Clinical and Histological Implications of Genotyping in Crohn’s Disease

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MD Thesis

2011
Acknowledgements

I would like to thank Dr James Henry for his help with the pathology and his invaluable teaching on the histological features of Crohn’s disease. I thank Prof. Burt for his help with the pathology and his teaching. Dr Pete Donaldson I would like to thank for his advice and direction. I thank Dr T Booth for the use of the microscope and Dr Gordon Beakes for his help in photographing pathology sections. I thank Phil Kelly at the Queen Elizabeth Hospital, Gateshead for cutting the sections.

I am indebted to Dr Satish Keshav for directing the laboratory work and for the use of his laboratory and to Dr Sanjay Lala for his endless patience in teaching me the technique of in situ hybridization.

I apologise to any one I have not directly thanked, I am grateful for all the help and assistance I have received during the course of this project.

I am grateful to the Margaret Simm legacy, the National Association for Colitis and Crohn’s disease and the Special Trustees for their financial support.

And finally I would like to thank Dr John Mansfield for his monumental support and forbearance. I am indebted to him for his unfailing encouragement, his criticism and his sound advice.
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<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>CARD15</td>
<td>Caspase associated recruitment domain 15</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HD-5</td>
<td>Human alpha defensin 5</td>
</tr>
<tr>
<td>HD6</td>
<td>Human alpha defensin 6</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-23</td>
<td>Interleukin-23</td>
</tr>
<tr>
<td>IL-23R</td>
<td>Interleukin-23 receptor</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithmic of odds</td>
</tr>
<tr>
<td>LREC</td>
<td>Local regional ethics committee</td>
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<tr>
<td>LZM</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatability complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerization domain</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Riboxynucleic acid</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>sPLA₂</td>
<td>Secretory phospholipase A₂</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>WTCCC</td>
<td>Wellcome trust case control consortium</td>
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Chapter 1 Introduction

Crohn’s disease is a common inflammatory bowel disease affecting approximately 1 in 1000 of the population in the UK. Surgery for Crohn’s disease is common with the majority of patients requiring surgery at some point in the course of the disease. Both genetic and environmental factors influence Crohn’s disease. Smoking significantly influences the disease course in Crohn’s disease with more frequent relapses and increased need for surgery. Recent research had concentrated on the genes predisposing to the development of Crohn’s disease. In 2001 the CARD15 (NOD2) gene was identified and since then ~30 genes have been found to be associated with Crohn’s disease. This thesis aimed to investigate the influence of CARD15 (NOD2) on the time to second operation in terminal ileal Crohn’s disease.

Crohn’s disease affects all parts of the gastrointestinal tract but particularly the terminal ileum. A particular cell type, the Paneth cell, has been implicated in the pathogenesis of Crohn’s disease. Paneth cells are located at the base of Crypts of Liberkuhn throughout the small intestine but are found in greatest numbers in the terminal ileum. Paneth cells contain and secrete antimicrobial peptides in response to bacterial products. They have been found to express CARD15 (NOD2). The expression of antimicrobial peptides and the CARD15 (NOD2) genotype were investigated using the technique of in situ hybridization.

There are well characterized histological features of Crohn’s disease. There are no known histological features of Crohn’s disease associated with the CARD15 (NOD2) genotype. These features were investigated.

Osteoporosis is an important complication of Crohn’s disease and it’s treatment. Predictors of those at risk for the development of Crohn’s disease would be clinically useful in targeting therapy. Genetic influences on bone mineral density and Crohn’s disease were investigated.

Recent publications have furthered our knowledge of the genetic factors influencing the development of Crohn’s disease. The Newcastle cohort of patients contributed to this knowledge.
1.1 History

Inflammatory bowel disease is a common chronic condition with a current UK prevalence of 240 000 (Hellier, Sanderson et al. 2006). It is a group of inflammatory diseases affecting the alimentary tract including Crohn’s disease and ulcerative colitis. The incidence of Crohn’s disease is around 8 per 100 000 and that of ulcerative colitis 14 per 100 000 (Rubin, Hungin et al. 2000). Crohn’s disease causes significant morbidity with the cost of care estimated at £3000 per patient per year (Hellier, Sanderson et al. 2006). The majority of people with Crohn’s disease will require surgery at some time in their lives (Forbes 1997). The pathogenesis of Crohn’s disease is thought to be a complex interaction between genetic predisposition, the immune system and environment.

The disease that was to become known as Crohn’s disease was first described in the medical literature as a chronic interstitial enteritis by T. K Dalziel in 1913 (Dalziel 1913). Probably the first case recorded in formal literature was that of a 20 year old man who died of a perforated terminal ileum in the 17th Century. This was described by Giovanni Battista Morgagni (1682-1771) the Italian anatomist as granulomatous enterocolitis (Morgagni 1769). Dalziel wrote of a number of cases in which a varying amount of the small and large intestine were involved. The histopathological appearance however was similar between all cases. In the British Medical Journal in 1913 Dalziel described the case of a fellow Doctor who suffered prolonged intestinal obstruction and then died (Dalziel 1913). The event occurred at the turn of the 20th Century. Preceding the terminal event he had suffered colic, diarrhoea and fever. Dalziel operated on the Professor and found that his internal organs were fixed; now known as “fat wrapping” with chronic inflammation and nodal enlargement. He noted that the samples taken were negative for tuberculosis. Dalziel went on to describe a further case from 1905. This patient had a palpable mass with symptoms of sub-acute obstruction. Dalziel operated on the patient and removed the “caput coli” (caecum) with the adjoining ileum. He described a number of cases in whom discrete segments of the bowel were involved. Dalziel described a characteristic pathology; “early vascular congestion and oedema with an infiltrate rich in polymorphs”. He noted that the lymphoid aggregates seen were pathology free, i.e normal unlike those seen in intestinal tuberculosis. There was loss of the epithelium with infiltration of the muscularis mucosae and necrosis. Eosinophils and giant cells were seen. In one
patient coliform bacteria were cultured from within the gut wall. Dalziel postulated that there was no known aetiology. He thought it was probably tuberculous, but the pathology was different with a smooth gut and notable absence of caseating granulomas. However, there was a marked similarity to Johne’s disease of cattle that had been described in 1895. No acid fast bacilli were found in any of Dalziel’s cases. Dalziel thought that many previous cases had been miss-diagnosed as intestinal tuberculosis were actually this disease of hyperplastic enteritis. The only treatment option available at that time was surgery. A ‘regenerative process’ was remarked upon.

Interestingly, the similarity to cases of Johne's disease of cattle noted by Dalziel was not novel. Johne’s disease is a chronic enteritis of cattle due to *mycobacterium paratuberculosis*. However, in the human cases, the non caseous nature of the inflammation was distinguished. The condition had been called *hyperplastic enteritis* by human pathologists for a number of years prior to Dalziel’s description.

In 1932 in the Journal of the American Medical Association Burrill B Crohn described a regional ileitis (Crohn, Ginzberg et al. 1932) after whom Crohn’s disease is named. A case series of 14 was published. The paper described disease affecting the terminal ileum with fever, diarrhoea and emaciation leading to obstruction (Crohn, Ginzberg et al. 1932). At surgery they found disease affecting the ileocaecal valve extending towards the mouth for 20-30cm. The disease often led to fistula formation to the colon or abdominal wall. The disease was of unknown aetiology but followed a benign course. They described an association of right iliac fossa mass, fistula, emaciation and anaemia, previous appendectomy and intestinal obstruction. The disease was one of young adults with granulomas seen on microscopy with giant cells but no tuberculosis. They described the disease as only involving the terminal ileum, never the colon or perianal region. They hypothesized that the disease progressed from one of acute inflammation to ulceration and enteritis with symptoms of abdominal pain and diarrhoea. This progressed to stenotic disease with sub-acute and chronic small bowel obstruction. They stated however that stenotic disease could be the primary manifestation. The disease then progressed to fistula formation. The diagnosis could be made using a barium meal. Crohn suggested that this disease of the terminal ileum was a separate disease to that affecting the colon and that tuberculosis affecting just the terminal ileum was extremely rare. The treatment was surgical resection. Recurrence
was noted to be a problem occurring proximal to surgical anastomosis. It was thought to be the result of leaving disease when performing the original operation. Crohn and colleagues termed this disease regional enteritis. A number of benign granulomatous conditions had been described but the condition that Crohn described was one of inflammation of the terminal ileum. Crohn’s disease however is now understood to mean a chronic granulomatous inflammation of any of the small or large intestine.

These initial descriptions of Crohn’s disease highlight clearly a number of issues; the description and diagnosis of Crohn’s disease is difficult, a number of diseases can masquerade as Crohn’s disease thereby weakening associations and studies. Crohn identified that regional enteritis recurs unpredictably and often requires further surgery.

From the first descriptions in the medical literature the diagnosis of Crohn’s disease has presented a challenge. It is a heterogeneous condition with variable clinical and pathological features. This results in potential for diagnostic misclassification and leads to errors in association and outcome studies. Recognised criteria for the diagnosis of Crohn’s disease were published by Lennard-Jones in 1989, some 57 years after Crohn’s paper, Table 1. Crohn’s disease is diagnosed with 3 positive findings or one positive plus the finding of granuloma.

In summary, to meet Lennard-Jones criteria there should be;

Typical diarrhoea history for at least 2 months;

Radiological features of Crohn’s disease: segmental distribution, deep ulcerations or cobblestone pattern, thickened bowel wall, coarse mucosal relief, stenotic segments and fistulae;

Macroscopic diagnosis by endoscopy: patchy penetrating lesions, fissuring and strictures;

Fistulas and/or abscesses with typical intestinal disease
Table 1 Lennard-Jones anatomic criteria for the diagnosis of Crohn’s disease recognizable by clinical, radiological and pathologic examination. A diagnosis of Crohn’s disease requires 3 positive findings, or one positive finding with granulomas on histology.

<table>
<thead>
<tr>
<th></th>
<th>Clinical/Endoscopy</th>
<th>X-ray</th>
<th>Biopsy</th>
<th>Resected Specimen</th>
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<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Upper gut</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Anus</td>
<td>+</td>
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<td>+</td>
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<td>Discontinuous</td>
<td>+</td>
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<td>Transmural inflammation</td>
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<td>+</td>
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<tr>
<td>Abscess</td>
<td>+</td>
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<tr>
<td>Fistula</td>
<td>+</td>
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<tr>
<td>Fibrosis/Stenosis</td>
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<td>Lymphoid</td>
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<td>Ulcers</td>
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<td>Aggregates</td>
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<tr>
<td>Mucin Retention</td>
<td></td>
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</tr>
<tr>
<td>Granuloma</td>
<td></td>
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</table>
1.2 Clinical phenotypes and classification

A standardised classification system enables disease phenotype and outcome to be compared consistently by different groups in different studies. There are many clinical phenotypes with variable site and severity of disease; mild to severe, some requiring no intervention, some requiring multiple medical treatments and multiple surgeries. The challenge is to create a simple, easy to use, reliable classification system with little or no inter-observer variation that gives normally distributed groups for ease of analysis. There are a number of problems with creating a classification system for Crohn’s disease. Disease behaviour changes with time; it is a progressive unpredictable disease in the majority of people, relapsing and remitting. Additionally there are environmental influences such as smoking on the disease process.

An initial classification system published in 1975 by Farmer used an anatomical system only (Farmer 1975). This gave 3 subgroups based on the anatomical distribution of disease. This takes no account of the variability of disease behaviour in Crohn’s disease with time or between people with Crohn’s disease.

The International Working Party published the Rome classification system in 1992 (Sachar 1992). This was based around anatomical distribution with clinical behaviour; inflammatory, stricturing or stenotic, and operative history included. This created a classification system with 756 subgroups.

A classification system was published in 2000, known as the Vienna Classification Table 2 from the International Working Party at the World Congress of Gastroenterology, Vienna, 1998 (Gasche, Scholmerich et al. 2000). This uses three criteria to categorise Crohn’s disease; age, disease behaviour and disease location. The categories for age are very broad, either above or below the age of 40 years at diagnosis. Disease behaviour only allows one type of behaviour, when commonly in clinically practice more than one behaviour type is found. Disease location is relatively inflexible and does not include perianal disease. It may be included as a disease behaviour as “perforation” but this subgroup was intended for intestinal perforation, not isolated perianal disease. Although superficially simplistic there are 24 groups of disease which means that large study numbers are needed for phenotype studies. Upper GI disease is included as an independent location even if ileum or
colon are involved. The age of onset categories are very broad leaving no subgroup for childhood onset disease.

Table 2 Vienna Classification

<table>
<thead>
<tr>
<th>Category</th>
<th>A1</th>
<th>A2</th>
</tr>
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<tbody>
<tr>
<td>Age at diagnosis</td>
<td>&lt;40 years</td>
<td>&gt;40 years</td>
</tr>
<tr>
<td>Disease behaviour</td>
<td>B1</td>
<td>B2</td>
</tr>
<tr>
<td></td>
<td>Inflammatory</td>
<td>Stricturing</td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>Penetrating</td>
</tr>
<tr>
<td>Disease location</td>
<td>L1</td>
<td>L2</td>
</tr>
<tr>
<td></td>
<td>Terminal ileum</td>
<td>Colon</td>
</tr>
<tr>
<td></td>
<td>L3</td>
<td>L4</td>
</tr>
<tr>
<td></td>
<td>Ileocolon</td>
<td>Upper GI</td>
</tr>
</tbody>
</table>

The Vienna classification system does not lead to normally distributed populations. This leads to difficulties with statistical analysis. The Vienna classification system can be used to predict need for surgery and immunosuppression. The advantages of the Vienna classification are that it is internationally recognised and relatively straightforward providing consistent classification of disease.

The issue of the stability of disease classification over time is addressed in a study published in 2001 (Louis, Collard et al. 2001). Extensive review of over 1000 patient records resulted 6% of the patients having a change in diagnosis. As noted by Crohn in his 1932 paper, disease behaviour changes over time. Disease location remains relatively stable over time with only 16% of patients having a change in location over 10 years. Change in behaviour is more likely with 45.9% of patients having had a change in behaviour over 10 years. Most change was from inflammatory to stricturing disease in 27% or fistulising disease in 29%. A study of 2002 patients with Crohn’s disease in France had similar results (Cosnes, Cattan et al. 2002); only 12% were found
not to have stricturing and/or penetrating disease after 20 years follow-up compared with 48% at 5 years. In a further study of 231 patients (Smith, Arnott et al. 2004) only 8% had no stricturing or penetrating disease after 20 years compared with 46% at 5 years and 70% at diagnosis.

Table 3 Montreal classification system (Silverberg, Satsangi et al. 2005) *L4 is a modifier that can be added when concomitant upper gastrointestinal disease is present, †p perianal disease modifier, added to B1-3 when concomitant perianal disease present

<table>
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<td>Age at diagnosis</td>
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<tr>
<td>A1</td>
<td>&lt;16 years</td>
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<td>17-40 years</td>
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<td>A3</td>
<td>&gt;40 years</td>
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<td>Disease location</td>
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<td>L1</td>
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<tr>
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<td>Colonic</td>
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<tr>
<td>L3</td>
<td>Ileocolonic</td>
</tr>
<tr>
<td>L4*</td>
<td>Isolated upper gastrointestinal</td>
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<td>Disease behaviour</td>
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<tr>
<td>B2</td>
<td>Stricturing</td>
</tr>
<tr>
<td>B3</td>
<td>Penetrating</td>
</tr>
<tr>
<td>p†</td>
<td>Perianal disease modifier</td>
</tr>
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</table>

Most recently a working party at the 2005 Montreal World Congress of Gastroenterology revised the Vienna Classification system (Silverberg, Satsangi et al. 2005), Table 3. Early onset disease is classified separately. It was felt this change was needed as a number of genotypes are associated with childhood onset disease. Disease location was altered to allow upper gastrointestinal disease to co-exist with lower gastrointestinal disease, it was felt that as the upper gastrointestinal tract is more easily accessible with studies such as wireless capsule endoscopy the frequency of upper gastrointestinal disease is becoming more common (Satsangi, Silverberg et al. 2006).
Perianal disease, a common manifestation of Crohn’s disease, was added as a modifier in disease behaviour in the Montreal system. There remains a problem with disease variability over time. The number of relapses, need for surgery and other disease modifying drugs.
1.3 Genetics, the story so far

1.3.1 A history of Crohn’s disease genetics

As early as 1934 it was recognised that Crohn’s disease was found in multiple members of the same family. Crohn reported regional enteritis in two sisters (Crohn 1934). A further case report of regional enteritis in 3 siblings was published in 1939 (Brown and Scheifley 1939). In this paper it was stated that in their current patient cohort of 2500 patients with ulcerative colitis at the Mayo clinic there were only 15 familial pairs.

The genetic basis for Crohn’s disease has long been investigated. In 1971 Singer published a series of 646 patients with inflammatory bowel disease (Singer, Anderson et al. 1971). 113 of these had familial disease. This study looked only at inflammatory bowel disease and the frequency of familial disease. The number of patients with Crohn’s disease was not given. The conclusion in this paper was that inflammatory bowel disease had very little genetic basis.

A review in 1971 by McConnell (McConnell 1972) surmised that a family history of siblings with Crohn’s disease was more common than a vertical family history. A family history of inflammatory bowel disease was more common in patients with Crohn’s disease than those with ulcerative colitis. Familial disease was uncommon with only 1 in 15 to 1 in 50 having a first degree relative affected in this cohort. An association with ankylosing spondylitis and inflammatory bowel disease was seen. It was hypothesized that there was both a shared environment and shared genetic predisposition. The inheritance pattern was not Mendelian but a complex polygenic disorder with a combination of environmental and genetic factors determining the development of Crohn’s disease. It was suggested by the authors that there may be a number of genes in common between Crohn’s disease, ulcerative colitis and ankylosing spondylitis.

In 1989 Küster published a paper using the technique of complex segregation analysis to investigate the pattern of inheritance found in Crohn’s disease (Küster, Pascoe et al. 1989). This was carried out by collecting the pedigree of each proband with Crohn’s disease including first degree relatives, second degree relatives and cousins. All affected relative were contacted and the diagnoses confirmed making this a robust
study. Analysis of 265 probands with Crohn’s disease developed a hypothesis of a recessive gene with incomplete penetrance. By contrast ulcerative colitis was thought to represent the effects of a dominant gene with incomplete penetrance.

A further complex segregation analysis was published in 1993 (Orholm, Iselius et al. 1993). 662 patients with inflammatory bowel disease were included, but only 209 with Crohn’s disease. Using multi-factorial model the conclusion of this paper was that in Crohn’s disease there was a major recessive locus with complete penetrance with 7% of patients with Crohn’s disease carrying the gene. A considerable proportion of Crohn’s disease was considered sporadic.

Analysis of the Swedish twin registry published in 1988 (Tysk, Lindberg et al. 1988) revealed 8 of 18 monozygotic twins to be concordant for Crohn’s disease and 1 of 26 dizygotic twins. This gave a proband concordance rate among monozygotic twins of 58% in Crohn’s disease compared to 6% in ulcerative colitis. A coefficient of heritability was calculated to be 1.0 (95% confidence interval 0.34-1.0) for Crohn’s disease and 0.53 (95% confidence interval 0.24-1.0) for ulcerative colitis.

The low rate of familial disease is shown in the UK Thompson twin study (Thompson, Driscoll et al. 1996). Twins were identified by contacting patients through the National Association for Colitis and Crohn’s disease. 150 twin pairs were identified. 15 twins were concordant for disease. Monozygotic twins were significantly more likely to be concordant for diagnosis. The concordance rate was low at only 17% however, suggesting that there are a number of other significant factors influencing the development of Crohn’s disease.

An estimate of the risk to siblings for the development of a given disease can be expressed $\lambda_s$. This gives an estimate of the relative risk of development of the disease in siblings of affected people. The $\lambda_s$ for Crohn’s disease, ulcerative colitis and inflammatory bowel disease has been estimated to be 36.5, 16.6 and 24.7 from a study of relatives of people with inflammatory bowel disease in Oxford (Satsangi, Rosenberg et al. 1994). Compared to other complex genetic diseases the $\lambda_s$ for inflammatory bowel diseases is high. The $\lambda_s$ for type-1 diabetes estimated as 20, schizophrenia as 11, Grave’s disease as 6. However, the $\lambda_s$ of single gene disorders is much greater, haemophilia 3750 and cystic fibrosis 750.
Overall these studies suggest a significant genetic basis to Crohn’s disease with environmental modifiers to the development of disease. The mode of inheritance does not follow a simple Mendelian model but is polygenic typical of complex genetic disease.

1.3.2 **Association studies using candidate genes**

Some of the earliest genetic studies used candidate gene analysis. Candidate gene analysis uses an identified gene and compares the frequency of carriage of a known mutation or variant among cases and controls. It can be used to replicate positive findings from linkage studies in separate populations. Due to the small sample sizes of many studies, and therefore low power, false positive results are not unusual. For example, polymorphisms in the gene encoding the cytokine interleukin-1 receptor antagonist (IL-1RA) were thought to be associated with inflammatory bowel disease. This was first reported in 1994 (Mansfield, Holden et al. 1994). The positive association was only replicated in subgroup analyses (Bioque, Crusius et al. 1995; Bioque, Bouma et al. 1996; Heresbach, Alizadeh et al. 1997; Tountas, Casini-Raggi et al. 1999). Subsequently this positive association was refuted in the literature (Louis, Satsangi et al. 1996), Heresbach 1997, Hacker 1998, Stokkers 1998, Ferreira 2005, Mittal 2005 and Celik 2006) and by our own group, (Craggs, West et al. 2001).

There are a number of limitations in this method; it can only be used with known genes, multiple genes needs correction for multiple testing, there is publication bias with positive findings more likely to be published. There needs to be geographical and ethnic matching of cases and controls. The probability of false positive results is high.

The advantages of this technique are that a relatively small case-control population can be used and it is therefore good at finding genes with small effect, a potential advantage in complex diseases with multiple genes with small effect.

A number of genes have been investigated in this way, mainly genes thought to be involved in the regulation of immune response. In the 1970s a positive association with the major histocompatability complex (MHC) class I on chromosome 6 was found. The first investigation included 18 patients with Crohn’s disease, 16 patients with ulcerative colitis and 50 controls (Gleeson, Walker et al. 1972). In this very small study no association was found. Subsequently in a larger study of 51 patients with Crohn’s disease an association with the class I antigen B18 was found (van den Berg-
Loonen, Dekker-Saeys et al. 1977). Association has been found with HLA B44Cw5 (Purrmann, Bertrams et al. 1988), HLA A11 (Biemond, Burnham et al. 1986), HLA-DR4 and 5 (Fujita, Naito et al. 1984). A large number of HLA alleles have subsequently been investigated but only a small number replicated (Yap, Ahmad et al. 2004).

A modification of candidate gene analysis has subsequently been used to attempt to identify relevant genes within susceptibility loci highlighted in genome-wide linkage and genome-wide association studies. This will be explored later in 1.3.4.

1.3.3 **Linkage studies**

The genetics of inflammatory bowel disease was advanced by the development of genome-wide linkage studies. The first linkage map was published in 1992 using 814 polymorphic markers across the entire genome (Weissenbach, Gyapay et al. 1992). These markers cover 90% of the genome, but are spaced widely. This linkage map allows variation in nucleotides at these markers to be compared between disease groups and control groups using panels of affected sibling pairs and their first degree relatives. The main disadvantage is a lack of power; multiple tests are applied to the cohort. The gaps between the tested genetic markers are wide; therefore it is difficult to identify the location of the causative mutation.

The LOD score is calculated: a statistical estimate of linkage. A LOD score of >3.0 suggest that the likelihood of linkage between the marker of interest and a gene associated with disease is 1000x greater than if there was no linkage. In inflammatory bowel disease there have been only 5 significant LOD scores. The remaining LOD scores being only suggestive.

In 1996 Hugot published the first genome-wide search in inflammatory bowel disease (Hugot, Laurent-Puig et al. 1996). Two panels of multiply affected families were used in this investigation. 25 families in the first panel and 53 in the second independent panel were genotyped. This was done by initially studying 270 highly polymorphic markers spread over the entire genome. Four markers with the highest LOD score were then genotyped in the second panel, i.e. 4 markers on chromosomes 1 and 16 in this first panel had increased allele sharing between sibling pairs. An independent panel of 53 families were then used to try to replicate this finding. Two of these markers on chromosome 16 were confirmed to have increased allele sharing. To
narrow down the area on chromosome 16 further markers at closer intervals were used. The degree of allele sharing and the estimated ratio for the dominant genetic variant to the total genetic variance was consistent with the expected finding of a recessive mode of gene inheritance. This locus was termed IBD1, a susceptibility locus in the pericentromeric area of chromosome 16. This was to be identified later as CARD15 (NOD2) (Hugot, Chamaillard et al. 2001; Ogura, Bonen et al. 2001), see section 1.3.5.

A two-stage genome-wide-linkage search published in 1996 identified 3 further loci on chromosomes 3, 7 and 12 (Satsangi, Parkes et al. 1996). This investigation used a panel of 89 sibling pairs with inflammatory bowel disease and their first degree relatives. 260 markers were used in the initial panel. The markers with the highest probability of linkage were then studied in a further panel of 97 sibling pairs. In this data set the locus on chromosome 16 was not found to have significant linkage.

The pericentromeric region on chromosome 16 was confirmed in US genome-wide linkage study in 1999 (Cho, Nicolae et al. 1998). This study of 439 patients with inflammatory bowel disease from 174 families identified significant areas of linkage on chromosomes 1p, 3q, 4q and 5q. The previously reported areas of linkage on chromosomes 2, 3p and 12 did not show linkage in this scan. This study did find linkage with chromosome 16; however it was over a broad area.

Linkage was replicated further on chromosome 16 by Hampe in 1999 (Hampe, Schreiber et al. 1999). 353 affected sibling pairs were used in a linkage study. This scan highlighted areas of linkage on chromosomes 1, 6, 10 and 12. Interestingly the maximum LOD in this study was on the X chromosome.

A Canadian cohort used 183 affected sibling pairs with 312 markers and their available parents (Rioux, Silverberg et al. 2000). This genome wide search identified two novel loci in chromosomes 19p13 and 5q31. The previously reported linkage on chromosome 3p and 6p was replicated. The 5q31 haplotype had a significant LOD only in a sub-group of early onset Crohn’s disease.

A later genome-scan by van Heel (van Heel, Dechairo et al. 2003) used markers 9.4cM (137 relative pairs from 112 families) across the genome. This identified two areas with a significant LOD; chromosome 3q, LOD 2.1 and chromosome Xp, LOD 2.0. They then stratified the cohort by IBD5 haplotype. This revealed three further significant LOD scores; chromosome 3p, LOD 2.2, chromosome 12p, LOD 2.5 and
chromosome 19, LOD 2.4. The cohort was then stratified by CARD15 (NOD2) genotype. Interestingly there was an additional significant region found on chromosome 16 in CARD15 (NOD2) negative Crohn’s disease; 16q LOD 2.2. This indicates that there is likely another gene contributing to disease risk on chromosome 16. An area on chromosome 19 had a significant LOD (2.9) in CARD15 (NOD2) negative Crohn’s disease.

