but include diarrhoea, skin rashes and neutropenia. Again several mechanisms of action are described including scavenging of reactive oxygen species,[192] inhibition of leukotrienes,[193] inhibition of platelet activation,[194] and activating the gamma form of the peroxisome proliferator-activated receptors[195].

1.5.3.4 Azathioprine and 6-mercaptopurine (6-MP)

Azathioprine and 6-mercaptopurine (6-MP) are two commonly used immunosuppressive drugs used in the treatment of PIBD.[196] Although these drugs have a slow onset of action and are therefore seldom used to induce remission, their use in maintaining remission is commonplace. Azathioprine is a prodrug which is absorbed in the gut and converted to 6-MP via glutathione and similar compounds in the intestinal wall, liver and on red blood cells. It has been long thought that the incorporation of 6-thioguanine nucleotide metabolites of 6-

MP into leucocyte DNA was the main mechanism of these drugs but recent data has suggested that interaction with the GTP-binding protein RAC1 leads to increased T cell apoptosis, through the co-stimulation of CD28.[197] Furthermore, the precise mechanism of action of these drugs in the context of PIBD is not yet clear, with other potential pathways possibly yet to be elucidated.

Although the addition of 6-MP may have a steroid-sparing effect during remission induction,[198] overall data is lacking, and concerns over the risk of developing lymphoma[199] may be outweighed by the benefits of thiopurine therapy[200]. The side effect profile can also be troublesome, with between 25-50% of children suffering from adverse effects such as raised aminotransferase levels, leucopenia, nausea, pancreatitis and recurrent infections, leading to dose reduction or drug cessation.[201,202] The genetically determined activity of thiopurine *S*-methyltransferase (TPMT), the primary inactivating enzyme for the thiopurines, are now routinely used in most centres to explain variations in drug metabolite levels. Although there does not seem to be a clear relationship between TPMT genotype and adverse effects,[203] the direct measurement of TPMT can help inform initial dosing and dose adjustment to improve outcome.[204]

1.5.3.5 Methotrexate, Cyclosporin and Tacrolimus

Although there are published data regarding the use of methotrexate during induction of remission in CD (methotrexate has a shorter onset of action than the thiopurines),[202,205] methotrexate is often thought of as a second line therapy in PIBD[196]. Methotrexate is an antifolate drug that inhibits DNA and RNA synthesis in rapidly dividing cells and requires folic acid supplementation following administration. Taken orally or as a weekly subcutaneous preparation, nausea is a common side-effect, often requiring anti-emetics to aid toleration;[206] hepatotoxicity and bone marrow suppression can also occur during methotrexate treatment.

Both cyclosporin and tacrolimus work as calcineurin inhibitors, altering the transcription of interleukin-2 and leading to a reduction in T-cell activity.[207] Their use is mostly limited to second-line medication in the treatment of steroid-refractory acute severe ulcerative colitis,[208] or in children with fistulising CD[209]. Significant renal and neurological side-effects can occur with these drugs, therefore careful serum monitoring is required to ensure successful bridging to maintenance drugs such as azathioprine or biological agents.[185]

1.5.3.6 Biological agents

In the last decade, the arrival of monoclonal antibodies (such as anti-tumour necrosis factor-alpha [TNF-α]) in clinical practice has led to dramatic changes in IBD treatment.[210]

Although the indications and treatment regimens for these drugs are still under debate,[211,212] several of these drug therapies have proven efficacy with newer biologicals showing promising results in early trials[213,214]. Even the most effective of these biologicals (infliximab) only induces remission in around fifty percent of adult Crohn's patients,[215] although higher rates are reported in paediatric studies.[146,216] There are therefore renewed efforts to identify other cytokine pathways involved in IBD aetiopathogenesis with a myriad of cytokines likely to be involved in IBD, such as interleukin-21,[217] interleukin-22,[218] and interleukin-32[219]. Infliximab has been used to induce remission in those intolerant or refractory to EEN and steroids, and in those with frequent relapses 6-8 weekly infusions are becoming more common during maintenance therapy. The acute risks surrounding the use of biological therapies, such as infliximab and adalimumab, arise from infusion reactions and sepsis events,[220] with the long term risk of T-cell lymphoma of major concern[221]. Despite the known anti-inflammatory effects of these drugs, the exact mechanism of action within the gut, the variable response from patients with clinically similar disease severity and the optimal dosing regimens are still under debate.

1.5.4 Conclusion

It can be seen that although the genetic susceptibility and pathogenic mechanisms of disease in PIBD are similar to those of adult-onset IBD, a distinct clinical phenotype and treatment strategies lacking a clear evidence base exist. Further work is required to elucidate the epidemiology of PIBD, including incidence, prevalence and therapeutic utilisation, further characterisation of the genetic architecture of disease, new robust biomarkers to help screen children presenting with suspected bowel inflammation and deeper insights into the mechanism of action of commonly used drugs.

2. The rising incidence of paediatric inflammatory bowel disease in Scotland

2.1 Background and aims

2.1.1 Background

The identification of true rises in disease incidence, in conjunction with other population trends, have the potential to provide useful insights into disease pathogenesis, especially with regard to environmental influences. By contrast, trends in disease prevalence are invaluable for organising current health care provision, planning future service design and informing patients and the charities that support them.[222]

The incidence of paediatric inflammatory bowel disease (PIBD) has been shown to vary significantly worldwide.[223] Although a large body of evidence exists with regard to incidence figures, a significant proportion of these data lacks analyses of trends over time.[223] Within the United Kingdom (UK), Scotland has a long history of PIBD epidemiological research with incidence data spanning over 30 years showing an increase in PIBD.[134,224] Work from Wales has shown a modest rise, with an overall Welsh incidence of 2.6/100,000/year for PIBD (less than 17 years old) between 1995-1997[225] and a subsequent small prospective regional study in South Wales providing an incidence of 5.4/100,000/year (less than 16 years old) during 1996-2003[226]. England with its large geographical area and extensive network of healthcare trusts has yet to produce robust population-wide incidence rate trends for PIBD, although a few studies have presented some adolescent data nested within adult data in smaller regions.[227,228] In the same way, single centres in Ireland had previously contributed to larger epidemiological studies,[229] with robust PIBD data only recently becoming available[230]. To date the only prospective UK- and Ireland-wide PIBD incidence data is the 13-month British Paediatric Surveillance Unit (BPSU) survey carried out between 1998-1999, which demonstrated an overall PIBD incidence of 5.2/100,000/year across all regions (again in children diagnosed less than 16 years old); incidence rates ranged from 3.6/100,000/year in Northern Ireland, through 4.4/100,000/year in the Republic of Ireland to 6.5/100,000/year in Scotland.[231]

With regard to worldwide rates of PIBD incidence, a recent exhaustive systematic review presented data on 139 studies from 32 different countries, demonstrating a clear increase in worldwide trends.[223] Significant variation was apparent, with rates varying from 0.24/100,000/year in Libya to 13.3/100,000/year in Ontario, Canada. This review highlighted the differing methodologies utilised during case ascertainment, the lack of robust and

appropriate statistical analysis to assess trends over time (only performed in 20% of studies) and the paucity of data from developing nations in Asia, Africa and South America. Since this review a number of studies have continued to present data demonstrating the rising incidence of PIBD worldwide.[230,232,233]

There is now a pressing need to provide an accurate indication of the incidence of PIBD in Scotland within the last decade, as any continued rise in conjunction with other population trends may provide further insights into susceptibility factors and the mechanisms underlying disease pathogenesis.

2.1.2 Hypotheses and aim

Hypotheses:

1. The incidence of PIBD in Scotland has risen significantly between the periods 1990-1995 and 2003-2008.
2. The age at PIBD diagnosis has remained stable between the periods 1990-1995 and 2003-2008.

Aim: To compare the incidence of PIBD (diagnosed less than 16 years) in Scotland over a six year period (2003-2008), to incidence rates and other basic demographic data in an historical cohort (1990-1995).

2.2 Methods

2.2.1 Setting

Scotland covers an area of 30,500 square miles and has a population of just over 5 million people. The total population has risen steadily since 2002 and represents approximately 8.4% of the population of the UK.[234,235] The paediatric gastroenterology service within Scotland is coordinated through three tertiary centres, with academic paediatric centres and their associated district general hospitals combining to form regional networks. These networks currently provide nationwide coverage for a total population of approximately 915,000 children less than 16 years.[234]

2.2.2 Data acquisition

2.2.2.1 2003-2008 cohort

Prospective accrual of all new PIBD (diagnosed less than 16 years of age) cases in South East Scotland who were managed solely or in shared care with the PIBD team in Edinburgh

has been performed since 1997. This prospective database is held by Ms Pam Rogers (IBD Nurse Specialist) at the Royal Hospital for Sick Children, Edinburgh, and holds basic demographic information as well as details regarding diagnosis, therapies and surgery. A similar database, developed in Edinburgh for this project, was distributed to each of the two remaining regional networks (West of Scotland and North of Scotland) to allow the routine audit of their retrospective PIBD caseloads. Datasets of these retrospective cases were compiled through exhaustive examination of PIBD clinic lists, endoscopy lists, pathology reports and local team knowledge. As a result of this collaboration, the cohort of all new IBD diagnoses within the entire Scottish PIBD service between 1st July 2002 to 1st July 2009 was captured. Anonymised incidence datasets were then combined and examined in detail and to ensure the most robust recent cohort (hereafter referred to as cohort 2), the records of those patients diagnosed before their 16th birthday between 1st January 2003 and 31st December 2008 were retrieved. Of note, PIBD cases diagnosed in adult IBD services and who were not transferred to any of the Scottish PIBD centres were neither systematically sought nor enumerated. The combined database included demographic data and diagnosis, all recorded electronically using Microsoft Access 2007 (Microsoft Corporation, Redmond, WA, USA) following thorough case note review. Before database entry all case notes were reviewed at each centre by individuals trained specifically in PIBD phenotyping and all ambiguous cases discussed with senior clinicians (Dr Richard K Russell, Consultant Paediatric Gastroenterologist, Royal Hospital for Sick Children, Glasgow, and Prof. David C Wilson, Professor of Paediatric Gastroenterology and Nutrition, Royal Hospital for Sick Children, Edinburgh). The participating clinicians in the other regional networks were Dr Richard Hansen (NHS Grampian and the University of Aberdeen), Dr Fiona Cameron (NHS Greater Glasgow and formerly NHS Highland) and Dr Kostas Gerasimidis (NHS Greater Glasgow and University of Glasgow). Prof. David C Wilson, Dr Richard K Russell and Dr W Michael Bisset were the clinical leads for IBD in the South East, West of Scotland and North of Scotland regional networks respectively and oversaw local data collection. Diagnostic category was determined using standard clinical, histological and radiological findings in line with Lennard-Jones and Porto criteria.[147,236]

2.2.2.2 1990-1995 cohort

For the earlier cohort (hereafter referred to as cohort 1), all raw data on incident cases between 1st January 1990 and 31st December 1995 used by Dr Emma Reynish (nee Armitage) in a previously published study[134] were re-examined. These cases were originally identified from national coding records and validated by studying the pathology reports and case notes of all IBD patients less than 18 years old (to ensure complete ascertainment of those less than 16 years) in *all* hospitals throughout Scotland, encompassing all adult and

paediatric gastroenterology centres. The raw data from this study have been held in hand-written paper copy in the Gastrointestinal Unit based at the Institute of Genetics and Molecular Medicine, University of Edinburgh since the late 1990s. To allow for a more efficient analysis to be performed, over 1000 individual patient records were manually re-entered onto a newly created Microsoft Access 2007 database, with any queries cross-checked with the local hospital records or historical notes.

Within both the cohorts a small number of children originally diagnosed with UC or IBDU were subsequently re-classified as CD. Regardless of the timing of this diagnostic change only their most recent diagnosis was recorded to ensure a true representation of IBD distribution. All children who were not living in Scotland at the time of their diagnosis but subsequently moved to Scotland were excluded.

2.2.2.3 Methodological differences in cohort ascertainment

It should be noted that some differences in the data collected between the two cohorts exists. Firstly, during initial data collection for cohort 1 (the earlier cohort), the classification of IBDU was not well established in the literature, and so children with IBD colitis but without features of CD were almost universally placed in the diagnostic category of UC. In fact, the previously published historical cohort (comprising incident IBD cases from 1981-1995[134]) only included two children with the classification of IBDU (both children were diagnosed prior to 1990). To compensate for this, and to provide a meaningful analysis, the UC and IBDU categories in cohort 2 were combined to create a 'non-Crohn's colitis' (NCC) group. Secondly, within the entire historical cohort (1981-1995), the date of IBD symptom onset was used to generate the *original* incidence data, whereas our current database used to define cohort 2 uses the date of the diagnostic endoscopy. As children are generally admitted for their diagnostic procedures it was therefore felt that the date of the first IBD-coded admission (which was also recorded for the original historical cohort) was a more suitable proxy and more in line with current IBD epidemiological literature,[223] and this was therefore used to define the incident date in cohort 1. To further validate this, the median time from symptom onset to the date of first IBD-coded admission for cohort 1 was calculated and was 3 months (interquartile range [IQR] 1-8 months). This is comparable with the duration between symptom onset and diagnostic endoscopy previously reported in the literature.[237] The date of diagnostic endoscopy was used in cohort 2 because as a fixed point in time it was easily reproducible on a retrospective case note review. In addition, this made cohort 2 more comparable with other reported studies examining a similar time period.[136]

2.2.3 At-risk population and statistics

2.2.3.1 At-risk population

In Scotland the number of children under 16 years decreased from a mean of 1,025,360 per year between 1990-1995 to 926,668 per year between 2003-2008.[234] To allow for these changes in population structure between and within the cohorts, incidence figures and sex ratios for each year were first calculated from known population figures (**Table 2.1**) and then standardised to the 2001 Scottish Census population to enable direct comparison.[234] Age groups were defined as pre-school (0-5 years), primary school (6-10 years) and secondary school (11-15 years). Age-sex standardised rates were calculated using the direct method[238] and 95% confidence intervals (CIs) determined using a method based on gamma distribution.[239] All rates are presented as per 100,000 of the at risk population, that is those less than 16 years of age.

Table 2.1. At-risk Scottish population (all children less than 16 years old) for each year during the 1990-1995 and 2003-2008 epochs.

Year	Male	Female	Total
1990	522,036	498,252	1,020,288
1991	522,389	498,560	1,020,949
1992	522,413	499,775	1,022,188
1993	524,641	503,034	1,027,675
1994	527,337	505,166	1,032,503
1995	525,087	503,468	1,028,555
Total	**3,143,903**	**3,008,255**	**6,152,158**
2003	482,952	460,288	943,240
2004	478,706	456,750	935,456
2005	475,538	453,456	928,994
2006	471,729	450,104	921,833
2007	469,295	447,656	916,951
2008	467,572	445,962	913,534
Total	**2,845,792**	**2,714,216**	**5,560,008**

2.2.3.2 Statistics

p values for comparisons between time periods were obtained from Poisson regression models. Analysis was carried out using the epitools package in R version 2.9.2 (R Foundation for Statistical Computing, Vienna, Austria)[240] and Mann-Whitney tests using GraphPad Prism version 4.03 (GraphPad Software, CA, USA). R scripts outlining the methods used for age-sex adjustment and Poisson analysis are shown in **Figure 2.1**.

2.2.4 Ethics

Ethics approval was sought from the Lothian Research and Ethics Committee but was deemed unnecessary as this was an anonymous, observational study of patients already under the care of paediatric gastroenterology services and under the umbrella of the Paediatric-onset IBD Scottish Audit (PISA).

(a)
```
library(epitools)
cases<-c(15,30,96)
studypop<-c(1188750,988394,966759)
censuspop<-c(172404,160386,165459)
mult<-100000
mult*ageadjust.direct(count=cases,pop=studypop,stdpop=censuspop)
```

(b)
```
Period<-factor(c(rep("Early",6), rep("Late",6)))
Cases<-c(10,21,20,18,15,8,29,26,31,26,25,21)
Pop<-c(522036,522389,522413,524641,527337,525087,482952,478706,475538,471729,469295,467572)
minn.txt<-data.frame(Cases,Pop)
minn.fit<-glm(Cases~Period+offset(log(Pop)), family=quasipoisson, data=minn.txt)
summary(minn.fit)
exp(minn.fit$coefficients[2]); # Point estimate of incidence rate ratio
exp(confint(minn.fit)[2,]); # 95% Confidence interval for rate ratio
```

Figure 2.1. R scripts to determine age-sex standardised incidence rates (a) and the Poisson regression analysis of incidence rates (b).

2.3. Results

2.3.1 The incidence of PIBD, CD and UC has risen in Scotland

In total, 260 children under 16 years were diagnosed in the period 1990-1995 (cohort 1), compared to 436 children between 2003-2008 (cohort 2). The crude numbers of patients diagnosed each year by disease category and sex is shown in **Table 2.2**. Following age-sex standardisation, the incidence rates of IBD were 4.45/100,000/yr (95% CI 3.91 to 5.03) and 7.82/100,000/yr (95% CI 7.10 to 8.59) for cohorts 1 and 2 respectively revealing a 76% rise ($p<0.001$) in the adjusted number of cases in each period. The adjusted incidence of CD rose significantly from 2.86/100,000/yr [95% CI 2.45 to 3.33] to 4.75/100,000/yr [95% CI 4.19 to 5.36]) ($p<0.001$) and in UC from 1.59/100,000/yr [95% CI 1.28 to 1.94] to 2.06/100,000/yr [95% CI 1.70 to 2.47] ($p=0.023$).

Table 2.2. Crude number of PIBD cases diagnosed in Scotland during each six-year epoch by sex and IBD type.

Epoch	Persons at risk*	IBD	CD	UC	IBDU	NCC
			Male			
1990-1995	523,984	137	93	44	0	44
2003-2008	474,299	251	157	63	31	94
			Female			
1990-1995	501,376	123	74	49	0	49
2003-2008	452,369	185	108	52	25	77

* = Mean number of persons at risk (i.e. less than 16 years old) for each year of the cohort. IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic inflammatory bowel disease, type unclassified; NCC, non-Crohn's colitis (UC and IBDU combined).

In addition, comparing the NCC group in cohort 2 with the UC group in cohort 1, led to a more significant rise in non-Crohn's colitis (1.59/100,000/yr [95% CI 1.28 to 1.94] to 3.07/100,000/yr [95% CI 2.63 to 3.57]) ($p<0.001$). The percentage rise in each type of IBD can be seen in **Figure 2.2**.

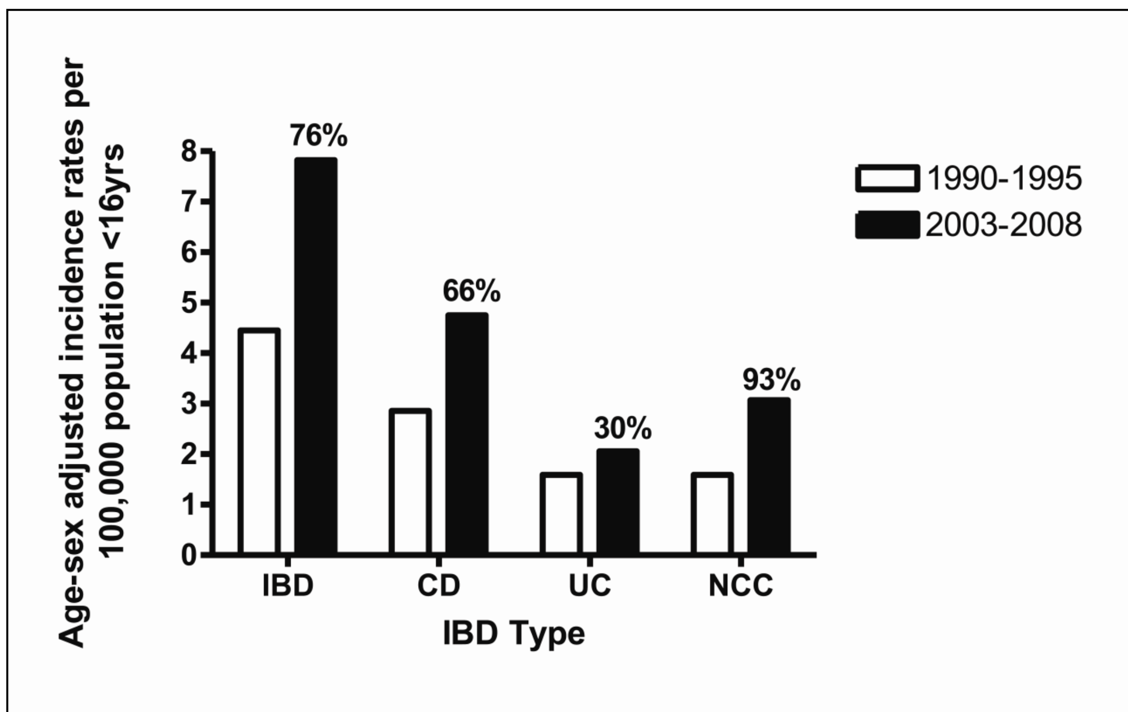


Figure 2.2. Graph showing percentage rise between the two six-year epochs for all inflammatory bowel disease and by inflammatory bowel disease type. IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; NCC, non-Crohn's colitis (comprising UC and IBDU).

2.3.2 The incidence of both males and females diagnosed with PIBD has increased

Analysing the incidence rates of all males under 16 years diagnosed with IBD, and the three IBD types (CD, UC and NCC) during the study periods, demonstrated a significant rise in the respective incidence rates within cohort 2 (**Table 2.3**). There was also a significant rise in females diagnosed with IBD, CD and NCC in cohort 2 (**Table 2.3**). Stratifying the results by age demonstrated that the adjusted incidence of IBD, CD, UC and NCC had risen significantly in males in the 6-10 year and 11-15 year age groups, most notably in the later group with an overall IBD incidence of 16.44/100,000/yr (**Table 2.4**). Although there were rising trends for all IBD types across all age-specific groups in females, only rises in total IBD and NCC in the 6-10 year age group reached statistical significance (**Table 2.5**). There was no significant change in those diagnosed less than 6 years in any IBD type in either sex.

Table 2.3. Age-adjusted incidence rates of PIBD in Scotland for all males and females by IBD type.

Cohort	1990-1995	2003-2008	Difference between cohorts (P value)
Male			
IBD	4.60 (3.86-5.44)	8.78 (7.73-9.94)	<0.0001
CD	3.14 (2.53-3.84)	5.48 (4.66-6.41)	<0.0001
UC	1.46 (1.06-1.96)	2.20 (1.69-2.82)	<0.01
NCC	1.46 (1.06-1.96)	3.30 (2.66-4.04)	<0.0001
Female			
IBD	4.29 (3.56-5.12)	6.80 (5.85-7.86)	<0.01
CD	2.58 (2.02-3.24)	3.97 (3.25-4.79)	<0.05
UC	1.71 (1.27-2.26)	1.91 (1.43-2.51)	0.394
NCC	1.71 (1.27-2.26)	2.83 (2.23-3.54)	<0.01

Figures are adjusted rates per 100,000 at risk population per year [95% CIs]. IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; NCC, non-Crohn's colitis (UC and IBDU combined). Significance = p<0.05.

2.3.3 PIBD continues to affect more males with a reduction in age at diagnosis

These results demonstrate that a male preponderance continues in the Scottish paediatric population; most clearly evident for CD. (**Table 2.2**). The adjusted male to female incidence ratio for all IBD in cohort 2 was 1.29, rising from 1.07 in cohort 1. This was mainly driven by an increase in the male to female incidence ratio in CD (1.22 to 1.38). In addition, the age at diagnosis was reduced in cohort 2. For total IBD the age at diagnosis decreased from 12.7 years to 11.9 years (p<0.01) driven by a decrease in the age at CD diagnosis (13.2 years to 12.1 years; p<0.001). There was no significant change in either UC or NCC.

Table 2.4. Age-adjusted incidence rates for males diagnosed with PIBD in Scotland by IBD type and age group.

Cohort	1990-1995	2003-2008	Difference between cohorts (*P* value)
	0 – 5 years		
IBD	0.76 [0.35-1.44]	1.81 [1.07-2.86]	0.143
CD	0.34 [0.09-0.86]	1.21 [0.62-2.11]	0.129
UC	0.42 [0.14-0.98]	0.40 [0.11-1.03]	0.636
NCC	0.42 [0.14-0.98]	0.60 [0.22-1.31]	0.772
	6 – 10 years		
IBD	3.44 [2.38-4.81]	8.37 [6.57-10.51]	<0.001
CD	2.12 [1.32-3.25]	4.75 [3.42-6.42]	0.011
UC	1.32 [0.70-2.25]	2.04 [1.21-3.22]	0.037
NCC	1.32 [0.70-2.25]	3.62 [2.46-5.11]	<0.001
	11 – 15 years		
IBD	9.72 [7.86-11.90]	16.44 [14.00-19.20]	<0.001
CD	7.03 [5.46-8.92]	10.65 [8.69-12.92]	0.009
UC	2.69 [1.76-3.94]	4.24 [3.04-5.75]	0.037
NCC	2.69 [1.76-3.94]	5.79 [4.37-7.52]	0.003

Figures are adjusted rates per 100,000 at risk population per year [95% CIs]. IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; NCC, non-Crohn's colitis (UC and IBDU combined). Significance = p<0.05.

Table 2.5. Age-adjusted incidence rates for females diagnosed with PIBD in Scotland by IBD type and age group.

Cohort	1990-1995	2003-2008	Difference between cohorts (*P* value)
	0 – 5 years		
IBD	0.88 [0.42-1.62]	1.48 [0.81-2.48]	0.292
CD	0.62 [0.25-1.27]	0.63 [0.23-1.38]	0.952
UC	0.26 [0.05-0.77]	0.53 [0.17-1.23]	0.434
NCC	0.26 [0.05-0.77]	0.84 [0.36-1.66]	0.184
	6 – 10 years		
IBD	4.33 [3.11-5.88]	7.37 [4.65-9.44]	0.039
CD	2.43 [1.54-3.65]	4.28 [3.00-5.92]	0.105
UC	1.90 [1.13-3.01]	1.90 [1.09-3.09]	0.999
NCC	1.90 [1.13-3.01]	3.09 [2.02-4.53]	0.039
	11 – 15 years		
IBD	7.78 [6.09-9.80]	11.80 [9.69-14.23]	0.052
CD	4.76 [3.46-6.39]	7.14 [5.52-9.09]	0.099
UC	3.03 [2.01-4.38]	3.36 [2.28-4.76]	0.701
NCC	3.03 [2.01-4.38]	4.65 [3.37-6.27]	0.092

Figures are adjusted rates per 100,000 at risk population per year [95% CIs]. IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; NCC, non-Crohn's colitis (UC and IBDU combined). Significance = p<0.05.

2.4. Discussion

This study has shown a clear rise in the incidence of both paediatric CD and UC in Scotland, representing a minimum 76% increase in the adjusted cases of IBD between the periods 1990-1995 and 2003-2008. It highlights and extends data regarding the continued male preponderance in paediatric disease in contrast to adult onset disease,[141] and also demonstrates a significant reduction in the age at diagnosis between the two cohorts.

The strengths of this study stem from the fact that the regional networks in Scotland have for many years collaborated effectively in combined research studies.[141,241,242] Other studies providing epidemiological data on PIBD have often made broad assumptions about population figures, based on smaller cohorts within a region[223] and over short periods of time[243]. This study presents data with robust case note review as the only method of case confirmation. It should be emphasised however that although the historical cohort is absolutely robust for those less than 16 years, with cases obtained from paediatric and adult clinical services, the more recent cohort represents a dataset generated from tertiary paediatric practice only. This is due to the fact that no attempt was made to ascertain cases of IBD diagnosed less than 16 years of age in adult centres in cohort 2 which historically have seen a significant number of paediatric cases.[244] However, there is anecdotal evidence that this has happened much less in the last decade during which regional PIBD services have been strengthened considerably. The selection bias that exists within cohort 2 therefore would be likely to produce an *underestimate* of that cohort's incident caseload of IBD by reduced acquisition of cases diagnosed by adult physicians. However, despite this, there has been a significant increase in incidence, therefore only minimal rises in incidence of IBD, CD, UC and NCC are reported here. Furthermore, due to the shorter follow up of the most recent cohort, fewer patients with IBDU would have had the opportunity to be reclassified as either UC or CD and so possibly further underestimating their true rise, although this of course would not change total IBD incidence.

There is a historical body of evidence with regard to PIBD incidence in Scotland. The first report by Barton et al. showed the incidence of CD to be 0.7/100,000/yr and UC 1.9/100,000/yr in 1968, demonstrating a rise in CD.[224] This study was however hampered by poor coding of hospital admissions in those under five years with UC, who were subsequently completely excluded. Armitage et al. later reported that between 1981-1995 there was an overall incidence of 2.6/100,000/yr for CD and a marginal fall in UC to 1.2/100,000/yr.[245] The current study has added significantly to this historical data and demonstrates the sharp rise, especially in CD, within the 2003-2008 cohort. This is clearly demonstrated in a summary graph (**Figure 2.1.3**) which has been constructed using the

adjusted rates of incident paediatric CD diagnosed in Scotland from previously published Scottish studies[134,224] in addition to the most recent cohort. (All raw data has been age-sex standardised to the 2001 Scottish population as described above). There has therefore been a 5-fold increase in incidence of paediatric CD over the last 34 years, representing a huge rise in this chronic childhood disease.

As mentioned above, another study from the UK which is of particular relevance is the prospective survey carried out by the BPSU which recorded all instances of new IBD diagnoses in those under 16 years over a 13 month period during 1998-1999.[231] This study showed that the overall incidence of PIBD UK-wide was 5.2/100,000/yr with Scotland producing the highest rate of 6.5/100,000/yr. This clearly shows an 'intermediate' incidence rate between the two epochs presented here (i.e. 4.45/100,000/yr and 7.82/100,000/yr), and further supports the current data. This current study has therefore provided the highest recorded incidence of PIBD in the UK to date, and is the first published from anywhere in the UK since 2003. However, it has yet to be seen if incidence rates in Scotland will continue to rise to the levels described in Canada[246] and Scandinavia[247] which have reported rates of 13.3/100,000/yr and 10.6/100,000/yr respectively.

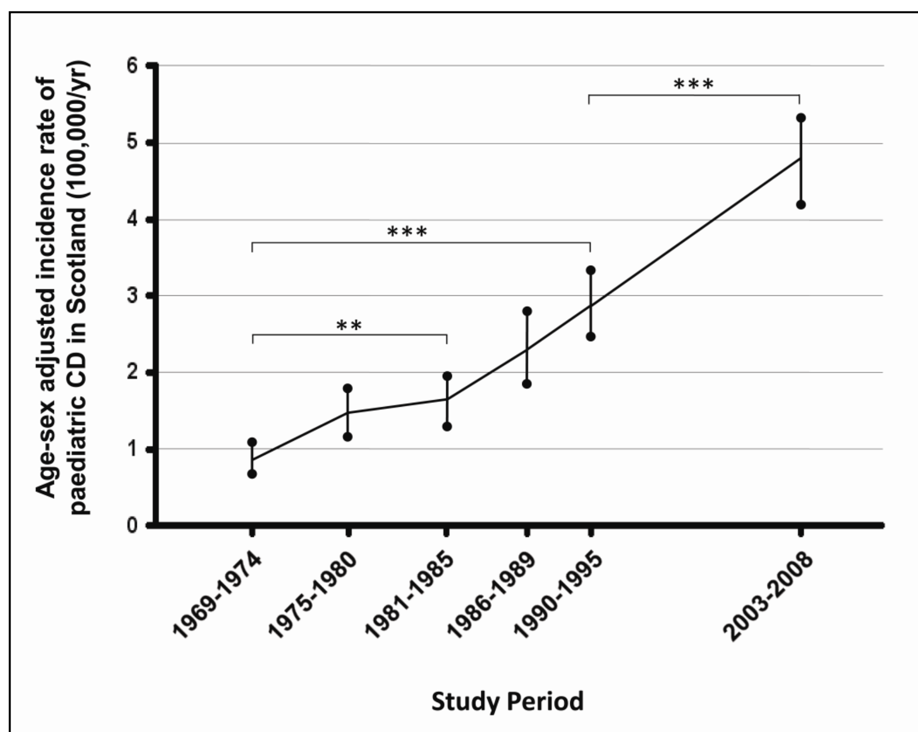


Figure 2.3. Summary graph showing age-sex adjusted incidence rates and 95% confidence intervals for paediatric Crohn's disease from various Scottish cohorts between 1969-2008.[134,224] All crude rates are age-sex adjusted to the 2001 Scottish Census data. In addition to the significance values pictured, significant increases in incidence rates exist between the 1986-1989 and the two earliest cohorts ($p<0.01$). ** = $p<0.01$; *** = $p<0.001$.

Also of interest is the recent study from Denmark which reported the incidence of UC as 3.1/100,000/yr suggesting that Scotland may yet see further rises in the UC patient population.[232] It is also noteworthy that a recent study from Northern California has demonstrated a stable incidence of CD and a significant rise in UC during a similar study period (1996-2006) to the new Scottish data presented above.[136] However, it should be noted that although this study demonstrated a robust method of case identification, calculations were based on children registered with a private healthcare insurer and therefore only included 30% of the population. In addition there was poor diagnostic agreement between physicians during a pilot study and unlike the current study no correction was made for statistical overdispersion (i.e. the presence of greater variability [statistical dispersion] in a data set than would be expected based on a given simple statistical model[248]).

Studies showing an increasing incidence of PIBD are often criticised as being due to an increased accrual of milder cases of IBD than occurred in the past; this is felt to be particularly true for the milder phenotypic cases of CD. There are three reasons why this may not be the case in the Scottish PIBD population. Firstly, the Scottish PIBD population has previously been shown to not only have significantly more extensive involvement of the intestinal tract at diagnosis than the Scottish adult IBD population, but also to have significant extension of location within the first few years after diagnosis;[141] the majority of the 2003-2008 cohort were included in this previous phenotypic publication. Secondly, specifically in the paediatric population, once infection has been excluded, chronic symptoms of abdominal pain, rectal bleeding, diarrhoea and growth failure[249] due to IBD will almost invariably have led to appropriate assessment at any time in the last 40 years by adult and paediatric gastroenterologists or by colorectal surgeons. Additionally, although newer diagnostic tests, most notably faecal calprotectin and capsule endoscopy, have been in more widespread use in recent years these tests have not been shown to significantly influence the number of new PIBD diagnoses. Finally, none of the CD cases in the 2003-2008 cohort were diagnosed by wireless capsule endoscopy alone, with this imaging modality having been suggested to potentially identify milder cases of CD.[250]

It is not clear from these studies which factors are contributing to this rise but several hypotheses have been suggested. The westernisation of lifestyle, such as the increased consumption of a low-fibre, high-sugar diet[137] and the rising rates of obesity have been suggested as a possible cause of the rising rates of IBD[138,251]. Other hypotheses regarding dysbiosis of the gut microbiota have been postulated and include the cold chain hypothesis,[123] the hygiene hypothesis[121] and the protective effect of breast-feeding.[111] However, it is not clear how significantly these factors have changed in recent decades in those populations with rising rates of PIBD. Interestingly one environmental aspect of

Scottish life which *has* demonstrated an increase in recent years is the number of families living outwith towns and cities.[252] This coupled with further Scottish work demonstrating that higher human density is protective for PIBD at diagnosis,[253] suggests that movement of urban populations back to more remote areas may contribute to the development of IBD. With regard to vitamin D and sunlight exposure (a topical subject in IBD pathogenesis[254]), although the recent PIBD epidemiology systematic review was unable to confirm a north-south gradient in incidence,[223] studies showing that vitamin D deficiency may play a role in disease development or prevention, and that sunlight may exert vitamin D independent beneficial effects on inflammation through nitric oxide production are certainly of interest[255-257].

Although the selection bias outlined above will have underestimated the rise in PIBD incidence this may also have potentially produced a false positive with regard to the demonstrated earlier age at onset. As discussed, during the 2003-2008 epoch a significant number of adolescents will have been treated in adult units, without any input from paediatric gastroenterologists. This fact alone may have led to the reduced age at onset seen in the analysis. In contrast, a recent study has in fact demonstrated a rise in the age at PIBD diagnosis, however this was not a population-based study, and may have been influenced by tertiary referral bias.[258] With regard to gender differences, early-onset disease predominantly occurs in males[141,259,260] in contrast to an overall female preponderance in adult disease[261,262]. The full picture however is more complex with CD showing a male bias and UC demonstrating equal gender frequencies until puberty.[263] The reason for this sex difference is not clear, however both genetic (e.g. males with a certain interleukin-6 [IL-6] genotype are prone to develop CD at a younger age) and environmental influences (e.g. increased risk relating to oral contraceptive use) are among several possible aetiological factors.[264,265] In addition to these risk factors, there are data to suggest that the clinical presentation differs with gender in PIBD. Gupta et al. showed that in 989 consecutive paediatric CD patients girls were more likely to have aphthous mouth ulcers and low serum albumin levels at diagnosis.[266] Although disease location did not differ, boys were less likely to develop erythema nodosum and pyoderma gangrenosum but more likely to have growth failure. A follow-up study by the same group demonstrated that males with paediatric CD have lower insulin-like growth factor-1 (IGF-1), with inflammation (as determined by ESR, CRP and albumin) more likely to affect IGF-1 levels and bone age in males.[267] Although gender differences exist at presentation in PIBD, studies have highlighted the similarities in disease severity and medication use in childhood,[268] with possible differences in adult life[269]. The gender differences in colorectal cancer risk in long-standing IBD is also under investigation.[270]

2.5. Conclusion

This study has demonstrated a minimum 76% rise in PIBD in Scotland through the turn of the millennium, however the reasons for this rise have yet to be elucidated. Many avenues of research are continuing to unravel the possible gene-environment interactions which may be implicated in IBD pathogenesis, in particular at the gut epithelial-microbiota interface,[105] with a greater understanding of the basic mechanisms underlying gut inflammation already uncovering the new molecular pathways involved.[271] Furthermore, well constructed, longitudinal, prospective studies examining the incidence of PIBD across a sufficient time period, in addition to secondary epidemiological research, are required to clarify the aetiological factors involved.

3. The epidemiology and natural history of paediatric inflammatory bowel disease in South-East Scotland: a prospective 14-year study

3.1 Introduction

In order to further understand the burden of disease on young people with paediatric inflammatory bowel disease (PIBD) several parameters should be closely examined: (1) a clearer appreciation of the prevalence of disease; (2) the location and behaviour of disease at diagnosis and its subsequent natural history; (3) the need for medical therapies (including biologicals) and (4) the requirement for surgery. To date, little data exists either in the United Kingdom (UK) or worldwide with regard to many of these aspects and with the provision of healthcare now high on the political agenda,[272] it is now more important than ever to fully determine the most likely future provision of services required and the potential morbidity faced by children with PIBD.

3.2 Background

Unlike disease incidence, disease prevalence is more valuable in the context of informing service design, identifying emerging healthcare costs and evaluating clinical pressures.[139] Although in relatively stable populations such as Scotland the rates of incident and prevalent PIBD cases are likely to fluctuate in parallel,[273,274] it is particularly important to fully understand the service requirements of the prevalent population to provide efficient management strategies. This is especially important with regard to PIBD as it is clear that with the increasing incidence demonstrated both in Scotland (**Chapter 2**) and worldwide,[223] a subsequent increase in overall disease burden will undoubtedly emerge in the paediatric population.

To date there has been a paucity of robust data on the prevalence of PIBD. Although there are several large studies presenting data on adult cohorts,[275,276] these have rarely provided a detailed analysis of the paediatric data within their research. However, there have been some figures of PIBD prevalence presented in both Europe and further afield. One of the first of these studies was in fact from Scotland where in a retrospective cohort of children from 1968-1989 a figure of 9.5/100,000 was provided for those under 16 years of age prevalent in 1983.[224] A study from a similar time period performed in Scandinavia gave figures of between 17.6/100,000 to 18.2/100,000 children less than 16 years.[277] These studies were

however hampered by difficulties ascertaining clear diagnoses in younger patients with colitis,[224] and lack of clear endoscopic evidence of PIBD[277]. More recently, studies from the United States (US) have been predominant in the literature with most studies based on data retrieved from insurance databases, with selection bias likely confounding the results;[278,279] although population-based studies have also been reported[280,281]. There is some consistency to these data with Crohn's disease (CD) prevalence figures of 39/100,000 (<20 years old; 2001),[280] 43/100,000 (<20 years old; 2003-2004)[279] and 57.8/100,000 (<20 years old; 2008-2009)[278] however it can be seen that two of these studies analysed the same database, with all studies using a relatively wide paediatric age range. The equivalent prevalence figures for UC in these studies were 42/100,000, 28/100,000 and 33.9/100,000 respectively. One of the largest studies to date carried out in five Canadian provinces demonstrated the large variation in IBD prevalence within a single country.[281] They reported paediatric prevalence rates of between 30.5 - 71.1/100,000 for CD and 17.5 - 30.7/100,000 for UC (less than 20 years of age), postulating that ethnicity and environmental risk factors played an important part in disease development and varying prevalence. Further data was published by Abramson et al. in which figures from Northern California collected from 1996-2006 were analysed.[282] Although again using a large health-plan database (with potential selection bias), they demonstrated age-standardised point prevalence rates of 12.0/100,000 for CD and 19.5/100,000 for UC, with the useful inclusion of rates of colonic IBD, type unclassified (IBDU) of 3.5/100,000 (less than 18 years of age). These figures were however low in contrast to the other US studies, possibly as a result of the much higher non-Caucasian population investigated.

As discussed in **Chapter 1** it has been clearly shown that PIBD presents with extensive anatomical involvement, with higher rates of pan-enteric CD[266,283] and pancolitis in UC[284,285] compared to adult disease[141,286]. Furthermore, heterogeneity within the paediatric population is evident, with those diagnosed before eight years of age showing significantly less ileal disease and more isolated colonic disease than patients with adult-onset disease.[141,287,288] Disease behaviour however has been shown to be similar to adult disease with progression from inflammatory disease to stricturing/penetrating complications,[141,289] with surgical intervention being more diverse.

With regard to PIBD phenotype at diagnosis and its natural history the most comprehensive data to date has come from Scotland and France. Van Limbergen and Russell et al. demonstrated that 31% of children with CD had pan-enteric disease (Montreal A1/L3+L4) at diagnosis with 44% after 2-years follow up.[141] With regard to CD behaviour, 24% of children developed stricturing or penetrating disease after 4 years from diagnosis in the same study. Similarly for UC, 75% had maximal colonic disease at diagnosis (Montreal A1/E3), with 82% having E3 disease at last follow up. This was in stark contrast to adult-onset disease

(Montreal A2/A3) were 3% of CD patients and 48% of UC patients had maximal disease location at last follow up. In France, the Registre des Maladies inflammatoires chroniques de l'Intestin du Nord Ouest de la France (EPIMAD), has provided data on an incident cohort of children diagnosed since 1988, allowing the publication of a large volume of research regarding the natural history of PIBD. Several overlapping studies involving children (less than 17 years old) newly diagnosed with PIBD from an at-risk population of 1.3 million have now been published, with the most recent study involving 309 CD patients with at least 5-years follow up.[259,290-294] Interestingly, this group report similar rates of pan-enteric CD (27% at diagnosis, 48% at follow up) as the Scottish data,[291] however their rates of pancolitis in UC were lower at diagnosis (37%) but with a more comparable extension of disease at a median of 77 months follow up (60%).[290] Further data has now been presented by the EUROKIDS registry, an inception cohort of children (less than 18 years) newly diagnosed with PIBD since 2004, highlighting that terminal ileal disease and stricturing behaviour are uncommon in children under 10 years old,[295] and the high prevalence of pancolitis and atypical phenotypes in UC[296]. Of further note, a recent population-based Scandinavian data-set (*n*=130; children <15 years old) possibly suggests a slightly milder phenotype at diagnosis for both CD and UC, although the data is unclear with regard to the total number of incident cases per centre, with no complete geographical case ascertainment.[232]

With the increasing use of biological agents such as infliximab and the growing impetus towards the use of exclusive enteral nutrition (EEN) in paediatric CD, there may have been significant changes in the use of immunomodulators and surgical rates in PIBD patients over recent years. Jakobsen et al. described the rate of immunomodulator use in a cohort of 65 paediatric CD patients (less than 15 years of age) with a median of 1.6 years follow up between 2007-2009.[232] This study showed that 61.5% of patients required azathioprine (AZA) or 6-mercaptopurine (6-MP), with 3.1% requiring methotrexate (MTX) and 24.6% requiring any biological. Van Limbergen and Russell et al. described the combined use of any immunomodulator (AZA, 6-MP, MTX or biological) in a larger cohort of 408 Scottish PIBD patients with extended 10 year follow up data showing that 11.5%, 40.5% and 38.4% of CD, UC and IBDU patients respectively had remained free from immunomodulators.[141] One of the most recent studies to evaluate therapeutic usage in PIBD was a survey of 164 children aged 8-17 years old attending a charity-run camp in the US.[297] This survey showed that after approximately 4 years of follow up 47.1% were on an immunomodulator with 36.3% on a biological; interestingly 5% were being treated with a biological agent and an immunomodulator. Other studies have shown the variable use of immunomodulators in both UC[298] and CD[299] most likely reflecting the length of follow up, the population studied and the historical use of biologicals in the study centres.

With regard to surgery in PIBD, Van Limbergen and Russell et al. demonstrated that after 5 years follow up 20.2% and 20.0% of children with CD and UC respectively had undergone resectional surgery.[141] Schaefer et al. in a study of 854 children with CD demonstrated a similar 17.7% CD-related surgery rate at 5 years.[300] Gupta et al. also described the rates of surgery in 989 consecutive CD patients less than 17 years old from six centres in the US.[301] They showed that the cumulative risk of surgery was 17% at 5 years from diagnosis, with the addition of female gender, initial diagnosis of UC, poor growth, and fistulating or stricturing CD increasing the risk of surgery in a multivariate model. Extended data from the EPIMAD registry demonstrated that 32% of CD patients required bowel resection at a median of 13 years of follow up with the probability of a second resection being 8%, 17% and 29% at 2, 5 and 10 years respectively.

It is clear that in patients diagnosed with IBD in childhood the disease burden is high.[302] However, an accurate indication of the need for therapies in larger geographically defined cohorts are required to ascertain the true extent of healthcare service use and disease morbidity.

3.3 Hypotheses and aims

Hypotheses:

1. The incidence and prevalence of PIBD in South-East Scotland are rising in-line with Scottish trends (**Chapter 2**).
2. Children diagnosed with PIBD have a severe disease phenotype at diagnosis which continues to extend within a short time from diagnosis.
3. PIBD medical therapies have been used more aggressively in recent years with the use of immunomodulators, especially biologicals, more commonplace.
4. A large proportion of children with PIBD require surgery while still in paediatric services.

Aims: To collate data from a prospective cohort of children diagnosed with PIBD in South-East Scotland from August 1997 to December 2011 to ascertain the natural history of disease, including (1) incidence and prevalence; (2) disease location and behaviour; (3) medical therapeutic use and (4) crude surgical rates.

3.4 Methods

3.4.1 Setting

The Department of Paediatric Gastroenterology and Nutrition at the Royal Hospital for Sick Children, Edinburgh (RHSCE) was initially established in August 1997 following the appointment of Prof. (then Dr) David C Wilson as a Paediatric Consultant Gastroenterologist. The hospital currently acts as a secondary referral centre for City of Edinburgh, East Lothian and Mid-Lothian (all NHS Lothian) as well as the a tertiary referral centre for three district general hospitals (DGH) (Borders General Hospital, NHS Borders; Victoria Hospital, NHS Fife; St. John's Hospital, West Lothian, NHS Lothian) in South-East Scotland (SES). Historically, the Department has managed all PIBD in paediatric services in SES, and also cared for the majority of PIBD patients referred from Dumfries and Galloway Royal Infirmary (NHS Dumfries and Galloway) and a sizeable minority of PIBD patients referred from Forth Valley Hospital (NHS Forth Valley) until recent changes in health board restructuring gradually came into full effect (April 2010). The Department currently employs four experienced paediatric consultants, three nurse specialists and two senior dietitians, as well as junior medical staff and administrative assistants. Serving a population of approximately 228,000 children less than 16 years,[303] RHSCE currently provides all the initial diagnostic evaluation, induction of remission and ongoing medical and surgical management of all children with PIBD. In 1998, Dr Wilson had contacted the paediatric services in all the DGHs and adult gastroenterology units in SES to confirm that RHSCE would manage all PIBD patients less than 16 years old in addition to any 16-18 year old teenagers still to complete puberty. This transition was aided by agreeing to provide endoscopy under general anaesthetic, outreach clinics in DGHs, and transition clinics in the main academic adult gastroenterology centre. All prevalent PIBD in RHSCE under the care of general paediatricians and paediatric surgeons were also transferred to the PIBD clinic. Therefore by 1999, there is a high degree of confidence that all SES PIBD patients were in paediatric services in RHSCE, however a small number of patients less than 17 years old may have remained in adult services without any paediatric involvement.

3.4.2 Patient cohort

Following the inception of the paediatric gastroenterology service in August 1997 all cases of incident and prevalent PIBD cared for at RHSCE were initially recorded retrospectively in paper copy. Ms Pam Rogers (Liver and IBD Nurse Specialist) was appointed in April 1998 and until November 1999 all prevalent cases were retrospectively entered on an hand-held database until the current prospective database was introduced in December 1999; this is

currently a custom-made Microsoft Access 2003 database (Microsoft Corporation, Redmond, WA, USA) with previous versions of Microsoft Access used prior to this. Children are entered in the database if they are diagnosed within the study centre with either CD, UC or IBDU using standard clinical, histological and radiological findings in-line with the Lennard-Jones and Porto criteria.[147,236] For those children who have moved into the area with a known diagnosis of PIBD, as much information as possible is compiled from transfer letters, histology reports, imaging and direct contact with their previous physician to confirm the diagnosis prior to database inclusion, with re-evaluation undertaken in cases where the diagnosis is in any doubt.

3.4.3 Data collection

Following a confirmed diagnosis of PIBD the basic demographics of each patient are entered into the departmental Access database (unique hospital number, surname, forename, date of birth, referral area and sex). Additionally, details of the patient's diagnosis (CD, UC or IBDU) and the date of diagnostic endoscopy are also entered. Following this initial entry, data regarding the commencement of AZA, 6-MP, MTX and biologicals are entered prospectively with the dates of initial prescription recorded. Evidence of perianal disease and details of surgery are also entered. The date of discharge/transfer/transition/death is also stipulated and the destination post-discharge (i.e. other hospital, adult services or moved out of area). Where there is any doubt as to an exact date the 15th day of the month is used as this would ensure the smallest margin of error when subsequent calculations are made.

To augment the current dataset several other sources of information were used to obtain as much clinical information on each patient as possible. Firstly, the incident cohort from 2003-2008 (**Chapter 2**) was used to ensure the accuracy of diagnosis and the patient's sex, date of diagnosis and date of birth. An even greater degree of clinical information was also obtained from the current Paediatric Inflammatory Bowel Disease Cohort and Treatment Study (PICTS) database currently held by Mrs Hazel Drummond at the Gastrointestinal Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh under the supervision of Prof DC Wilson, Dr RK Russell and Prof J Satsangi (co-investigators on PICTS). This comprehensive database holds a vast array of clinical data on over 800 children with PIBD (diagnosed less than 17 years old) cared for in Scotland, with diagnoses dating back as far as 1979. This database has been used in a number of previous studies,[141,241,304,305] with comprehensive case-note review providing detailed phenotyping of patients at diagnosis and at 2-yearly intervals. Data pertaining to medical therapies and surgical interventions is also collated on this database along with histology reports, environmental questionnaire results, genetic data and data regarding biomarkers and anthropometric data. With regard to

phenotypic classification, although the Paris classification (see **Chapter 1**) has now become the standard system for paediatric disease,[154] historically all data collected in Edinburgh has been recorded using the Montreal classification. There is currently an ongoing process to update these data to the Paris classification, however the current data-set presents only Montreal location and behaviour, which allows comparisons to cohorts derived over a similar period of time to be made. With regard to CD it should be noted that since commencing detailed phenotyping in Edinburgh, following the confirmation of histological granulomatous disease with macroscopic changes in any location, the presence of classical microscopic disease with granulomas elsewhere was then also recorded as a disease location (**Table 3.1**).

Table 3.1. Definitions of microscopic and macroscopic disease used in Edinburgh to classify paediatric Crohn's disease location using the Montreal classification.

Definite macroscopic CD	Non-diagnostic macroscopic CD
• Mucosal apthous ulcers • Linear or serpentine ulceration • Cobblestoning • Pseudopolyps • Stenosis/stricturing of bowel • Radiological or surgical – bowel wall thickening with luminal narrowing • Perianal CD – fistula(s), abscesses, anal stenosis, anal canal ulcers • Skip lesions	• Oedema • Erythema • Friability • Granularity • Exudate • Loss of vascular pattern • Single or very few isolated apthous ulcers • Perianal – fissures, skin tags
Definite microscopic CD	**Non-diagnostic microscopic CD**
• Non-caseating granuloma(s) – must be remote from ruptured crypt • Classical features of IBD – mucosal ulceration/erosion, crypt architectural changes, colonic Paneth cell metaplasia, focal chronic inflammation, transmural inflammatory infiltrate, submucosal fibrosis	• Granuloma adjacent to ruptured crypt • Non-specific inflammatory infiltrate in lamina propria

CD, Crohn's disease; IBD, inflammatory bowel disease

Despite the well validated sources of data described above there were occasions were data was incomplete or uncertain and in all of these cases the case-notes and computerised laboratory pathology reports were re-examined were possible to clarify the information provided. Within the cohort a small number of children had been re-classified as CD, UC or IBDU. Regardless of the timing of this diagnostic change only their most recent diagnosis

(taken on the 1st October 2012) was recorded to ensure a true representation of IBD distribution. The date of last follow up was taken to be the last clinical episode with paediatric services in SES, and follow up duration was taken from the date of diagnosis regardless of whether the child was diagnosed in SES.

3.4.4 At-risk population and statistics

In view of the differences in health board referral patterns and the fact that children over the age of 16 years old often continue to be managed in the PIBD service until transition in their late teenage years, there are in fact two separate cohorts evident within the current data. The first cohort is the complete prevalent case-load cared for since August 1997 and includes children from the core referral areas (i.e. NHS Borders, NHS Fife and NHS Lothian) as well as children from NHS Forth Valley, NHS Dumfries and Galloway and a small number of children from other areas such as NHS Highland and NHS Tayside (**Figure 3.1**); this cohort is hereafter referred to as Cohort 1.

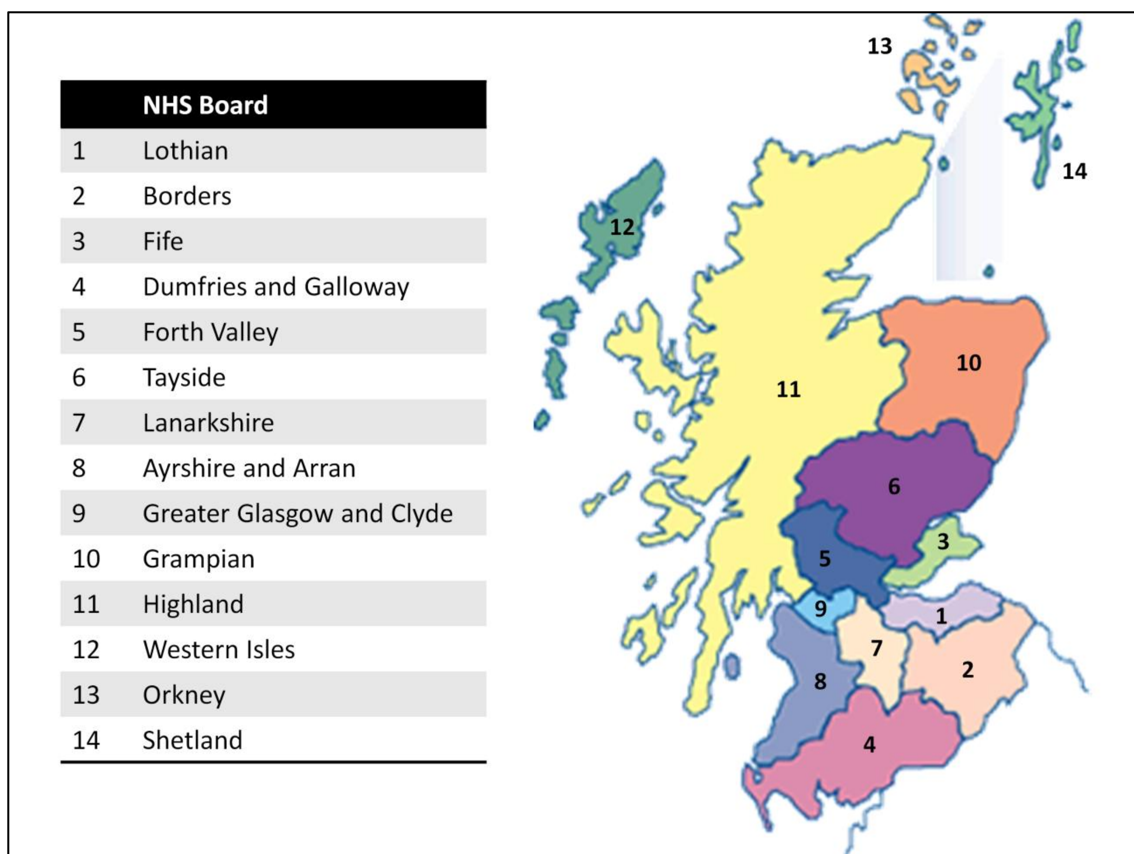


Figure 3.1. Map of Scotland showing the boundaries of each of the 14 NHS Health Boards.

The second cohort is the robust, prospective prevalent cohort consisting of those in Cohort 1 that were less than 16 years old and cared for in RHSCE between 1st January 2000 and 31st December 2011, importantly residing in one of the three core referral areas at the time of diagnosis (Cohort 2). Additionally those who moved into the area from outwith SES were also removed from this cohort.

To allow for changes in population structure within Cohort 2, incidence and prevalence figures for each year were first calculated from known population figures and then standardised to the 2001 Scottish Census population to enable direct comparison.[234] Although the most recent Scottish census took place in 2011 the figures are not yet available publicly and therefore mid-year estimates from 2000-2011 were used (**Table 3.2**). To allow for differences in age structure within the cohort age groups were defined as pre-school (0-5 years), primary school (6-10 years) and secondary school (11-15 years). Age-sex standardised rates were then calculated using the direct method[238] and 95% confidence intervals (CIs) determined using a method based on gamma distribution.[239] All rates are presented as per 100,000 of the at risk population, that is those less than 16 years of age. p values for comparisons between time periods were obtained from Poisson regression models. Analyses were carried out using the epitools package in R version 2.14.1 (R Foundation for Statistical Computing, Vienna, Austria)[240] and Mann-Whitney *U*, Chi-squared analysis using GraphPad Prism version 4.03 (GraphPad Software, CA, USA). A two-tailed p value of less than 0.05 was considered significant in all analyses.

Table 3.2. Numbers of children aged less than 16 years of age in the three NHS health boards that have consistently referred to the Royal Hospital for Sick Children, Edinburgh since 2000.

Year	NHS Borders	NHS Fife	NHS Lothian	Total
2000	20,765	71,145	145,007	236,917
2001	20,185	68,516	143,896	232,597
2002	20,165	67,612	142,081	229,858
2003	20,233	66,949	140,347	227,529
2004	20,271	66,524	139,625	226,420
2005	20,310	66,041	138,890	225,241
2006	20,131	65,478	139,013	224,622
2007	20,107	65,104	138,937	224,148
2008	20,126	65,028	139,314	224,468
2009	19,947	64,882	140,444	225,273
2010	19,880	64,833	141,376	226,089
2011	19,763	64,988	142,984	227,735

NHS, National Health Service

3.4.5 Ethics

Formal ethics approval was sought from the Lothian Research and Ethics Committee but was deemed unnecessary as this was an anonymous, observational study of patients already under the care of paediatric gastroenterology services and under the umbrella of the Paediatric-onset IBD Scottish Audit (PISA). For the children were data was obtained from the Medical Research Council-funded PICTS database, the patient and/or their parents provided full written consent which was approved by local ethics committees at all three participating Scottish clinical networks: South-East Scotland (LREC/2002/6/18), West of Scotland (YREC/P12/03) and North of Scotland (GREC/03/0273).

3.5 Results

3.5.1 Cohort characteristics

In total, 326 children with PIBD were cared for at RHSCE between 1st August 1997 and 31st December 2011. Of this complete cohort (Cohort 1) the median age at diagnosis was 11.7 years (range 0.5-13.4; interquartile range [IQR] 9.1-13.4), with approximately two thirds of children suffering from CD. In Cohort 2 the proportion of children with each PIBD type was similar and although there was a higher median age at diagnosis in this cohort this was not significantly different (p=0.376). There was a slight preponderance of males in both cohorts, driven by the (non-significant; p=0.169) higher proportion of males patients with CD. Details of the basic characteristics of both cohorts are shown in **Table 3.3**. Cohort 1 had a total follow up time of 1577 patient years, with a median of 4.4 years (IQR 2.7-6.3) per patient; the corresponding figures for Cohort 2 were 1075 patient years and 4.1 years (IQR 2.4-5.8) respectively. A total of 292 children (90%) were registered on the PICTS database in Cohort 1 and 240 children (98%) in Cohort 2. Seven patients (four CD and three UC) in Cohort 2 had been diagnosed outwith SES (three in the north of Scotland, two in mainland Europe and two in North America), with 26 discharged/transferred/transitioned to centres outside SES; 20 while still under 16 years old (therefore an overall negative net migration for Cohort 2). There was one death in a 12-year old girl following surgery for UC, this was suspected to be a volvulus in her rectal stump but no details of her final post mortem are available.

Table 3.3. Basic characteristics of the two study cohorts of children diagnosed with paediatric inflammatory bowel disease in South-East Scotland.

	IBD	CD	UC	IBDU
Cohort 1				
Total number	326	215	74	37
Male sex (%)	178 (55)	127 (59)	35 (47)	16 (43)
Median age at diagnosis (years [IQR])	11.7 (9.1-13.4)	11.6 (9.5-13.4)	12.0 (9.4-13.6)	11.5 (8.5-13.7)
Age range (years)	1.5-17.7	2.5-16.8	1.5-17.7	3.3-17.0
Cohort 2				
Total number	244	157	58	29
Male sex (%)	136 (56)	95 (60)	29 (50)	12 (41)
Median age at diagnosis (years [IQR])	12.2 (9.7-13.6)	12.1 (9.8-13.5)	12.4 (9.5-13.8)	11.8 (8.3-13.8)
Age range (years)	1.5-15.9	2.5-15.7	1.5-15.9	3.3-15.7

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic IBD, type unclassified; IQR, interquartile range.

3.5.2 South-East Scotland has the highest recorded incidence of paediatric inflammatory bowel disease in the UK and demonstrates a significant increase in ulcerative colitis

Although incidence figures for the entire Scottish population have been presented in **Chapter 2** it is important to determine if this effect is evident in constituent regions. The details of all children in Cohort 2 were therefore examined to determine those who were diagnosed in SES following referral from the three core SES health boards, leaving 237 incident cases of PIBD in SES over the 11-year period between 2000-2011. This incident cohort consisted of 153 CD, 55 UC and 29 IBDU patients of which 134 (57%) were male.

Table 3.4. Crude number of paediatric inflammatory bowel disease cases diagnosed in South-East Scotland during each six-year epoch by sex and IBD type.

Epoch	Persons at risk*	IBD	CD	UC	IBDU
			Male		
2000-2005	117,562	61	46	9	6
2006-2011	115,182	73	48	19	6
			Female		
2000-2005	112,198	50	31	11	8
2006-2011	110,206	53	28	16	9

* = Mean number of persons at risk (i.e. less than 16 years old) across each epoch. IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic IBD, type unclassified.

It can be seen in **Table 3.4** that there was an increase in the number of diagnoses of PIBD between the two six-year epochs (2000-2005 and 2006-2011) from 111 to 126. Interestingly this seems to have been driven by an increase in the number of children (both males and females) diagnosed with UC during the study periods.

Following age-sex standardisation, the incidence rates of PIBD were 8.00/100,000/year (95% CI 6.58-9.64) and 9.50/100,000/year (95% CI 7.91-11.31) for the 2000-2005 epoch and the 2006-2011 epoch respectively (p=0.325). The adjusted incidence of CD rose marginally from 5.55/100,000/year (95% CI 4.38-6.94) to 5.70/100,000/year (95% CI 4.49-7.14) (p=0.974) and significantly in UC from 1.44/100,000/year (95% CI 0.88-2.22) to 2.67/100,000/year (95% CI 1.86-3.71) (p=0.010). There was no significant rise in the incidence of IBDU from 1.01/100,000/year (95% CI 0.55-1.70) to 1.13/100,000/year (95% CI 0.63-1.87) across the two epochs respectively (p=0.827). Although there was a trend towards an increasing age-standardised incidence in both sexes across all PIBD types, none of these were significant (**Table 3.5**).

Table 3.5. Age-adjusted incidence rates of PIBD in South-East Scotland for all males and females by IBD type.

Cohort	2000-2005	2006-2011	Difference between cohorts (p value)
	Males		
IBD	8.59 (6.57-11.04)	10.80 (8.46-13.58)	0.462
CD	6.48 (4.75-8.65)	7.06 (5.20-9.37)	0.734
UC	1.26 (0.58-2.40)	2.84 (1.71-4.45)	0.104
IBDU	0.84 (0.31-1.84)	0.90 (0.33-1.97)	0.964
	Females		
IBD	7.39 (5.48-9.24)	8.14 (6.10-10.67)	0.731
CD	4.59 (3.12-6.51)	4.29 (2.85-6.21)	0.766
UC	1.62 (0.81-2.90)	2.48 (1.41-4.04)	0.337
IBDU	1.18 (0.51-2.33)	1.37 (0.63-2.62)	0.831

Figures are adjusted rates per 100,000 at risk population per year (95% CIs]). IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic IBD, type unclassified. Significance = p<0.05.

3.5.3 The South-East of Scotland has seen a significant rise in the point prevalence of PIBD since 2000

In areas with high incidence rates of PIBD (as demonstrated in SES) it is important to fully understand the current trends in disease prevalence. To first ascertain the overall service case-load experienced at RHSCE the crude number of PIBD cases in Cohort 1 was calculated on 30th August annually. **Figure 3.2** shows that the number of children with PIBD

under the care of the paediatric gastroenterology team has risen steadily, even accounting for the initial years of service establishment. In the last five years of the study (2007-2011) there were a median of 134 PIBD patients living in SES currently cared for at RHSCE.

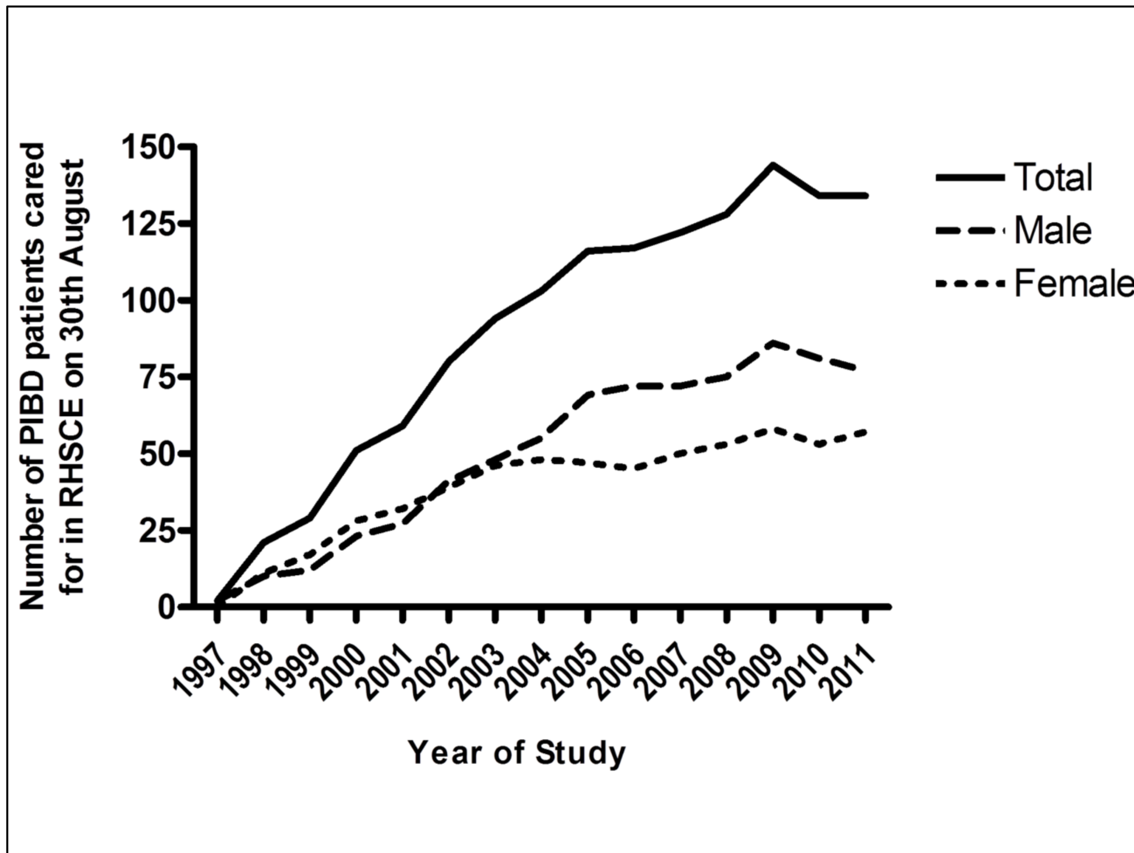


Figure 3.2. Graph showing the crude number of paediatric inflammatory bowel disease patients cared for by the paediatric gastroenterology service in RHSCE on the 30th August annually between 1997 and 2011. PIBD, paediatric inflammatory bowel disease; RHSCE, Royal Hospital for Sick Children, Edinburgh.

To further define the true trends in the prevalence of PIBD, age-sex standardised point prevalence figures on the 30th August annually were calculated for Cohort 2. To further increase the accuracy of these data, children were removed from the prevalence figures after their 16th birthday to ensure that only prevalence rates for those less than 16 years old were included. To take full account of service establishment, only figures from 2000 onwards are presented.

Following age-sex standardisation, the point prevalence rates of PIBD were 29.5/100,000 (95% CI 26.8-32.5) and 41.2/100,000 (95% CI 37.6-44.8) for the 2000-2005 epoch and the 2006-2011 epoch respectively (p=0.016). The adjusted point prevalence of CD rose from 21.1/100,000 (95% CI 18.7-23.6) to 27.5/100,000 (95% CI 24.8-30.5) (p=0.073) and

significantly in UC from 5.3/100,000 (95% CI 4.1-6.6) to 8.4/100,000 (95% CI 6.9-10.2) (p=0.004). There was also a significant rise in the point prevalence of IBDU from 3.2/100,000 (95% CI 2.7-4.3) to 5.2/100,000 (95% CI 4.1-6.6) across the two epochs respectively (p=0.021). **Table 3.6** presents the age-standardised point prevalence rates by sex showing an rise in all IBD types in both sexes with significant increases in all IBD types in males.

Table 3.6. Age-adjusted point prevalence rates of PIBD in Scotland by IBD type for males and females aged less than 16 years old.

Cohort	2000-2005	2006-2011	Difference between cohorts (p value)
Males			
IBD	31.1 (27.1-35.5)	48.7 (43.6-54.3)	0.001
CD	24.9 (21.4-28.8)	34.9 (30.6-39.6)	0.043
UC	3.5 (2.3-5.2)	9.3 (7.1-11.9)	0.003
IBDU	2.7 (1.6-4.2)	4.6 (3.1-6.6)	0.013
Females			
IBD	27.9 (24.1-32.2)	33.3 (29.0-38.0)	0.124
CD	17.1 (14.1-20.5	19.9 (16.6-23.6)	0.316
UC	7.1 (5.2-9.4)	7.6 (5.6-10.0)	0.813
IBDU	3.8 (2.5-5.6)	5.8 (4.1-8.0)	0.087

Figures are adjusted rates per 100,000 at risk population per year (95% CIs). IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic IBD, type unclassified. Significance = p<0.05.

3.5.4 Children with inflammatory bowel disease present with a severe phenotype and progress quickly to extensive disease

Using the data available in the RHSCE departmental database, in addition to the comprehensive PICTS database (and review of clinical and histological reports were necessary), data regarding CD Montreal classification disease location and behaviour was available for 212/215 (98.6%) of all patients. CD patients were followed up for a median of 4.9 years (range 0.5-15.4, IQR 3.0-6.9) before discontinuing their contact with paediatric services. It can be seen from **Table 3.7** that, in line with previous studies, children with CD presented with a severe phenotype, with over one third of children having pan-enteric disease at diagnosis (Montreal L3+L4) and 43.9% of children having L3+L4 disease at last follow up. In addition, a significant proportion of children presented with only oral and/or perianal disease (4.7%) with a small number (1.4%) remaining with this phenotype at follow up. With respect to behaviour, the vast majority of children presented with an inflammatory phenotype (95%) with 14% of children demonstrating perianal disease at diagnosis,

increasing to 20% at last follow up. However for those with B1 behaviour at diagnosis (+/- perianal disease), 30% had extended to either B2 or B3 disease at follow up. There was no difference in males and females with regard to the presence of pan-enteric disease at diagnosis (p=0.931), in those diagnosed less than or greater than 10 years of age (p=0.816) or the presence of perianal disease (p=0.755). The presence of any ileal disease (Montreal L1 or L3 +/- L4) was rare in children aged less than 8 years and 10 years old (13% and 30% respectively). There was also a significantly higher number of children aged less than 10 years old with pure colonic disease (L2) compared with older children (30.4% vs 9.7%; p<0.001)

Table 3.7. Montreal classification of Crohn's disease location and behaviour at diagnosis and last follow up for 212 children cared for in South-East Scotland between 1997-2011.

Montreal Location	At diagnosis (*n* [%])	At last follow up (*n* [%])	Montreal Behaviour	At diagnosis (*n* [%])	At last follow up (*n* [%])
L1	8 [3.8]	3 [1.4]	**B1**	173 [81.6]	137 [64.6]
L2	34 [16.0]	24 [11.3]	**B1p**	29 [13.7]	35 [16.5]
L3	38 [17.9]	44 [20.8]	**B2**	5 [2.4]	20 [9.4]
L4	2 [0.9]	0 [0.0]	**B2p**	1 [0.5]	5 [2.4]
L1+L4	5 [2.4]	4 [1.9]	**B3**	4 [1.9]	12 [5.7]
L2+L4	43 [20.3]	41 [19.3]	**B3p**	0 [0.0]	3 [1.4]
L3+L4	72 [34.0]	93 [43.9]			
Oral/perianal only	10 [4.7]	3 [1.4]			

With regard to cases of UC and IBDU, 107/111 (96.3%) had Montreal location data available at diagnosis and Montreal maximum extent at last follow up; the median follow up time for these patients was 3.9 years (range 0.1-11.3, IQR 2.1-5.4).

Table 3.8. Montreal classification location at diagnosis and maximal extent at last follow up for 107 patients with UC or IBDU cared for in South-East Scotland between 1997-2011.

Montreal Classification	At diagnosis (*n* [%])	Maximal Extent at follow up (*n* [%])
E1	16 [15.0]	11 [10.3]
E2	10 [9.3]	9 [8.4]
E3	81 [75.7]	87 [81.3]

Table 3.8 shows that three quarters of children with UC or IBDU presented with a pancolitis, with 81.3% having extensive disease at last follow up. Of the 26 children who presented with a more limited colitis (i.e. Montreal E1 or E2), eight (30.8%) had extended their disease at last follow up.

3.5.5 Immunomodulators and biologicals are used earlier in the disease course in children diagnosed in the more recent 2006-2011 epoch

With regard to the use of immunomodulators and biologicals, data regarding 'any exposure' of AZA, 6-MP, MTX or biologicals, including the date of first prescription, was available for all patients.

AZA was prescribed to a total of 217 of patients (70.0%) in Cohort 1 with no difference in the numbers of males and females prescribed the drug (p=0.991). With respect to the time of commencing AZA in Cohort 2, 101 children diagnosed between 2000-2005 were started on AZA at a median of 202 days from diagnosis (IQR 92-550); the corresponding values for those diagnosed between 2006-2011 (*n*=76) was 182 days (IQR 67-375), this was not significantly different (p=0.295). Looking at patients diagnosed before their 10th birthday in Cohort 1 there was a significantly longer period to their first AZA prescription (403 days [IQR 157-1164]) compared to those diagnosed greater than 10 years old (196 days [IQR 92-488]) (p=0.001). Additionally there was no difference in the proportion of younger (i.e. less than 10 years old at diagnosis) or older (i.e. over 10 years old at diagnosis) children prescribed AZA across the whole study period (p=0.767). A total of 20 children were prescribed 6-MP (50% male) at a median of 21 months following diagnosis (IQR 13-36). All these had previously been exposed to AZA and were converted to 6-MP for AZA intolerance confirmed on reproducible symptoms (nausea, headache, fatigue) on two separate exposures.

MTX was prescribed to a total of 85 patients (26.1%) in Cohort 1, with a slighter greater proportion of males (p=0.123). In Cohort 2 51 patients diagnosed between 2000-2005 commenced MTX at a median of 34 months (IQR 22-53) from diagnosis, which was significantly longer than those diagnosed more recently in the 2006-2011 epoch (22 months [IQR 15-27]) (p=0.001). Children diagnosed before 10 years of age were not more likely to receive MTX across the entire study period (p=0.604) and had a significantly longer time to first prescription (47 months [IQR 28-76]) than older children (25 months [IQR 16-35]) (p<0.001).

The use of biological therapy (such as infliximab and adalimumab) has increased in recent years, with preparations now licensed for both CD and UC. A total of 58 patients in the entire cohort received either infliximab, adalimumab or both during the study period (17.8%); 12 (20.7%) of whom had a diagnosis of UC. Fifty-seven patients were administered infliximab

and 14 adalimumab (only one patient was given adalimumab without previously encountering infliximab; a child with known juvenille idiopathic arthritis diagnosed prior to confirmed luminal CD) of which 34 were male and 24 female (p=0.594). Twenty-seven children in Cohort 2 diagnosed between 2000-2005 were given a biological at a median time from diagnosis of 47 months (IQR 22-58), with the 26 children diagnosed between 2006-2011 receiving their first dose of biological at a significantly shorter 20 months (IQR 4-37) (p<0.001). The same proportion of those diagnosed less than 10 years old were administered a biological as children diagnosed in later childhood (p=0.912); older children were more likely to receive a biological quicker (23 months [IQR 11-42]) than younger children (58 months [IQR 35-92]) (p=0.003). Overall, 67.4% (220/326) received any immunomodulator (including biologicals) during the study period.

3.5.6 Surgery

A detailed account of the surgeries performed in this cohort of children is outwith the scope of this study, however crude data on surgery was available through the departmental database and the PICTS database. A total of 65 patients (19.9%) had 73 IBD-related surgeries carried out while under the care of paediatric services (35 male, 30 female). **Figure 3.3** shows the total number of surgeries performed during the study period. It can be seen that the majority of children required a sub-total or total colectomy, with 15 requiring surgery for perianal disease. Eight children underwent perianal surgery prior to a confirmed diagnosis of IBD, with a further 11 undergoing surgery within one month of diagnosis. Of the remaining 44 children, the median time to first surgery was 3.3 years (IQR 1.8-4.6) and median time to first resection 2.4 years (IQR 1.3-4.6).

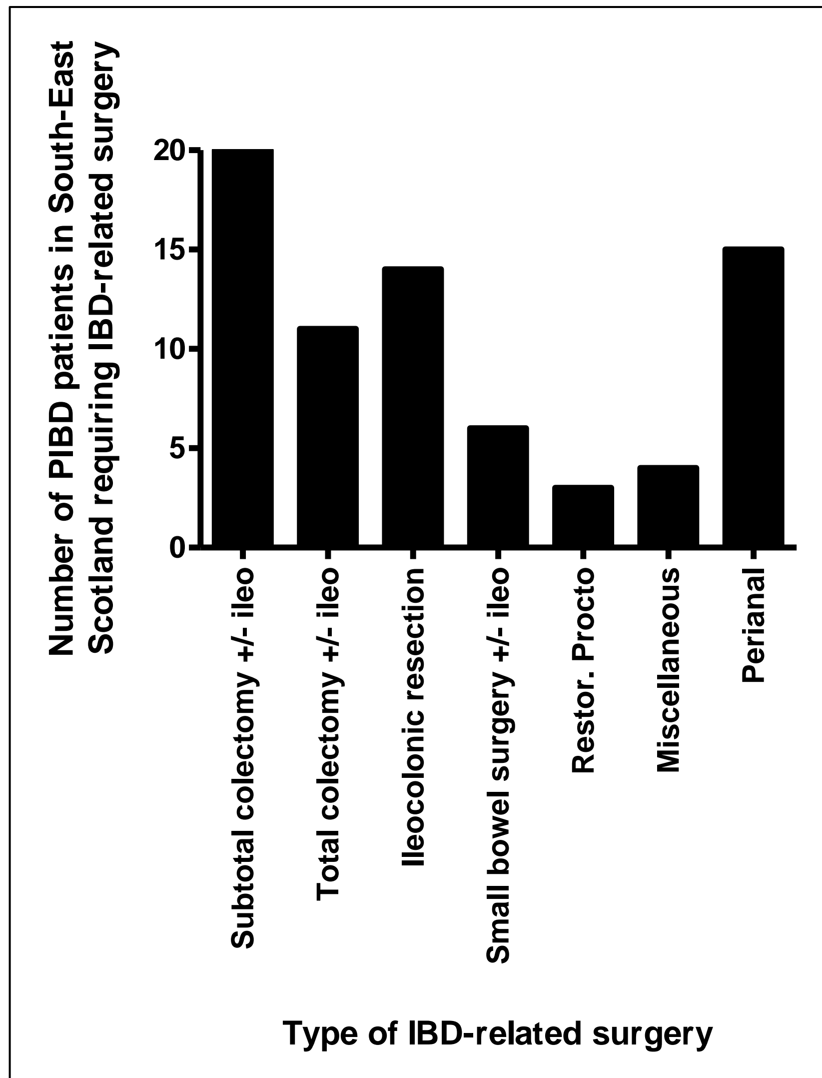


Figure 3.3. Graph showing the number of each type of IBD-related surgery performed in the South-East Scotland cohort while in paediatric services. PIBD, paediatric inflammatory bowel disease; ileo, ileostomy; Restor.Procto, restorative proctocolectomy.

3.6 Discussion

This study has demonstrated that SES has the highest recorded incidence rate of PIBD in the UK to date, with a significant increase in UC diagnoses since 2000 and rising prevalence rates in those under 16 years of age. It has also confirmed the severe disease phenotype both in CD and UC, and the high rates of immunomodulator use and surgery.

The data regarding the incidence and prevalence of PIBD in SES are certainly of interest. In the context of the figures presented in **Chapter 2**, the higher incidence rates of total PIBD of 8.00/100,000/year in the 2000-2005 cohort are higher than would have been expected, suggesting that other areas of Scotland may have lower rates. This would be in keeping with

previous work from Canada showing varying incidence rates of between 5.4 to 12.0 per 100,000 population per year for CD and between 3.2 and 4.7/100,000 for UC in five large regions.[281] The authors suggest several reasons for these differences including hospital coding[306] and changes in immigration and ethnic diversity. It is not clear however how these changes currently affect the Scottish population and certainly these regional variations may partly be explained by environmental factors.[253] It is also of interest that SES had higher incidence rates of CD and UC than the Scotland-wide data, evident between 2000-2005. This suggests that the incidence for both these IBD types has been higher for a greater length of time, again questioning the role of urban dwelling,[253] diet and standard of living,[307,308] migration patterns[273] and potential bacterial pathogens[309]. Specifically with relation to migration, publicly available data from the General Registrar Office shows that net migration of children 0-15 years old has remained stable but positive since 2001 Scotland-wide, with a mean of 3,400 children per year. However, regional variation exists with a decline in net migration in the NHS Borders and NHS Fife health boards and a dramatic increase in positive net migration in NHS Lothian from 2001-2011. This is unlikely to have significantly influenced the currently robust prevalent cohort given that there was an overall negative net migration of patients during the study period. An alternative explanation of this higher incidence rate in SES is the greater accrual of older teenagers (14-15 years old) in this region, which anecdotally is the certainly the case.

Despite the evidence suggesting increasing incidence rates of PIBD in both the UK (**Chapter 2**) and Ireland,[230] these figures do not fully encompass the burden of disease encountered by these young people. In the first comprehensive study of PIBD prevalence in the UK, it has been shown here that children with this chronic disease create a significant workload for the centres providing their care.[310] Not only can the prevalence figures presented provide a sound basis for the provision of future services (as with other conditions[311]) but they can also better inform the charities that support these young people[312]. A greater understanding of the prevalence and disease burden of PIBD may also help inform allied health services such as psychology and dietetics,[313,314] as well as helping to underpin the drive for translational research in the field of IBD[315]. The infrastructure for PIBD services has been brought into focus over recent years in the UK by the IBD audit commissioned by the Healthcare Quality Improvement Partnership.[316,317] The recent round of the audit demonstrated that although there had been an increase in the PIBD service workforce nationwide (especially with regard to PIBD nurse specialists) there were still inadequacies in the shared care pathways with primary care, annual review of patients in outpatient services, real time patient management systems and research network trial participation.[317] With accurate prevalence data now available, a stronger business case regarding funding for many of these issues may be put forward.

Besides the crude numbers of children living with PIBD, the disease severity evident even at diagnosis is notable. This current study has confirmed the work by Van Limbergen and Russell et al. (whose study included a large number of SES patients[141]), and others,[291] by demonstrating a high percentage of children with pan-enteric CD or pancolonic UC at diagnosis. It has also highlighted once again in CD the rarity of an ileal disease location in those less than 10 years old at diagnosis and the high prevalence of colonic disease in this age group.[318] In keeping with the differences between paediatric-onset and adult-onset disease outlined by the Scottish group, other groups have now shown similar results. Pigneur et al. evaluated 206 CD patients (aged less than 16 years) diagnosed at a median age of 13 years, comparing various parameters with 412 adult-onset CD patients. They reported that the paediatric-onset CD group consistently had a higher proportion of patients with active disease year-on-year, greater use of immunomodulators (including biologicals) and a trend towards a higher 25-year cumulative risk of permanent stoma, all resulting in lower mean adult final height in the early-onset group.[142] In a smaller Danish study by Jakobsen et al., they reported disease location in 49 PIBD and 173 adult-onset IBD cases.[319] Although the UC outcome data were consistent with previous observations (i.e. more severe disease within the first 2 years from diagnosis), there were concerns about the validity of the predominantly negative conclusions drawn from the small paediatric-onset CD cohort (n= 29).[320] Additionally Guariso et al. demonstrated that children (aged less than 18 years old) have a higher risk of perianal disease and extra-intestinal manifestations following diagnosis.[321]

With regard to perianal involvement in CD, the figure of 14% at diagnosis in the current dataset (and previous Scottish data[141]) is higher than reported by the EPIMAD group (9%).[291] It is unclear if pure methodological variation is responsible for this difference, but with disease location and ethnicity having been shown to influence the presence of perianal disease,[322] other studies that present a figure of 9-10% for perianal disease at diagnosis suggest that geographical variation may certainly play a part[295,323]. In terms of behaviour, the reasons for the low proportion of children presenting with non-inflammatory disease (i.e. Montreal B2 or B3) in Scotland is of interest. One reason for this difference could be age at diagnosis,[295] with the EPIMAD group consistently reporting a median age of 14 years,[291-293] and national (**Chapter 2**) and regional Scottish data (above) demonstrating a median age of 11.9 and 11.7 years respectively.

With respect to medication use, the overall combined use of immunomodulator and biological use is comparable to previous data showing that 68% of children diagnosed after 1995 in France, when immunomodulator use became common practice, were prescribed one of these drugs.[291] Specifically the use of biologicals in the current cohort is similar to other reports with a similar follow up period.[324] The basic surgery data for PIBD presented

here are also similar to previous studies at approximately 20%,[300,301] however these were mainly concerned with resectional surgery. With perianal disease included in the above figures, however, it is likely that removal of these non-resectional cases and a longer follow up into adult services would bring together any discrepancies in the data.

The strengths of this study are mainly due to the prospective nature of the data collection. Although for a short period during 1998-1999 children were retrospectively entered into a paper record, the numbers of PIBD patients under the care of the paediatric gastroenterology department at that time were small, therefore robust inclusion was likely. Prospective accrual of cases since late 1999 in this setting was vital and has permitted the accurate calculation of incident cases since 2000 in a geographically defined population. Restriction of cases residing in the three core health-board referral areas has enhanced the data further. Also with regard to incidence and prevalence rates, the availability of health-board specific population estimates, age-sex standardisation and correction for overdispersion increase the accuracy of the results. With the correct identification of all PIBD cases, the strict methods employed to ensure a correct IBD type is assigned, using case-note review, histology reports, endoscopy reports and multidisciplinary team discussion ensure a true reflection of PIBD epidemiology is given. The inclusion of data from the PICTS database has also refined the results with 'free-text' comments often providing useful and insightful information.

There are also potential limitations to this study, mostly with regard to medication use and surgery. At present, the departmental database does not hold sufficient information to ascertain the precise length of medication course, reasons for discontinuation, drug side effects or the achievement of disease remission; however much of this data is available from other research sources and is currently under analysis. These are important aspects to consider for not only immunomodulators such as AZA, but also for the biological agents. It can be seen that these drugs have been used more aggressively in recent years in SES and data pertaining to their efficacy may influence future practice and provide a clearer safety profile for these drugs. With regard to surgery, the minimal data available here does not convey the specific indications for surgery, the medical and psychosocial morbidity of the patients and their families,[325] or the long-term sequelae which may only become apparent following transition to adult services. As highlighted and discussed in detail in **Chapter 2.4**, due to the inability to accrue all cases of PIBD diagnosed less than 17 years in adult centres in the region, incidence and prevalence figures would in fact be under-estimates of the true figures.

3.7 Conclusion

In the first paediatric study of its kind in the UK, it has been shown that the incidence of PIBD may vary across Scottish regions with high rates of both CD and UC in the paediatric population. The prevalence of disease is also continuing to rise which will undoubtedly lead to changes in health provision and service development. With this increased prevalence comes additional morbidity in these young people, who have extensive disease at diagnosis with rapid progression. Further work is now required to determine the reasons behind this variation in incidence and to unravel the complex pathogenesis of this chronic, debilitating disease.

4. The genetics of paediatric inflammatory bowel disease

4.1 Introduction

There is some evidence that in those with IBD, a disease course that starts earlier in life may have a stronger genetic influence[288,326-328] although clear similarities in the genetic susceptibility to adult disease are also apparent[18]. This chapter will focus on the newly implicated genes described in the two exclusively paediatric genome-wide association studies (GWAS) and the associated dysregulated immunological pathways implicated in IBD pathogenesis.

In recent years the introduction of the Human Genome Project and other international collaborations have allowed the detailed examination of the entire genetic code.[329] This information, coupled with the creation of high-throughput genotyping platforms, has led to the ability to analyse up to 500,000 single nucleotide polymorphisms (SNPs) using hypothesis-free GWAS. (**Table 4.1**) This has led to an explosion of multiple candidate genes implicated in IBD pathogenesis.

Table 4.1. Table comparing genome-wide linkage analysis and genome-wide association studies.

Characteristic	Linkage Analysis	Association Study
Methods	Non-parametric linkage	High-throughput genotyping using information from the HapMap Project and Human Genome Project
SNPs analysed (*n*)	500-5000	300,000 – 500,000
Population (*n*)	Affected sibling pairs or small families (<500)	Single cases and healthy controls (500 – 4000)
Cost	Expensive	Relatively cheap
Significance	LOD score >3.6, or P-value $<2.2 \times 10^{-5}$	p values less than 5×10^{-8} (after Bonferroni correction for multiple testing)
Implicated genes per study (*n*)	< 3	5-10

SNP, single nucleotide polymorphism; LOD score, logarithm of the odds score

Before the first major CD meta-analysis published in 2008 by Barrett et al., only 16 loci had been identified,[330] with the meta-analysis confirming many of these and providing evidence for 21 additional SNPs[13]. There have now been over 15 GWAS performed in IBD

patients[331] with only two GWAS performed exclusively in early-onset disease. The most recent of these built on the first study by Kugathasan et al. in 2008[332] and involved 3,426 affected individuals and 11,963 genetically matched controls[18] (**Table 4.2**). These two international collaborations involving countries throughout Europe and North America identified seven new regions associated with childhood IBD susceptibility (**Table 4.3**), in addition to nominally replicating 29 of 49 previously recognised loci implicated in both adult-onset CD and UC.[13]

Table 4.2. Summary of the two early-onset genome-wide association studies performed to date.

Study (year)	Age range	Country	Platform	Discovery Cohort			Healthy Controls
				CD	UC	IBDU	
Kugathasan (2008)	< 19yrs	North America; Italy	Illumina 550k	647	317	47	4250
Imielinski (2009)	< 19yrs	North America; Canada; Scotland; Italy	Illumina 550k	1636	724	53	6158

CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic IBD, type unclassified.

Table 4.3. Summary of new risk loci for IBD identified by the two early-onset genome-wide association scans.

Locus	Best Candidate Gene	Best SNP	$P_{Combined}$	OR	Other Genes in Region
2q37	*CAPN10*	rs4676410	3.64×10^{-8}	1.41†	*GPR35, KIF1A, RNPEPL1*
10q22	*ZMIZ1*	rs1250550	5.63×10^{-9}	0.86	
16p11	*IL27*	rs8049439	2.41×10^{-9}	1.20	*SULT1A1, SULT1A2, EIF3C, CLN3*
19q13		rs10500265	4.26×10^{-10}	1.21	
20q13	*TNFRSF6B*	rs2315008 rs4809330	8.85×10^{-15} 1.62×10^{-14}	0.84 0.84	*STMN3, RTEL1, ARFRP1, ZGPAT, LIME1, SLC2A4RG, ZBTB46*
21q22	*PSMG1*	rs2836878	4.48×10^{-12}	0.90	
22q12	*MTMR3*	rs2412973	1.55×10^{-9}	1.18	*HORMAD2, LIF*

*Underlined genes found to have altered expression in IBD patients *vs* controls. SNP, single nucleotide polymorphism; $P_{Combined}$, Bonferroni corrected p-value for IBD combined; OR, odds ratio. †Odds ratio for UC only.

4.2 Is childhood-onset IBD genetically distinct?

It seems clear from recent GWAS that early-onset and adult disease share the majority of their risk loci. However, whether those developing IBD at an earlier age have an increased 'genetic risk' is still in dispute, with variability in family history and certain risk genotypes fuelling the debate. Several studies have shown the increased prevalence of a family history in those diagnosed in early life. Polito et al. demonstrated that those diagnosed with CD under the age of 20 years were more likely to have a positive family history of CD compared to those diagnosed over 40 years (29.9% *vs.* 13.6%).[333] These high rates of family history have also been demonstrated in UC (26% *vs.* 11%).[287] With regard to variations in adult-onset and early-onset genotype, inconsistencies still exist. One of the first indications of genotype variability came from a Canadian genome-wide scan which showed that a locus of genome-wide significance in the 5q31-q33 (IBD5) region contributed to CD susceptibility in families with early-onset disease.[334] Although this was not replicated in two independent UK studies,[335,336] IBD5 variants have been shown to be associated with growth indices in early-onset disease[337] and a more severe overall phenotype[338]. Several studies have also been performed looking at the frequency of *NOD2* mutations in early-onset disease. Although several of these have suggested differing allele frequencies, these studies have included small numbers and often lacked control cohorts.[17,339,340] One of these studies demonstrated that the 3020insC polymorphism in *NOD2* occurred more often in early-onset (less than 19 years old) than adult-onset CD.[328] Markers in the IBD1 locus have also been shown to be more common in early-onset disease in both Jewish[327] and non-Jewish[326] Caucasian populations.

Although some differences in the genetic susceptibility of childhood and adult-onset IBD exist, results from the recent early-onset GWAS have highlighted the overall similarities in these geenotypes with some notable exceptions. As mentioned above, 29 of the 49 known susceptibility loci where replicated including *PTPN22*, *MST1* and *IL-12B*.[18] Overall, 72% of known adult CD loci and 47% of adult UC loci were replicated suggesting more heterogeneity in the 'genetic risk' in UC. Of interest, two SNPs, rs3024505 (in the region of *IL10*) and rs917997 (in the region of *IL18R1, IL18RAP*) showed genome-wide significance for CD in the early-onset population and not in adult disease.[18] In addition, SNPs near *ORMDL3* and *ICOSLG* were shown to be significant for early-onset UC, having only previously been implicated in adult CD.[13] In addition to the known risk alleles common to both early-onset and adult-onset disease a recent study has also demonstrated no significant association between CD genetic risk score and age at onset.[341] This paper

illustrated that the genetic effect of established CD risk variants is similar in early and late-onset disease.

4.3 Dysregulated pathways implicated by the study of IBD genetics

The increasing success of genome-wide scans has led to the identification of large numbers of possible candidate genes with many involved in the same immunological pathways. These pathways broadly involve the control of intestinal barrier function, mucosal response to luminal bacteria and the secondary downstream immune response. Furthermore the analysis of the effect of multiple SNPs within these pathways has already been successful, most notably by the observed IL-12/IL-23 pathway-specific association signal in CD.[342]

4.3.1 Innate pattern recognition receptors (NOD2, TLR4, CARD9)

The innate immune system is the body's first-line, non-specific response to foreign antigen. Both transmembrane and intracellular pattern recognition receptors (PRRs) recognise conserved, microbe-specific molecules named pathogen-associated molecular patterns (PAMPs), leading to an appropriate immune response.[343] As mentioned above, the major breakthrough in IBD genetics came when fine mapping of the IBD1 locus on chromosome 16 by the French paediatrician JP Hugot identified the leucine-rich repeat (LRR) variants of the *NOD2* gene as conferring susceptibility to CD.[17] Since this discovery the *NOD2*mutations have been widely replicated in both adult- and early-onset disease.[13,332] This gene encodes for NOD2 which is an intracellular PRR which recognises muramyl dipeptide (MDP), a product of bacterial cell wall degradation. It has now been shown that NOD2 has expression limited to the Paneth cells[19] in the intestinal crypts and that the recognition of MDP is facilitated through the LRR domain of the protein[20]. Three main regulatory pathways have since been described for NOD2, namely through the activation of RIP2 and NF-κB,[344] preferential expression of a truncated NOD2 isoform (NOD2-S)[345] and 5' UTR splice forms linking NOD2 with the autophagy process[346]. Furthermore the important role of NOD2 in the regulation of commensal bacteria and viral recognition have also been demonstrated in recent studies, highlighting the complex role of this intracellular receptor.[21,23]

Besides *NOD2*, two further gene groups implicated in IBD and involving the innate immune response are the toll-like receptors (TLRs) and CARD9. TLRs are a family of transmembrane receptors which bind a myriad of ligands such as LPS, beta-defensins and heat shock proteins,[347] possibly through binding with specific hydrophobic portions of such molecules[348]. The *TLR4* Asp299Gly polymorphism has generated most interest[349] with

other molecules involved in the complex TLR4 pathway also under investigation[350,351]. The locus at 9q34.3 containing the *CARD9* gene (which contains the characteristic caspase-associated recruitment domain) has also been shown to confer susceptibility to CD and is linked to several mechanisms including TLR-NOD2 signalling and Th17 cell differentiation.[15] Although mutations in *TLR4 or CARD9* have yet to be confirmed as conferring disease susceptibility in the early-onset population, studies have shown that more *TLR4* Asp299 Gly variants were found in early-onset disease[352] and that carriers of *NOD2*/*TLR4* mutations present at an earlier age[353].

4.3.2 Th17 differentiation and IL-27

Following innate immune system signalling, a coordinated T helper (Th) cell response by the adaptive immune system leads to tissue-specific inflammation. The now outdated Th1/Th2 classification has been superseded by a multitude of T cell lineages including iTregs, Tr1 and T_{FH} subsets.[80] One of these recently described subsets are the Th17 effector cells, classified on the basis of intracellular transcription factors such as STAT3, RORγT and RORα and a cytokine profile including IL-17A, IL-21 and IL-22.[34] The association with CD of a germline variation of the *IL23R* (interleukin-23 receptor) gene in 2006 provided the initial stimulus to look more closely at the Th17 subset.[12] This association has been replicated in both adult-onset CD and UC,[354-356] as well as trios with both CD and UC in the index study[12]. Replication studies in the paediatric population have mainly demonstrated significant association for CD patients only, however the recent early-onset GWAS showed significance for both CD and UC.[18,241,357,358] Specifically the Arg381Gln variant in the *IL23R* gene has been shown to be protective for the development of IBD although other signals within the gene also confer disease susceptibility independently of this SNP.[35] Taylor et al. genotyped multiple SNPs in *IL23R* and identified *IL23R* risk and protective haplotypes demonstrating a substantial increase in population-attributable risk compared to Arg381Gln alone.[359] Raelson et al. also reported multiple significantly associated SNPs within the *IL23R* gene itself or within linkage disequilibrium (LD) blocks extending from the gene into 3' and 5' intergenic regions.[360]

IL-23 is a soluble type I cytokine member of the IL-12 family and signals through a heterodimeric receptor consisting of the IL-12β1 and specific IL-23R chains.[81] This receptor is expressed on T cells with IL-23 promoting the stabilisation of the Th17 subset after differentiation through various intracellular transcription factors such as STAT3. Furthermore, germline variations in the genes encoding STAT3 and the tyrosine kinase JAK2, have also been implicated in the recent CD GWAS meta-analysis,[13] however only SNPs in the region of *JAK2* gained genome-wide significance in the paediatric population[18]. Variations in the

gene encoding CCR6, which is involved in the recruitment of Th17 cells to the epithelium, has also been shown to predispose to CD[13], but again this has not been replicated in early-onset disease, possibly due to lack of power.

Earlier in the differentiation of the Th17 subset the molecule inducible co-stimulator ligand (ICOSLG) binds with its T cell ligand (ICOS) leading to T cell activation.[36] The gene encoding ICOSLG was suggested as the candidate gene at 21q22.3, however no replication or expression data has yet confirmed this association.[13] Barrett et al. demonstrated that the SNP at this locus (rs762421) conferred susceptibility to CD, however the recent paediatric GWAS showed significance in both CD and UC.[18] The interaction of ICOSLG with its ligand ICOS is not only essential in the early differentiation of naïve T cells but is likely to be important in the careful balance of IL-10 producing regulatory cells and the Th17 population.[37] (**Figure 4.1**)

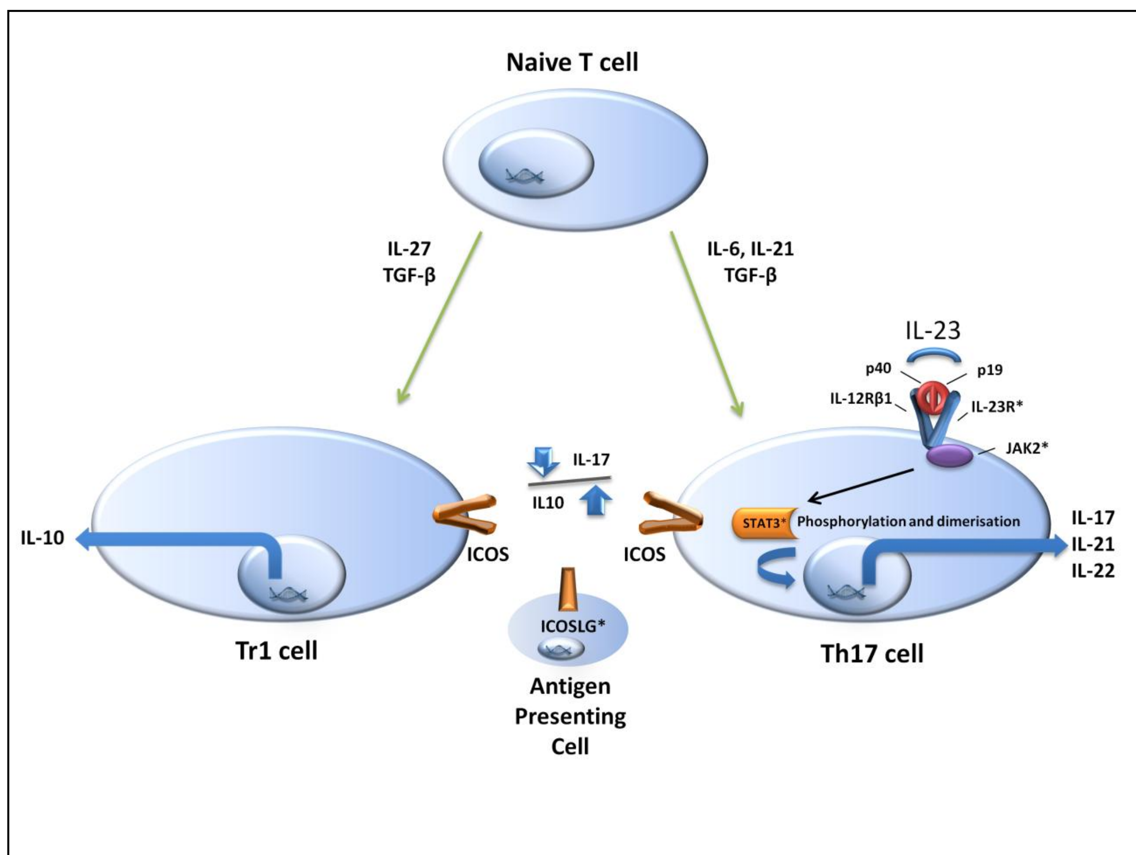


Figure 4.1. IBD susceptibility genes implicated in Th17 differentiation. (Molecules highlighted by *). Under the influence of IL-6, IL-21 and TGF-β Th17 cells differentiate from naïve T cells. IL-23 is not required for this initial differentiation, but instead stabilises the Th17 lineage and enhances the production of various cytokines including IL-17 and IL-21. In addition to this ICOSLG expressed on antigen presenting cells (such as dendritic cells and intestinal epithelial cells) interacts with ICOS, enhancing the balance of pathogenic Th17 cells and IL-10 producing Tr1 cells.

Functional assays in IBD patients show that IL-17 producing cells and the levels of the Th17-related cytokines IL-17 and IL-22 are increased in IBD patients.[361-363] It is also clear that Th17 cells are required during the inflammatory response with studies showing that mice with T cells lacking the gene encoding RORγT (which is required for efficient Th17 differentiation) are protected from chemically induced colitis in addition to experimental autoimmune encephalomyelitis.[364,365] Of further interest is work showing that priming of human dendritic cells by bacteria promotes IL-17 production through NOD2.[366] Coupled with evidence that signalling through the dectin-1-Syk-CARD9 pathway induces the maturation of dendritic cells capable of eliciting Th17 differentiation[367] and with dectin-1 levels elevated in IBD patients,[368] the interaction between known IBD susceptibility genes and the Th17 subset is growing.

The SNP rs8049439 identified in the recent paediatric GWAS at 16p11.2 lies in LD with the gene encoding the p28 subunit of the IL-12 cytokine family member IL-27.[18] The SNP identified reached genome-wide significance ($p<1 \times 10^{-7}$) for both CD and IBD combined, however a surrogate SNP (rs4788084) did not attain genome-wide significance in the recent, majority adult-onset CD meta-analysis.[13] With regard to *IL-27* mRNA expression, Imielinski et al. demonstrated a decrease of over 90% in lymphoblastoid cell lines from individuals homozygous for the risk allele and significantly reduced colonic expression in early-onset IBD patents compared to controls.[18] Other candidate genes in the region include *SULT1A1*, *SULT1A2* and *EIF3C* (**Box 4.1**).

IL-27 is a type 1 cytokine composed of the p28 subunit in addition to the EBV-induced gene 3 (EBI3)[369] and was first described by Pflanz et al. in 2002[370]. IL-27 mediates its effects through a heterodimeric receptor complex made up of the widely expressed gp130 and IL-27-specific WSX-1(TCCR) subunit.[371] The role of IL-27 has been controversial mainly due to its perceived contradictory anti-inflammatory and pro-inflammatory roles.[372] It is now recognised that IL-27 is required for the initial phase of Th1 differentiation by inducing the IL-12Rβ2 subunit which is then ligated by IL-12 which commits the naïve T cell to the Th1 lineage.[373] In this respect IL-27 has also been shown to upregulate the Th1 transcriptional factor TBET[374] and reduce the Th2 transcriptional factor GATA3[375] thus skewing the Th1/Th2 differential.

However it is the role of IL-27 in the careful balance of pathogenic Th17 cells and suppressive T cell subsets which is of most interest in the pathogenesis of IBD.[376] (**Figure 4.2**) As discussed above, Th17 cells are essential in the propagation of intestinal inflammation, with increased numbers of IL-17 producing cells seen in IBD patients.

Box 4.1. Other candidate genes at 16p11.

***SULT1A1* and *SULT1A2* (sulfotransferase family, cytosolic, 1A, phenol preferring member 1/2)**

These cytosolic sulfotransferase 1A family members have various known actions including the metabolism of phenolic substances,[377] drugs, oestrogens and the activation of procarcinogens[378]. These proteins have already been implicated in bladder[379], breast[380] and colorectal cancer risk[381]. SULT1A1 is highly expressed in differentiated enterocytes throughout the gut[382] and culture of colonic cell lines with TNFα increases colonocyte SULT1A1 activity[383].

***EIF3C* (eukaryotic translation initiation factor 3, subunit C)**

Encodes for the ubiquitous EIF3C subunit of the EIF3 complex, the largest of 12 eukaryotic initiation factors that interact with Met-tRNA$_i^{Met}$, mRNA and ribosomal subunits during translation initiation.[384] Although there is evidence of an interaction between the EIF3 complex and the autophagy regulator the mammalian target of Rapamycin (mTOR),[385,386] there has been no direct evidence of EIF3 dysregulation altering the autophagy process.

***CCDC101* (coiled-coil domain containing 101)**

Comprises a subunit of STAGA, a complex involved in gene activation.[387]

***CLN3* (ceroid-lipofuscinosis, neuronal 3)**

Function in humans has yet to be delineated but is known to be mutated in Batten disease and may be involved in mechanisms within the Golgi complex[388] and the regulation of the transcription factor SBF[389].

***NUPR1* (nuclear protein, transcriptional regulator, 1)**

Encodes a stress-induced protein and has a variety of functions, some of which apparently contradictory, and has been implicated in a wide variety of cancers including pancreatic,[390] brain[391] and thyroid[392].

In this respect IL-27 has been shown to directly influence the balance of these T cell subsets through direct actions on transcriptional factors such as STAT1 and RORγT. IL-27 has been shown to suppress the production of pro-inflammatory cytokines demonstrated by the fact that WSX-1 deficient mice (lacking the ability to ligate IL-27) over-produce pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and IL-6 from CD4+ T cells following *Trypanosoma cruzi* infection.[393] Of great interest was work carried out by Murugaiyan et al. which showed that, in the presence of IL-27, there was reduced IL-17 production by T cells (in addition to reduced *RORC* expression), IL-23R and CCR6/CCL20.[376] With regard to IL-27 during intestinal inflammation Troy et al. used IL-27R deficient mice to show a significant reduction in Th1 IFN-γ producing cells and an increase in Th17 cells in gut-associated lymphoid tissue.[394] In addition the IL-27R deficient mice showed earlier-onset and increased clinical severity of DSS-induced colitis. Besides the effect of IL-27 on the Th17 cell lineage other anti-inflammatory properties of IL-27 have been demonstrated. These mostly comprise the production of the anti-inflammatory cytokine IL-10 through mechanisms involving STAT3,[395] cMAF and ICOS,[396] although other pathways including the suppression of cFos,[371] B cell class switching[397] and enhanced TLR responsiveness[398] have also been described.

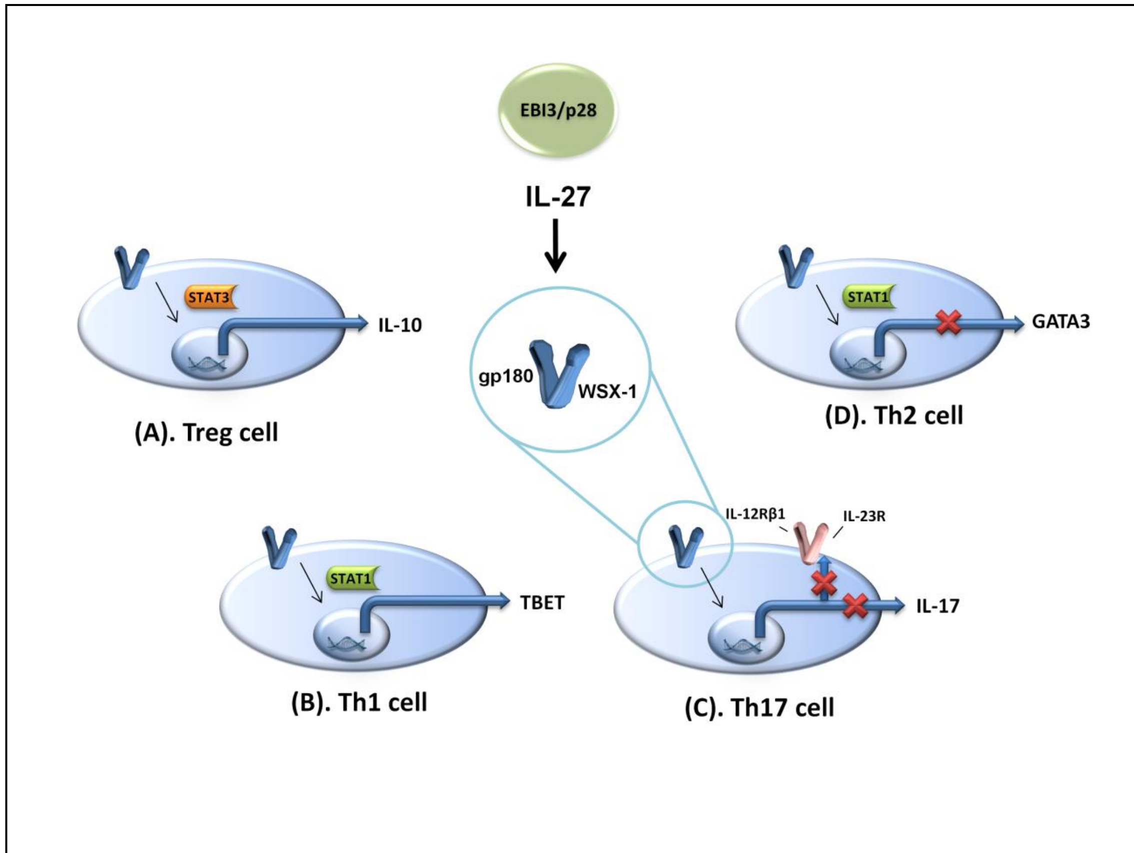


Figure 4.2. The effect of Interleukin-27 (IL-27) on various T cell lineages. (A) IL-27 exerts its effects on IL-10-producing T regulatory cells in a STAT3-dependent manner. (B) In Th1 effector cells IL-27 induces the transcription of TBET via STAT1. (C) In Th17 effector cells IL-27 down-regulates IL-17 production and IL-23R expression. (D) Through STAT1, IL-27 down-regulates GATA3 leading to a reduction in Th2 effector cell differentiation.

4.3.3 Autophagy and MTMR3

Along with the degradation of unwanted organelles, autophagy is responsible for the elimination of pathogenic micro-organisms gaining entry to the cytoplasm through mechanisms involving TLRs[399] and NOD2 signalling[30,31]. SNPs in the region of three genes encoding proteins involved in the process of autophagy have been shown to confer susceptibility to CD, namely *ATG16L1*,[26] *IRGM*[400] and *LRRK2*[13]. The association with *ATG16L1* and CD was first described by Hampe et al. and has since been widely replicated in both adult[13,401,402] and in most but not all paediatric populations[283,305,332,403]. ATG16L1 has been shown to be crucial for Paneth cell biology[25] with the associated CD coding variant driven by ileal disease[305,404]. A SNP in the region of *LRRK2*, which reached genome-wide significance for CD in both the majority adult GWAS meta-analysis[13] and the recent paediatric GWAS,[18] was implicated in the autophagy process through its association with another autophagy-mediated condition, Parkinson's disease[405]. Although its role in CD pathogenesis has yet to be defined, a recent study has shown its involvement in negatively

regulating the autophagy process with silencing of *LRRK2* increasing the cell's autophagic activity.[406] SNPs in the region of *IRGM*, a member of the p47 immunity-related GTPase family, although replicated in adult association studies as conferring susceptibility to both CD and UC,[407,408] do not seem to confer as strong a risk in several paediatric studies[18,332,409].

Box 4.2. Other candidate genes at 22q12.

***HORMAD2* (HORMA-domain protein 2)**

The SNP rs2412973 identified at the locus 22q12.2 lies within *HORMAD2*, an open reading frame with functions involved in the generation of haploid gametes. HORMA-domain proteins lead to efficient crossovers during meiosis with both *HORMAD1* and *HORMAD2* being implicated in double strand break (DSB) formation, the suppression of synaptonemal complex formation between non-homologous chromosomes and meiotic prophase checkpoint that monitors DSB repair.[410]

***LIF* (leukaemia inhibitory factor)**

Encodes LIF which is a member of the IL-6 cytokine family. This cytokine was first identified in the late 1980s as a factor that induced the differentiation of murine myeloid leukaemic cell lines[411] and is now known to signal through a receptor which shares one of its subunits (gp130) with IL-6. Since its discovery the major focus on this molecule has been in the fields of reproductive medicine, [412] stem cell research[413] and cancer[414]. Although the most recent paediatric GWAS did not demonstrate altered expression in colonic biopsies from IBD patients it should be noted that LIF and IL-6 have been implicated in regulating the balance between the recently described Th17 CD4+ lineage and FOXP3+ regulatory cells.[415] Also due to the ubiquitous nature of this molecule, the known increase in serum IL-6 in Crohn's disease patients[416,417] and the recent interest in IL-6-receptor monoclonal antibody for inflammatory disorders[418] the possibility of this molecule being dysregulated in IBD should not be discounted.

Coding-sequence variation has been excluded as a source of this association with adult IBD in the *IRGM* region.[400] However, a common 20-kb deletion polymorphism upstream of *IRGM* which affects IRGM expression has been shown to be in perfect LD (r^2=1.0) with the mostly strongly associated CD SNP.[408] In the recent paediatric GWAS the SNP at locus 22q12.2 (rs2412973) showed genome-wide significance in the combined discovery IBD cohort but again, as with the SNP at 16p11, not in the adult CD meta-analysis.[13,18] The only gene in this region which was shown to differ in colonic expression was *MTMR3.* Other candidate genes at the 22q12 locus are summarised in **Box 4.2**. This gene showed significantly reduced expression in colonic biopsies in UC patients compared to controls. Further details of *MTMR3* and the other genetic susceptibility genes implicating the autophagy pathway are discussed in detail in **Chapter 9**.

4.3.4 The ubiquitin-proteasome system and PSMG1 (21q22)

The SNP rs2836878 identified in the paediatric GWAS by Kugathasan et al. resides at 21q22 in a small region of linkage disequilibrium with no known genes.[332] However, in their studies of gene expression Kugathasan et al. did observe an increase in the closest gene *PSMG1* in the colon of IBD cases compared to controls. *PSMG1* (also known as *PAC1* or *c21-LRP*) encodes the protein proteasome (prosome, macropain) assembly chaperone 1 which is most closely associated with the clinical entity of Down syndrome (trisomy 21), where the gene is more commonly named Down syndrome critical region 2 (*DSCR2*). This gene was first described in 1998 and found to contain a potential open reading frame predicting a 288-amino acid protein[419] with functions relating to cell proliferation[420]. In 2005 PSMG1 was found to directly act as a chaperone involved in the assembly of the four heteroheptameric rings of the 20S proteasome.[421] The ubiquitin-proteasome system (UPS) is responsible for the degradation of over 80% of intracellular components (autophagy accounting for the remaining 20%) with Ubiquitin used as the initial signal for protein-targeting and the proteasome ensuring the efficient removal of denatured and misfolded proteins.[422] Proteasomes have become of particular interest to those studying inflammatory conditions mainly due to the observation that ubiquination and proteasomal degradation of IκB leads to NFκB activation and the subsequent upregulation of proinflammatory cytokines.[423] In addition, other immunoregulatory functions such as the generation of peptide antigen presented to MHC class I molecules[424] and the regulation of T-cell receptor and co-stimulatory molecule signalling[425] are controlled by the UPS system. These studies have prompted a growing interest in the therapeutic role of proteasomal-inhibitors in several conditions including pancreatitis[426] and rheumatoid arthritis[427]. It is also of note that certain subunits (beta1i and beta2i) of the 20S core particle have been shown to be up regulated in CD compared to UC patients[32] and that the probiotic and commensal bacteria *Lactobacillus plantarum* acts as a proteasomal inhibitor through the IκB-NFκB pathway[428].

4.3.5 The secondary immune response and TNFRSF6B, ZMIZ1 and Smad3

Following the interaction between pathogenic material and the innate immune machinery, certain cells present these antigens leading to the production of soluble mediators, chemotactic elements and immune cell recruitment. Several genes involved in this complex mechanism have been implicated in adult-onset disease with varying degrees of replication in the paediatric population. The Human Leukocyte Antigen (HLA) region on chromosome 6p (IBD3) reached significance in the meta-analysis by van Heel et al. with several SNPs also reaching nominal significance in the GWAS meta-analysis by Barrett et al.[13,429] Although the SNPs previously identified were not replicated in the early-onset GWAS, a SNP in the region

of HLA-DRA (recently confirmed in adult-onset disease[430]) was shown to be significant in early-onset UC[18]. Furthermore, SNPs in other genes involved in the secondary immune response were replicated in the childhood-onset GWAS.[18] These included *IL-12B*, a gene encoding the p40 subunit shared by the cytokines IL-12 and IL-23, and *MST1* which encodes macrophage-stimulating protein (MSP) that regulates the innate immune response to several bacterial ligands such as LPS.[15,431] Two SNPs shown to be only nominally significant in the recent CD GWAS meta-analysis showed significant association with early-onset CD, namely the IL18R1-IL18RAP locus on 2q12 (rs917997) and the C-C motif chemokine (CCL) gene cluster on 17q12 (rs991804). IL-18 is of particular interest in CD pathogenesis with IL-18 expression increased in the mucosa of CD patients[432] and IL-18 blockade demonstrated to ameliorate murine colitis[433].

The association between *TNFSF15* (tumour necrosis factor superfamily member 15; also known as TL1A or VEGI) at 9q33 has been widely replicated in adult CD and encodes a TNF-like factor expressed in endothelial cells and professional antigen presenting cells.[13,434-436] Interaction of this molecule with the IL-12/IL-18 pathway has been shown to induce the secretion of IFN-γ[437] and can promote the proliferation of Th17 cells[438]. Although SNPs in this region were not shown to be significant in either early-onset GWAS, another member of this receptor family (TNFRSF6B – tumour necrosis factor receptor superfamily 6B) gained genome-wide significance for IBD combined with levels of mRNA expression correlating with the degree of mucosal inflammation within the colon.[332]

The 20q13 signal suggested by the SNPs rs2315008 and rs4809330 lies in a complex telomeric region of LD, the central block of which contains multiple genes, most interestingly *TNFRSF6B*.[332] Other genes in the region are summarised in **Box 4.3**. After gaining genome-wide significance in the first early-onset GWAS it has since been replicated in an independent paediatric study.[439] As mentioned above, differing expression and its correlation with inflammation made it the most promising candidate gene at this locus.

The protein product of *TNFRSF6B*, decoy receptor 3 (Dcr3) has three known ligands, Fas ligand (FasL), LIGHT (also known as tumour necrosis factor [ligand] superfamily, member 14 [TNFSF14]) and TL1A (see above).[440] Interaction with these ligands influences a number of mechanisms, mostly involving T cell expansion and apoptosis. Dcr3 has been shown to reduce FasL-induced[441] and TL1A-induced apoptosis of lymphocytes with the addition of reducing TL1A-induced lymphocyte proliferation[442]. The interaction between Dcr3 and LIGHT is more complex with soluble Dcr3 blocking[443] and solid Dcr3 enhancing[444,445] T cell costimulation. Kugathasan et al. showed that serum Dcr3 was significantly increased in childhood-onset IBD (UC > CD)[332] which had previously been demonstrated in adult CD patients[446].

Box 4.3. Other candidate genes at locus 20q13.

***STMN3* (stathmin-like 3)**

Also known as SCLIP, STMN3 is a member of the stathmin family of cytosolic phosphoproteins which is involved in microtubule dynamics.[447] It is known to colocalise with STMN2 in the Golgi apparatus[448] and is ubiquitously expressed throughout human tissues including the small intestine[449].

***RTEL1* (regulator of telomere elongation helicase 1)**

A helicase which antagonises homologous recombination by promoting the disassembly of recombination intermediates which is required for genome stability and tumour avoidance.[450] It has been further shown to protect telomeres through the shelterin component TRF1[451] and has been implicated in the genetic susceptibility of gastrointestinal tumours[452] and gliomas[453,454].

***ARFRP1* (ADP-ribosylation factor related protein 1)**

A membrane associated GTPase that regulates intracellular protein trafficking[455] and is essential for embryonic development by stabilising the egg cylinder during gastrulation[456]. ARFRP1 is involved in anterograde transport form the *trans*-Golgi network[457] and is colocalised with ARL1 to the Golgi membrane[455]. Of specific interest, in the enterocytes of *ARFRP1* -/- mice there is mistargeted E-cadherin expression to the intracellular as opposed to lateral membranes.[458] [The E-cadherin gene *CDH1* has recently been shown to be linked to the genetic susceptibility of Crohn's disease, in addition to those with the disease-risk haplotype showing increased E-cadherin cytoplasmic accumulation[459]]

***ZGPAT* (zinc finger, CCCH-type with G patch domain)**

The 5'-flanking region of *ARFRP1* contains the first exon of ZGPAT on the opposite strand[460] which is a G patch domain-containing protein and has been shown to be involved in the repression of gene transcription[461].

***LIME1* (Lck interacting transmembrane adaptor 1)**

A transmembrane adaptor protein primarily expressed in haematopoetic cells and the lung.[462] It has been shown to associate with phophatidylinositol 3-kinase thus activating ERK1/2 with overepxression shown to induce the transcriptional activation of the IL-2 promotor.[462] It has been found to be involved in T cell[463] and B cell[464] activation through the TcR and BcR respectively.

***SLC2A4RG* (SLC2A4 regulator)**

A transcriptional regulator also known as *GEP* and *HDBP1* which has been shown to regulate the transcription of the human *GLUT4* promoter[465] and the Huntington disease (*HD*) gene[466].

***ZBTB46* (zinc finger and BTB domain containing 46)**

A CACC *cis*-regulatory zinc finger protein which regulates gastrin gene expression by interfering with the transactivation of the ubiquitous zinc finger protein Sp1.[467]

Of note, elevated serum concentrations of Dcr3 have also been demonstrated in other inflammatory conditions such as rheumatoid arthritis[468] and systemic lupus erythematous[469]. Several studies have now demonstrated that both lamina propria T cells and intestinal epithelial cells in both UC and CD are resistant to apoptosis, with Dcr3 shown to be significantly over-expressed in the ileum of CD patients.[446,470,471] The hypothesis that IBD patients lack effective T cell apoptosis is further strengthened by the likely mechanism of action of newer biological therapies, most notably Infliximab.[472] Although the precise pharmacodynamics of anti-TNF alpha antagonists is not yet clearly understood, induction of

apoptosis is almost certainly involved. Three independent studies have now demonstrated an increase in apoptotic lamina propria T cells and peripheral blood monocytes of CD patients after Infliximab infusion, with the timing of apoptotic-induction ranging from four hours to four weeks.[473-475] It is clear that confirmation of mutations in *TNFRSF6B* conferring susceptibility to IBD would be critical in our understanding of IBD pathogenesis. Not only would it allow a deeper understanding of the mechanistic dysregulation at the protein level, but it would further enhance our understanding of the pharmacodynamics of current biological therapies.

The SNP rs1250550 lies at 10q22 within the gene *ZMIZ1* and was associated with early-onset CD and IBD combined in the recent early-onset GWAS.[18] *ZMIZ1* was initially named *hZIMP10* (human zinc finger-containing, Miz1, PIAS-like protein on chromosome 10) and was identified as a novel androgen receptor (AR)-interacting protein that augmented AR-mediated, ligand-dependent transactivation in prostate cells.[476] This gene has since been shown to act as a transcriptional co-regulator, possibly through chromatin-remodelling,[477] and is known to play a role in vascular development[478] and the co-activation of the p53 tumour suppressor[479]. However, it is the role of *ZMIZ1* in the modulation of transforming growth factor-beta (TGF-β) through the transcriptional activity of Smad3 (mothers against decapentaplegic homolog 3) which is of most interest in the pathogenesis of IBD. Li et al. showed that the altered expression of ZMIZ1 affected Smad3-mediated transcription, in addition to using a Smad4-negative cell line to demonstrate that ZMIZ1 may mediate Smad3 activity by interacting with the Smad3/Smad4 transcriptionally active complex.[480]

Importantly a SNP in the region of *Smad3* at 15q22 was also found to confer susceptibility in the early-onset IBD discovery cohort providing further evidence of dysregulation in this pathway.[18] Following ligation of the heterodimeric TGF-β receptor, phosphorylation of Smad2 and Smad3 occurs leading to a Smad2-Smad3-Smad4 complex which translocates to the nucleus where it participates in the transcriptional control of target genes.[481] Targeted disruption of Smad3 has been shown to result in diminished cell responsiveness to TGF-β with Smad3 mutant mice demonstrating massive intestinal T cell infiltration and impaired mucosal healing.[482-484] Of possible therapeutic interest is the role of Smad7 which interferes with TGF-β signalling by preventing the activation of the Smad2/Smad3 complex.[485] Smad7 has been found to be increased in the lamina propria mononuclear cells (LPMC) of IBD patients with recent work demonstrating that IBD LPMCs were resistant to regulatory T cell suppression which could be reversed using Smad7 antisense treatment.[486] With ZMIZ1 playing a role in Smad3 transcription and the importance of the TGF-β pathway in immune tolerance,[487] the consequence of possible risk alleles in both *ZMIZ1* and *Smad3* need to be further delineated.

4.3.6 Endoplasmic reticulum stress and CAPN10

The endoplasmic reticulum (ER) is contiguous with the nuclear envelope and contains a membranous network of tubules and sacs. Its main functions are the biosynthesis, folding and assembly of soluble proteins and it also operates as a dynamic calcium store. Endoplasmic reticulum stress occurs as a result of dysregulated homeostatic pathways in secretory cells (such as Paneth and goblet cells[488]) leading to the accumulation of misfolded or unfolded proteins.[489] The unfolded protein response (UPR) is a mechanism that allows cells to cope with conditions of ER stress,[490] however during sustained ER stress the common final pathway is apoptosis (programmed cell death). Three proximal effectors of the UPR exist in an inactive state under homeostatic conditions – inositol-requiring 1α (IRE1α), double-stranded RNA-dependent protein kinase-like ER kinase (PERK) and (activating transcription factor 6 (ATF6).[491] In the presence of misfolded proteins the ER chaperone immunoglobulin-heavy-chain-binding protein (BiP) senses these changes and releases the three effectors from inhibition, leading to an increase in cell-coping mechanisms.[492] The genes targeted by the UPR are involved in aminoacid biosynthesis and transport, the oxidative stress response and ER-stress-induced apoptosis.[493] The most conserved of the UPR pathways is IRE1α, activation of which leads to the splicing of the protein XBP1, which in turn leads to a myriad of cell survival processes.

Dysregulation in the cell's ability to control the processing of redundant proteins efficiently was first realised as a possible pathway in IBD pathogenesis following the discovery that *Xbp1*-deficient intestinal epithelial cells led to a spontaneous enteritis.[488] In addition to this, the same paper described deep sequencing of the *Xbp1* locus showing a number of rare alleles, two of which being nonsynonymous variants present only in IBD patients. ER stress is now becoming increasingly recognised as a primary mechanism in intestinal inflammation[488] in addition to a secondary consequence[494-496]. With regard to the generic inflammatory response, two ER-pathway-induced mechanisms have been proposed to lead to NFκB activation. The PERK-EIF2α attenuation of IκB and the recruitment of IκB kinase by the IRE1α-TRAF2 complex both lead to translocation of NFκB to the nucleus where it induces the transcription of numerous inflammatory genes.[491] Furthermore, a SNP in the region of *ORMDL3* on 17q12 was shown to be significant in the adult CD GWAS meta-analysis and replicated in both CD and UC in the early-onset GWAS.[13,18] Although *ORMDL3* was previously known to encode a transmembrane protein in ER,[497] a recent paper has shown its specific role in altering ER-mediated calcium homeostasis and the facilitation of the UPR[498].

The recent paediatric GWAS identified a susceptibility locus at 2q37.3 that yielded genome-wide significance in UC patients from a combined analysis of the discovery and replication

cohorts.[18] The SNP rs4676410 lies within *GPR35* in an LD block containing the genes *CAPN10, GPR35, KIF1A* and *RNPEPL1.* However, following the analysis of normalised colonic expression of each candidate gene, only *CAPN10* was found to be significantly altered (downregulated) in UC patients. The remaining genes at the locus are summarised in **Box 4.4**.

Box 4.4. Other candidate genes at locus 2q37.

GPR35

A G-protein coupled receptor (GPR) that has been shown to be highly expressed in the human stomach, small intestine and colon.[499] It is involved in the tryptophan metabolic pathway and has been shown to agonised by kynurenic acid [500] with dendritic cells expressing indoleamine 2,3-dioxygenase (IDO) shown to be involved in immune tolerance through tryptophan catabolism[501]. *GPR35* has been implicated in various conditions such as pseudopseudohypoparathyroidism[502] and gastric cancer[503] with other members of the GPR family already implicated in intestinal inflammation[504,505]

KIF1A

Encodes one of the kinesin superfamily proteins which is a motor protein involved in the transport of organelles and macromolecules along microtubules.[506]

RNPEPL1

RNPEPL1 is a member of the M1 family of zinc metallopeptidases with ubiquitous tissue distribution;[507] a SNP at the same locus has been implicated in bipolar disorder[508].

The gene *CAPN10* encodes calpain-10, a member of a superfamily of cytoplasmic cysteine proteases with several known isoforms in the form of splice variants.[509,510] Calpain-10 is a 672 aminoacid protein which is expressed ubiquitously[511,512] and hydrolyses substrates important in calcium-regulated signalling pathways[513]. With regard to complex polygenic disease pathophysiology, *CAPN10* was the first gene to be identified to confer susceptibility to type 2 diabetes mellitus by positional cloning.[510] Dysregulation in *CAPN10* has also been implicated in polycystic ovary syndrome,[514] in determining serum cholesterol levels[515] and more recently the risk of colorectal carcinoma[516]. Although the calpains have been implicated in various cellular processes, including cell differentiation and proliferation,[517,518] the most interesting pathway with regard to inflammatory bowel disease is endoplasmic reticulum stress.

Calpain-10 is key in the regulation (via proteolysis) of ORP150 (Grp170)[519] which is another ER chaperone known to be upregulated during the UPR, overexpression of which has been shown to reduce ER stress[520]. ORP150 has been shown to act as a nucleotide exchange factor for BiP, hydrolysing ATP to release energy for conformational changes within the BiP protein.[521] (**Figure 4.3**) Of particular interest with regard to CD pathogenesis

was the finding that cigarette smoke extract induced ER stress (albeit in bronchial epithelium) with this effect ameliorated by the overexpression of ORP150.[522] With previous work on the role of *XBP1* in intestinal inflammation, the replication of *ORMDL3* and the finding of genetic polymorphisms in the *CAPN10* gene in childhood IBD, the argument for a dysregulated ER stress response in IBD has certainly been strengthened.

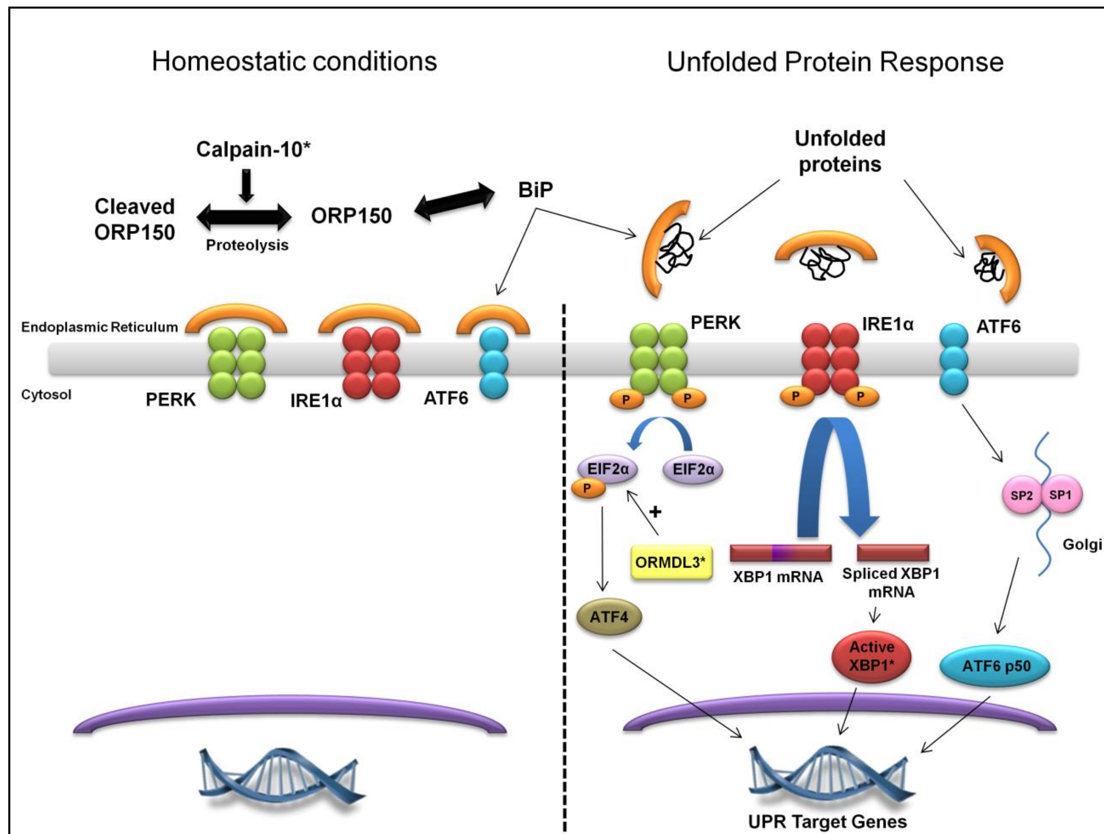


Figure 4.3. The role of calpain-10 in endoplasmic reticulum stress. Calpain-10 is involved in the regulation of ORP150 via proteolysis, with ORP150 acting as a nucleotide exchange factor for BiP. During homeostatic conditions BiP acts to inhibit activation of the three proximal effectors IRE1α, PERK and ATF6 (left panel). During the unfolded protein response BiP preferentially binds to unfolded proteins within the ER lumen releasing the proximal effectors from their inhibition (right panel). Homodimerisation and *trans*-phosphorylation of PERK leads to cytosolic phosphorylation of EIF2α leading to the translation of ATF4. Autophosphorylation of IRE1α directs the splicing of XBP1 mRNA leading to an activated form of XBP1. Once released from BiP, ATF6 translocates to the Golgi where it is cleaved by SP1 and SP2 producing an active fragment (ATF6 p50). These three active components (ATF4, active XBP1 and ATF6 p50) migrate to the nucleus where they activate the transcription of the UPR target genes. ORMDL3 has also been shown to positively regulate the phosphorylation of EIF2α. (Molecules implicated in intestinal inflammation/IBD are indicated by a *)

4.4 The future

Although there are clear differences in the phenotypic characteristics of early-onset and adult-onset,[141] the influence of genetic factors on these variations is likely to be small. With clear similarities in the genetic risk of both phenotypes, and parallel dysregulated pathways

implicated, the aim of future research should undoubtedly focus on the confirmation of the shared associated mutations. The functional implications of these mutations will then not only lead to a greater understanding of IBD pathogenesis, but also allow the generation of newer therapeutic modalities and genotype-tailored treatment plans.

5. The role of ICOSLG in inflammatory bowel disease - genetic susceptibility and mucosal mRNA expression

5.1 Introduction

As discussed in **Chapter 4**, recent progress in determining the genes (or more often the loci) involved in the susceptibility to IBD has been especially notable. Meta-analyses of genome-wide association studies have now significantly increased the number of novel risk variants to over one hundred,[523,524] with the International IBD genetics consortium Immunochip project having already increased this number further in recent months[525,526]. One of these loci, identified in both adult and paediatric populations, is situated on chromosome 21q22 in the region of the gene encoding inducible T cell co-stimulator ligand (ICOSLG),[13,18] a protein expressed on antigen presenting cells and intimately involved in T cell differentiation.

The dysregulation of mucosal[527] and peripheral[528] T cells is likely to play a key role in the pathogenesis of IBD, resulting in secretion of pro-inflammatory mediators, accumulation of activated inflammatory cells and tissue damage[529]. However, the stimuli responsible for the initiation and maintenance of signal-mediated lymphocyte activation in IBD remain somewhat elusive. The 'two-signal' model of lymphocyte activation was first proposed by Bretscher and Cohn in 1970[530] and has since been widely explored providing a clearer understanding of the initial stages of T cell differentiation[531,532]. The two-signal model has now been refined and superseded by a three-signal process with antigen-specific activation of T cell receptors (TcR) (signal one), interaction of co-stimulatory molecules on antigen-presenting cells (APC) with ligands on T cells (signal two) and cytokine interaction (signal three).[33] It has also been shown that in the absence of signal two (i.e. co-stimulation), lymphocytes fail to respond effectively and are rendered anergic.[533] Co-stimulation is therefore of pathogenic and therapeutic interest as the manipulation of co-stimulatory signals may provide a means to either enhance or terminate immune responses.[534,535]

Much research has been carried out over the last 25 years to identify the molecules responsible for this co-stimulatory signal. To date, cluster of differentiation (CD) 28 remains the best-characterised co-stimulatory molecule and is constitutively expressed on the surface of T cells.[33] The natural ligands for CD28 have been identified as B7 molecules and are present or inducible on the surface of antigen-presenting cells.[536-538] CD28-B7 interaction results in an enhanced T cell proliferation, cytokine production, and resistance to apoptosis. Recent investigation has further delineated a role of CD28-B27 interactions in

promoting peripheral T cell tolerance.[539,540] Previous studies have also demonstrated that CD28-B7 interactions play an important role in several immune-mediated diseases, with over-expression of B7 molecules found in affected clinical material from patients with multiple sclerosis, rheumatoid arthritis and psoriasis.[541] There have also been reports of increased expression of B7 molecules in the mucosa of IBD patients.[542,543]

In 1999 Hutloff et al. described another member of this family of co-stimulator molecules, namely inducible T-cell co-stimulator (ICOS).[544] This disulphide-linked dimer with around 40% similarity to CD28 at the protein level is upregulated on T cells after activation.[545,546] The ligand for ICOS (known as ICOSLG, or CD275, ICOS-L, B7RP-1 and B7h) was described later the same year by two independent groups[545,547] and splice variants later replicated elsewhere (B7H2,[548] GL50[549]). ICOS expression is stimulated on T cells by both TcR and CD28 signals. Importantly however, ICOS expression is not *solely* dependent on CD28 signals, as studies blocking ICOS in CD28-deficient mice further inhibited Th1/Th2 differentiation.[550] Although ICOS engagement was shown initially to selectively produce high levels of IL-10 and IL-4, *in vivo* studies in several different models have demonstrated that ICOS can stimulate production of both Th1 and Th2 cytokines during initial priming and during effector T cell responses.[544,551]

5.2 ICOS and ICOSLG expression

ICOS is present on the T cell surface with ICOSLG present on many antigen presenting cells and lymphoid tissue. ICOSLG has also been found on endothelial cells and the coronary microvasculature,[552] as well as endothelial cells,[553] testes[554] and most recently thyroid follicular cells[555]. With regards to epithelial expression De Haij et al. found that ICOSLG was constitutively expressed on both proximal renal tubular epithelial cells and renal epithelial cell lines,[556] with several groups showing expression on lung epithelial cells[557-559]. With regard to intestinal epithelial cell expression there is currently a paucity of data. Nakazama et al. looked in detail at ICOS and ICOSLG expression on a intestinal cancer cell line (HT29) as well as a small number of surgical resection specimens from IBD patients (*n*=10) and controls (*n*=6).[78] This group demonstrated that ICOSLG mRNA and protein was constitutively expressed on epithelial cell lines and colonic epithelial cells from clinical samples. They also demonstrated that ICOSLG expression was moderately increased in those with IBD, more-so for CD.

5.3 Overview of other genes at the 21q locus

The single nucleotide polymorphism (SNP) identified as varying significantly between IBD cases and controls through genome-wide association studies (GWAS) (rs762421) is situated on 21q22.3 approximately 25kb upstream from *ICOSLG*. Also within this region are genes encoding DNMT3L, AIRE, PWP2H, C21orf33 and PFKL; all genes with known protein products. **Figure 5.1** illustrates the locus region with the position of SNP rs762421 highlighted. Although this SNP lies in an intergenic region in close linkage disequilibrium (LD) with *ICOSLG*, other genes in the region have the potential to be influenced by germline variations at this loci.

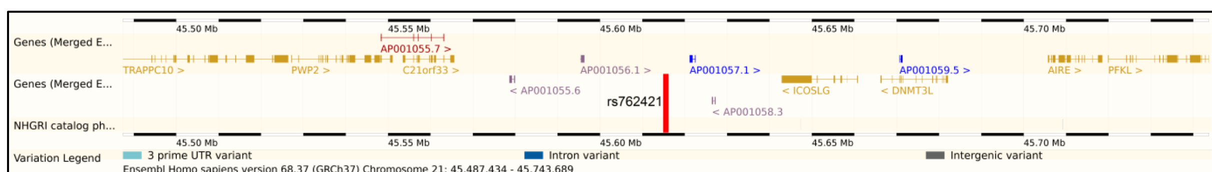


Figure 5.1. Graphical representation of 21q locus region. Image taken from Ensembl genome browser 68.37 (GRCh37) (http://www.ensembl.org)

5.3.1 *DNMT3L*

The gene encoding DNA (cytosine-5-)-methyltransferase 3-like gene (*DNMT3L*) lies closest to *ICOSLG* on chromosome 21. This DNA methyltransferase does not contain intrinsic DNA methyltransferase activity but instead regulates other methyltransferases (such as DNMT3a) through epigenetic mechanisms.[560] Currently, DNMT3L has no defined function in IBD pathogenesis, however it is interesting to note that a recent genome-wide scan demonstrated a CD susceptibility loci in the region of *DNMT3A*.[523] Considerable evidence now exists that epigenetic alterations are of importance in regulating the integrity and development of both innate and acquired arms of the immune system, including Th1/2 cell differentiation,[561-563] and have stimulated a real interest in defining the contribution of these alterations in immune-mediated diseases. The importance of epigenetic changes, such as DNA methylation, histone acetylation, and microRNA synthesis in the pathogenesis of CD and UC is likely to emerge as a key area of interest over the next decade.[564-567] Recent data demonstrated that epigenetic alterations may contribute to concordance or discordance of disease phenotype in twin pairs,[568,569] thereby potentially accounting for a proportion of the apparently hidden heritability. Intriguingly there is now data implicating *NOD2* as a regulator of epigenetic alterations, including microRNA synthesis.[570] Studies in other diseases,

notably systemic lupus erythematosus,[571] and asthma[572] have provided a further focus for the studies of epigenetics in immune-mediated disorders. Specifically in relation to DNMT3a, several papers have also alluded to its involvement in idiopathic thrombocytopenic purpura,[573] hepatitis B,[574] epithelial tumours[575] and endometriosis[576]. Mutations in DNMT3L have also been found to affect DNA methylation during egg development.[577]

5.3.2 Human *PWP2* and *AIRE*

The human *PWP2* gene is a homologue of the yeast periodic tryptophan protein 2 (PWP2) gene.[578] Mapping and linkage analysis near this gene led to the possible role of this region in autoimmune polyglandular disease type 1.[579,580] This autosomal recessive disease results in failure of the parathyroid glands, adrenal cortex and pancreatic β-cells along with alopecia and mucocutaneous candidiasis. However, in 1997 two groups using positional cloning identified the underlying gene as *AIRE* (autoimmune regulator),[581] a transcriptional regulatory factor. More recent evidence has suggested a role for AIRE in T cell tolerance[582] and as a result there has been some interest in its possible role in IBD. Torok et al. looked at the correlation between mutations in two exons of *AIRE* and serum autoantibodies in IBD.[583] Evaluation of 234 IBD patients did not detect any of the three polymorphisms and therefore could not subsequently comment on the relationship with autoantibodies. A more recent study proposed that AIRE represents an early molecular switch imposing a suppressive mesenchymal stem cell phenotype in a model of chronic colitis.[584]

5.3.3 *c21orf33* (*HES-1*/*ES-1*)

The chromosome 21 open reading frame 33 (*c21orf33*) gene has two aliases *HES-1* and *ES-1*. *HES-1* (Hairy and Enhancer-of-split 1) has been implicated in endocrine disease and is also involved in Notch signalling, important in endoderm development and subsequent neurogenesis.[585] Murine studies have demonstrated that loss of *HES-1* leads to neural tube defects due to premature neurogenesis.[586] Under the alias of *ES-1* (esterase 1), *c21orf33* may be involved in the basic function of mitochondria with elevated levels in the foetal brains of children with Trisomy 21.[587] Again there is a paucity of evidence for the involvement of *HES-1* in inflammatory bowel disease, although a possible role has been established in psoriasis,[588] which shares several susceptibility loci with CD[589].

5.3.4 *PFKL*

PFKL (phosphofructokinase, liver) lies downstream of *AIRE* and no clear role in disease pathogenesis has yet been determined. Studies have suggested a role in the glycolytic

pathway in animal models[590] with some evidence that *PFKL* is a susceptibility locus for bipolar disorder[591,592].

5.4 Hypotheses and aims

Hypotheses:

1. The paediatric IBD susceptibility gene identified through genome-wide scanning at locus 21q22.3 is *ICOSLG*.[18]
2. Expression of *ICOS* and *ICOSLG* mRNA on primary biopsy material from patients with IBD varies significantly from non-IBD patients.

Aims: To perform a detailed family-based analysis of *ICOSLG* using a gene-wide haplotype-tagging approach to determine if variations in this gene are responsible for increased susceptibility to IBD in the paediatric population, and if so, to delineate the region of strongest association to guide future deep sequencing studies. Additionally to evaluate differences in co-stimulatory molecule mRNA expression on mucosal biopsies using previously acquired data from an adult cohort of IBD and non-IBD patients.