In a study of 92 Finnish affected relative pairs with IBD, 12q23 was found to be significantly associated with Crohn’s disease (Paavola-Sakki, Ollikainen et al. 2002). There was very little linkage with chromosome 16. A second Finnish study identified a region on chromosome 11p12-q13 (Lappalainen, Paavola-Sakki et al. 2006).

A meta-analysis of the 10 published genome-wide linkage scans in 2004 (van Heel, Fisher et al. 2004) combined the genotyping data from 1952 affected relative pairs with inflammatory bowel disease. This enabled a much larger cohort than each individual study. The Genome Scan Meta-Analysis allowed for different genotype analysis methods and needed no assumptions of inheritance models. However this technique lacks precision and divided the genome into 34cM bins. Linkage was detected on chromosome 16 with Crohn’s disease which acted as a positive control. Areas suggestive of linkage with Crohn’s disease were found on chromosomes 2q, 3q, 12q 17q and 19p, of which the linkage on chromosomes 2q and 17q were novel findings. Significant linkage was found with chromosome 19q and within the HLA region 6p. Table 4 has a summary of candidate gene findings.

These studies have been able to identify areas of the genome that are likely to contain genes that influence the development and/or disease behaviour. The genes within these most of these areas still await identification. There is great heterogeneity between studies with little reproducibility. It became clear that a new method of investigating complex disease genetics needed to be developed.
<table>
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<th>Date</th>
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<th>Association</th>
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<td>2005</td>
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<td>None</td>
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<td>Date</td>
<td>Loci</td>
<td>Association</td>
<td>Gene</td>
<td>CD/control</td>
<td>Replication</td>
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<td>Takagawa (Japan)</td>
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<td>IL-18 promoter</td>
<td>205/212</td>
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<td>King (UK)</td>
<td>2003</td>
<td>16p11</td>
<td>None</td>
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<td>Glas (Germany)</td>
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<td>PTPN22</td>
<td>569/812</td>
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<td>11p12</td>
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<td>19q</td>
<td>Positive</td>
<td>TUCAN (CARD8)</td>
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<td>2006</td>
<td>7p14.3</td>
<td>None</td>
<td>NOD1 (CARD4)</td>
<td>645/760</td>
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<td>Van Limbergen (UK)</td>
<td>2007</td>
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<td>None</td>
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<td>2007</td>
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<td>None</td>
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1.3.4 Genome wide association studies

Despite the evidence that Crohn’s disease has a strong heritability up until 2006 there were only two genetic loci that could be reliably replicated; \textit{CARD15 (NOD2)} and 5q31 with a third on chromosome 10q23 with less consistent replication together with the MHC region on chromosome 6. The combined, modest effect of these genetic loci is not sufficient to account for the genetic load in Crohn’s disease. It was put forward in 1996 that large-scale testing by association analysis was going to be necessary to detect the multiple genes of modest effect implicated in complex diseases (Risch and Merikangas 1996). The first published genome-wide association scan was in 2005 (Yamazaki, McGovern et al. 2005).

Genome wide association studies use a case-control method to investigate association between disease and thousands of SNPs throughout the genome. Technological progress through the human genome project and the development of the HapMap has enabled this technique. The HapMap is a schematic of the human genome in which a minimal number of SNPs can be used to tag common haplotype blocks, rather than using every known SNP throughout the genome. The technique can use either SNPs evenly spaced across the entire genome or use the HapMap to tag common haplotypes. Allele frequencies are then compared between cases and controls. Large sample size and well matched ethnic and geographical controls are needed due to the multiple testing employed and the modest increase in disease risk conferred by any variant. Statistical methods have had to be developed to enable interpretation of the large number of results produced and replication in a similar number of cases and controls.

The first genome-wide association study published in 2005 (Yamazaki, McGovern et al. 2005) was an investigation in Japanese and European populations. The most reliably replicated genetic associations in European cohorts; \textit{CARD15 (NOD2)} and 5q31 haplotype have not been found in Japanese patients with Crohn’s disease (Yamazaki, Takazoe et al. 2002; Negoro, McGovern et al. 2003; Yamazaki, Takazoe et al. 2004; Tosa, Negoro et al. 2006). This investigation initially identified SNP loci in 94 patients with Crohn’s disease and 752 controls. The 1888 SNP were then investigated in a second cohort of 484 Crohn’s disease. 22 SNP with \( p<10^{-4} \) were identified, the highest number around the 9q32 locus. High density SNP map was then used to attempt to identify the causative SNP. The most significant association was in
TNFSF15 gene at the 9q32 locus. 10 markers across this region were then investigated in two Caucasian cohorts from the UK and a positive association was found. This investigation used a relatively small number of Crohn’s disease cases. It has previously been established that the genetic associations in people of European Caucasian ancestry is different to those with Japanese ethnicity, therefore the validity of replicating association within a different ethnic group could be questioned.

The next genome-wide association study was performed on 567 people with ileal Crohn’s disease with non-Jewish European ancestry (Duerr, Taylor et al. 2006) in North America. Two loci were identified within the CARD15 (NOD2) gene, p 2.86 x 10^{-9}, p 8.82 x 10^{-10}. A third (Arg381Gln) was within the interleukin 23 receptor (IL23R) gene, p 5.05 x 10^{-9}. Nine other SNPs were identified around chromosome 1p31 and the IL23R gene with less significant association. This association was then replicated in an independent case-control panel of 401 Crohn’s disease and 433 controls. It was found in a panel of 833 IBD nuclear families that there was a significant distortion in allele transmission. Combined data from all three groups showed a highly significant association of 10 IL23R markers with IBD. Interestingly the glutamine allele at position 381 was found to be protective against the development of Crohn’s disease; it is significantly under transmitted from heterozygous parents to affected offspring.

A genome-wide association study in Germany (Hampe, Franke et al. 2007) of 735 Crohn’s disease and 368 controls identified a gene within chromosome 2q37; ATG16L1. This group used non-synonymous SNPs spread across the genome. The initial positive results with p <0.01 were then investigated in a second panel of 380 trios, 498 sporadic Crohn’s disease cases and 1032 healthy controls.

Libioulle published a large genome-wide association study of 547 Crohn’s disease and 928 controls with replication in 1266 Crohn’s disease and 559 controls (Libioulle, Louis et al. 2007). The Arg381Gln SNP of IL23R was significantly associated with Crohn’s disease as was CARD15 (NOD2). The association with ATG16L1 was replicated, but no association with OCTN, DLG or TNFSF15 was found. A novel association was reported within the gene desert of chromosome 5p13.1. An attempt was made to identify the causal variant using haplotype linkage disequilibrium (LD). The area of highest significance was closest to PTGER4 as well as CARD6, and 3 complement factors.
In an extension of the genome-wide association study by Duerr 988 Crohn’s disease and 1007 controls were used in the initial panel (Rioux, Xavier et al. 2007). ~300 000 SNPs were used. The results were replicated in 530 trios then an independent case-control study of 353 Crohn’s disease and 207 controls. The two most significant loci corresponded to CARD15 (NOD2) and IL23R. The 5q31 haplotype was not among the top loci in this study. By examining the LD structure and fine mapping it there was found to be significant association at 2q37. One corresponding to the previously reported ATG16L1 gene. Novel associations were found at 10q21.1 an intergenic region, 4p13 possibly implicating PHOX2B, 22q31.1 NCF4 and 16q24.1.

The significant associations of IL23R and ATG16L1 were replicated in the UK study in June 2007 (WTCCC 2007). Again the CARD15 (NOD2) gene was positively associated with Crohn’s disease. This very large study of 7 common disease used ~2000 cases of each disease and 3000 controls. Further areas of significant association were identified 10q21, 5p13, 5q33, 3p21, 10q24 and 19p11. No gene has been identified for the area of association within 10q21. The area of association with 5p13.1 occurs within a gene desert. There were four novel associations reported. 5q33.1 includes several SNPs around IRGM. The areas on 3p21 and 10q24 involve a number of possible genes. The association on 18p11 was found upstream of PTPN2. A number of other associations were found with significance levels p<10^{-7} including the previously reported association with TNFSF15, the MHC region on chromosome 6, an area on the X chromosome and a number of areas of interest with genes known to interact with the CARD15 (NOD2) pathway. The positive findings were replicated in an independent Crohn’s disease cohort (Parkes, Barrett et al. 2007).

A further genome-wide association study was performed on a genetically isolated population in Canada, originating from 17th Century French migrants (Raelson, Little et al. 2007). The initial panel included 383 trios with a second replication panel of 477 trios with 2 additional independent cohorts of 521 German trios, 750 cases and 828 controls. Four novel loci were identified, 3p21.3 BSN, 4p16.1 JAKMIP1, 17q11.1 haplotype and 17q22-23 haplotype in strong LD. These loci were replicated in the German cohort suggesting that they are not unique to the genetically homogeneous Quebec population.

A meta-analysis of three genome-wide association studies from European derived Crohn’s disease populations combined to a total of 3230 Crohn’s disease and 4829
controls (Barrett, Hansoul et al. 2008). This identified and replicated 21 novel loci to bring the current total of significant association to ~30. 526 SNPs within this study were found to have p value <5x10^{-5} within 74 genomic loci. The combined power of the three studies enabled a significant association to be found for odds ratio of 1.2, this highlights the problem of significance level in these large studies with multiple testing. 32 of the 526 associations SNPs correlated with known non-synonymous SNPs. 21 areas have no known genes. It may well be that this meta-analysis does not include a large enough case or control population to identify significant genes albeit with small effect. There is current work proceeding to establish an international consortium to include 20 000 patients with Crohn’s disease.

The above genome-wide association study (GWAS) have consistently identified three genes associated with Crohn’s disease; IL23R, ATG26L1 and IRGM. A significant problem with the genome-wide association study is the identification of the causal variant and/or gene product of the SNPs identified from these investigations, rather than the tagging SNP which is positive for the association. In the case of CARD15 (NOD2) the OR of the homozygotes is 30-40 when the causative SNPs were examined, but only 3 when the GWAS data is examined. A predisposition to Crohn’s disease may result from altered transcription or expression of a gene product rather than a gene mutation per se. The current genome-wide association studies do not incorporate methods for identifying copy number variants as disease modifiers but such an experiment is in progress under the umbrella of the WTCC, results in 2009.

To date the genetics studies have identified genes which predispose to Crohn’s disease. The clinical benefit has come from identifying the pathophysiological pathways in the development of Crohn’s disease rather than in predicting the course of the disease. Interestingly the GWAS have highlighted a number of genes important in autophagy, innate immunity and functioning IL23 pathway. Autophagy is an ancient mechanism by which cell organelles, cell breakdown products and bacteria are engulfed and disrupted. IL23 is important in regulation of T regulatory cells and control of inflammation. See Chapter 6 Genetics and Appendix for more detail.
1.3.5 CARD15 (NOD2)

The area of linkage on chromosome 16 has been investigated in order to identify the gene within this region associated with Crohn’s disease. By candidate gene analysis interleukin-4 (Olavesen, Hampe et al. 2000) and CD19 and CD43 (Hugot 1999) were found not to be associated with Crohn’s disease.

In 2001 back to back papers (Hugot, Chamaillard et al. 2001; Ogura, Bonen et al. 2001) were published in Nature identifying the gene associated with Crohn’s disease located within the susceptibility locus on chromosome 16. Hugot et al (Hugot, Chamaillard et al. 2001) used a positional cloning technique, initially 26 microsatellite markers spaced 1cM apart over the pericentromeric region of chromosome 16. Then linkage analysis in 77 affected relative pairs and transmission disequilibrium testing identified a region containing the CARD15 (NOD2) gene. SNPs were then investigated within this gene and three were found to be carried more frequently in Crohn affected patients. These regions were sequenced and compared with known characterised genes. 11 SNPs (1-11) from within these regions were then subsequently searched for in families with Crohn’s disease. Significant excess transmission was found with most of the SNPs investigated. Four of these SNPs (5-8) and an addition two SNPs (12 and 13) were found to be within a single gene. It was subsequently found that four (2, 4, 5 & 6) of the original SNPs were not transmitted independently of SNP13. These final 8 SNPs within a single gene were found to identify 41 haplotypes, three of which were preferentially transmitted to people with Crohn’s disease. Each of these contained an allele of either SNP 8, 12 or 13. There was found to be a gene dosage effect with carriage of two SNPs being associated with the greatest risk. This is again consistent with the recessive model of inheritance in Crohn’s disease. Each of these SNPs is functional resulting in a change in the nature of the protein product.

Ogura et al (Ogura, Bonen et al. 2001) used candidate gene analysis of CARD15 (NOD2) showing that a frame shift mutation 3020insC is associated with Crohn’s disease. CARD15 (NOD2) was known to be involved in recognising bacterial components and was therefore hypothesized to have a role in the pathogenesis of Crohn’s disease. The CARD15 (NOD2) gene was then sequenced in a number of families with Crohn’s disease as well as controls. An insertion SNP was found that resulted in truncation of the CARD15 (NOD2) protein. The transmission of the
mutation was investigated among families with Crohn and families with ulcerative colitis. There was an excess of transmission only in families with Crohn’s disease. This mutation was then searched for in a number of unrelated Crohn’s disease cases in a case-control fashion. This confirmed the association of this SNP with 8.2% frequency in patients with Crohn’s disease (n=412) and a frequency of 4% in controls (n=287). Two other SNPs in CARD15 (NOD2) were mentioned in this paper as being associated with Crohn’s disease.

The association of Crohn’s disease with the CARD15 (NOD2) gene was found by two different groups by differing methods. Positional cloning using microsatellite markers and SNPs in multiply affected families and candidate gene analysis. At around the same time another European group also identified a functional mutation in the CARD15 (NOD2) gene that was significantly associated with Crohn’s disease (Hampe, Cuthbert et al. 2001). All of the SNPs found in the CARD15 (NOD2) gene are individually rare. The three SNPs that are occur most commonly in association with Crohn’s disease are SNP8 R702W, SNP12 G908R and SNP13 L1007fs. These SNPs only very rarely occur on the same haplotype (<0.2%) (Cuthbert, Fisher et al. 2002). NOD2 is synonymous with CARD15 (capsase recruitment domain 15) (Human Genome Organisation approved name) NOD2 (nucleotide oligomerization domain 2) and has been identified as part of a large family of intracellular pattern recognition receptors. The association of mutations in the CARD15 (NOD2) and Crohn’s disease has been replicated in many large studies.

There has been intense investigation of the CARD15 (NOD2) gene and its association with Crohn’s disease. A mutational analysis on the CARD15 (NOD2) was undertaken by Lesage et al in 2002 (Lesage, Zouali et al. 2002). This identified 67 sequence variations in 483 unrelated patients with Crohn’s disease of whom 287 had familial disease. 31 of these variations were considered to be potential disease causing mutations as they could result in functional changes in the peptide gene product. The three previously reports SNPs were confirmed to be associated with Crohn’s disease in this population. 13% of Crohn’s disease chromosomes had a rare sequence variation compared with 6% of control chromosomes independent of the three main disease causing mutations. In total at least one mutation was found in up to 50% of patients with Crohn’s disease. Of note no difference in allele frequencies was found between familial and sporadic Crohn’s disease. Phenotypic study of this cohort revealed an
association between stenotic disease behaviour and younger age at diagnosis and carriage of two \textit{CARD15} (\textit{NOD2}) mutations. Colorectal disease was found to be less common in Crohn’s disease patients with \textit{CARD15} (\textit{NOD2}) mutation. Stenotic disease behaviour remained significantly associated with \textit{CARD15} (\textit{NOD2}) mutations even after correction for disease location. There was no significant difference in surgical interventions between the groups. R702W, G908R and L1007fs represent 81% of \textit{CARD15} (\textit{NOD2}) mutated alleles.

The contribution to disease risk of each of these three \textit{CARD15} (\textit{NOD2}) SNPs was investigated in a large study of 688 unrelated people with Crohn’s disease (Cuthbert, Fisher et al. 2002). This was the first study that investigated the phenotype of Crohn’s disease associated with \textit{CARD15} (\textit{NOD2}) mutations. R702W, G908R and 3020ins C (L1007fs) SNPs were genotyped and the relative risk calculated. The highest risk for heterozygotes was associated with carriage of G908R SNP with an odds ratio of 6.3. Homozygotes and compound heterozygotes were found to have similar risk with a combined odds ratio of 23.4 and a population attributable risk of 26%. In this cohort 35.8% of patients were carriers of at least one \textit{CARD15} (\textit{NOD2}) mutation, with 8.0% carrying two mutations. 7 people were found to be homozygous for \textit{CARD15} (\textit{NOD2}) mutation but had a diagnosis of ulcerative colitis. The phenotype of Crohn’s disease related to \textit{CARD15} (\textit{NOD2}) genotype was investigated in 444 cases of Crohn’s disease. The frequency of \textit{CARD15} (\textit{NOD2}) mutation was significantly increased in patients with ileal and ileocolonic disease over colon only disease. The \textit{CARD15} (\textit{NOD2}) allele frequency of patients with colon only Crohn’s disease approached that of controls. Carriage of a \textit{CARD15} (\textit{NOD2}) mutation was associated with ileal disease with an odds ratio of 2.04.

In a study published at the same time of 244 people with Crohn’s disease the highest risk was associated with L1007fs SNP not G908R SNP (Ahmad, Armuzzi et al. 2002). However, a similar overall population attributable risk was calculated of 27%. It is not clear in the methodology of this paper whether all patients were unrelated. This may account for the higher overall frequency of \textit{CARD15} (\textit{NOD2}) mutations in this study compared with the Cuthbert paper. A significant association was again found between carriage of a \textit{CARD15} (\textit{NOD2}) mutation and ileal disease. Stenotic disease was also found to be associated with \textit{CARD15} (\textit{NOD2}) mutations; however this was not independent of ileal disease. 158 patients required surgery for stenotic disease of
whom 77 had a second operation. This was not found to be associated with \textit{CARD15 (NOD2)} mutations. \textit{CARD15 (NOD2)} mutations were not found to be associated with fistulising disease. In a further study of 97 people with Crohn’s disease in 2002 (Radlmayr, Török et al. 2002) carriage of \textit{CARD15 (NOD2)} L1007fs mutation was found to be associated with fistulising and fibrostenotic disease and also need for surgical intervention. This study is again small with only 39 patients having had surgery.

The association of \textit{CARD15 (NOD2)} mutations with ileal Crohn’s disease is well established and has been replicated many times. However association of \textit{CARD15 (NOD2)} mutations with disease behaviour is variable. A number of studies have found there to be an association with stricturing and/or fistulising disease (Ahmad, Armuzzi et al. 2002; Lesage, Zouali et al. 2002; Radlmayr, Török et al. 2002; Brant, Picco et al. 2003; Helio, Halme et al. 2003; Abreu and Yang 2004) this is not always consistent (Hampe, Grebe et al. 2002; Louis, Michel et al. 2003; Esters, Pierik et al. 2004). \textit{CARD15 (NOD2)} mutations have been associated with ileal location (Cuthbert, Fisher et al. 2002; Bünning, Genschel et al. 2004) and fibrostenosing (Abreu, Taylor et al. 2002) phenotype.

A meta-analysis of the phenotypic effects of \textit{CARD15 (NOD2)} was published in 2004 (Economou, Trikalinos et al. 2004). This meta-analysis included studies of the common variants of \textit{CARD15 (NOD2)}: R702W (SNP8), G908R (SNP12) and L1007fs (SNP13), termed high-risk alleles, and their frequency in Crohn’s disease, Ulcerative colitis and healthy controls. Studies that investigated the association between alleles and Crohn’s disease phenotype were included. The meta-analysis investigated prevalence of \textit{CARD15 (NOD2)} mutation, the association between \textit{CARD15 (NOD2)} and disease susceptibility, phenotype and dose response. Familial disease and carriage of \textit{CARD15 (NOD2)} risk allele and colonic disease and healthy controls was investigated. Odds ratio with 95% confidence intervals (CI) were used. 42 studies were included.

There was a significant variation in high-risk allele prevalence between ethnic groups. In healthy non-Jewish Caucasians the high-risk allele frequency was 6.7-19.3% in Jewish population 23.2% (95% CI 16.7-31.2%). Overall in the non-Jewish population carriage of at least one high-risk allele gave an OR of 3.15 for Crohn’s disease (95% CI 2.76-3.59), in Jewish population OR 1.55 (95% CI 0.99-2.42), limited data was
available. Carriage of SNP13 conferred the highest OR 4.09 (95% CI 3.23-5.18), SNP8 2.20 (95% CI 1.84-2.62), and SNP12 2.99 (95% CI 2.38-3.74). There was found to be a dose response effect with carriage of one high-risk allele giving an OR of 2.39 (95% CI 2.00-2.86) and two high-risk alleles an OR of 17.1 (95% CI 10.7-27.2).

Carriage of \textit{CARD15 (NOD2)} was associated with familial disease; 1.41 (95% CI 1.17-1.69), small bowel involvement 2.53 (95% CI 2.01-3.16) and stricturing disease; 1.94 (95% CI 1.61-2.34). Carriage of \textit{CARD15 (NOD2)} was overall found to be associated with colonic Crohn’s disease 1.49 (1.18-1.87) in contrast to other studies (Ahmad, Armuzzi et al. 2002; Cuthbert, Fisher et al. 2002). It is not clear in this study whether the association with stricturing disease is independent of the small bowel association.

In a long-term follow-up study of patients with Crohn’s disease in Edinburgh carriage of \textit{CARD15 (NOD2)} mutation was found to be associated with late progression of disease, considered to be no stricturing or penetrating disease after 3.9 year follow-up (Smith, Arnott et al. 2004). No association was found between \textit{CARD15 (NOD2)} carriage and evolution of disease behaviour after 5 year follow-up (Louis, Michel et al. 2003). \textit{CARD15 (NOD2)} mutation carriage has been associated with both ileal resection and reoperation at the terminal ileum (Büning, Genschel et al. 2004), however this is not independent of the association of \textit{CARD15 (NOD2)} with ileal disease. An Italian study of 193 patients with ileal Crohn’s disease found that \textit{CARD15 (NOD2)} carriage was associated with no stricturing or penetrating disease but with ileal resection (Laghi, Costa et al. 2005).

Carriage of \textit{CARD15 (NOD2)} has been associated with younger age at disease diagnosis (Büning, Genschel et al. 2004; Laghi, Costa et al. 2005; Onnie, Fisher et al. 2008).

The association of \textit{CARD15 (NOD2)} with Crohn’s disease varies over geographical populations. The frequency of \textit{CARD15 (NOD2)} mutations is lower in Northern Europe in both Crohn’s disease and controls, and is much more rare in Scandinavia. This was first noted by Hampe et al 2002 (Hampe, Cuthbert et al. 2001; Hampe, Grebe et al. 2002) in a study of German and Norwegian Crohn’s disease populations. The estimated haplotype frequencies in Norwegian compared to German populations was significantly lower (p<0.001). In the Finnish population (Helio, Halme et al. 2003) overall \textit{CARD15 (NOD2)} carriage was 15.7% in Crohn’s disease.
and 6.7% controls. There was no significant difference in allele frequency of G908R or R702W between Crohn’s disease and controls. Carriage of L1007fs allele was significantly higher in Crohn’s disease than in controls. CARD15 (NOD2) was again associated with ileal disease. Although a total of 271 people with Crohn’s disease were included in this study, due to the low carriage of CARD15 (NOD2) mutations data regarding phenotype association has low power and may be unreliable. A summary of international CARD15 (NOD2) frequencies is in Table 5.

Crohn’s disease is more common in Caucasian populations. Inflammatory bowel disease has a 2-8 times greater prevalence in Jewish population compared to non-Jewish (Sandler and Golden 1986; Yang, McElree et al. 1993). There is a two-four fold increased incidence of Crohn’s disease among the Jewish population. A study with 64 Ashkenazi-Jewish families and 144 non-Jewish Caucasian families and an independent case control panel of 112 Ashkenazi-Jewish patients and 116 non-Jewish Caucasian patients with Crohn’s disease identified a novel haplotype (JW1) associated with Crohn’s disease in Ashkenazi-Jewish patients with Crohn’s disease (Sugimura, Taylor et al. 2003). It was not associated with Crohn’s disease in the non-Jewish population. JW1 is situated in the non coding region of the CARD15 (NOD2) gene therefore it is unclear at present whether it exerts a direct effect on gene expression or is merely a marker for an as yet unknown gene mutation. In this study R702W was not found to be associated with Crohn’s disease in the Jewish population. Further study of Ashkenazi and Sephardic Jewish populations has shown that Ashkenazi Jews with Crohn’s disease have an increased carrier rate of CARD15 (NOD2) mutations compared with Sephardic Jews (Karban, Waterman et al. 2004). Carriage of all three SNPs was increased in Crohn’s disease compared to controls. The L1007fs was almost nonexistent with the Sephardic Jewish population in this study. Carriage of CARD15 (NOD2) was not significantly associated with ileal disease in this population. Sephardic Jews in this study were much more likely to be smokers than those of Ashkenazi origin. This suggests a different environmental role for the pathogenesis of Crohn’s disease in this population. This further demonstrates the genetic heterogeneity in Crohn’s disease.

In a study of CARD15 (NOD2) within families with Crohn’s disease (Esters, Pierik et al. 2004) 570 people with Crohn’s disease and 762 first degree relatives were studied, along with 165 healthy controls and 173 people with ulcerative colitis. A significant
number of healthy first degree relatives were carriers of *CARD15 (NOD2)* mutations, 37.3% one mutation and 3.1% two mutations. There was no difference found in the frequency of *CARD15 (NOD2)* among sporadic, familial or mixed IBD families. Segregation analysis was performed on 137 families in which genotypes were known for all members. Maternal transmission of the *CARD15 (NOD2)* variant allele was associated with a lower proportion of affected individuals compared to paternal transmission. This study is interesting as it clearly shows that there are other influences on the development of Crohn’s disease other than *CARD15 (NOD2)*, both genetic and environmental. Carriage of *CARD15 (NOD2)* does not explain the concordance found in Swedish twins (Halfvarson, Bresso et al. 2005), indicating that there are other genes associated with Crohn’s disease other than *CARD15 (NOD2).*
Table 5 Summary of allele frequency for international studies in Crohn’s disease, *includes single rare mutations not used in other studies *Belgium, Denmark, France, Germany, Ireland, Italy, Spain and Sweden

<table>
<thead>
<tr>
<th>Author Year</th>
<th>Country</th>
<th>n=</th>
<th>Crohn’s disease</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Any variant</td>
<td>R702W</td>
</tr>
<tr>
<td>Cuthbert 2002</td>
<td>England</td>
<td>429</td>
<td>35.8%</td>
<td>9.1%</td>
</tr>
<tr>
<td>Ahmad 2002</td>
<td>England (Oxford)</td>
<td>244</td>
<td>38.5%</td>
<td>12.5%</td>
</tr>
<tr>
<td>Abreu 2002</td>
<td>USA</td>
<td>201</td>
<td>35.3%</td>
<td>16.4%</td>
</tr>
<tr>
<td>Lesage 2002</td>
<td>Europe*</td>
<td>453</td>
<td>49.4%</td>
<td>11%</td>
</tr>
<tr>
<td>Hampe 2002</td>
<td>Germany</td>
<td>552</td>
<td>28.9%</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>Norway</td>
<td>55</td>
<td>7.0%</td>
<td>n/a</td>
</tr>
<tr>
<td>Heliö 2003</td>
<td>Finland</td>
<td>271</td>
<td>15.5%</td>
<td>3.3%</td>
</tr>
<tr>
<td>Brant 2003</td>
<td>USA</td>
<td>275</td>
<td>39.3%</td>
<td>n/a</td>
</tr>
<tr>
<td>Louis 2003</td>
<td>Belgium</td>
<td>163</td>
<td>45.5%</td>
<td>n/a</td>
</tr>
<tr>
<td>Esters 2004</td>
<td>Belgium</td>
<td>570</td>
<td>46.3%</td>
<td>12.9%</td>
</tr>
<tr>
<td>Weiss 2004</td>
<td>Israel (Jewish population)</td>
<td>144</td>
<td>37.5%</td>
<td>11%</td>
</tr>
<tr>
<td>Karban 2004</td>
<td>Israel Jewish population</td>
<td>180</td>
<td>41.1%</td>
<td>5%</td>
</tr>
<tr>
<td>Smith 2004</td>
<td>Scotland</td>
<td>218</td>
<td>23.8%</td>
<td>n/a</td>
</tr>
<tr>
<td>Büning 2004</td>
<td>Germany</td>
<td>250</td>
<td>35.6%</td>
<td>7.2%</td>
</tr>
<tr>
<td>Laghi 2005</td>
<td>Italy</td>
<td>239</td>
<td>33%</td>
<td>10%</td>
</tr>
<tr>
<td>Cruyssen 2005</td>
<td>Belgium</td>
<td>156</td>
<td>49.3%</td>
<td>15%</td>
</tr>
<tr>
<td>Crawford 2005</td>
<td>USA (South)</td>
<td>254</td>
<td>32%</td>
<td>9%</td>
</tr>
</tbody>
</table>
1.3.6 **IBD5**

A risk haplotype was identified in 2000 on chromosome 5; 5q31 (Rioux, Daly et al. 2001) by a genome wide search in Canadian families with inflammatory bowel disease. A significant LOD score (3.9) was found when the cohort was stratified by early onset disease. The locus identified spanned 18cM. Using 256 family trios, high density mapping revealed a common haplotype spanning 250kb containing a cytokine gene cluster. The causative gene was not identified.

Association with the 5q31 haplotype and Crohn’s disease has been replicated in Caucasian (Armuzzi, Ahmad et al. 2003; Mirza, Fisher et al. 2003; Negoro, McGovern et al. 2003; Noble, Nimmo et al. 2005; Török, Glas et al. 2005; Latiano, Palmieri et al. 2006), but not Japanese populations (Negoro, McGovern et al. 2003; Yamazaki, Takazoe et al. 2004; Tosa, Negoro et al. 2006). Carriage of 5q31 seems to be associated with early age of onset (Rioux, Daly et al. 2001; Mirza, Fisher et al. 2003; Török, Glas et al. 2005; Onnie, Fisher et al. 2008). There are a number of reports of association with perianal disease (Armuzzi, Ahmad et al. 2003; Vermeire, Pierik et al. 2005; Onnie, Fisher et al. 2008) and one study associated carriage of the 5q31 haplotype with a reduced need for surgery (Török, Glas et al. 2005).

Attempts have been made to identify the causative mutation within the haplotype. Mutations in the SLC22A4 gene and SLC22A5 promoter region have been suggested to be causative (Peltekova, Wintle et al. 2004). This gene encodes for an organic cation transporter. Association with SNPS within SLC22A4/5 have not been consistent with some studies showing no association with SNPs within SLC22A4/5 independent of the 5q31 haplotype (Noble, Nimmo et al. 2005; Vermeire, Pierik et al. 2005).
1.4 Smoking

Smoking was suggested to play a role in the aetiology of inflammatory bowel disease in 1982 when a series of papers noted that non-smoking was a feature of ulcerative colitis (Harries, Baird et al. 1982; Benoni and Nilsson 1984; Logan, Edmond et al. 1984). In 1984 (Somerville, Logan et al. 1984) a series of 252 patients with inflammatory bowel disease were studied. 52% of patients with Crohn’s disease were smokers compared with 8% of patients with ulcerative colitis. A meta-analysis in 1989 established the relationship between smoking and an increased likelihood of Crohn’s disease (Calkins 1989). There is thought to be up to a 4.1 matched relative risk of Crohn’s disease in smokers as compared with non-smokers (Tobin, Logan et al. 1987). There appears to be a dose response to number of cigarettes smoked and risk of developing Crohn’s disease with those smoking over 20 cigarettes a day at greatest risk, odds ratio 2.8 compared with 1.2 for smoking less than 10 per day (Lindberg, Tysk et al. 1988; Calkins 1989; Corrao, Tragnone et al. 1998). Smoking is associated with a worse clinical course and more frequent relapses, including re-operation (Cosnes, Carbonnel et al. 1999; Cosnes, Beaugerie et al. 2001; Ryan, Allan et al. 2004), although the mechanism through which smoking exerts this effect is unknown. Cessation of smoking results in improvement of relapse rate to that of non-smokers (Cosnes, Beaugerie et al. 2001), Figure 5.

Smoking has been associated with fistulising and stricturing Crohn’s disease (Louis, Michel et al. 2003; Picco and Bayless 2003) although this finding is not consistent (Smith, Arnott et al. 2004). Smoking has been found not to be associated with Crohn’s disease in Jewish patients in Israel (Reif, Lavy et al. 2000), however the effect of smoking on the clinical course of Crohn’s disease in this population is not known.

A higher concordance 72% vs. 34% for smoking has been found in siblings with Crohn’s disease than in healthy siblings of people with Crohn’s disease, suggesting that smoking interacts with genetic predisposition for Crohn’s disease (Brignola, Belloli et al. 2000). In siblings with both ulcerative colitis and Crohn’s disease the smokers tended to develop Crohn’s disease and the non-smokers ulcerative colitis (Bridger, Lee et al. 2002). This provides further evidence that a genetic predisposition may be influenced by environmental factors such as smoking.
Figure 1 From (Cosnes, Beaugerie et al. 2001); Kaplan-Meier estimates of the risk of flare-up in the 3 groups. The numbers above the curves indicate the numbers of patients at risk in each group at 18 and 36 months after inclusion, respectively. $P$ value refers to comparison between quitters and continuing smokers.

The interaction between smoking and \textit{CARD15 (NOD2)} genotype was investigated in a cohort of 275 patients with Crohn’s disease in a multicentre study in the USA (Brant, Picco et al. 2003). 43 patients classified as having inflammatory disease were excluded from this study as they had a disease duration of less than 8 years. Age at diagnosis was not found to be significantly associated with \textit{CARD15 (NOD2)} until adjustments for attained age at study entry were made. Ileal disease and non-inflammatory behaviour, i.e. stricturing and fistulising disease were found to be associated with two \textit{CARD15 (NOD2)} mutations. It was found by logistic regression with ileal disease as a constant that \textit{CARD15 (NOD2)} mutations increased the risk of stricturing or fistulising disease with an odds ratio of 1.88 for each mutant allele. Ileal disease was associated with shortest time to first surgical resection, as was smoking and age at diagnosis. Carriage of two mutant \textit{CARD15 (NOD2)} alleles was not associated with shorter time to first surgery. It is not stated in this study how many patients had surgery for ileal disease (217 had any ileal disease) and nor the number of these who carried one or more \textit{CARD15 (NOD2)} mutations. Stricturing and fistulising disease were considered together as complications of disease. Smoking was found to be a risk factor for ileal disease independent of \textit{CARD15 (NOD2)} genotype.
Several other risk factors in the development of Crohn’s disease have been studied including oral contraceptive use, appendectomy, antibiotic usage and various markers for childhood hygiene (Corrao, Tragnone et al. 1998; Sicilia, Miguel et al. 2001; Card, Logan et al. 2004)
1.5 Human antimicrobial mechanisms

The gastrointestinal tract contributes one of the largest proportions of the human immune system. Immune mechanisms can be divided crudely into the innate and adaptive immune systems. The innate immune system comprises mechanical, chemical and cellular mechanisms to maintain generic protection against pathogens prior to the generation of specific immune response. The adaptive immune system is mediated by both T and B cells. The immune response is directed to specific antigens with differentiated lymphocytes and antibody response. There is necessity for mechanism to differentiate self from commensal and pathogenic organisms.

There is a mechanical barrier to pathogens in the gastrointestinal tract in the form of the mucus layer and the epithelial cell layer with tight junctions between. So called pattern recognition receptors (PRR) within the epithelial cell barrier and lamina propria recognise pathogen associated molecular patterns (PAMP) present in the majority of microorganisms, but not in the mammalian host, with the resultant triggering of signalling pathways to eliminate microbial pathogens. The signalling cascade may result in the secretion of antimicrobial peptides from Paneth cells including lysozyme, sPLA2 and the human defensins as well as the process of autophagy. PAMP include lipopolysaccharide, present in the cell wall of gram negative bacteria, and muramyl dipeptide (MDP), the minimal bacterial cell wall motif. PRR include the toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD) proteins. TLR comprise a family of extracellular receptors for a number of unique microbial antigens. NOD proteins are intracellular. In the gastrointestinal tract NOD1 (CARD4) and CARD15 (NOD2) are receptors for bacterial peptidoglycan. CARD15 (NOD2) is expressed in Paneth cells and is a receptor for MDP. A further microbicidal method of killing is that employed by neutrophils by the generation of hydrogen peroxide.

Paneth cells can be found in small numbers in the normal right colon, but are only found in the transverse and left colon in inflammatory bowel disease. They can be found in the stomach only with intestinal metaplasia, not in the normal stomach (Day, Jass et al. 2003). Paneth cells do not proliferate (Troughton and Trier 1969) but are derived from undifferentiated stem cells of the crypt, they normally migrate downwards to the base of the crypt (Cheng 1974; Mathan, Hughes et al. 1987).
Human Paneth cells have secretory eosinophilic granules containing antimicrobial products, that include defensins (Jones and Bevins 1992; Jones and Bevins 1993).

Defensins were first identified from purified rabbit granulocytes (Selsted, Szklarek et al. 1984; Selsted, Brown et al. 1985). Similarly structured peptides were then identified from human neutrophils and named human defensins (Ganz, Selsted et al. 1985). Defensins are small molecules with a molecular weight of <3500 containing 29-35 amino acid residues. There is a highly conserved nucleotide region at the 5’ end of defensin genes (Jones and Bevins 1992; Jones and Bevins 1993). Defensins 1-3 extracted from human neutrophils, and have been found in vitro to be bactericidal against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*; fungicidal to *Cryptococcus neoformans*, and have antiviral activity against herpes simplex virus (Ganz, Selsted et al. 1985), by selectively disrupting membranes (Kagan, Ganz et al. 1994). In the Ganz et al study 1985, by immunogold transmission electron microscopy, the defensins 1-3 were shown to be localized to azurophil granules in human neutrophils. A 4th neutrophil defensin has been characterized (Wilde, Griffith et al. 1989).

1.5.1 **Intestinal defensins**

Defensins have been localised to epithelial cells in the mouse small intestine (Ouellette, Greco et al. 1989) and trachea (Diamond, Zasloff et al. 1991). Jones and Bevins in 1992 (Jones and Bevins 1992) used Southern blot techniques and genomic clone analysis to investigate whether there were more than four defensin like genes in the human genome. This is possible due to the presence of a highly conserved nucleotide sequence, between all species and tissues, in the defensin gene. Numerous areas were found within the human genome containing the conserved defensin sequence. A screen was then performed of tissue cDNA and genomic DNA using one of the new identified defensin gene probes using a PCR reaction and subsequent hybridization with a labelling probe. This showed that the identified gene, defensin 5, was found in intestinal tissue cDNA. The defensin gene was then sequenced fully. The cellular localization of the defensin 5 mRNA was then investigated by in situ hybridization. This revealed the defensin 5 mRNA to be localised to Paneth cells of the small intestine. A second Paneth cell defensin, defensin 6, was identified in 1991 (Jones and Bevins 1993). Screening of cDNA from human intestine has not revealed
any further defensins (Mallow, Harris et al. 1996), this is in direct contrast to other mammals, such as mice, where at least 16 defensins type peptides are found in the small intestine (Ouellette, Hsieh et al. 1994).

Expression of defensin 5 and 6 mRNA coincides temporally with Paneth cell detectability in the human fetal intestine (Mallow, Harris et al. 1996). Neonates have significantly less defensin activity than adults, premature neonates less than full term, possibly increasing vulnerability to gastrointestinal infections such as necrotising enterocolitis (Salzman, Polin et al. 1998). However in cases of necrotising enterocolitis levels of HD-5 and HD-6 expression per Paneth cell are increased to three times that in controls (Salzman, Polin et al. 1998), this may be a function of reduced Paneth cell number. Human defensin-5 is expressed at a ratio of approximately 4:1 to human defensin-6. HD-5 is expressed in normal Paneth cells of the terminal ileum but is also seen in metaplastic Paneth cells found in inflammatory bowel disease in the colon (Cunliffe, Rose et al. 2001).

The human defensin 5 and 6 genes are expressed in Paneth cells (Jones and Bevins 1992; Jones and Bevins 1993). Paneth cells store defensins as propeptides in large isoforms (Porter, Poles et al. 1998; Cunliffe, Rose et al. 2001). Defensins undergo posttranslational processing during or after secretion (Cunliffe, Rose et al. 2001) by matrilysin, a metalloproteinase, to the biologically active form (Wilson, Ouellette et al. 1999; Cunliffe, Rose et al. 2001). This was shown in an investigation of mice α-defensins in which it was found that matrilysin cleaved the pro segment from the precursor form in vitro. By subsequently generating matrilysin deficient mice, it was shown that intestinal peptide preparations had reduced antimicrobial activity. Bacterial preparations administered orally to the matrilysin deficient mice survived in greater numbers when compared to bacteria in wild-type mice. The matrilysin deficient mice succumbed more rapidly and to lower numbers of the bacterium Salmonella typhimurium. Subsequently it has been shown that HD-5 is processed by trypsin in Paneth cells (Ghosh, Porter et al. 2002). It is possible to isolate defensins from urine obtained from surgically constructed bladders made from ileum (Porter, Poles et al. 1998). The defensin peptides isolated from the urine were shorter that those isolated from tissue. Comparison of these isolates to recombinant defensins suggests that there is posttranslational processing of these propeptides to shorter microbicidal peptides (Porter, Poles et al. 1998). Recombinant HD-5 has been found to be active in vitro
against a variety of organisms including *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium* as well as against the yeast *Candida albicans* (Porter, van Dam et al. 1997). Trypsin fails to inactivate this peptide, suggesting that HD-5 would remain biologically active in the lumen of the small intestine.

Murine Paneth cells have been shown to secrete defensins in response to both whole bacteria and bacterial products (Ayabe, Satchell et al. 2000). Intact crypts were isolated from mouse small intestine. Cholinergic response of the crypt was maintained. The crypts were then exposed to various stimuli. It was found that Paneth cells degranulated and secreted their contents into the crypt lumen in response to whole bacteria *Salmonella typhimurium*, *Escherichia coli* and *Staphylococcus aureus*. The secreted products had high microbicidal activity. Secreted products from matrilysin deficient mice had significantly lower microbicidal activity than that of wild-type mice indicating that the majority of microbicidal activity was due to defensin activity. The antimicrobial activity was significantly attenuated by direct antibody to the murine defensins. A variety of bacterial antigens stimulated Paneth cell secretion including lipopolysaccharide, lipid A, lipoteichoic acid, liposomes of phospholipid and muramylidipeptide. Fungal pathogens *Candida albicans* and *Cryptococcus neoformans* did not induce Paneth cell secretion of antimicrobial products. This study indicates that Paneth cell are able to secrete antimicrobial products with sufficient microbicidal activity to maintain crypts of Lieberkühn sterile. Paneth cells degranulation is modulated by a calcium-gated potassium channel that controls uptake of extracellular calcium (Ayabe, Wulff et al. 2002). Inhibitors of this channel were found to block Paneth cell degranulation.

Other defensins are found in human intestinal epithelial cells. It has been predicted by analysis of the human genome that there would be 28 β-defensins (Schutte, Mitros et al. 2002) of which 8 have been identified. Human β-defensins are expressed in epithelial cells (Lehrer and Ganz 2002). Human β defensin-1 is expressed constitutively in human colonic cells whereas human β-defensin 2 expression is upregulated by pro-inflammatory cytokines in colonic cell lines (Wekkamp, Fellermann et al. 2002). Human β-defensin was shown to be expressed in a greater proportion of biopsies from patients with inflammatory bowel disease and particularly biopsies from ulcerative colitis, than in biopsies from controls. Expression of human β-defensin 3 was found to be increased in biopsies from patients with ulcerative colitis.
Expression of human β-defensin 2 and 3 were found not to be increased in this study in biopsies from patients with Crohn’s disease suggesting a possible defect in inducible defensin expression. The expression of human β-defensin 3 and 4 in human small and large intestinal epithelial cells has been investigated (Fahlgren, Hammarstöm et al. 2004). Human β-defensin 3 is expressed at higher levels than human β-defensin 4. Expression was higher towards the base of crypts of Lieberkühn. In agreement with the Wehkamp 2003 study (Wehkamp, Harder et al. 2003), expression of human β-defensins 3 and 4 were increased in colonic epithelial cells from biopsies from patients with ulcerative colitis, with no increase in expression in biopsies from patients with Crohn’s disease compared to controls. The expression in small intestinal epithelial cells was not investigated.

It is thought that defensins exert their antimicrobial action by disruption of structural elements in the target cell membrane (Selsted and Ouellette 2005). This is evidenced by the cationic nature of the peptides that bind electrostatically to negatively charged constituents of the microbe surface membrane and induce an increase in permeability that correlates with antimicrobial activity. Interaction of defensins with antimicrobial membranes has been demonstrated with model membranes with human β-defensins and some animal defensins, but not with human α-defensin 5 and 6.

1.5.2 Tumour necrosis factor alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine implicated in the pathogenesis of Crohn’s disease. It has been suggested that TNF-α increases uptake of antigen in people with Crohn’s disease (Söderholm, Streutker et al. 2004). This was demonstrated using horseradish peroxidise as the antigen in a chamber experiment on resected human ileal tissue. When normal tissue was exposed to TNF-α uptake of horseradish peroxidise was increased. This could therefore be a mechanism by which inflammation in Crohn’s disease is potentiated. In a study of a cohort of children with Crohn’s disease, ulcerative colitis and normal controls, increased TNF-α was found in all intestinal biopsies of children with Crohn’s disease compared with those with normal intestine and a proportion of those with ulcerative colitis (MacDonald, Hutchings et al. 1990). Using antibody to TNF-α, TNF-α was found throughout the lamina propria and submucosa of the intestine and appeared to be localised in macrophages (Murch, Braegger et al. 1993). The number of cells secreting TNF-α has been found to be
increased (Breese, Michie et al. 1994) in Crohn’s disease. Increased levels of TNF-α were associated with mild inflammation, with significantly increased TNF-α found in biopsies from patients with Crohn’s disease compared with patients with ulcerative colitis and normal controls (Breese, Michie et al. 1994). Investigation of TNF-α expression using isotopic in situ hybridization revealed TNF-α mRNA in Paneth cells of mice (Keshav, Lawson et al. 1990) and human (Tan, Hsueh et al. 1993). TNF-α mRNA can be demonstrated in Paneth cells of normal small bowel biopsies (Tan, Hsueh et al. 1993), levels are then elevated in bowel from neonates with necrotising enterocolitis. In these cases, TNF-α expression can be demonstrated in eosinophils and macrophages as well as in Paneth cells.

Mouse-human chimeric antibody is an effective treatment in a proportion of people with Crohn’s disease (Hanauer, Feagan et al. 2002). Anti-TNF-α antibody treatment is costly and not without significant side-effects. A number of people with Crohn’s disease are non-responders to treatment. There are no known factors that predict response to treatment.

1.5.3 CARD15 (NOD2) and defensins

Carriage of functional mutations in the CARD15 (NOD2) gene, located on chromosome 16, have been well established to be associated with an increase susceptibility to Crohn’s disease (Hugot, Chamaillard et al. 2001; Ogura, Bonen et al. 2001), and particularly ileal Crohn’s disease (Ahmad, Armuzzi et al. 2002; Cuthbert, Fisher et al. 2002). Using monoclonal antibody to CARD15 (NOD2) it has been demonstrated that Paneth cells of the terminal ileum in patients with Crohn’s disease, ulcerative colitis and normal controls express CARD15 (NOD2) (Ogura, Lala et al. 2003). In this study CARD15 (NOD2) was also found in metaplastic Paneth cells of the colon in patients with inflammatory bowel disease. On microscopy, CARD15 (NOD2) was located in the cytosol in close proximity to the granules of Paneth cells. Minimal CARD15 (NOD2) was located in the villous epithelium. By further investigation using in situ hybridization CARD15 (NOD2) mRNA was found in Paneth cells of normal controls and in patients with Crohn’s disease (Lala, Ogura et al. 2003). CARD15 (NOD2) mRNA was localised in the same investigation to monocytes, but not macrophages or in granulomas. CARD15 (NOD2) is found in the cytosol of Paneth cells not in the secretory granules (Ogura, Bonen et al. 2001; Lala, Ogura et al. 2003).
An investigation using paediatric colonic tissue and CARD15 (NOD2) antibody however demonstrated expression in macrophages and epithelioid giant cells within granulomas (Berrebi, Maudinas et al. 2003). This group found CARD15 (NOD2) in epithelial cells in severely inflamed Crohn’s disease resection sections. On close inspection the cells could be said to look like Paneth cells.

In an investigation using biopsies from human ileum it was shown that biopsies taken from inflamed areas of ileum in patients with Crohn’s disease had less α-defensins than in biopsies from normal and unaffected ileum by real-time PCR (Wehkamp, Harder et al. 2004). It has recently been suggested that the expression of human defensins 5 and 6 (Wehkamp, Salzman et al. 2005) are reduced in ileal Crohn’s disease and particularly in those with CARD15 (NOD2) mutations (Wehkamp, Harder et al. 2004). However a more recently published investigation has not replicated these findings and suggests that the reduced α-defensins found in Crohn’s disease is an artefact of inflammation rather than a primary process (Simms, Doecke et al. 2008).
1.6 Histological features of Crohn’s disease

The histological features of Crohn’s disease are varied. The characteristic features of Crohn’s disease are considered to be skip lesions and focal ulceration with transmural inflammation, lymphoid aggregates and granulomas (Day, Jass et al. 2003). Pyloric metaplasia is a well recognised feature of Crohn’s disease (Koukoulis, Ke et al. 2002; Day, Jass et al. 2003).

Paneth cells were first described as granular cells found at the base of crypts of Lieberkühn in the epithelial cell layer of the small intestine by Joseph Paneth in 1888 (Paneth 1888) Figure 2. They are thought to develop from stem cells mid-way up the crypt and migrate to the crypt base (Troughton and Trier 1969). Paneth cells express genes for a number of antibacterial products, including lysozyme (Chung, Keshav et al. 1988), secretory phospholipase A₂ (Keshav, McKnight et al. 1997), and the α-defensins human defensin 5 (Jones and Bevins 1992) and human defensin 6 (Jones and Bevins 1993). They have been found to secrete α-defensins in response to bacteria in crypts (Ayabe, Ashida et al. 2004). CARD15 (NOD2) has been found to be expressed in Paneth cells (Lala, Ogura et al. 2003; Ogura, Lala et al. 2003). The product of CARD15 (NOD2) is involved in bacterial sensing and NF-κB response (Bonen, Ogura et al. 2003; Kobayashi, Chamaillard et al. 2005; Maeda, Hsu et al. 2005). Results from real-time polymerase chain reaction (PCR) experiments have suggested that α-defensin secretion alters with CARD15 (NOD2) genotypes (Wehkamp, Harder et al. 2004). Normal small intestine crypts contain three to five Paneth cells (Rubio and Nesi 2003). The number and distribution of Paneth cells has been found to be increased in murine small intestine with infection by Trichinella spiralis (Kamal, Wakelin et al. 2001). The distribution has been suggested to be abnormal in CARD15 (NOD2) homozygotes (Abeya, Bromfield et al. 2005). In humans Paneth cell numbers have been shown to be increased in patients with Crohn’s disease (Kelly, Feakins et al. 2004) and further increased in patients with two CARD15 (NOD2) mutations (Abeya, Bromfield et al. 2005).

Paneth cell deficient mice can be generated by the introduction of cell-type specific genes that code for cytotoxic gene products (Garabedian, Roberts et al. 1997). When the cell lineage of interest differentiates it is therefore ablated by the cytotoxic gene product. In the mouse there seems to be little effect, however, there have been a
number of case reports of patients with absent goblet cells, enteroendocrine and Paneth cells. Interestingly, the description in humans is of severe diarrhoea with enterocolitis (Moore, Xu et al. 1995; Shaoul, Hong et al. 2005).

Pyloric metaplasia also known as glands of the “ulcer associated cell lineage” (UACL) can be identified without difficulty on haematoxylin and eosin stained sections, Figure 3. Pyloric metaplasia is a recognised histopathological feature of Crohn’s disease first described by A. F Liber in 1942 (Liber 1951). Pyloric metaplasia is not specific to Crohn’s disease but occurs in around 78% of patients with Crohn’s disease (Lee 1964). It is found much more commonly and extensively in Crohn’s disease than other diseases. Little is known about the factors that affect the presence and amount of pyloric metaplasia in different patients. UACL glands have been found to be associated with increased submucosal neomuscularisation (Borley, Mortensen et al. 2001). Pyloric metaplasia is thought to be part of the regeneration process in the mucosa, however, in a study of cell proliferation the glandular cells were negative for the cell proliferation marker Ki-67 (Noffsinger, Unger et al. 1998). In this 1998 study the glandular buds appeared to arise from the overlying crypts. The cells express a number of growth factors including epidermal growth factor (Wright, Poulsom et al. 1990) and trefoil peptides (Longmann, Douthwaite et al. 2000). It has been suggested that the presence of pyloric metaplasia in terminal ileal biopsies is associated with the diagnosis of Crohn’s disease (Koukoulis, Ke et al. 2002).