5.5 Methods

5.5.1 Subjects

A total of 1331 individuals were initially genotyped, however 56 were immediately removed from the analysis as they were either not part of a case-parent trio (e.g. grandparents) or had been re-phenotyped and deemed not to be definite PIBD. Further individuals were also removed as they: (1) failed genotyping completely as a result of poor DNA quality (n=55); (2) demonstrated Mendelian error during genotyping (n=9); or (3) were diagnosed after their 17th birthday (n=5). Therefore a total of 1206 individuals were included in the final analysis, consisting of 448 PIBD patients (i.e. diagnosed before their 17th birthday) and their parents (n=758). Parents not part of a complete case-parent trio were used to inform the analyses within the liability-threshold model as described below; genotyping of parents provided 230 complete parent-child trios. All subjects were recruited in Scotland between October 2002 and July 2009 as part of research carried out by Dr Richard Russell (NHS Great Glasgow, formerly University of Edinburgh), Dr Johan Van Limbergen (University of Edinburgh), and latterly through the Medical Research Council (MRC)-funded Paediatric Inflammatory Bowel Disease Cohort and Treatment study (PICTS) held by Prof. David C Wilson (University of Edinburgh), Dr Richard Russell (Royal Hospital for Sick Children, Glasgow) and Prof. Jack

Satsangi (University of Edinburgh) (**Figure 5.1**). IBD was diagnosed according to standard clinical, histological and radiological findings[147,236] and rigorous phenotyping carried out as previously described;[141] the basic demographics of the PIBD group are shown in **Table 5.1**. It should be noted that where parents had a confirmed diagnosis of IBD (22 Crohn's disease [CD], 14 ulcerative colitis [UC], 2 colonic IBD, type unclassified [IBDU]), disease coding was altered appropriately during each analysis.

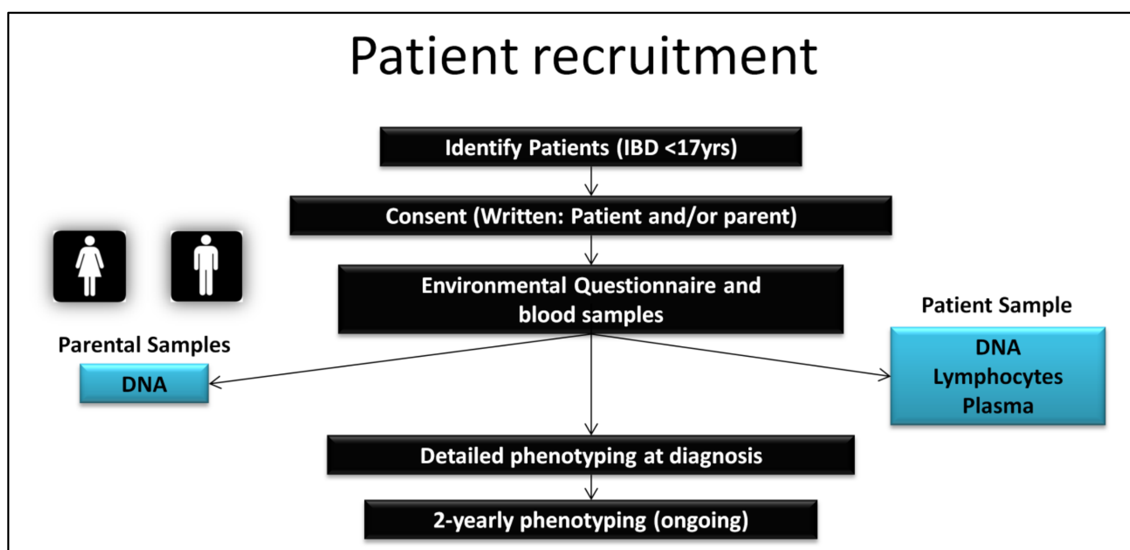


Figure 5.1. Flow diagram showing process of recruitment of Scottish paediatric inflammatory bowel disease patients into genetic studies since 2002.

Table 5.1. Characteristics of paediatric inflammatory bowel disease patients analysed in the family-based association study to determine the role of ICOSLG as a susceptibility gene.

Patient Characteristic	IBD	CD	UC	IBDU
Number (%)	448	301 (67)	107 (24)	40 (9)
Age at diagnosis (years [IQR])	11.5 [8.9-13.3]	11.8 [9.3-13.4]	11.0 [8.4-12.9]	10.6[8.6-12.2]
Male sex (%)	256 (57)	179 (59)	55 (51)	22 (55)
White European (%)	430 (96)	290 (96)	102 (95)	38 (95)

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic IBD, type unclassified; IQR, interquartile range.

5.5.2 Single nucleotide polymorphism selection

The *ICOSLG* gene is located on chromosome 21 between positions 45,642,874 and 45,660,849 (Ensembl Release 68 - ENSG00000160223; Build 37). *ICOSLG* tagging SNPs

were selected using genotypic data from the CEU study (30 trios with Northern and Western European ancestry, collected by the Centre d'Etude du Polymorphisme Humain [CEPH]), available from the HapMap project.[329] SNPs were identified using Haploview software version 4.2 (http://www.broad.mit.edu/mpg/haploview)[593] based on solid spine of linkage disequilibrium (LD) (r^2 >0.8, haplotype frequency >5%, minor allele frequency >10%). This process identified four tagging SNPs spanning a 19kb region containing two haplotype blocks: rs8126734, rs2838529, rs4818890 and rs2070558 (**Figure 5.2**). The original SNP (rs762421) identified through genome-wide scanning was also genotyped. Details of all genotyped SNPs are presented in **Table 5.2**. For all cases and their parents SNPs conformed to the Hardy–Weinberg equilibrium (p>0.001).

Table 5.2. Details of the five single nucleotide polymorphisms used to tag ICOSLG

dbSNP Name*	Chromosome position†	Location	MAF	Alleles	Hardy-Weinberg p value	Call rate	Detectable odds ratio‡
rs762421	45615561	Intergenic	0.400	A:G	0.0986	93.9	1.45
rs8126734	45636432	Intergenic	0.341	A:G	0.0208	96.3	1.46
rs2838529	45638152	5'UTR	0.194	G:C	0.4947	95.4	1.56
rs4818890	45646256	Exon 1	0.464	C:A	0.0015	94.2	1.45
rs2070558	45655658	Intronic	0.284	G:A	0.0821	93.5	1.49

*Based on NCBI Human Build 135; †Ensembl Release 68 - July 2012 ; ‡based on individual minimum allele frequency (MAF) with an 80% power to detect an effect. UTR, untranslated region.

5.5.3 DNA extraction and genotyping

DNA was extracted from venous whole blood from patients and parents by either a modified salting-out technique[594] or by using the Nucleon BACC2 kit (Gen-Probe Life Sciences, Manchester, UK) and resuspended in 1× Tris-EDTA buffer (10mM Tris, pH 7.5, and 1mM EDTA) at a final concentration of 100ng/µL. Genotyping was carried out on the Illumina platform by TaqMAN® analysis (detected on an ABI PRISM 7900HT instrument with genotype calling utilising SDS v2.1 software) at the Wellcome Trust Clinical Research facility on the Western General Hospital campus, Edinburgh. DNA extraction was performed by Dr Richard K Russell (Royal Hospital for Sick Children, Glasgow and formerly of University of Edinburgh), Dr Johan Van Limbergen (Department of Child Life and Health, University of

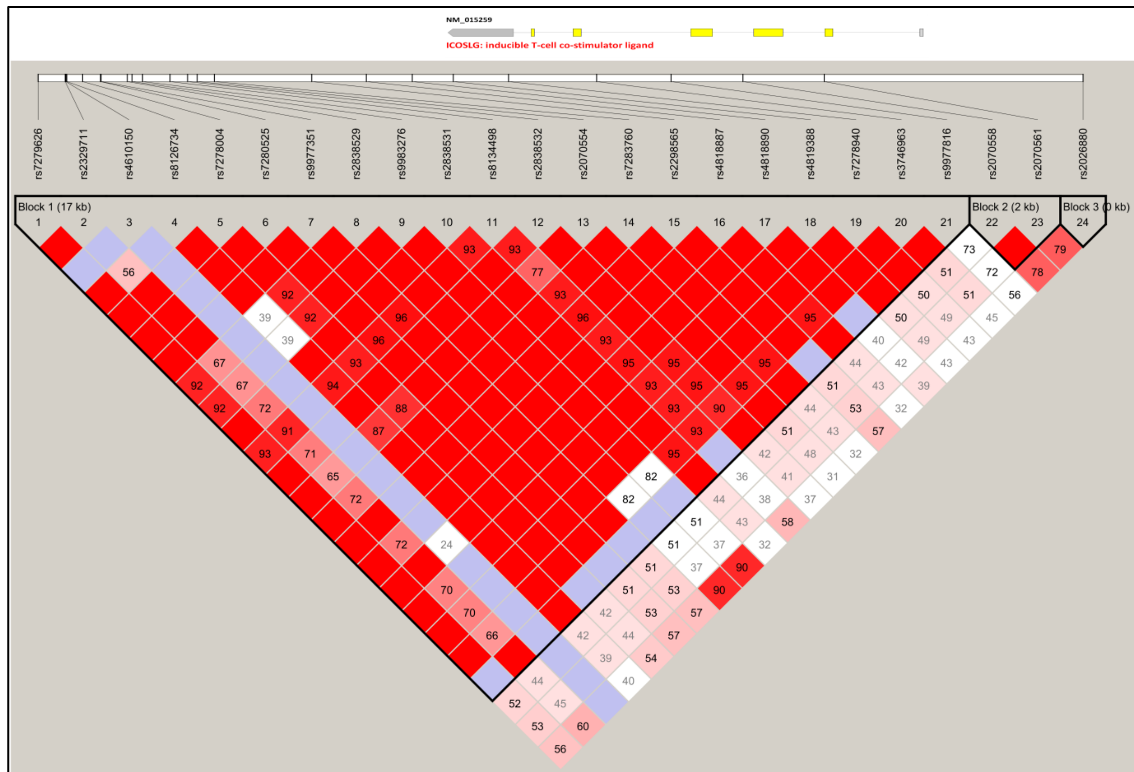


Figure 5.2. View of the genomic region containing ICOSLG using Haploview 4.2 software generated from the CEU study available through the HapMap project. *ICOSLG* can be seen to be positioned across two haplotype blocks spanning approximately 19kb. Haplotype blocks were generated using solid spine of LD. Tagging SNPs are identified in the schematic as SNPs 4 (rs8126724), 8 (rs2838529), 17 (rs4818890) and 22 (rs2070558).

Edinburgh) and Sean O'Neil (Medical Research Council [MRC] Human Genetics Unit, University of Edinburgh) as part of previous PhD projects and latterly in conjunction with PICTS. All genotyping was carried out in June 2009; primer sequences are shown in **Table 5.3.** Mendelian errors were identified using Haploview software for the current dataset in addition to errors identified during a previous genetic association study performed on this patient group.[242]

Table 5.3. Primer sequences for the five single nucleotide polymorphisms used to tag the ICOSLG gene on chromosome 21q.

dbSNP*	Position	Strand	Sequence
rs762421	45615561	Rev	ACATTCACACACCCACAAAAATCAA**[A/G]**AGAGCAGATTTTCCAAAAATAGGTG
rs8126734	45636432	Rev	TTAGGATCCTGATTTTAATGTCAGG**[A/G]**CTGGCCAGCTGTGCCTCAACTCTGA
rs2838529	45638152	Rev	ACTGGGGAGAAAGGAGGAAAGAAAG**[C/G]**CCGTGTTAAAACACAGTTCGAAGCT
rs4818890	45646256	For	GGACCGCCCCATCTGCCAGCCTTGA**[A/C]**TGGAGCCTCCCTGGTGGATGTTTAC
rs2070558	45655658	For	AAGCCAGTCCCAGTAGCCAGGGACC**[A/G]**CTGAGCCTGTCGGGCAGTGACTGGC

*Based on NCBI Human Build 135. Rev, reverse; For, forward .

5.5.4 Transmission disequilibrium test analysis

Transmission disequilibrium test (TDT) analyses were carried out using Haploview version 4.2[593] by utilising the parenTDT function[595]. Briefly, this liability-threshold-model approach can incorporate parental phenotypes (whether part of a complete trio or not) and is based on the between-within-sibship association model presented by Fulker et al.[596] For the TDT analysis all p values are reported as corrected following permutation analysis (*n*=100,000 permutations), unless otherwise stated. Calculation of the 95% confidence intervals for odds ratios (OR) was achieved using the formula:

$$e^{lnOR} \pm 1.96 \times \sqrt{(}\left(\frac{1}{transmitted}\right) + \left(\frac{1}{untransmitted}\right))$$

In all analyses a two-sided p value of less than 0.05 was considered significant.

5.5.5 Transmission disequilibrium test power calculation

Using QUANTO (version 1.2.4; http://hydra.usc.edu/gxe),[597] and based on the most recent prevalence data of Scottish PIBD presented in **Chapter 3** (41.2/100,000; keeping in mind this is data for those less than 16 years old not 17 years old) the IBD TDT analysis had 80% power to detect an effect with an OR between 1.45 and 1.56 depending on the individual SNP (ORs for each SNP are shown in **Table 5.2**).

5.5.6 Analysis of gene-wide susceptibility signal for *ICOSLG* using an international genome-wide association meta-analysis

As a member of the United Kingdom Inflammatory Bowel Disease (IBD) Genetics Consortium and the larger International IBD Genetics consortium (IIBDGC) it was possible to gain access to novel genetic data generated from large case-control studies. Although a meta-analysis of CD genome-wide association scans (GWAS) has previously been published,[598] a new improved meta-analysis of GWAS data imputed with the 1000 genomes reference set (first available in September 2011) was interrogated (http://www.1000genomes.org). To assess variants in the region of *ICOSLG* for association with CD susceptibility, SNP-specific p values generated from the analyses of seven individual (majority adult) CD datasets encompassing 5,956 CD patients and 14,927 healthy controls were obtained from data collated by Dr Stephan Ripke and held jointly by the Analytical and Translational Genetics Unit at Massachusett's General Hospital and the Broad Institute (http://www.broadinstitute.org/mpg/ricopili). p values obtained were generated with principal component analyses as covariates, therefore correcting the whole analysis for population stratification and inter-study differences. The scatterplot of results was generated

using the ggplot2 package[599] in R version 2.14.1 (R Foundation for Statistical Computing, Vienna, Austria).

5.5.7 mRNA expression microarray data

In order to identify differences in gene expression of *ICOSLG* and *ICOS* between IBD patients and controls, raw data (in the form of a large Microsoft Excel 2003 spreadsheet) was obtained from Dr Colin Noble (NHS Lothian, formerly University of Edinburgh). These raw data in their entirety had been acquired, analysed and published by Dr Noble (lead author, in conjuction with the Gastrointestinal Unit, University of Edinburgh) as two separate case-control studies (one examining CD[600] and the other UC[601]), and had formed part of his completed PhD thesis. Precise details of how this data was obtained is outlined in each manuscript, however a brief summary of the methods used are given here.

Using the Agilent microarray platform (Agilent Technologies, Santa Clara, CA), expression of mRNA from pinch biopsies taken from IBD patients or controls in South East Scotland was assessed. Biopsies were taken from the terminal ileum, ascending colon, descending colon or sigmoid colon of adult patients with CD (99 biopsies from 53 patients), UC (129 biopsies from 67 patients) or controls [i.e. non-IBD patients] (73 biopsies from 31 patients). Of the controls, 23 had histologically normal biopsies, while only eight had inflamed colonic biopsies. Biopsies were defined as inflamed or uninflamed from paired samples graded by experienced gastrointestinal pathologists at the Department of Pathology, Western General Hospital, Edinburgh; details of the IBD patients and controls are given in **Table 5.4** and **Table 5.5**. Biopsies were analysed and data presented as the $\log_2$ mRNA expression relative to a control reference marker (Stratagene universal human reference [Agilent Technologies, Santa Clara, CA]). The three Agilent probes evaluated were A_23_P371215 (ICOS), A_23_166280 (ICOSLG) and A_23_317667 (ICOSLG).

Table 5.4. Characteristics of adult inflammatory bowel disease patients used in the microarray analysis of ICOS and ICOSLG mRNA expression

Characteristic	Crohn's disease	Ulcerative colitis
Number of subjects	53	67
Male sex (%)	26 (49)	33 (49)
Median age at diagnosis (years)	28.6	37.0
Current smoker (%)	11 (21)	6 (9)
Family history of IBD (%)	12 (23)	5 (7)
5-ASA therapy (%)	21 (40)	40 (60)
Corticosteroid therapy (%)	4 (8)	10 (15)
Immunosuppressant therapy (%)	13 (25)	11 (16)

IBD, inflammatory bowel disease; 5-ASA, 5-aminosalicylic acid.

Table 5.5. Characteristics of adult non-inflammatory bowel disease controls used in the microarray analysis of ICOS and ICOSLG mRNA expression

Characteristic	
Number of subjects	31
Male sex (%)	11 (35)
Median age at endoscopy (years)	43.0
Normal biopsy and indications (*n*)	Irritable bowel syndrome (9), colon cancer screening (6), other (7)
Abnormal, inflamed biopsy and histological findings (*n*)	Microscopic colitis (2), scattered lymphoid aggregate (2), pseudomembranous colitis (1), diverticulitis (1), amoebiasis (1), eosinophilic infiltrate (1)

From these data a non-parametric *t* test (Mann-Whitney *U*) was performed to determine statistically significant differences in expression within the entire biopsied segment of specific areas of the bowel. Additionally, a non-parametric one-way ANOVA (Kruskal-Wallis test) was used to determine the differences in expression throughout the intestine in cases versus controls. Statistical analyses were performed in GraphPad Prism v4.03 (GraphPad Software, San Diego, USA; www.graphpad.com), with a two-tailed p value of <0.05 considered to be statistically significant. Advice on the statistical analyses used was obtained directly from Dr Alex Abbas (Department of Bioinformatics, Genentech, South San Francisco, USA), the bioinformatician on both of Dr Noble's original papers. An outline of the processes involved in the microarray analysis (carried out by Dr Colin Noble) are shown in **Figure 5.3**.

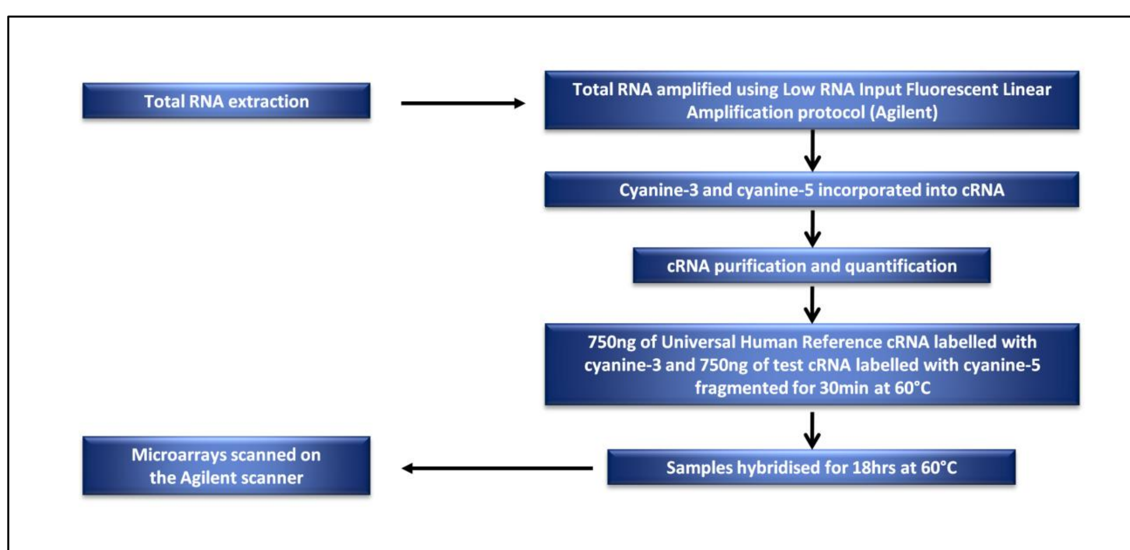


Figure 5.3. Outline of processes involved in microarray analysis following total RNA extraction from gastrointestinal biopsy material. (Performed by Dr Colin Noble)

5.5.8 Ethics

For the case-parent TDT analyses all patients and/or their parents provided full written consent as part of the MRC-funded PICTS cohort which was approved by local ethics committees at all three participating Scottish clinical networks: South-East Scotland (LREC/2002/6/18), West of Scotland (YREC/P12/03) and North of Scotland (GREC/03/0273). For the adult microarray analyses written informed consent was previously obtained by Dr Colin Noble from all subjects with the Lothian Local Research Ethics Committee approving the study protocol (REC 04/S1103/22).

5.6 Results

5.6.1 TDT analyses - the single SNP rs8126734-A is overtransmitted in IBD and CD patients

Analysing the entire cohort of PIBD patients and their parents demonstrated significant overtransmission of the rs8126734-A SNP following correction (p=0.0467; OR 1.48 [95% CI 1.12-1.94]) (**Table 5.6**) This result was strengthened in the CD TDT analysis with the same SNP being similarly overtransmitted (p=0.0084; OR 1.85 [95%CI 1.30-2.64]) (**Table 5.6**). No single SNP was significantly overtransmitted in the UC TDT analysis (**Table 5.6**).

5.6.2 The AGGC haplotype is significantly undertransmitted in CD patients

Focussing the remaining analyses on CD case-parent trios only and evaluating the four-marker haplotype spanning from the intergenic SNP rs762421 to rs4818890 (the final tagging SNP in the first *ICOSLG* haplotype block) demonstrated that only one haplotype was significantly undertransmitted after correction (**Table 5.7**). This haplotype was in keeping with the single SNP results and the previous GWAS (CD-associated risk allele rs762421G) in that the haplotype contained rs762421-A and rs8126734-G and was protective for CD (p=0.0069, OR 0.55 [95% CI 0.39-0.79]). The converse SNP combination (rs762421-G - rs8126734-A) was nominally significant (p=0.0407 prior to correction, OR 1.43 [95% CI 1.04-1.97]).

Table 5.6. Transmission Disequilibrium Test Analysis of individual single nucleotide polymorphisms tagging ICOSLG for paediatric IBD, Crohn's disease and ulcerative colitis.

dbSNP*	Overtransmitted Allele	Trans : Untrans	Uncorrected p Value	Corrected p Value	Odds ratio (95% CI)
		Inflammatory bowel disease			
rs762421	G	132:106	0.0837	0.3222	1.25 (0.96-1.61)
rs8126734	A	127:86	0.0100	**0.0467**	1.48 (1.12-1.94)
rs2838529	C	75:71	0.8736	1.0000	1.06 (0.76-1.46)
rs4818890	A	141:102	0.0220	0.0998	1.38 (1.07-1.78)
rs2070558	G	102:90	0.4378	0.9142	1.13 (0.85-1.50)
		Crohn's disease			
rs762421	G	92:68	0.0673	0.2582	1.35 (0.99-1.85)
rs8126734	A	87:47	0.0023	**0.0084**	1.85 (1.30-2.64)
rs2838529	N/A	50:50	0.9223	1.0000	1.00 (0.68-1.48)
rs4818890	A	97:62	0.0141	0.0543	1.56 (1.14-2.15)
rs2070558	G	68:61	0.5439	0.9675	1.11 (0.79-1.58)
		Ulcerative colitis			
rs762421	G	33:30	0.4669	0.9464	1.10 (0.67-1.80)
rs8126734	G	33:27	0.4386	0.9029	1.22 (0.73-2.03)
rs2838529	G	19:17	0.5271	0.9707	1.12 (0.58-2.15)
rs4818890	A	34:32	0.6326	0.9921	1.06 (0.66-1.72)
rs2070558	G	23:21	0.8815	1.0000	1.10 (0.61-1.98)

*Based on NCBI Human Build 135. Corrected p values were generated by permutation analysis (*n*=100,000) as described in Methods. Trans, transmitted; Untrans, untransmitted; CI, confidence interval; N/A, not applicable. Significant p values are highlighted in bold text.

Table 5.7. Crohn's disease TDT haplotype analysis of the four-marker haplotype spanning from rs762421 to rs4818890 in the region of ICOSLG.

Markers*	Haplotype	Freq	Trans : untrans	Odds ratio (95% CI)	Uncorrected p value	Corrected p value
1,2,3,4	GAGA	0.341	90.8:63.5	1.43 (1.04-1.97)	0.0407	0.1686
	AGGC	0.276	48.0:86.9	0.55 (0.39-0.79)	0.0027	**0.0069**
	AACC	0.153	39.4:36.6	1.08 (0.69-1.69)	0.9202	1.0000
	AAGA	0.117	34.8:27.1	1.28 (0.78-2.12)	0.4778	0.9910
	AGCC	0.038	12.0:11.6	1.03 (0.46-2.32)	0.9732	1.0000
	GGGC	0.026	5.0:8.3	0.60 (0.20-1.83)	0.3724	0.9746
	AAGC	0.021	7.1:2.9	2.45 (0.62-9.60)	0.1230	0.5214
	GAGC	0.017	8.4:5.3	1.58 (0.53-4.70)	0.4044	0.9821

*Marker numbers correspond with the SNP order shown in Table 5.2. Corrected p values were generated by permutation analysis (*n*=100,000) as described in Methods. Trans, transmitted; Untrans, untransmitted; Freq, frequency; CI, confidence interval. Significant p values are highlighted in bold text.

5.6.3 Sliding 2-marker haplotype analysis suggests a strong CD-risk signal at the 3-prime untranslated region of *ICOSLG*

Using a sliding 2-marker haplotype analysis to further define the extent of the CD-association signal from the intergenic rs762421 SNP, through the 3' untranslated region (UTR) to the 5' end of the *ICOSLG* coding sequence (rs2070558-G/C is located in intron 3), it can be seen that although a signal is present across the entire gene, the strongest signal is evident across the two marker haplotype rs762421-A / rs8126734-G (p=0.0072, OR 0.57 [95% CI 0.40-0.80]) (**Table 5.8**), suggesting that the 3'UTR may be targeted for deep sequencing.

Table 5.8. Sliding 2-marker haplotype analysis spanning from rs762421 to rs2070558 in the region of ICOSLG for paediatric Crohn's disease case-parent trios.

Markers *	Haplotype	Freq	T : U	Uncorrected p value	Corrected p value	Odds ratio (95% CI)
1,2	GA	0.368	94.6:65.6	0.0273	0.0657	1.44 (1.05-1.98)
	AG	0.317	50:88.3	0.0036	**0.0072**	0.57 (0.40-0.80)
	AA	0.288	69.5:55.2	0.3009	0.6323	1.26 (0.88-1.79)
	GG	0.028	4.6:9.5	0.1892	0.4423	0.48 (0.16-1.47)
2,3	AG	0.490	101.8:61.9	0.0048	**0.0114**	1.64 (1.20-2.26)
	GG	0.308	51.4:91.1	0.0034	**0.0080**	0.56 (0.40-0.79)
	AC	0.167	41.6:40.8	0.9397	0.9998	1.02 (0.66-1.57)
	GC	0.036	10.5:11.6	0.6990	0.9708	0.91 (0.39-2.09)
3,4	GA	0.457	100.8:65.1	0.0139	**0.0359**	1.55 (1.13-2.11)
	GC	0.340	54.1:89.8	0.0101	**0.0294**	0.60 (0.43-0.84)
	CC	0.202	50:50	0.9223	1.0000	1.00 (0.68-1.48)
4,5	AG	0.410	99.8:65.8	0.0150	**0.0363**	1.52 (1.11-2.07)
	CG	0.302	49.8:76.5	0.0345	0.0883	0.65 (0.46-0.93)
	CA	0.242	51.3:62.7	0.3429	0.7032	0.82 (0.57-1.18)
	AA	0.046	16.8:12.8	0.5903	0.9269	1.31 (0.63-2.72)

*Marker numbers correspond with the SNP order shown in Table 5.2. Corrected p values were generated by permutation analysis (*n*=100,000) as described in Methods. T, transmitted; U, untransmitted; Freq, frequency; CI, confidence interval. Significant p values are highlighted in bold text.

5.6.4 Genome-wide imputed meta-analysis data confirms the strong signal at the 3'UTR of *ICOSLG* for CD susceptibility

A total of 1495 SNPs in the 21q22 locus were tested from the imputed international GWAS data and the SNP-specific p values obtained. A total of 30 SNPs attained genome-wide significance ($p<5x10^{-8}$), all of which lay in the intergenic region at the 3'UTR end of the *ICOSLG* gene. A plot of the SNPs in the region is presented in **Figure 5.4**.

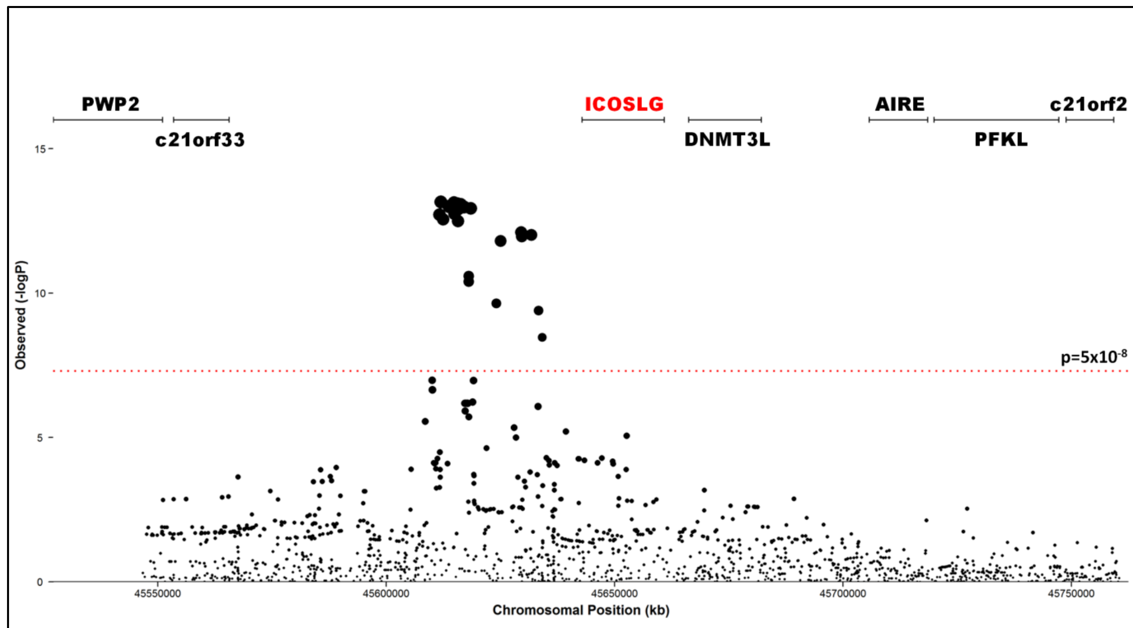


Figure 5.4. Results of a meta-analysis of seven CD genome-wide association studies imputed with the 1000 genomes reference set. Scatterplot showing -logP values in the region of *ICOSLG* for 1495 single nucleotide polymorphisms.

5.6.5 mRNA expression of *ICOS* and *ICOSLG* differs significantly across different intestinal sites

Evaluating the results for all biopsies taken from CD patients showed no difference in expression of *ICOS* or *ICOSLG* (with either probe) compared to non-IBD biopsies (**Table 5.9**). However, to delineate whether or not differences in either molecule were evident at 'baseline', uninflamed biopsies were compared across all sites and at each intestinal location. This showed significant downregulation of *ICOS* expression across all sites and the sigmoid colon, in addition to downregulation of *ICOSLG* expression in the ascending colon and upregulation in the descending colon. Similar analyses were performed comparing UC patients with non-IBD patients demonstrating differences in *ICOS* expression in all biopsy sites only (**Table 5.10**).

Table 5.9. Differences in ICOS and ICOSLG mRNA expression (expressed as p values) in biopsy material from Crohn's disease patients and non-inflammatory bowel disease patients.

Probe	All biopsies	Uninflamed Biopsies				
		All sites	Terminal Ileum	Ascending colon	Descending colon	Sigmoid Colon
ICOS	0.4206	**0.0123**	0.5338	0.0648	0.7595	**0.0251**
ICOSLG (166280)	0.0905	0.0655	0.2343	**0.0226**	0.3584	0.0610
ICOSLG (317667)	0.2348	0.8018	0.2343	0.2322	**0.0351**	1.0000

Significant differences between cases and non-inflammatory bowel disease patients are highlighted in bold. Data in this table is derived from *in silico* analysis of microarray data obtained by Dr Colin Noble.

Table 5.10. Differences in ICOS and ICOSLG mRNA expression (expressed as p values) in biopsy material from ulcerative colitis patients and non-inflammatory bowel disease patients.

Probe	All biopsies	Uninflamed Biopsies			
		All sites	Ascending colon	Descending colon	Sigmoid Colon
ICOS	**0.0143**	0.3369	0.5680	0.3935	0.8171
ICOSLG (166280)	0.2191	0.7707	0.3110	0.8843	0.5500
ICOSLG (317667)	0.7766	0.7556	0.5433	0.3115	0.1969

Significant differences between cases and non-inflammatory bowel disease patients are highlighted in bold. Data in this table is derived from *in silico* analysis of microarray data obtained by Dr Colin Noble.

5.6.6 Variation in *ICOS* and *ICOSLG* mRNA expression throughout the intestine differs in IBD patients

To assess the variation in mRNA expression throughout intestine in CD patients versus non-IBD controls, one-way ANOVA analyses of mRNA values from all four uninflamed sites (terminal ileum, ascending colon, descending colon and sigmoid colon) was performed showing that no significant variation in *ICOS* expression existed in controls (p=0.0542) but that variation did exist in CD patients (p=0.0414); no differences between the variation of both *ICOSLG* probes between CD cases and controls was observed (ICOSLG-166280 in controls [p=0.0119], CD patients [p=0.0063]; ICOSLG-317667 in controls [p=0.1524], CD patients [p=0.4940]). Similarly for UC, variation across three uninflamed sites (ascending colon, descending colon, and sigmoid colon) was different in UC patients (p=0.0143) but not controls (p=0.0994). However, the significant variation of *ICOSLG* seen in non-IBD patients (p=0.0331) was lost in UC (p=0.3110) (probe ICOSLG-166280); no difference in variation

was demonstrated in the ICOSLG-317667 probe between non-IBD (p=0.2219) or UC patients (p=0.1534).

5.7 Discussion

In this first family-based association analysis of *ICOSLG* in PIBD the results indicate that the signal at the 21q22 locus is most likely to be due to germline variation in *ICOSLG*, making it less likely that other genes in the region (such as *PWP2* or *AIRE*) contribute significantly to inherited IBD or CD susceptibility. The single SNP analysis shows that variations in rs8126734 provided the strongest signal, suggesting that the 3' UTR of *ICOSLG* may be the area of interest. Additionally, the more recent data from the meta-analysis of seven CD genome-wide association scans (GWAS) confirmed this signal. mRNA gene expression analysis also demonstrated that variations in *ICOSLG* and its ligand *ICOS* exist in uninflamed IBD biopsies compared to non-IBD controls; differences in the variation of the expression of these genes throughout the intestine strengthen the case for further investigation of these molecules in IBD pathogenesis.

Due to the strong record in paediatric IBD phenotyping in Scotland,[141,154] the resource of well-defined case-parent trios[602] and Scotland's involvement in international IBD genetics studies[18,603] this haplotype-tagging study has several strengths. Significant findings generated through TDT analysis, which are robust against population substructure, are generally accepted as implying both linkage and association.[604] Additionally, case-parent trios have a greater power to detect differences in less prevalent diseases, with TDT analysis avoiding false positives that arise when association is present but linkage is not.[604] This coupled with strict coding of parents with IBD, stringent permutation analysis and meticulous attention to changes in IBD phenotype over time (e.g. IBDU to CD) strengthen this study further. The use of data from a large meta-analysis of seven GWAS and a well executed microarray analysis also supports the results.

Despite these strengths, the study design and methodology still present difficulties with interpretation. Firstly, although the TDT analyses was adequately powered to detect an effect, the use of relatively small numbers during genetic analysis does allow the possibility of false positives, although less so during TDT analyses. Also, as this study used a haplotype-tagging approach (rather than deep-sequencing), only inferences can be made as to the possible regions that may contribute to IBD susceptibility. With regard to the microarray analysis additional problems arise. Firstly, the biopsy material analysed would have undoubtedly contained a myriad of cell populations, meaning that differences in expression of *ICOS* and *ICOSLG* on different lineages may have been masked. However, it

should be noted that previous work has shown that ICOS is significantly upregulated in both lamina propria mononuclear cells (LPMC) and enterocytes in patients with CD[605] and UC[606]. Secondly, many of the analyses relied on a relatively small number of biopsies from each location, and although median values (rather than mean values) were assessed, the potential for false positives still exists. Another factor which may have influenced the microarray results are the disease controls used. Although these patients may have in fact contributed to the specificity of the results for IBD, there is also the possibility that intestinal disease may have acted as a confounding factor. Also, without the knowledge of the *ICOSLG* genotype of the patients involved, no real conclusions can be drawn on the influence of genotype on gene expression. Similarly, disease activity was not taken into account.

As discussed above, the role of co-stimulatory molecules in a number of different immune disorders has been reported. Windhagen et al. investigated the expression of cytokines and co-stimulatory molecules in central nervous system specimens from multiple sclerosis (MS) suffers (MS is an inflammatory demyelinating disease that shares many susceptibility loci with IBD)[607] and controls using reverse-transcription polymerase chain reaction and immunocytochemistry.[608] They observed an upregulation of B7-1 and B7-2 in acute MS plaques, particularly from early-onset disease cases. Similarly, Verwilghen et al. analysed the expression and function of co-stimulatory molecules in the synovial membranes of patients with rheumatoid arthritis and psoriatic arthritis (again diseases that share IBD susceptibility genes),[607] in contrast to normal controls.[609] They found that B7 was highly expressed on synovial fluid T cells, and that these cells produced a five-fold increase in T cell proliferation when used as stimulator cells compared with paired peripheral blood T cells. Furthermore, studies looking specifically at the role of ICOS and ICOSLG in the pathogenesis of lung mucosal injury have demonstrated a key role for this co-stimulator. Gonzalo et al. investigated the role of ICOS and CD28 in regulating Th2-mediated mucosal inflammatory responses in murine lung epithelial cells.[559] They demonstrated that ICOS plays the predominant role in regulating Th2 effector cell activation and that ICOS-mediated signalling contributes to the inflammatory response through the regulation of IL-4 and upregulation of the chemokine receptors CCR3, CCR4 and CCR8. Stanciu et al. studied the role and expression of ICOSLG in lung epithelial cells on unstimulated and Respiratory Syncytial Virus (RSV)-infected cells by flow cytometry.[558] They found that ICOSLG was moderately expressed on unstimulated cells and was upregulated on bronchial and alveolar cells following RSV infection. In addition they showed that in RSV-infected alveolar cells had decreased expression of ICOSLG when treated with IFN-γ and IL-4. A similar study by Qian et al. showed that an alveolar type II cell line abundantly expressed ICOSLG with upregulation by both TNF-α alone and in combination with INF-γ and LPS.[557] Evidence also

suggests that the ICOS-ICOSLG interaction is likely to be involved in other (auto)immune diseases such as coeliac disease,[610] type I diabetes mellitus,[79] inflammatory neuropathies[611] and most recently allergic asthma[612].

There is increasing evidence that co-stimulatory molecules may play an important role in inflammatory bowel disease. Rugtveit et al. showed that B7.1 was upregulated compared to B7.2 on mucosal macrophage subsets in IBD compared to controls.[543] Similarly, Scarpa et al. showed that CD86 expression was not only increased in IBD patients but correlated with disease activity and CRP levels.[613] With regard to other co-stimulatory molecules a review by Danese et al. outlined the important role of CD40(L) in IBD.[614] They presented evidence that CD40 and its ligand are upregulated in the intestinal mucosa of IBD patients and that levels of soluble CD40L are increased in the serum of those affected. With activation of the CD40 pathway leading to the production of proinflammatory cytokines it is clear that CD40 may play a crucial role in the propagation of IBD. In experimental colitis models there have been interesting, but often conflicting, results. Grose et al. looked at the expression of B7+ T cells in a dextran sulphate sodium (DSS)-induced model of murine colitis.[615] Although there was no difference in expression in the initial stages of the disease, there was a 20-fold increase in both B7.1 and B7.2 expression at seven days. Similarly Eri et al. used B7.1 blocking peptide to mediate protection against experimental colitis, felt to be due to skewing of the Th2 cytokine response.[616] Interestingly, however, Kim et al. induced colitis in mice lacking either B7.1 or B7.2 and found that the disease was greatly accelerated in both cases.[617] Clearly the role of co-stimulatory molecules in the gut is complex and an over-riding hypothesis for their role in IBD pathogenesis is yet to be proposed.

With regard to the role of ICOS-ICOSLG pathway in inflammatory bowel disease, several groups have looked at the aetiology of chronic intestinal inflammation, mostly in animal models. Totsuka et al. used a murine colitis model to demonstrate the effect of anti-ICOS monoclonal antibody on chronic intestinal inflammation.[618] After injecting $CD4^{+}CD45RB^{high}$ T cells intraperitoneally into severe-combined immunodeficiency (SCID) mice they then assessed ICOS expression and clinical effects of anti-ICOS mAb versus rat IgG. They showed that there was upregulation of ICOS expression on infiltrating T cells within the mucosa. Also mice treated with anti-ICOS mAb did not exhibit signs of colitis, with good weight gain and no bowel wall thickening. However, they also tested anti-B7RP-1 (i.e. ICOSLG) mAb with no effect on colitic involvement suggesting that the ICOS-B7RP-1 interaction may not in itself propagate colitis. These findings suggest that the effect of treatment with anti-ICOS mAb may be a result of elimination of ICOS-expressing pathogenic T cells. Another interesting find of this study was that interferon-gamma produced by lamina propria $CD4^{+}$ cells was decreased in the anti-monoclonal antibody mice which may significantly influence T cell differentiation. Similarly de Jong et al. found that ICOS was

markedly upregulated on T cells involved in the murine colitis model, but their data suggested that only blockade of CD28 and ICOS had an effect on colitic changes.[619] Work carried out in a rat model induced colitis through the administration of DSS to sterile rats and again injected anti-ICOS mAb using mouse IgG as a control.[620] Using anti-CD3 mAb the researchers showed that ICOS was widely expressed regardless of CD3 status. They also found that ICOS expression on $CD4^+$ LPMC was markedly increased following DSS administration.

With respect to work involving human colitis, Sato et al. obtained both inflamed and uninflamed intestinal mucosal samples from 54 patients with CD and 46 patients with ulcerative colitis.[621] These were compared to normal controls (colonic cancer patients) and disease controls (acute colitis); disease activity was assessed using the CDAI and Truelove-Witts criteria for CD and UC respectively.[622,623] Again monoclonal antibody to anti-human ICOS, B7h, CD3 and CD28 were used to assess expression. They found that ICOS expression on LPMC was markedly increased in those with CD and UC compared with normal controls. Of clinical importance they also found that ICOS expression on $CD4^+$ LPMC cells was related to disease activity. There were significantly higher proportions of $CD4^+$ $ICOS^+$ LPMC in inflamed areas of mucosa in CD and UC patients, similarly for $CD4^+$ $B7h^+$ LPMC.

One of the most interesting recent developments in the investigation of ICOS/ICOSLG in inflammatory disease (and especially IBD) is the recognition that these co-stimulatory molecules are crucial for the development of Th17 cells[624] and suppressive T regulatory cell function[625]. As discussed in detail in **Chapter 4.3.2** the role of Th17 cells in IBD pathogenesis has gained much attention in recent years.[626,627] Much of the current evidence does not pertain directly to IBD, rather most research has focussed on the role of these molecules in allergic disease.[628,629] However, one study does describe ICOS and IL-17 producing cells during colonic inflammation.[630] This work demonstrated a direct correlation between ICOS expression and IL-17 production in colonic intra-epithelial cells (cIELs) from interleukin-10 (IL-10) deficient mice (conventionally-housed IL-10 deficient mice spontaneously developed enterocolitis[98]). ICOS was expressed only on cIELs from IL-10 deficient mice with IL-17-producing cells consisting of CD4+ and CD8+ cIELs; however, CD4+ cells were the predominant IL-17-producing cell population. ICOSLG was also shown to be up-regulated on colonic epithelial cells and on a population of large granular leukocytes during inflammation. Culture of cIELs with ICOSLG+ dendritic cells enhanced IL-17A production from normal cIELs but failed to do so using cIELs from ICOS deficient mice. Significantly, *in vivo* treatment of IL-10 deficient mice with antibody to ICOS resulted in a significant reduction in colonic pathology. These findings implicate ICOS as an activational signal of Th17 cells during chronic intestinal inflammation, and suggest that under some

conditions, control of ICOS expression may help to suppress chronic intestinal inflammation. With regard to regulatory T cells a very recent paper has identified ICOS as a pivotal effector molecule in the early decision between tolerance and immunity.[631] This study showed that ICOS did not act like most other co-stimulatory molecules in that it was induced during the immune response, but not by T cells activated under tolerogenic conditions. When expressed, ICOS selectively caused a massive (21:1) expansion of the T effector population of CD4+ cells, leaving the regulatory T cell compartment almost untouched, thus markedly affecting the balance between effector and regulatory T cells during inflammation.

5.8 Conclusion

Through robust, adequately powered TDT analysis in a well-phenotyped cohort of PIBD patients, the susceptibility signal at 21q22 has been shown to be likely due to variations in the 3'UTR of *ICOSLG*; this result was also confirmed using GWAS data from mainly adult studies. Also, using mRNA microarray analysis it has been demonstrated that *ICOS* and *ICOSLG* gene expression varies across different intestinal sites in uninflamed biopsies in IBD cases versus controls. The molecules involved in co-stimulation and the subsequent differentiation of T cells are clearly intricately involved in many inflammatory disease processes, including IBD. Although not required to initiate the response to antigen, their presence plays a vital role in the propagation of the inflammatory response. Given the genetic association with molecules such as *ICOSLG* in diseases such as IBD and coeliac disease, the work outlined above demonstrates that these molecules and other co-stimulatory signals are likely to be important therapeutic targets for chronic immune diseases in the future.

6. An exploration of C-reactive protein in paediatric inflammatory bowel disease

6.1 Introduction

C-reactive protein (CRP) is an acute phase reactant produced predominantly in response to pro-inflammatory cytokines.[632,633] First identified in the plasma of patients with *Streptococcus pneumoniae* infection, it was named following the observation that it possessed the ability to precipitate the somatic C-fraction of the pneumococcal cell wall.[634] During the acute-phase response to infection or inflammation, basal CRP levels (<10mg/L) can rise rapidly following induction by interleukin-6 (IL-6), interleukin-1-beta (IL-1β) and tumour necrosis factor-alpha (TNF-α).[635,636] Primarily produced in hepatocytes, it is here that CRP monomers are assembled into a cyclic homopentameric structure inside the endoplasmic reticulum prior to secretion into the plasma, via the transcription factors CCAAT/enhancer-binding protein beta (C/EBPβ) and the p50 subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB).[637] Initial work indicated that binding of p50 to the nonconsensus kappaB site enhanced and stabilised the binding of C/EBPβ to the CRP promoter, with the binding of both these proteins to their overlapping cognate sites required for the efficient induction of CRP by IL-6.[638] This study was later expanded to show that the binding of these proteins displaces a complex containing CCAAT/enhancer-binding protein zeta (C/EBPζ) and recombination signal binding protein for immunoglobulin kappa J region (RBP-Jκ).[639] It has now been shown that the CRP promoter has a low level of occupancy of these and other transcription factors (such as STAT3, cRel, Oct1, HNF-1 and HNF-3), with C/EBPβ the most important factor in the presence of a cytokine signal.[640-642]

With regard to the immunological function of CRP, the highly conserved amino acid sequence (with no known human variants) suggests a critical and non-redundant role.[643] CRP has been shown to interact with many molecules,[644] including C1q (activating the classical complement cascade[645]), factor H (regulating the alternative complement pathway[646]), FcγRI and FcγRII (leading to phagocytosis[647,648]), and FcαRI (promoting the further surface expression of FcαRI, phagocytosis and TNF-α secretion[649]), in addition to its primary ligand, phosphocholine, which is exposed on damaged biological membranes, including bacterial cell walls[650,651]. Additionally a significant role for CRP in the clearance of apoptotic cells and bacteria has also been postulated.[652,653] An outline of the pathways involved in CRP formation, induction and function are shown in **Figure 6.1**.

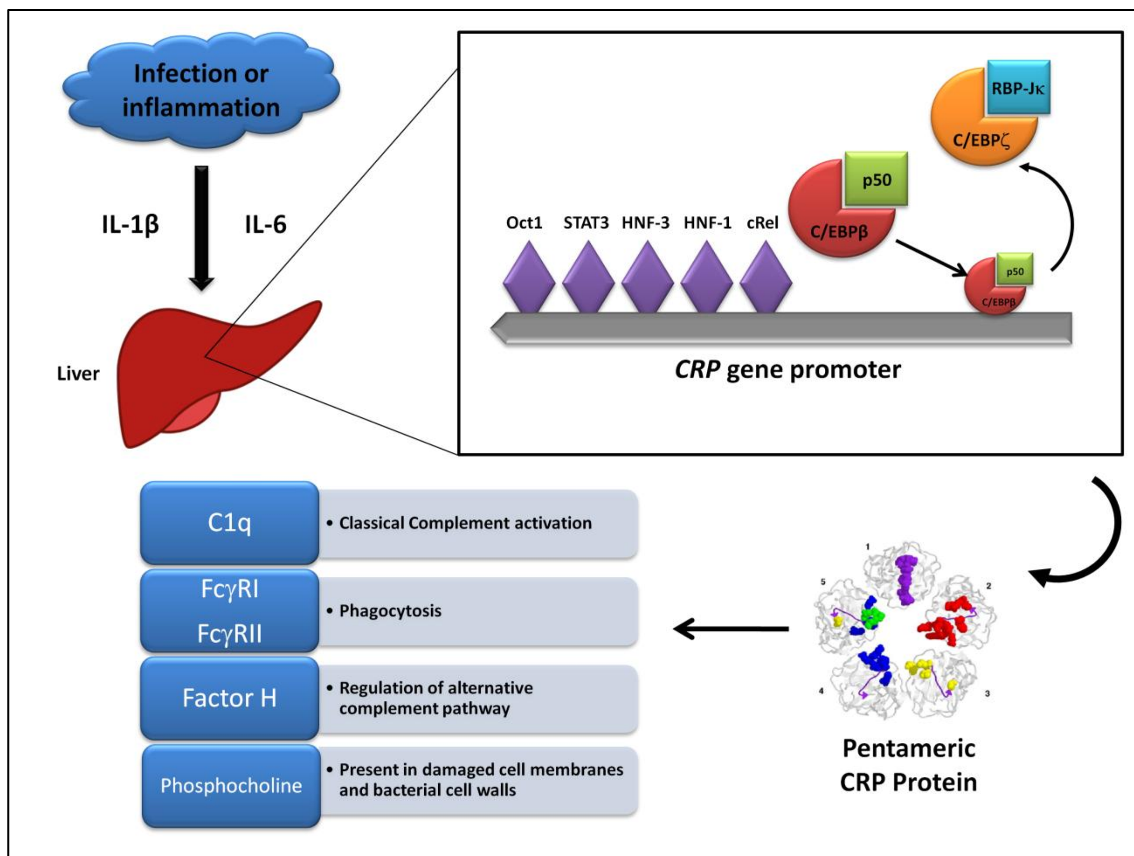


Figure 6.1. Outline of the pathways involved in CRP induction, formation and function in response to infection and/or inflammation. Following an infectious or inflammatory stimulus, the production of IL-1β and IL-6 leads to CRP synthesis in the liver. A complex arrangement of transcription factors are modified at the CRP gene promoter, with the displacement of the C/EBPζ-RBP-Jκ complex by the C/EBPβ-p50 complex the most critical step. Following the synthesis of the pentameric molecule, serum CRP binds to various ligands in order to interact with immune modulation and apoptosis.

Clinically, the rise in serum CRP levels allows its routine use as a marker of disease presence,[654] disease activity[655] and to subsequently determine the effect of therapeutic intervention[656]. However, it is commonly accepted that the absence of a rise in serum CRP does not always indicate lack of infection or inflammation.[654] Additionally, an individual patient's serum CRP response to the initial infection may be 'inappropriately' low given the clinical history,[657] with the response to treatment also varying widely[658]. The reasons for these person-to-person variations are not yet clear.

Variations in the gene encoding CRP have previously been shown to influence baseline serum CRP levels in healthy individuals,[659] leading to further work demonstrating that *CRP* genotype may affect the susceptibility to conditions with an inflammatory component such as acute myocardial infarction[660] and systemic lupus erythematosus (SLE)[661]. The recent genome-wide association study (GWAS) in paediatric inflammatory bowel disease (PIBD)[18]

demonstrated similar susceptibility loci as adult studies,[13] implicating pathways such as innate immunity, the adaptive T cell response and autophagy in disease pathogenesis. Although *CRP* was not a clear positional candidate in these case-control studies (*CRP* is a relatively small gene, which has not been tagged robustly on the genotyping platforms used to date) there is now increasing interest in the concept that the CRP protein itself may play an active role in inflammatory disease pathogenesis through the mechanisms outlined above.

With regard to IBD in clinical practice, serum CRP has been shown to have some limited utility at diagnosis in cases suspected of having IBD and to correlate to a degree with disease activity in those with known disease.[657,662] Of greater interest is the finding that adult patients with a high serum CRP level show a superior response to biological therapies,[662,663] in addition to a possible correlation of CRP levels with Crohn's disease phenotype[664].

6.2 Hypotheses and Aims

Hypotheses:

1. Variations in the *CRP* gene contribute to PIBD susceptibility in a robustly phenotyped cohort of Scottish PIBD patients.
2. Variations in the *CRP* gene influence PIBD phenotype, including age at onset, disease location and disease behaviour.
3. Serum CRP levels at PIBD diagnosis are influenced by *CRP* genotype.
4. *CRP* genotype and serum CRP levels are association with the ability of patients to respond to anti-tumour necrosis alpha (TNF-α) therapy.

Aims: To use a family-based, haplotype-tagging approach in cohort of PIBD patients and their parents, in addition to meta-analysis data from seven GWAS in adult CD patients, to determine if *CRP* is a candidate susceptibility gene for PIBD. Additionally, following strict phenotyping of patients, the influence of *CRP* genotype on disease phenotype, the influence of serum CRP levels and *CRP* genotype on CRP levels at diagnosis and the ability to attain disease remission using anti-TNF-α therapy will be assessed.

6.3 Methods

It should be noted that the methodologies used for this study were similar to those used in **Chapter 5**, therefore only the details of relevant differences or similarities in the approaches used in the current chapter are highlighted below.

6.3.1 Subjects

A total of 1331 individuals consisting of 466 early-onset IBD patients (i.e. diagnosed before their 17th birthday) and their parents (*n*=865) were included. Genotyping of parents provided 242 complete parent-child trios. PIBD was diagnosed according to standard clinical, histological and radiological findings[147,236] and rigorous phenotyping carried out as previously described;[141] the basic demographics of the PIBD group are shown in **Table 6.1**. It should be noted that 37 parents also had a confirmed diagnosis of IBD (22 Crohn's disease, 13 ulcerative colitis and 2 colonic IBD-unclassified [IBDU]) and therefore disease coding was altered appropriately during each analysis.

Table 6.1. Characteristics of paediatric inflammatory bowel disease patients analysed in the family-based association study to determine the role of CRP as a susceptibility gene.

	IBD	CD	UC	IBDU
Number [%]	466 [100.0]	311 [66.7]	113 [24.3]	42 [9.0]
Median age at diagnosis (Years [IQR])	11.5 [9.0-13.2]	11.7 [9.2-13.4]	11.0 [8.9-12.9]	10.3 [8.5-12.4]
Male sex (*n*, [%])	266 [57.1]	184 [59.2]	59 [52.2]	23 [54.8]
White European (*n*, [%])	450 [96.5]	301 [96.7]	108 [95.6]	41 [97.6]

IQR, interquartile range; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic IBD, type unclassified.

6.3.2 Single nucleotide polymorphism selection

The *CRP* gene is located on chromosome 1 between positions 159,682,079 and 159,684,379 (Ensembl Release 68 - ENSG00000132693; Build 37) flanked by two genes, *APCS* and *DUSP23*, which are 124kb and 68kb downstream and upstream of *CRP* respectively. *CRP* tagging single nucleotide polymorphisms (SNPs) were selected using genotypic data from the CEU study available from the HapMap project[329] and identified using Haploview software version 4.2[593] based on solid spine of linkage disequilibrium (LD) (r^2 >0.8, haplotype frequency >5%, minor allele frequency >10%) (**Figure 6.2a**). This process identified five tagging SNPs spanning a 46kb region: rs1935193, rs1130864, rs1205,

rs1417938 and rs11265263 (**Figure 6.2b**). It is important to note that three of these SNPs (rs1130864, rs1205, rs1417938) were specifically chosen as tagging SNPs as they had previously been demonstrated to influence basal CRP levels.[665-667] An additional SNP previously shown to influence serum CRP levels, rs1800947,[665] was also genotyped. Details of all genotyped SNPs are presented in **Table 6.2**. For all patients and their parents SNPs conformed to the Hardy–Weinberg equilibrium.

Table 6.2. Details of the six single nucleotide polymorphisms genotyped to tag CRP.

Marker Number	dbSNP Name*	Chromosomal position†	Location	MAF	Alleles	Detectable odds ratio‡
1	rs1935193	159664090	Upstream	0.301	A:T	1.48
2	rs1205	159682233	3' flanking	0.329	C:T	1.47
3	rs1130864	159683091	3' flanking	0.299	G:A	1.48
4	rs1800947	159683438	Exon 2	0.063	C:G	1.94
5	rs1417938	159684186	Intronic	0.298	T:A	1.48
6	rs11265263	159710517	Downstream	0.072	C:A	1.88

*Based on NCBI Human Build 135; †Ensembl Release 68 - July 2012; ‡based on individual minimum allele frequency (MAF) with an 80% power to detect an effect.

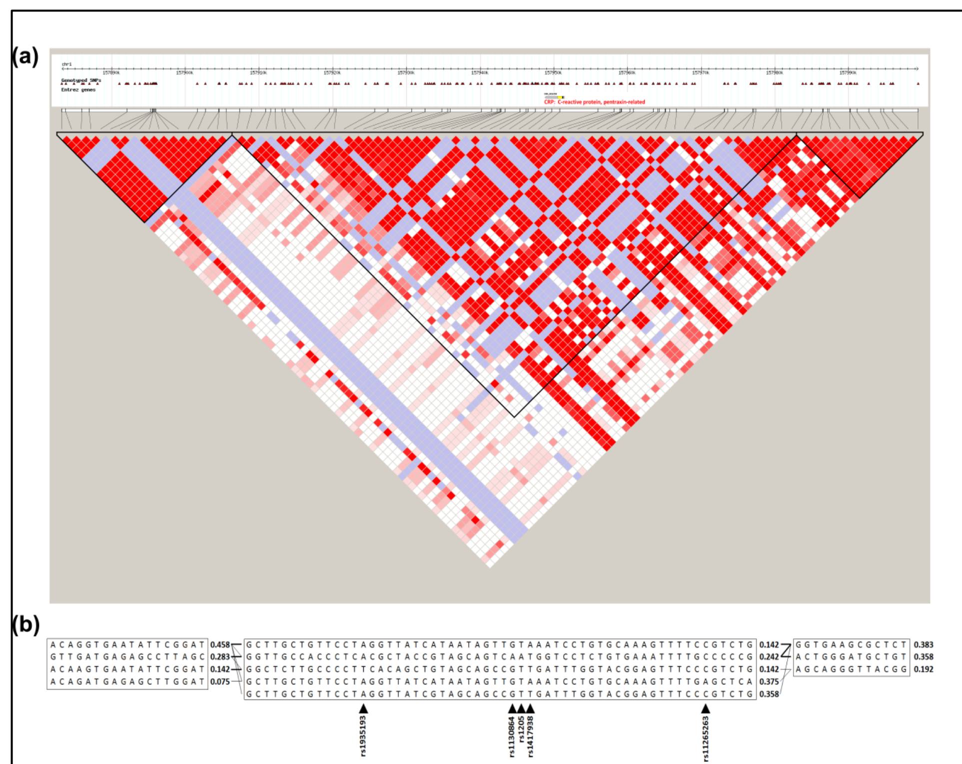


Figure 6.2. Genomic region and single nucleotide polymorphisms tagging CRP. (a) view of region generated from the CEU study available through the HapMap project data using Haploview 4.2 software; (b) the haplotype block containing *CRP* is tagged by five single nucleotide polymorphisms.

In order to assess the contribution of genes encoding various *CRP* transcription factors, the GWAS data described below was examined to determine if the regions containing SNPs tagging these genes had been genotyped. Using the "tag SNP picker" function on the HapMap project website (http://hapmap.ncbi.nlm.nih.gov/), eight SNPs tagging three genes (*CEBPZ* [C/EBPζ], *HNF1A* [HNF-1] and *POU2F1* [Oct-1]) were identified. It was then ensured that these SNPs, or a suitable proxy (i.e. D'=1, r^2=1.0), was present on the HumanHap 550 BeadChip (Illumina, San Diego) and therefore had full results available within the GWAS data. Suitable proxies were determined using SNAP (http://www.broadinstitute.org/mpg/snap/index.php)[668]; brief details of all eight SNPs are shown in **Table 6.3**.

Table 6.3. Summary of eight SNPs tagging three genes encoding transcription factors known to reside at the CRP promoter.

Gene tagged	dbSNP*	Chromosome	Location†
CEBPZ	rs10206380	2	37454286
	rs2041840	2	37467264
HNF1A	rs735396	12	121438844
	rs7310409	12	121424861
POU2F1	rs7532692	1	167250080
	rs4656538	1	167259755
	rs7355118	1	167319763
	rs7522390	1	167348685

*Based on NCBI Human Build 135; †Ensembl Release 68 - July 2012.

6.3.3 DNA extraction and genotyping

Primer sequences of the six SNPs tagging *CRP* are shown in **Table 6.4**; all genotyping was carried out in August 2009. The mean genotyping call rate for all SNPs in all patients/parents was 98.6%. Mendelian errors were identified using Haploview software for the current dataset in addition to the errors identified during a previous genetic association study performed on this patient group.[242] Additional genotyping data was also obtained from a previous genome-wide scan carried out on a nested cohort of the Scottish paediatric IBD patients. This genotyping was performed using the Illumina Infinium™ II HumanHap550 BeadChip technology (Illumina, San Diego), at the Center for Applied Genomics at the Children's Hospital of Philadelphia. This data was previously used in the first early-onset GWAS and published by Imielinski et al. in 2009.[18] This data was obtained in the form of a BeadStudio (Illumina, San Diego) file from Dr Johan Van Limbergen (University of Edinburgh) and converted to the updated GenomeStudio (Illumina, San Diego) format. The

data contained genotyping results on a representative sample of 330 of the patients described above (239 CD, 91 UC and 29 IBDU) for approximately 550,000 SNPs on the platform.

Table 6.4. Primer sequences for the five single nucleotide polymorphisms used to tag the **CRP** ***gene on chromosome 1.***

dbSNP*	Position†	Strand	Sequence
rs1935193	159664090	Rev	CAAGATACATTTTTAGAGAAATGCC**[A/T]**ATGATTTTTTAAACTACCTGTGATC
rs1205	159682233	For	ACTTCCAGTTTGGCTTCTGTCCTCA**[C/T]**AGTCTCTCTCCATGTGGCAAACAAG
rs1130864	159683091	For	CCTCAAATTCTGATTCTTTTGGACC**[A/G]**TTTCCCAGCATAGTTAACGAGCTCC
rs1800947	159683438	For	AGATGGTGTTAATCTCATCTGGTGA**[C/G]**AGCACAAAGTCCCACATGTTCACAT
rs1417938	159684186	Rev	CCCCACCCCCATACCTCAGATCAAA**[A/T]**CTCTCCCATAGCCTGGGGTGGCCCT
rs11265263	159710517	Rev	AGCTCTAGAGGCTGGGAAGGAAGTA**[A/C]**AAGATCAAGGTGGCAGAAGGTTTAG

*Based on NCBI Human Build 135. †Ensembl Release 68 - July 2012.

6.3.4 Replication using international case-control data

To assess variants in *CRP* for association with CD susceptibility SNP-specific p values, odds ratios (OR) and standard errors, generated from the analyzes of seven individual CD datasets imputed with the 1000 genomes reference set, encompassing 5,956 CD patients and 14,927 healthy controls (http://www.broadinstitute.org/mpg/ricopili/) were obtained. p values were generated with principal component analysis as covariates, therefore correcting the whole analysis for population stratification and inter-study differences. Data obtained through the HapMap project (CEU study)[329] and Haploview software were then used to visualise the haplotype structure (using solid spine of linkage disequilibrium) surrounding *CRP* to determine the areas of strongest signal.

6.3.5 Serum CRP level at diagnosis

As part of the standard phenotyping study protocol, serum CRP levels are recorded in the PICTS database (held and maintained by Mrs Hazel Drummond, Database Manager, Gastrointestinal Unit, University of Edinburgh) at the time of diagnosis (i.e. during the initial workup of symptoms suggestive of PIBD and closest to the primary diagnostic endoscopy). As CRP reference ranges have differed marginally both geographically and with time throughout Scotland, in addition to minor variations in biochemical assay, results are simply recorded as 'raised' or 'normal' (i.e. above normal range or not) at diagnosis. The highest normal cut-off value in use at any time was 10mg/L. For children with no results available through PICTS, case-notes and/or laboratory systems were searched.

6.3.6 Response to anti-TNF-α monoclonal antibody

Physician global assessment was used to assess initial remission of patients naïve to anti-TNF-α monoclonal antibody during induction, regardless of whether or not remission was then sustained (as worsening disease and other factors may contribute to loss of response). Remission was recorded at 10-12 weeks after commencement of the induction course of infliximab (three infusions at weeks 0, 2 and 6 - unless infusions discontinued for severe infusion reaction) or adalimumab (six fortnightly doses at weeks 0-10 unless treatment discontinued for severe adverse effects or absolute lack of response; UK paediatric clinical experience has been that many children and young people need longer than four weeks to judge induction response with adalimumab[669]). In some cases, the paediatric CD activity index[670] or paediatric UC activity index[671] score for CD and UC/IBDU respectively were also used to define remission; a senior clinician with experience of providing the routine care of all these patients (Prof. David C Wilson, University of Edinburgh) made the final decision on whether remission was achieved. All six SNPs were tested in a multivariate model to determine any effect of *CRP* genotype on response, with all analyses corrected for IBD type (categorical) and age at diagnosis (quantitative) if appropriate. Serum CRP results taken at the time of the first anti-TNF-α induction dose were also obtained retrospectively from patient notes and local laboratory databases throughout the three regional paediatric gastroenterology networks in Scotland.

6.3.7 Statistical analysis

Transmission disequilibrium test (TDT) analyses were carried out using Haploview v4.2[593] by utilising the parenTDT function.[595] Additional analyses were carried out using SNPStats[672] and R version 2.14.1 (R Foundation for Statistical Computing, Vienna, Austria). For the TDT analysis all p values are reported as corrected following permutation analysis (*n*=100,000 permutations), unless otherwise stated. The meta-analysis of the TDT and international GWAS data, and quantitative trait single SNP analysis was carried out using PLINK (http://pngu.mgh.harvard.edu/purcell/plink).[673] Chi-squared, Fisher's exact and Mann-Whitney *U* tests were carried out using R version 2.14.1 where appropriate. A two-sided p value of 0.05 was considered significant. R was used to generate the GWAS SNP scatterplot using the ggplot2 package.[599]

6.3.8 Power calculation

Using QUANTO (version 1.2.4; http://hydra.usc.edu/gxe),[597] and based on the most recent prevalence data of Scottish paediatric IBD presented in **Chapter 3** (41.2/100,000 of the population less than 16 years old[674]) the IBD TDT analysis had 80% power to detect an

effect with an odds ratio (OR) between 1.47 and 1.94 depending on the individual SNP (ORs for each SNP are shown in **Table 6.3**).

6.3.9 Ethics

All patients and/or their parents provided full written consent as part of the MRC-funded PICTS cohort which was approved by local ethics committees at all three participating clinical networks: South-East Scotland (LREC/2002/6/18), West of Scotland (YREC/P12/03) and North of Scotland (GREC/03/0273).

6.4 Results

6.4.1 Variations in *CRP* genotype increase the susceptibility to paediatric IBD

TDT analyses of all case-parent trios demonstrated that the A allele of two SNPs showed significant over-transmission to affected IBD patients after correction (rs1417938, p=0.006, OR 1.67 [95% confidence interval (CI) 1.22-2.29] and rs1130864, p=0.015, OR 1.56 [95% CI 1.16-2.08]) (**Table 6.5**). Over-transmission of the A allele of SNP rs1935193 was nominally significant (uncorrected, p=0.015). Haplotype analysis across the entire gene demonstrated that the six-marker haplotype (ACACAC) showed significant distortion of transmission to affected individuals ($p=8x10^{-4}$, OR 1.75 [95% CI 1.30-2.36]) (**Table 6.6a**). Two-marker sliding haplotype analysis revealed the strongest signal across a small 750 base region spanning the intron and exon 2 which incorporated rs1800947-C and rs1417938-A (p=0.001, OR 1.64 [95% CI 1.22-2.21]) with the substitution of rs1417938-A with rs1417938-T across the same two marker haplotype conferring significant protection (p=0.017, OR 0.69 [95% CI 0.52-0.91]) (**Table 6.6b**).

6.4.2 Variations in *CRP* genotype increase the susceptibility to paediatric CD

TDT analysis in paediatric CD produced similar results to the PIBD group. The A allele of the three SNPs detailed above (i.e. rs1417938, rs1130864 and rs1935193) showed significant distortion of transmission (**Table 6.5**). Similarly, the 6-marker haplotype ACACAC and the two-marker haplotype rs1800947C - rs1417938A produced the strongest signal. A similar TDT analysis was carried out in the cohort of UC patients (*n*=113) and their parents, however no significantly over-transmitted SNPs (**Table 6.5**) or haplotypes were identified.

6.4.3 Replication of the *CRP* locus association in international case-control data

A total of 905 SNPs in the haplotype block containing *CRP* and the two flanking blocks, spanning 169kb across the three blocks, were first assessed using imputed meta-analysis data available from the International IBD Genetics Consortium Crohn's disease data. A total of 52 SNPs across the entire region attained a nominal p value of <0.05, 25 of which lay in the haplotype block containing *CRP*; 16 SNPs in the haplotype block containing *CRP* achieved a nominal p value of <0.005. A plot of the SNPs in the region is presented in **Figure 6.3**.

Table 6.5. Transmission Disequilibrium Test analysis of individual single nucleotide polymorphisms tagging* CRP *for paediatric IBD, Crohn's disease and ulcerative colitis.

dbSNP*	Overtransmitted Allele	Uncorrected p value	Corrected p value	Odds ratio (95% CI)
Inflammatory Bowel Disease				
rs1935193	A	0.0153	0.0686	1.42 (1.05-1.90)
rs1205	C	0.2098	0.5899	1.20 (0.91-1.58)
rs1130864	A	0.0032	**0.0149**	1.56 (1.16-2.08)
rs1800947	C	0.2413	0.6808	1.36 (0.81-2.27)
rs1417938	A	0.0015	**0.0066**	1.67 (1.22-2.29)
rs11265263	C	0.2113	0.6406	1.37 (0.83-2.25)
Crohn's Disease				
rs1935193	A	0.0089	**0.0444**	1.60 (1.11-2.30)
rs1205	C	0.2191	0.6503	1.24 (0.90-1.72)
rs1130864	A	0.0056	**0.0321**	1.70 (1.18-2.44)
rs1800947	C	0.1011	0.4025	1.65 (0.90-3.01)
rs1417938	A	0.0025	**0.0145**	1.89 (1.26-2.83)
rs11265263	C	0.1489	0.5310	1.53 (0.86-2.72)
Ulcerative colitis				
rs1935193	A	0.8864	1.0000	1.00 (0.29-3.45)
rs1205	C	0.2743	0.7517	1.45 (0.82-2.56)
rs1130864	A	0.1985	0.6030	1.41 (0.76-2.63)
rs1800947	G	0.7389	1.0000	1.59 (0.87-2.91)
rs1417938	A	0.1317	0.4807	1.25 (0.34-4.66)
rs11265263	N/A	1.0000	1.0000	1.04 (0.59-1.82)

*Based on NCBI Human Build 135. Corrected P values were generated by permutation analysis (*n*=100,000) as described in Methods. CI, confidence interval; N/A, not applicable. Significant p values are highlighted in bold.

Table 6.6. Complete haplotype and 2-marker sliding haplotype analysis from the transmission disequilibrium test analysis of all PIBD case-parent trios

(a) Complete haplotype					
Markers*	**Haplotype**	**Trans:untrans**	**Odds ratio (95% CI)**	**Uncorrected p value**	**Corrected p value**
1,2,3,4,5,6	ACACAC	121:69	1.75 (1.30-2.36)	0.0002	**0.0008**
	TCGCTC	79.2:103.8	0.76 (0.57-1.02)	0.0591	0.2598
	ATGCTC	82:94.8	0.86 (0.64-1.16)	0.3795	0.8916
	ACGCTC	30:29.6	1.01 (0.61-1.68)	0.8580	1.0000
	ATGGTA	24:34	0.71 (0.42-1.19)	0.1892	0.7119
(b) Two-marker sliding haplotype					
Markers*	**Haplotype**	**Trans:untrans**	**Odds ratio (95% CI)**	**Uncorrected p value**	**Corrected p value**
1,2	AC	136.9:87.7	1.56 (1.19-2.04)	0.0010	**0.0031**
	AT	96:114.5	0.83 (0.63-1.09)	0.2296	0.0547
	TC	78.6:109.3	0.71 (0.53-0.96)	0.0209	0.4677
2,3	CG	89.5:113.5	0.78 (0.59-1.04)	0.0917	0.2224
	TG	96:115	0.83 (0.63-1.09)	0.2172	0.4500
	CA	118:75	1.57 (1.17-2.10)	0.0026	**0.0087**
3,4	GC	87.1:121	0.71 (0.54-0.94)	0.0229	**0.0461**
	AC	118:75	1.57 (1.17-2.10)	0.0026	**0.0042**
	GG	25:34.1	0.73 (0.43-1.22)	0.2368	0.4204
4,5	CT	83.9:120.9	0.69 (0.52-0.91)	0.0120	**0.0170**
	CA	117.2:71.1	1.64 (1.22-2.21)	0.0010	**0.0011**
	GT	25:34.1	0.73 (0.43-1.22)	0.2368	0.4248
5,6	TC	84.9:121.8	0.69 (0.52-0.91)	0.0127	**0.0219**
	AC	115.8:70	1.65 (1.22-2.22)	0.0010	**0.0013**
	TA	28.6:37.5	0.76 (0.47-1.24)	0.2731	0.4918

*Marker numbers correspond with those given in Table 6.2. Corrected p values were generated by permutation analysis (*n*=100,000) as described in Methods. Trans, transmitted; untrans, untransmitted; CI, confidence interval. Significant p values are highlighted in bold.

The two significant SNPs identified in the TDT analysis described above (rs1417938 and rs1130864) both attained p values of 0.004. (Notably, the triallelic SNP rs3091244, which has previously been associated with serum CRP levels,[675] was also significant [p=0.004]). Using the CD TDT analysis (discovery cohort) and the international GWAS data (replication cohort), combined p values and ORs were calculated using PLINK. This demonstrated that the risk alleles of all six SNPs provided an association signal in the same direction, with two SNPs (rs1417938 and rs1130864) achieving significant p values after correction (**Table 6.7**).