Figure 2 (a) Schematic of Paneth cells, shaded, situated at the base of crypts of Lieberkühn. B Haematoxylin and eosin staining of human small intestine highlighting the eosinophillic granules within Paneth cells (arrow).
Lymphoid aggregates are commonly found throughout the bowel wall in Crohn’s disease. They form the characteristic “Crohn’s Rosary” when distributed along the outside of the smooth muscle coat of the bowel wall (Morson Colour Atlas of Gastrointestinal Pathology). They can be defined as “pathological lymphoid cell clusters visible as distinct aggregates” (Borley, Mortensen et al. 2000). The number of lymphoid aggregates has been found to correlate with the degree of fat wrapping found macroscopically in Crohn’s disease. Fat wrapping correlates directly with stricture formation. CARD15 (NOD2) genotype is associated with stricturing disease (Abreu, Taylor et al. 2002; Hampe, Grebe et al. 2002; Lesage, Zouali et al. 2002; Brant, Picco et al. 2003). As CARD15 (NOD2) mutations result in defective bacterial sensing it was postulated that the number of lymphoid aggregates would be different between genotypes.

Figure 3 Pyloric metaplasia on Haematoxylin and eosin staining of human small intestine (arrows).

Granulomas are considered pathognomonic of Crohn, Figure 4. They are found in 30 to 70% (Shaoul, Karban et al. 2004), (Chambers and Morson 1979; Anseline, Wlodarczyk et al. 1997) of patients with Crohn’s disease, most commonly in the large bowel and rectum (Chambers and Morson 1979) and are more common in children than adults (Schmitz-Moormann and Schäg 1990). There is conflicting evidence of an
association with the CARD15 (NOD2) genotype. No association was found by Pierik et al in 161 surgical Crohn specimens (Pierik, de Hertogh et al. 2005) or by Shaoul et al in 230 specimens (Shaoul, Karban et al. 2004), although this was a mix of biopsy and surgical tissue. Heresbach et al did however find a significant correlation between CARD15 (NOD2) genotype, particularly carriage of the R702W allele, and presence of granulomas (Heresbach, Alexandre et al. 2005) as did Lesage and Onnie (Lesage 2002, Onnie, Fisher et al. 2008). CARD15 (NOD2) has been found to be expressed in monocytes at the periphery of granulomas (Lala, Ogura et al. 2003) and found to be diffusely expressed in granulomas by another group (Berrebi, Maudinas et al. 2003). There is a variable association between granulomas and surgical recurrence (Wolfson, Sachar et al. 1982; Anseline, Wlodarczyk et al. 1997).

Figure 4 Haematoxylin and eosin stained section of human small bowel showing (a) giant cell (x20) and (b) epitheloid granuloma (x5) (arrows)
1.7 Complications

1.7.1 Short bowel syndrome

Short bowel syndrome carries a significant morbidity and mortality (Schalamon, Mayr et al. 2003). There is no accurate data on the incidence of short bowel syndrome in Crohn’s disease. The prevalence of short bowel syndrome overall has been estimated to be 2-5 per million (Koffeman, van Gemert et al. 2003’). Crohn’s disease is a common cause of short bowel syndrome due to multiple surgeries or development of fistula. Up to 60% of some cohorts with short bowel have been attributed to Crohn’s disease (Nightingale, Lennard-Jones et al. 1992).

1.7.2 Osteoporosis

Osteoporosis is an important cause of morbidity in patients with Crohn’s disease with a fracture prevalence of up to 22% in those who are osteoporotic (Klaus, Armbrecht et al. 2002) and an overall increase in low energy fracture rate compared to the general population (Vestergaard, Krogh et al. 2000). As the majority of osteoporotic fractures are asymptomatic at the time of fracture (Klaus, Armbrecht et al. 2002), low bone mineral density (BMD) provides a useful marker for risk of later symptomatic fracture (van Staa, Cooper et al. 2003). Osteoporosis is more common in people with Crohn’s disease than those with ulcerative colitis (Marshall, Johnell et al. 1996).

The pathogenesis of the reduced BMD found in people with Crohn’s disease is multifactorial. Various risk factors have been identified for reduced BMD including low body mass index (Jahnsen, Falch et al. 2004; Siffledeeen, Fedorak et al. 2004; Taylor, Schreiner et al. 2004), disease duration (Schulte, Dignass et al. 1998), age (Robinson, al-Azzawi et al. 1998) and smoking (Pollak, Karmeli et al. 1998). In addition several genes have been implicated in the pathogenesis of osteoporosis in other at risk populations. Twin studies indicate that 60-80% of BMD may be genetically determined (Andreassen, Hylander et al. 1999).

Finding a genotype that is a marker for those at risk of lower BMD could enable targeted therapy before osteoporosis develops. Twin studies have linked polymorphisms of the vitamin D receptor (VDR) gene and BMD in healthy women (Silvennoinen, Lehtola et al. 1996), although these findings have not always been reproducible (Ralston 2003). A meta-analysis of 16 studies suggest a weak effect for
VDR polymorphisms (Spector, Keen et al. 1995). The vitamin D receptor is an important regulator of calcium metabolism and bone cell function. In addition VDR polymorphisms have been found to influence calcium absorption from the intestine (Hustmyer, Peacock et al. 1994). VDR polymorphisms have also been implicated in susceptibility to Crohn’s disease (Cooper and Umbach 1996).

The pro-inflammatory cytokine interleukin-6 (IL6) has also been implicated in the pathogenesis of osteoporosis. IL6 influences osteoclast function and stimulates bone resorption (Dawson-Hughes, Harris et al. 1995). Studies have found a link between polymorphisms in the IL6 gene and BMD in otherwise healthy women (Simmons, Mullighan et al. 2000) and in people with inflammatory bowel disease (Manolagas 1995).

Collagen type 1α1 gene (COL1A1) mutations are responsible for the condition osteogenesis imperfecta (Murray, McGuigan et al. 1997). Less dramatically, polymorphisms in this gene have been found to influence BMD in previous studies in the healthy population (Willing, Pruchno et al. 1992; Schulte, Dignass et al. 2000). Polymorphisms in the COL1A1 gene have been found to alter bone strength by altering binding affinity for the transcription factor Sp1 (Uitterlinden A G, Burger H et al. 1998). To date there are no published studies of the effect of COL1A1 on BMD in inflammatory bowel disease.

Polymorphisms in the CARD15 (NOD2) gene on chromosome 16 have been identified as susceptibility markers for Crohn’s disease (Hugot, Chamaillard et al. 2001) and are known to be associated with ileal disease rather than purely colonic disease (Cuthbert, Fisher et al. 2002).
1.8 Surgery and recurrence after surgery

Crohn’s disease is characterised by its relapsing-remitting disease course leading to intermittent abdominal pain, diarrhoea and malabsorption. Relapses are unpredictable with variable frequency between patients. There are few reliable predictors of relapse but smoking habit is well recognised as worsening disease the disease course and increasing the frequency of relapses (Cosnes, Carbonnel et al. 1999; Cosnes, Beaugerie et al. 2001; Picco and Bayless 2003; Ryan, Allan et al. 2004), see 1.4. Requirement for surgery is common with over 50% of patients with Crohn’s disease undergoing surgery at some point in their disease course (Forbes 1997). People with terminal ileal Crohn’s disease more often require surgery than those with disease in other locations (Bernell, Lapidus et al. 2000; Louis, Michel et al. 2003; Smith, Arnott et al. 2004), with a relative risk for surgery of 3.2 compared to patients with purely colonic Crohn’s disease (Bernell, Lapidus et al. 2000). Requirement for multiple surgeries has implications in terms of cost, morbidity and mortality and the development of short bowel syndrome with the potential to require long term parenteral nutrition. The risk of reoperation following ileocaecal resection has been found to be up to 89% after 15 years, with a clinical recurrence rate of 94% (Greenstein, Sachar et al. 1975). In a cohort of 1424 patients with Crohn’s disease in Sweden, 74% had had at least one surgical operation (Bernell, Lapidus et al. 2000). In ileocaecal Crohn’s disease 83% of patients have had surgery 10 years from diagnosis (Bernell, Lapidus et al. 2000). The cumulative reoperation rate is high with up to 89% reoperative rates in some studies (Greenstein, Sachar et al. 1975). Surgery has implications for patient and health service in terms of associated morbidity and health related costs.

Smoking increases recurrence rates (Cosnes, Carbonnel et al. 1999) and smokers are more likely to have reoperations than non-smokers (Ryan, Allan et al. 2004). It would be important to be able to predict those people in whom re-operation is likely in order to target interventions and more aggressive medical therapies to those at greatest risk.

In a study of age at operation (De Dombal, Burton et al. 1971) 168 patients with 30 year follow-up, 79 with small bowel disease were investigated. There was a very high perioperative mortality, with 9 of 182 dying in the immediate post-operative period. This study found a ~34% crude reoperation rate, however duration of disease and follow-up time introduces bias, with younger patients there is inevitably a longer
follow-up and therefore more chance of further operation. The conclusion was that small bowel disease is more likely to recur post surgery. No difference was found between urgent or emergency operation vs. elective surgery. It was found that the extent of disease at operation and location of disease was predictive of recurrence. Small bowel disease was more likely to recur with 60% requiring re-operation. A biphasic pattern of recurrence was described with peaks at 1-2 years or 5-15 years.

Younger age at diagnosis has been found to be a risk factor for surgery (Bernell, Lapidus et al. 2000). Perianal disease and long segment resection in this study of 907 patients were risk factors for recurrence. Surgery for ‘palpable mass or abscess’ had a lower risk for recurrence. There was no effect seen for gender, age at diagnosis or presence of granulomas in this study. Clinical recurrence rates of 94% are seen after surgery for Crohn’s disease (Greenstein, Sachar et al. 1975). Endoscopic recurrence is seen in 73% of patients after only 1 year and 85% of patients at 3 years (Rutgeerts, Geboes et al. 1990).

Factors other than smoking have been suggested to influence the rate of relapse in patients with Crohn’s disease. In a study of 541 people with Crohn’s disease from one referral centre in the Netherlands it was found that female patients have second surgery for Crohn’s disease within a shorter time than male patients (Wagtmans, Verspaget et al. 2001). Ileocaecal resections were also performed more frequently in female patients with Crohn’s disease. Women have been found to have a slightly increased risk of recurrence post resection than men (Bernell, Lapidus et al. 2000). The more surgery a patient has, the more likely they are to relapse (Rutgeerts, Geboes et al. 1990). This may well be an indication of more severe and aggressive disease.

In a recent cohort study of 207 patients with ileocaecal resections for Crohn’s disease in Rome, early surgery was found to be associated with shorter time to clinical recurrence, as defined by symptomatic plus endoscopic or radiological recurrence (Aratari, Papi et al. 2007). However, early surgery, that is surgery at the time of diagnosis, was not found to be associated with longer surgical remission. No difference has been previously reported for early age at diagnosis and risk of recurrence (Bernell, Lapidus et al. 2000).

CARD15 (NOD2) has been shown to be associated with terminal ileal Crohn’s disease (Ahmad, Armuzzi et al. 2002), stricturing (Abreu, Taylor et al. 2002) and fistulising
disease (Brant, Picco et al. 2003). CARD15 (NOD2) is associated with terminal ileal Crohn’s disease (Cuthbert, Fisher et al. 2002). A number of studies have shown that carriage of one or more mutations in the CARD15 (NOD2) gene is associated with more severe disease i.e. more frequent recurrence and fistulising and stenotic disease (Abreu, Taylor et al. 2002; Hampe, Grebe et al. 2002; Radlmayr, Török et al. 2002; Brant, Picco et al. 2003; Helio, Halme et al. 2003; Büning, Genschel et al. 2004; Alvarez-Lobos, Arostegui et al. 2005). It is therefore hypothesized that carriage of one or mutations in the CARD15 (NOD2) gene will be associated with more frequent re-operations and less time to re-operation than those people with wild-type CARD15 (NOD2). See section 1.3.5. Mutations within the 5q31 haplotype; SLC22A4 and SLC22A5 have been associated with reduced need for surgery (Török, Glas et al. 2005).

A number of people present acutely with severe disease. These people often have surgery within one year of diagnosis. It would be useful to know whether this cohort of people with Crohn’s disease are likely to require multiple surgeries and therefore may benefit from early immunosuppressive therapy.

Although not an intervention study, a proportion of people have received immunosuppressive therapy with thiopurines such as azathioprine and 6-mercaptopurine. The association between use of these drugs and reoperative rate was investigated.

Diversion of the faecal stream post terminal ileal resection prevents recurrent disease (Rutgeerts, Peeters et al. 1991) suggesting that microbial and environmental factors play a large role in the development of recurrent disease. Infusion of ileal fluid has been found to precipitate an inflammatory response in people with Crohn’s disease (D’Haens, Geobes et al. 1998). The diversity of intestinal microflora (Eckburg, Bik et al. 2005) has been implicated. Recurrence has been prevented with nutritional therapy (Esaki, Matsumoto et al. 2005), however the long term use of enteral nutritional therapy is limited by understandably limited patient compliance.

It has been suggested that Crohn’s disease recurs at the anastomosis due to residual disease at the resection margin, however, a number of studies have confirmed that residual disease after resection does not influence recurrence (Rutgeerts, Geboes et al. 1984; Kotanagi, Kramer et al. 1994). It has been suggested that the type of surgery,
e.g. stricturoplasty vs. resection influences the time to relapse. No difference has been found between the type of surgery and recurrence of Crohn’s disease (Poggioli, Pierangeli et al. 2002). In a retrospective study of 100 patients with first resection for Crohn’s disease, increased anastomotic recurrence rate was investigated in relation to evidence of microscopic disease at resection margins (Kotanagi, Kramer et al. 1994). No association between disease at the resection margin and anastomotic recurrence was found.

### 1.8.1 Definitions of recurrence

The definition of relapse post resection in Crohn’s disease is variable. Relapse can be defined clinically, endoscopically, radiologically, surgically and histologically. Clinical relapse can be defined as the return of obstructive symptoms of: nausea, vomiting, colicky abdominal pain, abdominal distension and constipation. Up to 70% of patients with have endoscopic recurrence within 6 months to 1 year of resection (Rutgeerts, Geboes et al. 1990; Bourreille, Jarry et al. 2006). However 80% may remain asymptomatic despite evidence of endoscopic recurrence (Rutgeerts, Geboes et al. 1990). Not many centres routinely endoscope patients who are asymptomatic after resection.

The availability of endoscopic investigation has increased greatly in the last 20 years. A 72% endoscopic recurrence at 1 year has been found post surgery (Rutgeerts, Geboes et al. 1984). In a more recent study of endoscopic recurrence, 73% had endoscopic recurrence at 3 months, 93% at 1 year and 100% at 3 years, whereas symptomatic recurrence was 33% at 3 months, 37% at 1 year and 86% at 3 years (Olaison, Smedh et al. 1992) raising the question of the relevance of endoscopic recurrence.

The definition of recurrence varies between studies. It may be based on symptoms and clinical findings, radiological investigation and endoscopic findings or a combination. Clinical practice and referral rates for surgery vary between clinicians. This study inevitably includes patients from different clinicians. This could be considered a confounding factor. However, in a longitudinal review of the literature over the last 40 years, disease outcome and recurrence rates have not changed (Wolters, Russel et al. 2004).
In a study of a cohort of 907 patients with ileocaecal Crohn’s disease in Sweden it was found that the overall resection rate was 83% at 10 years with a recurrence rate of 36% at 10 years after first surgery (Bernell, Lapidus et al. 2000). Recurrence was defined as symptoms consistent with “and later verified as, recurrent Crohn’s disease”. It is not clear what methods were used to verify recurrent disease. 43% were said to have a symptomatic recurrence at a median of 6.8 years. A history of perianal disease, and a long segment resection (>50cm) increased the risk of relapse. There was no association with gender, age at diagnosis, duration of disease, type of resection or presence of granuloma with increased recurrence.

Radiological recurrence can be considered when there is evidence of anastomotic stricture on barium studies or anastomotic inflammation on white cell scan. However radiological investigations involve a not insignificant radiation dose therefore they tend to be done only after clinical evidence of recurrent disease and therefore will provide a bias towards longer time to relapse.

Rates of surgery over time have not changed with the increasing use of immunosuppressants (Cosnes, Nion-Larmurier et al. 2005), reiterating the need for parameters that may predict recurrence.

1.8.2 Disease definitions

It appears that misdiagnosis is common in studies of Crohn’s disease. By its nature it can be difficult to diagnose clinically. There is therefore the potential for errors (Silverberg, Daly et al. 2001) and misdiagnosis with the inclusion of people without proven Crohn’s disease. In order to minimize the chances of these errors histopathological confirmation of diagnosis on surgical resection specimens was required in this investigation for confirmation of a diagnosis of Crohn’s disease. This therefore provides a robust definition of the recurrence of Crohn’s disease. The symptoms of clinical recurrence are non-specific and not always due to active Crohn’s disease, for example, surgical adhesions may cause obstruction. To be consistent in the definition of relapse and to have a reproducible definition of relapse, second ileal resection was taken as the definition of relapse in this investigation. It has been said that the most relevant clinical outcome after intestinal resection for Crohn’s disease is the requirement for second surgery (Penner, Madsen et al. 2005). Many factors have been postulated to effect recurrence of Crohn’s disease after surgery.
In 2000 there was a large retrospective cohort study of 1424 patients with at least one intestinal resection in Stockholm county, Sweden, (Bernell, Lapidus et al. 2000). Recurrence was taken to be the first presentation with symptoms after surgery that was verified by a diagnostic procedure, or required treatment. All surgery for Crohn’s disease was investigated, rather than dividing by location. Female sex, ileocolonic disease and perianal fistulae were found to be significant predictors of further surgery. In a further study by the same group of 907 with primary ileocaecal Crohn’s disease, taken from the same cohort of patients with Crohn’s disease, only presence of perianal disease and a long initial segment of resection were found to be predictors of relapse (Bernell, Lapidus et al. 2000).
2 Chapter 2 Terminal ileal resections and Crohn’s disease

2.1 Introduction and aims

The aim of this study was to investigate survival to second operation in terminal ileal Crohn’s disease. The effect of environmental factors including smoking and exposure to thiopurines was investigated. In particular the effect of CARD15(NOD2) genotype and carriage of the 5q31 haplotype was studied.
2.2 Patients and methods

Patients were identified from clinical coding databases using the codes for ilectomy, right hemicolecetomy and Crohn’s disease in order to identify those who had undergone small bowel resection for Crohn’s disease. Patients were also identified during routine clinic visits. Consent for a venous blood sample for DNA extraction was obtained. Patients were also asked for consent to return to small bowel specimens held in the hospital archives. A smoking and family history were taken. Follow-up was defined as date of last clinic attendance. Local region ethics committee (LREC) approval was sought and granted in 2002. Consent forms and information sheets can be found in the appendix.

Data was collected from the hospital notes onto standardised forms. 10ml venous blood was obtained from each patient into commercial plastic tubes containing EDTA using standard precautions. Blood samples were then labelled with a unique code and frozen at –20°C until DNA extraction. Blood samples were kept frozen while transported to the Genetics Department, Guys, Kings and St. Thomas’ School of Medicine, London.

2.2.1 DNA Extraction

10ml of blood was defrosted, rolled constantly and placed on ice. Blood was then decanted into a clearly labelled 50ml falcon tube and made up to 45ml with lysis buffer. The falcon was inverted to mix for 1 minute. The lysed blood was then centrifuged using a desk-top centrifuge for 20 minutes at 3000rpm at 4°C. The supernatant was then poured off. The excess was then removed using a plastic Pasteur pipette ensuring the visible white cell pellet was not disturbed. 4.5ml 1X SET was then added and the pellet broken up pipetting up and down. 250µl 10% SDS and 100µl proteinase K were then added, briefly mixed and incubated at 37°C overnight.

Once the suspension had cleared, if it was not clear after an overnight incubation, a further 100µl proteinase K was added and incubated for 2-3 hours at 37°C, made up to 7.5ml with 1X SET. 2.5ml prewarmed saturated salt solution was then added. An equal volume of 1:24 isoamylalcohol:chloroform solution was added and mixed by inversion for 1 minute. This was then centrifuged for 20 minutes at 3000rpm at 4°C. The clear supernatant was then transferred using a Pasteur pipette to a clean clearly
labelled falcon taking care not to transfer any of the interface. Two volumes of ice cold absolute ethanol was then added to precipitate the DNA. For each sample two 1.5ml eppendorfs were prepared; one with 1ml 70% ethanol, the second with 50-400µl (depending on the quantity of DNA precipitated) TE buffer. The DNA precipitate was removed using an inoculating loop and washed in 70% ethanol, air dried then dissolved in TE buffer. The DNA was then incubated at 60°C for 1 hour to ensure homogeneity and then stored at 4°C.

This was performed either in Newcastle or on arrival at Prof C G Mathew’s laboratory, Guy’s King’s and St. Thomas’ School of Medicine, London by myself or by a member of his team.

2.2.2 Genotyping

Samples were genotyped for the 3 common CARD15 (NOD2) SNPs and the IGR2063b_1 SNP of the 5q31 haplotype in Prof. C G Mathew laboratory, Guy’s, King’s and St. Thomas’ School of Medicine, London using published techniques (Cuthbert, Fisher et al. 2002).

2.2.3 Analysis

Results were analysed using SPSS software for Windows, version 12. Chi squared, Kaplan-Meier survival analysis and one-way ANOVA were used. Survival was measured in months to second operation. Data was censored when follow up ceased: most recent clinic appointment/patient death before the second operation occurred.
2.3 Results

2.3.1 Demographic details, Table 6

346 patients with terminal ileal Crohn’s disease and ileal resection for Crohn’s disease were included. Mean number of surgeries per patient was 1.8, median 1. 94 people had two operations, 64 people had three operations, 11 people had four resections, 5 patients had five resections and one person had seven resections.

2.3.2 Genotyping results; Table 7, Table 8

282 patients with terminal ileal Crohn’s disease had complete genotyping for CARD15 (NOD2). 250 wild type, 12 heterozygote for G908R genotype, 0 homozygote for G908R genotype, 1 heterozygote G908R/L1007fs, 3 heterozygote G908R/R702W, 19 heterozygote L1007fs, 4 homozygote L1007fs, 4 heterozygote L1007fs/R702W, 43 heterozygote R702W and 4 homozygote R702W.
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Table 7  
CARD15 (NOD2) genotyping results; wild type =198, heterozygotes =72, homozygotes/compound heterozygotes (2 mutations) =16.

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Table 8 5q31 haplotype carriage results

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<td>Heterozygote</td>
<td>80</td>
</tr>
<tr>
<td>Homozygote</td>
<td>39</td>
</tr>
</tbody>
</table>
2.3.3 **Cohort survival; Table 9, Figure 5**

For the entire cohort the mean time to second operation was 261 months (95% confidence interval 241-280 months). Median time to second operation was 236 months (95% CI 220 to 251 months), Figure 5.

![Time to second operation for cohort](image)

Figure 5 Kaplan Meier survival curve for cohort, n=347. Time in months to second operation. Crosses represent censored data. Dotted line represents 50% cumulative survival. Mean survival 225 months. Median survival 135 months.

2.3.4 **Gender and time to second small bowel resection**

There was no difference seen in time to second small bowel resection between male and female patients.
Table 9 Summary of Kaplan Meier survival times. Follow up months from first operation, defining event; second operation. Significant difference *p=0.002, §p=0.001, ¦p=0.001, ∆p=0.000

<table>
<thead>
<tr>
<th>Factor</th>
<th>n=</th>
<th>Mean Estimate (months)</th>
<th>95% Confidence interval</th>
<th>Median</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>222</td>
<td>220</td>
<td>177</td>
<td>265</td>
<td>123</td>
</tr>
<tr>
<td>Male</td>
<td>124</td>
<td>200</td>
<td>165</td>
<td>235</td>
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<td>Never</td>
<td>127</td>
<td>279*</td>
<td>208</td>
<td>350</td>
<td>203*</td>
</tr>
<tr>
<td>Current</td>
<td>125</td>
<td>148*</td>
<td>116</td>
<td>179</td>
<td>113*</td>
</tr>
<tr>
<td>Ex</td>
<td>88</td>
<td>193*</td>
<td>152</td>
<td>233</td>
<td>140*</td>
</tr>
<tr>
<td>Current Smoking</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>215</td>
<td>261§</td>
<td>211</td>
<td>311</td>
<td>173§</td>
</tr>
<tr>
<td>Yes</td>
<td>125</td>
<td>148§</td>
<td>116</td>
<td>179</td>
<td>113§</td>
</tr>
<tr>
<td>Age</td>
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</tr>
<tr>
<td>A1 (&lt;40y)</td>
<td>271</td>
<td>229</td>
<td>191</td>
<td>268</td>
<td>135</td>
</tr>
<tr>
<td>A2 (&gt;40y)</td>
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<td>198</td>
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</tr>
<tr>
<td>Disease behaviour</td>
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<td>259</td>
<td>353</td>
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</tr>
<tr>
<td>Stenotic</td>
<td>206</td>
<td>244¦</td>
<td>198</td>
<td>290</td>
<td>139¦</td>
</tr>
<tr>
<td>Fistulising</td>
<td>116</td>
<td>159¦</td>
<td>128</td>
<td>190</td>
<td>112¦</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At diagnosis</td>
<td>88</td>
<td>231</td>
<td>172</td>
<td>290</td>
<td>139</td>
</tr>
<tr>
<td>Post diagnosis</td>
<td>258</td>
<td>195</td>
<td>168</td>
<td>222</td>
<td>133</td>
</tr>
<tr>
<td>Presentation to surgery</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute &lt;12m</td>
<td>177</td>
<td>216</td>
<td>173</td>
<td>260</td>
<td>126</td>
</tr>
<tr>
<td>Chronic &gt;12m</td>
<td>169</td>
<td>199</td>
<td>162</td>
<td>237</td>
<td>140</td>
</tr>
<tr>
<td>Thiopurine use</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>176</td>
<td>242Δ</td>
<td>208</td>
<td>276</td>
<td>184Δ</td>
</tr>
<tr>
<td>Current</td>
<td>85</td>
<td>141Δ</td>
<td>107</td>
<td>174</td>
<td>103Δ</td>
</tr>
<tr>
<td>Previous</td>
<td>82</td>
<td>167Δ</td>
<td>112</td>
<td>221</td>
<td>113Δ</td>
</tr>
</tbody>
</table>
Table 10 Summary table of Kaplan-Meier survival times for \textit{CARD15 (NOD2)} genotype and 5q31 haplotype carriage. Follow up months from first operation, defining event; second operation.

<table>
<thead>
<tr>
<th>Factor</th>
<th>n=</th>
<th>Mean Estimate (months)</th>
<th>95% Confidence interval Lower</th>
<th>95% Confidence interval Upper</th>
<th>Median Estimate (months)</th>
<th>95% Confidence Interval Lower</th>
<th>95% Confidence Interval Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{CARD15 (NOD2)} genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>198</td>
<td>177</td>
<td>149</td>
<td>205</td>
<td>123</td>
<td>105</td>
<td>141</td>
</tr>
<tr>
<td>heterozygote</td>
<td>72</td>
<td>219</td>
<td>146</td>
<td>292</td>
<td>129</td>
<td>81</td>
<td>177</td>
</tr>
<tr>
<td>2 mutations</td>
<td>16</td>
<td>208</td>
<td>135</td>
<td>280</td>
<td>203</td>
<td>150</td>
<td>256</td>
</tr>
<tr>
<td>\textit{5q31 haplotype}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>44</td>
<td>195</td>
<td>141</td>
<td>248</td>
<td>140</td>
<td>21</td>
<td>259</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>80</td>
<td>212</td>
<td>140</td>
<td>284</td>
<td>133</td>
<td>85</td>
<td>181</td>
</tr>
<tr>
<td>Homozygote</td>
<td>39</td>
<td>228</td>
<td>157</td>
<td>299</td>
<td>158</td>
<td>58</td>
<td>258</td>
</tr>
</tbody>
</table>
2.3.5 **Age at diagnosis and time to second small bowel resection**

There was no significant difference in time to second operation and age at diagnosis by the Vienna classification, Figure 6.

![Kaplan-Meier survival curve of Vienna age at diagnosis and time (months) to second operation.](image)

Figure 6 Kaplan-Meier survival curve of Vienna age at diagnosis and time (months) to second operation. There is no difference in time to second operation between groups. A1=age at diagnosis <40 years, A2=age at diagnosis >40 years. Dotted lines represent 50% cumulative survival.

2.3.6 **Disease behaviour and time to second operation**

Inflammatory disease behaviour had significantly longer mean time to second operation than stricturing or fistulising disease behaviour, p=0.001, this group was too small to derive median survival. There was no significant difference in time to second operation and stricturing or fistulising disease behaviour.
2.3.7 **Smoking and time to second small bowel resection, Table 11.**

Smoking reduced time to second small bowel resection. Patients who had never smoked had an estimated median time to second operation of 203 months (16.9 years) compared to 113 months (9.4 years) in current smokers and 140 months (11.6 years) in ex-smokers, p=0.002.

Smoking was significantly associated with second operation chi squared p=0.02, with 58% of smokers having had 2 or more resections compared to 45% of non smokers.

**Table 11 Smoking behaviour at follow up and 2 or more resections, chi squared test for significance p=0.02.**

<table>
<thead>
<tr>
<th>Smoking behaviour</th>
<th>Second_operation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One resection</td>
<td>2 or more resections</td>
</tr>
<tr>
<td>Non smoker</td>
<td>118</td>
<td>98</td>
</tr>
<tr>
<td>Smoker</td>
<td>52</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>170</td>
<td>171</td>
</tr>
</tbody>
</table>
Figure 7 Kaplan-Meier survival curve for smoking and time to second operation. Smoking significantly reduces time to second operation \( p=0.002 \). Mean time to second operation from first operation; 279 months-never smoked, 193 months-ex-smokers, 148 months-current smokers. Median time to second operation from first operation: 203 months-never smoked, 140 months-ex-smokers, 113 months-current smokers. Dotted lines represent 50\% cumulative survival.
2.3.8 **Time to second operation and timing of the first resection**

There was no significant difference in time to second operation if surgery was performed at the time of diagnosis rather than later in the disease process Figure 8. There was no significant difference in time to second operation if first surgery was performed within 12 months of diagnosis, Figure 9.

![Surgery at diagnosis and time to second operation](image)

**Figure 8** Kaplan-Meier survival curve of surgery at the time of diagnosis of Crohn’s disease and surgery later in diagnosis. There is no significant difference in time to second operation.
Figure 9 Kaplan-Meier survival curve for operation within 12 months of diagnosis and time to second operation. There is no significant difference in time to second operation. Dotted line represents 50% cumulative survival.
2.3.9 Azathioprine and time to second small bowel resection

Azathioprine use was significantly associated with shorter survival to second small bowel resection, p=0.000, Figure 10.

Figure 10 Kaplan-Meier survival curve for thiopurine use and time to second operation, p=0.000. Thiopurine use was significantly associated with shorter time to second. Mean time to second operation from first operation; 242 months-no thiopurine use, 167 months –previous thiopurine use, 141 months-current thiopurine use. Median time to second operation from first operation: 184 months-no thiopurine use, 113 months –previous thiopurine use, 103 months –current thiopurine use. Dotted lines represent 50% cumulative survival.
2.3.10 *CARD15 (NOD2)* and time to second small bowel resection

There was no difference in time to second small bowel resection between *CARD15 (NOD2)* genotypes, Figure 11.

![Kaplan-Meier survival curve for *CARD15 (NOD2)* genotype and time to second operation.](Image)

Figure 11 Kaplan-Meier survival curve for *CARD15 (NOD2)* genotype and time to second operation. There is no difference in survival.

2.3.11 *CARD15 (NOD2)*, smoking and time to second small bowel resection

Smoking significantly reduced survival to second small bowel resection in all *CARD15 (NOD2)* genotypes, *p*=0.011, Table 13.
Figure 12 Kaplan Meier survival curve for carriage of 5q31 haplotype and time to second operation (months). There is no difference in survival.

2.3.12 5q31 and time to second small bowel resection

There was no difference in survival to second small bowel resection between carriage of 5q31 haplotypes, Figure 12.

2.3.13 5q31, smoking and time to second small bowel resection

Smoking reduced survival in wild-type, heterozygotes and homozygotes for 5q31 haplotype, Table 14.
2.3.14 Decade of first operation and time to second small bowel resection

There was no significant difference in decade of first operation and time to second small bowel resection $p=0.065$, Figure 13. *CARD15* (NOD2) genotypes were normally distributed in each decade of first operation, Figure 14.

Figure 13 Kaplan-Meier survival curve of decade of first small bowel resection and time to second small bowel resection (month). There is no significant difference in time to second small bowel resection over the decade of first small bowel resection.
Table 12 Summary table of Kaplan-Meier survival times for decade of first small bowel operation. Follow up months from first operation, defining event; second operation. There is no significant difference in survival to second operation.

<table>
<thead>
<tr>
<th>Decade</th>
<th>n=</th>
<th>Mean Estimate (months)</th>
<th>95% Confidence Interval</th>
<th>Median Estimate (months)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td>Lower</td>
</tr>
<tr>
<td>1950</td>
<td>2</td>
<td>90.000</td>
<td>31.200</td>
<td>148.800</td>
<td>60.00</td>
</tr>
<tr>
<td>1960</td>
<td>7</td>
<td>314.917</td>
<td>229.352</td>
<td>400.481</td>
<td>332.00</td>
</tr>
<tr>
<td>1980</td>
<td>70</td>
<td>138.920</td>
<td>119.345</td>
<td>158.496</td>
<td>123.000</td>
</tr>
<tr>
<td>1990</td>
<td>63</td>
<td>107/359</td>
<td>96.562</td>
<td>118.155</td>
<td>115.000</td>
</tr>
<tr>
<td>2000</td>
<td>6</td>
<td>56.263</td>
<td>49.639</td>
<td>62.887</td>
<td></td>
</tr>
</tbody>
</table>

Bar Chart of decade of first operation and CARD15 (NOD2) genotype

Figure 14 Bar chart displaying CARD15 (NOD2) genotype distribution over the decade of first small bowel operation. The genotypes are normally distributed.
Table 13 Smoking and *CARD15* (*NOD2*) genotype, follow up time, defining event second operation. Time to second operation, mean, median and 95% confidence intervals, *p*=0.013.

<table>
<thead>
<tr>
<th><em>CARD15</em> (<em>NOD2</em>) genotype</th>
<th>Smoking status</th>
<th>n=</th>
<th>Mean Estimate (months)</th>
<th>95% Confidence Interval Lower</th>
<th>95% Confidence Interval Upper</th>
<th>Median Estimate (months)</th>
<th>95% Confidence Interval Lower</th>
<th>95% Confidence Interval Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Non smoker</td>
<td>118</td>
<td>196*</td>
<td>157</td>
<td>234</td>
<td>140*</td>
<td>95</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>Smoker</td>
<td>75</td>
<td>144*</td>
<td>104</td>
<td>184</td>
<td>104*</td>
<td>82</td>
<td>126</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>Non smoker</td>
<td>46</td>
<td>250*</td>
<td>156</td>
<td>345</td>
<td>146*</td>
<td>58</td>
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<td>196</td>
</tr>
<tr>
<td>2 mutations</td>
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<td>237</td>
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<td>144</td>
<td>262</td>
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<tr>
<td></td>
<td>Smoker</td>
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<td>208</td>
<td>37</td>
<td>135</td>
<td>221</td>
<td>44</td>
<td>398</td>
</tr>
</tbody>
</table>

Table 14 Smoking and 5q31 haplotype carriage and time to second operation, follow-up, defining event second operation. Mean, median and 95% confidence interval and time to second operation, *p*=0.009.

<table>
<thead>
<tr>
<th>5q31 haplotype</th>
<th>Smoking status</th>
<th>n=</th>
<th>Mean Estimate (months)</th>
<th>95% Confidence Interval Lower</th>
<th>95% Confidence Interval Upper</th>
<th>Median Estimate (months)</th>
<th>95% Confidence Interval Lower</th>
<th>95% Confidence Interval Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
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<td>173*</td>
<td>107</td>
<td>239</td>
<td>140*</td>
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<td></td>
<td>Smoker</td>
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<td>132</td>
<td>306</td>
<td>221*</td>
<td>39</td>
<td>403</td>
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<tr>
<td>heterozygote</td>
<td>Non smoker</td>
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<td>269*</td>
<td>169</td>
<td>269</td>
<td>171*</td>
<td>104</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>Smoker</td>
<td>29</td>
<td>105*</td>
<td>76</td>
<td>134</td>
<td>97*</td>
<td>74</td>
<td>120</td>
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<tr>
<td>homozygote</td>
<td>Non smoker</td>
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<td>318*</td>
<td>224</td>
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<td>332*</td>
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</tr>
<tr>
<td></td>
<td>Smoker</td>
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<td>122*</td>
<td>74</td>
<td>170</td>
<td>117*</td>
<td>96</td>
<td>138</td>
</tr>
</tbody>
</table>
2.4 Discussion

This is a large investigation of the time to second operation in people who have undergone terminal ileal resections for Crohn’s disease. The influence of gender, age at diagnosis, time to first surgery, disease behaviour, smoking habit and azathioprine/6-mercaptopurine use was investigated. The influence of CARD15 (NOD2) genotype and 5q31 haplotype carriage on time to second operation in people with Crohn’s disease was explored, although the number of people carrying two CARD15 (NOD2) mutations was small. Data was collected from case notes and by direct interview on age at diagnosis, age at first operation, age at second and subsequent operations, smoking behaviour and treatments for Crohn’s disease. The relationship between modifiable variables and time to second operation was then investigated using Kaplan-Meier survival curves. Previous outcome studies have investigated the influence of CARD15 (NOD2) with in all Crohn’s disease and not investigated purely ileal Crohn’s disease.

No difference was found in time to second operation when analysed by gender or age at diagnosis. This study demonstrates that CARD15 (NOD2) genotype does not influence time to second operation in terminal ileal Crohn’s disease. Carriage of the 5q31 haplotype was not associated with shorter survival to second operation. Smoking was associated with a significantly shorter time to second operation. Thiopurine use was associated with a shorter time to second operation.

The finding of no association between outcome and CARD15 (NOD2) genotype is in contrast to previous published studies that have shown that CARD15 (NOD2) genotype is associated with a shorter time to reoperation (Büning, Genschel et al. 2004). The Büning study however was a much smaller study with only 41 patients with terminal ileal resection for Crohn’s disease; twelve of 29 CARD15 (NOD2) negative patients had a second operation and two of only 12 CARD15 (NOD2) positive patients had a second operation. In a further study of 193 patients with ileal Crohn’s disease in Italy, 187 of which had had at least one operation (Laghi, Costa et al. 2005), carriage of one or more CARD15 (NOD2) mutation was associated with shortened time to second operation. They found no effect of smoking on survival to second operation in patients with CARD15 (NOD2) mutations, of which there were 34. A German investigation by Seiderer (Seiderer, Schnitzler et al. 2006) found that carriage of the 1007fs mutation
was associated with surgery and an increased risk of restenosis, however this was a subgroup analysis of 19 patients.

These studies showing a positive association of CARD15 (NOD2) with shorter time to second operation were small, low powered studies. By taking an unselected cohort of patients with Crohn’s disease, there will be a bias of patients with ileal disease having CARD15 (NOD2) mutations. These patients are also more likely to have surgery for Crohn’s disease. Therefore there is bias towards a positive association with CARD15 (NOD2). By taking a cohort of patients all of whom have had resections for Crohn’s disease, this bias is reduced and the influence of CARD15 (NOD2) can be more accurately investigated.

The effect of smoking on time to second operation in terminal ileal Crohn’s disease is not unexpected. Smoking significantly reduces the median time to second operation in patients with resection for terminal ileal Crohn’s disease from 203 months in those who have never smoked to 113 months in those who are currently smoking. Patients who were considered to be ex-smokers at follow-up also had a reduced median time to second operation of 140 months, however this is not significant and the survival of these patients to second operation closely matches that of non-smokers. This further increases the evidence that it is current smoking habits that influence disease behaviour and that by stopping smoking patients can significantly improve their disease progression (Cosnes, Beaugerie et al. 2001). The effect of smoking on time to second operation is maintained in all CARD15 (NOD2) genotypes.

5q31, IBD5, is a haplotype with an established association with Crohn’s disease (Ma Y, Ohmen et al. 1999; Duerr, Barmada et al. 2000). The 5q31 haplotype has been found to be associated with perianal Crohn’s disease (Armuzzi, Ahmad et al. 2003) although this is not consistent (Noble, Nimmo et al. 2005). In this cohort of 374 patients with Crohn’s disease, carriage of the 5q31 haplotype was found to be associated with progression of disease to a fistulising phenotype and was significantly associated with a need for surgery. Therefore we investigated whether 5q31 haplotype carriage was a risk factor for reduced time to second operation in terminal ileal Crohn’s disease. In our cohort of patients with terminal ileal Crohn’s disease, there was no significant effect found with carriage of 5q31 haplotype and time to second operation. This may because this study does not have the power to detect a weak effect of this haplotype. However that weak effect is unlikely to be of clinical significance.
Smoking reduced time to second operation in all patients, whether they carried one or two of the disease associated haplotypes.

A study in 2007 of patients from two centres in Rome, a cohort of 635 patients revealed that people operated on at the time of diagnosis of Crohn’s disease had a longer surgical remission than people with more delayed surgery (Aratari, Papi et al. 2007). In contrast this investigation has shown the opposite effect. Surgery either at diagnosis, or within 12 months of diagnosis was not associated in this cohort with a shorter time to second operation. This may be related to different operative practices between different countries, requirement for early surgery may be a marker for more severe disease process in this cohort of patients, as this cohort was specifically chosen as all had had terminal ileal resection for Crohn’s disease.

Thiopurine use has been shown to improve disease outcome in Crohn’s disease (Lémann, Mary et al. 2005). The use of thiopurines in this cohort was associated with a shorter time to second operation. One explanation for this is that thiopurine use tends to be reserved for those with more severe disease. Its use in this context is therefore a surrogate marker for severity and or rapidity of disease progression.

In a population based cohort study of 907 patients with ileocaecal Crohn’s disease a younger age at diagnosis was associated with a lower resection rate (Bernell, Lapidus et al. 2000). In this investigation, no significant difference was found in between Vienna age at diagnosis and survival to second operation.

Surgery for Crohn’s disease is more likely in patients with stricturing or fistulising/penetrating disease. In this cohort only 24 patients were defined as having purely inflammatory disease. These categories are rather clumsy. Disease behaviour has been shown to change over the time course of the disease with 56 % of patients initially presenting with inflammatory disease eventually developing stricturing or fistulising complications (Louis, Collard et al. 2001). As disease behaviour in this cohort was taken at a single time point, i.e. at the time of surgery, there will be bias towards stricturing and fistulising disease behaviour. Fistulising behaviour was taken to be fistula seen on imaging before surgery, seen at the time of surgery or present in the resection specimen. Stricturing behaviour was taken as being the presence of obstructive symptoms leading to surgery with the presence of stricturing disease on imaging or at the time of surgery in the absence of fistulising disease. Most often
strictures and fistulae occur together in the same patient and it is rather artificial to divide the two.

The strengths of this study are its large size, robust diagnostic criteria and length of follow-up data. The study includes 346 people with terminal ileal resections for Crohn’s disease, all of whom have a robust histological diagnosis of Crohn’s disease. The mean length of follow up was 213 months, range 4 to 663 months. The median survival to second operation was 135 months (95% confidence interval 113 to 157 months). Lennard-Jones stated that a “follow-up study excludes any diagnostic uncertainty as it concerns only patients with chronic regional ileitis who needed surgical treatment. It therefore enables clear-cut, valuable data to be presented on the recurrence rate and mortality of Crohn’s disease” (Lennard-Jones and Stalder 1967). This investigation fulfills his suggestions.

In conclusion, this investigation has confirmed the association with increased frequency of second operation in smokers with a significantly reduced time to second operation. No significant difference in survival to second operation was found and age at diagnosis or timing of first surgery. No association has been found in this cohort with shorter time to second operation and CARD15 (NOD2) genotype or carriage of 5q31 haplotype. This data and lack of association with CARD15 (NOD2) genotype can be considered to be robust due to the size and histological confirmation of diagnosis of Crohn’s disease.
Chapter 3 Paneth cells and defensins

3.1 Introduction and aims

This study investigated the expression of antimicrobial peptides in archival sections of terminal ileum from patients with terminal ileal Crohn’s disease and controls with colorectal cancer. Expression of mRNA was detected using a non isotopic in situ hybridization technique and quantified using computed image analysis. It was hypothesized that there would be no difference in expression of lysozyme or sPLA2 mRNA between Crohn’s disease sections and controls. It was hypothesized that there would be a reduction in expression of human defensin 5 and 6 mRNA in Crohn’s disease sections compared to control. It was anticipated that there would be no difference between expression of lysozyme, sPLA2 and TNF-α mRNA and CARD15 (NOD2) genotypes and that there would be a reduction in expression of human defensin 5 and 6 mRNA with the number of CARD15 (NOD2) mutations carried.

There was expected to be increased TNF-α mRNA expression in Crohn’s disease sections compared to controls and that there would be no difference in TNF-α mRNA expression between CARD15 (NOD2) genotypes. TNF-α mRNA expression and quantitation was used as a positive control for the technique.
3.1.1 In situ hybridization

The technique of non radioactive *in situ* hybridization allows specific nucleic acid sequences to be detected in morphologically preserved tissue sections. The cellular location of the labelled mRNA can be demonstrated with light microscopy. Anti-sense RNA probes labelled with digoxigenin to the target mRNA are produced. Antibody to digoxigenin in an immunohistochemical reaction enables staining of the labelled probe. The degree of staining can then be measured by densitometry by specific computer software. The technique of *in situ* hybridization allows the measurement of staining density in specific cell types.

Using densitometry the expression of mRNA in Paneth cells can be estimated and compared between Crohn’s disease sections and between *CARD15 (NOD2)* genotypes. This study investigated the expression of mRNA for antimicrobial peptides in Paneth cells by *in situ* hybridization.
3.2 Materials and methods

Cases were identified from pathology databases at the Queen Elizabeth Hospital, Gateshead using the words Crohn’s disease, ileal and right hemicolecotmy. Cases were identified at the Freeman Hospital and Royal Victoria Infirmary, Newcastle upon Tyne by searching clinical coding databases using the words Crohn’s disease, ilectomy, right-hemicolecotmy and hemicolecotmy. Cases notes were then reviewed to establish the diagnosis of Crohn’s disease and the details of operation. Cases were included if Crohn’s disease in the terminal ileum was confirmed and resection of the terminal ileum had been performed.

Controls were identified who had undergone right-hemicolecotmy for caecal carcinoma and were only included if disease did not extend into the ileum. The normal ileal sections were used as the control sections.

Written informed consent was obtained from all participants. The consent forms and information sheets can be found in the appendix.

10ml venous blood was obtained from each patient into commercial plastic tubes containing EDTA using standard precautions. Blood samples were then labelled with a unique code and frozen at –20°C until DNA extraction. Blood samples were kept frozen while transported to the Genetics Department, Guys, Kings and St. Thomas’ School of Medicine, London.

DNA was extracted using the salting out method, as previously described 2.2.1 above.

Probes specific for lysozyme (LYZ), sPLA2, human defensin 5 (HD-5), human defensin 6 (HD-6) and TNF-α were generated in a bacterial culture. Probes were purified. Sections were incubated with probe then detected with anti-mouse antibody. This was then detected using anti-dig antibody and stained with peroxidase.

3.2.1 Genotyping

Samples were genotyped for the three common CARD15 (NOD2) SNPs (R702W, G908R and 3020insC) in Prof. C G Mathew laboratory, Guy’s, King’s and St. Thomas’ School of Medicine, London using published techniques (Cuthbert, Fisher et al. 2002).

Groups of four were created with each genotype plus one control; i.e. wild-type, heterozygotes, homozygotes and normal section. These groups were created by JCM
keeping CH blind to the genotype. Paraffin blocks were obtained from each case from the hospital pathology library. 6 micron sections were cut onto poly-L-lyscine or APES prepared slides (VWR).

### 3.2.2 Probe generation

**Table 15 Probe generation**

<table>
<thead>
<tr>
<th>Replication of DNA and cloning and transfection done by SK, SL and CK.</th>
<th>Probe length (base pairs)</th>
<th>Restriction endonuclease</th>
<th>RNA polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construct (Parent vector/subcloned cDNA sequence)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-LYZ (pGEM-3/lysozyme) 3605bp</td>
<td>738 (corresponding to nucleotide positions 1 to 738; NCBI accession number J03801)</td>
<td>EcoRI, HindIII</td>
<td>Ssp6, T7</td>
</tr>
<tr>
<td>pBS-PLA₂ IIa (pBluescript II KS +/sPLA₂ IIa) 3363 bp</td>
<td>402 corresponding to nucleotide positions 525 to 927; NCBI accession number NM_000300)</td>
<td>SstI, HindIII, HindIII</td>
<td>T3, T7, T3</td>
</tr>
<tr>
<td>pBS HD5 (pBluescript II KS +/HD5) 3204 bp</td>
<td>204 (corresponding to nucleotide positions 94 to 997; NCBI accession number BC069690)</td>
<td>Pst I, HindIII</td>
<td>T3, T7</td>
</tr>
<tr>
<td>pBS HD6 (pBluescript II KS +/HD6) 3278 bp</td>
<td>278 (corresponding to nucleotide positions 136 to 413; NCBI accession number BC069728)</td>
<td>Pst I, HindIII</td>
<td>T3, T7</td>
</tr>
</tbody>
</table>

### 3.2.3 Multiplication of DNA

10ml selective starter cultures were inoculated from DH5α glycerol stocks. These were then incubated overnight at 37° on a shaking platform in 50ml falcon tubes. 2ml were then added to 200ml selective LB medium into 500ml baffle flasks. These were then incubated overnight at 37° on a shaking platform. The DNA was then extracted.
using QIAGEN 500 maxiprep kit with colour indicator. Bacterial pellets were formed by centrifuging balanced tubes at room temperature for 15 minutes at 4000 rpm (Beckman Table Top centrifuge). Pellets were then resuspended in 10ml by pipetting up and down to break up bacterial clumps in buffer P1 (RNase and colour indicator). This was then decanted into Oakridge 30ml centrifuge tubes. Samples were then lysed using 10ml P2 lysis buffer by inversion of the tubes until the colour indicator turned blue, around 15 inversions until blue colour distributed evenly. The tubes were then incubated at room temperature for 5 minutes. 10ml of pre-chilled to 4° buffer P3 was then added to neutralise and precipitate protein. This turned the indicator clear and colourless. Centrifuge tubes were inverted until all the blue colour had dispersed. Tubes were then centrifuged at 20000g for 30 minutes at 4°, tubes were balanced by the addition of H₂O as necessary. Supernatant was then decanted in to a second set of labelled Oakridge 30ml centrifuge tubes. These were then centrifuged at 20000g for 30 min at 4° to further clear particulate matter.

10ml buffer QBT was then applied to labelled QIAGEN tips to equilibrate and allowed to drain freely into 50ml falcon tubes. The supernatant from the Oakridge centrifuge tubes was then applied to the tips, care was taken that flow through did not build up and so the flow through was regularly discarded. Tips were then washed with two lots of 30ml buffer Q. Care was again taken not to let flow-through build up.

DNA was then eluted off each tip into 30ml glass Corex centrifuge tubes using 15ml buffer QF. DNA was precipitated by adding 10.5ml room temperature isopropanol to each Corex tube. Solutions mixed by inversion. Tubes were then centrifuged at 15000rpm for 30 minutes at 4°. The supernatant was decanted, opaque DNA pellet visible on side of Corex tube. DNA was then washed with 5ml room temperature 70% ethanol. Tubes were then centrifuged again at 15000rpm, 4° for 10 minutes. Supernatant was then decanted and the pellet air dried. Then pellet was then resuspended in 400µL DEPC H₂O. DNA was then quantified by gel electrophoresis and UV spectrometry.
3.2.3.1 DNA estimation using UV spectrometry;

10µL DNA diluted to 1mL in DEPC H₂O, OD measured at 260 and 280nm. The 260/280 ratio was calculated to determine purity. The DNA concentration was calculated:

\[ [\text{DNA}] \mu g/ml = \text{OD}_{260} \times \text{dilution factor} \times 50 \mu g \text{ DNA/ml} \]

DNA was generated for lysozyme, secretory PLA₂, human defensin 5 and human defensin 6. SL provided TNF DNA.

DNA was then linearized in preparation for transcription using the following enzymes and buffers.