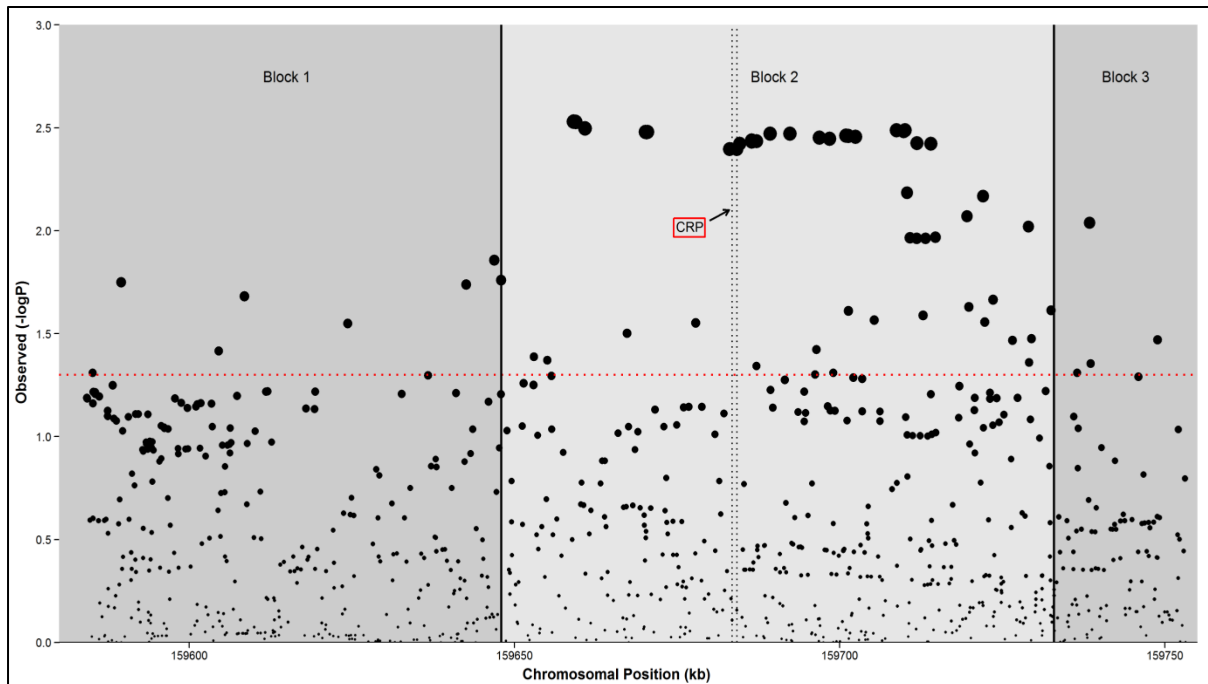


Figure 6.3. Results of a meta-analysis of seven CD genome-wide association studies imputed with the 1000 genomes reference set. Scatterplot showing -logP values in the region of *CRP* for 905 single nucleotide polymorphisms. Vertical dotted lines represent the boundaries of the *CRP* gene and solid lines the limits of each haplotype block. The logP value corresponding to p<0.05 is represented by the horizontal (red) dotted line.

Table 6.7. Meta-analysis of Scottish transmission disequilibrium test (discovery) and international GWAS (replication) for six SNPs tagging CRP on chromosome 1 for Crohn's disease.

		Scottish CD TDT Analysis		IIBDGC Meta-analysis		Combined Results		
dbSNP*	**Risk Allele**	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**	**Corrected p value**
rs1935193	A	1.60 (1.11-2.30)	0.009	1.03 (0.98-1.08)	0.274	1.04 (0.99-1.09)	0.158	0.948
rs1205	C	1.24 (0.90-1.72)	0.219	1.04 (1.00-1.09)	0.077	1.05 (1.00-1.10)	0.052	0.312
rs1130864	A	1.70 (1.18-2.44)	0.006	1.07 (1.02-1.13)	0.004	1.08 (1.03-1.14)	0.001	**0.006**
rs1800947	C	1.65 (0.90-3.01)	0.101	1.02 (0.91-1.13)	0.779	1.03 (0.93-1.15)	0.572	1.000
rs1417938	A	1.89 (1.26-2.83)	0.003	1.07 (1.02-1.13)	0.004	1.08 (1.03-1.14)	0.001	**0.006**
rs11265263	C	1.53 (0.86-2.72)	0.149	1.00 (0.91-1.11)	0.937	1.02 (0.92-1.12)	0.746	1.000

*Based on NCBI Human Build 135. CD, Crohn's disease; TDT, transmission disequilibrium test; IIBDGC, international inflammatory bowel disease genetics consortium; OR, odds ratio; CI, confidence interval. Results in bold text highlight significant results.

6.4.4 The influence of *CRP* genotype on CRP levels at diagnosis

Although four of the SNPs genotyped have previously been associated with variations in *basal* CRP levels, the influence of *CRP* genotype on the presence of an elevated CRP response at diagnosis was assessed. To achieve this, potential confounders identified in the PIBD cohort (i.e. IBD type and age at diagnosis) were used as covariates in a multivariate model. Using raised/normal serum CRP at diagnosis as a binary outcome, a representative sample of 342 IBD patients (229 CD [67%], 84 UC [25%] and 29 IBDU [8%]) had full data available. In this analysis rs1205, rs1130864 and rs1417938 showed nominal association with CRP levels (in a dominant model); however none of these SNPs remained significant after Bonferroni correction (**Table 6.8**). It should be noted that the presence of the risk alleles (rs1417938-A and rs1130864-A) identified in the TDT and GWAS data were associated with a raised serum CRP at diagnosis.

Table 6.8. Results of single nucleotide polymorphisms that demonstrated nominal significance in a dominant model when assessed for influence on normal/raised serum CRP levels at PIBD diagnosis.

dbSNP*	Genotype	CRP=Normal (%)	CRP=Raised (%)	OR (95% CI)	Uncorrected p value
rs1417938	T/T	49.5	38.4	1.67 (1.01-2.77)	0.045
	A/T - A/A	50.6	61.6		
rs1130864	G/G	50.6	38.9	1.73 (1.06-2.83)	0.029
	A/G - A/A	49.4	61.1		
rs1205	C/C	43.0	51.3	0.57 (0.34-0.94)	0.025
	C/T - T/T	57.0	48.7		

*Based on NCBI Human Build 135. CD, OR, odds ratio; CI, confidence interval.

6.4.5 Patients with paediatric CD and UC differ with regard to *CRP* genotype and serum CRP at diagnosis

Although CD and UC have been shown to share many susceptibility loci there are also clear differences in the candidate genes and postulated pathogenic pathways involved in these IBD types.[607] Evaluating the genotypic differences between CD and UC patients only (i.e. excluding all patients with IBDU) there was an increased frequency of the T allele of rs1205 in CD patients (34.2% and 22.6% in CD and UC respectively; **Table 6.9**). Haplotype analysis of these two disease groups also demonstrated significant differences in haplotype structure, with the strongest difference seen in the six-marker haplotype ATGCTC (**Table 6.9** and **Figure 6.4**). Additionally, serum CRP results were available for 229 CD patients and 84 UC patients at diagnosis as detailed above. These results showed that a greater proportion

of CD patients compared to UC patients had an elevated CRP level at diagnosis (62.8% versus 23.8%, p<0.0001).

Table 6.9. Analysis of the differences in paediatric Crohn's disease and ulcerative colitis with regard to six SNPs in the CRP gene and their associated haplotype structure.

dbSNP*	Associated Allele	Allele freq in CD (%)	Allele freq in UC (%)	Uncorrected p value	Corrected p value
rs1935193	A	74.2	65.4	0.0156	0.0656
rs1205	T	34.2	22.6	0.0020	**0.0085**
rs1130864	G	67.4	63.3	0.2903	0.7331
rs1800947	G	6.2	5.7	0.7991	0.7990
rs1417938	T	67.2	63.1	0.2959	0.7404
rs11265263	C	93.2	92.7	0.8105	0.9992
Haplotype†	**Haplotype freq (%)**	**Haplotype freq in CD (%)**	**Haplotype freq in UC (%)**	**Uncorrected p value**	**Corrected p value**
ACACAC	33.6	32.6	36.2	0.3512	0.8409
TCGCTC	28.1	25.8	34.2	0.0214	0.0819
ATGCTC	24.3	27.5	15.6	$6x10^{-4}$	**0.0024**
ACGCTC	7.0	7.3	5.9	0.4938	0.9346
ATGGTA	6.0	6.1	5.7	0.8245	1.0000

*Based on NCBI Human Build 135. †Based on the SNP order above. Significant results are highlighted in bold.

To further elucidate the possible reasons for the clear differences in serum CRP at diagnosis between CD and UC patients, tagging SNPs of three genes encoding *CRP* transcription factors (*POU2F1* [OCT1], *CEBPZ* [C/EBPζ] and *HNF1A* [HNF-1] were also evaluated. This demonstrated that none of the eight individual SNPs was over-represented in either CD or UC. Similarly, none of the haplotype analyses demonstrated differences between CD and UC.

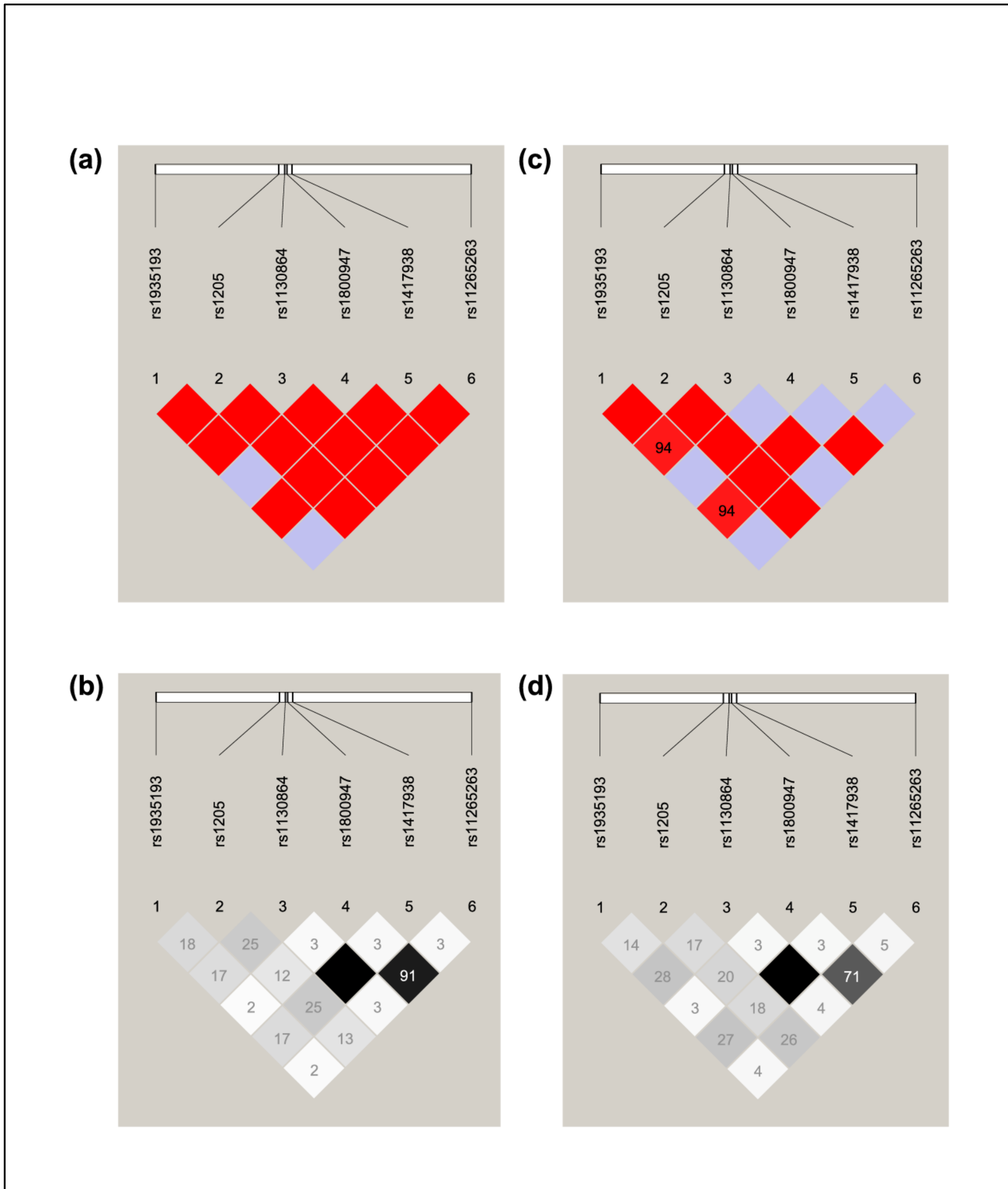


Figure 6.4. A linkage disequilibrium map showing the D' and r2 values for paediatric Crohn's disease patients and paediatric ulcerative colitis patients. (a,b) Crohn's disease; (c,d) Ulcerative colitis. For figures 2a and 2c, bright red squares equate to D'=1 and LOD≥2 and blue squares to D'<1 and LOD<2 .

6.4.6 *CRP* genotype may influence age at diagnosis in early-onset IBD

Recent consensus guidelines have suggested that PIBD phenotype differs between those diagnosed aged 0-9 years of age (Paris A1a) and between 10-16 years (Paris A1b), in addition to work presented in **Chapter 3**.[154] The genotypic differences between those diagnosed in these two age brackets was therefore assessed. Using greater than or equal to

10 years or less than 10 years at diagnosis as binary outcomes, the analyses revealed that, correcting for IBD type (CD, UC and IBDU), SNP rs1205 was nominally significant in an additive model (T/T>C/T>C/C) (uncorrected, p=0.022). Interestingly the haplotype ATGCTC, which differed significantly between those children with CD and UC, was also associated with age at diagnosis less than 10 years in CD patients, with a nominal p value of 0.040. There were no significant SNPs when either age at symptom onset or age at diagnosis was evaluated as a quantitative trait across for either the IBD or CD group.

6.4.7 *CRP* genotype does not influence CD location or behaviour

Due to the strong TDT results for CD the effect of *CRP* genotype on major CD phenotypes was evaluated. To achieve this clinical parameters from the PICTS database in a nested cohort of CD patients with full location and behaviour data available at diagnosis (*n*=286), and a second cohort who had at least 4 years of follow-up data available since diagnosis (*n*=189) was obtained; details of these sub-groups are shown in **Table 6.10**. There were no significant differences in *CRP* genotype (either individual SNPs or haplotypes) in CD patients with or without ileal disease (i.e. +/- Montreal L1 or L3), upper GI disease (i.e. +/- Montreal L4) or pure colonic disease (i.e. +/- Montreal L2) either at diagnosis, or at last follow up. Similarly CD patients with stricturing or penetrating disease behaviour (Montreal B2 or B3) did not differ with respect to *CRP* genotype compared to those with inflammatory disease only (i.e. Montreal B1) after at least 4 years follow up. A total of 229 CD patients had a 'normal/raised' CRP result available at diagnosis. The three phenotypes analysed above (i.e. with or without ileal, upper or pure colonic disease) did not differ with respect to normal/raised CRP levels at diagnosis (p=0.113, p=0.504, p=0.264, respectively).

Table 6.10. Characteristics of Crohn's disease subgroups used to determine the influence of CRP genotype on disease location or behaviour.

	At diagnosis	At last follow up
Number	286	189
Male sex (*n*, [%])	169 [59]	112 [59]
Median age at diagnosis (Years [IQR])	11.7 [9.1-13.4]	11.1 [8.4-12.9]
White European (*n*, [%])	277 [97]	183 [97]
Ileal disease (i.e. L1 or L3) (*n*, [%])	163 [57]	129 [68]
Upper GI disease (i.e. L4) (*n*, [%])	138 [48]	119 [63]
Pure colonic disease (i.e. L2) (*n*, [%])	64 [22]	28 [15]
Median follow up duration (Years [IQR])	N/A	6.1 [4.0-8.1]
Stricturing/penetrating behaviour* at last follow up (*n*, [%])	N/A	50 [26]

*B2 and/or B3. IQR, interquartile range; GI, gastrointestinal; N/A, not applicable

6.4.8 The influence of *CRP* genotype and serum CRP level on anti-TNF-α response

Clinical data was available regarding initial remission achieved during anti-TNF-α therapy in 83 anti-TNF-α naïve early-onset IBD patients (71 CD, 9 UC, 3 IBD-U; 81 received infliximab, 2 received adalimumab). Of the 83 patients, 52 (63%) patients achieved initial remission as defined in the methods above. Using a multivariate model incorporating IBD type and age at diagnosis, only rs1205 genotype was found to have a significant effect on remission rates in an over-dominant model (p=0.020, OR 4.62 [95% CI 1.55-13.74]) (**Figure 6.5**). Those patients who were heterozygous for the rs1205 SNP showed a remission rate of 79% compared to 51% in those homozygous for the C or T allele (p=0.021). Fisher's exact testing across all three rs1205 genotypes (CC:CT:TT) was also significant (p=0.032). No haplotypes were identified that significantly differed between those achieving or not achieving remission.

Additionally, serum CRP levels taken in the seven days prior to the first dose of anti-TNF-α agent (*n*=73) demonstrated that, comparable to a previously published adult study,[662] patients gaining clinical remission had similar median serum CRP levels than those who did not (16mg/L versus 12mg/L respectively, p=0.999). However, patients with an elevated serum CRP level at anti-TNF-α induction (i.e. >10mg/L) had higher remission rates than those with normal levels (67% versus 58%), although this was not statistically significant (p=0.612).

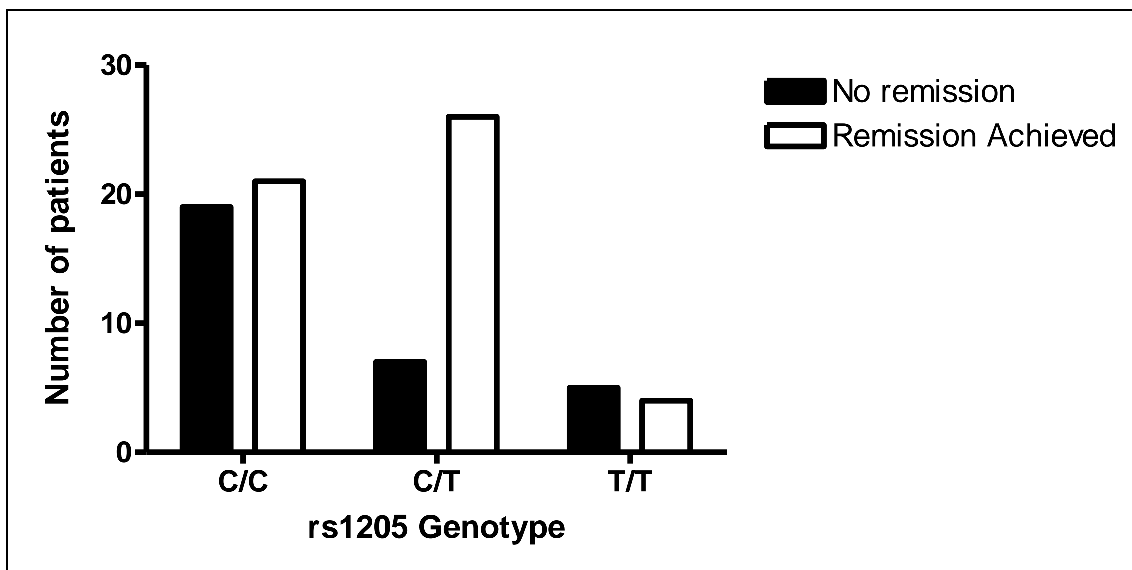


Figure 6.5. Graph showing the number of children with each rs1205 genotype achieving primary remission following anti-TNF-α therapy.

6.5 Discussion

Using family-based TDT analyses it has been shown that *CRP* is a susceptibility gene for IBD and CD in the Scottish paediatric population, with replication in CD using data from a large international case-control meta-analysis. It has also been demonstrated that *CRP* genotype has potential effects on age at diagnosis, but no discernible effect on major disease phenotypes in a sub-group of CD patients. Additionally *CRP* genotype (especially haplotype structure) and serum CRP at diagnosis differ between CD and UC patients in the same Scottish PIBD population, with *CRP* genotype influencing the ability of these patients to gain initial disease remission during their first course of anti-TNF-α therapy.

With regard to the design of the genetic susceptibility analysis, there are research benefits resulting from the utilisation of family-based studies such as improved recruitment, better recall and documentation of possible environmental influences, accurate population stratification and a reduction in population heterogeneity, although a degree of inherent bias still occurs.[676] The ability to rigorously phenotype a distinct population of paediatric IBD patients, and to follow their progress over long periods has also enabled detailed analysis of IBD sub-groups, in addition to the collection of laboratory parameters[159] and response to therapies[177].

The use of nonparametric linkage analysis and newer GWAS techniques[677] have now led to the identification of over 100 IBD susceptibility loci[526] with candidate genes involved in the innate immune response, autophagy and the interleukin-12/interleukin-23 pathways.[523,524] The impetus for carrying out a candidate susceptibility gene analysis on *CRP* in this Scottish PIBD population were four-fold. Firstly, several studies in inflammatory conditions have identified *CRP* as a susceptibility gene[667,678]. Secondly, we have observed, along with others,[657] that children across all IBD types differ significantly in their ability to produce a serum CRP response, even in the presence of overt clinical disease. Interestingly, recent evidence has shown that mesenteric fat (hyperplasia of mesenteric fat is characteristic of CD) is an important source of CRP, with CRP production by mesenteric adipocytes triggered by bacterial and inflammatory stimuli such as *E.coli* and IL-6.[633] Thirdly, with regards to cardiovascular risk and SLE, studies have suggested that failure to respond appropriately to inflammation with a rise in serum CRP may be a factor in disease pathogenesis.[637,679] This may be especially important in light of the fact that paediatric CD and UC patients differ significantly with regard to *CRP* genotype and serum CRP response at diagnosis. Finally, recent evidence has suggested that adult IBD patients with a high baseline CRP level at induction demonstrate a better primary response to biological

therapies, with early normalisation of CRP levels correlating with sustained long-term response.[662,680]

It is important to note that although the results of this paediatric study demonstrate a positive TDT outcome with replication in a mainly adult meta-analysis, recent correspondence with members of the IIBDGC have revealed that the *CRP* locus did not show a susceptibility signal in the recent IBD ImmunoChip experiment.[525] This study of approximately 14000 CD cases and 16000 controls demonstrated a p value of 0.83 for the rs1130864 SNP (Dr Jeffrey Barrett, Wellcome Trust Sanger Institute, personal communication), therefore the risk of a false positive result in this smaller family-based cohort may be significant. However, although the region containing *CRP* may not have been a proven susceptibility locus in these adult studies, it is possible that the signal may be specific to the paediatric population (e.g. *MTMR3*[18]) or be as a result of geographical variation in risk loci or allele frequencies. It is of interest to note that in the original Wellcome Trust case control study in CD, a number of risk alleles showed significant variation across the United kingdom,[508] with previous work in Scotland showing that the contribution of the three common *NOD2* mutations to disease susceptibility in the Scottish paediatric population is modest, with a population attributable risk lower than in most other populations studied[242].

With respect to the role of *CRP* genotype and inflammatory disease pathogenesis the evidence is substantial but often contradictory. The first stimulus to dissect the effect of *CRP* genotype with basal CRP levels came following the observation of a strong correlation between CRP levels and cardiovascular disease risk.[681] Although recent evidence has suggested that no causal link is likely to exist between *CRP* genotype, plasma CRP and cardiovascular disease,[682] extensive efforts to examine this link have provided a detailed insight into how inherited *CRP* variants influence basal CRP levels.[637] The role of CRP in inflammatory bowel disease in particular has been mostly detailed from a clinical perspective, however two studies have evaluated the contribution of *CRP* genotype to disease phenotype and treatment response. Thalmaier et al. evaluated the role of the rs1800847 (+1059G/C) polymorphism in CD susceptibility and disease phenotype in 241 adult patients with CD and 199 healthy controls.[664] They reported that C allele homozygosity was associated with ileal disease involvement at diagnosis, with no effect on disease susceptibility; this study was limited by evaluating only one SNP without replication. The second study evaluated the role of three SNPs in *CRP* (rs2794521, rs1130864 and rs1205) with regard to response to infliximab in 189 anti-TNF naive adult patients.[683] They concluded that none of these polymorphisms had a significant effect on either biological (as defined as a decrease in serum CRP) or clinical response to infliximab or indeed any effect on CRP levels prior to commencing treatment.

With regard to the clinical use of serum CRP in patients with inflammatory bowel disease, studies have looked at its utility at diagnosis, during the assessment of disease activity,[156] response to biological therapies[684] and association with disease outcomes such as surgery[685]. Although this study was unable to replicate adult studies in which patients with an elevated CRP responded better to biological therapies,[684] this may reflect the smaller numbers presented, the extensive disease phenotype of early-onset patients,[141] or differing response rates to biological therapies in children[686].

With respect to study limitations, although the TDT analysis had sufficient power to detect associations within case-parent trios, the sub-phenotypic analyses may have been underpowered; this is especially true with regard to certain SNPs with small MAF within the anti-TNF-α response data. Additionally, the biological plausibility of the increased response in those heterozygous for rs1205 is difficult to justify, although positive molecular heterosis (i.e. when subjects heterozygous for a specific genetic polymorphism show a significantly greater effect for a trait than subjects homozygous for either allele) may be involved.[687]

6.6 Conclusions

This study has demonstrated that *CRP* is a candidate susceptibility gene for PIBD and paediatric CD, with clear differences in CD and UC *CRP* genotype and potential effects on age at onset and response to anti-TNF-α agents. Further functional work is now required to assess the role of CRP in IBD, likely mimicking the previous work undertaken with regard to cardiovascular disease risk. It has already been shown that *CRP* genotype influences basal serum CRP levels, however the complex relationship between *CRP* genotype, CRP level and IBD phenotype should now be explored using 'Mendelian Randomisation', as this is more likely to reveal the true influence of CRP to IBD pathogenesis;[682] further insights into the role of transcription factors and the serum CRP response may prove fruitful with this respect. The evidence that CRP bridges both innate and adaptive immune pathways is intriguing in the context of our current knowledge of disease pathogenesis, and with CRP already widely used in the clinical setting, there is great potential to optimise the utilisation of this biomarker during the care of patients with IBD.

7. The diagnostic accuracy of faecal calprotectin during the investigation of suspected paediatric inflammatory bowel disease

7.1 Introduction

Work presented in **Chapter 2** and **Chapter 3** has clearly shown that both the incidence and prevalence of paediatric inflammatory bowel disease (PIBD) has risen significantly in recent years, with similar trends in incidence also evident worldwide.[223] These epidemiological changes not only raise questions regarding disease pathogenesis, but also have implications for the provision of PIBD services in paediatric gastroenterology, especially during the process of diagnostic workup for suspected disease.

In routine clinical practice, the initial investigation of children with suspected bowel inflammation commonly includes haematological and biochemical biomarkers to identify those patients warranting further evaluation. However, to date sparse evidence exists to support their diagnostic accuracy and therefore their overall value is not entirely clear. Beattie et al. looked at referrals for colonoscopy over an 18 month period to assess the usefulness of routine blood parameters in determining the presence of organic disease.[688] In a group of 91 patients referred, 26 had confirmed Crohn's disease (CD) and 13 patients were diagnosed with ulcerative colitis (UC). None of the CD patients and one of the UC patients had a full set of normal blood results (which included haemoglobin [Hb], erythrocyte sedimentation rate [ESR], albumin, platelet count and C-reactive protein [CRP]). A similar study by Cabrera-Abreu et al. looked at 153 children less than 18 years referred to a tertiary centre with symptoms suggestive of PIBD.[689] They demonstrated a sensitivity of 90.8% (95% CI 83.3 - 95.7) and a specificity of 80.0% (95% CI 65.7 to 89.8%) for a combination of low haemoglobin or raised platelet count (i.e. one of two abnormal), demonstrating poor diagnostic utility of the other commonly measured parameters. A systematic evaluation of CRP and ESR in a large cohort of UC patients demonstrated that these parameters had some correlation with disease activity, but that normal values were evident in a large proportion of patients with mild to moderate disease activity.[657] A Scottish study (incorporating a South-East Scotland PIBD cohort) evaluating a similar panel of blood tests, but with the addition of the faecal marker calprotectin, also showed that all 48 of the IBD patients studied had one abnormal blood test and/or calprotectin, but the absence of a control group did not allow the diagnostic accuracy of the markers to be evaluated.[159]

7.2 Background of faecal calprotectin

Faecal calprotectin (FC) is a calcium-binding protein found in neutrophilic granulocytes,[690] monocytes,[691] macrophages,[692] and epithelium[693]. A 24 kilodalton heterodimer composed of two calcium-binding proteins belonging to the S100 group of proteins (S100A8 and S100A9),[694] it constitutes between 40-60% of the cytosolic protein in human neutrophils.[690,695] FC has demonstrated bacteriostatic actions against a number of bacteria including *Listeria monocytogenes*,[696] *Borrelia burgdorferi*,[697] *Staphlococcus aureus*,[698] and *Escherichia coli*[699]. Additionally, FC has been shown to be fungicidal against several species.[699-701] The main mechanism of action for this microbial inhibition is the chelation of zinc and manganese leading to 'starvation' these organisms.[698,702] Although some bacteria such as *Salmonella typhimurium* can overcome this metal binding leading to symptomatic gut inflammation,[703] the efficient chelation of zinc and/or manganese (via histidine-rich regions of the calprotectin molecule[704]) and the enhancing the sensitivity of bacteria to superoxides can often produce an effective anti-inflammatory response[702].

FC has previously been shown to be markedly raised in children and adults with IBD,[705,706] with its stability for seven days at room temperature allowing the convenient collection of this non-invasive marker in both inpatient and outpatient settings.[695] In PIBD FC has been shown to correlate with endoscopic severity at colonscopy,[707] disease activity in UC[708] and has also demonstrated usefulness in predicting disease relapse.[709] There are currently only a small number of published studies that have evaluated FC during the *initial* investigation of paediatric bowel inflammation (i.e. prior to endoscopic confirmation of IBD).[161,247,705,710-712] These studies have often included small numbers of IBD patients or have not provided a detailed analysis of sub-phenotypic characteristics in a truly representative group of potential PIBD patients.[161,712] A recent meta-analysis evaluating the role of FC during the initial investigation of suspected IBD concluded that FC was a useful screening tool to identify patients requiring endoscopic assessment,[162] though the discriminative power to safely exclude IBD was significantly better in adults than in children.

7.3 Hypothesis and Aims

Hypotheses:

1. The diagnostic accuracy of FC in suspected PIBD is equivalent to endoscopy and superior to six commonly used blood parameters.
2. FC does not vary significantly between patients in each PIBD type.

3. The location of disease at diagnosis (e.g. upper intestinal disease) does not influence FC levels.

Aims: To describe the differences in FC levels at diagnosis between the three IBD types (CD, UC and colonic IBD, type unclassified [IBDU]) and a group of non-IBD disease patients who were initially and contemporaneously referred to paediatric gastroenterology services for suspected bowel inflammation. Additionally, to evaluate six commonly used blood parameters in the same cohort to assess their utility at diagnosis compared to FC.

7.4 Methods

7.4.1 Setting

The paediatric gastroenterology department based at the Royal Hospital for Sick Children in Edinburgh (RHSCE) (as described in detail in **Chapter 3**) provides a regional service for a population of approximately 228,000 children in South-East Scotland (SES). This tertiary hospital acts as a referral centre for the three district general hospitals with paediatric services, in addition to two adult academic gastroenterology services in Edinburgh and all adult gastroenterology departments within the district general hospitals. Children presenting or referred with suspected bowel inflammation currently have all their primary investigations and initial follow up carried out at RHSCE by four experienced paediatric gastroenterologists. The biochemistry department based at the Western General Hospital (WGH) in Edinburgh has routinely processed all FC samples in the SE Scotland region since October 2004 (under the supervision of Dr Kathleen Kingstone), with access to all tests carried out in primary care and all hospital-based services.

7.4.2 Faecal calprotectin measurements

All FC measurements carried out between 1st January 2005 and 31st December 2010 in patients born after the 1st January 1987 (to ensure the inclusion of all patients potentially undergoing endoscopy before 18 years of age) in SES were obtained retrospectively from the biochemistry department laboratory records (through correspondence with Dr Kathleen Kingstone, Biochemist) as a large Microsoft Excel 2003 database (Microsoft Corporation, Redmond, WA). These data included patient demographics (name and date of birth), the unique patient identifier and unique specimen number, the location code which specifies the sample origin (e.g. general practice, outpatient department), the sample date and FC concentration in micrograms per gram (μg/g) of stool for all patients. The data also specified if samples were taken but were insufficient for processing (marked as 'Insuff' on the

database). Faecal calprotectin was measured by the PhiCal™ Test (Calpro AS, Lysaker, Norway) according to the manufacturer's instructions in the biochemistry laboratory at the WGH; the local assay analytical range is currently 20 to 2500 µg/g. A normal FC value was taken to be <50 µg/g stool with the biochemistry laboratory routinely reporting FC results as "possible gastrointestinal (GI) inflammation" if between 51-100 µg/g, "GI inflammation" if between 101-200 µg/g and "active GI inflammation" if >200 µg/g. In order to aid analysis of FC levels, samples reported as <20 µg/g and >2500 µg/g were converted to 20 µg/g and 2500 µg/g respectively.

7.4.3 IBD patients

All incident cases of PIBD diagnosed by standard clinical, histological and radiological findings[147,236] since August 1997 have been collected prospectively by Prof. David C Wilson (Consultant Paediatric Gastroenterologist) and Ms Pam Rogers (IBD and Liver Nurse Specialist) and recorded on a departmental database using Microsoft Access 2003 (Microsoft Corporation, Redmond, WA, USA) - see **Chapter 3**. From this database patients who had a FC measured as part of their initial diagnostic work-up during the 6 year period of FC data from 2005-2010 were identified (hereafter referred to as the IBD group). Detailed phenotypic characteristics[141] for these patients were also available through the Scotland-wide Medical Research Council funded Paediatric Inflammatory Bowel Disease Cohort and Treatment Study (PICTS) database, details of which can be found in **Chapter 3** and **Chapter 5.6.1**.

7.4.4 Non-IBD (control) patients

Through the electronic departmental clinical records containing the details of over 5,600 children referred to paediatric gastroenterology services since 2001, a computerised search using the remaining FC sample list determined those patients who had previously had contact with the service. This was achieved using a Microsoft Word macro (Microsoft Corporation, Redmond, WA, USA) developed by Dr Nick Kennedy (Clinical Fellow, University of Edinburgh) that interrogated the local RHSCE shared folder (known locally as GISecs - referring to the gastroenterology secretarial records) to pull out the name and date of birth of each child seen by the service. These hospital records and departmental endoscopy lists (generated once or twice weekly by the departmental secretaries) were used to identify all patients undergoing both upper and lower endoscopy due to the clinical suspicion of bowel inflammation, but where PIBD was subsequently excluded based on histological and radiological evidence (hereafter referred to as the control group).

7.4.5 Exclusion criteria

Exclusion criteria for both the IBD and control groups were: (1) insufficient stool sample provided (usually less than 1 gram of stool, although the laboratory often attempted to provide FC levels in samples of between 0.5-1.0 grams); (2) aged less than one year or greater than 18 years of age on the endoscopy date (children less than six year old have previously been shown to have elevated FC levels[713-715]); (3) greater than a six month delay between the FC sample and the endoscopy date; (4) FC sample taken after endoscopy; (5) any previously known, hospital-diagnosed, GI disease; (6) previous upper or lower GI endoscopy for any indication.

7.4.6 Blood parameters

Blood results (taken within six months of endoscopy and closest to the date of the FC sample) were also obtained to compare the diagnostic utility of FC with commonly used blood parameters, namely: Hb, platelet count, total white cell count (WCC), ESR, serum albumin and CRP. The regional paediatric biochemistry-haematology laboratory normal values by age and sex range are shown in **Table 7.1**. Within one referral centre (Victoria Hospital, Kirkcaldy), plasma viscosity (PV) is routinely used as an alternative to ESR, therefore in six patients within the control group PV results were used as a proxy for ESR with a reference range of 1.50-1.72 mPa/s.

Table 7.1. South-East Scotland regional paediatric biochemistry-haematology laboratory normal values by age and sex for the six tests evaluated

Parameter	1-2yrs	2-6yrs	6-12yrs	12-18yrs
Haemoglobin (g/L)	113-141	115-135	113-155	Male 130-160 Female 120-160
Total white cell count (x10^9/L)	6.0-17.5	5.0-17.0	4.5-14.5	4.5-13.0
Platelets (x10^9/L)	150-450			
Erythrocyte sedimentation rate (mm/hr)	<20			
Albumin (g/L)	33-50			
C-reactive protein (mg/L)	<10			

Yrs, years

7.4.7 Data recording and statistics

Demographic information, details of endoscopic assessment, FC and blood results, phenotypic information, prescribed medications at the time of the FC sample and final diagnosis were recorded electronically using Microsoft Access 2007 (Microsoft Corporation, Redmond, WA, USA) following thorough case-note review. Statistical analysis was

performed using R version 2.14.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism version 4.03 (GraphPad Software, CA, USA). Pearsons's chi-square, Kruskal-Wallis and Mann Whitney U tests were used where appropriate; multivariate analysis was achieved using multiple logistic regression. The R packages epiR[716] and pROC[717] were used for further analysis (carried out by Dr Nick Kennedy, University of Edinburgh). Hb and total WCC were standardised to the reference range used for 12-18 year olds for the purpose of ROC curve generation. Youden Index was calculated as sensitivity + (specificity -1).[718] Statistical testing between ROC curves was performed using the DeLong[719] and bootstrap[720] methods. Statistical significance was taken to be a two-tailed p value of less than 0.05.

7.4.8 Ethics

Ethics approval was sought but deemed unnecessary as this was an anonymous, observational study of patients already under the care of PIBD services and under the umbrella of the Paediatric-onset IBD Scottish Audit (PISA).

7.5 Results

7.5.1 Group characteristics

A flow diagram outlining the patient selection process is shown in **Figure 7.1**. In total 91 IBD patients and 99 non-IBD controls met the inclusion criteria; the baseline characteristics of both groups are outlined in **Table 7.2**. Using univariate analysis the IBD group demonstrated an older age at endoscopy, a shorter time between their FC sample and endoscopy and higher terminal ileum (TI) intubation rates. However, only age at endoscopy and time between FC sample and endoscopy remained significant on multivariate analysis suggesting that differences in TI intubation rates between the groups was possibly a result of a higher age at endoscopy in the IBD group (with endoscopy being less technically demanding in older children). Additionally, the TI intubation rate was not significantly different between those children with and without a FC result available at endoscopy (p=0.437).

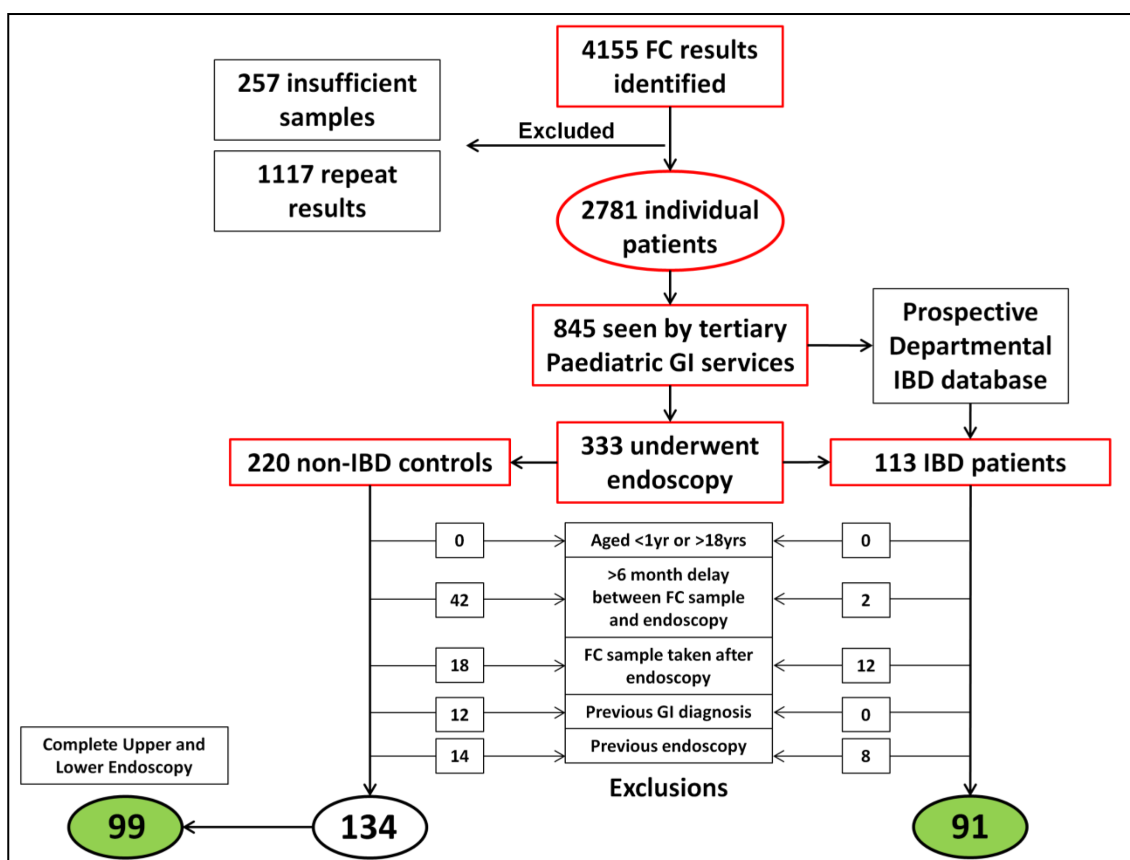


Figure 7.1. Flow diagram showing the retrospective selection of study participants. FC, faecal calprotectin; GI, gastrointestinal; Yr, year; IBD, inflammatory bowel disease.

Table 7.2. Characteristics of the inflammatory bowel disease and control groups

Characteristic	IBD group	Control group	Difference between groups (p value)*
Number of cases	91	99	
Male sex [n (%)]	56 (62)	55 (56)	0.403
Median age at endoscopy [years (IQR)]	12.6 (9.5-14.0)	9.3 (5.2-12.7)	<0.001
Terminal ileal biopsy obtained [n (%)]	57 (63)	46 (46)	0.037
Median time from FC result to endoscopy [days (IQR)]	18 (7-44)	48 (29-87)	<0.001
Median time from FC result to blood result [days (IQR)]	3 (0-9)	9 (3-30)	N/A
Median time from blood result to endoscopy [days (IQR)]	13 (2-37)	56 (29-93)	N/A

*p values as determined with Chi squared or Mann Whitney *U* tests. IQR, interquartile range; GI, gastrointestinal; LGIE, lower GI endoscopy; N/A, not applicable.

The IBD group consisted of 62 CD, 21 UC and 8 IBDU patients. In IBD patients without a TI biopsy obtained (*n*=34), 88% had small bowel imaging carried out within a median time of 46 days (interquartile range [IQR] 22-71 days) from endoscopy. All but one of the IBD group (99%) are currently recruited to the PICTS cohort; the remaining patient died prior to approach for possible consent.

The diagnostic categories of the control group are shown in **Table 7.3** and reflect their definitive diagnosis after follow up of at least 12 months from the date of endoscopy (median follow up 41 months [IQR 24-53 months]). All children presented with one or more symptoms suggestive of bowel inflammation (**Table 7.4**); 37% had two recorded symptoms and 23% had three or more. All controls were followed up to at least definitive diagnosis or to discharge from paediatric services. Of those with no pathology identified (*n*=11), all have been discharged from further follow up on no medications. To our knowledge none of the control group have subsequently been diagnosed with PIBD by the end of October 2012. Unless now in adult services or moved away from SE Scotland, all potential PIBD diagnoses will have been re-referred to our service.

Table 7.3. Non-IBD (control) diagnostic categories and median faecal calprotectin values

Diagnostic category	Number	Median FC (IQR)
Irritable bowel syndrome	32	48 (21-123)
Non-specific colitis	12	68 (25-258)
No pathology identified	11	20 (20-275)
Post-infectious enteropathy	11	45 (20-440)
Cow's milk/wheat intolerance	8	67 (41-115)
Threadworms	7	110 (29-275)
Allergic enteropathy	5	80 (50-1508)
Coeliac disease	3	350 (N/A)
Miscellaneous	10	177 (61-292)
Total	99	

FC, faecal calprotectin; IQR, interquartile range.

Table 7.4. Symptoms and signs in control patients suggestive of bowel inflammation/inflammatory bowel disease

Symptom/Sign	Percentage
Altered bowel habit	76
Abdominal pain	55
Rectal bleeding	48
Growth distortion	10
Vomiting	10
Recurrent mouth ulcers	3
Iron deficient anaemia	2

7.5.2 Children with IBD have a significantly elevated FC at diagnosis compared to controls undergoing endoscopy

The median FC at diagnosis for the IBD group was 1265 µg/g (IQR 734-2024 µg/g, range 26-2500 µg/g), which was higher ($p<0.001$) than the control group with a median FC of 65 µg/g (IQR 20-235 µg/g, range 20-2500 µg/g). Within the IBD group only two patients had a FC <50 µg/g recorded at initial presentation, an 11-year-old girl with colonic CD (Montreal L2 at diagnosis) who has since required immunomodulators and adalimumab, and a 4-year-old boy with pancolitic IBDU who is currently stable on a maintenance 5-ASA preparation. Only one child in the control group had a FC >2500 µg/g, a 2-year-old girl with allergic enteropathy who settled on dietary restrictions and who continues to have no evidence of PIBD during ongoing review.

The diagnostic accuracy for FC at different cut-off levels is shown in **Table 7.5**. Using the manufacturer's normal cut-off of >50 µg/g gives an excellent sensitivity (0.98), but a poor specificity (0.44) for IBD. This specificity increases steadily with increasing FC levels until the "pay-off" between sensitivity and specificity (calculated by the Youden Index) plateaus at around 200-300 µg/g. As discussed above it can be seen that the IBD group had a higher rate of TI biopsy, therefore to ensure that this was not confounding the results regarding diagnostic accuracy, children with an available TI biopsy (n=103) were analysed separately. This demonstrated that using a cut-off of >50 µg/g gave almost identical results as when the entire cohort was evaluated (i.e. sensitivity 0.98 [95% CI 0.91-0.99]; specificity 0.46 [95% CI 0.31-0.61]; negative predictive value 0.95 [95% CI 0.77-0.99]; positive predictive value 0.69 [95% CI 0.58-0.79]). It is important also to note that 34% (n=63) of FC results returned after the endoscopic assessment was performed, and secondly that 14% (n=27) of FC results were not known in the preceding two weeks prior to endoscopic assessment. Given the minimum two week delay in RHSCE to elective GI endoscopy over the full six year period, these 48% (n=90) of FC results could not have influenced the decision making with regards to performance of endoscopy, nor the occurrence of TI intubation in 34% of patients. Furthermore, even for the 52% of patients where FC results were known in the two weeks prior to the endoscopic assessment, none were cancelled after confirmation of procedure based on FC result, nor had procedures expedited based on FC result - this occurred only for rapid clinical deterioration.

Table 7.5 Measures of diagnostic accuracy for increasing levels of faecal calprotectin and commonly measured blood parameters in children with suspected inflammatory bowel disease

Faecal calprotectin						
Cut-off (µg/g)	**Sens (95% CI)**	**Spec (95% CI)**	**NPV (95% CI)**	**PPV (95% CI)**	**Youden Index* (95% CI)**	**LR+ve (95% CI)**
>50	0.98 (0.92-1.00)	0.44 (0.34-0.55)	0.96 (0.85-0.99)	0.62 (0.53-0.70)	0.42 (0.27-0.55)	1.8 (1.5-2.1)
>100	0.97 (0.91-0.99)	0.59 (0.48-0.68)	0.95 (0.86-0.99)	0.68 (0.59-0.76)	0.55 (0.38-0.68)	2.3 (1.8-3.0)
>200	0.93 (0.86-0.98)	0.74 (0.64-0.82)	0.92 (0.84-0.97)	0.77 (0.67-0.84)	0.67 (0.50-0.80)	3.6 (2.5-5.0)
>300	0.89 (0.81-0.95)	0.83 (0.74-0.90)	0.89 (0.81-0.95)	0.83 (0.74-0.90)	0.72 (0.54-0.84)	5.2 (3.3-8.0)
>800	0.73 (0.62-0.81)	0.95 (0.89-0.98)	0.79 (0.71-0.86)	0.93 (0.84-0.98)	0.68 (0.52-0.80)	14.5 (6.1-34.4)
Blood Parameters (using normal paediatric values outlined in Table 7.1)						
Parameter	**Sens (95% CI)**	**Spec (95% CI)**	**NPV (95% CI)**	**PPV (95% CI)**	**Youden Index* (95% CI)**	**LR+ve (95% CI)**
Haemoglobin	0.64 (0.53-0.73)	0.79 (0.63-0.87)	0.69 (0.59-0.78)	0.75 (0.64-0.84)	0.43 (0.23-0.61)	3.1 (2.0-4.7)
Total WCC	0.09 (0.04-0.17)	0.99 (0.94-0.99)	0.53 (0.45-0.60)	0.89 (0.52-1.00)	0.08 (-0.02-0.18)	8.2 (1.0-6.4)
Platelets	0.43 (0.32-0.54)	0.92 (0.85-0.97)	0.62 (0.53-0.70)	0.84 (0.71-0.94)	0.35 (0.17-0.50)	5.6 (2.6-11.8)
ESR	0.67 (0.57-0.77)	0.89 (0.81-0.95)	0.74 (0.64-0.82)	0.86 (0.75-0.93)	0.56 (0.37-0.72)	6.1 (3.4-11.2)
Albumin	0.22 (0.14-0.33)	0.99 (0.94-0.99)	0.56 (0.49-0.64)	0.95 (0.76-1.00)	0.21 (0.08-0.33)	20.4 (2.8-149.1)
CRP	0.55 (0.44-0.66)	0.91 (0.83-0.96)	0.67 (0.58-0.76)	0.86 (0.74-0.94)	0.46 (0.28-0.62)	6.3 (3.1-12.5)

*Youden Index = Sensitivity + (Specificity-1); Sens, sensitivity; Spec, specificity; NPV, negative predictive value; PPV, positive predictive value; CI, confidence interval; LR+ve, positive likelihood ratio; WCC, white cell count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Additional analysis within the complete cohort (*n*=190) showed that the pre-test probability of IBD was 0.48 (i.e. 91/190) and that utilising a positive result of >200 µg/g provided a post-test probability of 0.77 (111 patients had a FC >200 µg/g with 85 having IBD), an increase of 60%.

7.5.3 FC levels in children with IBD are not influenced by sex, age, IBD type or disease location

Including all 91 children with IBD and categorising the entire IBD group by sex showed no difference between median FC levels in males (1265 µg/g [IQR 658-1864 µg/g]) and females

(1250 μg/g [IQR 890-2070 μg/g]) (p=0.695). There were also no differences between the sexes when the CD (p=0.508), UC (p=0.859) and IBDU (p=0.999) groups were analysed separately. Although comparing age with FC level for the entire IBD group demonstrated a significant correlation (p=0.037, Spearman's rho=0.245), this was lost when analysed in a multivariate model which included ESR, CRP and albumin (p=0.375), likely reflecting the fact that disease severity was a confounder

To determine if disease type or location influenced FC levels, detailed phenotypic information was collected from the PICTS database; their Montreal[2] classification for location at diagnosis is shown in **Table 7.6**.

Table 7.6. Montreal classification of disease location at diagnosis for all IBD patients (n=91).

IBD type	Number
Crohn's Disease (*n*=62)	
L1	1
L1+L4	2
L2	8
L2+L4	8
L3	12
L3+L4	29
(Oral/perianal disease only)*	(2)
Ulcerative colitis (*n*=21)	
E1	2
E2	1
E3	18
IBDU (*n*=8)	
Pancolitis	7
Limited distal colitis	1

* Oral and/or perianal disease alone without luminal GI involvement is not recognised as a location phenotype in the Montreal Classification[2] but represents 5% of Scottish paediatric CD patients at diagnosis[141]. IBDU, colonic IBD, type unclassified

There was no difference (p=0.710) between CD, UC and IBDU patients, with these three types having median FC levels of 1258 μg/g (IQR 710-1671 μg/g), 1250 μg/g (IQR 925-2200 μg/g) and 1463 μg/g (IQR 898-2125 μg/g) respectively. There was no difference in median FC levels between those with upper intestinal CD location (1440 μg/g [IQR 885-2034 μg/g]) and those without (1220 μg/g [IQR 400-1340 μg/g]) as defined as the presence or absence of Montreal L4 disease (p=0.077), nor any difference observed when comparing median FC levels in CD patients with any ileal (L1 ± L4 or L3 ± L4) disease (1266 μg/g [IQR 875-1757

µg/g]) and those without (1295 µg/g [IQR 494-1832 µg/g]) (p=0.694). To allow meaningful numeric comparison of UC and IBDU disease location both groups were combined and each patient re-classified according to the newly described Paris classification of PIBD.[154] This demonstrated no difference (p=0.536) between those with extensive pancolonic disease (Paris E4, disease proximal to the hepatic flexure) and those with more limited disease (Paris E1-E3, all with disease of varying extent from the rectum yet distal to the hepatic flexure) who had median FC levels of 1480 µg/g (IQR 978-2135 µg/g) and 963 µg/g (IQR 691-2135 µg/g) respectively. Similarly, to evaluate all colonic IBD, CD patients with isolated colonic or ileo-colonic disease (L2 or L3 only) had a similar median FC level of 1230 µg/g (IQR 408-1421 µg/g) compared to those with UC and IBDU combined (1300 µg/g [IQR 925-2200 µg/g]) (p=0.324).

7.5.4 FC does not vary significantly between the diagnostic categories within the control group

The median (IQR) for each of the control group diagnostic categories is shown in **Table 7.3**. The miscellaneous group comprised children with functional abdominal pain (*n*=2), colonic polyps (*n*=2), gastritis (*n*=2), Meckel's diverticulum (*n*=1), pancreatic insufficiency (*n*=1), perianal abscess (*n*=1) and an as yet undiagnosed growth restriction syndrome (*n*=1). There was no difference in median FC values between the diagnostic categories (p=0.575) with all median values < 200 µg/g (except for the coeliac disease group which only had 3 members). The high third quartile within the allergic enteropathy group was as a result of the high FC level obtained in the 2-year-old girl mentioned above. As bleeding per rectum (PR) often leads to a differential diagnosis of PIBD the median FC in controls presenting with (*n*=48) and without (*n*=51) a history of PR blood was assessed, revealing no difference in median FC values of 60 µg/g (IQR 20-218 µg/g) and 65 µg/g (IQR 25-275) respectively (p=0.512). Additionally, no difference was demonstrated when correlating age with FC levels across all control group diagnostic categories (p=0.051, Spearman's rho=-0.197).

7.5.5 Medications do not seem to influence FC levels

Within each group 24% of PIBD cases and 33% of controls were on any oral medication at the time of FC sampling (p=0.219). Combining all patients, there was no difference (p=0.519) in median FC between those currently prescribed (275 µg/g [IQR 40-1265]) or not prescribed (385 µg/g [IQR 60-1275 µg/g]) any medication. Within the control group the median FC level of patients prescribed proton pump inhibitors (PPIs) (*n*=10) was 108 µg/g (IQR 20-240 µg/g) which was similar to those not on PPIs (*n*=89) (60 µg/g [IQR 21-240 µg/g]) (p=0.906).

7.5.6 FC performs better than commonly used blood parameters as a diagnostic biomarker during the evaluation of children with suspected IBD

To compare the performance of FC with six commonly used blood parameters, blood results taken at a similar time as the FC measurement were analysed (median time difference 6 days [IQR 1-28 days]; see **Table 7.2**). The availability of each blood parameter for each group is shown in **Table 7.7**.

Table 7.7. Availability of blood parameters measured in the IBD and control groups

	IBD group	Control group
Availability of individual tests [*n* (%)]		
Haemoglobin	89 (98)	91 (92)
Total white cell count	89 (98)	91 (92)
Erythrocyte sedimentation rate	87 (96)	83 (84)
Platelets	85 (93)	90 (91)
Albumin	89 (98)	83 (84)
C-reactive protein	87 (96)	85 (86)
All six blood parameters available	84 (92)	71 (72)

IBD, inflammatory bowel disease

The diagnostic accuracy of each blood parameter in comparison to FC is outlined in **Table 7.5**. **Figure 7.2** demonstrates that the area under the curve (AUC) for FC was greater than all six blood parameters at 0.93 (95% CI 0.89-0.97), and significantly higher than ESR ($p=0.011$), CRP ($p=0.006$), total WCC ($p<0.001$), Hb ($p<0.001$) and platelet count ($p<0.001$), but was not significantly greater than albumin ($p=0.374$). Further analysis of albumin as a predictor for IBD revealed that the optimum threshold was in fact 41g/L (within the normal paediatric reference range of 33-50 g/L), with the relevant diagnostic specificity using our normal lower limit of normal being far inferior (**Table 7.5**). However, by combining FC and serum albumin (using an optimised formula of [60 + FC/100 µg/g] - [serum albumin in g/L]) it can be seen that the AUC is improved at 0.96 (95% CI 0.93-0.99); however this was not significantly different to FC alone ($p=0.227$). Using the above formula and utilising a cut-off of 20 produced the following diagnostic accuracy indicators: sensitivity 0.97 (95% CI 0.90-0.99), specificity 0.81 (95% CI 0.71-0.89), negative predictive value 0.96 (95% CI 0.88-0.99) and positive predictive value 0.84 (95% CI 0.76-0.91).

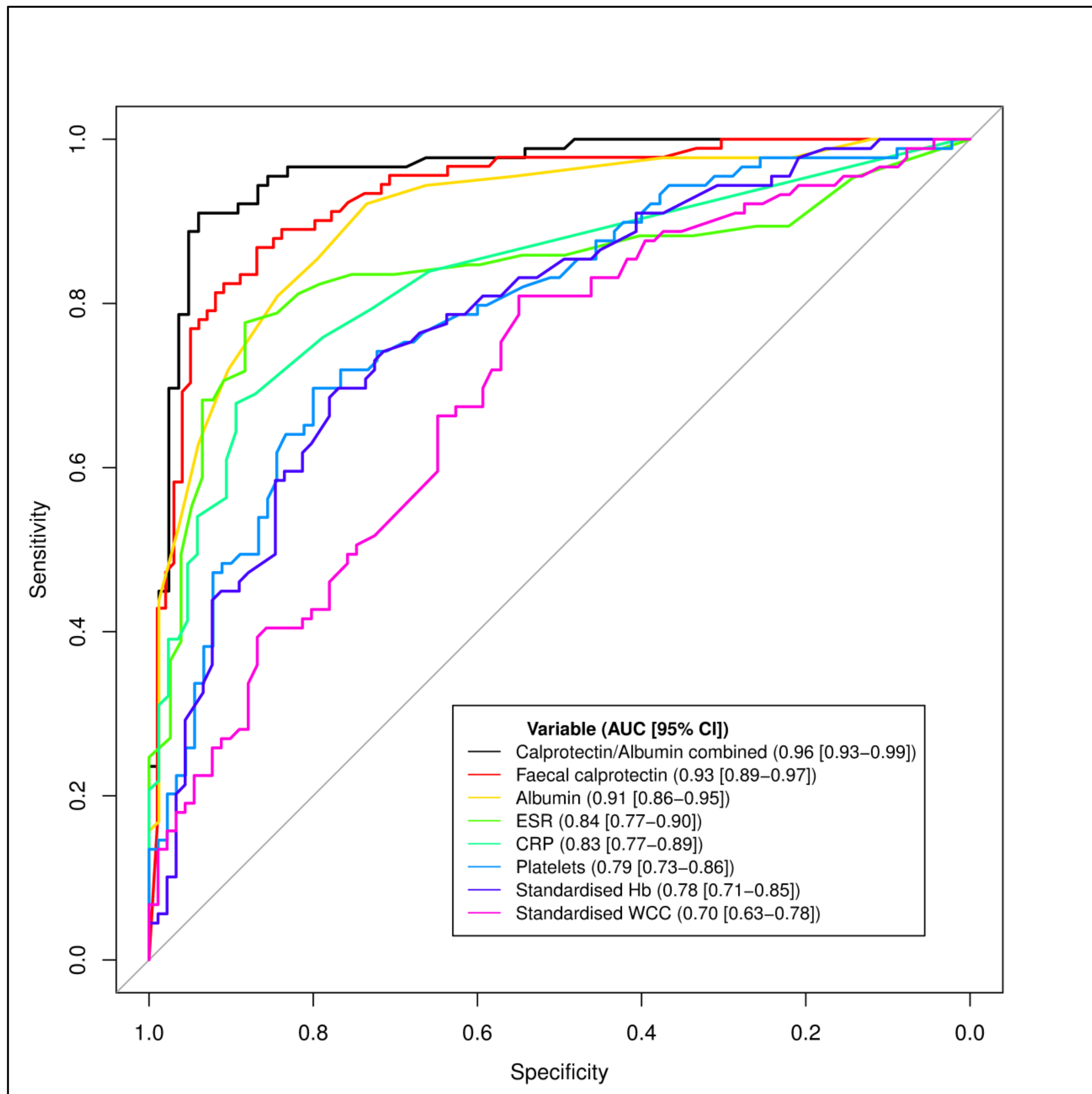


Figure 7.2. Receiver operating characteristic (ROC) curves and corresponding area under the curve (AUC) for faecal calprotectin and commonly measured blood parameters in paediatric patients with suspected IBD. AUC, area under the curve; CI, confidence interval; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; Hb, haemoglobin; WCC, white cell count.

7.6 Discussion

The results of this study demonstrate that FC is a highly useful biomarker during the initial investigation of suspected PIBD. Using only children presenting with suspected bowel inflammation, without any known GI diagnosis, and subsequently undergoing their first full endoscopic investigation, it has clearly been shown that FC is markedly raised in those with IBD, with no influence of IBD type or location. The study has also provided evidence that FC performs better than all commonly used blood parameters with an AUC of 0.93 (95% CI 0.89-0.97).

Although some would possibly argue that FC is not needed in a classical presentation of CD or UC, this data shows the utility of FC across the whole clinical spectrum of all types of IBD (e.g. those without luminal CD or with mainly extraintestinal symptoms) and, even more importantly, shows the potential utility of FC in deciding which children and teenagers presenting with possible gut inflammation may not need endoscopic assessment.

The extensive clinical use of FC as a biomarker in GI-related disease for over seven years within RHSCE leads to this study having several strengths in relation to study design and the analyses performed. Firstly, the regional IBD cohort is representative of the larger Scottish nationwide cohort with regard to demographic composition and disease location at diagnosis.[141] Previous studies often excluded younger children,[708,721] those with a high suspicion of IBD[711] and those on medications,[161] potentially skewing subsequent analyses. This is reflected in the modest pre-test probability in this study of 0.48 which is lower than reported in two recently published relevant papers on FC usage.[161,247] Additionally, previous groups often used a control group of patients with no known GI symptoms or signs to generate sensitivity and specificity,[708,712] which is certainly not applicable in a real world clinical setting of a paediatric GI service. In fact within this study a selection bias does occur from the entire cohort assessed using FC levels, but this bias works against the test as only sicker children with increased FC levels are likely to be referred to specialist GI services and undergo endoscopy. Secondly, only including those undergoing full endoscopy, and the reporting of small bowel imaging within the IBD group, has ensured robust phenotyping of all patients; many studies include only patients undergoing colonoscopy[705] or provide no details of endoscopic investigation[712]. Thirdly, the large study group presented here (n=190) has allowed further meaningful examination of particular sub-groups. Previous studies have frequently combined those with previously confirmed IBD[708] (therefore presenting a heterogeneous group with both *de novo* disease and established PIBD), or presented small numbers of each IBD type[161].

The retrospective design of this study does however produce potential limitations. Firstly, as the clinical utility of FC was not evaluated during the initial decision to perform endoscopic assessment it cannot be determined whether or not the FC level contributed to the individual gastroenterologist's choice to perform endoscopy. Similarly, the influence of the various blood parameters on the ultimate decision to perform endoscopy could not be elucidated. This is an important factor when assessing the usefulness of any biomarker in this context as prior knowledge of the FC result may have led to the avoidance of unnecessary endoscopic procedures, or conversely the delay in PIBD diagnosis (which may have occurred in two of the patients if FC level had been used in isolation at diagnosis). Secondly, during the study datawas collected within a time period of 6 months prior to endoscopy with other prospective studies standardising the sampling time (e.g. one week prior to

endoscopy) to potentially eliminate a confounding effect of variable disease activity on their results.[707] Although useful during the initial phases of determining the diagnostic accuracy of a certain biomarker, the approach taken here is more comparable with current clinical practice, with children often attending general practice and/or a general paediatrician with non-specific GI symptoms prior to assessment by a paediatric gastroenterologist. Delay to endoscopic assessment of suspected paediatric PIBD has been a major issue in many countries including the United Kingdom (UK), although changes in National Health Service (NHS) service design in Scotland has dramatically reduced this waiting time over the period from 2005 to 2011. Thirdly, another potential difference from previous studies is the use of a relatively low upper limit of FC assay. For example Perminow et al.[247] were able to demonstrate a significantly higher FC level in those with CD versus UC (1181 µg/g, range 11-6123 µg/g; 1250 µg/g, range 13-8625 µg/g respectively) due in all likelihood to their (undisclosed) higher reference range. Finally, two potential confounding factors in this study were the differing rates of TI biopsy in the IBD and control groups and the effect of oral medications. With regard to these aspects the analyses have shown that TI intubation rates were likely a result of the higher age of the IBD group, that removal of those without a TI biopsy did not change the main results of diagnostic accuracy and that TI intubation rates did not differ between those with and without a FC level available at endoscopy; it is acknowledged however that this study was likely not powered to look at the effects of drugs on FC levels.

Acknowledging the relative weakness of retrospective study design, there is confidence that (1) the robust choice of inclusions (i.e. only children presenting with suspected bowel inflammation, without known GI diagnosis, and subsequently undergoing their first ever endoscopic investigation); (2) the evaluation of all of the FC levels performed in the region over the six year study period; (3) the ability to review and follow up all suspected paediatric cases of gut inflammation in a defined geographical region (one child was initially placed in the "no pathology identified" control group but was very recently diagnosed as non-specific colitis and was re-assigned as such); and (4) the knowledge of all new regional cases of PIBD (within paediatric services in primary, secondary and tertiary care) to the end of October 2012, when taken together, add considerable confidence to the generalisability of these findings to all PIBD services worldwide.

Based on the clinical experience at RHSCE, laboratory guidance and relevant literature,[708] a FC cut-off of 200 µg/g has been routinely used as the threshold for the suspicion of a new PIBD diagnosis, accepting the need for full clinical history, examination and other blood tests. This has been validated by the results with the Youden index suggesting a FC level between 200-300 µg/g providing an optimum sensitivity/specificity. Although several newly described serum markers have been identified as possible markers of IBD, these are often

present at higher levels in certain sub-phenotypes[722] or are only available as research tools[723]. Commonly measured blood parameters remain the investigations of choice, with ESR[155] and CRP[724] currently providing the best indication of possible IBD, but have a relatively poor diagnostic accuracy, especially specificity. As discussed above, previous work by a group in SES demonstrated that the use of common blood tests could be enhanced significantly by the inclusion of FC in a "panel" of inflammatory markers during the investigation of suspected IBD.[159] This has been further enhanced with the development of a combined FC and serum albumin score described above, which provided a better (although not statistically significantly) AUC than FC alone.

GI endoscopic assessment is difficult for children, with the need for hospital or day-case admission. For the children this involves fasting, bowel preparation (if undergoing lower GI evaluation) and anaesthesia/sedation (admission to a day-case unit and total intravenous anaesthesia usage is the current design for elective procedures at RHSCE). For their parents, anxiety and time away from employment or dependent younger children is also a potential issue. It is also expensive, with a wide variation in training and resources across different countries.[725] By comparison FC can be obtained by providing the family with instructions, a sample pot, a prefilled laboratory request form and suitable packaging for postage to the appropriate IBD unit. It is relatively cheap in the UK; currently, the NHS laboratory pays £485 per kit (PhiCal™ Test) equating to £6.70 per sample. NHS requests are charged at £24 per test as this currently used test remains relatively labour intensive (Dr Kathleen Kingstone, personal communication).

7.7 Conclusion

This study has shown that FC is significantly raised in children with IBD compared with non-IBD, scoped controls, and that FC provides greater diagnostic accuracy than other commonly used blood parameters. The characteristics of both groups and the timing of their investigations represent a true reflection of the investigative procedures carried out in children with suspected bowel inflammation and it is suggested that FC should now be used routinely during the initial assessment of these children. Further studies are now required to fully determine the effect of FC measurement on endoscopy rates, with the potential to reduce the number of children undergoing endoscopic assessment for suspected IBD, therefore reducing costs,[726] streamlining paediatric GI endoscopy services, and reducing both child and family distress and inconvenience.

8 Faecal calprotectin for the diagnosis of paediatric inflammatory bowel disease: a systematic review and meta-analysis

8.1 Introduction

In **Chapter 7** it was demonstrated that faecal calprotectin (FC) performs better than any of the commonly used blood parameters (i.e. C-reactive protein, erythrocyte sedimentation rate, platelet count, haemoglobin, total white cell count and albumin) with regard to diagnostic accuracy in paediatric inflammatory bowel disease (PIBD). A previous meta-analysis evaluating the role of FC during the initial investigation of suspected IBD concluded that FC was a useful screening tool to identify patients requiring endoscopic assessment, however the discriminative power to safely exclude IBD was significantly better in adults than in children.[162] Pooled sensitivity in adults was 0.93 (95% confidence interval [CI] 0.85-0.97) and pooled specificity 0.96 (95% CI 0.79-0.99); the corresponding values in children were 0.92 (95% CI 0.84-0.96) and 0.76 (95% CI 0.62-0.86) respectively. In this analysis the included paediatric studies presented FC levels in a total of 226 IBD patients, with several of the included patient cohorts containing a combination of both new and established cases of PIBD. Since the publication of this meta-analysis, whose literature search ended in October 2009, several studies assessing the diagnostic accuracy of FC in PIBD have been published, including the data described in **Chapter 7**.

8.2 Hypothesis and Aim

Hypothesis: The addition of newly identified studies of diagnostic accuracy of FC published since October 2009, in addition to the strict inclusion of only patients undergoing their *primary* investigation for suspected PIBD, will improve the pooled sensitivity and specificity of FC in PIBD diagnosis.

Aim: The aim of this study was therefore to re-evaluate the diagnostic accuracy of FC for IBD in the paediatric population by robust systematic review and meta-analysis.

8.3 Methods

8.3.1 Literature search

Inclusion criteria were retrospective or prospective case-control studies utilising FC (index test) during the diagnostic workup of children with suspected bowel inflammation who underwent at least colonoscopy. It should be noted however that for PIBD both ileocolonoscopy and upper endoscopy are recommended[154] and therefore during the assessment of methodological quality this was considered the reference standard. An electronic search was conducted using OVID Medline (1946 – May Week 3 2012) and Embase (1974 - Week 25 2012) using a detailed, comprehensive search strategy outlined in **Table 8.1**. In addition to this, relevant keywords in PubMed (www.ncbi.nlm.nih.gov/pubmed), Google Scholar™ (www.scholar.google.co.uk) and the Cochrane Library were also used to ensure the robust identification of articles still undergoing indexing. A hand search of articles was also performed, drawn from reference lists of retrieved articles, personal collections and meeting abstracts; only papers published in full were considered for inclusion. There was no English language restriction and, to ensure no relevant study was excluded, all foreign language papers were retrieved in full text and translated using Google Translate™ (www.translate.google.co.uk). No paediatric filters were used in the initial search.

8.3.2 Study identification and data acquisition

Following the completion of the initial literature searches and study selection, the potentially relevant citations were imported into Endnote version X2.0.4 (Thomson Reuters, NY, USA) and the full papers obtained. Studies were then evaluated independently for eligibility by two reviewers (Dr Paul Henderson and Prof. David C Wilson, University of Edinburgh) with any discrepancy resolved by discussion. From each included study the following data was extracted and entered into a customised database in Microsoft Access 2007 (Microsoft Corporation, WA, USA): first author, year of study publication, country of origin, language, age range of study subjects, number of IBD cases (sub-divided into Crohn's disease [CD], ulcerative colitis [UC] and colonic IBD, type unclassified [IBDU]), number of non-IBD cases, FC assay used and FC normal cut-off value. To allow the robust inclusion of all appropriate studies corresponding authors were contacted via email to clarify certain parameters if uncertainty existed, especially in relation to the construction of diagnostic 2x2 tables. Following confirmation of the number of patients presenting with *newly suspected* bowel inflammation, this data was also entered into the results database.

Table 8.1. Detailed Medline search strategy used to gain high sensitivity and precision for detecting diagnostic accuracy studies of faecal calprotectin.

1	exp inflammatory bowel diseases/
2	ibd.mp.
3	exp ileitis/
4	(ulcerative colitis or proctocolitis or proctosigmoiditis or rectocolitis or rectosigmoiditis or ulcerative rectocolitis or ulcerative proctocolitis or hemorrhagic ulcerative or hemorrhagic proctocolitis or proctitis or haemorrhagic ulcerative or haemorrhagic proctocolitis or crohn$ disease or ileitis or regional enteritis or granulomatous ileocolitis or ileocolitis or granulomatous colitis).mp.
5	4 or 1 or 3 or 2
6	exp Leukocyte L1 Antigen Complex/ or faecal calprotectin.mp. Included Entry terms: • Calcium-Binding Myeloid Protein P8,14 • Calcium Binding Myeloid Protein P8,14 • Calgranulin • Calprotectin • Migratory Inhibitory Factor-Related Protein MRP • Migratory Inhibitory Factor Related Protein MRP • Myelomonocytic Antigen L1 • Antigen L1, Myelomonocytic • L1 Antigen • Antigen, L1 • 27E10 Antigen • Antigen, 27E10 • Leukocyte L1 Protein • L1 Protein, Leukocyte
7	calprotectin.mp.
8	calgranulin.mp.
9	6 or 7 or 8
10	5 and 9

8.3.3 Assessing methodological quality of included studies

In order to assess the methodological quality of the individual studies a modified version of the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool was used,[727,728] which is recommended by the Cochrane Diagnostic Reviewers' Handbook[729]. This modified version of the QUADAS tool consists of 11 items concerning methodological characteristics that have the potential to introduce bias. From these 11 items the most relevant 8 sections were coded. Also, in line with the Cochrane Collaboration recommendations,[729] three relevant supplementary items were added. All items were coded as 'positive' (i.e. no bias), 'negative' (i.e. potential bias) or 'unclear'; disagreements were resolved by consensus. No summary quality score was calculated in line with previous concerns regarding their validity.[730] Details of the included and additional items, and how each was coded, are shown in **Box 8.1**.