**Table 16 DNA linearization enzymes**

<table>
<thead>
<tr>
<th>DNA</th>
<th>Enzymes (Invitrogen)</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>Eco RI</td>
<td>React 3</td>
</tr>
<tr>
<td></td>
<td>HindIII</td>
<td>React 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>React 2 (double digest react 2)</td>
</tr>
<tr>
<td>sPLA₂</td>
<td>HindIII</td>
<td>React 2</td>
</tr>
<tr>
<td></td>
<td>Sst I</td>
<td>React 2</td>
</tr>
<tr>
<td>HD5</td>
<td>Pst I</td>
<td>React 2</td>
</tr>
<tr>
<td></td>
<td>HindIII</td>
<td>React 2</td>
</tr>
<tr>
<td>HD6</td>
<td>Pst I</td>
<td>React 2</td>
</tr>
<tr>
<td></td>
<td>HindIII</td>
<td>React 2</td>
</tr>
<tr>
<td>TNF</td>
<td>Eco RI</td>
<td>React 3</td>
</tr>
<tr>
<td></td>
<td>Ecl</td>
<td></td>
</tr>
</tbody>
</table>

It was aimed to linearize ≈8µL DNA each of LZM, PLA₂, HD5 and HD6 in order to generate enough probe for the entire *in situ* experiment.
In a 50µL reaction volume the following quantities were used in 1.5mL labelled eppendorf;

- DNA 3µL
- 10x buffer 5µL
- Enzyme 4µL
- DEPC H₂O 38µL

The digest was then incubated at 37° overnight. Digest run on 1% agarose gel to check for linearization.

Linearized DNA was then extracted using phenol precipitation method and stored as an ethanol precipitate at -20°; the volume of digest was made up to 100µL with DEPC H₂O. Then an equal volume i.e. 100µL of phenol:chloroform:IAA (25:24:1) was added. This was mixed by vortexing, then spun at 13 000 rpm (16100g) for 5 minutes at room temperature. The aqueous layer was then transferred to a fresh eppendorf containing 100µL chloroform. This was again mixed by vortexing and spun at 13000 rpm for 5 minutes at room temperature. The aqueous layer was then aspirated to a fresh ependorf, volume ≈100µL. 1:10 volume (10µL) 3M sodium acetate was then added and mixed by vortexing. 2x volume (250µL) 100% ethanol was then added and mixed by vortexing. This mix was then stored at –20° overnight to precipitate (an alternate is to precipitate the DNA at –70° for 20minutes). This was then mixed and spun at 14 000 rpm for 15 minutes at 4°. An opaque pellet is formed on the outer edge of the eppendorf. The supernatant was decanted and the excess pipetted off. This was then washed with 2x volume (200µL) 70% ethanol, the pellet being dislodged off by vortexing. The DNA was then spun again at 14 000 rpm for 15 minutes at 4°, a pellet forming at the outer edge of the eppendorf. The supernatant was again discarded. The pellet was centrifuged at 14 000 rpm for 1 minute at room temperature and the excess ethanol pipetted off. The pellet was then air dried. DNA was resuspended in 100µL DEPC H₂O by gentle pipetting.
Table 17 RNA transcription enzymes

<table>
<thead>
<tr>
<th>Template</th>
<th>Polymerase</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA₂ HindIII</td>
<td>T7</td>
<td>Antisense</td>
</tr>
<tr>
<td>PLA₂ Sst I</td>
<td>T3</td>
<td>Sense</td>
</tr>
<tr>
<td>LZM HindIII</td>
<td>T7</td>
<td>Antisense</td>
</tr>
<tr>
<td>LZM Eco RI</td>
<td>T3</td>
<td>Sense</td>
</tr>
<tr>
<td>HD5 HindIII</td>
<td>T7</td>
<td>Antisense</td>
</tr>
<tr>
<td>HD5 Pst I</td>
<td>T3</td>
<td>Sense</td>
</tr>
<tr>
<td>HD6 HindII</td>
<td>T7</td>
<td>Antisense</td>
</tr>
<tr>
<td>HD6 Pst I</td>
<td>T3</td>
<td>Sense</td>
</tr>
<tr>
<td>TNF Ecl</td>
<td>T7</td>
<td>Antisense</td>
</tr>
<tr>
<td>TNF Eco RI</td>
<td>T3</td>
<td>Sense</td>
</tr>
</tbody>
</table>

3.2.4 Transcription

RNA probes were generated by *in vitro* transcription using reagents from Roch RNA Labelling Kit (Roche 1175025). The transcription reaction products for each probe were pooled in order to minimize variation in probe transcription reactions.

In a 40µL reaction volume:

- PLA₂ DNA: 29µL
- LZM DNA: 3µL
- HD5 DNA: 3µL
- HD6 DNA: 5µL
- TNF DNA: 7µL
- Nuclease free H₂O: 0
- 10x dig mix: 4µL
- 10x buffer: 4µL
- RNAse inhibitor: 1µL
- RNA polymerase: 2µL

The reaction was then incubated at 37° for 1 hour
A further 1 µL RNA polymerase added and the reaction was incubated for a further 1h at 37° giving a final volume of 41 µL.

2 µL was removed to run on 1.2% agarose gel, 2 µL DNase I added to digest any remaining template. 4 µL removed for quantification gel and dot blot.

The reaction was then terminated by the addition of 0.2M EDTA (Sigma). 5 µL 10 mg/mL tRNA (Roche 109495) was then added. The total volume was then made up to 100 µL by the addition of 56 µL nuclease free water (Invitrogen 10977). 10 µL 3M sodium acetate was then added with 250 µL 100% ethanol to form a precipitate giving a total volume of 360 µL. This was then stored at –20°. Care was taken throughout transcription reaction not to contaminate the experiment with RNase. All equipment was either dedicated to RNA work or decontaminated by baking at 220° for 12 hours.

2 µL of the reaction was run on a 1.2% gel with a DNA ladder. The relative densities of the gel bands were then compared to estimate the quantity of RNA probe generated.

1.2% gel 50 ml TBE buffer (Invitrogen), 0.60g agarose (Sigma) run at 60 mV for 45 minutes.

Figure 15 Transcription gel left to right; (a) DNA ladder, TNF-α (x2), (b) DNA ladder, HD5 (x2), HD6 (x2), (c) DNA ladder, lysozyme (x4), sPLA₂ (x4), HD5 (x4)

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3.2.4.1 Dot blot quantitation; Figure 16

RNA was quantitated using immunological detection of nucleases. A 10 x 12 cm section of membrane was cut and marked with sections. A dilution series was set up using and initial control RNA at concentration of 100ng/mL.

2µL of transcription RNA or control added to initial eppendorf and vortexed, 5µL then removed and added to 45µL and vortexed, 5µL removed and added to 45µL and vortexed and so on. 2µL of each dilution was then aliquoted on to the membrane starting with the most dilute solution on to the right of the membrane.

<table>
<thead>
<tr>
<th>i</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5</td>
<td>45</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>+ 198</td>
<td>+ 45</td>
<td>+ 45</td>
<td>+ 25</td>
<td>+ 25</td>
<td>+ 25</td>
<td>+ 25</td>
<td>+ 25</td>
</tr>
</tbody>
</table>

1:100 1:10 1:10 1:2 1:2 1:2 1:2

1000pg 100pg 10pg 5pg 2.5pg 1.25pg 0.625pg 0.3pg

The membrane was then equilibrated with 20mL Detection buffer. 10mL Colour substrate was then freshly prepared and the membrane was then kept static in the dark overnight. The reaction was halted by washing in distilled H₂O.

The probe concentration was then estimated by comparison of dot density to control.
Figure 16 Dot blot quantitation of mRNA probes
3.2.5 *In situ* hybridization

3.2.5.1 Groups

Sections were run in groups matched for, but blinded to, genotype together with a normal control and positive jejunum control. Each group therefore comprised lysozyme; 4 sections (control, Crohn’s disease *CARD15* (*NOD2*) wild-type, Crohn’s disease *CARD15* (*NOD2*) heterozygote, Crohn’s disease *CARD15* (*NOD2*) homozygote), PLA2, 4 sections (control, Crohn’s disease *CARD15* (*NOD2*) wild-type, Crohn’s disease *CARD15* (*NOD2*) heterozygote, Crohn’s disease *CARD15* (*NOD2*) homozygote), HD6, 4 sections, (control, Crohn’s disease *CARD15* (*NOD2*) wild-type, Crohn’s disease *CARD15* (*NOD2*) heterozygote, Crohn’s disease *CARD15* (*NOD2*) homozygote) and HD5, 5 sections, (jejunum control, control, Crohn’s disease *CARD15* (*NOD2*) wild-type, Crohn’s disease *CARD15* (*NOD2*) heterozygote, Crohn’s disease *CARD15* (*NOD2*) homozygote). The density of staining could then be compared through each run to the jejunum control. Care was taken throughout *in situ* not to contaminate experiment with RNase.

3.2.5.2 *In situ* hybridization

Sections were placed in Schiefendecker staining jars holding 100ml total volume. Sections were de-waxed in serial xylene (VWR) washes at room temperature on a shaking platform; 3 washes of 20 minutes each. Then rehydrated in serial alcohols at room temperature on a shaking platform, 100% ethanol for 5 minutes, 95% ethanol for 5 minutes, and 70% ethanol for 5 minutes. The sections were then washed twice in DEPC H₂O for 5 minutes. Sections were then fixed using 4% paraformaldehyde in PBS prewarmed to 25° before use for 20 minutes on a shaking platform. Sections were then washed twice in sterile PBS for 5 minutes at room temperature on a shaking platform. To improve probe penetration sections were then treated with 200mM HCl (1.72mL cHCl made up to 100mL with DEPC H₂O) for 20 minutes at room temperature on a shaking platform. Sections were then washed twice in DEPC H₂O for 5 minutes at room temperature. Sections were then equilibrated with proteinase K buffer for 5 minutes at room temperature. Sections were then digested for 17 minutes at 37° at 45rpm in a shaking water bath in proteinase K buffer with 40μg/mL proteinase K (Sigma-Aldrich P2308) (timing for proteinase K digestion optimised by experimentation with timings from 15 minutes to 25 minutes). Sections were then
washed in proteinase K buffer for 5 minutes at room temperature on a shaking platform. Sections were then washed twice in sterile PBS at room temperature on a shaking platform. Sections were then dehydrated in serial alcohols; 20% ethanol for 5 minutes, 40% ethanol for 5 minutes, 60% ethanol for 5 minutes, 80% ethanol for 5 minutes and 100% ethanol for 5 minutes, each at room temperature on a shaking platform.

**Table 18 in situ protocols**

<table>
<thead>
<tr>
<th>Transcription date</th>
<th>Probe</th>
<th>[probe] ng/µL</th>
<th>ng of probe used</th>
<th>Vol of ethanol precipitate</th>
<th>70% ethanol to 300µL</th>
<th>Hybe mix/section (total)</th>
<th>No slides</th>
<th>Vol NF H2O for pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>17/11/05</td>
<td>LZM T7</td>
<td>1.4</td>
<td>840</td>
<td>40</td>
<td>260</td>
<td>150 (600)</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>17/11/05</td>
<td>PLA2 T7</td>
<td>1.0</td>
<td>600</td>
<td>46</td>
<td>254</td>
<td>150 (600)</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>17/11/05</td>
<td>HD5 T7</td>
<td>1.0</td>
<td>900</td>
<td>58</td>
<td>242</td>
<td>150 (750)</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>17/11/05</td>
<td>HD6 T7</td>
<td>1.0</td>
<td>600</td>
<td>58</td>
<td>242</td>
<td>150 (600)</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>24/1/06</td>
<td>TNF T3</td>
<td>0.8</td>
<td>320</td>
<td>136</td>
<td>164</td>
<td>60 (480)</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>24/1/06</td>
<td>TNF T7</td>
<td>0.6</td>
<td>1280</td>
<td>560</td>
<td>440</td>
<td>60 (1920)</td>
<td>32</td>
<td>80</td>
</tr>
</tbody>
</table>

Prepared hybe master mix was used in 180µL aliquots, enough for 1mL total mix, stored at –20° and thawed prior to use. 50% Dextran sulphate was heated to 65° and 200µL/ml added to the mix and vortexed thoroughly. 500µL deionised formamide (Sigma-Aldrich F9037) was then added and vortexed. The mix was then incubated on ice for 10 minutes and centrifuged at 13 000rpm, 4° for 10 minutes. The supernatant was the pipetted into clean eppendorfs. RNA probes were used as follows for 1 run of 17 sections, for the TNF in situ hybridization one run comprising all Crohn’s disease sections (32) plus HD5 jejunum control was done, Table 18.

The RNA was extracted from the ethanol precipitate by the addition of a volume of 70% ethanol to make a total volume of 300µL. This was then centrifuged at 13 000 rpm, 4° for 15 minutes. The supernatant decanted and the pellet air dried. The RNA was then resuspended in nuclease free H2O. The probe was then heated to 80° for 5
minutes and snap cooled on ice. The probe was then added to the hybe mix. 150µL hybe mix was then pipetted over each section. The slides were then incubated in a humid container at 42° overnight.

3.2.5.3 Post hybridization
Sections were then placed in Schiefendecker staining jars. Sections were washed twice in 2X SSC buffer at 37° for 15 minutes in a shaking water bath. Sections were then placed in glass staining rack kept specifically for RNase washes and rinsed in NTE buffer then incubate in NTE buffer containing 20µg/mL RNase A (Sigma-Aldrich R4642) for 30 minutes at 37° in a shaking water platform. Sections were then rinsed in NTE buffer. Sections were then placed back into the Schiefendecker staining jar and rinsed twice with 0.2X SSC buffer at 37° for 30 minutes.

3.2.5.4 Immunological detection
Sections were washed in PBS for 10 minutes at room temperature. Sections were then transferred to Sequenza coverslips and washed with PBS. Sections were then blocked with PBS blocking solution for 30 minutes at room temperature. Primary antibody was added; 1:250 dilution mouse anti-DIG antibody in PBS blocking solution was added to the sections and left to incubate for 90 minutes at room temperature. Sections were then washed for 5 minutes with PBS. Sections were then blocked with PBS blocking solution for 30 minutes at room temperature. Secondary antibody was added; 1:200 biotinylated anti-mouse antibody in 1.5% horse serum in PBS was added to the sections and incubated for 30 minutes at room temperature. Sections were then washed for 5 minutes with PBS. Sections were then blocked with PBS blocking solution for 30 minutes at room temperature. ABC reagent was then added to sections and incubated for 30 minutes at room temperature. Sections were then washed for 5 minutes with PBS. Sections were then removed from the Sequenza coverslips.

200µL freshly prepared DAB-Peroxidase substrate solution was then pipetted over each section. The reaction was monitored microscopically. The reaction was then stopped by washing with distilled H₂O.

Sections were then counterstained for 10 seconds with haematoxylin and washed in running tap water. Sections were then washed in 70% ethanol for 2 minutes then
100% ethanol for 2 minutes then in 3 xylene washes for 2 minutes. Sections were mounted with DPX and dried overnight.

3.2.5.5 Microscopy and photography

All sections were imaged using Zeiss Axioskop 2 microscope with the following lenses; Plan-Neofluar 10x, 20x, 40x. Images were captured using Zeiss Axiocam MRc 0.63x 2/3”. The standard image set up was; Axiocam MRc with Plan NeoFLUA 40x lens at point 5 on light source.

3.2.5.6 Densitometry

Images were processed using the Automatic measurement programme written using the programme writing wizard, programme named 191205.xml (Axiovision re1.4.4 Axiovision automatic measurement programme generation. Software; Axiovision Rel. V4.4 Zeiss UK (06-2005) Sp1). No changes were made to contrast, sigma, shading correction or delineation of objects. Density is measured in arbitrary units as light transmission, therefore the lower the measurement unit the more intense the density of stain and therefore expression of mRNA.

Colour phases were obtained interactively and the outline filled. Objects were separated automatically. Artefacts and background were deleted manually. Each picture was given an identity. The area of density measured. Maximum, minimum and mean density of fields measured in red, blue and green together with the overall mean for all of the image and for regions within the image. Density was measured in amount of light transmitted, therefore the darker the picture the less light is transmitted.

The picture was then saved with the regions measured outlined. Jejunum standard was measured twice for indication of consistency. The background density was measure in a similar way using 2 interactive phases for DAB (brown-red) staining and haematoxylin (blue) staining. Programme backgroundmeasurement.xml. All images were saved as .zvi (Zeiss format) and .tif files. Data files saved as text – comma separated files .csv and converted to Excel for import into SPSS.

Results are demonstrated as crude density measurement and corrected to the jejunum standard. Correction to the jejunum standard was performed to allow for between run variation in probe binding and digoxigenin staining.
All images were analysed blind to genotype.

### 3.2.5.7 Analysis

Analysis of mean density was by ANOVA using SPSS software.

### 3.3 Results

Lysozyme, sPLA₂, human defensin 5 and human defensin 6 were all found within the secretory granules of Paneth cells, Figure 17 and Figure 19, TNF-α was found within Paneth cells, Figure 20.

![Image](image-url)  

**Figure 17** In situ hybridization with anti sense probes showing labelling of mRNA in Paneth cells, arrows, images of control (ileum) sections at x40. (a) lysozyme (b) sPLA₂ (c) human defensin 5 (d) human defensin 6
Figure 18 In situ hybridization using sense probes showing no mRNA labelling. This is the negative control. Images at x40 (a) lysozyme (b) sPLA2 (c) human defensin 5 (d) human defensin 6

10 in situ hybridization runs were performed for lysozyme, sPLA2, human defensin 5 and human defensin 6. A total of 40 sections were probed for lysozyme, sPLA2 and human defensin 6 and 50 sections for human defensin 5. There were a total of 10 jejunum standards. Sections from paraffin blocks dating back to 1977 were used. Successful staining however was not achieved on the oldest 1977 sections for any of the probes used.
Figure 19 In situ hybridization images of jejunum controls showing labelling of mRNA in Paneth cells. Probes: (a) lysozyme (b) sPLA2 (c) human defensin 5 (d) human defensin 6
3.3.1 Lysozyme densitometry

There was no significant difference in mean density of lysozyme staining between 
CARD15 (NOD2) genotype groups. There was no significant difference in the total 
lysozyme staining between CARD15 (NOD2) genotype groups. There was no 
significant difference in the mean or total lysozyme staining between controls and sections from patients with Crohn’s disease, Figure 23, Figure 25. This applied to 
crude analysis and correction of density to jejunum standard Figure 21 and Figure 22.

3.3.2 sPLA₂ densitometry

There was no significant difference in mean density of sPLA₂ staining between 
CARD15 (NOD2) genotype groups, Figure 21 and Figure 22. There was no significant 
difference in the total sPLA₂ staining between CARD15 (NOD2) genotype groups. 
There was no significant difference in the mean or total sPLA₂ staining between controls and sections from patients with Crohn’s disease, Figure 23, although there was a trend to an increase in sPLA₂ staining in the Crohn’s disease sections, Figure 25.
Figure 21 Mean density (arbitrary units) for RNA probes studied compared over CARD15 (NOD2) genotypes. There was no difference in mean density between sections. Density is uncorrected to the standard.
3.3.3 Human defensin 5 densitometry

Human defensin 5 was found in Paneth cells in all sections. There was no significant difference in mean density of human defensin 5 staining between \textit{CARD15} (\textit{NOD2}) genotype groups, Figure 21 and Figure 22. There was no significant difference in the total human defensin 5 staining between \textit{CARD15} (\textit{NOD2}) genotype groups, Figure 23. There was no significant difference in mean or total human defensin 5 staining between controls sections and sections from patients with Crohn’s disease, Figure 25.
Figure 23 Total density measured for each section for each RNA probe compared between *CARD15 (NOD2)* genotypes
Figure 24 Sum of density corrected to jejunum standard for each RNA probe compared between CARD15 (NOD2) genotypes

3.3.4 Human defensin 6 densitometry

Human defensin 6 was found to be expressed in Paneth cells in all sections. There was no significant difference in mean density of human defensin 6 staining between CARD15 (NOD2) genotype groups, Figure 21. There was no significant difference in mean density of human defensin 6 staining between CARD15 (NOD2) genotype groups, Figure 22. There was no significant difference in the total human defensin 6 staining between CARD15 (NOD2) genotype groups, Figure 23. There was no significant difference in mean or total human defensin 6 staining between controls sections and sections from patients with Crohn’s disease, Figure 25.
Figure 25 mean density of each RNA probe compared between control sections and Crohn’s disease sections
Figure 26 Mean density of RNA probes corrected to jejunum standard between control sections and Crohn’s disease sections
Figure 27 Total density of RNA probes compared between control sections and Crohn’s disease sections
Figure 28 Total density corrected to jejunum standard compared between control sections and Crohn’s disease sections
Figure 29 Mean density (arbitrary units) of mRNA probe and age at operation. There is no significant association between mRNA probe density and age at operation.
3.3.5 TNF-α densitometry

There was significantly more TNF-α in the Crohn’s disease sections than the control sections as measured by mean densitometry p=<0.001, Figure 30. There was no significant difference in mean density in CARD15 (NOD2) genotype groups, Figure 31. Three sections from patients with Crohn’s disease did not demonstrate TNF-α expression. These sections had previously demonstrated positive in situ reaction to lysozyme, sPLA2 and human defensin 5 and 6.

![Figure 30: Mean TNF-α density between control sections and Crohn’s disease sections. There is a significant difference in expression of TNF-α in Crohn’s disease sections compared to controls, p=<0.001.](image-url)

Error Bars show 95.0% CI of Mean
Figure 31 Mean density for each section for RNA probe for TNF-α. There is reduced light transmission indicating increased TNF expression in Crohn’s disease sections.
3.4 Discussion

In order to investigate a possible link between Paneth cells, *CARD15 (NOD2)* and Crohn’s disease *in situ* hybridization technique was used to estimate the relative quantity of mRNA for the antimicrobial peptides in Paneth cells of normal terminal ileum and that of patients with Crohn’s disease. In addition the possible relationship to *CARD15 (NOD2)* genotype was investigated. TNF-α is a proinflammatory cytokine that is found in Paneth cells (Keshav, Lawson et al. 1990). It is implicated in the pathogenesis of Crohn’s disease. Treatment with anti-TNF antibody is known to be of benefit in Crohn’s disease.

This investigation has confirmed that Paneth cell secretory granules contain mRNA for antimicrobial peptides; lysozyme, sPLA₂ and human α-defensins 5 and 6. TNF-α mRNA can be demonstrated in Paneth cells, although not in secretory granules. This study aimed to further investigation the expression of lysozyme, sPLA₂ and human defensins 5 and 6 mRNA in Paneth cells of paraffin imbedded sections of ileum taken at time of surgery for terminal ileal Crohn’s disease, the most normal ileum from each resected section was used in order to maximise architectural preservation. The expression of TNF-α and important pro-inflammatory cytokine expressed in Paneth cells was also investigated. The expression of mRNA can be detected archival material preserved in paraffin wax, however tissue of 30 years old does not have reliable detection.

There was no difference in expression of lysozyme, sPLA₂ and human defensin 5 and 6 mRNA between control and Crohn’s disease sections as measured by computerized densitometry. There is no difference in expression with age. By this method no difference in human defensin 5 and 6 expression could be shown between *CARD15 (NOD2)* genotypes. No difference in mRNA expression of lysozyme, sPLA₂ could be demonstrated between *CARD15 (NOD2)* genotypes.

TNF-α mRNA expression was demonstrated in Paneth cells. There was an increase in TNF-α expression in sections from patients with terminal ileal Crohn’s disease compared to controls. No difference was seen between *CARD15 (NOD2)* genotypes. Of interest three sections from three different patients with terminal ileal Crohn’s disease Crohn’s disease did not express TNF-α. This represents 10% of the total number of people with Crohn’s disease in this study.
It has previously been shown by quantitative real-time PCR techniques that expression of α-defensin is reduced in biopsies from human ileum with Crohn’s disease, and reduced still further in patients who carry two mutations in the CARD15 (NOD2) gene (Wehkamp, Harder et al. 2004; Wehkamp, Salzman et al. 2005). A study published in 2005 (Wehkamp, Harder et al. 2004; Wehkamp, Salzman et al. 2005) demonstrated a decrease in human defensin 5 and 6 expression between healthy controls and people with Crohn’s disease, and a further decrease was seen in people with two CARD15 (NOD2) mutations. A more recent investigation however has not confirmed these findings (Simms, Doecke et al. 2008). Instead it suggests that the reduced human defensins found in the previous Wehkamp studies was a function of destruction of Paneth cell and therefore a reduction in cell products, as a result of inflammation rather than genotype effect.

The increase in TNF-α gene expression in people with Crohn’s disease relative to controls is an expected finding and concurs with current literature. This validates the technique used in this investigation. It shows that densitometry can be used semi-quantitatively to demonstrate a difference in mRNA expression in archival tissue sections.

An advantage of this technique was that it enabled use of archival tissue and thereby increased the pool of available tissue from patients carrying CARD15 (NOD2) mutations. All patients used in this study had confirmed Crohn’s disease. As each section was examined by hand, areas with the least inflammation were identified and used for the in situ process. This therefore reduces the confounder of inflammation and suggests any difference found would be potentially related to genotype rather than a function of inflammation and therefore Paneth cell number. The sections were grouped therefore minimizing intra-experimental variation.

In situ hybridization is a relatively insensitive method for detection of differential expression of mRNA, however it would be expected to demonstrate a significant deficiency in defensin expression that was suggested by the Wehkamp group. The difference in results seen may be accounted for by the fact that the method of in situ hybridization is highlighting defensin mRNA expression within secretory granules and therefore may be a marker of propeptide formation. Defensins undergo post-translational processing on release from the secretory granules to the active peptide
form. There may still be a defect in this process leading to a deficiency in functional peptide formation that would not be shown by this technique.

There was an absence of TNF-α expression in a number of sections from people with Crohn’s disease. This warrants further investigation. If a number of patients with Crohn’s disease do not have a TNF-α dependent pathway of inflammation, or have a reduced level of TNF-α expression this could potentially be used to predict who may respond to anti-TNF-α therapy.
4 Chapter 4 Histology phenotypes

4.1 Introduction and aims

We hypothesized that there would be different histological phenotypes associated with CARD15 (NOD2) mutations and smoking status. We therefore investigated whether the number and distribution of Paneth cells is altered in CARD15 (NOD2) genotypes, whether the presence of pyloric metaplasia is associated with CARD15 (NOD2) genotype and whether there is an increased area of pyloric metaplasia in CARD15 (NOD2) mutants. We investigated the association between granulomas and CARD15 (NOD2) and lymphoid aggregates and CARD15 (NOD2).
4.2 Materials and methods

Cases were identified from clinical coding and pathology databases using the terms ilectomy, right-hemicolectomy, hemicolectomy and Crohn’s disease. All were surgical cases, no biopsies were used. Patients were also identified during routine clinic visits by review of clinical notes. All patients had a confirmed diagnosis of Crohn’s disease according to standard clinical, radiological and histological methodology. Controls were identified as recent cases at the routine Newcastle Hospitals Multidisciplinary meeting having had right hemicolecetomy for colorectal cancer. Patients were all resident of the Newcastle-Gateshead area. Venous blood from patients with Crohn’s disease was obtained for genotyping. All patients gave written informed consent to participate to return to small bowel specimens held in the hospital archives, for forms, see appendix. Ethical approval was granted by Newcastle and South Tyneside research ethics committee and Gateshead Local research ethics committee.