Box 8.1. Details of QUADAS scoring based on the Cochrane Handbook[729]

<u>Included items</u>

1. Representative spectrum

Recruitment of children less than 18 years of age presenting with symptoms suggestive of bowel inflammation and undergoing primary investigation with no previous diagnosis of PIBD should have occurred. Inclusion from the right patient group above should have yielded a representative sample of patients who would receive the test in practice. That is, a majority of patients with Crohn's disease, the inclusion of patients with IBDU and a male preponderance.[141,731]

- Yes - both the right patient group was recruited and a representative sample of patients yielded following recruitment
- No - either the right patient group and/or a representative sample not present
- Unclear - insufficient information to determine spectrum

2. Reference standard

Upper <u>and</u> lower endoscopy with or without terminal ileum intubation should have been performed (consensus reference standard being the Porto Criteria[147]). Although the Porto Criteria state that ileocolonoscopy is essential they recognise that this is not possible in 100% of cases and emphasise that ileal intubation should be 'attempted'.

- Yes - both upper and lower endoscopy performed
- No - only upper OR lower endoscopy performed
- Unclear - insufficient information to determine the reference standard used

3. Disease progression

In view of the chronic nature of PIBD, the frequent delay in diagnosis[231] and diverse/non-specific presenting symptoms and signs, a delay of 3 months between calprotectin sampling and endoscopy was deemed acceptable.

- Yes - time between index and reference tests is shorter than 3 months, or at least for an acceptably high proportion of patients
- No - time between index and reference tests was longer than 3 months for an unacceptably high proportion of patients
- Unclear - Information of the timing of tests is not given or no clear

4. Partial verification

The entire cohort (or random selection) of patients (that is all patients suspected of bowel inflammation) underwent both upper and lower endoscopy.

- Yes - all included patients underwent upper and lower endoscopy
- No - not all patients underwent upper and lower endoscopy
- Unclear - not clear if all patients received reference test

5. Differential verification

Patients should have received the same reference standard (i.e. upper and lower endoscopy) irrespective of the index test result.

- Yes - same reference standard used in all patients
- No - choice of reference standard varied between individuals
- Unclear - unclear what reference standards were used

6 and 7. Test and diagnostic review
The endoscopy results were interpreted without knowledge of the calprotectin results and the calprotectin results interpreted without knowledge of the endoscopy results?
- Yes - test results were interpreted blinded to the results of the other test
- No - clear that one set of test results was interpreted with knowledge of the other
- Unclear - unclear whether blinding took place

8. Withdrawals
All patients initially eligible for the study (i.e. patients <18yrs with suspected bowel inflammation with a stool sample obtained for faecal calprotectin) were accounted for.
- Yes - clear what happened to all patients
- No - not all patients accounted for
- Unclear - it is not clear how many patients entered the study and hence whether there were any withdrawals

Additional items

9. Clear definition of a positive result
The study provided a clear definition of what was considered to be a 'positive' result?
- Yes - clear definition of a positive result given
- No - no clear definition of a positive result
- Unclear - not clear what a positive result would mean in the context of the study

10. Treatment withheld until index test and reference standard performed
Treatment was withheld until both the index test (faecal calprotectin) and the reference standard (upper and lower endoscopy) were performed.
- Yes - treatment withheld until both tests performed
- No - treatment started prior to both tests being performed
- Unclear - not clear if treatment started prior to both tests being performed

11. Was the study free from commercial funding?
Evidence that commercial funding or influence was present during the study, especially with relation to the calprotectin assays used.
- Yes - no evidence of commercial funding during the study
- No - evidence of commercial funding during the study
- Unclear - no statement of funding given

8.3.4 Statistical analysis

Following the robust construction of the diagnostic 2x2 tables, specificity, sensitivity and 95% CI for each of the included studies was calculated using the epiR package in R v2.14.1 (R Foundation for Statistical Computing, Vienna, Austria).[716] A hierarchical summary receiver operating curve (ROC) model was fitted by the HSROC package[732] in R to provide a summary ROC curve, and to allow derivation of pooled sensitivity and specificity estimates. This Bayesian model is of similar form to that proposed by Rutter and Gatsonis,[733] but uses a probit link rather than a logit link function and is also fitted using a Gibbs sampling approach. The Gibbs protocol was to collect 100,000 iterations after a

10,000 iteration burn-in period, and then to estimate the model's parameters as the median of those obtained from sampling every hundredth realisation. Trace plots (the 1000 realisations of each parameter returned by the Gibbs sampler) were checked to ensure that the Markov Chain had achieved equilibrium, and therefore that the median estimate was appropriate in each case. Vague priors (Uniform distributions with wide ranges) were used for between-study parameters. This analysis was carried out by Dr Niall Anderson (Centre for Population Health Sciences, University of Edinburgh). As suggested by the Cochrane Diagnostic Test Accuracy group (http://srdta.cochrane.org/), no analysis of study heterogeneity was performed, as these tests do not account for heterogeneity explained by phenomena such as positivity threshold effects.

8.4 Results

8.4.1 Study selection

Using the detailed search strategy outlined above, 289 abstracts were identified from the OVID Medline/Embase search and 381 abstracts retrieved from a detailed search of PubMed, Google Scholar™, the Cochrane Library and the hand search of references. Following a thorough review of these abstracts and the exclusion of duplicates a total of 106 papers were imported into Endnote for evaluation. The majority of these papers were published in English (*n*=96) with three in Polish,[734-736] two in French,[737,738] two in Spanish[739,740] and one each published in Dutch,[741] Japanese[742] and Russian[743]. After the identification and exclusion of papers not presenting primary data (*n*=7) the full text of all 99 remaining articles were obtained through the University of Edinburgh or NHS Knowledge Network library resources, or by direct contact with the corresponding author(s). Following review of the full text articles a total of 14 studies[160,705,707,708,710,711,715,721,739,744-748] were considered for potential inclusion.

In six of the 14 potential studies it was unclear if the subjects represented only children undergoing their initial diagnostic work up for suspected PIBD,[707,708,715,721,745,748] therefore *all* corresponding and/or senior authors were contacted via email for clarification. Authors representing three studies kindly provided their complete raw study data,[707,708,745] however insufficient detail was present to allow the robust identification of children with *de novo* disease and these studies were therefore excluded (after agreement with their respective authors). Although the 2007 study by Fagerberg et al.[721] included both known and *de novo* PIBD patients (therefore considered for potential inclusion pending the availability of the raw data) it was deemed after discussion with the authors that this study contained an unknown number of patients from their earlier publication (Fagerberg et al. 2005[710]) and it was

therefore excluded on this basis. The authors of the remaining two studies were contacted and kindly responded but were unable to provide their primary data and these were therefore also excluded.[715,748] An outline of the study selection process is given in **Figure 8.1**.

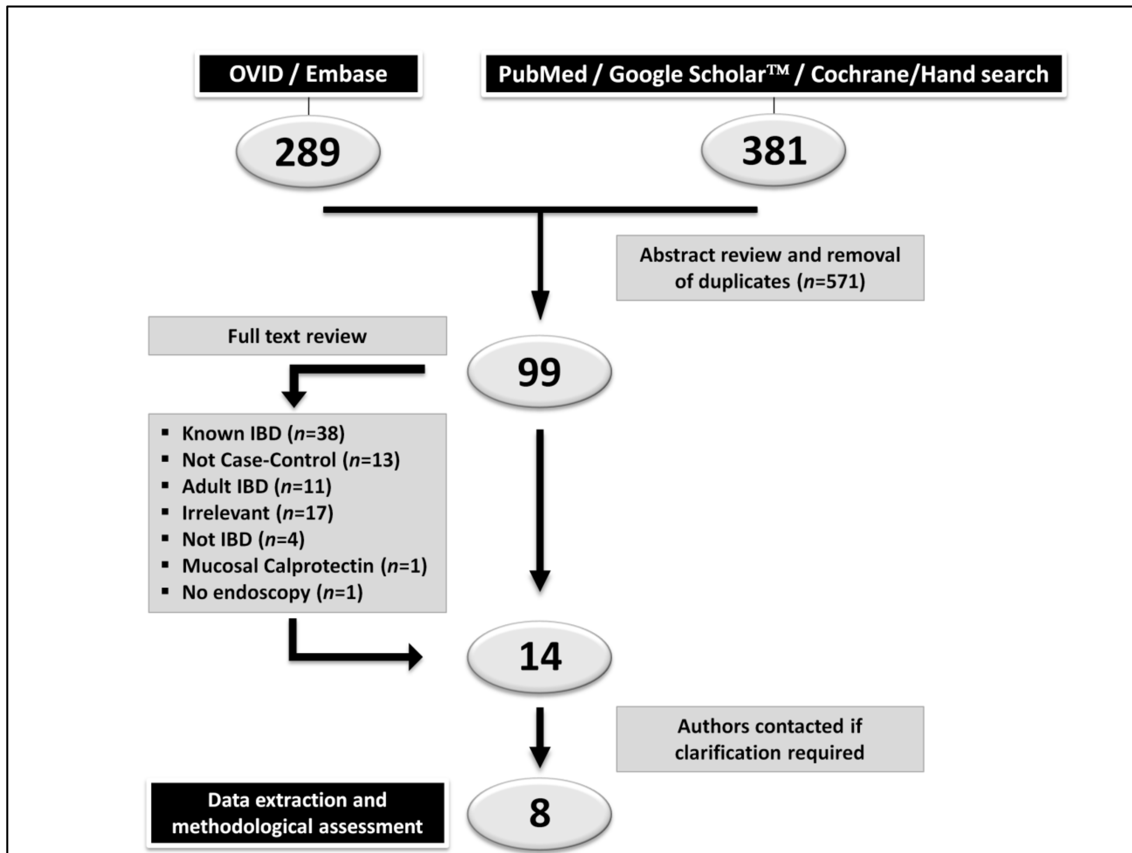


Figure 8.1. Flow diagram showing the process of selecting paediatric studies of diagnostic accuracy of faecal calprotectin that included only children undergoing their primary investigation for suspected inflammatory bowel disease.

8.4.2 Baseline data of included studies

The eight remaining studies included five of the seven studies from the previous meta-analysis[160,710,711,746,747], one non-English language manuscript[739] and two larger studies published since 2009,[705,744] which included the data presented in **Chapter 7**. Baseline data was extracted from the manuscripts or obtained directly from the authors; the details of each study are presented in **Table 8.2**. (It should be noted that this data represents all the patients in each study, not necessarily those with FC results available; this is discussed below). All 8 studies (containing a total of 715 cases and controls) were published in the last seven years in countries with developed health care services (Europe and Australia) with seven studies performed in large tertiary referral centres and one population-based study.[746] The median

number of PIBD cases in each study was 34 (range, 12-117; IQR 25-69) and the median number of total study participants was 55 (range, 36-197; IQR 45-123). All studies used either the Phical® (Calpro AS, Lysaker, Norway) or Calprest® (Eurospital, Trieste, Italy) FC assay with normal cut-off values of either 50μg/g or 100μg/g.

Table 8.2. Details of the eight faecal calprotectin diagnostic accuracy studies (715 cases and controls) included in the meta-analysis.

First Author (Year of Publication)	Country of Origin	PIBD cases (Prev [%])	PIBD Type			Cont	Age range (Years)	Assay Type	FC cut-off (μg/g)
			CD	UC	IBDU				
Fagerberg (2005)	Sweden	20 [55]	10	7	3	16	6.0 - 17.0	Phical	50
Berni Canani (2006)	Italy	27 [60]	17	10	0	18	6.5 - 18.0*	Phical	50
Bonnín Tomàs (2007)	Spain	12 [28]	10	2	0	31	0.4 - 15.3	Calprest	50
Sidler (2008)	Australia	31 [51]	30	1	0	30	2.2 - 16.0	Phical	50
Ashorn (2009)	Finland	37 [77]	13	21	3	11	2.7 - 19.9	Phical	100
Perminow (2009)	Norway	62 [62]	39	19	4	38	0.8 - 17.9	Phical	50
Diamante (2010)	Italy	117 [59]	49	68	0	80	1.0 - 18.0	Calprest	100
Henderson (2012)	Scotland	91 [48]	62	21	8	99	1.3 - 17.7	Phical	50

*Data obtained directly from the authors as not presented in the original manuscript. PIBD, paediatric inflammatory bowel disease; Prev, PIBD prevalence; CD, Crohn's disease; UC, ulcerative colitis; IBDU, colonic inflammatory bowel disease, unclassified; Cont, controls; FC, faecal calprotectin.

8.4.3 Assessment of methodological quality of included studies

The eight studies underwent quality assessment using the QUADAS tool as discussed above; a summary of the results are presented in **Figure 8.2**. Only three studies were deemed to have a representative spectrum of patients, with the inclusion of a majority of CD patients and patients with IBDU. Similarly, three studies described upper and lower endoscopy in all patients (with one study presenting terminal ileum intubation rates), with three studies not clearly defining the type of endoscopy performed. Interestingly only two studies reported delay in FC sampling, and only two demonstrated blinding of both reference standard to index test and vice versa. All but two papers explained withdrawals (one of which was the population-based study) and only one had no clear definition of a positive

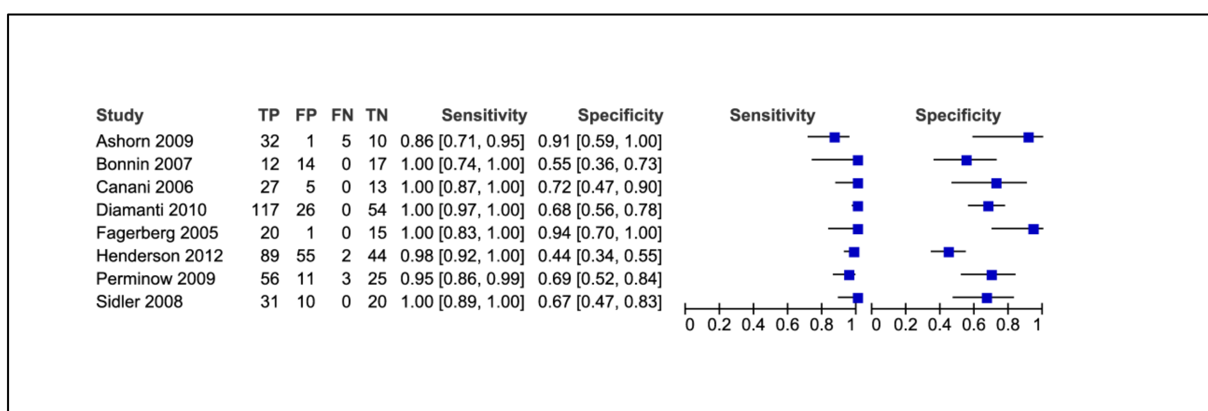
result evident in the manuscript. Of note, only one paper commented on whether FC samples were obtained prior to commencing treatment, which may be a major confounder in reports of diagnostic accuracy. There was no evidence of commercial funding in any of the studies, however three manuscripts did not explicitly have a funding statement to verify this.

	Representative spectrum?	Acceptable reference standard?	Acceptable delay between tests?	Partial verification avoided?	Differential verification avoided?	Reference standard results blinded?	Index test results blinded?	Withdrawals explained?	Clear definition of a positive result?	Treatment withheld until both tests performed?	Free from commercial funding?
Ashorn 2009	−	+	?	+	+	?	?	−	+	?	+
Bonnin 2007	−	?	?	?	?	?	?	+	+	?	?
Canani 2006	−	?	?	?	?	?	?	+	+	?	?
Diamanti 2010	−	?	?	−	+	+	+	+	+	?	?
Fagerberg 2005	+	−	+	−	+	+	+	+	+	?	+
Henderson 2012	+	+	+	+	+	?	−	+	+	+	+
Perminow 2009	+	−	?	−	−	?	?	−	−	?	+
Sidler 2008	−	+	?	+	+	?	?	+	+	?	+

Figure 8.2. A summary of the methodological assessment of the eight included case-control studies that evaluated the diagnostic accuracy of faecal calprotectin during the investigation of suspected paediatric inflammatory bowel disease. +, no bias, -, potential bias, ?, unclear.

8.4.4 Diagnostic 2x2 tables and individual study results

As each of the eight studies now included only children presenting with *de novo* disease, the original manuscripts were examined to determine the number of true positives, false positives, true negatives and false negatives. For three of the studies exact data extraction was either not possible[746] or not clear[711,747] despite thorough examination of the manuscript and therefore the authors were contacted directly by email to clarify the number of PIBD and non-PIBD cases with abnormal FC levels (i.e. >50µg/g). All queries in this respect were answered fully with reference to their raw data by the relevant three authors and 2x2 diagnostic tables were constructed. In one study the authors highlighted that the original published figure depicting their calprotectin results was in fact inaccurate and therefore provided the exact data for clarification.[711] It should be noted that in the population-based study by Perminow et al. a total of five patients did not have a FC level available (three PIBD patients and two non-PIBD controls) and therefore the sensitivity and specificity calculations for this study include only 95 of the original 100 patients in the cohort.[746] The numbers of children used to populate the 2x2 diagnostic tables is presented in **Figure 8.3** together with a forest plot of the sensitivities and specificities corresponding to each individual study.



Study	TP	FP	FN	TN	Sensitivity	Specificity
Ashorn 2009	32	1	5	10	0.86 [0.71, 0.95]	0.91 [0.59, 1.00]
Bonnin 2007	12	14	0	17	1.00 [0.74, 1.00]	0.55 [0.36, 0.73]
Canani 2006	27	5	0	13	1.00 [0.87, 1.00]	0.72 [0.47, 0.90]
Diamanti 2010	117	26	0	54	1.00 [0.97, 1.00]	0.68 [0.56, 0.78]
Fagerberg 2005	20	1	0	15	1.00 [0.83, 1.00]	0.94 [0.70, 1.00]
Henderson 2012	89	55	2	44	0.98 [0.92, 1.00]	0.44 [0.34, 0.55]
Perminow 2009	56	11	3	25	0.95 [0.86, 0.99]	0.69 [0.52, 0.84]
Sidler 2008	31	10	0	20	1.00 [0.89, 1.00]	0.67 [0.47, 0.83]

Figure 8.3. 2x2 diagnostic accuracy table and forest plot for the eight included studies evaluating the diagnostic accuracy of faecal calprotectin during the investigation of suspected paediatric inflammatory bowel disease. TP, true positive; FP, false positive; FN, false negative; TN, true negative. Numbers in brackets represent 95% confidence intervals.

8.4.5 Diagnostic accuracy meta-analysis

The total number of children in the final meta-analysis was 715, which included 394 PIBD patients and 321 non-PIBD controls. This represented a 74% increase in the number of included PIBD patients and a 93% increase in the total number of patients (i.e. both PIBD and non-PIBD controls) compared to the previous meta-analysis.[749] Pooled sensitivity and

specificity for the diagnostic accuracy of FC in suspected PIBD were 0.978 (95% CI 0.947-0.996) and 0.682 (95% CI 0.502-0.863) respectively. The positive likelihood ratio was 3.074 and negative likelihood ratio 0.0324. **Figure 8.4** presents the diagnostic values of the studies in a hierarchical summary receiver operating characteristic graph.

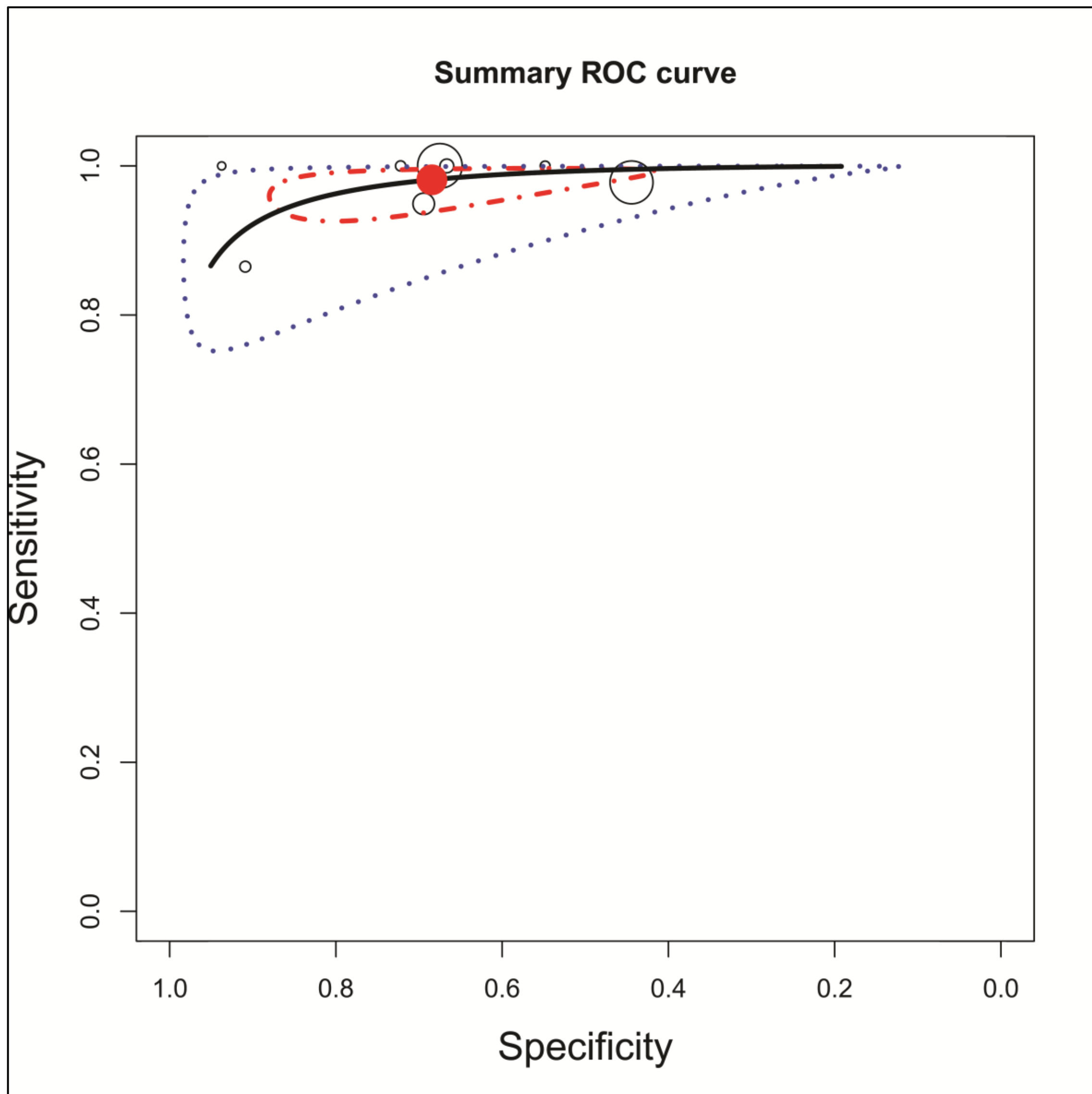


Figure 8.4. Hierarchical summary receiver operating characteristic graph for the eight included studies evaluating the diagnostic accuracy of faecal calprotectin during the investigation of suspected paediatric inflammatory bowel disease. Solid red dot, pooled sensitivity and specificity; red dotted line, 95% credible region (Bayesian equivalent of confidence interval); blue dotted line, 95% prediction region for future sensitivity/specificity values of studies in this context.

8.5 Discussion

Through robust systematic review and appropriately performed meta-analysis it has been shown that FC has extremely high sensitivity (97.8%) and modest specificity (68.2%) for PIBD during the investigation of children with suspected bowel inflammation. With regard to the initial hypothesis, the inclusion of two larger studies published since October 2009 and the strict selection of only children undergoing their primary investigation for PIBD, has in fact increased the sensitivity but marginally reduced the specificity compared to the original meta-analysis by van Rheenen et al.[162] The assessment of methodological quality determined that there were deficiencies in all the studies evaluated, but especially with regard to important aspects such as the use of a representative spectrum of patients, an acceptable reference standard (upper and lower endoscopy) and the poor reporting of current treatment modalities in use during FC sampling.

With regard to improving PIBD diagnosis, biomarkers present in serum and faeces have attracted the most attention to date.[750] In **Chapter 7** it was demonstrated that FC performed better than all of the commonly used blood markers; previous work in a smaller number of the same cohort showed that FC was also better than all the blood markers combined.[159] As alluded to briefly in **Chapter 1**, there is currently no consistent body of work on any biomarker during PIBD diagnosis, although small case series and markers such as ANCA, ASCA and lactoferrin have been intermittently trialled. Without doubt the clear advantage of a quality biomarker such as FC is that screening is performed in conjunction with the initial clinical assessment and often before invasive procedures such as endoscopy and radiological imaging.

This study has several strengths with regard to its methodology and analysis. Using a robust electronic search strategy in multiple databases (with the augmentation of the search through the use of PubMed and Google Scholar),[751] the use of free text words within these databases,[752,753] and the inclusion of non-English language manuscripts[754] allowed the identification of the appropriate studies for inclusion (i.e. high sensitivity). In addition the detailed email discussions with the authors of potential papers ensured that the included studies contained only paediatric patients with *de novo* disease rather than cohorts of new and established PIBD cases, ensuring the robustness of the final pooled data. In line with current practice, summary scores during the QUADAS assessment were not used and the statistical analysis involved in calculating pooled sensitivity and specificity also took into account different cut-off values for defining a positive test during the meta-analysis.[755]

It is also recognised that several potential limitations exist in the analysis, arising mainly from the variable quality of the included studies discussed above. The composition of study

cohorts was inconsistent, often with the inclusion of children less than one year,[715,739,746] or the exclusion of children with very-early onset disease (i.e. less than six years old)[710,711]. Also the PIBD groups contained either an overwhelming majority of one IBD type such as CD[747] or no patients with IBDU[705,711,739,747]. The prevalence of PIBD in each study was also variable (28-77%) suggesting that bias was present at study recruitment; tertiary centre referral bias was also likely to have influenced outcome.[756]

To date there have been two meta-analyses of the diagnostic accuracy of FC during the investigation of suspected IBD.[162,757] van Rheenen et al. performed a comprehensive meta-analysis of FC diagnostic accuracy studies in both adults and children with a literature search that ended in October 2009.[162] Using a robust method of identifying relevant studies and sound statistical methods they identified 13 eligible studies (six adult and seven paediatric) published between 2000 and 2009. A thorough electronic search strategy and clear definitions of their QUADAS scoring were presented, however despite claims that authors were contacted in cases where information was missing (specifically regarding 2x2 tables), two paediatric studies that included children with known PIBD were included in the final analysis.[707,745] Additionally, of the remaining five studies, anomalies in the 2x2 tables were also apparent (based on our detailed discussions with all authors outlined above). The conclusions of the paediatric meta-analysis (i.e. sensitivity 92%, specificity 76%) were therefore erroneous, and the reported statistical superiority of the pooled specificity of the adult data over the paediatric data may have therefore not existed. Prior to this analysis, von Roon et al. had performed a systematic review and meta-analysis of studies published to March 2006 that compared FC with a histological diagnosis of CD, UC or colo-rectal cancer.[757] Again the authors used appropriate search strategies but sensitivity and specificity were pooled separately; additionally studies that included a healthy control group (which has been shown to increase the accuracy of a diagnostic study[758]) were included in the final analysis. Combining all studies of diagnostic accuracy and studies that compared FC levels of known IBD patients from non-IBD patients they concluded that in children a FC cut-off of 50μg/g gave a sensitivity of 0.83 (95% CI 0.73-0.90) and specificity of 0.85 (95% CI 0.77-0.91); comparable results for a cut-off of 100μg/g were 0.98 (95% CI 0.94-1.00) and 0.97 (95% CI 0.92-0.99) respectively. However, in view of the limitations discussed and the heterogeneity of the populations studied, the accuracy of this analysis should certainly be questioned.

As discussed briefly in **Chapter 7** the effect of the use of FC on endoscopy is certainly of interest. van Rheenen et al. calculated that the use of FC as a screening tool for IBD would reduce endoscopy rates by 67% in adults and 35% in children.[162] In another recently published study, referring clinicians were blinded to the calprotectin result in 68 children presenting with a clinical suspicion of PIBD.[759] Their analysis revealed that using the 'clinical

eye' of the paediatrician would have resulted in 38% of children undergoing IBD-negative ileo-colonoscopy, with this figure dropping to 32% if a FC level of 50μg/g was used, and reducing further still to 22% using a FC level of 50μg/g in the absence of GI infection. The authors however acknowledged the limitations to their study with regard to the lack of reference standard in all patients and the paucity of data regarding drug use in the study participants. Mention is also given to the difficulties surrounding false positives and false negatives. It has been documented that calprotectin can be elevated in other conditions such as cystic fibrosis[760] and enthesitis-related arthritis[761] and therefore a negative endoscopy for IBD does not always indicate failure of FC as a screening test. With regard to cost analysis, there have been no comprehensive studies to date, however limited data on actual or potential endoscopy reduction suggest a significant benefit.[726,759]

Besides the use of FC during diagnosis there is also a growing body of literature with regard to its use in disease activity monitoring. Although studies have shown the lack of correlation of FC levels with disease location (**Chapter 7**),[762] and clinical disease activity scores,[762,763] there is certainly evidence that FC does have some utility in distinguishing endoscopic activity and mucosal healing.[763,764] Extrapolating from this data it is likely that, with increasing use in the clinical setting, that a combination of symptoms and signs, serum markers and calprotectin will prove the most fruitful with regard to assessing ongoing intestinal inflammation in IBD patients.

8.6 Conclusion

Following on from work presented in **Chapter 7** it can be seen that, despite some limitations in the case-control studies to date, a robust meta-analysis of 715 patients undergoing their primary investigation of suspected PIBD has demonstrated that FC has a very high sensitivity (98%) and modest specificity (68%) for PIBD at diagnosis. With work from other groups already showing that FC is a useful tool for the clinician with regard to screening children with suspected bowel inflammation, especially in the context of other clinical findings, its routine addition to the investigations carried out in children with suspected intestinal inflammation is likely to increase. However, it has yet to be seen how this biomarker will perform with regard to reducing endoscopy rates and the subsequent cost-benefit, and how it will fare during the assessment of children with known PIBD.

9. The role of autophagy in Crohn's disease

9.1 Introduction

Major CD susceptibility pathways uncovered through recent genome-wide association studies (GWAS) implicate the innate immune response (e.g. *NOD2*), the more specific, acquired T cell response (e.g. *IL23R*, *ICOSLG*) and autophagy (e.g. *ATG16L1, IRGM*).[598,765] Examination of the disease-associated microbiome has also alluded to several potentially contributory organisms, most notably adherent-invasive *E.coli* strains (AIEC), which have been isolated by independent investigators in both adult[766] and paediatric[767] CD patients. This chapter will discuss the emerging role of autophagy in CD, with particular focus on enteric *E.coli* strains with an adherent and invasive phenotype (AIEC).

9.2 (Macro)-Autophagy

Two cellular processes, the ubiquitin-proteasome pathway and autophagy are involved in the removal of redundant, misfolded and harmful proteins.[768,769] Unlike the proteasome, autophagy can target large protein aggregates and also has the ability to degrade deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and lipids.[770] Ultimately all autophagic pathways converge at the lysosome, a central, acidic organelle that harbours lysosomal hydrolases such as peptidases and nucleases.[771] Three major types of autophagic mechanisms have been described in mammalian cells, namely chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. CMA is estimated to degrade approximately 30% of cytosolic proteins, specifically targeting proteins bearing a motif in their amino acid sequence biochemically related to the pentapeptide KEFRQ.[772] This pathway is mediated by heat shock cognate 70 and degradation occurs following interaction with lysosomal-associated membrane protein 2.[773] Microautophagy refers to the direct engulfment of the cytoplasm by the lysosome.[774] This process has been shown to occur in five main stages, namely microautophagic invagination, followed by vesicle formation, expansion, scission and degradation.[774] Although discovered in the 1960s,[775] many of the mechanisms involved in microautophagy are still unclear and its role in disease is still to be determined. Although several other forms of autophagy are now being investigated,[774] it is the process of macroautophagy which has gained most attention in the field of disease pathogenesis.[776] Macroautophagy (hereafter referred to as autophagy) differs from CMA and microautophagy in that it relies on specialised double membrane vesicles called autophagosomes that form *de novo* through the assembly of protein and lipid constituents.

Enigmatic for many years, it is now clear that autophagosomes originate from the surface of membranes of various cell organelles, including the endoplasmic reticulum, mitochondria and the plasma membrane.[777]

Autophagy is upregulated in response to many stimuli including starvation, genotoxic stress and microbial infection,[778] and is controlled by the ATG proteins (or autophagy-related proteins). The process can be divided into three distinct stages: vesicle nucleation, vesicle elongation and fusion of the autophagosome with a lysosome (**Figure 9.1**). The further detailed and complex molecular machinery involved in autophagosome formation is discussed further in **Chapter 11** , however a summary of the major pathways are outlined in **Figure 9.2**.

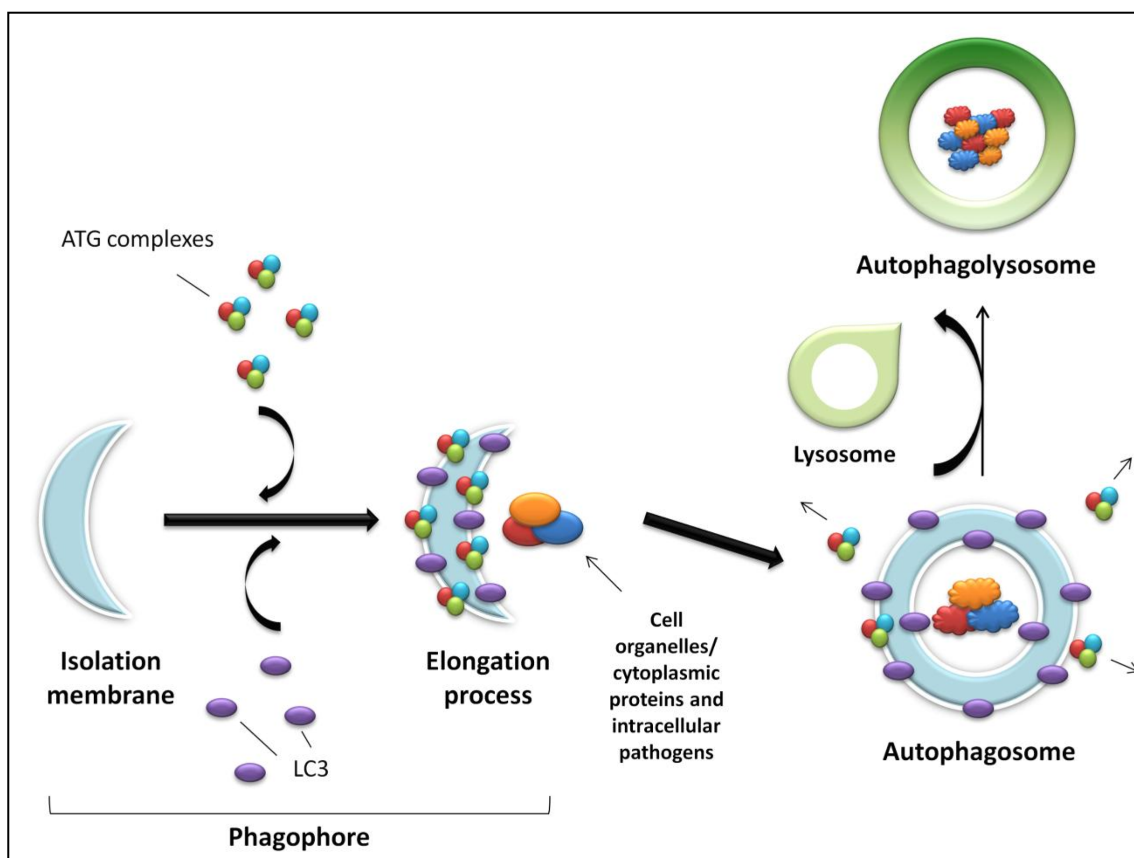


Figure 9.1. Schematic representation of autophagy. An expanding membrane sac (the phagophore) sequesters cytosolic material forming a double membrane vesicle (the autophagosome) enclosing proteins, organelles or pathogens to be degraded. The autophagosome then fuses with the acidic lysosome forming the final autophagolysosome.

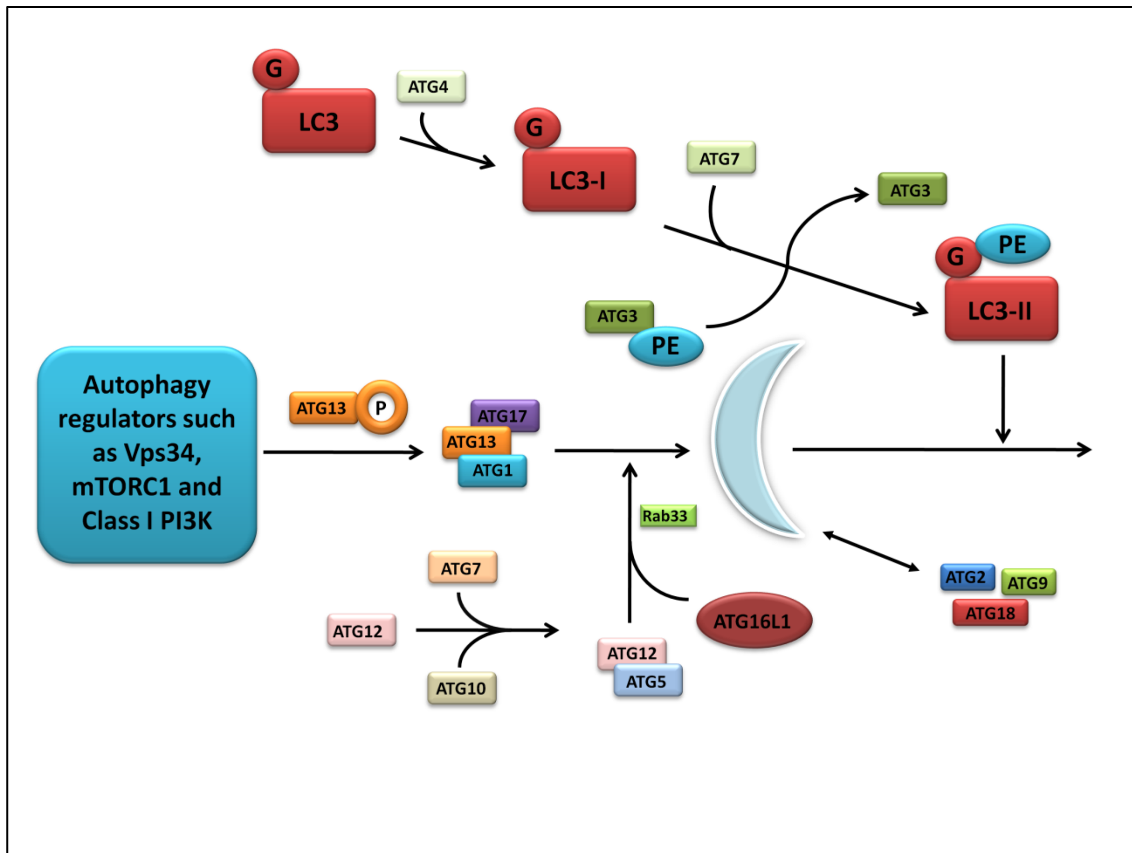


Figure 9.2. Molecular machinery of autophagosome formation. Two ubiquitin-like protein conjugation systems (the LC3/ATG8 and ATG12 systems) mediate membrane formation and expansion. PE, phosphatidylethanolamine; G, glycine

9.3 Antimicrobial autophagy (xenophagy)

Autophagy is activated in response to diverse stress or stimuli, including infection and is a crucial part of the innate immune defence against micro-organisms.[779] Antimicrobial autophagy, also referred to as xenophagy can selectively target microbes, including viruses, protozoa and bacteria for degradation in lysosomes.[779] Host cell defence against infection is complex and involves the convergence of several pathways. Pathogens invading host cells are initially detected by pattern recognition receptors (PRR), which include Toll-like receptors (TLR) on the surface of cells and interior of endosomes, and cytosolic NOD-like receptors (NLR). PRRs detect the presence of microbes through specific microbe-associated molecular patterns (MAMPs) such as peptidoglycan and lipopolysaccharide which are major constituents of bacterial cell walls.[343] The recognition of MAMPs by PRRs triggers the activation of several signalling cascades, among them NFκB an important regulator of inflammatory cytokine production, as well as antimicrobial mechanisms to clear the infection. Mounting evidence supports a role for autophagy as an antimicrobial mechanism

downstream of TLR and NLR signalling. For example, stimulation of NOD2 by muramyldipeptide (MDP), a constituent of peptidoglycan, induces autophagy in dendritic cells in a receptor-interacting serine-threonine kinase 2 (RIPK-2) dependent manner[30] (discussed in more detail in a latter section of this Chapter), while stimulation of TLR2, 4 and 7 with their specific ligands has also been shown to stimulate autophagy.[780,781] In addition to its essential role in innate immune defence against infection, autophagy plays a role in the adaptive immune response. Autophagosomes can deliver pathogen protein fragments (peptides) to MHC class II molecules. Once the MHC class II molecule is bound to the pathogen fragment it is transported to the surface of the cell where it triggers an appropriate CD4+ T-cell response.[782]

Recently, cargo receptor proteins or adaptors such as p62/SQSTM, NBR1 and NPD52 that contain both ubiquitin-binding and LC3-binding domains have been shown to detect intracellular bacteria.[783] Bacteria are heavily ubiquitinated in the host cell cytosol,[784] therefore by simultaneously binding to ubiquitinated cargo and LC3-containing autophagosomes, cargo receptors can target invading microbes to lysosomes for degradation. For example, p62 and NPD52 have recently been shown to target the intracellular pathogen *Salmonella typhimurium* to the autophagic machinery.[785] Additionally, recent work has demonstrated that phosphorylation of the autophagy receptor optineurin by protein kinase TANK binding kinase 1 (TBK1) enhanced the LC3 binding affinity and autophagic clearance of ubiquitin-coated cytosolic *Salmonella enterica.*[786] While it is clear that both PRRs and cargo receptors contribute to the detection and elimination of cytosolic bacteria via autophagy, it remains to be determined how these factors functionally interconnect, and whether they constitute a general anti-microbial defence mechanism or a more specific defence against pathogens.

9.4 Genetic variants in autophagy pathway genes and Crohn's disease susceptibility

Before discussing the autophagy-related proteins implicated in CD aetiopathogenesis it is first important to recognise the genetic research that ignited the current interest in autophagy within the study of IBD. The first study to link the autophagy pathway with CD pathogenesis was a genome-wide association study (GWAS) by Hampe et al. carried out in 2007.[26] Using 735 CD patients and 368 controls, single nucleotide polymorphisms (SNPs) with a p value <0.01 were followed up in three independent cohorts. This revealed a disease association with SNP rs2241880 in the autophagy-related 16-like 1 (*ATG16L1*) gene on chromosome 2q37.1. Resequencing of exons, splice sites and promotor regions concluded that the entire

association signal was likely due to this threonine to alanine substitution (Thr300Ala or T300A), however very recent data has suggested additional variants, independent of rs2241880, could implicate any of the coiled-coil domain, the WD (tryptophan-aspartic acid, Trp-Asp or WD40 repeat) domain and/or the 3' untranslated region, in CD susceptibility.[787] Almost immediately following this initial finding of association with *ATG16L1*, confirmation of this association was published by the same group in an independent cohort[788] and within 12 months multiple research groups had replicated the discovery in a variety of cohorts both in Europe and further afield.[400,402,789-791] Subsequently, further individual genetic association studies have confirmed this finding in Caucasian populations,[792] in addition to validation by several meta-analyses.[13,793] However replication in Asian populations has not been forthcoming despite various attempts.[793-796] Interestingly several paediatric studies have sought to confirm the association in childhood-onset CD however results have been conflicting, with two smaller studies confirming the association[283,403] while two larger studies proved negative,[305,797] possibly reflecting the strong association with ileal Crohn's disease (a phenotype not as common in childhood-onset disease[141]).

Another gene on chromosome 5q33.1, immunity-related GTPase family, M (*IRGM*), was linked with CD susceptibility through a genome-wide association scan by the Wellcome Trust Case Control Consortium (WTCCC), again in 2007.[400] In a case-control study involving 1748 CD patients and 2938 controls, SNPs achieving a p value $< 10^{-5}$ (below the stringent threshold of their original study that incorporated several major diseases[508]) were followed up. Using a new panel of over a thousand Caucasian CD patients, allele frequency comparisons were made between CD cases and nearly six thousand non-autoimmune WTCCC cases (which included patients with bipolar disorder, coronary artery disease and hypertension) as well as independent population controls from the 1958 British Birth Cohort. The strongest replication adjacent to a known gene was for SNPs rs13361189 and rs4958847 immediately flanking the *IRGM* gene on chromosome 5. Resequencing of *IRGM* in 48 CD patients revealed three novel SNPs which were subsequently genotyped in an independent case-control cohort. This demonstrated that only the silent 313T>C variant was associated with CD. This SNP was in strong linkage disequilibrium with the original rs13361189 suggesting that the causal variant may lie in a regulatory region adjacent to *IRGM* thus affecting transcription. Since this initial finding, the association of SNPs in *IRGM* have been replicated in other adult CD populations,[13,407,798] but again not in early-onset cohorts[18,409].

A subsequent meta-analysis of three GWAS with 3230 adult CD cases and 4829 controls demonstrated that the A allele of a SNP on chromosome 12q12 (rs11564258) was over-represented in CD patients, tagging a 0.89Mb region containing both Leucine-rich repeat serine/threonine protein kinase 2 (*LRRK2*) and mucin 19, oligomeric (*MUC19*),[13] this was

later replicated in a paediatric GWAS[18]. Although the susceptibility gene at this locus has been shown to be most likely *LRRK2* (rather than *MUC19*),[799] replication in subsequent individual studies has been inconsistent[800,801]. A further SNP (rs2412973) at locus 22q12 has shown association with childhood-onset CD patients.[18] With regards to colonic expression, the only gene in this region which was shown to differ between the two major IBD types (CD and ulcerative colitis) and healthy controls was myotubularin-related protein 3 (*MTMR3*). This gene showed significantly reduced expression in colonic biopsies in UC patients compared to controls and was also significantly associated with CD in the meta-analysis of discovery and replication cohorts. To date there have been no attempts to replicate this finding in either adult or paediatric populations.

Along with the original discovery of *ATG16L1* variants, SNPs in the region of protein tyrosine phosphatase, non-receptor type 2 (*PTPN2*) were also described.[400] This was again replicated in several populations, including paediatric cohorts.[802-804] Genetic association studies have also identified SNPs in unc-51-like kinase 1 (*ULK1*),[805,806] with conflicting results with regard to the association of variations in neutrophil cytosolic factor 4 (*NCF4*)[789,807,808].

9.5 Autophagy proteins implicated in Crohn's disease pathogenesis

9.5.1 ATG16L1 and NOD2

Following the initial identification and confirmation of *ATG16L1* as a CD susceptibility gene in 2007,[26,789,790] and its relationship with an ileal disease phenotype,[788] further studies began to unravel the potential biological relevance to this protein in CD pathogenesis. ATG16L1 is a member of a large group of ATG and ATG-related proteins which are intimately involved in autophagosome biogenesis.[809] Along with ATG12 and ATG5, ATG16L1 forms a 800kDa complex in a 2:2:2 stoichiometry; and although this ATG12 system has no deconjugating enzyme, the complex is formed constitutively irrespective of nutrient conditions.[809,810] During autophagosome formation the complex localises to the outer surface of the isolation membrane and dissociates following completion of the autophagosome.[811]

One of the first studies that began to outline the precise functional role of ATG16L1 was by Fujita et al.[810] Using a variety of mammalian cell culture techniques they demonstrated that the ATG12-ATG8-ATG16L1 complex was involved in LC3-lipidation, and interestingly that overexpression of ATG16L1 (specifically the coiled-coil region) disrupted the subunit stoichiometry leading to reduced autophagy. Also, the importance of correct localisation of ATG16L1 to sites of LC3-lipidation (such as the plasma membrane) was also shown to be

vital for appropriate autophagosome formation. The same group later described the interaction of the Golgi-resident GTPase Rab33B with ATG16L1, however the precise pathways involved during autophagy are still to be determined.[812] More relevant to CD pathogenesis, Saitoh et al. demonstrated that ATG16L1-deficient macrophages produced high amounts of the inflammatory cytokines interleukin 1-beta (IL-1β) and interleukin-18 (IL-18) following stimulation with the Toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS).[813] They also showed that this increased IL-1β production was due to Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF)-dependent activation of caspase-1 in ATG16L1-deficient cells. Additionally they utilised mice with ATG16L1-deficient haematopoetic cells to highlight their increased susceptibility to dextran sulphate sodium-induced colitis, which was in turn alleviated by treatment with IL-1β and IL-18 antibodies.

Cadwell et al. extended this work by initially generating mice and murine embryonic fibroblasts that were hypomorphic for ATG16L1 (using gene trap-mediated disruptions of *ATG16L1*).[25] Using this method allowed the detailed investigation of the consequences of low-expressing ATG16L1, avoiding the lethal effect of gene deletion.[814,815] They demonstrated that rapamycin-induced degradation of the adaptor protein p62 and LC3-II was diminished in ATG16L1 hypomorphic fibroblasts, which was restored by expressing ATG16L1. Additionally they showed that hypomorphic mice expressed approximately 25% of the expected levels of ATG16L1 in their ileum which equated to an increase in LC3-I:LC3-II ratio and p62. However, more importantly, these mice also showed striking abnormalities in Paneth cell morphology (Paneth cells are felt to be a key site in CD pathogenesis[816]) including lack of mucal lysozyme staining, reduced and disorganised granules, degenerating mitochondria and absence of apical microvilli. Following this, these changes in Paneth cell biology were confirmed in ileocolic resection specimens from CD patients carrying the *ATG16L1* risk allele. Overall these data provided the first indication that ATG16L1 had a specific role in humans and mice in regulating the specialised properties of Paneth cells.

In 2010 there were several publications which intriguingly brought together two of the major pathways implicated in CD pathogenesis, namely the innate response (through NOD2) and autophagy. The innate immune system is the body's first-line, non-specific response to foreign antigen. Both transmembrane and intracellular pattern recognition receptors (PRRs) recognise conserved, microbe-specific molecules named pathogen-associated molecular patterns (PAMPs; also known as microbe-associated molecular patterns (MAMPs)) such as LPS and peptidoglycan, leading to an appropriate immune response.[343] The NLR family are a major group of PRRs with a characteristic domain architecture comprising a central nucleotide binding and oligomerisation domain (NOD), an N-terminal effector binding (CARD) domain and C-terminal leucine-rich repeats (LRR) through which they detect MAMPs.[817] A key breakthrough in both CD and complex disease pathogenesis came in

2001 when fine mapping of the IBD1 locus on chromosome 16 identified the LRR variants of the *NOD2* gene as conferring susceptibility to CD.[17] Since this discovery these *NOD2* mutations have been widely replicated in both adult- and early-onset disease,[13,18] with functional studies beginning to unravel the role of NOD2 in CD.[818,819] Although initially identified as a cytosolic protein, recent studies have shown that NOD2 also resides at the plasma membrane.[820] This membrane localisation suggests that NOD2 may function at the sites of bacterial entry to directly engage with pathogens and signal an appropriate inflammatory and antimicrobial response.[820] Furthermore, it is proposed that NOD2 can recognise danger associated molecular patterns (DAMPs) which are exposed when there is disruption of host cell membranes.[821] Some pathogens, such as *Salmonella typhimurium* and enteropathogenic *E.*coli (EPEC) use type-3-secretion systems (T3SS) to inject effector proteins into the cytosol of host cells to mediate adhesion and invasion, and this may act as a DAMP recognised by NOD2.

The first of the seminal papers involving ATG16L1 and NOD2 demonstrated that NOD2 stimulation by its ligand muramyl dipeptide (MDP) induced autophagy in human, monocyte-derived dendritic cells (DCs) and also influenced bacterial handling and antigen presentation.[30] This was shown to be independent of TLR4 signalling and required the NOD2 signalling mediator RIPK-2 in addition to PI3K and the autophagy proteins ATG5, ATG7 and ATG16L1. Additionally, they demonstrated that DCs isolated from individuals with any of the three known CD-associated *NOD2* or T300A *ATG16L1* variants exhibited defective autophagy. Also cells carrying a *NOD2* variant failed to localise *Salmonella enterica* and CD-associated adherent-invasive *E.coli* species to autophagosomes, with this effect reversed by artificially inducing autophagy using rapamycin. At the same time a study led by Dana Philpott in Toronto similarly used bone marrow-derived macrophages and macrophages from NOD2-deficient mice to demonstrate NOD2-dependent autophagy induction with MDP.[31] Interestingly this group highlighted RIPK-2 independent autophagy induction, with colocalisation of NOD2 and ATG16L1 at the plasma membrane, with only cells homozygous for the T300A mutation effecting autophagy. NOD2 and ATG16L1 were also found to surround invading pathogens at the entry foci with mutant NOD2 proteins failing to do so. Homer et al. further showed that MDP-activated autophagy and NFκB signalling, in addition to increased *Salmonella* killing, was dependent on NOD2 and ATG16L1 expression, specifically in intestinal epithelial cells; this response was again shown to be altered by known CD-associated *NOD2* mutations.[822] The same group has most recently show the dual role of RIPK-2 in NOD2-induced autophagy with a positive signal through activation of p38 MAPK and reduced autophagy mediated by the phosphatase PP2A.[823] Kuballa et al. also showed abnormal capture of internalised *Salmonella* within autophagosomes in epithelial cells carrying the T300A mutation.[824] A summary of the role of

ATG16L1 and NOD2 in autophagy and the defects demonstrated when CD-associated mutations are present are shown in **Figure 9.3**.

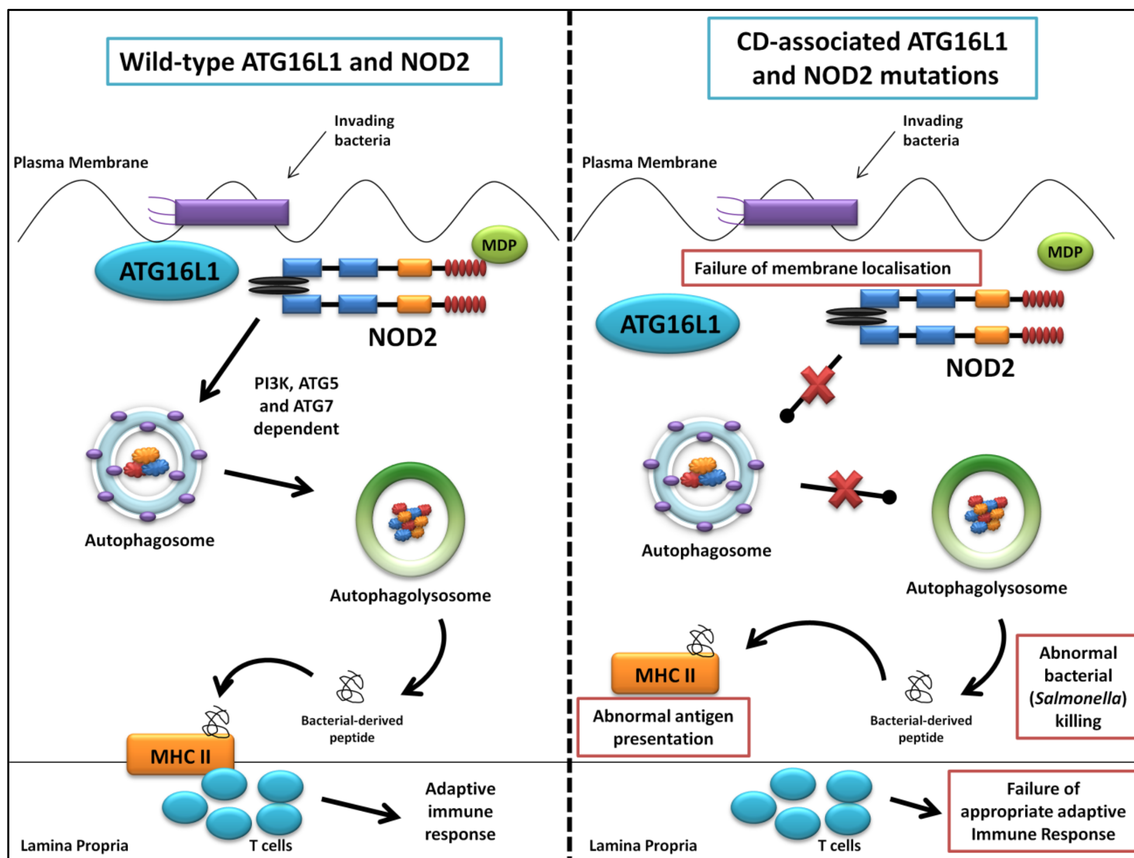


Figure 9.3. Summary diagram showing the role of ATG16L1 and NOD2 in the autophagy pathway and the defects observed when CD-associated mutations are present. The left panel shows the normal autophagy response with NOD2 recruiting ATG16L1 to the plasma membrane, normal autophagosome and autophagolysosome formation, followed by MHC II antigen presentation and an appropriate adaptive immune response. The right panel demonstrates abnormal membrane localisation of ATG16L1 with the LRR and T300A mutations resulting in abnormal bacterial killing and defective antigen presentation.

Further work has now shown that ATG16L1 is intimately involved in autophagosome formation with Ravikumar et al. showing that ATG16L1 interacts with the heavy chain of clathrin (a protein that plays a major role in the development of coated vesicles[825]) during the formation of ATG16L1-positive pre-autophagosomes[826]. This was shown to occur at the plasma membrane, although no obvious disruption of the clathrin-ATG16L1 interaction was observed with the T300A mutation. The inflammatory cytokine profile of cells possessing the T300A mutation has also been investigated with MDP-stimulation of peripheral blood cells harbouring T300A showing increased IL-1β , IL-6 and IL-10,[827,828] in addition to a reduction

in IFN-γ production[829]. Lee et al. went on to show that increased IL-1β production was a result of increased p62 levels in ATG16L1-deficient cells.[830] The T300A mutation has also recently been shown to produce hyperstable interactions between DCs and T cells at the immune synapse, with increased T cell activation, specifically the T-helper 17 cell response.[831] Work highlighting the role of ATG16L1 during *Helicobacter pylori* infection as well as murine Norovirus has also been presented.[832,833]

9.5.2 IRGM

The immunity-related GTPase (IRG) family of proteins, first described in the 1990s, can be separated into IRGA, IRGB, IRGC, IRGD and IRGM subfamilies based on homology across their GTP-binding domain, however only two *IRG* sequences, both transcribed, are present in humans, namely *IRGC* and *IRGM.*[834] IRGC is not induced by interferons (unlike IRGM) and seems to be very tissue-specific, having been shown to be mainly expressed in the testes.[834] To date, five different 3'-splicing isoforms have been identified for human *IRGM* with the protein products of these isoforms predicted to have molecular weights of between 19-24kDa.[835]

Interferon-gamma (IFN-γ) has long been recognised as important cytokine during the cellular response to bacterial invasion.[836] One of the first indications that the 47-kDa GTPase family, including IRGM, was involved in this response was the observation that genes encoding these proteins were upregulated during IFN-γ stimulation.[837,838] Culture of murine macrophage and fibroblast lines in medium containing high concentrations of IFN-γ showed a significant number of cDNA fragments belonging to both the 65-kDa and 47-kDa GTPases, including *Irgm1* (also known as *LRG-47*, the murine homologue of *IRGM*). Following this, work by MacMicking et al. demonstrated that *Irgm1* null mice failed to control *Mycobacterium tuberculosis* replication, with defective bacterial killing of *mycobacterium*-containing phagosomes in null macrophages.[839] MacMicking et al. also observed impaired maturation of *Mycobacterium tuberculosis*-containing phagosomes suggesting a critical role of Irgm1 in vacuolar trafficking; this work was validated in a similar study by Feng et al.[840] A further study investigated this role showing that there was an increase in autophagic vacuoles and the maturation of mycobacterial phagosomes in murine macrophages transfected with *Irgm1*, with Irgm1 partially co-localising with small LC3-positive organelles.[841] Singh et al. used a number of autophagic markers such as monodalsyncadaverine, Lyso-Tracker red and Mito-Tracker red to demonstrate early autophagic vacuoles, vacuole acidity and the presence of mitochondrial material in vacuoles respectively, induced by over-expressing Irgm1 in murine macrophages.[842] However, more interestingly, they also demonstrated that human IRGM participates in autophagy induced

conventionally (by the use of rapamycin or serum starvation) and by IFN-γ in human macrophages, and confirmed an increase in mycobacterial survival using siRNA to *IRGM*. More recent work has also shown that IRGM participates in virus-induced autophagy, with siRNA knockdown of *IRGM* decreasing the number of autophagosomes induced by measles virus, hepatitis C virus and HIV proteins.[843] Although many other studies have confirmed the importance of IRGM in cellular resistance to a number of pathogens[844,845] some conflicting evidence still exists[28].

9.5.3 LRRK2

Leucine-rich repeat serine/threonine protein kinase 2 (LRRK2; also known as PARK8) is a multi-domain protein of 2527 amino acids.[846] This large ubiquitous protein with multiple functional domains, namely kinase, ROC GTPase, Cor, leucine-rich repeat, ankyrin and WD40[847] has been linked to CD through the association of a SNP on chromosome 12q12[523]. LRRK2 is expressed in a large number of murine tissues, including lung, heart, kidney and small intestine.[848] In addition, recent work has demonstrated that full length LRRK2 is a common constituent of human peripheral blood mononuclear cells such as CD14+ monocytes, CD19+ B cells and CD8+ and CD4+ T cells.[849]

To date, little work has focussed on the potential functional role of LRRK2 in CD pathogenesis. However, due to mutations in LRRK2 having been consistently associated with Parkinson's disease (PD) (a neurodegenerative disease characterised by progressive disturbances in motor, autonomic and psychiatric functions[850]) an increasing number of studies are now beginning to unravel the complex functions of this protein. The best characterised mutation in *LRRK2* (G2019S - a glycine to serine substitution at amino acid 2019) was initially demonstrated in several familial cases of PD[851] and subsequently common variations in *LRRK2* have been shown to contribute to the risk of sporadic PD[852,853]. This, coupled with the previously established importance of autophagy in a number of neurodegenerative diseases,[854] has focussed current research on the role of LRRK2 during the autophagic process.

LRRK2 has been shown to localise to vesicular granular structures of the late endosomal-lysosomal pathways.[406,855,856] Several membrane microdomains such as the neck of caveolae, microvilli/filopodia and intraluminal vesicles of multivesicular bodies (MVBs) were shown to contain LRRK2, as well as cytoplasmic puncta corresponding to MVBs and autophagic vacuoles.[855] A recent study demonstrated that overexpression of wild-type LRRK2 increased autophagy through activation of a calcium-dependent protein kinase kinase-β (CaMKK-β)/adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway.[857] This effect was through nicotinic-acid adenine dinucleotide phosphate (NAADP)

receptors causing calcium efflux and partial alkalinisation of lysosomal store pH. Increased LC3 punctae were also visualised with overexpression of full-length LRRK2, but not the mutant protein, suggesting involvement of the kinase domain. Further work has provided evidence that the presence of the G2019S-mutant protein in neuronal cells leads to the accumulation of autophagic structures, with an impaired autophagic balance also demonstrated in non-neuronal and yeast cells,[857] induced pluripotent stem cells from patients with the G2019S mutation also show an accumulation of autophagic vacuoles[858]. Similarly, aged LRRK2-null mice show increased LC3-II and p62 in the kidney with a subsequent inflammatory response.[859]

More pertinent to CD pathogenesis, work by Liu et al. showed that LRRK2-deficient mice had significantly poorer clinical outcomes in an experimental (DSS-induced) colitis model compared to their wild-type counterparts, manifested as increased weight loss and diarrhoea.[860] A further study by Gardet et al. in human subjects used peripheral blood mononuclear cells and intestinal biopsies from IBD patients to further delineate the role of LRRK2.[861] Stimulation of macrophage-differentiated THP-1 cells with IFN-γ showed an increase in both LRRK2 mRNA and protein, with peripheral blood mononuclear cells from healthy controls showing a similar response in CD3+ T cells, CD19+ B cells and CD11b+ monocytes. LRRK2 mRNA expression was also shown to be increased six-fold in inflamed CD biopsies compared to paired non-inflamed biopsies, with histological specimens showing LRRK2-positivity in CD206 macrophages, CD103+ dendritic cells and CD20- B cells. Gardet et al. also showed, using luciferase-based reporters, that LRRK2 can activate NFκB pathways dependent on the IKK complex. Particularly relevant to CD they also demonstrated that LRRK2 co-localised with *S. typhimurium* in the cytosol of RAW macrophages, with LRRK2 siRNA knockdown reducing reactive oxygen species after IFN-γ stimulation and increased *S. typhimurium* survival in a gentamicin-protection assay. Extending this work, Hakimi et al. showed that LRRK2 is present in a subset of circulating leucocytes with the expression of ^{R1441C}mutant LRRK2 in a primary, non-neural cell model revealing an autophagy defect manifested as a reduction of LC3-II levels.[849] Stimulation of murine bone-marrow derived macrophages by a variety of pattern-recognition receptor ligands (which are likely to be pivotal in CD pathogenesis[862]) produced up-regulation of LRRK2 mRNA with respect to LPS, R837 and CpG and down-regulation when stimulated with Pam3CSK4.

9.5.4 MTMR3

MTMR3 encodes a protein of the tyrosine phosphatase superfamily.[863] The MTMR proteins are involved in the regulation of phosphatidylinositol (PtdIns) and its derivatives, accounting for around 10% of the total lipid in eukaryotic membranes.[864] This family of

proteins (a subgroup of the PtdIns(3)*P* phosphatases) has 15 members, only nine of which (including MTMR3) possess the active phosphatase domain that specifically dephosphorylates PtdIns(3)*P*.[865] PtdIns(3)*P* is highly enriched as a component of the elongating isolation membrane and autophagosome membranes in yeast,[866] with MTMR3 ubiquitously expressed and shown to hydrolyse PtdIns(3)*P* to its derivative PtdIns[867]. Roles in autophagosome size and constitutive autophagy initiation in epithelial cells,[868] coupled with hVps34 (a class III PI 3-kinase) being intimately involved in autophagy and the suppression of autophagy by the PI 3-kinase inhibitor Wortmannin, makes this group of proteins of particular interest[869,870]. PtdIns(3)*P* has specifically been shown to bind to the autophagy protein ATG18[871-873] and this ATG18-PtdIns(3)*P* interaction has now been recognised as an essential component for the efficient progression of both selective and non-selective autophagy[874]. It is likely that the ATG18-PtdIns(3)*P* complex exerts this role through the interaction of the ATG18-ATG2 complex with ATG9 which is the only known integral membrane ATG protein.[875] Further work has now extended the role of MTMR3 specifically in autophagy. Work in alveolar basal epithelium (A549 cells) by Taguchi-Atarashi et al. showed that overexpression of mutant *MTMR3* (*MTMR3*^{C413S}) produced an increase in GFP-LC3 and GFP-ATG5 which was not seen with wild-type overexpression.[869] Also, this increase in autophagy was dependent on PtdIns(3)*P*, with siRNA knockdown of *MTMR3* producing a similar response, suggesting MTMR3 as a negative regulator of autophagy. The 22q12 locus harbouring *MTMR3* has now also been associated with lung cancer,[876] with specific *MTMR3* mutations discovered in gastric and colorectal cancer,[877] all of which may be important in the context of the emerging role of autophagy in cancer biology[878].

9.5.5 PTPN2

The *PTPN2* gene is located on chromosome 18p11 and codes T cell protein tyrosine phosphatase (TC-PTP, also known simply as PTPN2), one of over 100 PTP proteins capable of removing phosphate moieties from tyrosine residues of protein substrates.[879] First cloned in 1989,[880] this ubiquitous protein exists as two splice variants, one localised to the nucleus and the other to the endoplasmic reticulum[879]. Much of the functional work surrounding this protein has been through investigation of *PTPN2* knockout mice that demonstrate a progressive systemic inflammation and abnormal cytokine environment.[881] TC-PTP has also been shown to be involved in haematopoiesis and autoimmune disease.[879]

Much of the work specifically relating to the role of *PTPN2* and CD has been performed by Scharl et al. The first of these papers studied the role of PTPN2 in intestinal barrier function. They demonstrated that PTPN2 was over-expressed in biopsies from CD patients, and that

stimulation of an intestinal epithelial cell (IEC) line with IFN-γ increased PTPN2 levels.[882] Also, *PTPN2* knockdown increased STAT1 and STAT3 phosphorylation after IFN-γ stimulation and increased epithelial permeability as determined by FITC-Dextran flux across IEC monolayers. Similar increases in IL-6 and macrophage chemoattractant protein 1 (MCP-1) were seen with *PTPN2* knockdown in a monocyte cell line in a subsequent study.[883] Other work showed comparable results when IECs were stimulated with TNF-α.[884] Very recent research has now shown a role for PTPN2 in autophagy with siRNA knockdown in IECs reducing autophagosome formation as well as autophagy induction as determined by persisting *Listeria* infection; this was replicated in colonic lamina propria fibroblasts from patients with CD carrying the *PTPN2* variant.[885] Additionally a novel variant in *PTPN2* (rs1893217) was also shown to impair autophagosome formation, as well as elevating IFN-γ levels in response to MDP.[886]

9.5.6 NCF4 and ULK1

One of the first GWAS to identify the association of autophagy genes with CD susceptibility postulated *NCF4* as a candidate gene.[789] Although this finding was replicated soon afterwards,[807] several studies have also proved negative[808,887]. NCF4 (also known as p40phox) is a member of the cytoplasmic SH3-domain proteins involved in the activation of gp91phox during phagocytosis.[888] Along with other phox proteins (e.g. p67phox) NCF4 is involved in the generation of reactive oxygen species (ROS) by the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which is critical in the antimicrobial functions of phagocytic cells.[889] In addition to *NCF4*-deficient mice showing defective killing of *S.aureus,*[889] ROS have also been shown to be important during autophagy induction[890]. A limited number of studies have demonstrated an association with an ileal[807,891] or perianal[892] phenotype with one functional study showing a reduction in reduced ROS in GM-CSF-primed granulocytes from CD patients harbouring the *NCF4* mutation[893].

To date only two studies have evaluated the role of the well-characterised autophagy protein ULK1 in CD pathogenesis. The first of these demonstrated that the SNP rs12303764 near *ULK1* was significantly associated with CD in a case-control study, replicated in a transmission disequilibrium test in 335 case-parent trios.[805] This association with *ULK1* variants was then recently replicated in an independent case-control study in New Zealand.[806] Although the association with rs12303764 was not present in their population, several other SNPs in the region of *ULK1* showed positive association, however the replication for these SNPs in larger GWAS meta-analysis data was variable. The *ULK1* gene, consisting of 28 exons, with a transcript length of 5211 basepairs and a translation

length of 1050 residues, is localised on chromosome 12q24. The two known mammalian orthologs of yeast Atg1, ULK1 and ULK2, form a stable complex with ATG13, FIP200, ATG101 and mTORC1. Although this complex is intimately involved in autophagy induction, in view of its currently weak association with CD a detailed discussion of the role of ULK1 in autophagy is outwith the scope of this chapter, however several excellent reviews of its biological function are available.[894,895]

It has yet to be seen what direct role CD-association variants in *NCF4* and *ULK1* will play, if any, in CD pathogenesis. However future research will undoubtedly endeavour to illicit any further association given the vital role of ULK1 in the mTORC1 complex and the intriguing importance of ROS in bacterial handling. A summary of the postulated autophagy-related pathways influenced by the lesser-studied CD susceptibility proteins are outlined in **Figure 9.4**.

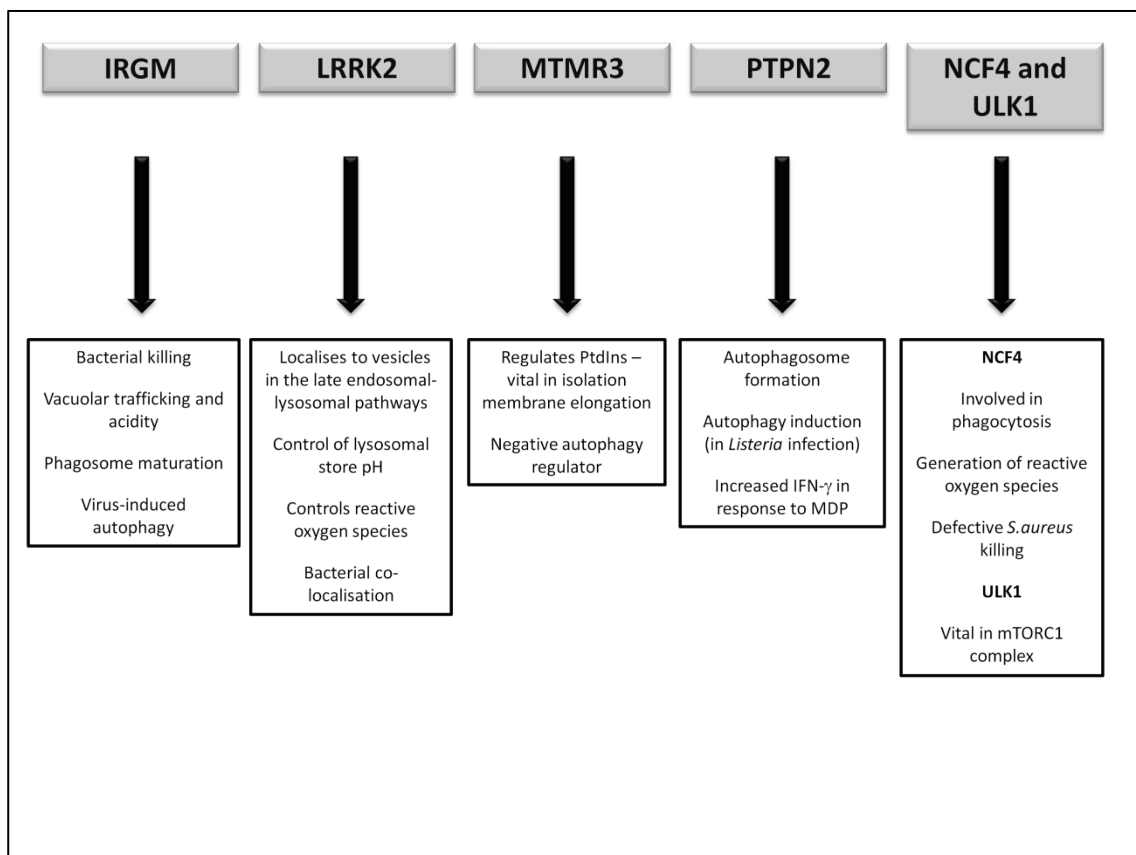


Figure 9.4. Postulated autophagy-related pathways influenced by CD susceptibility proteins currently under more detailed investigation.