Genotyping was undertaken for the three main CARD15 (NOD2) SNPs (R702W, G908R, and 3020insC) by Prof. C G Mathew’s team at the Department of Genetics, Guy’s, King’s and St. Thomas’ School of Medicine, London, by published methodology (Cuthbert, Fisher et al. 2002).

Sections were obtained from Queen Elizabeth Hospital, Gateshead and Newcastle Acute Hospital NHS trust encompassing the Royal Victoria Infirmary, Freeman Hospital and Newcastle General Hospital. All specimens had been routinely processed in hospital pathology. Archival haematoxylin and eosin sections were retrieved along with the paraffin embedded blocks where available. Groups were matched for CARD15 (NOD2) genotype; 2 mutations, 1 mutation and wild-type. In addition a cohort of control sections was used with healthy terminal ileum obtained at right hemicolecetomy for colorectal cancer.

Clinical characteristics were recorded on each patient from patient notes and direct interview; smoking history, age, gender, immunomodulatory drug usage, including azathioprine/6-mercaptopurine and infliximab, onset of symptoms and diagnosis and date of operation.
4.2.1 Scoring of histological features

All sections were scored using an Olympus CH-2 microscope. Each set of sections were evaluated for degree of inflammation based on the scoring system by Borley et al 2000, see Table 19 (Borley, Mortensen et al. 2000).

**Table 19** Adapted from (Borley, Mortensen et al. 2000) histological scoring of inflammatory features in surgically resected specimens

<table>
<thead>
<tr>
<th>Score</th>
<th>Lymphoid aggregate score</th>
<th>Granuloma Score</th>
<th>Inflammatory score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>-</td>
<td>Structural change only</td>
</tr>
<tr>
<td>1</td>
<td><em>Mucosa</em></td>
<td>No granuloma</td>
<td>Chronic inflammation</td>
</tr>
<tr>
<td>2</td>
<td><em>Submucosa</em></td>
<td>Mucosal and/or submucosal granuloma Or &gt;10 granuloma present</td>
<td>Lamina propria neutrophils</td>
</tr>
<tr>
<td>3</td>
<td><em>Muscularis propria</em></td>
<td>Granuloma in all bowel layers with or without granuloma in mesenteric lymph nodes</td>
<td>Crypt destruction</td>
</tr>
<tr>
<td>4</td>
<td><em>Serosa</em> or extramural fat</td>
<td>-</td>
<td>Erosions or ulcers</td>
</tr>
</tbody>
</table>

4.2.2 Paneth cell identification haematoxylin and eosin

Paneth cells were identified by their autofluorescent granules with routine haematoxylin and eosin (Rubio and Nesi 2003) and the absolute number in each well oriented crypt counted (Kamal, Wakelin et al. 2001). The height of the furthest Paneth cell from the base of the crypt was counted in epithelial cell nuclei in all well oriented crypts (Figure 32). Paneth cells height was also measured in nm using Axiovision software. Paneth cells were considered to have migrated if they were separated from the other Paneth cells at the base of the crypt by columnar epithelial cells.
4.2.3 Lysozyme immunohistochemistry

6 µm sections for immunohistochemistry were cut into poly-l-lyscine slides. Sections were dewaxed by three 5 minute washes in xylene on a shaking platform. Sections were then rehydrated in graded ethanol washes for 5 minutes each; 100% ethanol, 95% ethanol, 70% ethanol. Sections were then washed twice in distilled water for 5 minutes. Endogenous peroxidase activity was blocked by washing in 3% hydrogen peroxide in methanol for 10 minutes. This was neutralised by two 5 minute washes in dH₂O followed by one 5 minute wash in PBS. Sections were then loaded into Sequenza coverslips and washed with PBS for 5 minutes.

Sections were blocked for 30 minutes in 3% bovine albumin and 20% goat serum in PBS. Sections were then incubated for 90 minutes with 1 in 500 primary antibody rabbit antihuman lysozyme. Sections were washed twice for 5 minutes in PBS. Sections were then incubated with secondary biotinylated antibody, 1 in 100 goat anti-rabbit antibody in 3% bovine albumin, 1% goat serum. Sections were washed twice for 5 minutes in PBS. Sections were then incubated with ABC reagent for 30 minutes. Sections were washed for 5 minutes in PBS. Sections were removed from the coverslips and dried.

Sections were then covered with DAB-H₂O₂. Development of the colour reaction was monitored and stopped by washing in dH₂O.

Sections were then counterstained with haematoxylin for 10 seconds washed in cold running water then washed in 30% ethanol for 1 minute, 70% ethanol for 1 minute and 100% ethanol for 1 minute. This was followed by three sequential xylene washes for 1 minute and mounted using DPX.
4.2.4 **Pyloric metaplasia**

Pyloric metaplasia was identified according to published morphology (Lee 1964; Day, Jass et al. 2003). The total area of pyloric metaplasia was measured. The areas were photographed using a Leica DMR photomicroscope and captured using a JVC camera and QWin software (Leica Microsystems, Heidelberg). In order to correct for the differences in the size of each section on the section the surface area of each section was measured. Each section was photographed using an Olympus E1 digital SLR camera using a macro lens and scale. Images were rescaled and processed using Adobe Photoshop v7.0. Leica Qwin was calibrated to the scales used and the images imported. Then the areas of pyloric metaplasia and the total section area was calculated by manually outlining the areas within QWin.

In order to attempt to replicate the initial results all available histopathology reports for patients who had undergone terminal ileal resection for Crohn’s disease were reviewed. The presence, absence or no mention of pyloric metaplasia was recorded. An association with **CARD15** (**NOD2**) genotype and ATG16L1 genotype was investigated.

4.2.5 **Lymphoid aggregates**

The number of lymphoid aggregates below the *muscularis mucosa* were counted for each ileal section. Lymphoid aggregates were defined as pathological lymphoid cell clusters visible as distinct aggregates at 40x magnification (Borley, Mortensen et al. 2000). For areas of confluent lymphoid follicles each germinal centre was counted as one aggregate. This count was then corrected for total section surface area therefore reducing bias for the variation in section number.

4.2.6 **Granulomas**

The number of epithelioid granulomas were counted in each ileal section. Granulomas were defined as 5 or more epithelioid cells in close association visible at 100x magnification according to published morphology (Borley, Mortensen et al. 2000). This count was then corrected for total section surface area therefore reducing bias for the variation in section number.
Assessment of each patients’ sections was performed blind to genotype, smoking history and order of resection. Sections were preferably from the first small bowel resection or initial right hemicolectomy, however the second resection was used where the first was unavailable. Scores for each feature were then compared between first and second resection to ensure the validity of using the second resections. Only one resection was included for each patient. Scores were then compared with respect to CARD15 (NOD2) genotype and smoking using analysis of variance, t-test and chi-squared.

In order to confirm the initial findings all available histopathology reports were read and the presence or absence/no mention of granuloma was recorded. This was then compared among CARD15 (NOD2) and ATG16L1 genotypes.

### 4.3 Results

#### 4.3.1 Demographics

Sections for full histological scoring were from 59 patients, 49 patients with Crohn’s disease and 10 controls. Patient demographics are shown in Table 13. Genotyping results are shown in Table 20. There was no difference in inflammatory scores between CARD15 (NOD2) genotypes. A mean of 24 (5 to 62) well orientated crypts were counted per patient. An additional 10 sections were included in the Paneth cell counting group. 33 patients had had one operation only, 15 patients had had two resections, 8 patients had had three resections and 2 patients had had four resections.

<table>
<thead>
<tr>
<th>Characteristics of Crohn’s disease Patients</th>
<th>n=49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at operation (range)</td>
<td>34.7 (14 to 66) years</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>15/34</td>
</tr>
<tr>
<td>Smoking (Yes/no)</td>
<td>32/17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Controls</th>
<th>n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at operation (range)</td>
<td>73 (53 to 81) years</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>4/6</td>
</tr>
</tbody>
</table>
Table 21 *CARD15 (NOD2)* genotype, one genotype R702W/Rare SNP. 26 wild-type, 19 heterozygotes and 13 homozygotes/compound heterozygotes.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>R702W</th>
<th>G908R</th>
<th>L1007fs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td>26</td>
</tr>
<tr>
<td>R702W</td>
<td>11</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>G908R</td>
<td>5</td>
<td></td>
<td>0</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>L1007fs</td>
<td>3</td>
<td></td>
<td></td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

4.3.2 First and second resections

There was no significant difference in the mean Paneth cell count, mean Paneth cell height, and corrected pyloric metaplasia score and order of resection, Table 22, Figure 34. Pyloric metaplasia was significantly more likely to be present in the first resection than the second, Table 23.

![Mean Paneth cell count lysozyme and H&E identification](image)

Figure 33 Comparison between mean Paneth cell count and lysozyme and H&E staining identification. (PCC=Paneth cell count, H&E=haematoxylin and eosin staining, Lyz=lysozyme immunohistochemistry)
## Table 22 Comparison of assessment scores over successive resections

<table>
<thead>
<tr>
<th></th>
<th>Mean (95% confidence interval)</th>
<th>First resection</th>
<th>Second resection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammatory score</strong></td>
<td></td>
<td>0.6 (0.16 to 1.0)</td>
<td>0.6 (0.04 to 1.1)</td>
</tr>
<tr>
<td><strong>Corrected pyloric metaplasia score</strong></td>
<td></td>
<td>145.36 (18.08 to 272.65)</td>
<td>495.43 (-485 to 1476)</td>
</tr>
<tr>
<td><strong>Granuloma score</strong></td>
<td></td>
<td>0.0022 (-0.0017 to 0.0061)</td>
<td>0.001 (-0.0002 to 0.0022)</td>
</tr>
<tr>
<td><strong>Lymphoid aggregate score</strong></td>
<td></td>
<td>0.034 (0.019 to 0.049)</td>
<td>0.056 (0.019 to 0.094)</td>
</tr>
</tbody>
</table>
Figure 34 Error bars showing 95% confidence intervals indicating no significant difference in Paneth cell count (top) and Paneth cell height (bottom) between successive resections. (PCC= Paneth cell count, PCH=Paneth cell height, H&E= haematoxylin and eosin stain, Op_No= operation number)
Table 23 Comparison of features over successive resections. *Significant difference

<table>
<thead>
<tr>
<th></th>
<th>Resection number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Paneth cell migration</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>5</td>
</tr>
<tr>
<td>Absent</td>
<td>2</td>
</tr>
<tr>
<td>Pyloric metaplasia*</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>7</td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Granuloma</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>3</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
</tr>
</tbody>
</table>
4.3.3 Paneth cell counts

More Paneth cells were counted when H&E (mean 6.3) was used to identify Paneth cells compared with lysozyme (mean 5.3), Figure 33.

Figure 35 Mean Paneth cell count compared between control and Crohn’s disease section. H&E (a) p=0.014, lysozyme (b) p=0.1. (PCC= Paneth cell count)

There were significantly more Paneth cells counted by H&E in Crohn’s disease sections compared to controls, Figure 35.
The controls were significantly older than the Crohn’s disease patients. There was no significant correlation between age and Paneth cell count (mean) in the Crohn’s disease patients, Figure 36.

![Mean paneth cell count and age at operation - Crohn's disease](image)

Figure 36 Scatterplot with linear regression plot showing no significant association between Paneth cell count (mean) and age in Crohn’s disease, F 3.211, p=0.08.

There was no significant association between age and Paneth cell count (mean) in controls, Figure 37.
Figure 37 Scatterplot with linear regression plot showing no significant association between age and Paneth cell count (mean) in controls, \( F_{2.021}, p = 0.193 \).

There was no significant difference in mean Paneth cell count and \textit{CARD15 (NOD2)} genotype, Figure 38, Table 24.
Mean Paneth cell (lysozyme) count and NOD2 genotype

Error Bars show 95.0% CI of Mean

Control  wild-type  heterozygote  homozygote/compound heterozygote

Figure 38 Mean Paneth cell count using H&E (a) and Lysozyme (b) identification and CARD15 (NOD2) genotype. There is no significant difference between genotypes. (PCC=Paneth cell count)
Table 24 Summary of Paneth cell counts for Crohn’s disease and controls and \textit{CARD15} (\textit{NOD2}) genotype. *p=0.014. H&E=haematoxylin and eosin staining, Lysozyme=lysozyme immunohistochemical identification of Paneth cells.

<table>
<thead>
<tr>
<th></th>
<th>Mean Paneth cell count</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H&amp;E (n=)</td>
<td>Lysozyme (n=)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>4.9(^{a}) (10)</td>
<td>4.4(6)</td>
</tr>
<tr>
<td>Crohn</td>
<td></td>
<td>6.5(^{a}) (57)</td>
<td>5.5(33)</td>
</tr>
<tr>
<td>\textit{CARD15}(\textit{NOD2}) genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td>6.8 (26)</td>
<td>5.6 (6)</td>
</tr>
<tr>
<td>Heterozygote</td>
<td></td>
<td>6.6 (18)</td>
<td>5.7 (13)</td>
</tr>
<tr>
<td>Homozygote/Compound heterozygote</td>
<td></td>
<td>5.9 (12)</td>
<td>5.3 (9)</td>
</tr>
<tr>
<td>smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex</td>
<td></td>
<td>5.8 (13)</td>
<td>5.9 (10)</td>
</tr>
<tr>
<td>Never</td>
<td></td>
<td>6.9 (21)</td>
<td>5.5 (13)</td>
</tr>
<tr>
<td>Current</td>
<td></td>
<td>6.7 (22)</td>
<td>5.3 (10)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>6.6 (16)</td>
<td>6.1 (13)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>6.5 (40)</td>
<td>5.2 (20)</td>
</tr>
</tbody>
</table>
4.3.4 Paneth cell height

No significant difference was found in Paneth cell height when H&E was used to identify Paneth cells compared with lysozyme, Figure 39.

![Mean Paneth cell height lysozyme and H&E identification](image)

Figure 39 Comparison between Paneth cell height (cells) and H&E and lysozyme identification (PCH=Paneth cell height).

Paneth cells were found significantly higher up the crypt by cell height in Crohn’s disease compared to controls, p=0.015, Figure 40.
Mean Paneth cell (H&E) height and disease status

![Graph (a)]

Mean Paneth cell (lysozyme) height and disease status

![Graph (b)]

Mean Paneth cell (lysozyme) height (nm) and disease status

![Graph (c)]

Figure 40 Mean Paneth cell height between Crohn’s disease and control measured in cell height (a) H&E, (b) lysozyme and nanometres (c) (PCH=Paneth cell height)
There was no difference in Paneth cell height and \textit{CARD15 (NOD2)} genotype, Figure 41, summary Table 25.

![Mean Paneth cell (H&E) height and NOD2 genotype](image)

Figure 41 Mean Paneth cell height and \textit{CARD15 (NOD2)} genotype (a) H&E staining, (b) lysozyme immunohistochemistry.

### 4.3.5 Paneth cell migration

There was no significant association between \textit{CARD15 (NOD2)} genotype and Paneth cell migration. No Paneth cell migration was seen in controls. There was no significant association between smoking and Paneth cell migration.
Table 25 Summary of mean Paneth cell height. *p=0.015. H&E=haematoxylin and eosin staining, Lysozyme=lysozyme immunohistochemical identification of Paneth cells.

<table>
<thead>
<tr>
<th></th>
<th>Mean Paneth cell height (cells)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H&amp;E (n=)</td>
<td>Lysozyme(n=)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>4.3* (10)</td>
<td>4.9 (6)</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td></td>
<td>7.8* (57)</td>
<td>7.3 (33)</td>
</tr>
<tr>
<td><strong>CARD15 (NOD2) genotype</strong></td>
<td>Wild type</td>
<td>8.6 (10)</td>
<td>7.8 (13)</td>
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<tr>
<td></td>
<td>Heterozygote</td>
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<td>7.7 (9)</td>
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<tr>
<td></td>
<td>Homozygote/Compound</td>
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<td>6.2 (11)</td>
</tr>
<tr>
<td></td>
<td>heterozygote</td>
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<td></td>
</tr>
<tr>
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<td>8.5 (10)</td>
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<td>No</td>
<td>7.2 (21)</td>
<td>5.9 (13)</td>
</tr>
<tr>
<td></td>
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<td>7.8 (13)</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>8.0 (40)</td>
<td>6.9 (20)</td>
</tr>
</tbody>
</table>
4.3.6 **Pyloric metaplasia**

There was no significant difference in area of pyloric metaplasia between *CARD15* (*NOD2*) genotypes as measured by this technique, Figure 42. A single small area of pyloric metaplasia was seen in one control. In a pilot study, pyloric metaplasia was significantly more likely to be present in compound heterozygotes/homozygotes and heterozygotes than in wild type Crohn’s patients, Figure 43. There was no significant difference in pyloric metaplasia between smokers and non smokers.

![Corrected pyloric metaplasia score](image)

**Figure 42** There was no significant difference in the mean pyloric metaplasia score between *CARD15* (*NOD2*) genotypes (Cor_PM= corrected pyloric metaplasia score).
Figure 43 Pyloric metaplasia was significantly more likely in CARD15 (NOD2) compound heterozygotes/homozygotes than wild type (p=0.024) Crohn’s patients. Pyloric metaplasia was significantly more likely in CARD15 (NOD2) heterozygotes than wild type Crohn’s patients (p=0.024).

In order to confirm the initial findings, pathology reports for the entire cohort of patients with terminal ileal resections were reviewed. A total of 115 patients had reports and genotyping results available. No difference in was seen among CARD15 (NOD2) genotypes and the number of patient with pyloric metaplasia mentioned in the histopathology report, Figure 44. There was no difference when the number of patients who had ever had pyloric metaplasia was investigated. No difference in was seen among ATG16L1 genotypes (n=42) and the number of patient with pyloric metaplasia mentioned in the histopathology report, Figure 45.
Figure 44 Bar chart to demonstrate the finding of pyloric metaplasia (PM) in Crohn’s disease sections as mentioned in the pathology report. There is no significant difference in the presence of pyloric metaplasia and the number of NOD mutations.

Figure 45 There was no significant difference in ATG16L1 genotype and the presence, as mentioned in the histopathology report, of pyloric metaplasia in Crohn’s disease sections.
4.3.7 **Lymphoid aggregates**

There was no significant difference in number of lymphoid aggregates and *CARD15 (NOD2)* genotype. No lymphoid aggregates were found in controls. There was no significant difference in number of lymphoid aggregates between smokers and non smokers.

4.3.8 **Granulomas**

Granulomas were not associated with *CARD15 (NOD2)* genotype. No granulomas were found in controls. There was no significant difference in number of granulomas between smokers and non smokers.

A total of 115 patients had reports and genotyping results available. No difference in was seen among *CARD15 (NOD2)* genotypes and the number of patient with granuloma mentioned in the histopathology report, Figure 46. There was no difference when the number of patients who had ever had granuloma was investigated. No difference in was seen among ATG16L1 genotypes (n=42) and the number of patient with granuloma mentioned in the histopathology report, Figure 47.

![CARD15 (NOD2) and granuloma](image)

Figure 46 There was no significant difference in the presence of granuloma in pathology reports and *CARD15 (NOD2)* genotype.
Figure 47 There was no significant difference in ATG16L1 genotype and the presence of granuloma in the pathology report.
4.4 Discussion

It was hypothesized that there would be a histological phenotype associated with CARD15 (NOD2) genotype. There are a number of typical features of Crohn’s disease including transmural inflammation, lymphoid aggregates and granulomas (Day, Jass et al. 2003). Pyloric metaplasia is a recognised feature of Crohn’s disease (Koukoulis, Ke et al. 2002; Day, Jass et al. 2003). CARD15 (NOD2) mutations are associated with ileal (Cuthbert, Fisher et al. 2002; Büning, Genschel et al. 2004) and fibrostenosing (Abreu, Taylor et al. 2002) phenotype. CARD15 (NOD2) is expressed in Paneth cells (Lala, Ogura et al. 2003; Ogura, Lala et al. 2003) and may influence Paneth cell morphology (Abeya, Bromfield et al. 2005).

Smoking is associated with a higher risk of developing Crohn’s disease (Lindberg, Tysk et al. 1988; Calkins 1989). It is associated with a worse clinical course and more frequent relapses (Cosnes, Carbonnel et al. 1999; Ryan, Allan et al. 2004) although the mechanism through which smoking exerts this effect is unknown. It is possible that smoking would be associated with a more severe histological phenotype.

People with Crohn’s disease had significantly more Paneth cells than controls. There was no significant association with age and Paneth cell count in controls or in people with Crohn’s disease suggesting a genuine effect. This is suggested by a previous investigation in which more Paneth cells were found in zone 2 of the small intestinal crypt in a small cohort of people with Crohn’s disease (Kelly, Feakins et al. 2004). There is evidence to suggest in intestinal metaplasia in the stomach there is deregulation of Paneth cell proliferation (Wong, Stamp et al. 2000). Much work has been done to investigate the control of Paneth cell differentiation. Migrated Paneth cells in mice have been found not to express Frizzled-5 (van Es, Jay et al. 2005). Paneth cells localised to the crypt base still express Frizzled-5. The migrated Paneth cells lack expression of the Wnt signal transducer nuclear β-catenin, and the Wnt target genes EphB3, cryptidin-1 and cryptidin-6 (Batlle, Henderson et al. 2002). It has been found that there is an increase in cell proliferation markers in Crohn’s disease (Noffsinger, Unger et al. 1998) with an increase in staining for Ki-67, a cell proliferation marker in the basal one-third of crypts. Through an unknown mechanism CARD15 (NOD2), possibly through bacterial handling, mutations appear to alter Paneth cell proliferation. The deregulated Paneth cell proliferation found in intestinal
metaplasia demonstrated by Wong et al 2000 could suggest a mechanism by which Paneth cells in Crohn’s disease are increased (Wong, Stamp et al. 2000).

Pyloric metaplasia was initially found to be significantly associated with \textit{CARD15} (\textit{NOD2}) homozygotes/compound heterozygotes and heterozygotes compared with wild type Crohn’s patients. As expected only one very small area of pyloric metaplasia was found in the control population. Pyloric metaplasia is not found exclusively in Crohn’s disease. There was no significant difference in the area of pyloric metaplasia in \textit{CARD15} (\textit{NOD2}) homozygotes/compound heterozygotes than heterozygotes and wild type Crohn’s patients. In an attempt to replicate these findings all available histopathology reports were reviewed. No significant difference was found. This may be because pyloric metaplasia, while known to be a feature, is not pathognomonic of Crohn’s disease and therefore may not be specifically mentioned in reports. However it would be likely that a genuine difference would be maintained between populations. In order to minimize this effect the number of patients who had ever had pyloric metaplasia mentioned in any histopathology report was investigated and no difference found. This potential finding is interesting however and warrants further detailed review of a larger number of histology sections with the specific aim of identifying pyloric metaplasia.

Pyloric metaplasia has been found to be associated with increased smooth muscle content (Borley, Mortensen et al. 2001). This increase in smooth muscle content associated with pyloric metaplasia may explain the association between carriage of \textit{CARD15} (\textit{NOD2}) mutation and fibrostenosing disease phenotype. Growth factors are produced in areas of pyloric metaplasia (Longmann, Douthwaite et al. 2000; Pera, Heppell et al. 2001) and this could influence Paneth cell numbers. There was no evidence to suggest there was more severe inflammation and ulceration in \textit{CARD15} (\textit{NOD2}) patients.

Consistent with other published data there was no association with granulomas and \textit{CARD15} (\textit{NOD2}) genotype (Pierik, de Hertogh et al. 2005). No association was found between the number of lymphoid aggregates and \textit{CARD15} (\textit{NOD2}) genotype. This is not surprising as \textit{CARD15} (\textit{NOD2}) has not been found in lymphocytes but is found almost exclusively in monocytes and Paneth cells.
This was a straightforward investigation of the histological features of Crohn’s disease and Paneth cell morphology. The features of Crohn’s disease, pyloric metaplasia and Paneth cells were identified reliably without the need for special stains. An advantage of this is the subsequent availability of archival tissue. Cases were selected with a robust diagnosis of Crohn’s disease in a relatively homogenous population. All people had severe disease Crohn’s disease requiring surgery. This investigation was cheap and simple using H&E and lysozyme for Paneth cell and histological features identification. There was an equal proportion of smokers and non-smokers. The investigator (CH) was taught to identify features of Crohn’s disease in patient whom the diagnosis is already established rather than ability to assess the histological features to make a diagnosis.

There are a number of weakness in this investigation. Sections studied were of a longitudinal 2-dimensional nature with a single cut through crypt which is a 3-dimensional structure. This may well not be representative of entire crypt. Often partial cells are seen which is an artefact of the sectioning process. H&E identifies Paneth cells but does not differentiate them from intermediate cells, these may be what we are seeing higher up the crypts. This may be indicated in the higher number of Paneth cells counted with H&E over lysozyme. Lysozyme is not found in intermediate cells, only mature Paneth cells (Calvert, Bordeleau et al. 1988). Measuring the height distribution of Paneth cells in number of cells is a simple measure that allows for errors in magnification. However this may not be representative of entire crypt due to 3-dimensional structure. It assumes that the Paneth cells lie around the base of the crypt equally all the way round. A number of patients had received disease modifying therapy and this was not controlled for. There were a limited number of sections due to tissue availability. This is a small study that therefore limits power to detect a small effect.

A better way to investigate Paneth cell morphology would be by microdissection of the entire crypt, and then count whole crypt Paneth cells. This would necessitate fresh tissue sampling. Ideally all Paneth cells would be isolated for counting. Immunohistochemistry to human defensin-5 or other unique Paneth cell product could be used to more accurately identify the Paneth cells. A more simple method would be to count multiple sections through the entire crypt. This would then be more
representative. This study used pre-cut sections, i.e. those that had been originally used on clinical grounds post operation.

Pyloric metaplasia was again identified just using H&E staining. To take this forward, the finding of an association between pyloric metaplasia and the carriage of CARD15 (NOD2) mutation needs replication in a larger cohort of patients. If the finding holds true a further investigation would be to investigate the 3-dimensional structure of pyloric metaplasia. It would be better to use a specific marker e.g. trefoil peptide to identify areas of pyloric metaplasia. Again a 2-dimensional method was used to attempt to quantify a 3-dimensional structure. It would be better to section the entire area to quantify volume rather than area of pyloric metaplasia.

Lymphoid aggregates are common, easily identifiable on H&E staining. The volume of lymphocyte load may be more useful than absolute number. The nature of lymphocytes could be useful. It would be worth exploring in future studies with cell markers to identify the cell lineages found in lymphoid aggregates to see if there is over-representation of various T cells.