9.6 Adherent invasive E.coli

E.coli is normally a harmless commensal micro-organism that can become a highly-adapted pathogen through the acquisition of specific virulence factors. To date eight pathovars have been extensively studied and implicated in a wide range of diseases, of which enteropathogenic *E.coli* (EPEC), enterohaemorrhagic *E.coli* (EHEC), enterotoxinogenic *E.coli* (ETEC) and diffusely adherent *E.coli* (DAEC) cause gastroenteritis in humans.[896] In addition there are several recently described *E.coli* pathovars, of which AIEC are the most relevant to CD. The association of AIEC with CD was first reported by Darfeuille-Michaud and colleagues in the late nineties,[897,898] and since then AIEC have been isolated from independent studies involving adult and paediatric CD patients[766,767,899]. The high prevalence of AIEC strains observed in the ileal mucosa of CD patients has been attributed to their ability to adhere to the glycosylated carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), which is overexpressed on the surface of epithelial cells in patients with CD.[109] In addition the ER-stress response chaperone Gp96 is highly expressed in epithelial cells of patients with CD and has been demonstrated to act as a host cell receptor for AIEC.[108] Recently, complete genome sequencing of the reference strain of CD-associated AIEC LF82 has shown that this strain contains several virulence factors including the *lpf* operon, which encodes long polar fimbriae (LPF).[900] AIEC have also been shown to interact with Peyer's patches, specialised cells of the intestinal epithelium via LPF, and the prevalence of AIEC strains containing the *lpf* operon was shown to be markedly higher in CD-patients compared to controls.[901]

Following adhesion AIEC can invade the intestinal epithelium, breaching the epithelial barrier and are subsequently found in the lamina propria of patients with ileal CD. A recent study investigated the role of autophagy in the replication and survival of *E.coli* in epithelial cells.[27] Specifically, the authors investigated whether cells deficient in the CD-associated autophagy proteins IRGM and ATG16L1 affected intracellular replication and survival of AIEC strains. This study demonstrated that the AIEC strain LF82 have enhanced replication and survival in both IRGM and ATG16L1 deficient cells. Remarkably, autophagy deficiency did not interfere with the replication and survival ability of other *E.coli* strains tested, including non-pathogenic, environmental, commensal, or pathogenic strains involved in gastroenteritis, suggesting a specific role for autophagy in restraining AIEC. AIEC can translocate the epithelium into the lamina propria where they can replicate and survive within macrophages. In a subsequent study the same group investigated whether defects in autophagy affected the replication and survival of AIEC in macrophages.[902] AIEC were shown to recruit the autophagy machinery to sites of entry into host cells and autophagy

limited intramacrophagic replication. Impaired IRGM or ATG16L1 expression resulted in increased AIEC in macrophages and significantly this was accompanied by increased secretion of the pro-inflammatory cytokines IL-6 and TNF-α. Further studies demonstrated that a family of micro-RNA (miRNAs) miR-196 that are expressed in the intestinal epithelium of individuals with CD down regulate the expression of IRGM in those with the protective variant (c.313C).[903] The resulting impairment of the autophagy response resulted in increased intracellular replication and survival of AIEC. Taken altogether these studies suggest that stimulation of autophagy, perhaps using small molecule inhibitors of mTORC1, would be a therapeutic strategy to restrain AIEC replication and dampen the exacerbated inflammatory response that leads to chronic inflammation and granuloma formation observed in patients with CD.

9.7 Autophagy pathway-phenotype correlations in Crohn's disease

A number of studies have now endeavoured to correlate CD phenotypes with autophagy gene risk variants in an attempt to translate knowledge of the autophagy pathway to clinical application. The most replicated finding in this respect is the positive association of *ATG16L1* and *IRGM* variants with ileal disease phenotype. Along with Prescott et al. who initially reported this finding for *ATG16L1* in a cohort from the United Kingdom,[788] groups in Australia[404] and Portugal[801] have recognised this relationship, with the Portuguese group also showing this association for *IRGM*. Similarly carriage of the *ATG16L1* risk variant has been shown to correlate with a reduction in extra-intestinal manifestations, however the study was likely unpowered to detect an effect.[904] Another reported positive association was with *IRGM* variants and the need for ileocolectomy, however the number of patients in the study again make this difficult to interpret.[905] Several recent studies have attempted to define the association of autophagy gene risk variants with the presence of granulomas on intestinal biopsy samples, with conflicting results. (A granuloma is an inflammatory lesion containing epithelioid cells, macrophages, and lymphocytes, and is considered one of the pathological hallmarks of CD, however detection at endoscopy is variable[906]). Wolfkamp et al. in a cohort of approximately 200 patients demonstrated no association of granulomas with CD-risk variants in *ATG16L1* or *IRGM.*[907] SNPs in *NCF4* and *ATG4A* have also shown negative correlation with the presence of granulomas in an Israeli cohort.[908] A larger study from Belgium, which included 464 patients, did find both a positive and negative association of variations in four autophagy-related genes (ATG2A, ATG4A, ATG4D and FNBP1L); to date these genes have not been shown to confer a susceptibility risk to, or altered protein expression in, CD.[909]

The role of the microbiome in the pathogenesis of CD has become more prominent in recent years,[910] with a small number of studies aiming to determine the relationship between autophagy risk-variants and microbial components. Murdoch et al. looked at the presence of four antimicrobial antibodies stratified by CD-associated variants.[911] They showed that in a cohort of over 600 CD patients, that increased seropositivity for anti-Saccharomyces cerevisiae antibody (ASCA) was associated with carriage of the *ATG16L1* variant and that the *IRGM* CD-risk variant was associated with increased anti-flagellin seropositivity. Frank et al. looked at DNA extracted from intestinal specimens from 35 CD patients to determine the microbial composition and its relationship with genotype.[912] 16S rRNA sequence analysis revealed that patients with the *NOD2* or *ATG16L1* variants had significantly altered microbiota, although the authors did acknowledge that the study was underpowered to determine any genera-specific effects.

9.8 Conclusions

It can be seen that following the emergence of autophagy as a pathogenic mechanism in CD that some progress has been made to unravel the functional aspects of these complex pathways. To date much of the research surrounding this topic has been focussed on genetic susceptibility and despite the field of autophagy progressing at a rapid pace, much is still unknown regarding the precise molecular abnormalities that may underlie the disease process. It is likely that further knowledge of the autophagy proteins involved specifically in CD and a deeper insight into the role of xenophagy (especially in the context of AIEC and the ileal disease phenotype) will be required before effective intervention can be implemented to alter the disease course. Functional work is now required to determine the potential therapeutic modulation of both the autophagy pathway itself, in addition to targeting specific bacterial subgroups such as AIEC, although these therapies will most likely only be beneficial in a small number of patients.

10. Laboratory Methods

In order to fully assess the NOD2 stable cell lines and to evaluate autophagy induction and MTOR activation outlined in **Chapter 11** and **Chapter 12**, the following laboratory methods were employed.

10.1 Cell lines and tissue culture

Human Embryonic Kidney 293 (HEK293) cells (American Type Culture Collection, ATCC) and HEK293 cells stably expressing green-fluorescent protein (GFP)-labelled light chain 3 (LC3) (a kind gift from Dr Craig Stevens, University of Edinburgh and Napier University) were grown in high glucose (4.5g/L) Dulbecco's Modified Eagle Medium (DMEM) (Gibco). HCT116 cells (a colorectal carcinoma cell line; ATCC) and HCT116 stably expressing GFP-LC3 (also a kind gift from Dr Craig Stevens, University of Edinburgh and Napier University) were grown in McCoy's 5A medium (Gibco). SW480 cells and HT29 cells (ATCC) were grown in Leibovitz and DMEM media respectively. All media were supplemented with L-Glutamine, 10% FCS (Invitrogen) and Penicillin-Streptomycin (Gibco). Cells were incubated at 37ºC in high relative humidity (95%) and controlled CO_2 level (5%), except SW480 cells that were grown at 37ºC in sealed flasks. Cells were maintained in 75cm^2 culture flasks (Costar®), passaged twice-weekly using trypsin (Gibco) and cells checked monthly for mycoplasma infection at the Institute for Molecular Medicine, University of Edinburgh using the MycoAlert™ mycoplasma detection kit (Lonza). Cells from two confluent 75cm^2 flasks of each cell line were also frozen after suspension in 12 x 1ml aliquots of freezing mix (1.8ml dimethyl sulfoxide [DMSO], 10.2ml FCS), placed in a Nalgene "Mr Frosty" freezing container (Thermo Scientific) and immediately stored at -80ºC; cells were then transferred to liquid nitrogen storage within 48hrs. Fresh aliquots of cells were thawed and re-seeded approximately every 8-12 weeks to ensure the culture of robust, healthy cell populations; cell viability was routinely assessed by trypan blue (Gibco) exclusion.

10.2 Western blot procedure

Cells were grown on 6-, 12- or 24-well culture plates (Greiner Bio One; all culture plates were purchased from this manufacturer unless otherwise stated). Following the appropriate treatments, culture media was removed and cells scraped into 1ml of phosphate-buffered saline (PBS), placed in a 1.5ml eppendorf tube and centrifuged at 1200rpm for five minutes;

supernatants were then carefully removed. Pellets were then incubated for 30 minutes on ice in the presence 30-50µl of protein lysis buffer (see **Table 10.1**) and supplemented with 10µl of phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich) per 1ml of lysis buffer. Lysates were centrifuged in standard 1.5ml eppendorfs at 13,000rpm for 15 minutes, and protein quantification for each sample was determined using the Qubit™ fluorometer (Invitrogen) as per the manufacturer's instructions.

Table 10.1. Buffers and solutions used during laboratory experiments

Buffer or Solution (volume)	Components
Tris pH 6.8/8.0/8.8 (1000ml)	• 121.14g Trizma base (Sigma-Aldrich) • 1000ml ddH_2O (pH adjusted accordingly using HCl and verified using a standard digital pH meter)
Protein lysis buffer (40ml)	• 6ml of 150mM sodium chloride solution • 2ml of 50mM tris(hydroxymethyl)aminomethane (Tris) solution (pH 8.0) • 200µl of 0.5% Tergitol-type nonyl phenoxypolyethoxylethanol (NP-40) (Sigma-Aldrich) • One tablet of EDTA-free complete protease inhibitor cocktail (Roche) • 31.8ml ddH_2O
10x transfer buffer (1000ml)	• 144g Glycine (Promega) • 30g Trizma base (Sigma-Aldrich) • 1000ml ddH_2O
10% SDS solution (1000ml)	• 100g SDS (Sigma-Aldrich) • 1000ml ddH_2O
10x SDS buffer (1000ml)	• 900ml 10x transfer buffer • 100ml 10% SDS solution
Phosphate-buffered saline/Tween® 0.1% solution (400ml)	• 400ml phosphate-buffered saline • 400µl Tween® 20 (Sigma-Aldrich)
Milk powder solution 5% or 10% (10ml)	• 0.5g or 1g Marvel dried milk powder (Premier Foods) • 10ml 0.1% PBS/Tween® solution (Sigma-Aldrich)

ddH_2O, double distilled water; SDS, sodium dodecyl sulphate

Samples were placed in a heat block at 95ºC for approximately 10 minutes after the addition of 10µl of 3x SDS to each sample. Cell lysates were separated by sodium dodecyl sulphate (SDS) gel electrophoresis on 6-15% gels dependent on the size of the protein(s) being investigated. The volumes of double distilled H_2O (ddH_2O), Tris (pH 8.8), Protogel 40% (National Diagnostics), and 10% SDS for each percentage gel are shown in **Table 10.2**.

Table 10.2. Reagents and volumes used to prepare western blot gels for protein electrophoresis.

Reagent (units)	Percentage Gel					
	6%	7%	8%	10%	12%	15%
ddH_2O (ml)	3.87	3.8	3.6	2.84	2.17	1.17
Tris pH 8.8 (ml)	3.73	3.73	3.73	3.73	3.73	3.73
Protogel 40% (ml)	2.0	2.3	2.7	3.3	4.0	5.0
10% SDS (ml)	100	100	100	100	100	100

ddH_2O, double distilled water

In addition, 40µl of 25% ammonia persulphate and 10µl of Tetramethylethylenediamine (TEMED; Sigma-Aldrich) were added to polymerise the gel solutions. The gel solution was then poured between two glass plates, left to set for approximately 10 minutes and a stacker with similar components (with the exception of the substitution of Tris pH 8.8 for Tris pH 6.8) poured on top. Cell lysates were resolved using gel electrophoresis (Mini-PROTEAN Tetra, Bio-Rad) with running buffer (100ml 10x SDS buffer and 900ml deionised water - **Table 10.1**) and run for approximately 2hrs at 120V/400mA depending on protein size. To allow the accurate assessment of protein size during blotting, 5µl of protein standard (PageRuler Plus, Fermentas) was placed in at least one lane of each gel. Proteins were then transferred to mixed ester nitrocellulose membrane (Hybond-C Extra, GE Healthcare) in transfer buffer (120ml 10x transfer buffer, 130ml methanol, 900ml deionised water) for between 90-120 minutes at 400mA.

Following transfer, membranes were placed in trays and stained with Ponceau S (Sigma-Aldrich) for several minutes to confirm successful protein transfer. Following ponceau staining, membranes were de-stained with PBS. To minimise non-specific binding of antibody, membranes were placed in trays on a roller in 10% milk powder solution for 30 minutes prior to incubation with antibody. Primary antibody at a concentration of between 1:500-1:4000 (depending on the experimental protocol) was then added to 5% milk powder solution and incubated overnight in a tray at 4ºC on a rocker.

Following incubation with primary antibody, membranes were washed for five minutes (x3) in 0.1% PBS/Tween®. Secondary antibodies (10µl anti-rabbit IgG-HRP or 2.5µl anti-mouse IgG-HRP [Dako]) were placed in 5% milk powder solution and incubated with membranes for approximately 90 minutes at room temperature. Before developing, membranes were again washed for five minutes (x3) in 0.1% PBS/Tween®. Each membrane was then placed face-down on Saran wrap (Dow Chemical Company) with between 100-2000µl of ECL Western

Blot Analysis fluid (GE Healthcare) which was prepared as per manufacturer's instructions. Membranes were bathed in analysis fluid for approximately 5 minutes, the excess fluid removed, and then placed on a generic acetate transparency sheet, covered in Saran wrap (ensuring all bubbles expelled) and secured in autoradiography cassettes (Research Products International). For development, appropriately cut BioMax® XAR film (Kodak) was placed in the cassettes for between 10 seconds and 30 minutes (as required for each antibody) and inserted into a standard film developer (Agfa Curix 60, Agfa-Gevaert N.V, Mortsel, Belgium).

10.3 Immunofluorescent microscopy

Cells were seeded on 19-mm borosilicate glass coverslips (Fisher Scientific) in 6- or 12-well plates at a density of approximately $2x10^4$ cells. After 24 hours, if required, cells were transfected with the appropriate constructs for a further 16 hours. Cells were washed in PBS, placed on a custom-made chamber (with coverslips placed on a moist sponge to avoid desiccation) then fixed with 200µl of 4% paraformaldehyde for 15 minutes. Cells were then washed in PBS and permeabilised with 200µl of PBS/0.2% Triton™ X-100 (Dow Chemical Company) for 10 minutes before being blocked for 30 minutes with 200µl of PBS containing 10% foetal calf serum (FCS) (Gibco). For protein detection, 1-5µl of primary antibody was added to 10ml of PBS containing 1% FCS and incubated overnight at 4ºC. The following day cells were incubated for 1 hour at room temperature with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) conjugated anti-mouse or anti-rabbit secondary antibodies (Invitrogen). Cells were then fixed to standard microscope slides with adhesive containing 4',6'-diamidino-2-phenylindole (DAPI) (VECTASHIELD®, Vector Laboratories) to allow the easy identification of cell nuclei. Images were captured using an Axioskop 2 fluorescent microscope (Carl Zeiss) and, if required, analysed using Image J software (National Institutes of Health).

10.4 Lipofectamine transfection

Cells were seeded in a standard 12-well culture plate for at least 16 hours, or until approximately 70% confluent. For each well to be transfected, 250µl of Opti-MEM® reduced serum medium (Gibco) was placed in two 1.5ml eppendorfs. In one eppendorf, the appropriate amount of DNA was placed (usually between 0.5-2.0µg); in the other, 2µl of Lipofectamine 2000 (Invitrogen) was used for every 1µg of DNA. These two solutions were left at room temperature for 5 minutes then mixed thoroughly. Following another 25 minute

period at room temperature, this solution was added dropwise onto the cell culture monolayer and placed at 37ºC in high relative humidity (95%) and controlled CO_2 level (5%). Transfected cells were harvested (or treated with the appropriate substance) after 16-18 hours, then lysed and processed for either western blot or immunofluoroescent microscopy as detailed above.

10.5 Transformation and details of vectors

For the transformation of constructs, *Escherichia coli* DH5α bacteria were used (a kind gift from Dr Craig Stevens).[913] These bacteria and the vector to be transformed were first placed on ice in suitable containers. 20mls of agar was then liquefied in a standard microwave oven for approximately two minutes and the agar left to cool slightly before the addition of Ampicillin (Gibco) at a 1:1000 concentration; two Petri dishes were then filled with 10ml of agar. Two 1.5ml eppendorfs were filled with 100µl of bacteria suspension and approximately 50ng of construct DNA was then placed in the second eppendorf, leaving the first eppendorf as a (blank) control; these solutions were then left on ice. After 30 minutes the solutions were placed at 42°C for two minutes then back on ice for two minutes. 300µl of Lysogeny Broth (LB) was then added to each sample and incubated for 1-2 hours at 37°C. Each solution was added to one agar plate and evenly spread using sterile glass beads. Plates were then placed inverted into a standard tissue culture incubator overnight at 37°C. The following day, 200ml of LB was placed in a large canted neck flask. After supplementation with 1:1000 Ampicillin (from 100mg/ml stock), a sterile pipette tip was used to pick a single colony of bacteria for inoculation of the LB solution, and then cultured overnight at 37°C with gentle agitation. (As an intermediate step, to make glycerol stock to thus avoid the need for further transformations of the same vector, 600µl of LB bacteria solution was placed in a CryoTube [Thermo Scientific] before the addition of 600µl glycerol and stored at -80°C.) DNA was then extracted using the QIAGEN Plasmid Midi Kit (QIAGEN) as per the manufacturer's instructions. DNA concentration and purity were then assessed using a Nanodrop spectrophotometer (Thermo Scientific). A ratio of absorbance above 1.8 at 260nm and 280nm was deemed acceptable.[914]

The generation of haemagglutinin-tagged (HA) wild type NOD2, HA-NOD2 (1-693) lacking the C-terminal LRR domain and HA-NOD2 (1-247) comprising only the two N-terminal CARD domains were created using site directed mutagenesis (Stratagene); further details of their generation have previously been published.[915] The 1-693 and 1-247 mutants were produced by the introduction of a stop codon at amino acids 694 or 248. These variants had previously been generated in the laboratory of Professor Jack Satsangi (Gastrointestinal

Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh) and were a kind gift from Dr Elaine Nimmo and Dr Craig Stevens. In addition to the NOD2 vectors, GFP-LC3 and HA-tagged S6K were also previously generated and available in the laboratory of Prof. Satsangi at the start of the project.

10.6 Immunofluorescent microscopy and western blot analysis

For immunofluorescent microscopy analysis, especially in relation to the assessment of cells undergoing autophagy, captured raw images were first loaded in Image J (National Institutes of Health), which was downloaded from http://rsbweb.nih.gov/ij/download.html. In order to accentuate the presence of autophagosomes (depicted by bright punctate green foci) the Process>Shadows>North function was used. Following this, the Plugins>Analyze>Cell counter function was utilised to count the cells of interest. These values were recorded in a custom-made Excel 2007 (Microsoft) spreadsheet that automatically calculated overall percentages. In all immunofluorescent microscopy analysis of autophagy induction, at least 100 cells were evaluated taken from five random fields of view at 40x magnification.

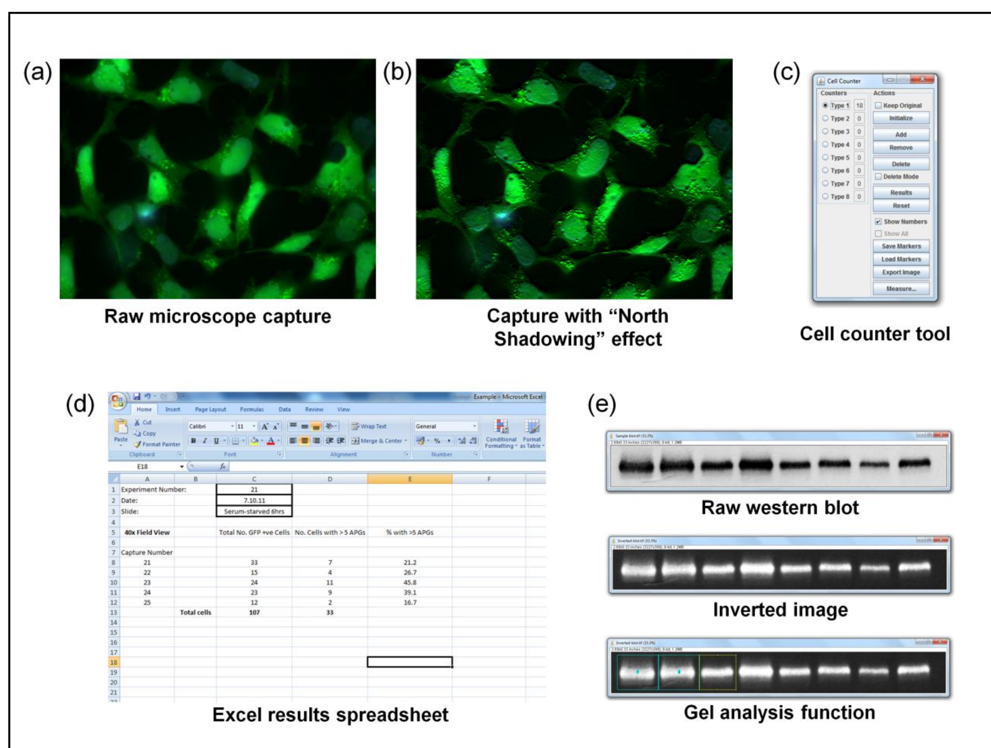


Figure 10.1. Examples depicting the use of ImageJ and Microsoft Excel 2007 to analyse captured microscopy pictures and western blot gels. (a) Raw microscope capture of cells undergoing autophagy. (b) 'North' shadowing effect used in ImageJ to accentuate the LC3 punctae. (c) Cell counter tool used to manually count punctae in cells. (d) Microsoft Excel 2007 spreadsheet

used to automatically calculate percentage of cells counted compared to total cell number. (e) Progression of analysis of raw western blots using the gel analysis function following inversion.

For densitometry analysis of western blots, films containing the blots were first scanned as TIFF images using an Epson Perfection 1660 Photo flat-bed scanner (Epson). These TIFF images were then loaded in Image J and after inverting the image (to achieve a "positive" value), the Analyze>Gels function was used to outline each lane with the scale set to 'pixels'. After each lane was plotted, the 'magic wand' tool was used to outline the graphical representation of each blot and the area analysed using the Analyze>Measure function. Each lane was analysed in turn and, if applicable, β-actin blot densitometry also performed to adjust for any discrepancies in protein loading. The numbers corresponding to each blot density were then copied into Excel 2007 (Microsoft) and the protein:actin ratio calculated using simple formulae. An outline of these methods are depicted in **Figure 10.1**.

10.7 Antibodies

All antibodies were kept at -20ºC or 4ºC as per the manufacturer's instructions. Several antibodies were frozen in aliquots to avoid repeated freeze-thaw cycles. In addition, antibody-milk powder solutions were often frozen and used up to three times in order to avoid repeated purchases. Details of each antibody used during all the experiments are given in **Table 10.3**.

Table 10.3. Details of antibodies used during laboratory experiments

Antibody Name/description	Manufacturer (ID)	Host	Dilution
Atg7	Cell Signaling (#2631)	Rabbit	1:1000
β-Actin	Santa Cruz (sc-69879)	Mouse	1:4000
FLAG M2	Sigma-Aldrich (F3165)	Mouse	1:4000
Green fluorescent protein	Abcam (ad290)	Rabbit	1:1000
HA.11 (16B12)	Covance (MMS-101P)	Mouse	1:4000
LC3B	Abcam (ab48394)	Rabbit	1:1000
NOD2 (2D9)	Santa Cruz (sc-56168)	Mouse	1:1000
NOD2 Polyclonal (28-301)	Cayman (160777)	Rabbit	1:1000
NOD2 Polyclonal (28-301)	ABR (MA1-16611)	Mouse	1:1000
PARP	Cell Signaling (#9542)	Rabbit	1:1000
p62	BD Biosciences (610833)	Mouse	1:1000
Phospho-S6 Ribosomal Protein (Ser 235/236) (2F9)	Cell Signaling (#4856)	Rabbit	1:1000
Phospho-p70 S6 Kinase (Thr389) (1A5)	Cell Signaling (#9206)	Mouse	1:1000
p70 S6 Kinase	Cell Signaling (#9202)	Rabbit	1:1000
S6 Ribosomal Protein (54D2)	Cell Signaling (#2317)	Mouse	1:1000
Vimentin (3B4)	Abcam (ab28028)	Mouse	1:1000

10.8 Drug treatments

A list of drug and substances used during all the laboratory experiments, including their stock solution, working concentrations and manufacturers are shown in **Table 10.4**.

Table 10.4. Details of drugs and substances used during all laboratory experiments.

Drug/substance	Stock solution	Working conc.	Manufacturer/Supplier
Azathioprine	100mM	30-90µM	Tocris Bioscience
Bafilomycin A1	100µM	10µM	Invivogen
CFDA-SE	5mM	500µM	eBioscience
Chloroquine	100mM	100µM	InvivoGen
Cycloheximide	10mg/ml	10µg/ml	Supleco
Dimethyl sulphoxide	Neat	Equivalent volume	Sigma-Aldrich
FITC-MDP	10mg/ml	1µg/ml	InvivoGen
Geneticin	50mg/ml	0-1500mg/ml	Invitrogen
Infliximab	100mg/ml	3-30µg/ml	Centocor
L18-MDP	10mg/ml	1-75µg/ml	InvivoGen
6-thioguanine	300mM	30-90µM	Tocris Bioscience
Methotrexate	100mM	33-99µM	Tocris Bioscience
3-methyladenine	1mg/ml	10µl/ml	Sigma-Aldrich
MG132	20mg/ml	10µM	Calbiochem
Rapamycin	10mM	10µM	InvivoGen
U0126	100mM	10µM	Cell Signaling
Withaferin-A	10mg/ml	2-10µM	Sigma-Aldrich

10.9 Flow cytometry

For flow cytometry, cells were labelled with 5-(and -6) – carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (eBioscience). The stable incorporation of CFDA-SE into cells allows for tracking of cell proliferation (up to eight cell divisions) as its intracellular concentration is sequentially halved. To achieve this labelling, 179.4µl of DMSO was added to CFDA-SE stock giving a 5mM solution. This was converted to a 1:10 dilution (500µM) and 10µl of solution added per ml of cells (i.e. ~1x10^6 cells in 1ml). The cell suspension was then placed at 37°C in the dark for approximately 8 minutes and then 3-4 volumes of ice cold complete medium (DMEM) added; cells were then plated as per the experiemental protocol. Following the completion of the experiment, cells were washed three times via 5 minute centrifugation at 1200prm with ice cold complete medium. The final cell pellet was then resuspended in 4% paraformaldehyde for 15 minutes to fix then washed (3x at 1200rpm) prior to resuspension in FACS wash 1% FCS solution. Cell suspensions were analysed by

Dr Elizabeth Freyer (Institute of Genetics and Molecular Medicine) using an Aria II flow cytometer (Becton Dickinson), with the results analysed using FACSDiVa (BD Bioscience).

10.10 Polymerase chain reaction

RNA was extracted from HEK293 and HCT116 cells using RNeasy® kits (Qiagen). RNA (1 μg) was then transcribed to cDNA using the SuperScriptIII® Reverse Transcriptase cDNA kit (Invitrogen) with a 1:1 mixture of oligo-dT_{20} and random hexamers as primers. To produce standards, firstly NOD2 plasmid was diluted serially from 1M to 1:1000M and GAPDH (derived from SW480 cells) similarly diluted from 1:50 to 1:5000. PCRs for NOD2 and GAPDH were carried out on the Rotorgene®6000 (Qiagen). The PCR mix was: EXPRESS SYBR® GreenER qPCR Supermix (Invitrogen) with premixed ROX containing 1μM of each primer and template cDNA, with RNA-negative and water controls. NOD2 primers had previously been generated in the laboratory of Prof. Satsangi and were a kind gift from Dr Elaine Nimmo. Primers were selected using Primer3 and crossed exons. The qPCR cycle was as follows: 95°C for 2 minutes, then 40 cycles of 95°C for 10 seconds and 60°C for 30seconds.

10.11 Generation of NOD2 stable cell lines

Wild-type (WT) NOD2 and the frameshift mutant NOD2 (NOD2-L1007fs) had previously been sub-cloned into the p3XFLAG-myc-CMV-26 expression vector (Sigma-Aldrich) (**Figure 10.2**) in the laboratory of Prof. Satsangi by Dr Elaine Nimmo (Institute of Genetic and Molecular Medicine, University of Edinburgh). Once stably integrated into the host cell genome through Geneticin selection, this vector allows the expression of a dual tagged protein (FLAG-tagged N-terminus and *c-myc*-tagged c-terminus).

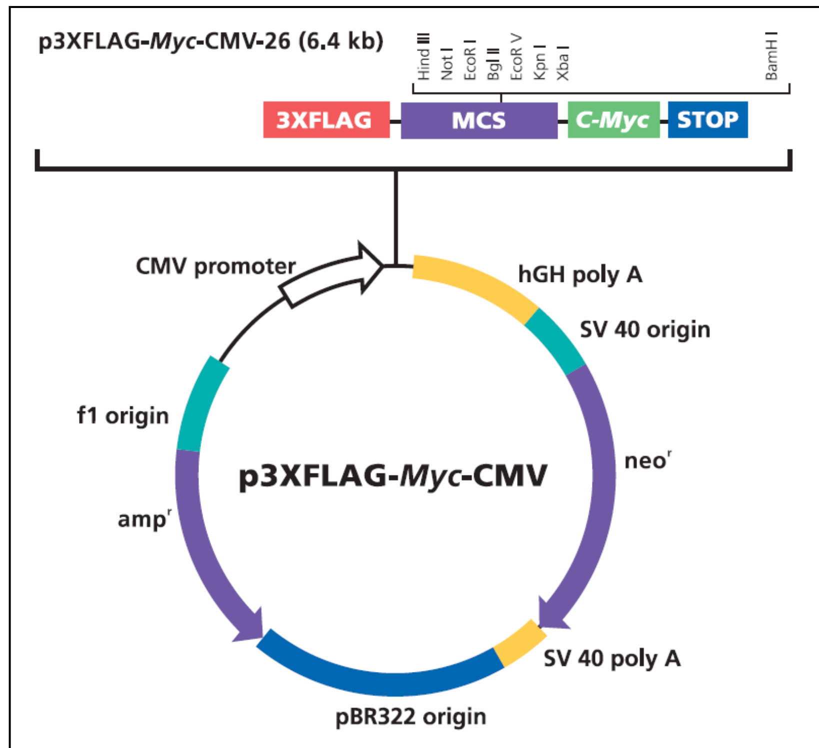


Figure 10.2. Details of the p3XFLAG-myc-CMV-26 expression vector.

HEK293 cells have previously been shown to express low levels of *NOD2*, with the HCT116 cell line showing intermediate expression and a known wild-type genotype.[916,917] Prior to deriving stable cell lines expressing the NOD2 and NOD2-L1007fs proteins an rtPCR was performed as outlined above. This was performed to compare *NOD2* expression in the HCT116 and HEK293 cells. This demonstrated very low expression of *NOD2* in the HEK293 cell line compared to the HCT116 cells (**Figure 10.3**). In addition, to confirm the well-recognised difficulties with detecting endogenous NOD2 protein, and to determine the best antibody for detecting over-expressed NOD2, an antibody 'screen' of the NOD2 antibodies currently held in the laboratory of Prof. Satsangi was also performed using western blot analysis. The HA-tagged NOD2 construct was used as a positive control. This demonstrated that the NOD2 polyclonal antibody purchased from Cayman Chemical was the only antibody to readily detected over-expressed NOD2 protein, with the ABR and Santa-Cruz antibodies showing non-specific bands at slightly smaller and larger sizes respectively (**Figure 10.4**).

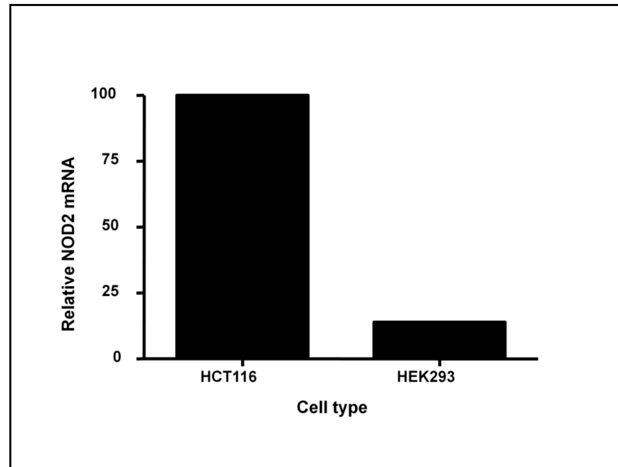


Figure 10.3. rtPCR experiment showing relatively low expression of NOD2 mRNA in the HEK293 cell line compared to HCT116 cells.

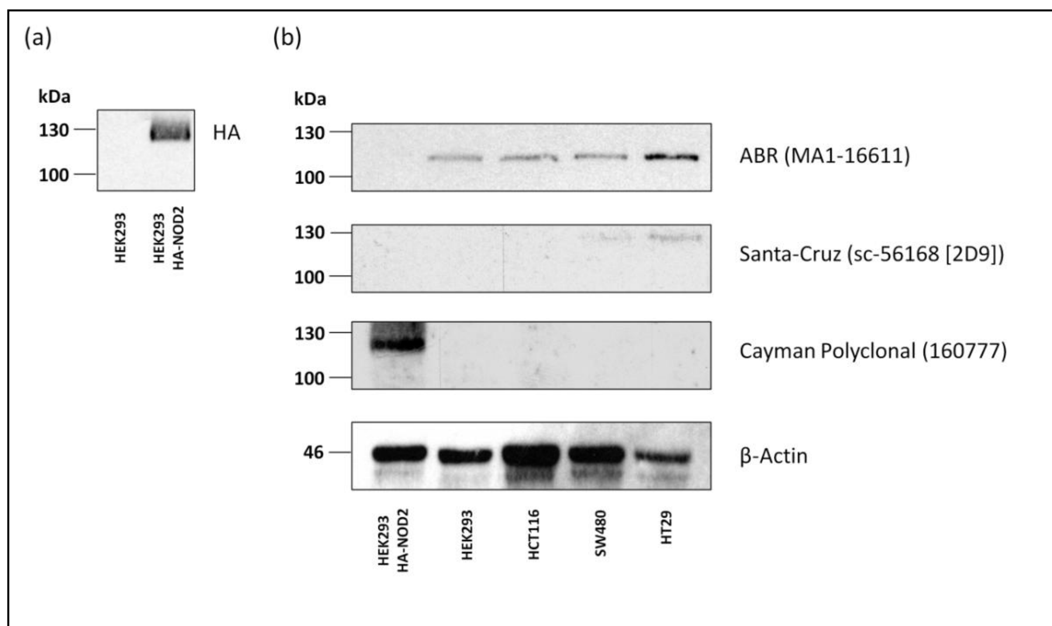


Figure 10.4. Results of a 'screen' of NOD2 antibodies performed on wild-type and HA-tagged HEK293 cells, in addition to three other gastrointestinal cell lines. (a) Wild type and HA-tagged HEK293 cells probed with HA antibody demonstrating expression of the over-expressed NOD2 protein. (b) Panel of three commercially available antibodies to NOD2 used to detect endogenous NOD2 and over-expressed NOD2 in HEK293 cells and three other gastrointestinal cells lines, HCT116, SW480 and HT29.

For stable NOD2-expressing cell line generation, parental HEK293 and HCT116 cells were first subjected to increasing concentrations of Geneticin antibiotic (Gibco). From this treatment it was determined that a concentration of 800μg/ml of Geneticin was sufficient to eliminate non-resistant cells from culture. Evidence of the effect of increasing Geneticin

concentrations on confluent HEK293 cell culture is shown in **Figure 10.5**; identical results were obtained for the HCT116 cell line.

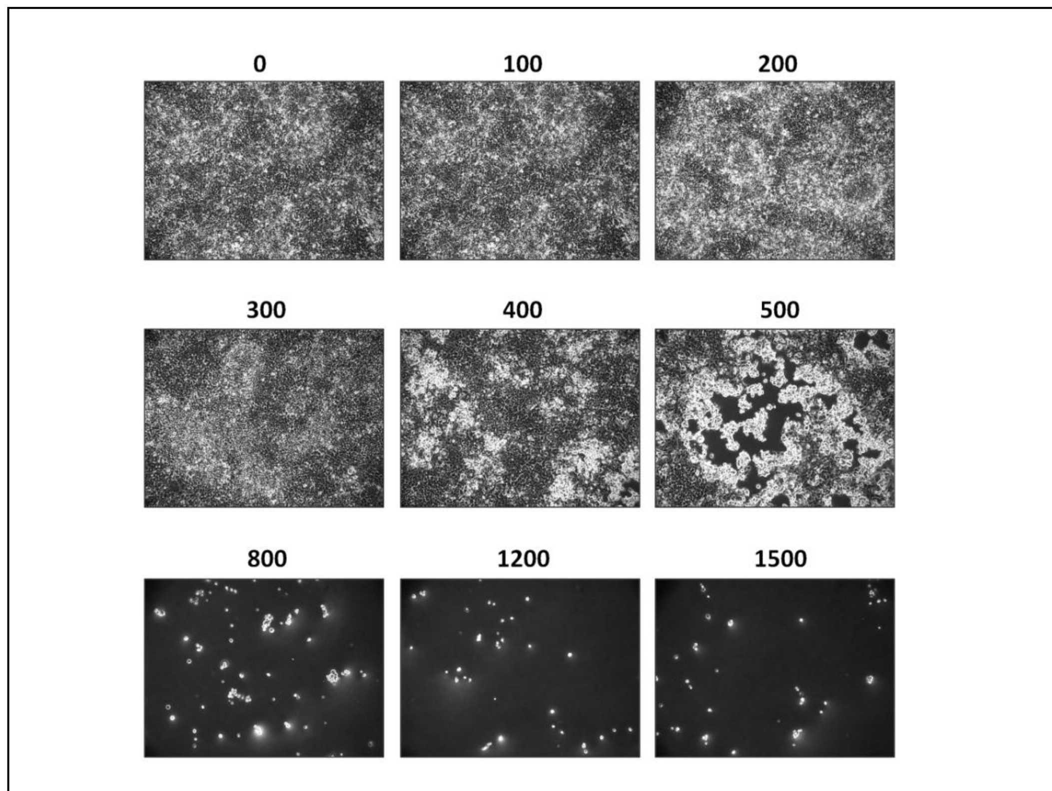


Figure 10.5. Geneticin 'kill curve' for the HEK293 cell line. Images from top to bottom, left to right show increasing concentrations of Geneticin antibiotic (µl/ml) added to culture medium for 5-7 days showing no clear benefit of using concentrations higher than 800µl/ml to achieve selection of non-resistant cells.

Multiple 10cm^2 culture plates of HEK293 and HCT116 cells were therefore transfected with the empty vector, NOD2 or NOD2-L1007fs vectors using Lipofectamine (Invitrogen) as described above. A control plate of each cell line was left untransfected to allow the determination of resistant colonies. Fourty-eight hours following transfection, all cultures were grown in DMEM or McCoy's complete medium supplemented with 800µg/ml Geneticin until the control plate for each cell line was void of all cells and there was clear expansion of resistant colonies (**Figure 10.6**). Approximately 14 days after Geneticin selection resistant colonies expressing empty vector, NOD2 and NOD2-L1007fs were lysed with trypsin and clones either pooled or carefully picked using a cloning ring.

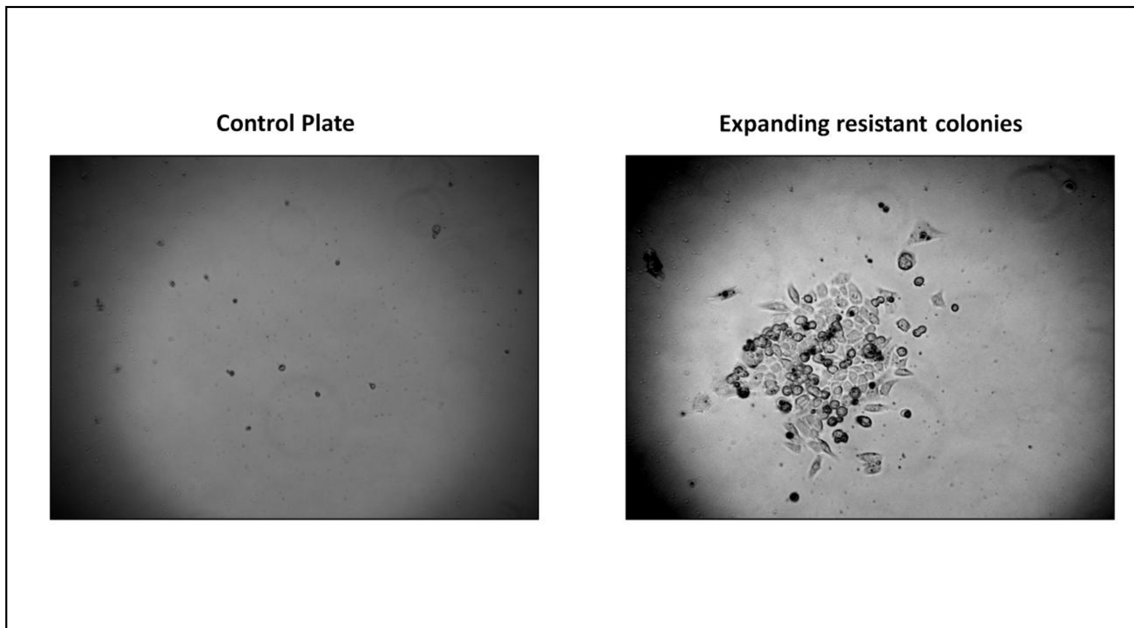


Figure 10.6. Example of HCT116 cells non-transfected and transfected with Geneticin-resistant wild-type NOD2 vector. Control plate (left panel) shows killing of non-resistant cells at 800µl/ml of Geneticin with expanding resistant colonies evident (right panel).

10.12 Analysis of gene-wide susceptibility signal for *Vim* using an international genome-wide association meta-analysis

As with Chapter 5 and Chapter 6, an improved meta-analysis of GWAS data imputed with the 1000 genomes reference set (http://www.1000genomes.org) was interrogated. To assess variants in *Vim* (the gene encoding vimentin on chromosome 10 between positions 17,270,258 - 17,279,592 [Ensembl Release 65 -_ENSG00000026025]; Build 37) for association with CD susceptibility SNP-specific p values generated from these analyses of seven individual CD datasets encompassing 5,956 CD patients and 14,927 healthy controls (http://www.broadinstitute.org/mpg/ricopili/) were obtained. Haploview software version 4.2 (http://www.broad.mit.edu/mpg/haploview)[593] was used to visualise the haplotype structure (using solid spine of linkage disequilibrium) surrounding *Vim* to determine the areas of strongest signal.

11. Characterisation of cells stably expressing wild-type NOD2 or the Crohn's disease-associated NOD2 frameshift mutation.

11.1 Introduction

As previously discussed, the innate immune system is the body's first-line, non-specific response to foreign antigen.[33] Both transmembrane and intracellular pattern recognition receptors (PRRs) recognise conserved, microbe-specific molecules named microbe-associated molecular patterns (MAMPs), leading to an appropriate immune response.[343] The nucleotide binding and oligomerisation domain (NOD)-like receptor (NLR) family are a major group of PRRs with a characteristic architecture comprising a central NACHT (or NOD) domain, an N-terminal effector binding domain and C-terminal leucine-rich repeats (LRRs);[817] the NOD2 protein is the prototype member of this group (**Figure 11.1**).

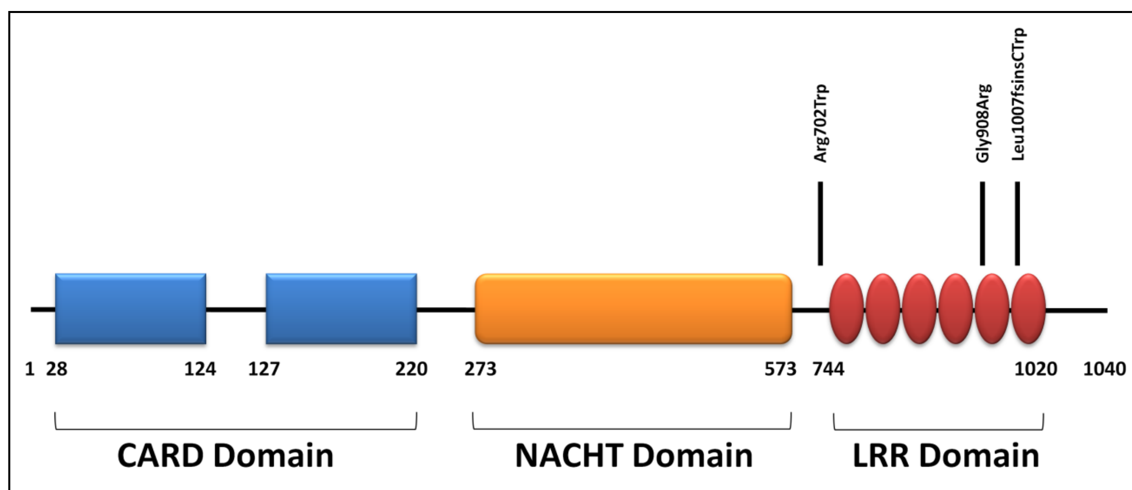


Figure 11.1. Schematic showing the three main domains of the NOD2 protein with corresponding residues. Additionally, the location of the three well-characterised Crohn's disease-associated mutations are shown. CARD, caspase activation recruitment domain; NACHT, NAIP, CIITA, HET-E, TP1; LRR, leucine-rich repeat.

As discussed in detail in **Chapter 4** and **Chapter 9**, although *NOD2* was the first Crohn's disease (CD) susceptibility gene to be described, polymorphisms in this gene on chromosome 16 remain the strongest risk signal to date.[15] Polymorphisms in *NOD2* have also been associated with an increased risk of graft versus host disease in allogeneic stem

cell and bone marrow transplantation, Blau's syndrome (a granulomatous disease affecting the eyes, skin and joints), allergic inflammation as well as other conditions.[607,918-922] There are currently three well-defined *NOD2* polymorphisms which confer a high degree of disease susceptibility for CD (**Figure 11.1**),[923] with additional rarer variants recently described[603]. In one meta-analysis, those carrying two risk alleles had a 17.1-fold (95% CI 10.7–27.2) increased risk of CD;[924] to date the *NOD2* frameshift (L1007fs) variant has consistently been shown to confer the highest individual risk of developing CD[923,924].

11.2 NOD2 pathway signalling

NOD2 has been shown to have cellular expression in antigen presenting cells such as dendritic cells and macrophages,[925] as well epithelial and Paneth cells[19,926]. Within these cell types it has been shown to interact with an array of different proteins,[927] however the exact mechanisms involved in the pathway from NOD2 to (1) NFκB and pro-inflammatory cytokine release; (2) antigen presentation through the major histocompatibility complex class II system; and (3) type I interferon release, remain ill-defined[23,928]. In addition, the precise pathways coordinating the autophagy response to muramyl dipeptide (MDP) sensing (described in **Chapter 9**) are still poorly understood.

Initial studies of NOD2 function demonstrated that MDP-stimulation through the LRR domain led to NFκB activation.[929] This process is initiated through a conformational change in NOD2 structure, leading to unfolding of the molecule to expose the CARD domain. Following this change, binding to the downstream adaptor protein RIPK-2 (or RICK) occurs, most likely at the plasma membrane.[930,931] The association of NOD2 and RIPK-2 leads to polyubiquination of RIPK-2 at lysine 209 which in turn requires the presence of an E3 ligase (most likely cIAP1/cIAP2), with other proteins such as ITCH also involved.[932-934] Other NOD2-interacting proteins include negative regulators such as Erbin,[935] Centaurin beta 1,[936] and angio-associated migratory cell protein,[937] in addition to Rac1 GTPase[938]. A number of NOD2 regulatory factors such as GRIM-19, CARD9 and Bid have also been described.[939-941]

The well-characterised CD-associated NOD2-L1007fs variant has been shown to truncate the terminal 33 amino acids of the protein, resulting in a marked loss in the capacity to activate NFκB.[925] This MDP recognition abnormality in CD is in contrast to the Blau's syndrome mutation where mutations in the NACHT/NOD region affect the activation of NOD2, rather than MDP recognition. Despite a significant volume of research evaluating the clinical aspects of carriage of the CD-associated *NOD2* variants, little is known about the

effect of NOD2 expression on cell morphology, proliferation, apoptotic response or protein stability.

With regard to cellular morphology, there are few studies to date evaluating the direct effect of NOD2 expression on cell size. Although researchers have directly imaged Paneth cells of patients with and without *NOD2* polymorphisms,[942] in addition to murine work assessing the morphology of Peyer's patches in *NOD2*-deficient mice,[943] little data exists demonstrating any *direct* effect of NOD2 expression (wild-type or variant) on cell (or nuclear) size. In work carried out by Cruickshank et al., *NOD2* mRNA levels in primary colonic epithelial cells (CEC) from C57BL/6 mice were measured.[944] This demonstrated that cells stained for the proliferation marker Ki67 contained significantly higher levels of *NOD2* mRNA than cells stained for differentiation (with alkaline phosphatase). Also, crypt length was assessed in *NOD2*+/+ and *NOD2* -/- mice, showing that *NOD2*-/- mice had shorter colonic crypt length (both pre- and post-infection with *Salmonella*) compared to their wild-type counterparts, although again this did not directly assess the cellular size, rather a proxy for cell proliferation. Further experiments *in vitro* showed that primary cultures of *NOD2*-/- CEC contained significantly higher numbers of apoptotic cells (as assessed by caspase 3 activity) and that cell numbers did not increase after 24 hour and 48 hour in culture, compared with *NOD2*+/+ CECs, although the data for these cultures at baseline was absent. Additionally, shRNA knockdown of NOD2 in colonic carcinoma cells lines (HT29 and SW480) demonstrated that reduced NOD2 protein expression led to a decline in cell survival, explained by decreased viability and increased apoptosis. Other studies evaluating the role of NOD2 in apoptosis have shown that MDP stimulated (FOXP3+) regulatory T cells were protected from death receptor Fas-mediated apoptosis.[22] However, despite evidence that NOD1 and other CARD-containing proteins are intimately involved in apoptosis through the caspases,[945,946] the over-riding mechanisms controlling apoptosis through NOD2 specifically remain elusive[947,948].

11.3 Hypotheses and aims

Hypotheses:

1. The stable over-expression of NOD2, but not the CD-associated NOD2-L1007fs variant influences basic cell morphology, early cell proliferation and baseline apoptosis.
2. NOD2 protein stability and half-life are regulated through the ubiquitin-proteasome pathway.
3. The NOD2-L1007fs variant has altered stability and half-life in mammalian cells.

Aims: to characterise and compare HEK293 cells stably expressing wild-type NOD2 (hereafter referred to as NOD2) or the CD-associated NOD2-L1007fs variant. More specifically to assess whether NOD2 affects cell morphology, proliferation, and baseline apoptosis. The regulation of protein stability and half-life of NOD2 and NOD2-L007fs variant through the proteasome and lysosome degradation pathways will be evaluated.

11.4 Results

11.4.1 HEK293 stably express both NOD2 and NOD2-L1007fs proteins with failure of the NOD2-L1007fs protein to interact with muramyl dipeptide

In order to assess the expression level of NOD2 and NOD2-L1007fs proteins in the derived HEK293 cell lines (see **Chapter 10.11**), western blot analysis was performed. **Figure 11.1** shows that the cell lines stably express proteins of the expected size (NOD2 ~130kDa), with the NOD2-L1007fs variant demonstrating a marginally smaller protein size in keeping with a truncated LRR domain.

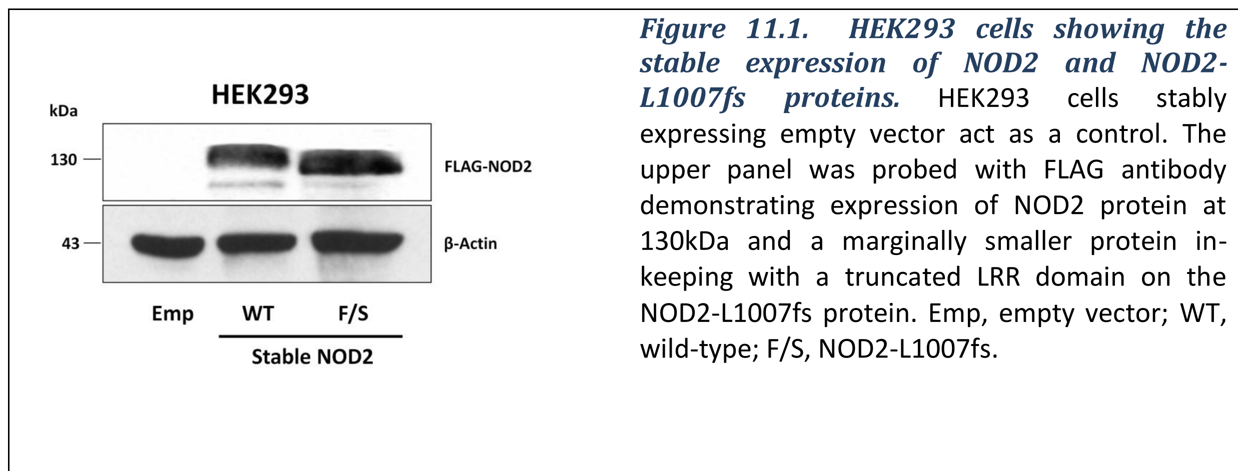


Figure 11.1. HEK293 cells showing the stable expression of NOD2 and NOD2-L1007fs proteins. HEK293 cells stably expressing empty vector act as a control. The upper panel was probed with FLAG antibody demonstrating expression of NOD2 protein at 130kDa and a marginally smaller protein in-keeping with a truncated LRR domain on the NOD2-L1007fs protein. Emp, empty vector; WT, wild-type; F/S, NOD2-L1007fs.

To confirm that the expressed proteins were NOD2, immunofluorescent microscopy was performed using the NOD2 (Cayman) primary antibody identified in the initial antibody screen (**Figure 10.4**). In addition, cells derived from pooled clones and single clones were evaluated for uniform NOD2 expression, to determine the appropriate cell lines to use in subsequent experiments (**Figure 11.2**). It can be seen that both the pooled clones of the NOD2 and NOD2-L1007fs cells expressed similar levels of NOD2 protein, with empty vector used as a negative control. (**Figure 11.2a**). Similarly, three separate NOD2 single clone cell lines also expressed similar levels of NOD2 protein for both the NOD2 and NOD2-L1007fs

(**Figure 11.2b**). It was therefore decided that the pooled clones would be used in subsequent experiments as clonal selected cells may have integrated the plasmid at an inappropriate location (therefore disrupting a gene important for the pathway being studied). Pooled clones would hopefully mask any unwanted phenotype in addition to avoiding the possibility of single clones expressing very high (or very low) levels of protein.

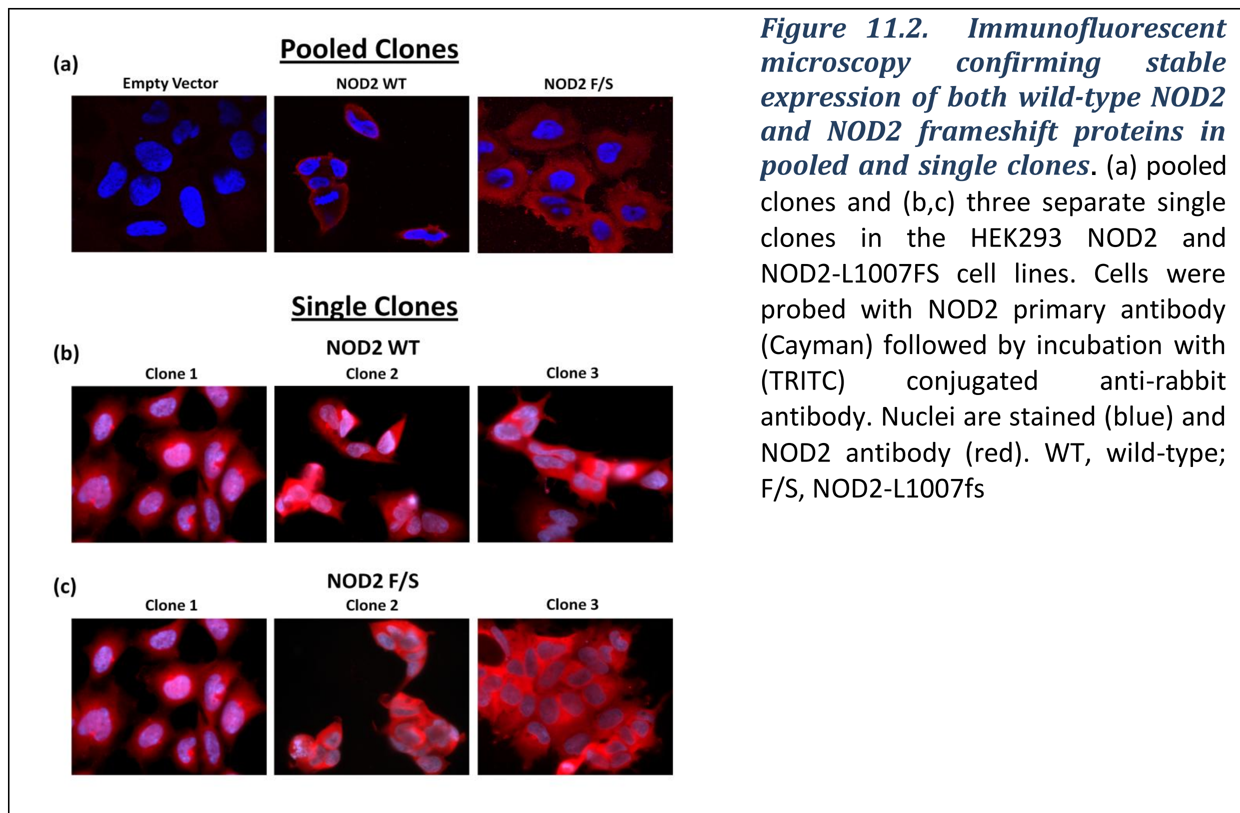


Figure 11.2. Immunofluorescent microscopy confirming stable expression of both wild-type NOD2 and NOD2 frameshift proteins in pooled and single clones. (a) pooled clones and (b,c) three separate single clones in the HEK293 NOD2 and NOD2-L1007FS cell lines. Cells were probed with NOD2 primary antibody (Cayman) followed by incubation with (TRITC) conjugated anti-rabbit antibody. Nuclei are stained (blue) and NOD2 antibody (red). WT, wild-type; F/S, NOD2-L1007fs

To assess the basic interaction of both the NOD2 and the NOD2-L1007fs proteins with MDP, cell lines were incubated for either 2 or 24 hours with FITC-labelled MDP. **Figure 11.3** shows that at 2 hours the MDP had penetrated the cell membrane and colocalised with the NOD2 protein (**Figure 11.3a**). This was more evident at 24 hours post MDP treatment (**Figure 11.3b**). In contrast, no colocalisation of MDP with NOD2-L1007fs was observed at either the 2 hour or 24 hour time points. (**Figures 11.3c** and **Figure 11.3d**).

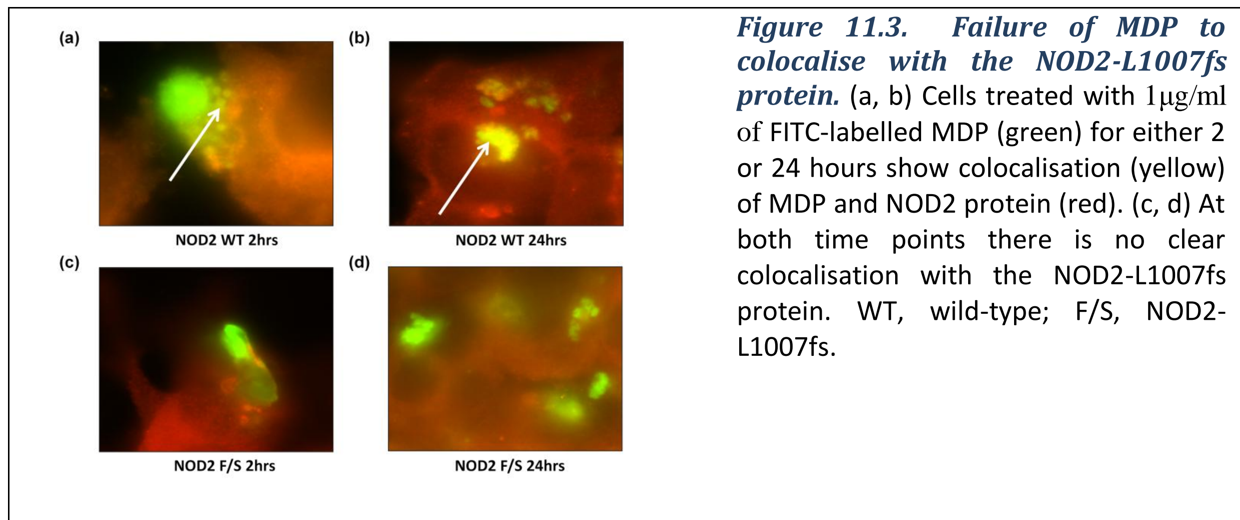


Figure 11.3. Failure of MDP to colocalise with the NOD2-L1007fs protein. (a, b) Cells treated with 1μg/ml of FITC-labelled MDP (green) for either 2 or 24 hours show colocalisation (yellow) of MDP and NOD2 protein (red). (c, d) At both time points there is no clear colocalisation with the NOD2-L1007fs protein. WT, wild-type; F/S, NOD2-L1007fs.

11.4.2 Overexpression of wild-type NOD2 or NOD2-L1007fs does not affect nuclear or cellular size

To assess the effect of overexpression of NOD2 and NOD2-L1007fs on nuclear size in the HEK293 stable cells, the three lines (empty vector, NOD2 and NOD2-L1007fs) were cultured for 24 hours on cover slips, fixed, permeabilised and stained with DAPI. Immunofluorescent microscopy was then used to produce five random images (at 40x magnification) of each cell line, with ImageJ used to measure the area of 100 randomly selected cells from each line. **Figure 11.4a** shows that no clear difference in nuclear size was evident on visual inspection; this was also confirmed statistically (**Figure 11.4b**).

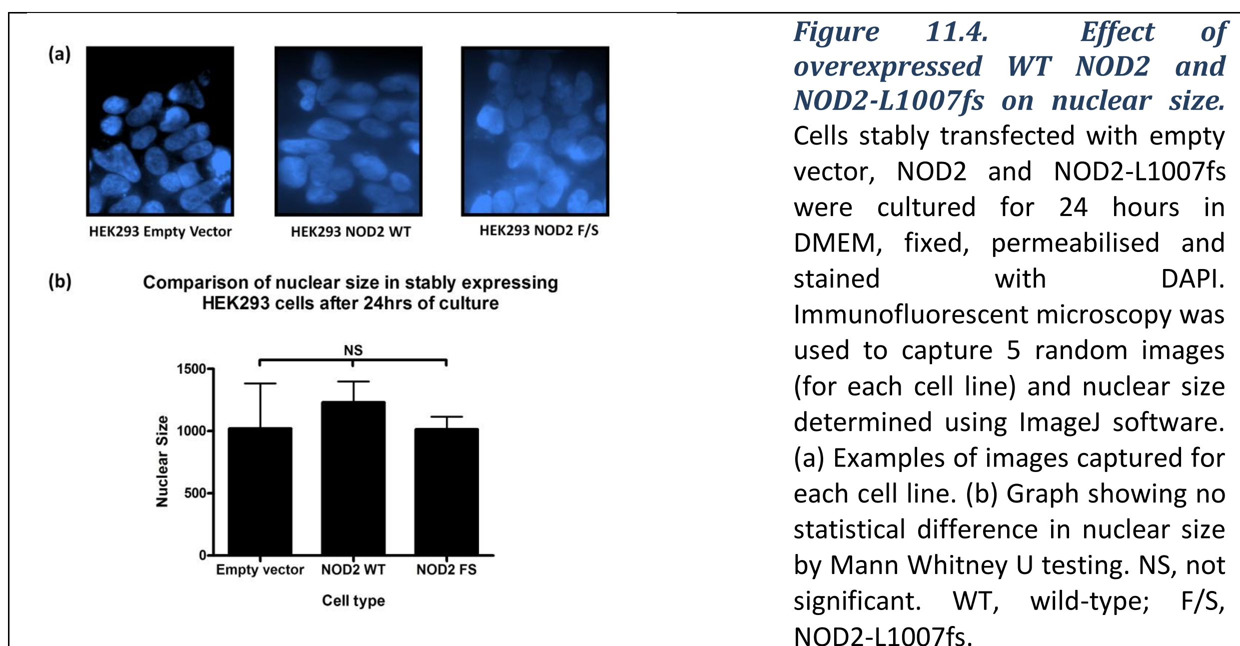


Figure 11.4. Effect of overexpressed WT NOD2 and NOD2-L1007fs on nuclear size. Cells stably transfected with empty vector, NOD2 and NOD2-L1007fs were cultured for 24 hours in DMEM, fixed, permeabilised and stained with DAPI. Immunofluorescent microscopy was used to capture 5 random images (for each cell line) and nuclear size determined using ImageJ software. (a) Examples of images captured for each cell line. (b) Graph showing no statistical difference in nuclear size by Mann Whitney U testing. NS, not significant. WT, wild-type; F/S, NOD2-L1007fs.

A similar process was carried out to assess any differences in cell size. The three HEK293 cell lines were cultured for 24 hours in standard 6 well plates. Following this, cells were placed under a standard light microscope and five random images taken of each cell line (at 40x magnification). Cell size was determined using ImageJ by measuring the area of 50 representative cells. **Figure 11.5** shows that no difference in cell size was evident.

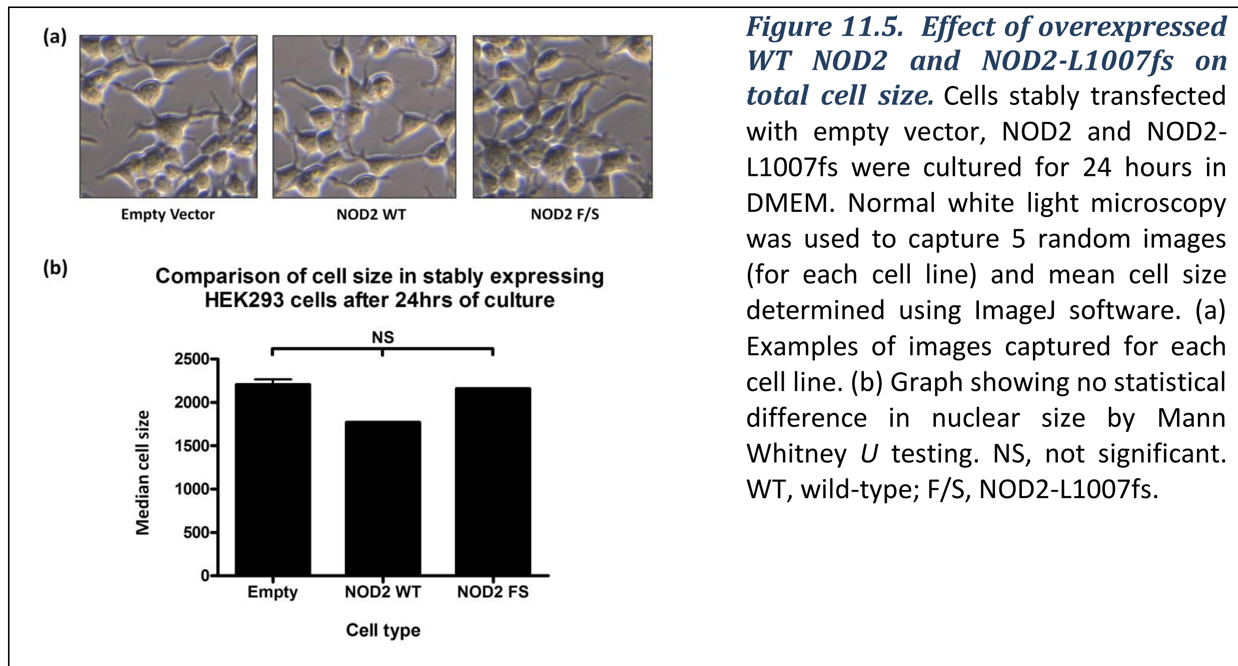


Figure 11.5. Effect of overexpressed WT NOD2 and NOD2-L1007fs on total cell size. Cells stably transfected with empty vector, NOD2 and NOD2-L1007fs were cultured for 24 hours in DMEM. Normal white light microscopy was used to capture 5 random images (for each cell line) and mean cell size determined using ImageJ software. (a) Examples of images captured for each cell line. (b) Graph showing no statistical difference in nuclear size by Mann Whitney *U* testing. NS, not significant. WT, wild-type; F/S, NOD2-L1007fs.

11.4.3 Presence of over-expressed NOD2 protein influences cell proliferation independent of wild-type or frameshift status

It had been observed during routine cell passaging that both the HEK293 NOD2 and NOD2-L1007fs cultures were proliferating at a faster rate than the empty vector stables. To assess if this was correct, and to assess if any difference in proliferation could be quantified, the three HEK293 stable cell lines were labelled with CFDA-SE. Cells were cultured for 72 hours, with a control (i.e. non-proliferating) CFDA-SE-labelled cell suspension analysed at time 0 for comparison for each cell line. It can be seen in **Figure 11.6** that even after 24 hours in culture both the NOD2 stable cell lines had already shown an increased rate of proliferation compared to the empty vector. This was further accentuated by 36 hours, when the geometric mean CFDA-SE level for the empty vector cell line had only dropped to 5% of the baseline (day 0) compared to 2% for both the NOD2 stable lines.

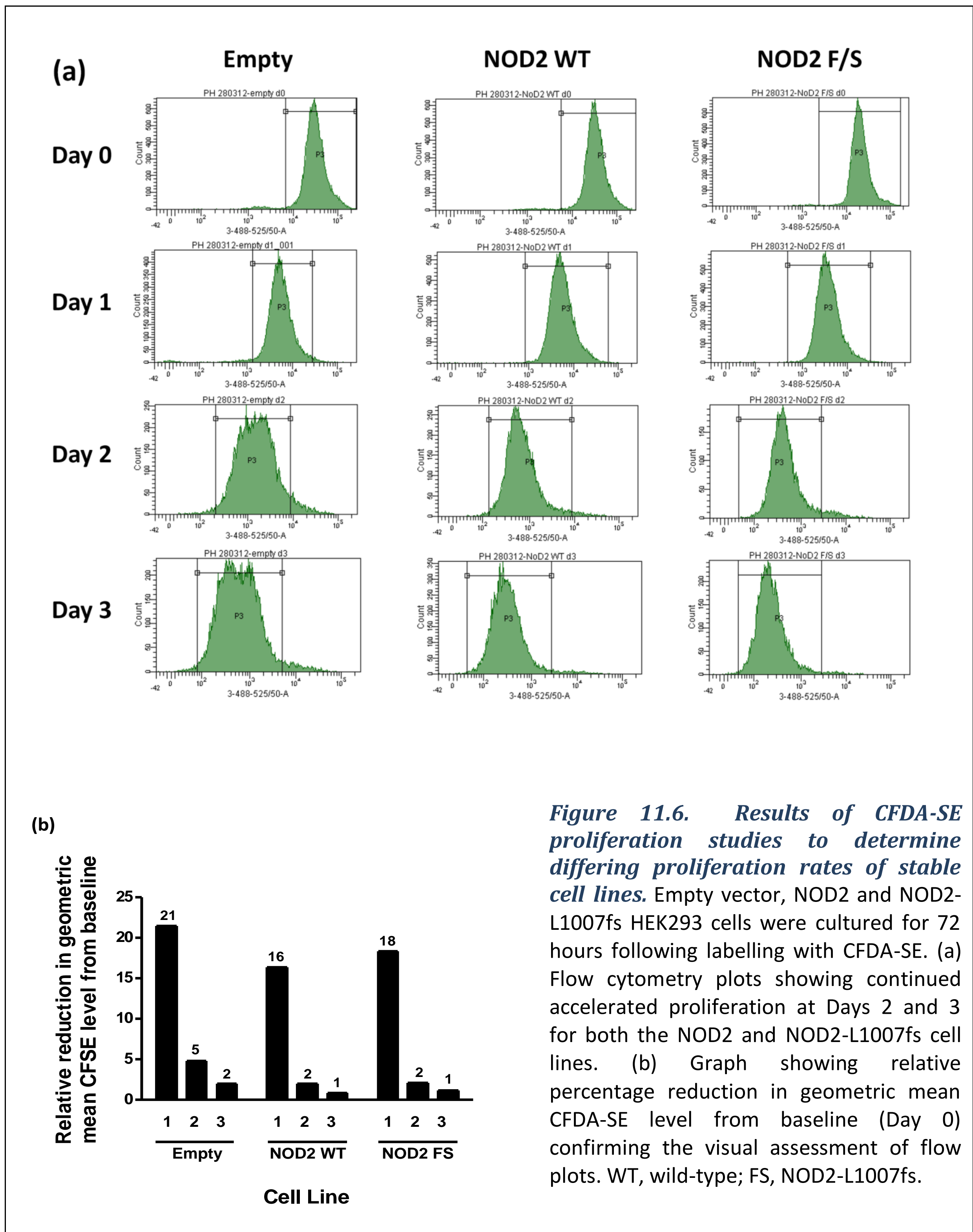


Figure 11.6. Results of CFDA-SE proliferation studies to determine differing proliferation rates of stable cell lines. Empty vector, NOD2 and NOD2-L1007fs HEK293 cells were cultured for 72 hours following labelling with CFDA-SE. (a) Flow cytometry plots showing continued accelerated proliferation at Days 2 and 3 for both the NOD2 and NOD2-L1007fs cell lines. (b) Graph showing relative percentage reduction in geometric mean CFDA-SE level from baseline (Day 0) confirming the visual assessment of flow plots. WT, wild-type; FS, NOD2-L1007fs.

11.4.4 The effect of stable NOD2 expression on apoptosis

To assess the baseline level of apoptosis, the three HEK293 stable cell lines were cultured in DMEM in standard 6-well culture plates for 24 hours. Following this period cell pellets

were collected and lysates probed with Poly ADP ribose polymerase (PARP) antibody looking for differences in cleaved PARP (a measure of apoptotic activity[948]). **Figure 11.7** shows that the basal level of PARP cleavage was similar in all three cell lines. To assess whether NOD2 influences the apoptotic response in response to stress (specifically serum-starvation and MDP treatment) cell lines were cultured in standard DMEM, Dulbecco's balanced salt solution (serum-starved) or treated with 5µg/ml of L18-MDP, all for 24 hours. **Figure 11.8** shows that both the NOD2 and NOD2-L1007fs cell lines had an increased apoptotic response to serum starvation at the 24 hour time point compared to the empty vector, with a stronger response seen in the wild-type NOD2-expressing cells. No clear differences in apoptosis were observed with L18-MDP treatment.

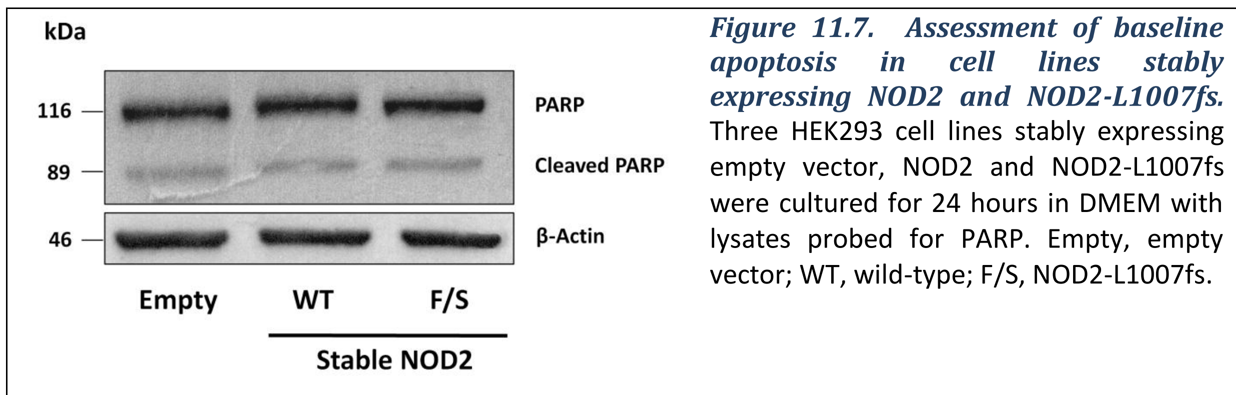


Figure 11.7. Assessment of baseline apoptosis in cell lines stably expressing NOD2 and NOD2-L1007fs. Three HEK293 cell lines stably expressing empty vector, NOD2 and NOD2-L1007fs were cultured for 24 hours in DMEM with lysates probed for PARP. Empty, empty vector; WT, wild-type; F/S, NOD2-L1007fs.

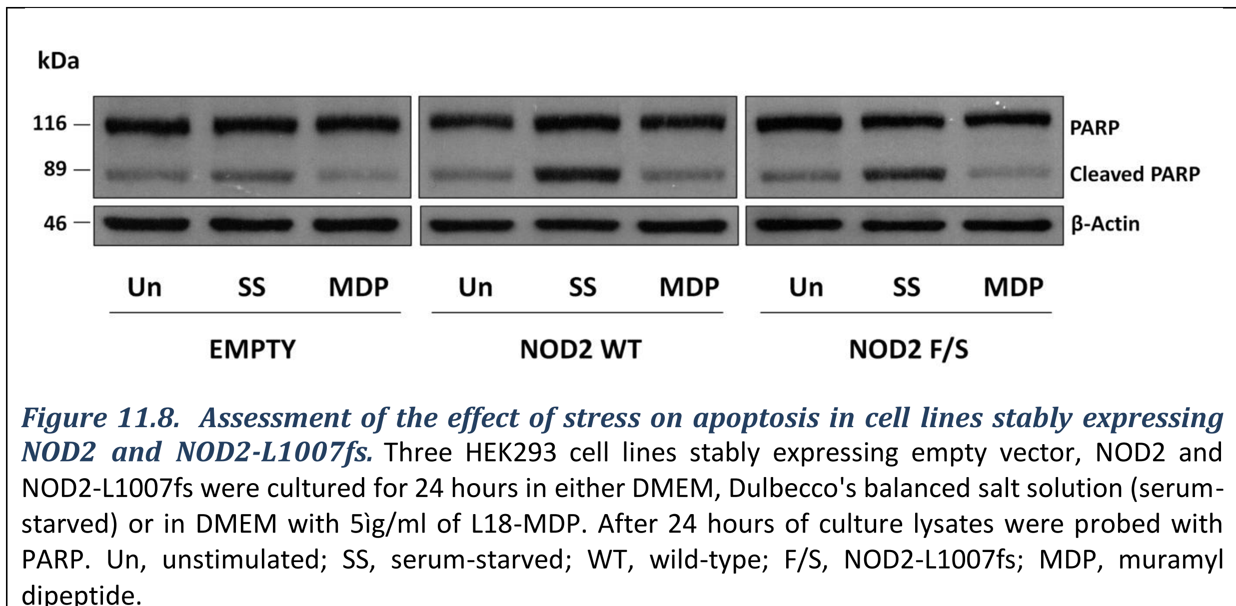


Figure 11.8. Assessment of the effect of stress on apoptosis in cell lines stably expressing NOD2 and NOD2-L1007fs. Three HEK293 cell lines stably expressing empty vector, NOD2 and NOD2-L1007fs were cultured for 24 hours in either DMEM, Dulbecco's balanced salt solution (serum-starved) or in DMEM with 5ìg/ml of L18-MDP. After 24 hours of culture lysates were probed with PARP. Un, unstimulated; SS, serum-starved; WT, wild-type; F/S, NOD2-L1007fs; MDP, muramyl dipeptide.

11.4.5 The NOD2 frameshift protein has a shorter half-life than wild-type NOD2 and is mainly degraded in a proteasome dependent manner, possibly through the CARD domain

To compare the half-lives of the NOD2 and NOD2-L1007fs proteins, cells from each stable line were cultured in 6-well culture plates and treated with cycloheximide (a well-characterised inhibitor of protein synthesis[949]) for 24 hours; cells were collected at 0, 8, 16 and 24 hours post treatment and lysates assessed for NOD2 protein levels by western blot using FLAG antibody. **Figure 11.9** shows that the NOD2-L1007fs protein has a shorter half-life (approx 11h) than the NOD2 protein (>24hrs).

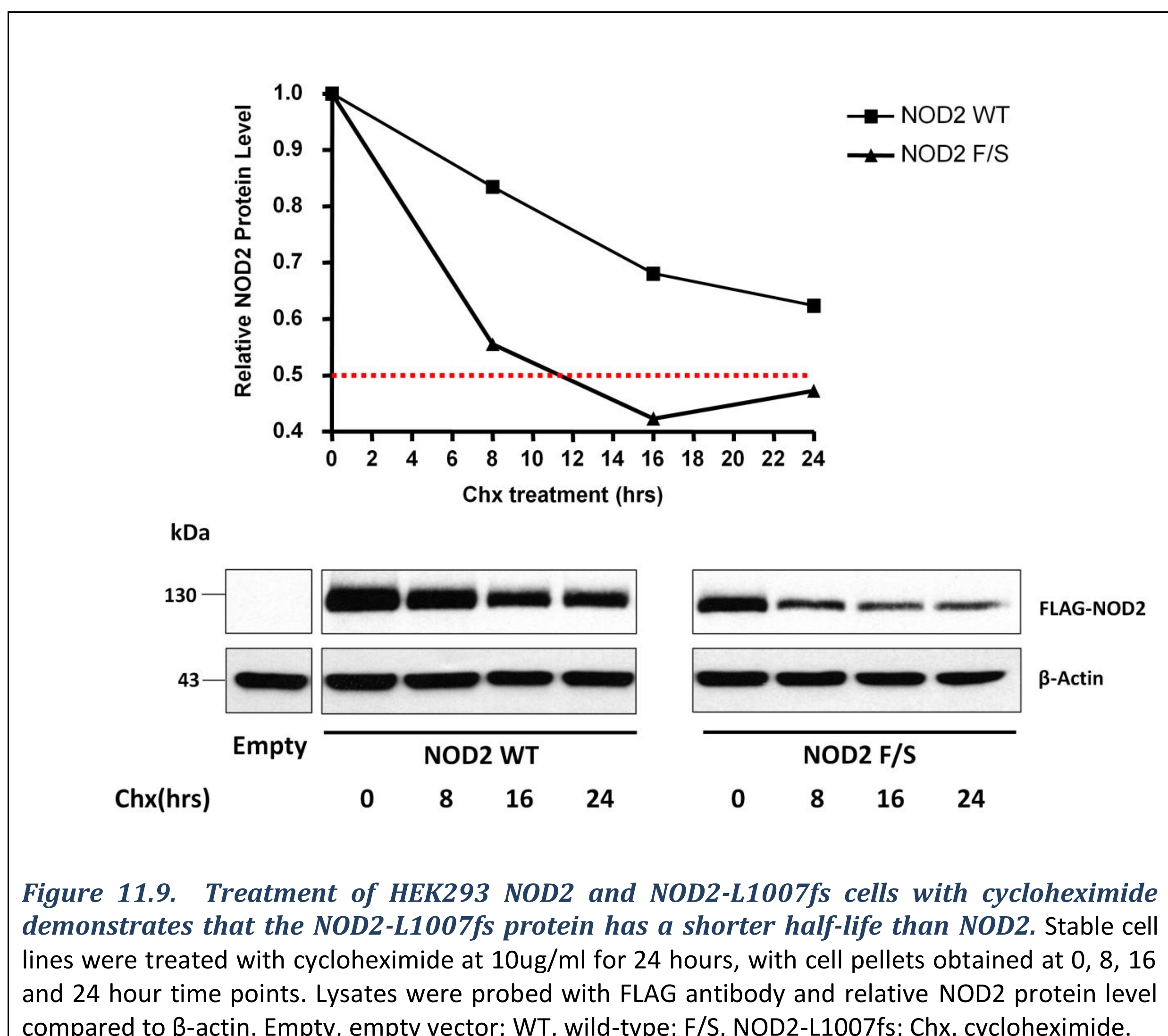


Figure 11.9. Treatment of HEK293 NOD2 and NOD2-L1007fs cells with cycloheximide demonstrates that the NOD2-L1007fs protein has a shorter half-life than NOD2. Stable cell lines were treated with cycloheximide at 10ug/ml for 24 hours, with cell pellets obtained at 0, 8, 16 and 24 hour time points. Lysates were probed with FLAG antibody and relative NOD2 protein level compared to β-actin. Empty, empty vector; WT, wild-type; F/S, NOD2-L1007fs; Chx, cycloheximide.

To further assess the mechanism by which the NOD2 protein is degraded, two well characterised inhibitors of the ubiquitin proteasome or lysosome degradation pathways were

utilised. MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal) is a natural peptide-aldehyde proteasome inhibitor derived from a Chinese medicinal plant.[950] This compound binds to the beta subunit of the 20S proteasome and therefore blocks the proteolytic action of the 26S proteasome complex.[951] MG132 therefore effectively inhibits the degradation of proteins disassembled by the ubiquitin-proteasome pathway. Conversely, chloroquine, a 4-aminoquinoline drug used mainly in the treatment of malaria, has been shown to inhibit the lysosome, thus reducing the degradation of proteins broken down via this pathway.[952] Chloroquine enters the acidic lysosome where deprotonation leads to trapping of the compound in the lysosomal compartment, leading to an increase in pH and reduced lysosomal pathway activity.[953] HEK293 cells were transiently transfected with HA-tagged NOD2 or NOD2-L1007fs DNA then treated with MG132 for 6 hours and with chloroquine for 16 hours in standard medium (DMEM) to assess the potential accumulation of each protein. **Figure 11.10** shows that both the NOD2 and NOD2-L1007fs proteins accumulated with MG132 treatment, with a characteristic high molecular weight polyubiquination 'smear' evident on the western blot.[954] Chloroquine treatment had no observable effect on NOD2 protein levels or migration.

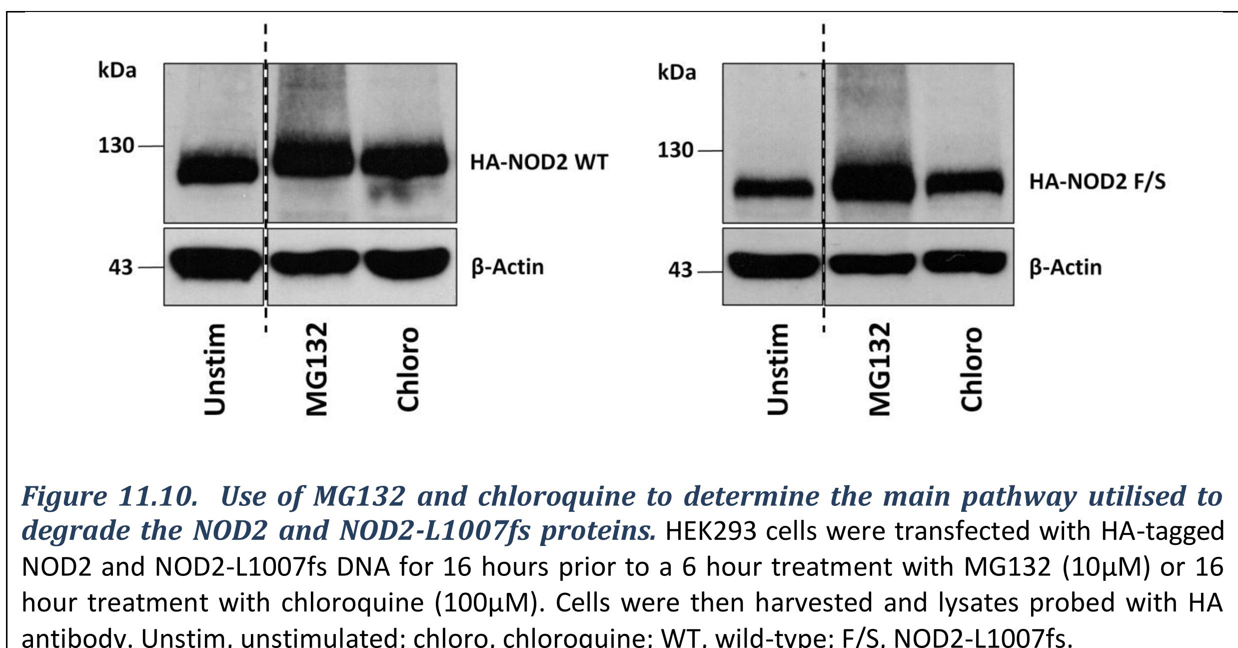


Figure 11.10. Use of MG132 and chloroquine to determine the main pathway utilised to degrade the NOD2 and NOD2-L1007fs proteins. HEK293 cells were transfected with HA-tagged NOD2 and NOD2-L1007fs DNA for 16 hours prior to a 6 hour treatment with MG132 (10µM) or 16 hour treatment with chloroquine (100µM). Cells were then harvested and lysates probed with HA antibody. Unstim, unstimulated; chloro, chloroquine; WT, wild-type; F/S, NOD2-L1007fs.

Having determined that the NOD2 protein is likely degraded through a proteasome-dependant pathway, the HA-tagged full-length NOD2 protein in addition to the NOD2 mutants with a truncated LRR domain (NOD2 [1-693]), or truncated LRR and NACHT domains (NOD2 [1-247]) (**Figure 11.11**) were subjected to a similar treatment with MG132 to

assess the accumulation of NOD2 protein. **Figure 11.12** shows that a ubiquitin smear was present with a sustained accumulation of protein evident with only the minimal CARD domain. This suggests that the amino acid residue(s) required for ubiquitination and subsequent targeting of NOD2 for proteasomal degradation may be situated in the first 247 residues of the NOD2 protein, but may also exist across other domains.

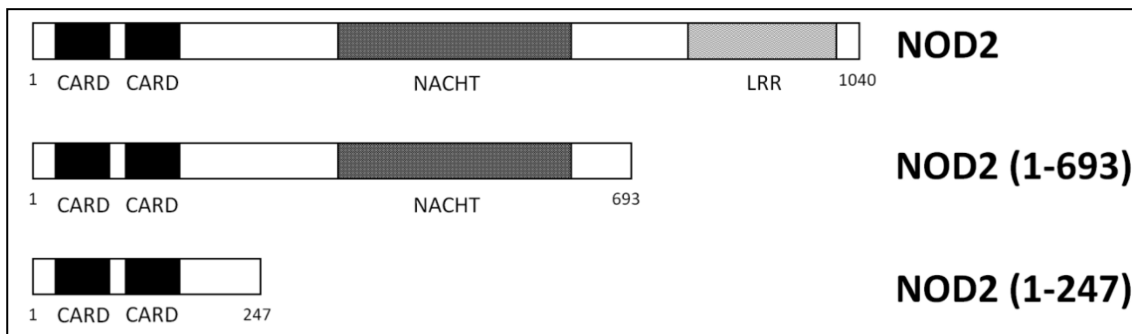


Figure 11.11. Schematic representation of the full length NOD2 protein and mutant NOD2 proteins used to evaluate the ubiquination site on the NOD2 protein. Site-directed mutagenesis was used to produce two NOD2 mutants with either the LRR, NOD2 (1-693), or the LRR and NACHT, NOD2 (1-247), truncated.

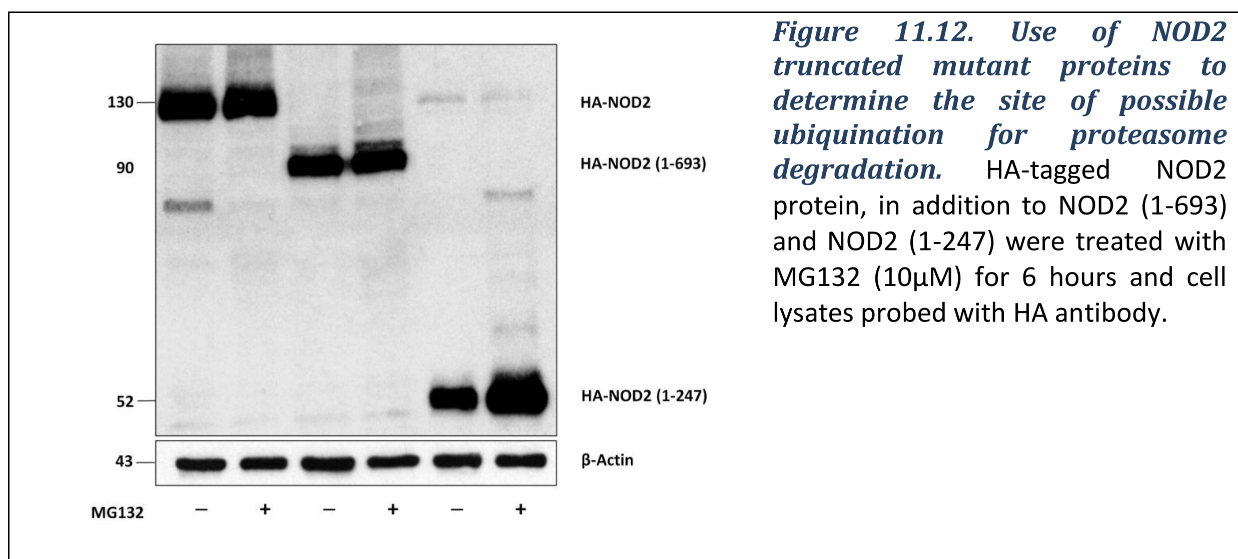


Figure 11.12. Use of NOD2 truncated mutant proteins to determine the site of possible ubiquination for proteasome degradation. HA-tagged NOD2 protein, in addition to NOD2 (1-693) and NOD2 (1-247) were treated with MG132 (10µM) for 6 hours and cell lysates probed with HA antibody.

11.5 Discussion

In HEK293 cells (that express low levels of *NOD2*), overexpression of NOD2 and NOD2-L1007fs proteins can be easily detected by western blot and immunoflourescence. This overexpression has been shown not to influence cell or nuclear size, but does seem to effect

cellular proliferation, independent of polymorphism status. Additionally, although no evidence of differences in baseline apoptosis were apparent, cells overexpressing NOD2 or NOD2-L1007fs had an accentuated apoptotic response to serum starvation, but not treatment with MDP, with the direct interaction of NOD2 with MDP impaired with the frameshift protein. Finally, using proteosome and lysosome inhibitors it has been determined that NOD2 protein stability is regulated through the ubiquitin-proteasome degradation pathway. Transient expression of truncated mutant NOD2 proteins revealed that stability is likely regulated through lysine amino acid residues present at least in the CARD domain.

With respect to cellular size there was no clear difference in cells expressing NOD2 or NOD2-L1007fs by simple light microscopy. Although this did not take into account cell volume *per se* (which would require layered imaging by confocal microscopy), it was clear visually that no discrepancy was evident. The processes controlling cell size are complex with nucleotides, amino acids, sugars and fatty acids making up the majority of structural cell mass.[955] Cell growth is tightly regulated by growth factors which control cell expansion through the phosphorylation of initiation factors, with nutrient availability and growth factors critical in maintaining steady growth, and the mammalian target of rapamycin (see **Chapter 12**) acting as the main intracellular regulator of biosynthesis.[956] The balance between cellular degradation (through processes such as autophagy) and cell expansion are tightly controlled,[957] and the lack of evidence relating to difference in size at baseline between the empty vector and NOD2 cell lines, suggests that unstimulated NOD2 does not have direct effects on growth signalling or specifically mTOR activity. With regard to the mechanisms determining nuclear size, there is as yet no consensus on the factors involved. Two current observations which may explain differences in size are the karyoplasmic ratio (i.e. nuclear size is directly proportional to cytoplasmic volume)[958] and the nucleoskeletal theory which states that the larger the genome, the larger the cell. The latter theory has been dissected over recent years but has come up against resistance due to the observations that nuclear size differs within the same organism,[959] in addition to a 16-fold increase in ploidy having no effect on nuclear size[960]. There are currently no direct reasons why over-expression of *NOD2* (either wild-type or L1007fs) would have influenced nuclear size, however with the endoplasmic reticulum (specifically endoplasmic reticulum stress) gaining more interest in CD pathogenesis (**Chapter 4.3.6**), and it's intimate role in the development of the nuclear envelope, further links between the NLRs and nuclear volume may become apparent.[492]

As discussed in the introduction, there is currently little direct evidence that NOD2 effects cellular proliferation, as crypt length has most often been used as a proxy.[944] Although Cruickshank et al. determined BrdU incorporation in NOD2+/+ and NOD2-/- murine colonic crypts, the marginal difference in the number of BrdU+ cells in the wild-type and null mice prior to infection with *Salmonella* may not have been clinically relevant, with no clear growth

in the null mice evident after infection suggesting only lack of growth during MDP stimulation. In the proliferation experiment outlined above, over the course of a 72 hour experiment, the HEK293 cells expressing either NOD2 or NOD2-L1007fs progressed through more cellular proliferation that the empty vector alone. Direct links between NOD2 and proliferation have not been forth-coming, however it is intriguing that the interaction between NOD2, RIPK-2 and cIAP1/cIAP2 (as outlined in the introduction) may play a part. Conceivably, the overexpression of NOD2 in the absence of MDP-sensing could affect the balance of cIAP1/cIAP2 activity leading to modulation of any number of cell mechanisms such as cell survival , proliferation or differentiation.[961] Additionally, other NOD2 interacting proteins such as GRIM-19 are closely linked to cell proliferation,[962] with binding of these proteins potentially reducing their cytoplasmic availability leading to a loss in the normal negative feedback required to control proliferation[963].

Previous studies assessing the role of NOD2 during apoptosis have mainly evaluated cells with or without NOD2 expression. In this study, the level of baseline apoptosis of cells overexpressing NOD2 or NOD2-L1007fs was also considered. It was evident that the expression of NOD2 had no effect on baseline apoptosis (as determined by the measurement of cleaved PARP) but that NOD2 expression did have a negative effect on stress induced by serum-starvation, but not MDP treatment. This single experiment does not offer any indication as to the dynamics of apoptosis activation in these cell lines, and the effect of MDP especially may have been transient, therefore evidence of apoptosis at earlier time-points may have been missed. However the increased degree of apoptosis with serum-starvation is certainly of interest. Serum-starvation is a well characterised inducer of apoptosis,[964,965] and there was some evidence in the HEK293 cells expressing empty vector that serum-starvation had activated the apoptotic pathway. The increased apoptotic response in the NOD2 cell lines (more so in the wild-type than L1007fs line) may reflect simply the processes involved in protein overexpression making the cells more sensitive to stress, however direct effects on apoptosis should certainly be considered. A number of NOD proteins have been shown to be involved in the apoptotic pathway, including APAF1,[966] NOD1,[967] DEFCAP,[968] and IPAF[969]. It has been recognised that these proteins often have a dual effect, signalling to capases and NFκB leading to simultaneous pro- and anti-apoptotic effects,[970] similar to the effect seen with toll-like receptors[971,972]. Potentially in the absence of MDP stimulation the imbalance of caspase activation and NFκB production may have led to an increased apoptotic response. Specifically with regard to the apoptotic response during serum-starvation, recent work showing that NOD2 binds ATP,[973] and the close link between ATP and energy metabolism may be relevant.

Several experiments carried out on the NOD2 protein demonstrated that there were significant differences in half-life between the NOD2 and NOD2-L1007fs proteins. Additionally, work also showed that the NOD2 protein is likely to be degraded through the ubiquitin-proteasome pathway through lysine residues on the CARD domain or other protein sites. Protein half-life is controlled by the balance of protein degradation and protein synthesis therefore the difference demonstrated between the wild-type and L1007fs proteins may reflect differences in either pathway. Given that subsequent experiments demonstrated the likely role of the ubiquitin-proteasome pathway in NOD2 degradation, with very recent work confirming this finding,[974] the relevance of the differing half-lives is of interest. In fact, a very recent study has shown that ectopically expressed NOD2 is ubiquitinated with K48-linked ubiquitin chains on the NOD domain followed by proteasomal degradation through functional TRIM27, but with proteins often displaying several ubiquitination sites on multiple domains,[975] it has yet to be seen if specific sites on the CARD or LRR domains will be determined. Specifically, the differing half-lives of the NOD2 and NOD2-L1007fs proteins may reflect the importance of residues on the LRR domain, and with recent work demonstrating the potential importance of this domain in proteasomal degradation in other NLR proteins,[969] further work is certainly warranted to delineate this relationship further.

11.6 Conclusion

It can be seen that overexpression of the *NOD2* or *NOD2-L1007fs* variant has no influence on cell or nuclear size, but does seem to effect cellular proliferation, independent of polymorphism status. Additionally, there is an accentuated apoptotic response to serum starvation with NOD2 overexpression with the NOD2 protein most likely degraded through the proteasome-ubiquination pathway. Further work is now required to determine the precise mechanisms connecting NOD2 with apoptotic and proliferation pathways, and how different lysine residues may be responsible for ubiquitination and subsequent proteasomal degradation, thus impinging on NOD2 activity and potentially CD pathogenesis.

12. Autophagy signalling in inflammatory bowel disease - mammalian target of rapamycin, vimentin and azathioprine

12.1 Introduction

In **Chapter 9.5.1** recent work linking NOD2 and autophagy was examined in detail, especially with relation to Crohn's disease (CD). These studies carried out by several independent groups demonstrated that muramyl dipeptide (MDP) induced autophagy through NOD2, that NOD2 colocalised with the major autophagy protein ATG16L1, and that CD-associated variants in *NOD2* influenced bacterial handling and antigen presentation.[30,31] Although these studies were a ground-breaking insight into NOD2 signalling, the authors were unable to determine the precise mechanisms involved in this MDP-induced autophagy, however the mammalian target of rapamycin (mTOR) was suggested as a possible major participant in the NOD2-autophagy pathway.[30] Additionally, with a number of novel NOD2-interacting proteins now identified (e.g. vimentin[915]) their role in this important pathway has yet to be elucidated. As discussed in **Chapter 1.5.3**, the mechanism of action of many therapeutic agents remains still unclear in relation to inflammatory bowel disease (IBD) pathogenesis, and with autophagy a potential therapeutic target in other inflammatory conditions,[976-978] as well as IBD,[979] the effect of these drugs on the autophagic machinery is of particular interest.

12.1.1 Mammalian target of rapamycin

Genetic screening of *Saccharomyces cerevisiae* identified two TOR genes (*TOR1* and *TOR2*), giving an early indication of the importance of TOR in the control of cell proliferation.[980] This discovery was facilitated by utilising rapamycin, an antifungal metabolite produced by the Gram-positive eubacteria *Streptomyces hygroscopicus*, isolated from a soil sample obtained on Easter Island in the early 1970s.[981] The mammalian orthologue mTOR is a protein encoded by the *MTOR* gene on chromosome 1p36.2 in humans;[982] this serine/threonine protein kinase with a large molecular mass (300kDa) is a member of the phosphatidylinositol kinase-related (PIKK) family.[983] Since its discovery, mTOR has been shown to be a key component in the regulation of a myriad of complex cellular processes including cell growth and autophagy.[984,985] Through these intricate processes mTOR also influences cell proliferation, cell motility, cell survival and protein synthesis and transcription.[986] The majority of research focusing on mTOR has been in relation to cancer pathogenesis and therapy,[987] however a growing volume of work is now

beginning to uncover important roles for mTOR in innate immunity,[988] the adaptive immune response (including T cell activation[989] and regulatory T cell function[990]), behaviour and intellect[991] and metabolic homeostasis[992]. Since its first description in 1991, mTOR has been shown to bind several proteins to form two distinct protein complexes, namely mTORC1 and mTORC2 (**Figure 12.1**).

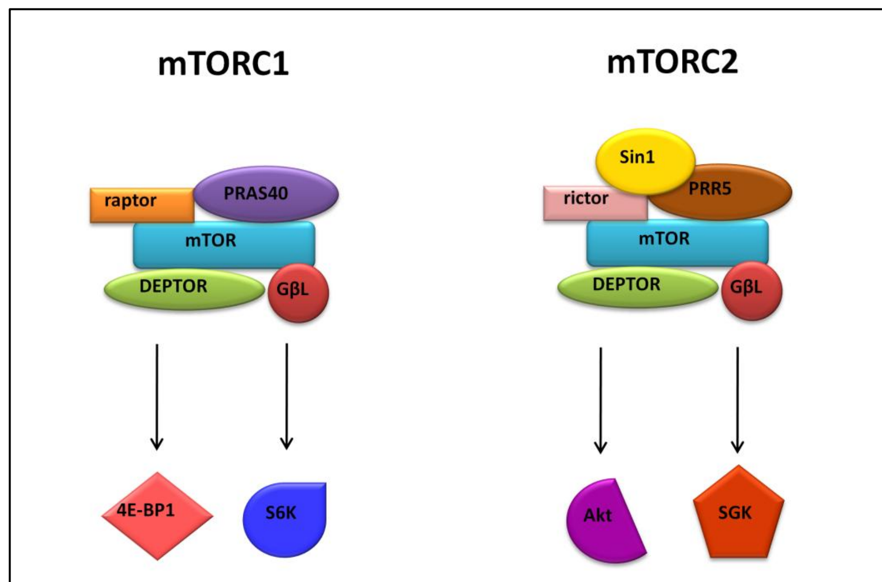


Figure 12.1. Schematic representation of the two known mTOR complexes. mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; PRAS40, proline-rich AKT substrate 40kDa; GβL, G protein beta subunit-like; 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; S6K, S6 kinase; Sin1, stress-activated protein kinase-interacting protein 1; PRR5, proline-rich protein 5; SGK, serum and glucocorticoid-regulated kinase.

Specifically related to autophagy, although the mTORC2 complex does have some influence on autophagy through transcription factors such as FoxO3, the current evidence suggests that the autophagy response is mainly controlled through mTORC1.[993,994] In conditions of abundant nutrients, growth factors and insulin, signalling through mTORC1 promotes cell growth and stimulates protein translation with concomitant inhibition of autophagy (**Figure 12.2a**). However during periods of starvation this inhibition through mTORC1 is released leading to autophagy induction (**Figure 12.2b**).

Although the mTOR pathway is extremely complex,[956] there are certain methods that can be utilised to artificially induce autophagy to allow its investigation. As alluded to above, the withdrawal of nutrients from culture medium is a well-recognised method of inducing autophagy *in vitro*.[995] Additionally, rapamycin (also known as Sirolimus) forms a complex

with FKBP12 (FK506-binding protein-12), which subsequently binds to mTOR within the mTORC1 complex inducing autophagy,[996] most likely through direct effects on the mTORC1 protein regulatory associated protein of mTOR (raptor).[997,998] Rapamycin has long been shown to possess immunosuppressive and antiproliferative properties, being used for many years in transplant medicine.[999] Although rapamycin has also been shown to have direct effects on the mTORC2 complex,[1000] these effects relate more to glucose metabolism,[1001] with only some evidence linking mTORC2 with autophagy[1002]. Within the autophagy pathway, bafilomycin A1 (a vacuolar-type H^+-ATPase inhibitor that inhibits the fusion of the autophagosome and lysosomes and therefore the subsequent accumulation of LC3-II[1003]) has also been used to accentuate the conversion of LC3-I to LC3-II and thus help to evaluate autophagy (**Figure 12.3**).

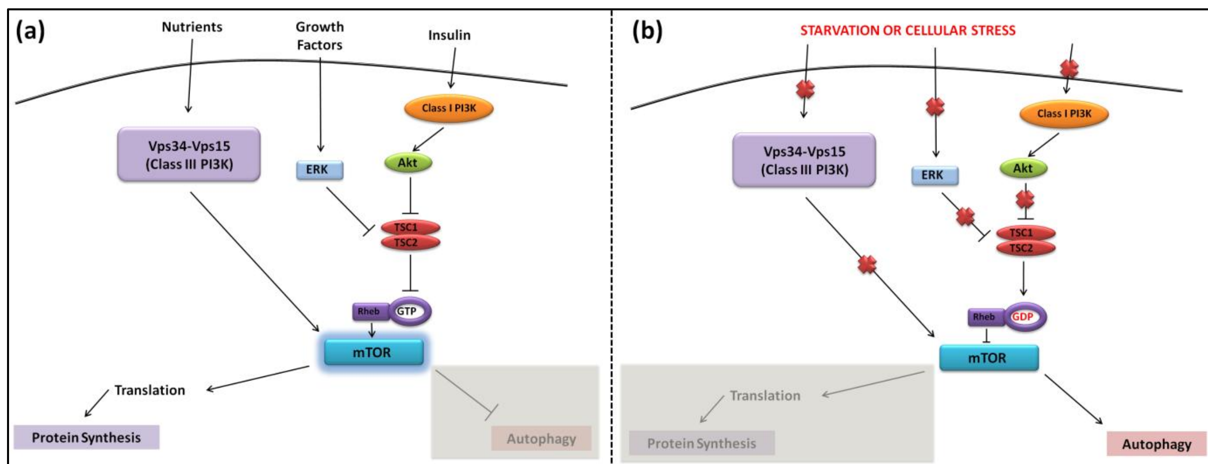


Figure 12.2. Basic mTORC1 signalling pathway demonstrating activity during periods with and without starvation and cellular stress. (a) During periods of abundant nutrients, growth factors and insulin, activation of mTORC1 leads to protein translation and protein synthesis. (b) During starvation (or cellular stress) inhibition of mTORC1 leads to autophagy induction through the TSC complex and class III PI3K pathways.

Following induction with either pharmcotherapy or other compounds, autophagy must then be quantified to allow meaningful studies to be performed.[1004] As there is no single, robust method employed to determine this quantification, recent guidelines on the measurement and interpretation of autophagy assays have suggested using a combination of different assays, primarily western blot of LC3-I and LC3-II, immunofluorescent microscopy of steady state green fluorescent-labelled protein LC3 (GFP-LC3), in addition to measurement of the phosphorylation of substrates directly downstream from mTORC1.[1005] With regard to these downstream substrates of mTOR, phosphorylation of S6 kinase (S6K) and ribosomal protein S6 kinase-1 (rpS6) are stimulated by insulin and growth factors in an mTORC1-dependent

manner.[994] Phosphorylation at both these sites (S6K at threonine 389 [T389] and rpS6 at serine 235/236 [S235/236]) are therefore well established readouts of mTORC1 activity *in vitro* and *in vivo* (**Figure 12.3**).[1005,1006]

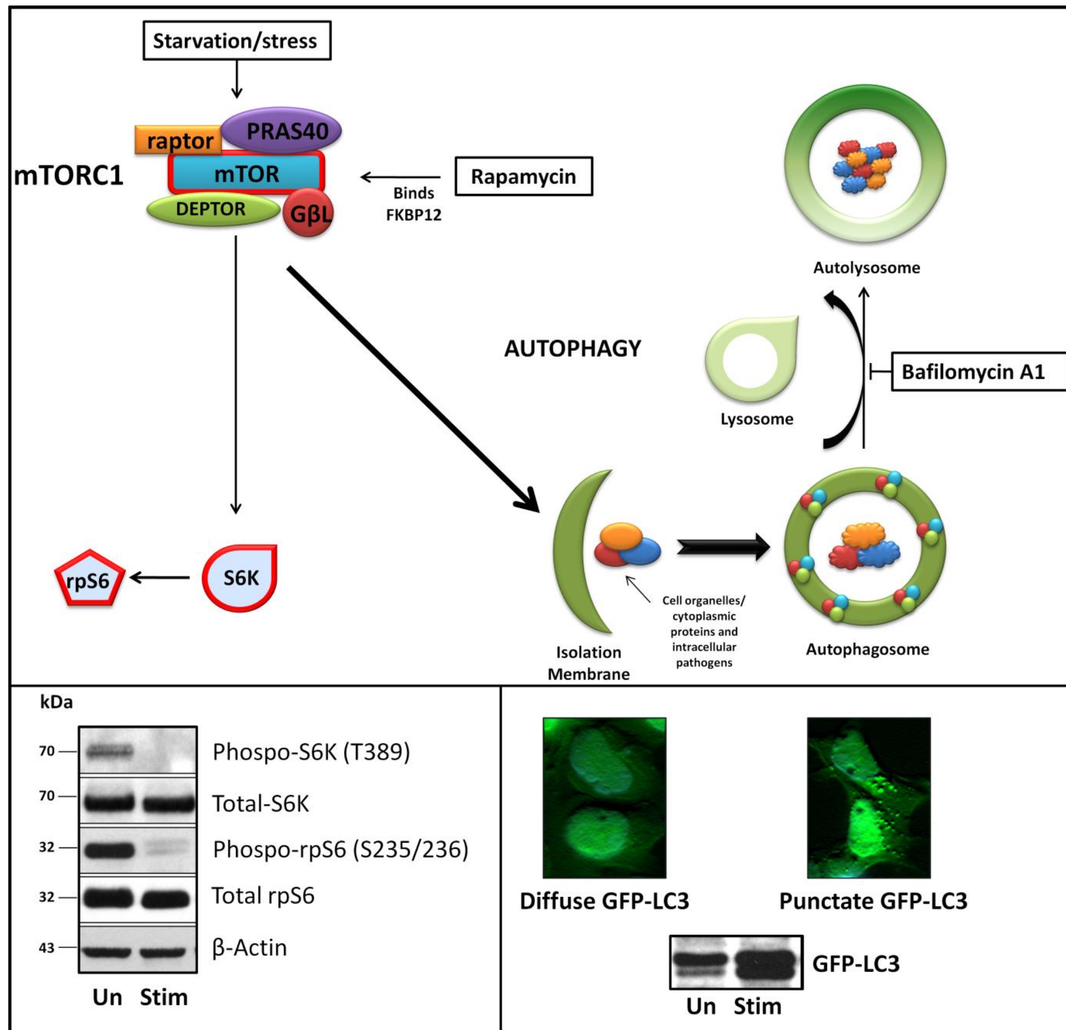


Figure 12.3. Diagrammatic representation of the process of autophagy induction and the assays used to determine these changes. Starvation/stress and rapamycin have been shown to act on the mTORC1 complex to induce autophagy (top panel). Due to the direct effects on mTORC1, substrates downstream (S6K, rpS6) are dephosphorylated which can be assessed using western blot (bottom left panel). Also, using GFP-LC3 (see **Figure 9.2** for further details on LC3 biogenesis) a diffuse pattern of GFP immunofluorescence can be observed, with punctate LC3 foci visible as the autophagosomes form. An increase in LC3-II (smaller sized band on GFP-LC3 western blot) can also be determined (bottom right panel). Un, unstimulated; Stim, stimulated.

12.1.2 The intermediate filament vimentin in CD pathogenesis

As discussed in **Chapter 11.2** only a small number of NOD2 interacting proteins have been identified to date,[927] and currently there is limited knowledge with regard to their functional effect on NOD2.[927] In a previous yeast-two-hybrid (Y2H) screen carried out in the laboratory

of Prof. Jack Satsangi (Gastrointestinal Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh), a novel interaction between NOD2 and the cytoskeletal protein vimentin was described.[1007] Vimentin is the major intermediate filament (IF) protein in cells of mesenchymal origin where it plays an important role in the maintenance of cell architecture.[1008] Along with actin filaments and microtubules the IF proteins constitute one of the three main cytoskeletal components in eukaryotic cells, with desmin, vimentin, peripherin and glial filament acidic protein (GFAP) making up the type III IF proteins.[1009] Analysis of IF protein primary structures has shown that despite enormous diversity, all these proteins share a common structure, namely a central alpha-helical domain flanked by a non-helical N-terminal and C-terminal domains.[1010]

Specifically, vimentin has been shown to be important for the correct positioning and trafficking of organelles within the cytoplasm and is emerging as an important factor linked to bacterial and viral pathogenicity.[1011,1012] Several recent studies have shown that vimentin is expressed on the surface of certain cell types, with this surface-expressed vimentin possessing lectin-like properties.[1013] Vimentin can bind to the monosaccharide *N*-acetylglucosamine (GlcNAc), which is cross-linked together with N-acetylmuramic acid to form peptidoglycan, a major constituent in the bacterial cell wall (MDP is the minimal peptidoglycan motif common to both Gram-positive and Gram-negative bacteria).[1014,1015] Furthermore, vimentin has been shown to be involved in the endocytosis of GlcNAc-conjugated liposomes suggesting that vimentin is actively involved in the internalisation of GlcNAc-bearing ligands on cell surfaces.[1013,1015] Cell surface-expressed vimentin has in the same way been identified as the binding target for several viruses such as Japanese encephalitis virus with vimentin structure and gene expression changing profoundly during infection.[1016] Most relevant to CD pathogenesis are recent reports that vimentin is expressed on the surface of human brain endothelial cells where it acts as a primary receptor for adherent-invasive *E.coli* strains expressing the virulence factor IbeA (see **Chapter 9.6**).[1011] Of particular significance is that the proper formation and distribution of autophagosomes depends on the integrity of intermediate filament networks,[1017,1018] and that autophagic vacuoles are found to be associated with vimentin[1019,1020].

12.1.3 The effect of inflammatory bowel disease therapeutic agents on autophagy

As discussed in **Chapter 9**, there have now been numerous studies evaluating the autophagy pathway-phenotype correlation in relation to IBD, especially CD. However, despite this keen interest, few studies have aimed to determine any potential manipulation of the autophagic process with currently utilised IBD therapies. Much of the current literature focuses solely on the genetic aspects of autophagy in IBD,[1021,1022] yet recent studies have alluded to the fact that increasing autophagy levels in CD patients may be of therapeutic

benefit[27,902]. One of the most intriguing reports of the potential of autophagy manipulation was published by Massey et al. in 2008. They presented the case of a 37-year-old woman with severe refractory colonic and perianal CD, who had lost response to second-line therapies. Following treatment with Sirolimus for six months there was a marked and sustained improvement in both her clinical symptoms and mucosal appearance at endoscopy. Another case report of the successful use of autophagy inducers in CD treatment has been published,[1023] as well as encouraging work in IL10-/- colitic mice,[1024] but clinical trials have yet to prove the efficacy of these drugs in large scale clinical practice.[1025]

12.2 Hypotheses and Aims

Hypotheses:

1. MDP-induced autophagy through NOD2 involves signalling via mTORC1 and this interaction is dysregulated by the CD-associated *NOD2* frameshift variant (NOD2-L1007fs).
2. The NOD2-interacting protein vimentin is involved in MDP-induced autophagy and the gene encoding vimentin (*Vim*) has an association signal for CD susceptibility.
3. Drugs commonly used during paediatric inflammatory bowel disease (PIBD) management induce autophagy through an mTORC1 dependent pathway.

Aims: To establish a robust system of measuring autophagy in both HEK293 and HCT116 cell lines in addition to a reliable read-out of mTORC1 activity during autophagy induction. To determine if mTORC1 and/or vimentin is involved in MDP-induced autophagy induction, in addition to assessing the association signal of the single nucleotide polymorphisms in the gene encoding vimentin (*Vim*) with regard to the genetic susceptibility to CD. To determine the effect on autophagy of drugs commonly used in PIBD management.

12.3 Results

12.3.1 The stable expression of green fluorescent-labelled protein LC3 is required for the robust identification of autophagy induction within HEK293 and HCT116 cell lines.

To first determine the potential utility of endogenous LC3 the parental HEK293 and HCT116 cell lines were treated with rapamycin, serum-starvation (using Dulbecco's balanced salt solution [DBSS]) or bafilomycin A1 (hereafter referred to as bafilomycin) for 4 hours and assessed for LC3 foci formation by immunofluorescent microscopy or for LC3 lipidation by western blot. **Figure 12.4** shows that although there was some formation of punctate LC3 foci in the rapamycin-treated HEK293 cells and rapamycin- and bafilomycin-treated HCT116 cells, this did not correlate well with the western blot analysis, nor did the predicted autophagic response to serum-starvation produce a useful readout.

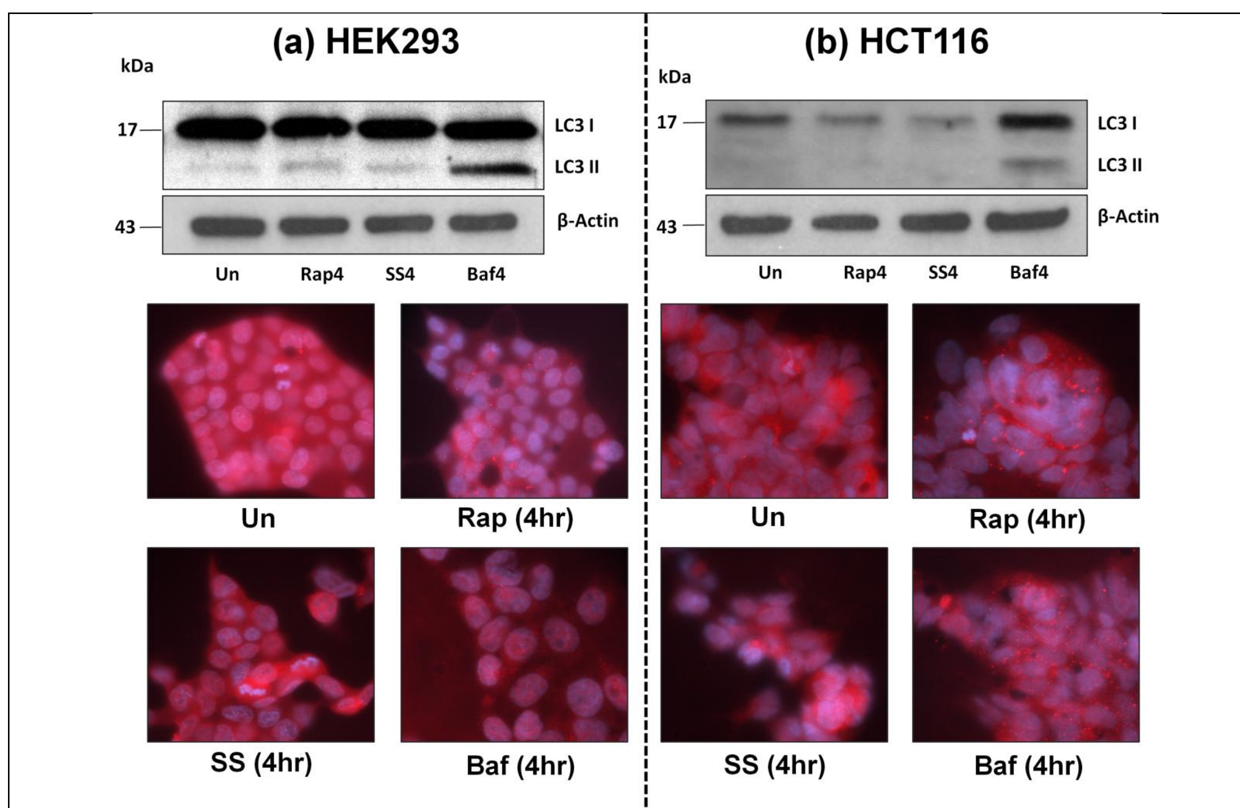


Figure 12.4. Use of endogenous LC3 to identify autophagy induction in HEK293 and HCT116 cell lines. HEK293 (a) and HCT116 (b) cell lines were left unstimulated or treated with rapamycin (100nM), serum-starvation (with DBSS) or bafilomycin (100nM) for 4 hours and the conversion of LC3-I to LC3-II assessed by western blot or the presence of punctate LC3 foci assessed by immunofluorescent microscopy (with secondary TRITC-labelled rabbit secondary). Un, unstimulated; Rapa, rapamycin; SS, serum-starved; baf, bafilomycin.

As this method of identifying autophagy induction was not robust, the HEK293 and HCT116 cell lines stably expressing GFP-LC3 were evaluated to determine if a more rigorous system could be established. It first needed to be established, as suggested by the recent guidelines,[1005] as to what constituted a 'positive' autophagy response in the current experimental system. To achieve this, HEK293 GFP-LC3 cells were treated with either rapamycin or DBSS for 2 hours. **Figure 12.5** shows that although four different methods used to evaluate autophagy induction exhibited some degree of measureable response, the use of greater than five autophagosomes (i.e. punctate LC3 foci) per cell allowed for basal levels of autophagy, while exhibiting significant increases in foci formation in response to both treatments and had the smallest overall error rate of all the methods.

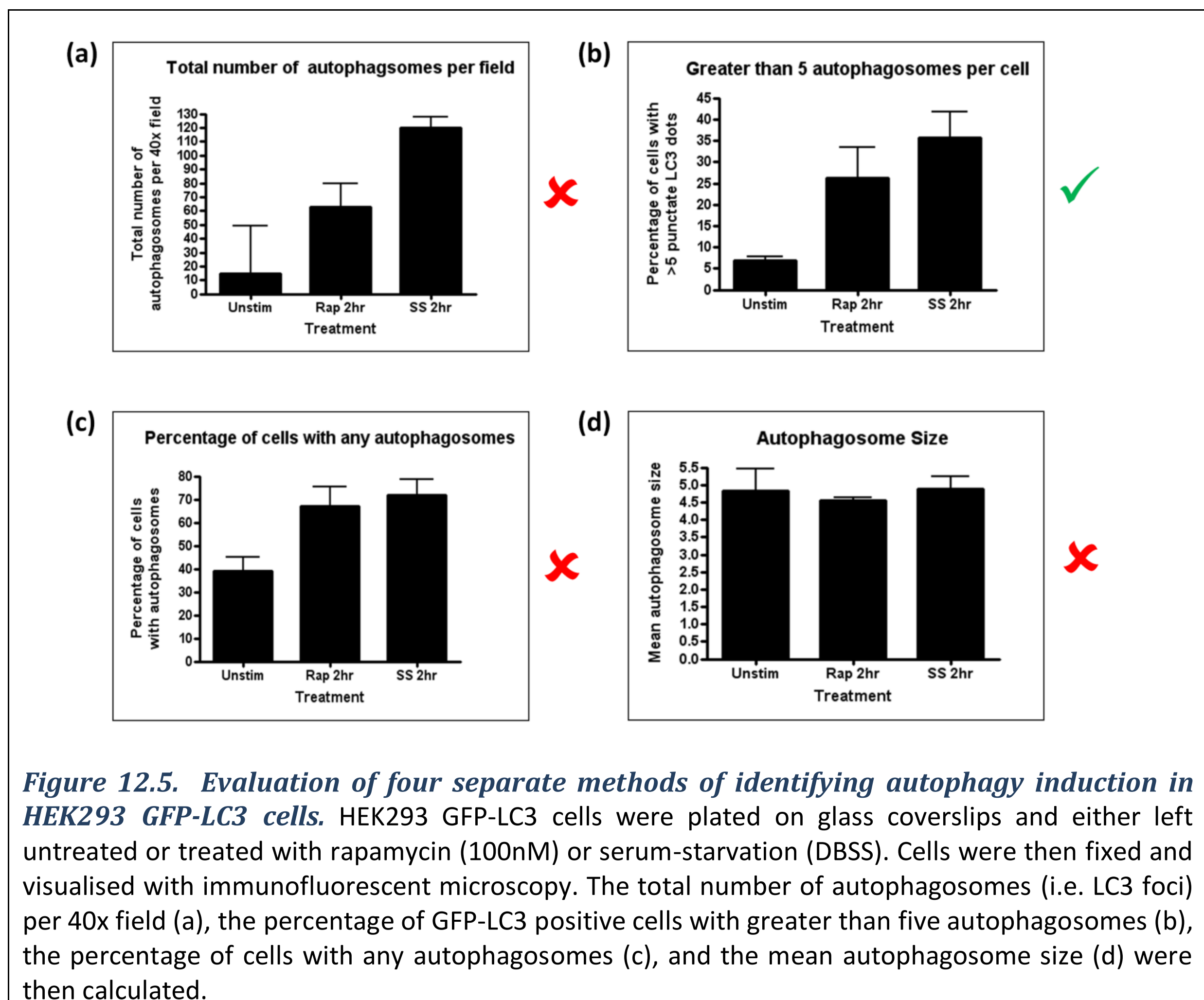


Figure 12.5. Evaluation of four separate methods of identifying autophagy induction in HEK293 GFP-LC3 cells. HEK293 GFP-LC3 cells were plated on glass coverslips and either left untreated or treated with rapamycin (100nM) or serum-starvation (DBSS). Cells were then fixed and visualised with immunofluorescent microscopy. The total number of autophagosomes (i.e. LC3 foci) per 40x field (a), the percentage of GFP-LC3 positive cells with greater than five autophagosomes (b), the percentage of cells with any autophagosomes (c), and the mean autophagosome size (d) were then calculated.

With a clear method of assessing autophagy by immunofluorescent microscopy established, the two GFP-LC3 stable cell lines were compared for their response to

treatment with rapamycin, serum-starvation (with DBSS) or bafilomycin for varying lengths of time. Cells were then visualised directly using immunofluorescent microscopy. **Figure 12.6** shows that both the HEK293 and HCT116 GFP-LC3 cells responded well to treatment with rapamycin and serum-starvation, with the HCT116 cell line showing a higher basal autophagy level.

To correlate the results of the immunofluorescent microscopy with the western blot analysis in the GFP-LC3 stable cell lines, a more limited set of treatments were applied to cultures of each cell line, with cell lysates immunoblotted with both LC3 antibody (therefore detecting LC3 bound to GFP), in addition to GFP antibody ('free' GFP is produced following expulsion of the LC3 complexes from the autophagosome[1026]). **Figure 12.7** demonstrates that detection of GFP-LC3 showed a clear increase in LC3-II response to bafilomycin treatment in both cell lines, and that the liberation of free GFP showed a consistent response (**Figure 12.6**) in the HEK293 cells. However, the free GFP response did not correlate well in the HCT116 cell line and overall the GFP-LC3 response was variable and confirmed that various methods of autophagy assessment are necessary as suggested by recent guidelines.[1005]

(a)

Unstimulated | Rapamycin 2hrs | Serum-starvation 2hrs | Bafilomycin A1 2hrs

% GFP-LC3 +ve cells with greater than 5 punctate LC3 dots

Unstim, Rapa(2hr), Rapa(4hr), Rapa(6hr), S-starved(2hr), S-starved(4hr), S-starved(6hr), Bafilo(1hr), Bafilo(2hr)

Treatment

(b)

Unstimulated | Rapamycin 2hrs | Serum-starvation 6hrs | Bafilomycin A1

% GFP-LC3 +ve cells with greater than 5 punctate LC3 dots

Unstim, Rapa(2hr), Rapa(4hr), Rapa(6hr), S-starved(2hr), S-starved(4hr), S-starved(6hr), Bafilo(1hr), Bafilo(2hr)

Treatment

Figure 12.6. Evaluation of autophagy response in HEK293 and HCT116 cells stably expressing GFP-LC3. HEK293 GFP-LC3 (a) and HCT116 GFP-LC3 (b) cell lines were treated with rapamycin (100nM), serum-starvation (with DBSS), bafilomycin (100nM), or left unstimulated. Figure shows representative immunofluorescent microscopy pictures and full results (graphs). Unstim, unstimulated; Rapa, rapamycin; Bafilo, bafilomycin. *=p<0.05, **=p<0.01, ***=p<0.001; NS, not significant by Mann-Whitney *U*.

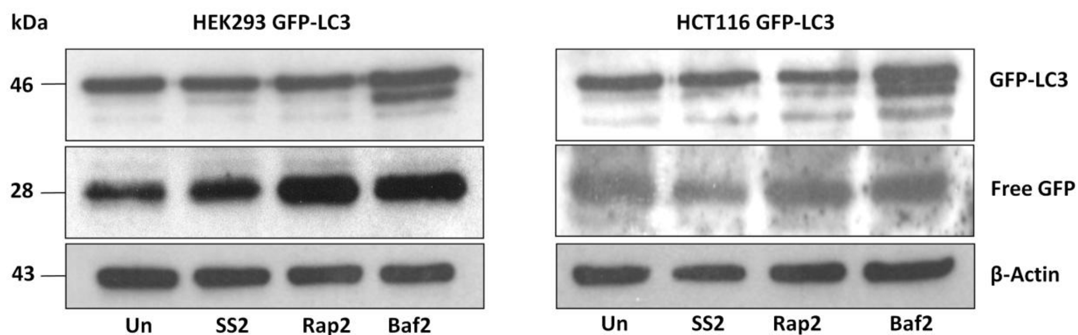


Figure 12.7. Assessment of GFP-LC3 and free GFP in HEK293 GFP-LC3 and HCT116 GFP-LC3 cell lines. Both cell lines were treated with rapamycin (100nM), serum-starvation (with DBSS) or bafilomycin (100nM) for 2 hours, or left untreated. Lysates were immunoblotted with LC3 antibody or GFP antibody. Un, unstimuated; SS, serum-starved; Rap, rapamycin; Baf, bafilomycin.

12.3.2 A reduction in phospho-S6K and phospho-rpS6 provide a robust readout of mTORC1 activity and autophagy induction in HEK293 and HCT116 cell lines.

In order to determine if a reduction in phosphorylated S6K (T389) and rpS6 (ser 235/236) provide a robust readout of mTORC1 activity (and therefore an indirect measurement of autophagy induction[1005]), HEK293 and HCT116 cells lines were treated with rapamycin, serum-starvation (with DBSS) or bafilomycin. Cell lysates were then immunoblotted with phospho-S6K and phospho-rpS6 antibodies by western blot. **Figure 12.8** shows that both cell lines responded appropriately to rapamycin treatment and serum-starvation, with the level of detectable phosphorylation of S6K and especially rps6 showing a significant reduction at all time points.

While there was a clear decrease in the level of endogenous phospho-S6K observed, it was less clear than the decrease observed in phospho-rpS6. To overcome this, an HA-tagged S6K construct (a kind gift from Dr Craig Stevens, University of Edinburgh and Napier University) was transiently transfected into both parental cell lines and a shortened experiment repeated. **Figure 12.9** shows that exogenous HA-S6K provides a much more robust reduction in phosphorylation at T389 than endogenous S6K.

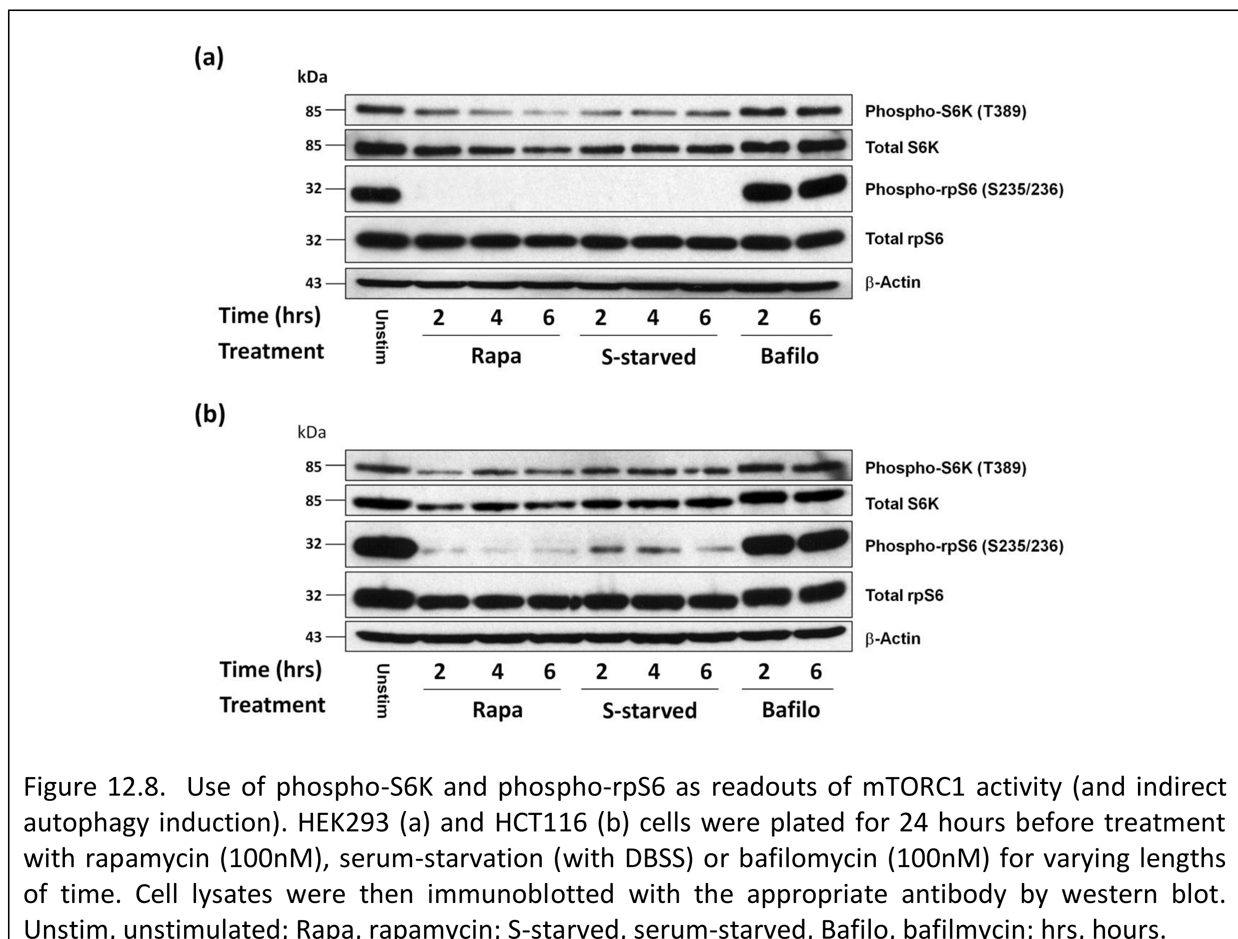


Figure 12.8. Use of phospho-S6K and phospho-rpS6 as readouts of mTORC1 activity (and indirect autophagy induction). HEK293 (a) and HCT116 (b) cells were plated for 24 hours before treatment with rapamycin (100nM), serum-starvation (with DBSS) or bafilomycin (100nM) for varying lengths of time. Cell lysates were then immunoblotted with the appropriate antibody by western blot. Unstim, unstimulated; Rapa, rapamycin; S-starved, serum-starved, Bafilo, bafilmycin; hrs, hours.

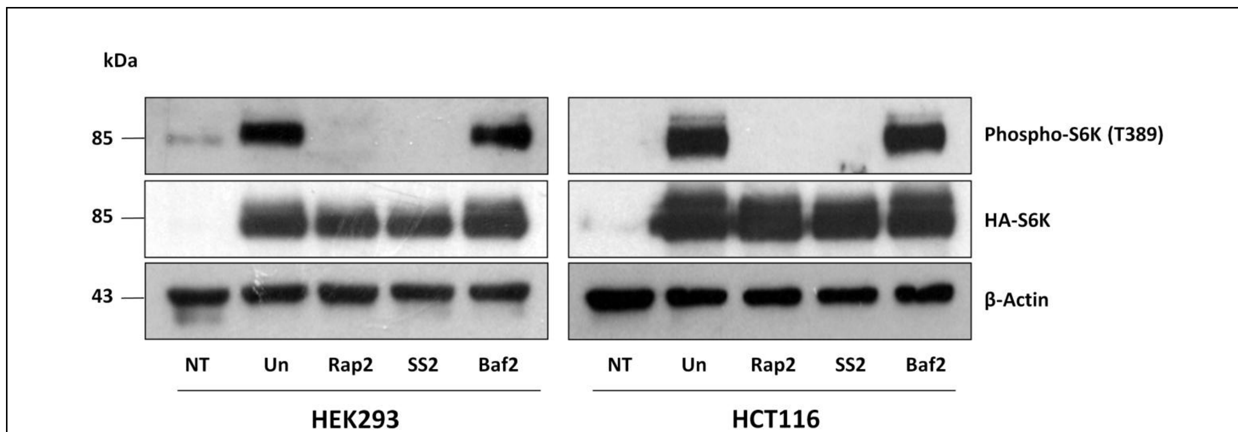


Figure 12.9. Use of transiently transfected HA-tagged S6K to more robustly assess the reduction of phospho-S6K during autophagy. HEK293 cells (left panel) and HCT116 cells (right panel) were plated in 6-well plates and after 24 hours of culture transiently transfected with 2µg of HA-S6K DNA for 16 hours. Following this, cells were treated with rapamycin (100nM), serum-starvation (with DBSS) or bafilomycin (100nM) for 2 hours. Cell lysates were immunoblotted with phospho-S6K (T389) and HA antibody by western blot. NT, not transfected; Un, unstimulated; Rap, rapamycin; SS, serum-starved; Baf, bafilomycin.

Finally, to determine if the previously established methods of autophagy induction (**Figure 12.6**) and mTORC1 readout (**Figure 12.8** and **Figure 12.9**) corresponded to true autophagy, two different methods were employed. Firstly, the autophagy inhibitor 3-methyadenine (3-MA) was used in HEK293 parental cells to reduce the level of autophagy to almost basal levels (**Figure 12.10**). 3-MA is a well-recognised, non-specific inhibitor of autophagy which works by impeding autophagy at the sequestration step by inhibiting class III PI3 kinase activity, without markedly affecting protein synthesis.[1027] It can be seen that the use of 3-MA does not restore mTORC1 activity, as although 3-MA is thought to act upstream of mTOR, it also has actions which are mTORC1-independent.[1028]

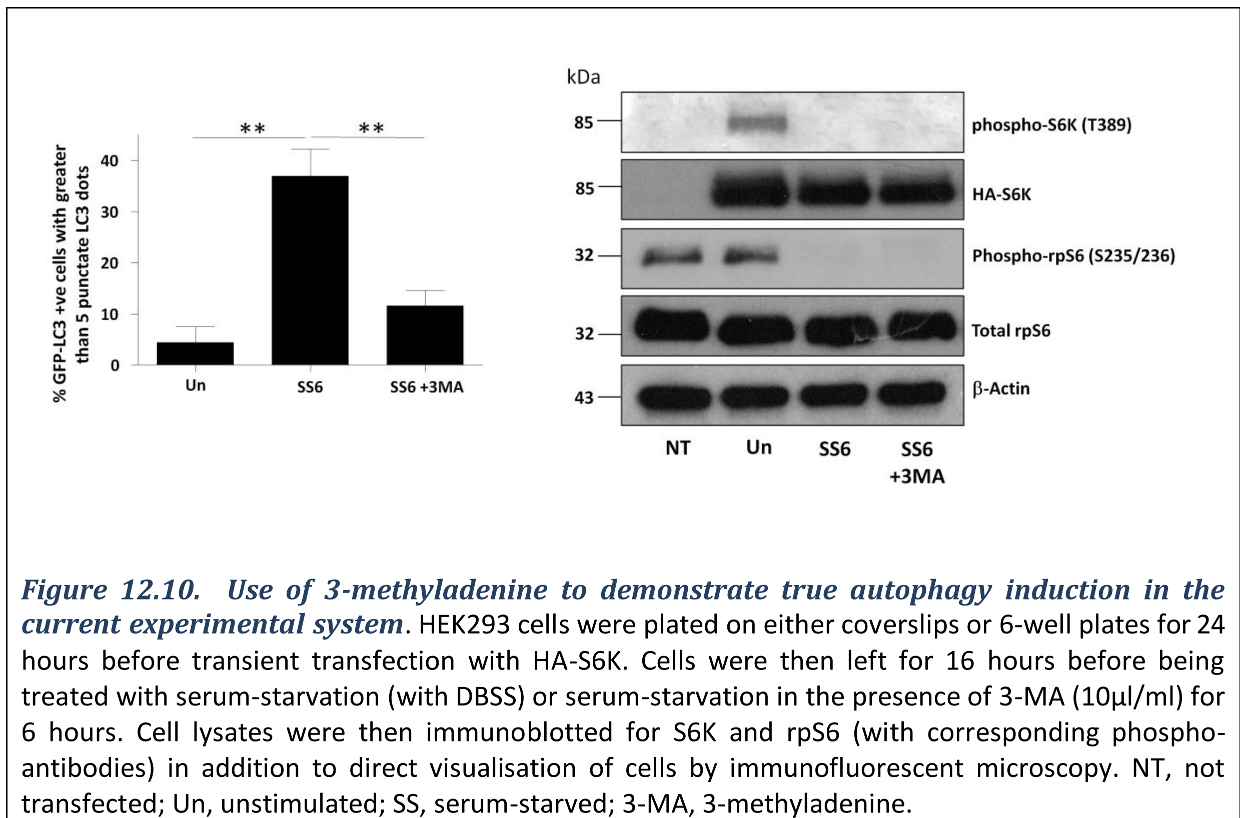


Figure 12.10. Use of 3-methyladenine to demonstrate true autophagy induction in the current experimental system. HEK293 cells were plated on either coverslips or 6-well plates for 24 hours before transient transfection with HA-S6K. Cells were then left for 16 hours before being treated with serum-starvation (with DBSS) or serum-starvation in the presence of 3-MA (10µl/ml) for 6 hours. Cell lysates were then immunoblotted for S6K and rpS6 (with corresponding phospho-antibodies) in addition to direct visualisation of cells by immunofluorescent microscopy. NT, not transfected; Un, unstimulated; SS, serum-starved; 3-MA, 3-methyladenine.

Additionally, small interfering RNA (siRNA) to the critical autophagy protein ATG7 (see **Figure 9.2**) was used to show that the autophagy response produced by serum-starvation was genuine and that this response could be significantly tempered with manipulation of the this key autophagy protein (**Figure 12.11**).

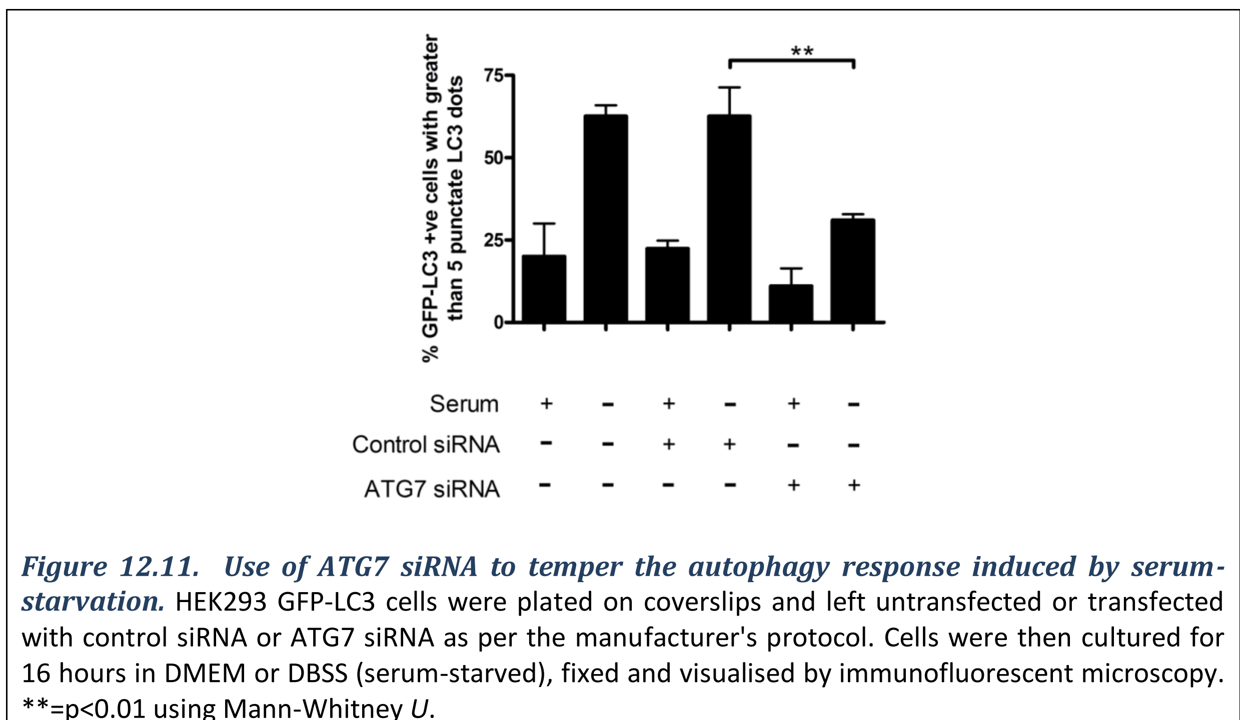


Figure 12.11. Use of ATG7 siRNA to temper the autophagy response induced by serum-starvation. HEK293 GFP-LC3 cells were plated on coverslips and left untransfected or transfected with control siRNA or ATG7 siRNA as per the manufacturer's protocol. Cells were then cultured for 16 hours in DMEM or DBSS (serum-starved), fixed and visualised by immunofluorescent microscopy. **=p<0.01 using Mann-Whitney *U*.

12.3.3 HEK293 cells stably expressing wild-type NOD2 and NOD2 frameshift proteins exhibit normal baseline autophagy and a normal response to autophagy induction through mTORC1

Before using the HEK293 NOD2 cell lines (expressing wild-type NOD2 or the CD-associated frameshift variant [NOD2-L1007fs]) generated in **Chapter 10.11** and characterised in **Chapter 11.4.1**, their baseline autophagy levels and autophagic response to various stimuli was assessed. In addition to the methods of detecting autophagy outlined above, another protein has been used to assess autophagic flux within cells, namely p62.[1029,1030] p62 accumulates when autophagy is inhibited and decreases when autophagy is induced giving another potential readout of autophagic activity,[1030] although this assay is possibly felt to be a more reliable read-out of autophagy inhibition than autophagy induction. **Figure 12.12** shows that transient transfection of GFP-LC3 into the three stable cell lines demonstrated no difference in baseline autophagy levels assessed by immunofluorescent microscopy, or by probing for p62 and GFP-LC3 by western blot.

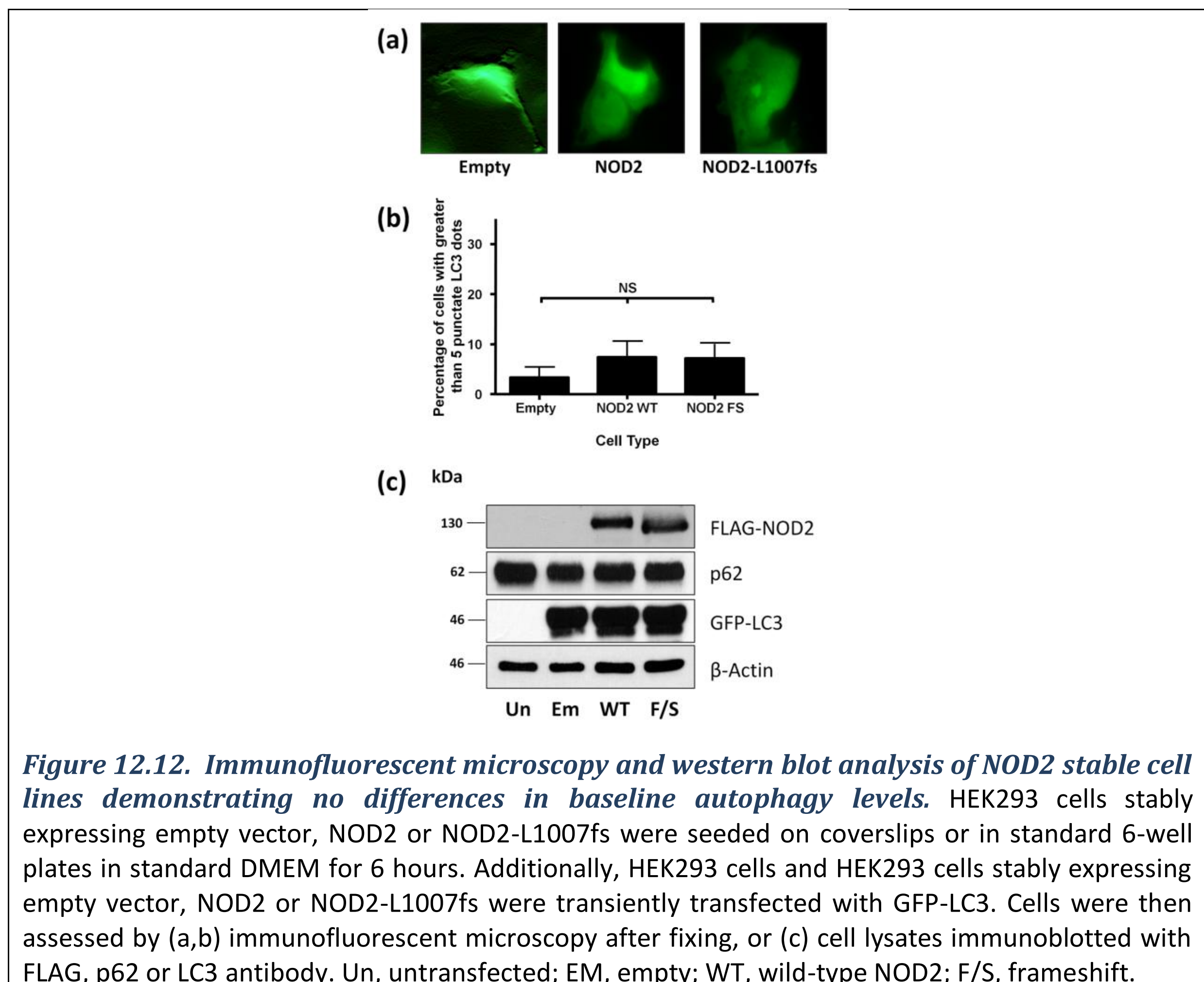


Figure 12.12. Immunofluorescent microscopy and western blot analysis of NOD2 stable cell lines demonstrating no differences in baseline autophagy levels. HEK293 cells stably expressing empty vector, NOD2 or NOD2-L1007fs were seeded on coverslips or in standard 6-well plates in standard DMEM for 6 hours. Additionally, HEK293 cells and HEK293 cells stably expressing empty vector, NOD2 or NOD2-L1007fs were transiently transfected with GFP-LC3. Cells were then assessed by (a,b) immunofluorescent microscopy after fixing, or (c) cell lysates immunoblotted with FLAG, p62 or LC3 antibody. Un, untransfected; EM, empty; WT, wild-type NOD2; F/S, frameshift.

Following the assessment of baseline autophagy levels in the NOD2 stable cell lines, similar experiments in these cell lines were conducted to determine the baseline mTORC1 activity by phosphorylation of downstream substrates of mTORC1 in response to rapamycin and serum-starvation. **Figure 12.13** demonstrates that comparable levels of S6K (T389) and rpS6 (S235/236) were apparent at baseline (**Figure 12.13a**), with both the NOD2 and NOD2-L1007fs cell lines demonstrating appropriate mTORC1 responses to rapamycin and serum-starvation (**Figure 12.13b** and **Figure 12.13c**).

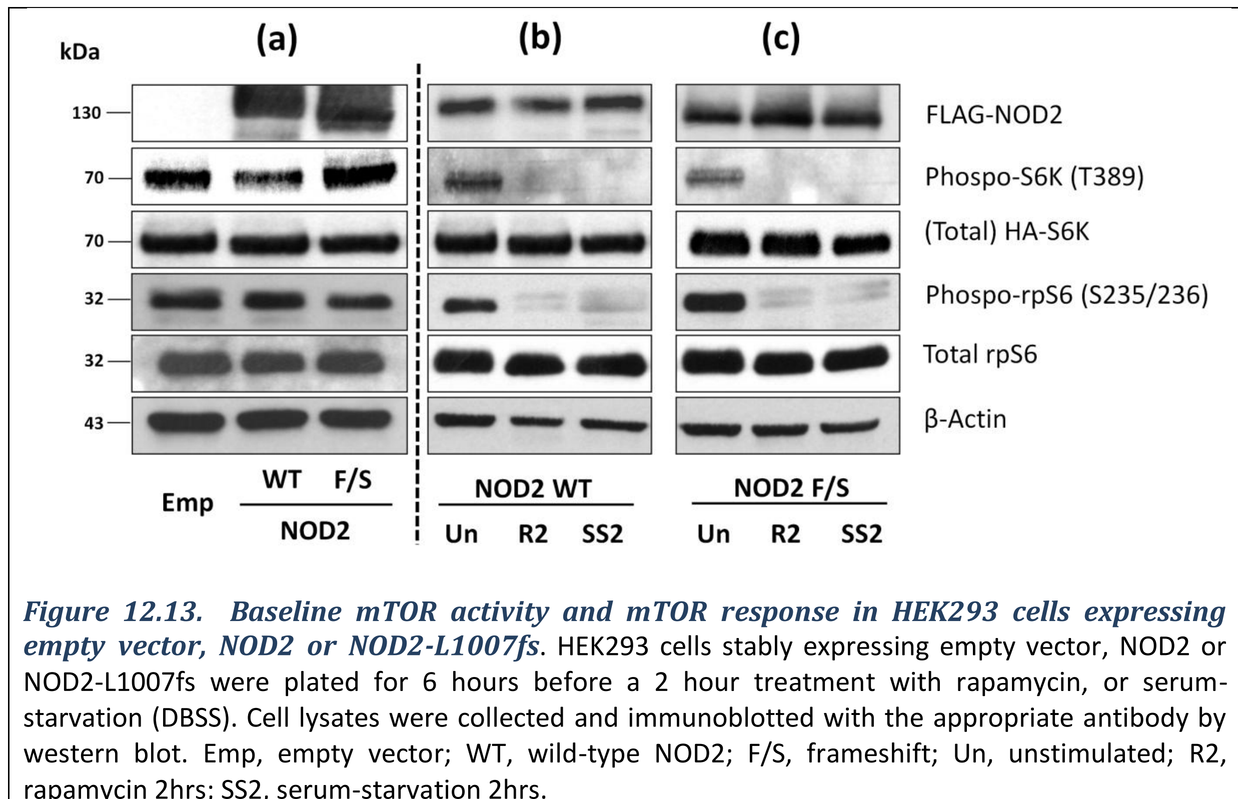


Figure 12.13. Baseline mTOR activity and mTOR response in HEK293 cells expressing empty vector, NOD2 or NOD2-L1007fs. HEK293 cells stably expressing empty vector, NOD2 or NOD2-L1007fs were plated for 6 hours before a 2 hour treatment with rapamycin, or serum-starvation (DBSS). Cell lysates were collected and immunoblotted with the appropriate antibody by western blot. Emp, empty vector; WT, wild-type NOD2; F/S, frameshift; Un, unstimulated; R2, rapamycin 2hrs; SS2, serum-starvation 2hrs.

12.3.4 MDP-induced autophagy induction in HCT116 GFP-LC3 cells does not signal through mTORC1

To establish if MDP-induced autophagy signals through mTORC1, HEK293 and HCT116 cells stably expressing GFP-LC3 were first treated with increasing concentrations of L18-MDP (a synthetic cell-permeable MDP derivative found to have greater potency that naturally occurring MDP[1031]) for six hours. **Figure 12.14** shows that in keeping with the NOD2 rtPCR results (**Figure 10.3**), HEK293 cells showed no response to MDP treatment, whereas HCT116 cells showed a dose-dependent increase in autophagy.

To further ascertain if this increase in autophagy was NOD2 dependent, HEK293 NOD2 and HEK293 NOD2-L1007fs stable cells, transiently transfected to express HA-S6K were

treated with 70μg/ml of L18-MDP. **Figure 12.15** shows that NOD2 stable cells showed robust autophagy induction, with no response observed in the NOD2-L1007fs cell line.

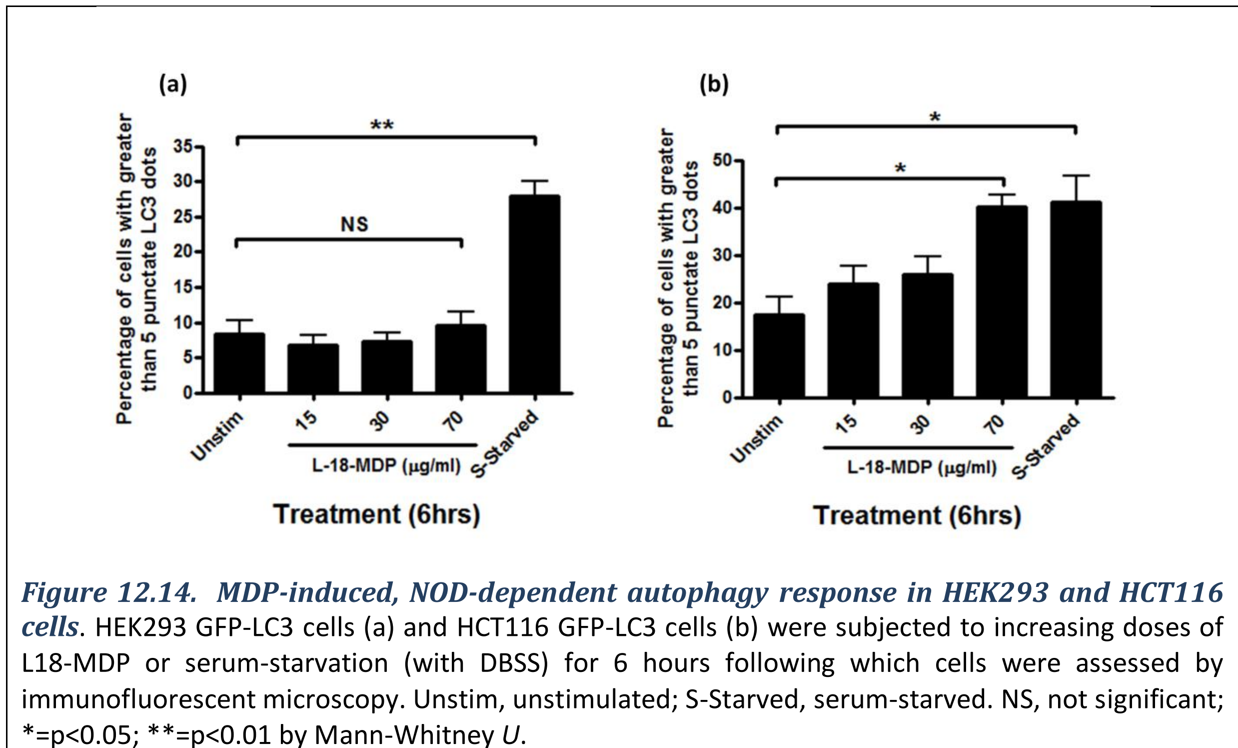


Figure 12.14. MDP-induced, NOD-dependent autophagy response in HEK293 and HCT116 cells. HEK293 GFP-LC3 cells (a) and HCT116 GFP-LC3 cells (b) were subjected to increasing doses of L18-MDP or serum-starvation (with DBSS) for 6 hours following which cells were assessed by immunofluorescent microscopy. Unstim, unstimulated; S-Starved, serum-starved. NS, not significant; *=$p<0.05$; **=$p<0.01$ by Mann-Whitney *U*.

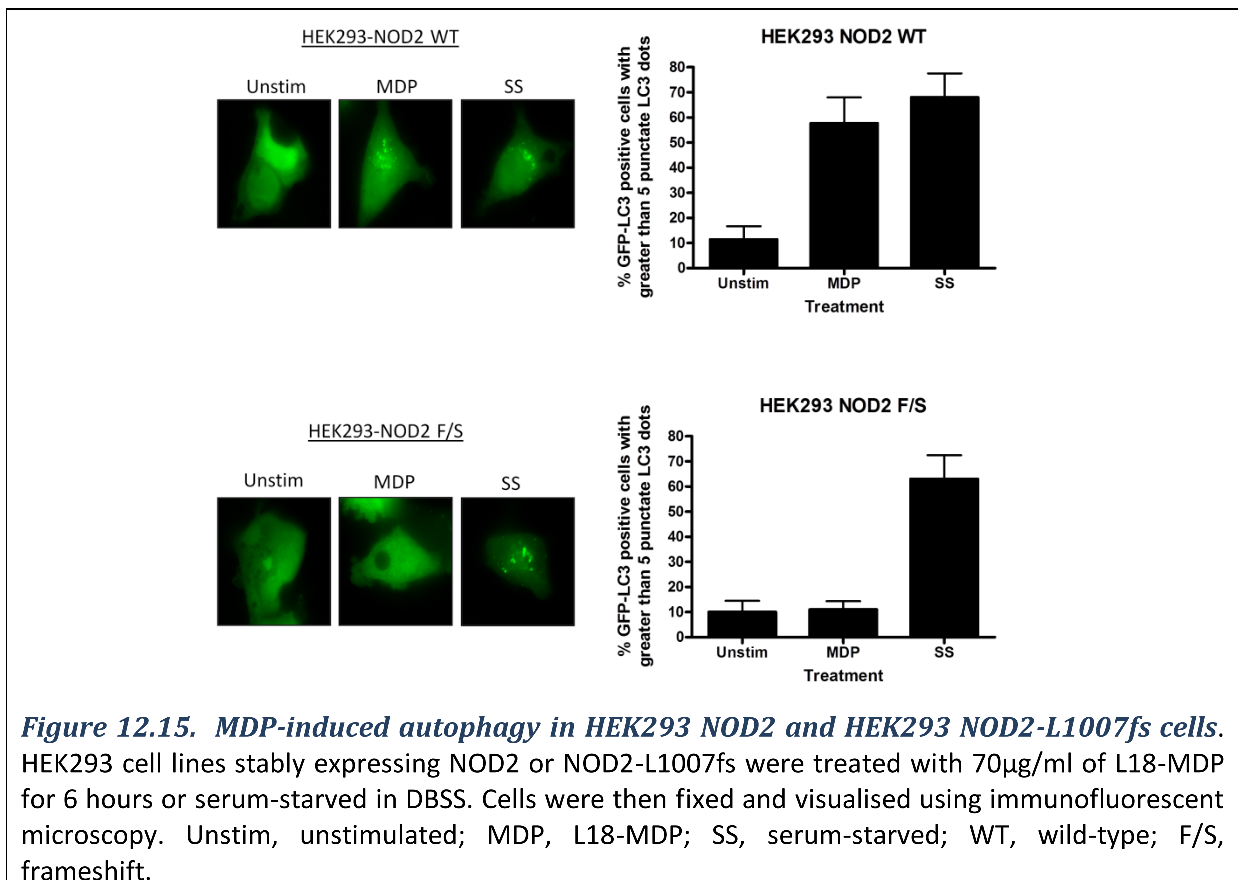


Figure 12.15. MDP-induced autophagy in HEK293 NOD2 and HEK293 NOD2-L1007fs cells. HEK293 cell lines stably expressing NOD2 or NOD2-L1007fs were treated with 70μg/ml of L18-MDP for 6 hours or serum-starved in DBSS. Cells were then fixed and visualised using immunofluorescent microscopy. Unstim, unstimulated; MDP, L18-MDP; SS, serum-starved; WT, wild-type; F/S, frameshift.

To then address the question regarding mTORC1 signalling in MDP-induced autophagy, cell lysates from the experiment shown in **Figure 12.15** were immunoblotted with S6K (T389) and rpS6 (S235/236) antibodies by western blot. **Figure 12.16** shows that there was no reduction in phosphorylation of either protein with MDP treatment, in contrast to treatment with serum-starvation which resulted in complete inhibition of protein phosphorylation.

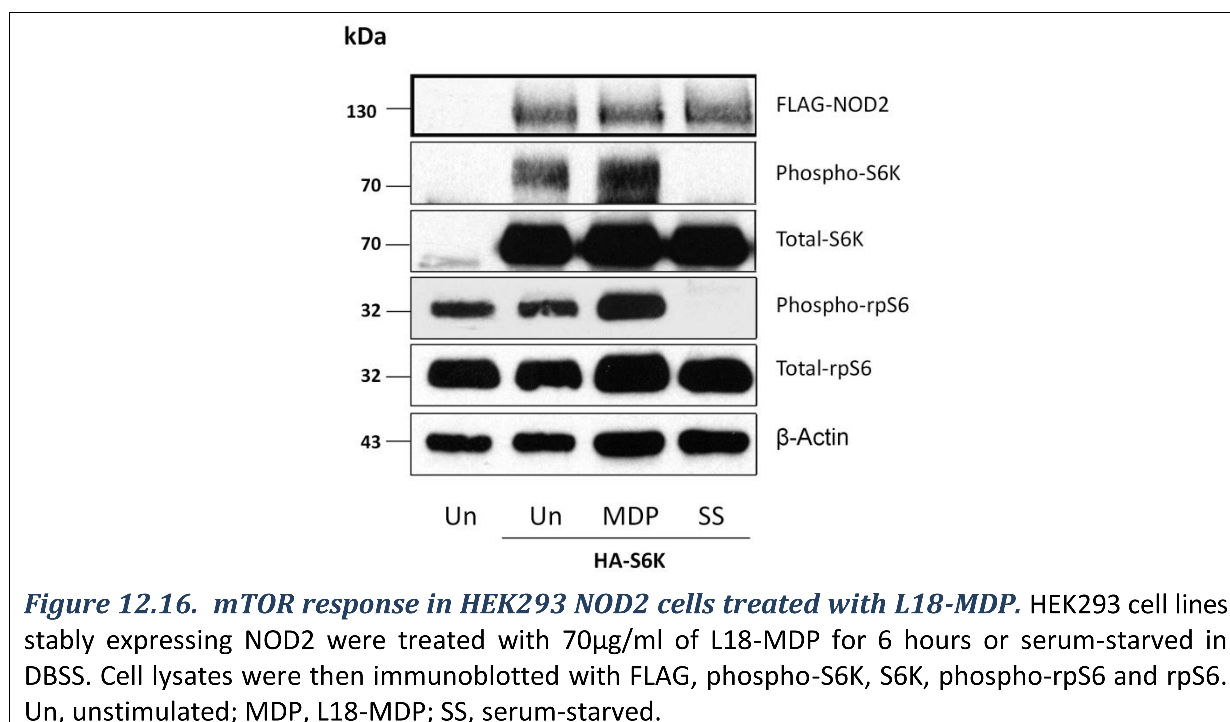


Figure 12.16. mTOR response in HEK293 NOD2 cells treated with L18-MDP. HEK293 cell lines stably expressing NOD2 were treated with 70µg/ml of L18-MDP for 6 hours or serum-starved in DBSS. Cell lysates were then immunoblotted with FLAG, phospho-S6K, S6K, phospho-rpS6 and rpS6. Un, unstimulated; MDP, L18-MDP; SS, serum-starved.

12.3.5 Withaferin A inhibits NOD2-dependent autophagy

The natural product Withaferin-A (WFA), a steroidal lactone from the plant *Withania Somnifera*, binds to and inhibits vimentin.[1032] To confirm the effects of WFA in the current experimental system, treatment of cells with 2µM WFA was shown to induce vimentin aggregation and cleavage (**Figure 12.17a** and **Figure 12.17b**) with only minimal effects on cell viability (**Figure 12.17c**). To test whether vimentin regulates the autophagic activity of NOD2, the effect of WFA on NOD2-dependent autophagy stimulated by L18-MDP was assessed. HEK293 cells stably expressing empty vector, NOD2 or NOD2-L1007fs were transiently transfected with GFP-LC3. L18-MDP efficiently stimulated autophagy in HEK293-NOD2 cells and this effect was inhibited by treatment with WFA (**Figure 12.18a**). As a control, the effect of MDP in HEK293 NOD2-L1007fs cells was also determined. As expected, L18-MDP had no effect on autophagy in these cells (**Figure 12.18b**).

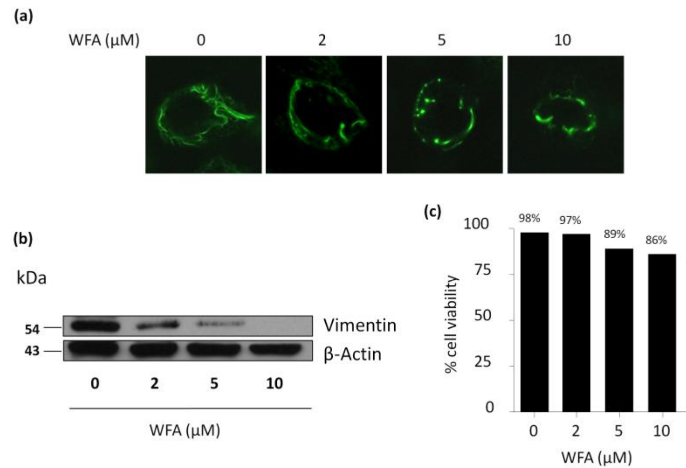


Figure 12.17. Effect of Withaferin-A on vimentin aggregation and cleavage. HEK293 cells were treated with increasing concentrations of WFA as indicated for 2 hours. Cells were then visualised by immunofluorescent microscopy (a) or immunoblotted (b) with vimentin antibody. The viability of cells treated with WFA for 2 hours at the indicated concentrations was evaluated using trypan blue exclusion. WFA, Withaferin-A (c).

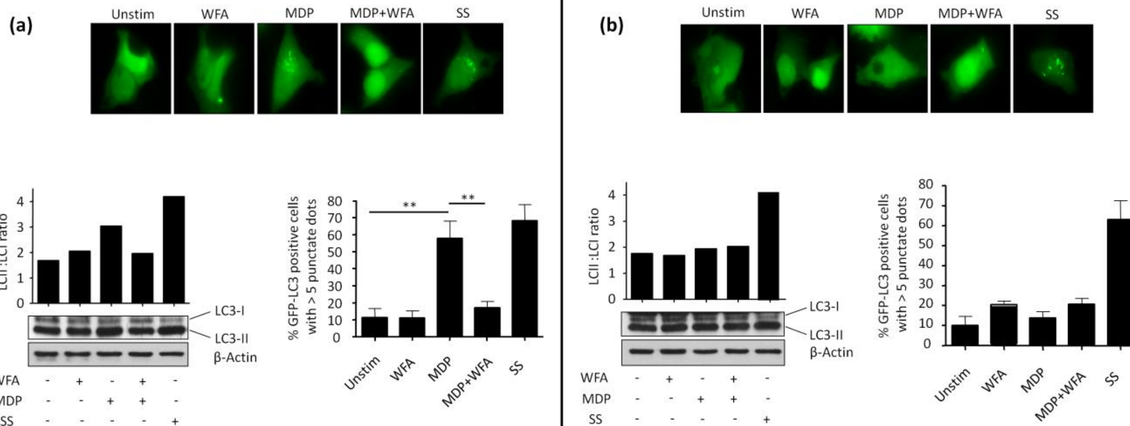


Figure 12.18. Withaferin-A inhibits NOD2-dependent autophagy. HEK293 cells stably expressing NOD2 (a) or NOD2-L1007fs (b) were transiently transfected to express GFP-LC3. Cells were pre-treated for 30min with WFA (2μM) before treatment for 8 hours with L18-MDP (50μg/ml). Cells were then fixed and visualised using immunofluorescent microscopy or cell extracts immunoblotted for LC3. The ratio of LC3-II to LC3-I was measured by densitometry. Unstim, unstimulated; WFA, Withaferin-A; MDP, L18-MDP; SS, serum-starved. ** = p<0.01 by Mann-Whitney *U*.

12.3.6 The haplotype block containing *Vim* is a susceptibility locus for CD

A total of 965 SNPs in the haplotype block containing *Vim* and the two flanking blocks, spanning 163kb, were tested. A total of 51 SNPs attained a p value of <0.001, with 47 of

these in the block containing *Vim*. The strongest SNP (rs1049341) is positioned in the ninth exon (ENSE00001906870) of the *Vim* gene and attained a p value of 4.67×10^{-5}. A plot of the SNPs in the region is presented in **Figure 12.19**.

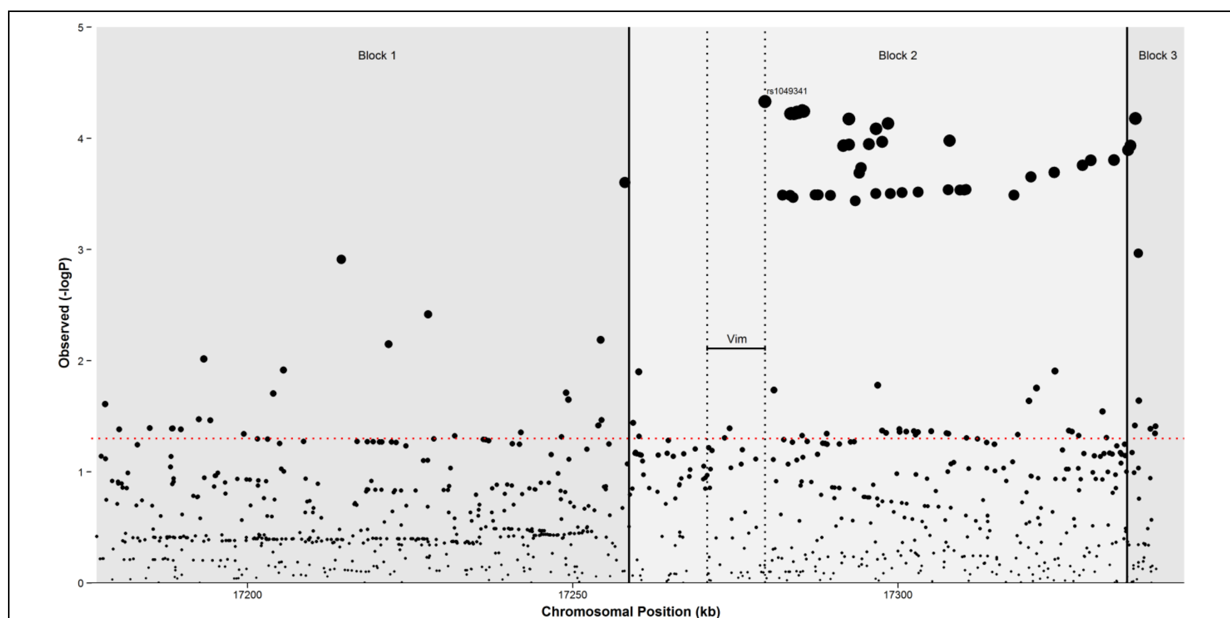


Figure 12.19. Results in the region of Vim of a meta-analysis of seven Crohn's disease genome-wide association studies imputed with the 1000 genomes reference set. Scatterplot showing -logP values in the 163kb region of *Vim* for 965 single nucleotide polymorphisms. Vertical dotted lines represent the boundaries of the *Vim* gene and solid lines the limits of each haplotype block. The -logP value corresponding to $p<0.05$ is represented by the horizontal (red) dotted line.

12.3.7 A number of drugs used in inflammatory bowel disease management induce autophagy, with azathioprine producing robust autophagy through mTORC1 in an ERK-independent manner

To first assess the effect of commonly used drugs on autophagy induction, HEK293 GFP-LC3 and HCT116 GFP-LC3 cells were treated with increasing doses of each drug and autophagic activity levels determined by immunofluorescent microscopy. To determine the most appropriate concentration of each drug to use, the literature was examined and either documented therapeutic blood levels or concentrations used in previous *in vitro* assays were used (see **Table 10.4**).[474,1033-1036] The four drugs that were easily accessible and chosen for evaluation were 6-thioguanine, azathioprine, infliximab and methotrexate; all drugs previously shown to influence IBD natural history.[1037] Although in practice 6-mercaptopurine is the thiopurine used in those intolerant to azathioprine (**Chapter 3.5.5**), 6-thioguanine measurement is now used in clinical practice to help identify inter-person variation that may underlie variation in efficacy and toxicity.[1038]

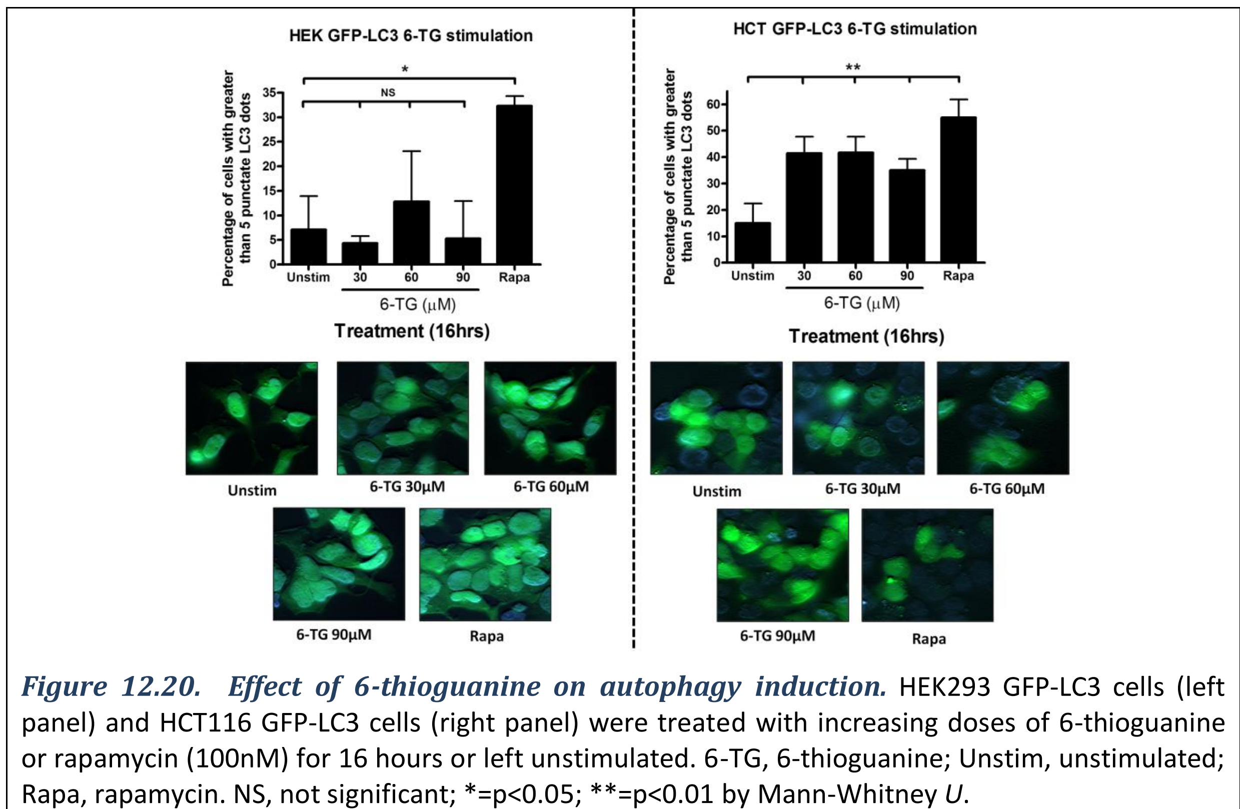


Figure 12.20. Effect of 6-thioguanine on autophagy induction. HEK293 GFP-LC3 cells (left panel) and HCT116 GFP-LC3 cells (right panel) were treated with increasing doses of 6-thioguanine or rapamycin (100nM) for 16 hours or left unstimulated. 6-TG, 6-thioguanine; Unstim, unstimulated; Rapa, rapamycin. NS, not significant; *=p<0.05; **=p<0.01 by Mann-Whitney *U*.

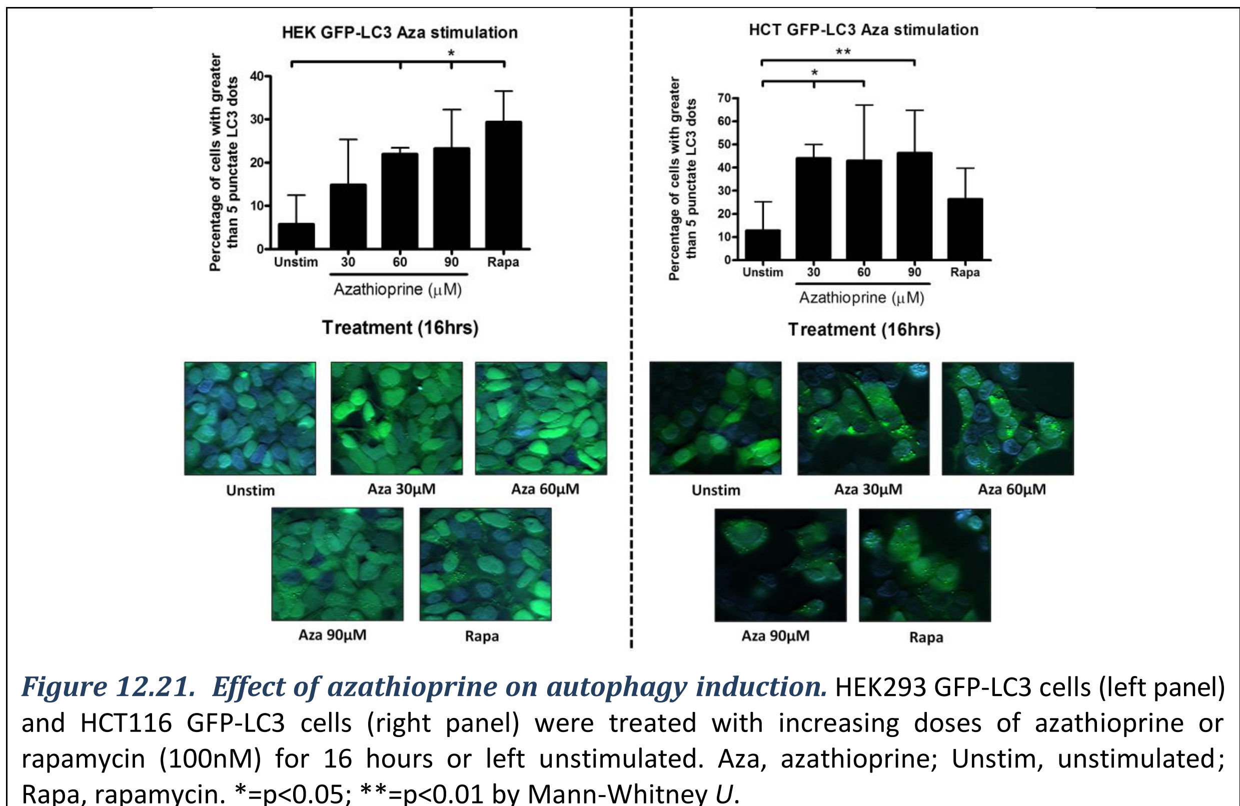


Figure 12.21. Effect of azathioprine on autophagy induction. HEK293 GFP-LC3 cells (left panel) and HCT116 GFP-LC3 cells (right panel) were treated with increasing doses of azathioprine or rapamycin (100nM) for 16 hours or left unstimulated. Aza, azathioprine; Unstim, unstimulated; Rapa, rapamycin. *=p<0.05; **=p<0.01 by Mann-Whitney *U*.

Figures 12.20, **12.21**, **12.22** and **12.23** show the results for increasing doses of 6-thioguanine, azathioprine, infliximab and methotrexate, in HEK293 GFP-LC3 and HCT116 GFP-LC3 cells respectively.

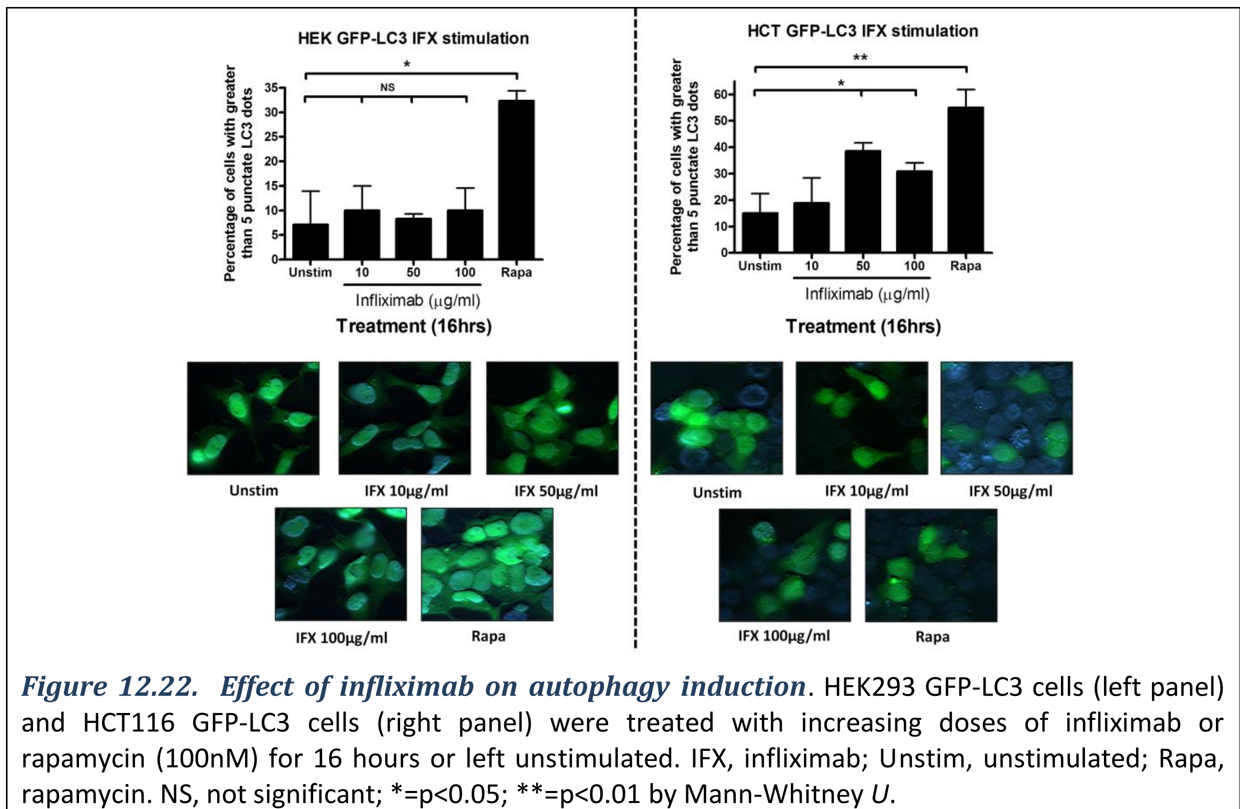


Figure 12.22. Effect of infliximab on autophagy induction. HEK293 GFP-LC3 cells (left panel) and HCT116 GFP-LC3 cells (right panel) were treated with increasing doses of infliximab or rapamycin (100nM) for 16 hours or left unstimulated. IFX, infliximab; Unstim, unstimulated; Rapa, rapamycin. NS, not significant; *=p<0.05; **=p<0.01 by Mann-Whitney *U*.

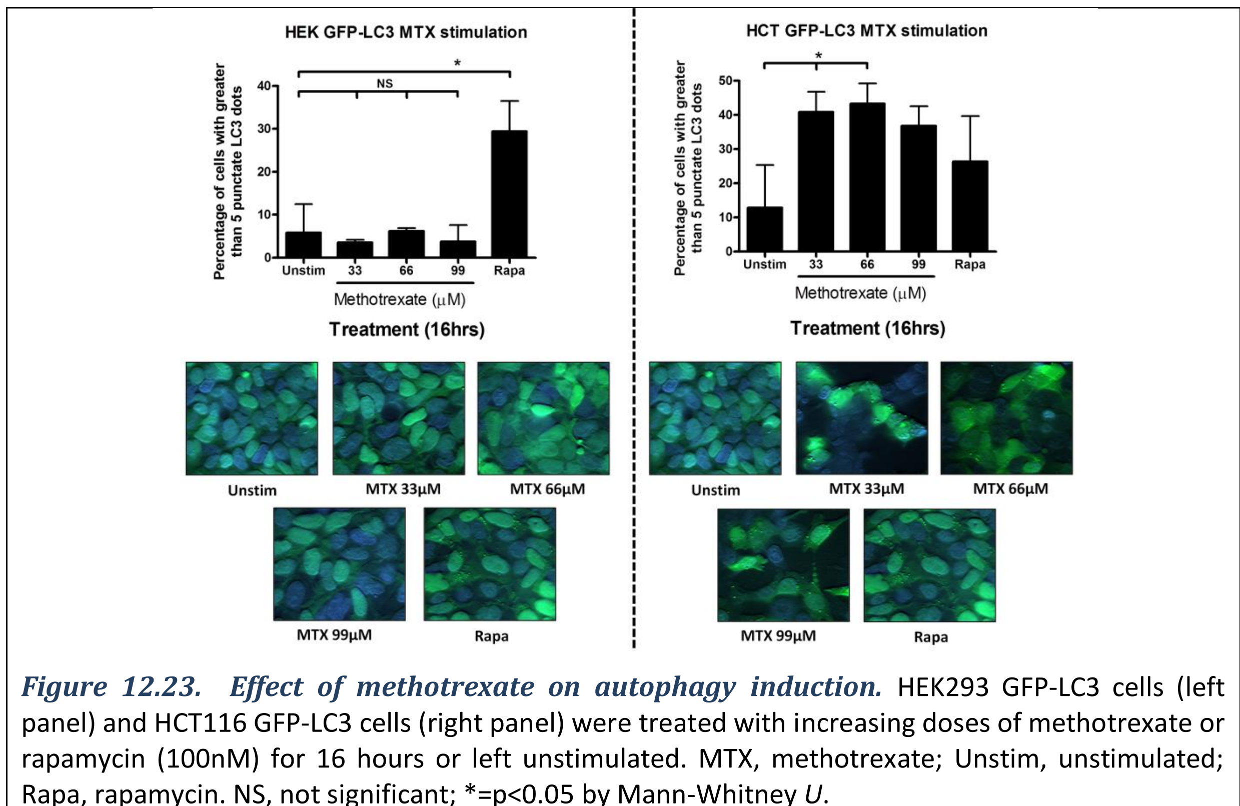


Figure 12.23. Effect of methotrexate on autophagy induction. HEK293 GFP-LC3 cells (left panel) and HCT116 GFP-LC3 cells (right panel) were treated with increasing doses of methotrexate or rapamycin (100nM) for 16 hours or left unstimulated. MTX, methotrexate; Unstim, unstimulated; Rapa, rapamycin. NS, not significant; *=p<0.05 by Mann-Whitney *U*.

It can be seen that treatment with all four drugs led to autophagy induction in the HCT116 cell line, but only azathioprine induced autophagy robustly in both cell lines. To complement the immunofluorescent microscopy results, HCT116 cells were treated with the

concentration of each drug producing the greatest autophagic response, lysed and immunoblotted for LC3, p62, rpS6 (S235/236) and total rpS6. Cells were also treated with rapamycin, serum-starvation or bafilomycin as controls. **Figure 12.24** shows that 6-thioguanine, azathioprine and methotrexate showed strong LC3-I to LC3-II conversion and a reduction in p62, with 6-thioguanine and azathioprine also showing a clear reduction in rpS6 (S235/236) phosphorylation suggesting that autophagy induction may be induced in response to repression of mTORC1 activity.

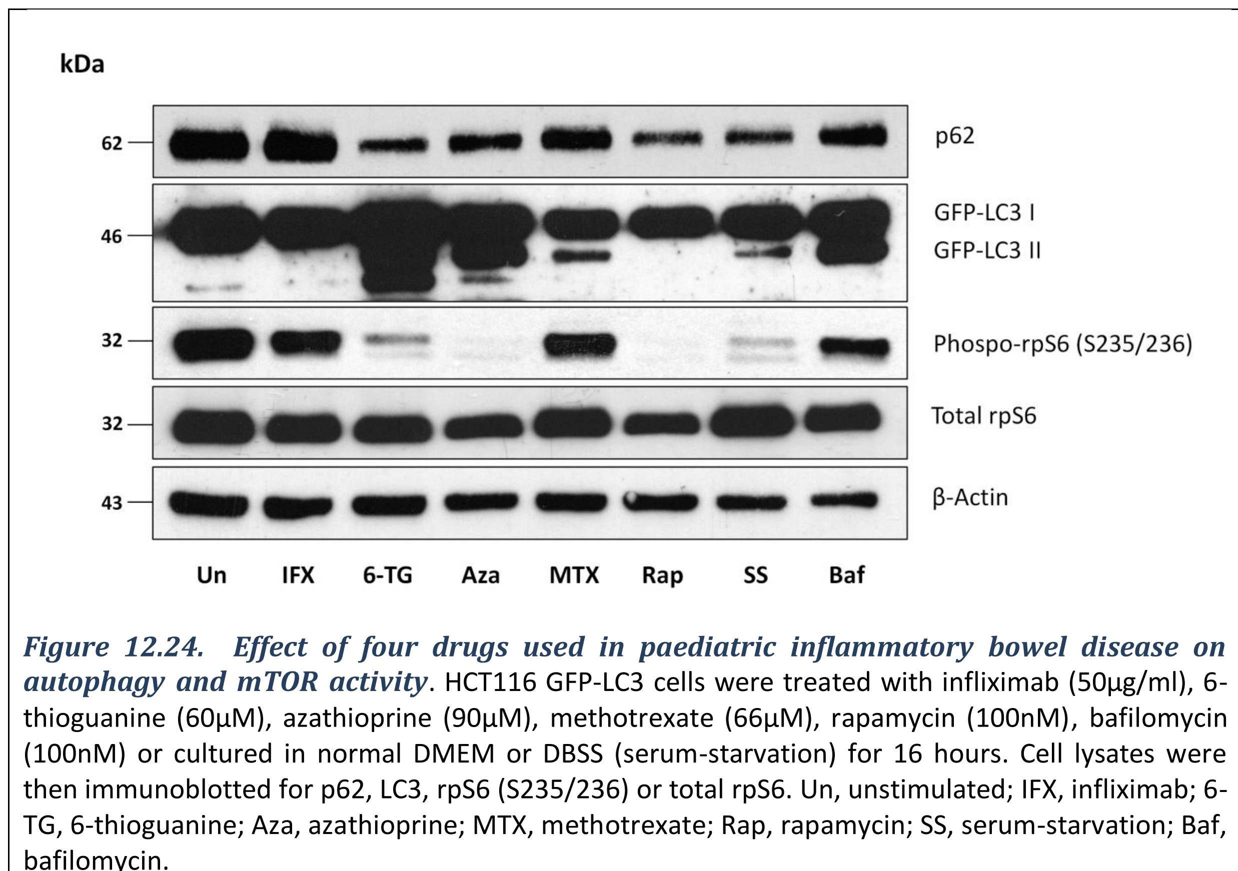


Figure 12.24. Effect of four drugs used in paediatric inflammatory bowel disease on autophagy and mTOR activity. HCT116 GFP-LC3 cells were treated with infliximab (50µg/ml), 6-thioguanine (60µM), azathioprine (90µM), methotrexate (66µM), rapamycin (100nM), bafilomycin (100nM) or cultured in normal DMEM or DBSS (serum-starvation) for 16 hours. Cell lysates were then immunoblotted for p62, LC3, rpS6 (S235/236) or total rpS6. Un, unstimulated; IFX, infliximab; 6-TG, 6-thioguanine; Aza, azathioprine; MTX, methotrexate; Rap, rapamycin; SS, serum-starvation; Baf, bafilomycin.

As azathioprine was found to be a potent inducer of autophagy in both HCT116 and HEK293 cells a further assessment of any dose dependent effect evident by western blot was undertaken. **Figure 12.25** shows that increasing doses of azathioprine led to a dose-dependent reduction in p62 levels, with 1µM enough to produce a robust reduction in rpS6 (S235/236) levels. It should be noted that trypan blue exclusion was performed on cells treated with each of the four drugs (Aza, IFX, 6-TG, MTX) with no significant effect on cell viability.

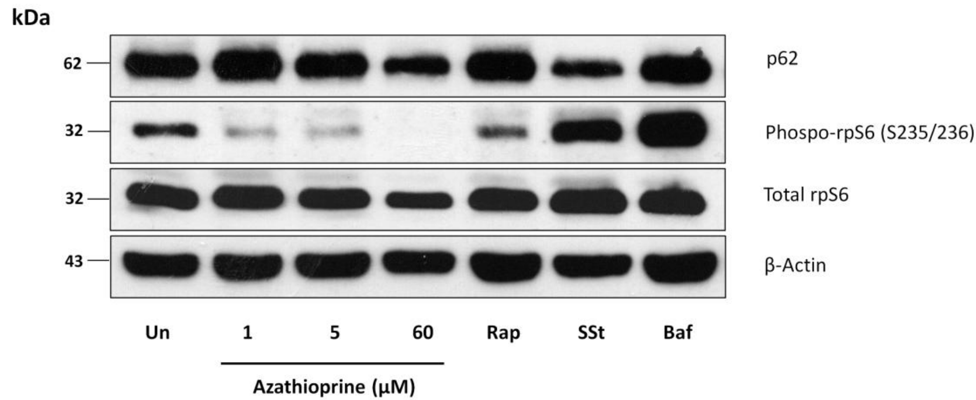


Figure 12.25. Dose-dependent effect of azathioprine on autophagy induction and mTORC1 activity. HCT116 GFP-LC3 cells were treated with azathioprine (1, 5 and 60μM), rapamycin (100nM), bafilomycin (100nM) or cultured in normal DMEM or DBSS (serum-starvation) for 16 hours. Cell lysates were then immunoblotted for p62, rpS6 (S235/236) or total rpS6. Un, unstimulated; Rap, rapamycin; SS, serum-starvation; Baf, bafilomycin.

To further delineate the pathways involved in azathioprine autophagy induction, cells were treated with the ERK inhibitor (U0126) or the non-specific autophagy inhibitor 3-MA in conjunction with azathioprine. **Figure 12.26** shows that neither U0126 or 3-MA had any effect on autophagy induction or mTORC1 activity, suggesting that azathioprine may exert its autophagic effect via an mTORC1-independent and ERK-independent pathway.

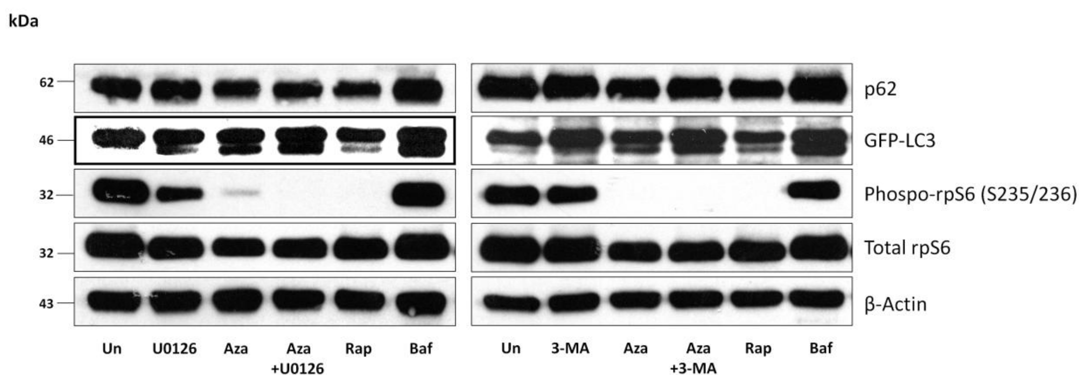


Figure 12.26. Effect of the ERK inhibitor U0126 and autophagy inhibitor 3-methyladenine (3-MA) on azathioprine-induced autophagy. HCT116 GFP-LC3 cells were treated with azathioprine (60μM) in the presence or absence of U0126 (10μM) or 3-MA(10μg/ml), in addition to treatment with rapamycin (100nM) or bafilomycin (100nM). Cells were also left untreated or cultured in DBSS (serum-starved). Cell lysates were then immunoblotted for p62, LC3, rpS6 (S235/236) and total rpS6. Un, unstimulated; Rap, rapamycin; SS, serum-starvation; Baf, bafilomycin.

12.4 Discussion

Using a robust system of autophagy induction in mammalian cells it has been shown that MDP-induced autophagy is unlikely to signal through mTORC1, that vimentin is intimately involved in the process of autophagy and that a number of drugs used in the treatment of inflammatory bowel disease induce autophagy, and that azathioprine may do so through a mechanism that involves the inhibition of mTORC1.

The landmark papers by Travassos et al. and Cooney et al. gave new insights into the NOD2-autophagy pathway, not only with regard to autophagy itself but also antigen presentation and bacterial handling,[30,31] with others further expanding this knowledge[822]. Since this work, surprisingly few studies have gone on to establish the exact mechanisms involved in MDP-induced autophagy. There has been some discrepancy in the previous literature regarding the requirement for NOD2 in MDP-induced autophagy, with Travassos et al. reporting the direct recruitment of the autophagic machinery to sites of bacterial invasion,[31] with other groups showing a NOD2-dependent pathway[30,822,1039]. Similarly the requirement for RIPK-2 has been conflicting, possibly reflecting the cell types and strains of bacteria involved in the different experimental systems.[30,31,822,1039] Recently, Homer et al. have published further work demonstrating that RIPK-2 tyrosine kinase activity plays an essential but dual-faceted role in NOD2-autophagy induction, providing both an activating signal through stimulation of MAPK p38 and the relief of PP2Ac-PPP2R1A-mediated repression of autophagy.[823] MAPK p38 has been shown to be involved in the mTORC1 pathway using p38-activating stresses (such as anisomycin),[1040] in addition to the manipulation of MAPK p38 altering the localisation of the critical autophagy protein ATG9[1041]. However, MAPK p38 has also been shown to positively or negatively regulate autophagy depending on the stimulus, and the processes underlying p38-regulated autophagy remain poorly understood.[1042,1043] Furthermore, Homer et al. demonstrated a role for MEKK4 during NOD2-induced autophagy,[823] with recent work showing that the phosphorylation of ATG5 by the MEKK4-p38 pathway inhibits autophagy[1044]. Although the current study suggests that there is no clear signalling through mTORC1 during autophagy, the complex nature of the mTORC1 pathway,[956] especially during autophagy[1045] requires further examination of this pathway during MDP stimulation, although the possibility that mTORC1-independent pathways exist should continue to be explored.

The origin of autophagosomes has recently gained intense interest in the field, and it is now clear that these double-membrane structures can originate from the surface of various cell organelles, including the endoplasmic reticulum, mitochondria and the plasma membrane.[1046] One of the major autophagy proteins ATG16L1 interacts with the heavy

chain of clathrin, and the plasma membrane has been shown to contribute to the formation of ATG16L1-positive pre-autophagic structures via endocytosis.[826] Additionally, there is now mounting evidence that NOD2 (previously thought to be purely a cytosolic protein[1047]) also resides and functions at the plasma membrane.[31,819,1048] This membrane localisation has lead to the suggestion that NOD2 functions at sites of infection to directly engage with pathogens and signal an appropriate inflammatory and antimicrobial response.[1048] This hypothesis is supported by a recent study demonstrating colocalisation of NOD2 and the major autophagy protein ATG16L1 at the plasma membrane with NOD2 recruiting ATG16L1 to sites of bacterial infection.[31] Further work has shown that a clathrin- and dynamin-dependent endocytic pathway regulates MDP internalisation and NOD2 activation.[1049]

The work presented above has demonstrated that vimentin is intimately involved in MDP-induced autophagy induction, as well as being a susceptibility gene for CD. This is in keeping with previous work (from our group) demonstrating that the ability of NOD2 to signal an appropriate inflammatory response, and to limit the invasion and survival of a CD-associated strain of AIEC, is impaired by treatment with the vimentin inhibitor WFA.[819] These findings are likely to be in part due to the additional observations that vimentin is important for the correct localisation of NOD2 to the plasma membrane, since CD-associated NOD2 variants are unable to interact with vimentin and are mislocalised to the cytosol.[819]

Manipulation of the host cell plasma membrane and associated proteins is an essential step for the uptake, survival and replication of pathogens.[1050] Vimentin structure changes profoundly during bacterial infection and up regulation of *Vim* gene expression has been observed during pathogenic *H.pylori* infection.[1051] Most relevant to CD pathogenesis are the recent reports that vimentin is expressed on the surface of human brain endothelial cells where it acts as a primary receptor for meningitis associated *E. coli* (MenPEC) strains expressing the virulence factor IbeA,[1011] with vimentin-mediated signalling required for IbeA+ *E.coli* invasion of these cells.[1011] The IbeA virulence factor is not restricted to MenPEC, and is also present in a variety of other *E.coli* pathovars, including 18.5% of AIEC strains and 17% of diarrhoeagenic *E.coli.*[107,1052] NOD2 activity has been shown previously to depend on the integrity of the actin cytoskeleton, with drugs disrupting actin having been shown to significantly increase the ratio of soluble to insoluble NOD2.[1053] Several bacterial pathogens manipulate the host cell cytoskeleton for their own intra- and inter-cellular motility[1054] with the invasion of epithelial cells with AIEC markedly reduced by cytochalasin D and colichicine indicating an important role for actin microfilaments and microtubules[107]. An important role for the cytoskeleton in the regulation of autophagy is also emerging.[1055] Of particular significance is that the proper formation and distribution of autophagosomes depends on the integrity of intermediate filament networks,[1017] and that autophagic vacuoles

are found to be tightly associated with vimentin[1019]. The results of the current study demonstrating that vimentin is important for NOD2-induced autophagy, together with recent studies linking NOD2 and the cytoskeleton with the regulation of autophagy, suggest that NOD2 has evolved to use the cytoskeletal network, possibly through cell surface-expressed vimentin, as a means to engage with pathogens. NOD2 may then use the intracellular cytoskeleton further to directly transport internalised pathogens via the autophagy machinery to lysosomes for degradation.

With regard to the mechanism of action of drugs used in the treatment of PIBD, there are still several pathways that have yet to be explored, especially with regard to autophagy. Work described here has shown that 6-thioguanine, azathioprine, infliximab and methotrexate all stimulate autophagy, however only azathioprine stimulated autophagy robustly in both HEK293 and HCT116 cell lines. 6-thioguanine has previously been shown to induce autophagy in several studies (by the same group), looking at DNA mismatch repair. Zeng et al. demonstrated that 6-thioguanine induced autophagy through a mTORC1-dependent pathway, mediated by Bcl2 and adenovirus E1B Nineteen-kilodalton Interacting Protein (BNIP3).[1056-1058] However, it should be noted that all these experiments were carried out in colonic cancer (HCT116 and HT29) cell lines. In addition, the study evaluating the mTORC1 pathway used low concentrations of rapamycin to inhibit mTOR over several days, to demonstrate that 6-thioguanine-induction of autophagy was reduced.[1057] However, it is unclear why low doses of rapamycin alone did not induce autophagy after 24 hours in culture, as rapamycin has clearly been shown to be an autophagy inducer.[1005]

Azathioprine, first described by Gertrude Elion in 1963,[1059] is the prodrug of 6-mercaptopurine. This commonly used purine analogue has been used widely in transplantation medicine and in the treatment of IBD for over 50 years.[1060,1061] Although its precise mechanism of action is still under investigation,[1062] it is thought that azathioprine is first rapidly cleaved non-enzymatically by sufhydryl-containing compounds (such as cysteine and glutathione[1063]) into two principal components, mercaptopurine and a imidazole derivative.[1062] Following this, three competing enzymes (TPMT, XO and HPRT) produce toxic and non-toxic metabolites. The main mechanism of action, mediated through 6-thiopurine active metabolites, are generally accepted to be the disruption of DNA, RNA and protein synthesis, with an increase in T cell apoptosis of particular relevance.[1064] The precise mechanisms of apoptosis induction are still under investigation, but the interaction of 6-ThioGTP with Rac1 (through Bcl-xL blockade), in a CD28-dependent manner are felt to be crucial steps.[197] Also, it has been shown that imidazole (a NADPH oxidase inhibitor) may produce reactive oxygen species, leading to cellular stress. There are therefore several mechanisms that may account for the autophagy induction seen in both the HEK293 and HCT116 cells treated with azathioprine: (1) a direct genotoxic effect from DNA disruption 6-

thioguanine metabolites; (2) the production of reactive oxygen species and (3) direct effects on the mTORC1 complex. With regard to the autophagic effects of infliximab, these results are also of interest. To date, no studies have directly evaluated the direct consequences of infliximab on cellular autophagy. However, work showing a reduction in bacterial translocation in acute pancreatitis with infliximab is notable,[1065] with the possibility that besides the obvious effects of pro-inflammatory cytokine disruption, increased xenophagy at the intestinal epithelium may be beneficial.

The strengths of the current study are that attempts were made to utilise several different methods of autophagy induction and autophagy quantification as per the recent guidelines.[1005] This is very relevant given the complexities of the autophagic machinery and the interaction of several pathways with similar end points. It is also notable that every effort was made to produce robust read-outs of both autophagy induction and mTOR activity, not only in colonic cancer cells (HCT116) but also the HEK293 cell line.

Although every endeavour was made to ensure robustness of the experimental systems, certain difficulties arose. Although most of the experiments used similar methods, the time points were often different and reflected the challenging work with regard to tissue culture, transfection, western blot analysis and the multitude of pathways involved. Ideally, all studies would have been performed in triplicate, using much more variable time-frames. However, this was not always possible and technical issues and unexpected pitfalls were encountered along the way. Additionally, there were problems throughout the experiments when attempting to correspond immunofluorescent microscopy results with immunoblot analysis, especially in relation to MDP-induced autophagy. Other aspects of the experimental work which may have influenced the results are the doses of drugs used, the lack of cell viability work during each experiment and the time pressures which did not allow the full determination of the pathways involved in the intriguing results obtained.

12.5 Conclusions

This study, using a robust system of autophagy induction in mammalian cells, has shown that MDP-induced autophagy is unlikely to signal through mTOR, that the intermediate filament vimentin is intimately involved in the process of autophagy and that a number of drugs used in the treatment of inflammatory bowel disease induce autophagy, with azathioprine doing so robustly through mTOR inhibition.

Further studies are now required to address the precise mechanisms involved in the MDP-NOD2-autophagy pathway in addition to the vimentin-NOD2 interaction within the context of CD patients. Further determination of the effect of commonly used IBD drugs on autophagy

is also required, especially with relation to the bacteria-epithelial interface in the intestinal lumen, that may allow the manipulation of bacterial translocation and dampen the subsequent immune response.

13. Conclusions and future directions

13.1 Paediatric inflammatory bowel disease epidemiology

It can be seen from the data presented in **Chapter 2** and **Chapter 3** that the incidence and point prevalence of paediatric inflammatory bowel disease (PIBD) has increased in Scotland, with supporting evidence suggesting an increasing incidence worldwide.[223] From this new data, Scotland now has the highest reported incidence of PIBD (< 16 years old) in the United Kingdom (UK) (7.8/100,000/year), with regional South-East Scotland (SES) incidence rates even higher at 9.5/100,000/year. Furthermore, the data also provides the only robust PIBD point prevalence data to date in the UK (41.2/100,000). It is not yet clear if this rise will continue to be evident in both developing or developed countries, or whether this rise will be sustained or plateau in future decades. Although there are those that suggest that this increase is purely a result of 'milder' forms of the disease, or the more rigorous investigation of those with gastrointestinal symptoms, there is certainly no evidence to imply that this is the case. In fact the phenotypic data collected prospectively in SES demonstrates that children with PIBD continue to present with extensive disease (pan-enteric CD in 34% and pancolitic UC in 76%) diagnosed during upper and lower endoscopy, therefore this true rise is likely to be in-keeping with the increase in other immune-mediated diseases.[132,1066] The potential regional variation in incidence rates is also of interest and suggests that environmental influences may be involved, with further dissection of these possible variations accompanied by detailed environmental data now fully warranted.

The rise in prevalent cases of PIBD is particularly of interest with respect to healthcare provision. With the increasing numbers of children cared for in paediatric services, and the high burden of disease demonstrated in this population, the cost of managing these often complex cases will rise in parallel. Additionally, with the increased use of immunosuppressive agents, especially maintenance therapy with costly biologicals, this cost will rise further and the provision of a multidisciplinary team to cater for these changes (e.g. PIBD nurse specialists and dietitians) will certainly need to be high on the political agenda.[317] Robust and comprehensive prevalence data not only assists in healthcare provision but also has direct effects on translational research. For example, studies of diagnostic accuracy and genetic association require accurate prevalence estimates in order to provide robust sample size and power calculations.[1067,1068]

The reasons for the rise in PIBD incidence remains elusive, with diet, lifestyle and other environmental factors most likely interacting with the gut microbiota and effecting genetic and epigenetic regulation in susceptible individuals.[1069] A recent large prospective cohort

study of over 72,000 women aged between 40-73 years used a diet and lifestyle assessment of vitamin D status at study entry using a validated model.[1070] The authors concluded that higher predicted plasma levels of vitamin D significantly reduced the risk of incident Crohn's disease (CD), but non-significantly for ulcerative colitis (UC). Animal studies have supported these findings, with vitamin D receptor (VDR) knock-out mice highly sensitive to dextran-sulphate-induced colitis and exogenously injected lipopolysaccharide.[1071] Additionally, vitamin D has been shown to ameliorate experimental murine colitis, possibly by changes in the T regulatory cell profile.[1072,1073] The biological plausibility in this protective effect has also been demonstrated, with 1,25-dihydroxyvitamin D(3) stimulating expression of *NOD2* in addition to genes encoding antimicrobial peptides.[255] With the interest in vitamin D now extending to both innate and adaptive immunity,[1074] and the observation that nearly 50% of children newly diagnosed with CD are vitamin D deficient,[1075] suggests that additional research is required to identify if vitamin D deficiency is a modifiable risk factor in the general population. Further work on the effect of urban living on the incident rates of PIBD is also justified. Previous work in Scotland has demonstrated that high human density is protective for PIBD.[253] However, with other studies demonstrating a similar[1076] or opposite effect[1077] it is likely that this relationship is more complex, with movement to and from urban areas associated with disease.

With regard to dysbiosis of the gut microbiota in IBD there has been a recent explosion of research in this field.[1078] Work from colleagues in Scotland has recently provided a comprehensive examination of the mucosal microbiota in children with newly-presenting, untreated IBD versus normal controls.[1079] Using pyrosequencing and confirmatory real-time PCR they demonstrated that no significant changes at phylum level among the *Bacteroidetes*, *Firmicutes*, or *Proteobacteria* were apparent. However, there was a significant reduction in bacterial alpha-diversity and an increase in Faecalibacterium in Crohn's disease versus controls; no disease-specific clustering was evident. Additional work has demonstrated differences in the microbiota of children with IBD, although much of this work has been performed on faecal (rather than mucosal) material of children on treatment, potentially influencing the results.[1080,1081] There are a myriad of hypotheses that aim to explain differences in the bacteria within the gut in health and disease, including caesarean delivery, breastfeeding, perinatal stress, probiotics, and antibiotics,[1082] however to date none of these have been confirmed as having robust association (or importantly causation) with PIBD. The differences described are certainly of interest, and with the overall role of the gut microbiota now being examined,[1083] further investigation is now required to determine the precise natural history of the gut microbiome from birth to PIBD diagnosis.

It is likely that the increasing incidence of PIBD will be as a result of some or all of these factors, possibly with population-specific and inter-population effects. Prospective,

longitudinal studies capturing incident cases of PIBD diagnosed by endoscopy and thorough case-note review are now required with parallel studies evaluating changes in suspected environmental triggers. Additionally, work addressing the issue of the severe phenotype seen in PIBD at diagnoses (and the subsequent rapid disease progression) should be performed. As with the rise in incidence, it is likely that multifactorial aetiologies will be evident in these studies, with hormonal, immunological, genetic and epigenetic factors all involved.

13.2 Inflammatory bowel disease genetics

Work presented in **Chapter 5** and **Chapter 6** has demonstrated that the use of case-parent trios in genetic association analysis is still plausible in an age of large case-control studies.[1084] By performing a family-based study in 230 affected offspring trios, the data showed that the region tagged by previous genome-wide association scans on chromosome 21q is likely to be *ICOSLG*. Despite having only a relatively small number of PIBD cases, the use of their parents, accurate power calculations based on robust prevalence data and correction for multiple testing confirmed the association with the 3' untranslated region of *ICOSLG*. Additionally, mRNA expression data highlighted the dysregulation of *ICOSLG* and its ligand *ICOS* in IBD patients, further strengthening the results. Although deep sequencing of this region is now required, the much broader role of co-stimulation in immune-mediated disease should also be explored.[1085] Specifically, the pivotal role of the ICOS:ICOSLG interaction may prove vital in the drive to control T cell differentiation.[1086] With this interaction having now been shown to be intricately involved in regulatory T cell populations,[625] and its importance in the early decision between tolerance and immunity,[631] further studies are now required to allow the manipulation of this pathway to control T cell numbers, fate and the ensuing cytokine environment.[1087]

As well as confirming already identified regions of genetic association, the use of transmission disequilibrium test analysis has allowed the identification of a new candidate susceptibility gene for PIBD, namely *CRP*. Although replication in large case-control studies was weak or non-existent, the possibility that this is a true association remains as the results may be population-specific or early-onset specific. C-reactive protein (CRP) has become an exciting molecule in recent years, moving from a simple non-specific laboratory test for infection and inflammation, to a molecule able to bridge the gap between innate and adaptive immunity. Although no clear genotype-phenotype correlations were apparent, the differences in *CRP* haplotype structure between paediatric CD and UC patients was of particular interest, especially given the significant differences in serum CRP levels at

diagnosis. Future studies are now required to determine if this finding is population or early-onset disease specific, in addition to studies to identify if genotype-biomarker-phenotype correlations exist. This method of Mendelian Randomisation has been utilised to confirm[1088] or refute[1089] causation of previously determined disease risk factors, and given the emerging importance of low-grade inflammation in immune-mediated disease,[1090] the assessment of CRP using this method would be of merit.

13.3 Faecal calprotectin

It has been clearly shown (in **Chapter 7**) that faecal calprotectin (FC) is markedly raised in children with IBD compared to scoped controls, with no influence of IBD type or location. Additionally, data has also provided evidence that FC performs better than six commonly used blood parameters (haemoglobin, white cell count, platelet count, CRP, erythrocyte sedimentation rate and albumin) with an area under the curve of 0.93 (95% CI 0.89-0.97), especially using a normal cut-off of 200µg/g. In **Chapter 8** a meta-analysis of robust data derived from previously published case-control studies also demonstrated the high sensitivity (98%) and modest specificity (68%) of this faecal marker for PIBD at diagnosis.

An interesting mechanistic action of FC is the chelation of zinc and manganese leading to the starvation of gastrointestinal pathogens. As there is some evidence to suggest that dysregulation of serum zinc is involved in intestinal inflammation in animal models,[1091] and human subjects[1092] (with some debate[1093]), it would certainly be of interest to examine this mechanism further in the context of IBD. Although the precise microbial composition apparent during IBD pathogenesis is not yet described, the *value* of raised calprotectin levels during intestinal inflammation should be determined. With further details of the complex relationship between FC, zinc-chelation and anti-microbial activity now being evaluated,[1094] and the delineation of microbial diversity in IBD under review,[1079] the utility of FC in IBD exacerbations (akin to the use of antibiotics such as metronidazole[1095,1096]) should be assessed.

Although the use of FC in patients suspected of bowel inflammation is becoming more commonplace, its effect on diagnostic delay, endoscopy rates and correlation with mucosal healing are still under debate. It is likely that FC will serve as a adjunct to other clinical findings (especially history and examination) to allow the efficient handling of the large volume of children who present in outpatient departments with gastrointestinal disturbance. Prospective studies and laboratory-based studies are now required to elicit (1) an accurate cost-benefit evaluation (based not only on the effect on endoscopy rates, but also the effect on radiological investigation, blood work-up and repeated outpatient attendances); (2) a

detailed analysis of the relationship between FC levels and clinical (and histological) disease remission; and (3) the potentially beneficial effects of FC during active inflammation and its anti-microbial mechanism of action. Furthermore, the reasons for false negative results in children with overt macroscopic disease, the inter-person variability in FC levels and the effects of commonly used medications should be examined further. With the National Institute for Health and Clinical Excellence now evaluating the role of FC in IBD (http://guidance.nice.org.uk/DT/12), with our research group actively involved, it is likely that some of these questions will be addressed in the near future.

13.4 NOD2 and autophagy

During the laboratory work performed during this research (presented in **Chapter 11** and **Chapter 12**), it was demonstrated that NOD2 overexpression did not influence cell or nuclear size, but seemed to effect early cellular proliferation, independent of CD-associated polymorphism status. Additionally, cells overexpressing NOD2 or NOD2-L1007fs had an accentuated apoptotic response to serum starvation, but not to treatment with MDP. The use of proteasome and lysosome inhibitors also determined that NOD2 protein stability is likely regulated through the ubiquitin-proteasome degradation pathway, possibly through lysine amino acid residues present at least in the CARD domain.

The effect of NOD2 on cellular proliferation may certainly be of future interest, from the position of Paneth cell morphology and function[1097] and their role in intestinal homeostasis[1098]. Experiments involving MDP stimulation should also be carried out to determine the effects of NOD2 ligation on cell size, nuclear size and cellular proliferation, as this may further identify NOD2 pathways important in Paneth cell and antigen-presenting cell function. With regard to the role of NOD2 in cellular apoptosis, this mechanism should also be fully explored, especially in relation to the cellular inhibitors of apoptosis proteins (cIAPs).[933] Detailed exploration is required to assess the relationship between serum-starvation, NOD2 signalling and the apoptotic machinery, with the possibility that an interaction with mammalian target of rapamycin (mTOR) is apparent. With regard to NOD2 degradation, the recent report of proteasomal degradation of NOD2 through TRIM27 is of great importance.[974] Despite a large volume of research focusing on NOD2 signalling, especially with relation to CD and MDP-ligation, the function and degradation of both wild-type and NOD2 variants need careful attention, given the diverse range of NOD2 pathways implicated in various diseases.[928,1099,1100]

Work presented here has shown that MDP-induced autophagy is unlikely to signal through mTOR, with the intermediate filament vimentin intimately involved in this process. A number

of drugs used in the treatment of inflammatory bowel disease were also shown to induce autophagy, with azathioprine doing so robustly in two separate cell lines through inhibition of mTOR.

The previous findings that vimentin is a NOD2 interacting protein,[915] and that the invasion and survival of a CD-associated strain of adherent invasive *E.coli* (AIEC) is disrupted with Withaferin-A treatment,[819] are of particular relevance with regard to the role of vimentin during autophagy. The importance of the cytoskeletal components continue to be described in relation to autophagosome biogenesis,[1101] with intermediate filaments other than vimentin having also now been shown to play a role in impaired autophagy[1018]. In view of the fact that impaired autophagy is associated with persistence of AIEC in macrophages,[902] mechanisms to increase cellular autophagy in some instances may be beneficial. The finding that several drugs known to alter the natural history of IBD[1037] increase autophagy, with robust results in the case of azathioprine, are certainly of interest. As these mechanisms may be cancer cell-specific, or be related purely to cell apoptosis, experiments clearly assessing apoptosis and autophagy in these cells following treatment are now required. Furthermore, the increase in autophagy demonstrated by the use of infliximab may be of further interest, although the possibility that this effect may be cell type specific within the gut (as is suggested in the case of apoptosis induction[1102]) may prove difficult to clarify.

13.5 Future research agenda

With regard to work relating to PIBD pathogenesis, epidemiology, biomarkers and therapeutic management, the following future research should be considered:

- Parallel prospective studies of PIBD incidence and robust environmental data collection, focusing strongly on prenatal, perinatal and potential triggering factors prior to diagnosis, in addition to gene-environmental and epigenetic interactions.
- A more complete understanding of the genetic architecture of PIBD should be addressed, focusing attention on the manipulation of pathways and genotype-phenotype correlations rather than genetic risk factors that may account for a small proportion of the overall pathogenetic mechanism.
- The use of genetic and clinical data to allow tailored treatment for individuals, based on genetic risk, immune response, the individual's intestinal microbiota and therapeutic response.

- More investigation into the role of certain biomarkers during disease progression and assessment, harnessing the potentially beneficial aspects of their mechanism of action.
- Dissection of the current drugs used in PIBD management with a focus on maximising their potential and identifying alternative mechanisms of action that may be further utilised or improved for patient benefit.

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