Only a very small amount of the resection specimen is cut therefore reducing the chances of finding all granulomas. This study assumed equal distribution of granuloma throughout specimen. There may have been a reduced sensitivity in identifying granuloma compared with experienced pathologists as granulomas are notoriously difficult to reliably identify. In order to ensure no granuloma were missed it would be better to sample and examine the entire resection specimen. In a clinical setting this is not so practical. It would be better to use an immunological marker of granulomas, e.g. CCR5- Th1 marker an epithelioid cell marker to accurately identify all granulomas.

There are no published studies investigating the histological phenotype and CARD15 (NOD2). Previous work has concentrated on the presence or absence of granulomas only. This work was carried out blind to genotype and smoking phenotype. Unfortunately there are only 11 homozygotes included in this study. We have demonstrated a difference in Paneth cell morphology in CARD15 (NOD2) genotypes that warrents further investigation. There may be an association between pyloric metaplasia and CARD15 (NOD2). This justifies further investigation of cell proliferation in Crohn’s disease.
Chapter 5 The Influence of IL-6, COL1A1, and VDR gene polymorphisms on bone mineral density in Crohn's disease

5.1 Introduction and aims

We investigated the potential relationship between well recognised, apparently functional single nucleotide polymorphisms (SNP) of VDR, IL6 and COL1A1 and bone mineral density (BMD) in a cohort of well characterised patients with Crohn’s disease.

The SNPs investigated were: The FokI and TaqI polymorphisms of VDR, the G to C SNP at position –174 of IL6 and a polymorphism at the Sp1 transcription factor binding site of COL1A1. The influence of CARD15 (NOD2) mutations and BMD were studied.

This project looking at the genetics of bone mineral density in Crohn’s disease started before the work for the MD. The patient recruitment was done by Dr Thompson, Dr Bartram and Dr Mansfield. The genotyping was performed by Dr A Sutherland-Craggs. The analysis of the data and the preparation of the manuscript undertaken by myself.
5.2 Materials and methods

5.2.1 Patients

Patients were recruited from the inflammatory bowel disease register at the Freeman Hospital and the Royal Victoria Infirmary, Newcastle upon Tyne, and the Queen Elizabeth Hospital, Gateshead. All patients had a diagnosis of Crohn’s disease and were under the care of either a surgical or medical gastroenterologist and were diagnosed by a combination of radiology, endoscopy and biopsy. Only patients aged between 24 and 70 years were included, and women who were pregnant or planning a pregnancy were excluded because of the potential risk from exposure to ionizing radiation during bone densitometry. Initial demographic data were collected by questionnaire including age, smoking history, reproductive and menstrual history in women. Details including duration and site of disease, corticosteroid use in months, surgical and drug history were obtained both through direct interview and from the medical notes. Prior to bone densitometry height and weight were measured and body mass index calculated. The Newcastle upon Tyne Joint Ethics Committee granted ethical approval.

For comparison, when testing the hypothesis that VDR genotype is associated with Crohn’s disease a geographically and racially matched cohort of healthy controls was recruited from in vitro fertilisation donors and healthcare workers.

5.2.2 Bone mineral density measurement

BMD was measured at the lumbar spine (LSP) and total left hip using dual energy x-ray absorptiometry (DEXA) as previously described (Bartram, Peaston et al. 2003). Osteoporosis is defined by WHO as a bone density 2.5 or more standard deviations below the mean value for young adults and osteopenia as a T score of -1 to -2.5 at any site (Kanis 1994). Classification as normal BMD, osteopenic or osteoporotic is dependent on the site with the lowest BMD T score.

5.2.3 Genotyping

5.2.3.1 Determination of VDR Taq1 genotype

A 340 bp fragment which included the site of the T to C polymorphism at codon 352 in exon 9 of the VDR gene on chromosome 12 was amplified in a 25µl reaction mixture
containing 200 µM each of dATP, dCTP, dGTP and dTTP (ABgene, Surrey, UK), 10mM tris-HCl pH 8.3, 50mM KCl, 0.01% gelatin, 1U Taq Polymerase (ABgene, Surrey, UK), 0.5 µM of each primer and 1µl (approx. 0.2 to 0.5 µg) template DNA on a Perkin-Elmer GeneAmp 9700. The alleles relating to this polymorphism are designated “t” (Taq I site present) or T (Taq I site absent).

The primers used were: 5’CAgAgCATggACAgggAgCAAg-3’ forward and 5’gggTgTACgTCTgCAgTgTg-3’ reverse (Oswel, Southampton, UK).

The PCR amplification conditions were as follows: initial denaturation at 94°C for 6 min followed by 35 cycles of, 94°C for 45 seconds, 63°C for 60 seconds, followed by 72°C for 75 seconds and a final extension at 72°C for 7 minutes.

Following amplification, 15µl of the product was digested with 2.5 units of Taq I (ABgene, Surrey, UK), at 65°C. Digested restriction fragments were separated on 2.5% (w / v) agarose (Sigma, Poole, Dorset, UK) gels, with 1x Tris-Borate-EDTA (TBE) running buffer (Sigma, Poole, Dorset, UK) Samples of known genotype were run as controls and a water negative (DNA free) control in each batch.

Bands were visualised on an Alpha Innotech Imager system and assigned as follows: T/T, 340 bp only, T/t, 340 bp, 293 bp and 47 bp, t/t, 293 bp and 47 bp.

5.2.3.2 Determination of VDR Fok1 genotype.

A 265 bp fragment which includes the site of a C to T polymorphism at the start codon was amplified as above. The alleles which result from this change are designated “f” (FokI site present) or “F” (FokI site absent).

The primers used were: 5’ACgTggCCCTggCACTgACTCTgCTCT-3’ forward and 5’ATggAAACACCTtgCTCTTCTCCCTC-3’ reverse (Oswel, Southampton, UK).

The PCR amplification conditions were as follows: initial denaturation at 94°C for 5 min followed by 32 cycles of, 94°C for 45 seconds, 58°C for 45 seconds, followed by 72°C for 45 seconds and a final extension at 72°C for 7 minutes.

Following amplification, 15µl of the product was digested with 4 units of FokI restriction endonuclease (ABgene, Surrey, UK), at 37°C. Digested restriction fragments were separated on 2% (w / v) agarose (Sigma, Poole, Dorset, UK) gels as
described above. Genotypes were assigned as follows: F/F, 265 bp only, F/f, 265 bp, 169 bp and 96 bp, f/f, 169 bp and 96 bp.

5.2.3.3 Determination of IL6 -174 genotype
A 431 bp fragment which includes the site of a G to C polymorphism at position –174 in the promoter region of the IL6 gene was amplified as above. On digestion with NlaIII, the wild-type sequence with G at position -174 is not digested, whereas a C at -174 creates a restriction site for NlaIII and is digested. The primers used were: 5’CAgAAgAACTCAgATgACTgg-3’ forward and 5’gTggggCTgATTggAAACC-3’ reverse (Oswel, Southampton, UK).

The PCR reaction conditions for amplification were as follows: initial denaturation at 95°C for 5 min followed by 5 cycles of, 95°C for 30 seconds, 58°C for 60 seconds, followed by 72°C for 60 seconds, followed by 25 cycles of, 95°C for 30 seconds, 56°C for 60 seconds, followed by 72°C for 60 seconds, followed by 5 cycles of, 95°C for 30 seconds, 54°C for 60 seconds, followed by 72°C for 60 seconds and a final extension at 72°C for 7 minutes.

Following amplification, 15µl of the product was digested with 2.5 units of NlaIII restriction endonuclease (ABgene, Surrey, UK), at 37°C. Digested restriction fragments were separated on 2% (w/v) agarose gels, as described above. For the various genotypes, the following band patterns were obtained: G/G 229 bp, 173 bp and 29 bp; G/C 229 bp, 173 bp and 122 bp; C/C 229 bp, 122 bp, 51 and 29 bp.

5.2.3.4 Determination of COL1A1 genotype
A 280 bp fragment which included the site of the G T polymorphism at the Sp1 binding site in the first intron of COL1A1 gene was amplified as above. The primers used were: 5’CTGGACTATTTGCGGACTTTTTG-3’ forward and 5’GTCCAGCCCTCATCCTGGCC-3’ reverse (Oswel, Southampton, UK).

The PCR reaction conditions for amplification were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 1 min.

Following amplification, 10µl of the product was digested with 1 unit of MscI restriction endonuclease (New England Biolabs), at 37°C. Digested restriction
fragments were separated by polyacrylamide gel electrophoresis on 10% polyacrylamide gels, with 1xTBE running buffer (Sigma, Poole, Dorset, UK). Samples of known genotype were run as controls, and water as a negative (DNA free) control in each batch.

Following electrophoresis for 4 hours at 160mV, bands were visualised on an Alpha Innotech Imager system. The patterns corresponding to the various genotypes were as follows: homozygous wild-type (GG) 280 bp only, heterozygous (GT) 280 and 260 bp and homozygous mutant (TT) 260 bp only.

5.2.3.5 CARD15 (NOD2) genotyping
This was performed as previously reported in Cuthbert et al 2002 (Cuthbert, Fisher et al. 2002) in Prof C. Mathew’s laboratory, Department of Medical and Molecular Genetics, Guy’s, Kings and St. Thomas’ School of Medicine, London.

5.2.3.6 Statistical analysis
To look for differences in BMD at the hip and LSP between genotypes analysis of variance (ANOVA) was employed. Where this was significant Tukeys test for multiple comparisons was used to compare differences between groups. This was carried out using SPSS for Windows v 11.1.
5.3 Results

5.3.1 Demographics

245 patients with Crohn’s disease were studied, 158 female, age range 24 years to 70 years mean age of 44.4 years, standard deviation 11.4 years. 96 (61%) patients were pre menopausal with 62 (39%) post menopausal at the time of the study. 75 (30%) had previously smoked, 92 (37%) had never smoked and 78 (32%) were current smokers. 73 (30%) were on oral corticosteroids at the time of the study, 144 (59%) had previously had oral corticosteroids and 28 (11%) had never had oral corticosteroid treatment. Mean duration of steroid use 45 months, range 0 to 372 months, median 21 months. Duration of disease range 6 months to 50 years mean 14.3 years, SD 9.5 years. 54 (22%) were osteoporotic at any site, 106 (43%) were osteopenic at any site and 160 (65%) had normal bone density.

There were no significant differences between any genotypes and age, BMI, pre/post menopausal, smoking or steroid use, data not shown.
5.3.2 Genotype

All genotypes were in Hardy-Weinberg equilibrium. Genotype distributions are shown in Table 26. Comparing genotype distribution of COL1A1, IL6 and VDR Fok1 with those of 257 healthy controls there were no statistically significant differences. There was no difference in VDR Taq1 genotype frequency between patients with Crohn’s disease and controls, p=0.10. Control genotype frequency CC 40 (15%), TC 115 (45%) and TT 102 (40%).

<table>
<thead>
<tr>
<th>VDR Taq1 genotype</th>
<th>CC</th>
<th>TC</th>
<th>TT</th>
</tr>
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<tr>
<td>n=244</td>
<td>37 (15%)</td>
<td>130 (53%)</td>
<td>76 (31%)</td>
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</table>

<table>
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<tr>
<th>VDR Fok1 genotype</th>
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<th>Ff</th>
<th>ff</th>
</tr>
</thead>
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<tr>
<td>n=240</td>
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<td>121 (50%)</td>
<td>33 (14%)</td>
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</tbody>
</table>

<table>
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<th>GG</th>
</tr>
</thead>
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<tr>
<td>n=213</td>
<td>32 (15%)</td>
<td>76 (36%)</td>
<td>104 (49%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>COL1A1 genotype</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=180</td>
<td>95 (53%)</td>
<td>49 (27%)</td>
<td>36 (20%)</td>
</tr>
</tbody>
</table>
5.3.3 Genotype and bone mineral density

5.3.3.1 VDR Taq1
There was no significant difference in BMD at the LSP or hip between VDR Taq1 genotypes. There was also no difference in T score at the LSP and hip and VDR Taq1 genotype, Table 27.

5.3.3.2 VDR Fok1
There was no significant difference in BMD at the LSP or hip between VDR Fok1 genotypes. There was also no difference in T score at the LSP and hip and VDR Fok1 genotype, Table 27.

5.3.3.3 IL6
At the LSP there was a significant difference in BMD between IL6 genotypes (p=0.041). This difference was between the GG and CC genotypes by pair-wise comparison allowing for multiple testing. The CC genotype having a higher mean BMD at 1.03 g/cm² than the GG genotype at 0.95 g/cm² respectively, 95% confidence interval for difference 0.0032 to 0.1524. There was only a trend towards significance between T score and genotype p=0.065, Table 27.

At the hip there was a significant difference in BMD between IL6 genotypes (p=0.014) (Table 27). The CC genotype had a higher mean BMD of 0.93 g/cm² compared to the GG mean BMD of 0.48 g/cm², 95% confidence interval for difference 0.0186 to 0.159. The difference between genotype groups when BMD was expressed as T score was statistically significant p=0.013. Mean T score was for CC genotype –0.709 and for GG genotype –1.37. The 95% confidence interval for the difference was 0.119 to 1.208.

5.3.3.4 COL1A1
There was no significant difference in LSP BMD between COL1A1 genotypes, p=0.144. There was also no significant difference in T score at the LSP between genotypes, p=0.14 and p=0.24, Table 27.

At the hip there was a significant difference in BMD between COL1A1 genotypes (p=0.034), (Table 27). This was between the homozygous wild-type (GG) and heterozygous (GT) genotypes. The mean BMD for the GG genotype was 0.88 g/cm²
and for GT 0.81 g/cm², with the 95% confidence interval for difference 0.0051 to 0.1307. There was also a significant difference in T scores between genotypes p=0.025. Again the GG genotype had a higher T score –1.03 than the GT genotype which was –1.59 with a 95% confidence interval for difference 0.066 to 1.063.

**Table 27 Bone mineral density g/cm², mean (standard deviation) for each genotype.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>LSP BMD</th>
<th>LSP T score</th>
<th>Total hip BMD</th>
<th>Hip T score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VDR Taq1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0.98 (0.13)</td>
<td>-0.73 (1.19)</td>
<td>0.86 (0.15)</td>
<td>-1.15 (1.20)</td>
</tr>
<tr>
<td>TC</td>
<td>0.98 (0.16)</td>
<td>-0.70 (1.47)</td>
<td>0.87 (0.15)</td>
<td>-1.12 (1.25)</td>
</tr>
<tr>
<td>TT</td>
<td>0.96 (0.15)</td>
<td>-0.95 (1.44)</td>
<td>0.84 (0.15)</td>
<td>-1.29 (1.17)</td>
</tr>
<tr>
<td><strong>VDR Fok1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ff</td>
<td>0.95 (0.18)</td>
<td>-1.04 (1.67)</td>
<td>0.85 (0.19)</td>
<td>-1.30 (1.54)</td>
</tr>
<tr>
<td>Ff</td>
<td>0.97 (0.15)</td>
<td>-0.80 (1.41)</td>
<td>0.85 (0.14)</td>
<td>-1.21 (1.15)</td>
</tr>
<tr>
<td>FF</td>
<td>0.99 (0.15)</td>
<td>-0.65 (1.35)</td>
<td>0.89 (0.15)</td>
<td>-1.05 (1.16)</td>
</tr>
<tr>
<td><strong>IL6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1.03 (0.15) †</td>
<td>-0.33 (1.41)</td>
<td>0.93 (0.14) †</td>
<td>-0.71 (1.03) †</td>
</tr>
<tr>
<td>GC</td>
<td>0.99 (0.17)</td>
<td>-0.69 (1.50)</td>
<td>0.87 (0.14)</td>
<td>-1.06 (1.09)</td>
</tr>
<tr>
<td>GG</td>
<td>0.95 (0.15)</td>
<td>-0.98 (1.37)</td>
<td>0.48 (0.15)</td>
<td>-1.37 (1.25)</td>
</tr>
<tr>
<td><strong>COL1A1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>0.98 (0.14)</td>
<td>-0.75 (1.33)</td>
<td>0.88 (0.15)</td>
<td>-1.03 (1.19)</td>
</tr>
<tr>
<td>GT</td>
<td>0.94 (0.15)</td>
<td>-1.15 (1.39)</td>
<td>0.81 (0.14) †</td>
<td>-1.59 (1.18) †</td>
</tr>
<tr>
<td>TT</td>
<td>0.99 (0.16)</td>
<td>-0.59 (1.51)</td>
<td>0.87 (0.15)</td>
<td>-1.08 (1.27)</td>
</tr>
<tr>
<td><strong>CARD15 (NOD2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.98 (0.15)</td>
<td>-0.77 (0.15)</td>
<td>0.87 (0.15)</td>
<td>-1.14 (1.22)</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>0.96 (0.17)</td>
<td>-0.89 (1.50)</td>
<td>0.85 (0.15)</td>
<td>-1.26 (1.14)</td>
</tr>
<tr>
<td>Homozygote</td>
<td>0.97 (0.16)</td>
<td>-0.95 (1.44)</td>
<td>0.88 (0.17)</td>
<td>-1.27 (1.23)</td>
</tr>
</tbody>
</table>

† indicates a significant effect, p<0.05
5.3.3.5 CARD15 (NOD2)

There was no significance in BMD or T score between CARD15 (NOD2) genotypes, table 2. The frequencies of CARD15 (NOD2) genotypes compared to controls are shown in Table 28. As expected there was a higher frequency of CARD15 (NOD2) mutations in the Crohn’s disease population than the control group.

Table 28 CARD15 (NOD2) genotype frequency

<table>
<thead>
<tr>
<th>CARD15 (NOD2) genotype</th>
<th>Wild Type</th>
<th>Heterozygote</th>
<th>Homozygote</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control n=104</td>
<td>77</td>
<td>7 (7%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(91%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease n=223</td>
<td>167 (75%)</td>
<td>46 (21%)</td>
<td>10 (4%)</td>
</tr>
</tbody>
</table>


5.4 Discussion

This is a large study of the relationship between genes and BMD in a well characterised population of patients with Crohn’s disease. When analysed by genotype there was no difference in duration of disease, steroid use, age, BMI or smoking status allowing valid comparisons to be made.

A significant association was found between \textit{IL6} polymorphisms and lower BMD at the LSP and hip in patients with Crohn’s disease. The largest difference being seen at the hip where the GG (49%) genotype had a 9.6% lower mean BMD than the CC (15%) genotype, at the LSP there was a 7.5% difference. From the analysis, the C allele appears to produce a protective effect as the GC heterozygote has significantly higher BMD than the GG homozygote.

The finding of an association between \textit{IL6} polymorphisms and BMD in Crohn’s is consistent with previous work (Schulte, Dignass et al. 2000). Schulte et al 2000 studied only 83 patients with a mixture of Crohn’s disease and ulcerative colitis. \textit{IL6} polymorphisms have also been associated with lower BMD in post-menopausal women (Ferrari, Garnero et al. 2001) and with lower peak bone mass in healthy males (Lorentzon, Lorentzon et al. 2000). Although in another small study of 75 patients with Crohn’s disease from a cohort of 105 patients with inflammatory bowel disease, no association was found between \textit{IL6} polymorphism and BMD (Schulte, Goebell et al. 2001). This last study however was a much smaller study than the present investigation and BMD was expressed as Z score only, rather than as absolute BMD. In addition the effect of \textit{IL6} seen in the present investigation is relatively small and the earlier study was significantly underpowered by comparison.

\textit{IL6} is thought to be involved in bone remodelling and hence development of osteoporosis through stimulation of osteoclast development and regulation of bone resorption (Manolagas 1995; Manolagas and Jilka 1995; Manolagas 1998). Higher levels of \textit{IL6} have been found in patients with Crohn’s disease (Pollak, Karmeli et al. 1998) and serum levels of \textit{IL6} have been found to predict bone loss in post-menopausal women (Scheidt-Nave, Bismar et al. 2001). Studies \textit{in vitro} have shown that the CC \textit{IL6} genotype results in lower stimulated \textit{IL6} promoter activity and \textit{in vivo} with lower levels of plasma \textit{IL6} (Fishman, Faulds et al. 1998). In post-menopausal women CC \textit{IL6} polymorphism was found to be associated with lower bone resorption and less of a
loss in BMD compared with GG genotype (Ferrari, Garnero et al. 2001). It is possible that in Crohn’s disease, a chronic inflammatory condition that results in higher serum levels of IL6, the CC genotype is protective against IL6 mediated bone resorption.

This study found a significantly lower BMD at the hip in Crohn’s disease patients with heterozygous COL1A1 (27%) genotype compared with homozygous wild-type (53%). At the hip the heterozygous genotype resulted in a 6% reduction in BMD. At the hip there was also a significant reduction in T score to a level below that where therapy is recommend in glucocorticoid induced osteoporosis (Eastell, Reid et al. 1998).

This study is in agreement with other published work on COL1A1 gene polymorphisms. COL1A1 gene polymorphisms have been associated with low BMD and fractures in other populations (Grant, Reid et al. 1996; Uitterlinden A G, Burger H et al. 1998). A recent meta-analysis including 6800 subjects also found a significantly lower BMD at the lumbar spine in the heterozygous group compared to homozygous wild-type but no significant difference in the homozygous TT group (Mann and Ralston 2003).

COL1A1 gene polymorphisms at the Sp1 binding site are thought to be functional, altering the level of transcription and production of collagen and therefore the physical properties of bone (Mann, Hobson et al. 2001). Osteoblasts derived from heterozygote subjects were found to have altered production of the collagen α1(1) chain relative to the α2(1) when compared to wild-type individuals. The same study found that bone strength was reduced in people with heterozygotes compared with wild-type subjects.

In this analysis there was no association between BMD and either two of the VDR SNPs studied. A meta-analysis of 16 studies in 1996 found a small effect for VDR polymorphisms (Cooper and Umbach 1996), with a 2.4% difference in BMD between genotypes. Such a small effect has limited clinical significance. The numbers in this study are adequate to detect a difference of between 4% and 5% between genotypes.

No association has been found in this study between VDR Taq1 polymorphism and susceptibility to Crohn’s disease. This is in contrast to previous published data of 245 patients with Crohn’s who were found to have an increased frequency of tt genotype (synonymous with CC in this study) (Simmons, Mullighan et al. 2000). The results here are in agreement with a small study of 95 patients with Crohn’s disease in which no association was found (Martin, Radlmayr et al. 2002).
The clinical application for genotyping data is to identify those patients with Crohn’s disease who are more at risk for developing osteoporosis. Multiple risk factors for development of osteoporosis have been identified including body mass index and steroid use (Siffledeeen, Fedorak et al. 2004). Development of risk stratification by genotype as well as clinical risk may enable targeted treatment with agents such as pamidronate (Bartram, Peaston et al. 2003) or other bisphosphonates (Adadchi, Bensen et al. 1997) or calcium supplements (Sambrook, Kotowicz et al. 2003) dependant on risk. Patients could be tested prior to starting corticosteroids for example and those at greater risk of developing osteoporosis be targeted with more aggressive screening and treatment. Crohn’s disease patients are younger than other disease groups previously studied therefore earlier identification of those at risk has potential to be therapeutically important.
6 Chapter 6 Genetics

It has long been recognised that Crohn’s disease has a strong genetic element. Crohn reported regional enteritis in two sisters (Crohn 1934). A further case report of regional enteritis in 3 siblings was published in 1939 (Brown and Scheifley 1939). The genetic basis of Crohn’s disease has been extensively investigated. A review in 1971 by McConnell (McConnell 1972) surmised that a family history of siblings with Crohn’s disease was more common than a vertical family history. It was hypothesized that there was both a shared environment and shared genetic predisposition. The inheritance pattern was not Mendelian but a complex polygenic disorder with a combination of environmental and genetic factors determining the development of Crohn’s disease. It was suggested by the authors that there may be a number of genes in common between Crohn’s disease, ulcerative colitis and ankylosing spondylitis.

Complex segregation analysis has suggested that Crohn’s disease follows a recessive pattern of inheritance (Küster, Pascoe et al. 1989) (Orholm, Iselius et al. 1993). By contrast ulcerative colitis was thought to represent the effects of a dominant gene with incomplete penetrance.

Some of the earliest genetic studies used candidate gene analysis. There are a number of limitations in this method; it can only be used with known genes, multiple genes needs correction for multiple testing, there is publication bias with positive findings more likely to be published. There needs to be geographical and ethnic matching of cases and controls. The probability of false positive results is high.

The advantages of this technique is that a relatively small case-control population can be used and is therefore good at finding genes with small effect, an advantage in complex diseases with multiple genes with small effect. A modification of candidate gene analysis has subsequently been used to attempt to identify relevant genes within susceptibility loci highlighted in genome-wide linkage and genome-wide association studies.

The genetics of inflammatory bowel disease was advanced by the development of genome-wide linkage studies. The first linkage map was published in 1992 using 814 polymorphic markers across the entire genome (Weissenbach, Gyapay et al. 1992).
The genome-wide scan published by Hugot in 1996 identified an area of linkage on chromosome 16 (Hugot, Laurent-Puig et al. 1996). This was to be identified later as \textit{CARD15 (NOD2)} (Hugot, Chamaillard et al. 2001; Ogura, Bonen et al. 2001).

A haplotype on chromosome 5q31 was identified in 2000 (Rioux, Silverberg et al. 2000) and association with Crohn’s disease has been replicated in other studies (Rioux, Daly et al. 2001; Negoro, McGovern et al. 2003; van Heel, Fisher et al. 2004; Vermeire, Pierik et al. 2005; Onnie, Fisher et al. 2006). DLG5 on chromosome 10q23 is a further area of interest (Stoll, Corneliussen et al. 2004) however association is variable in studies (Yamazaki, Takazoe et al. 2004; Daly, Pearce et al. 2005; Noble, Nimmo et al. 2005; Török, Glas et al. 2005; Vermeire, Pierik et al. 2005; Yamada and Yamamoto 2005; Büning, Geerdts et al. 2006; Ferraris, Torres et al. 2006; Friedrichs, Brescianini et al. 2006; Lakatos, Fischer et al. 2006; Medici, Mascheretti et al. 2006; Newman, Gu et al. 2006; Tremelling, Waller et al. 2006).

Despite the evidence that Crohn’s disease has a strong heritability up until recently 2006 there were only 2 genetic loci that could be reliably replicated; \textit{CARD15 (NOD2)} and 5q31 with a third on chromosome 10q23 with less consistent replication together with the MHC region on chromosome 6. The combined, modest effect of these genetic loci is not sufficient to account for the genetic load in Crohn’s disease. It was put forward in 1996 that large-scale testing by association analysis was going to be necessary to detect the multiple genes of modest effect implicated in complex diseases (Risch and Merikangas 1996). The technology; that is the human genome project, development of HapMap and large scale genotyping methods, however did not become available until recently and the first published genome-wide association scan was in 2005 (Yamazaki, McGovern et al. 2005).

Genome wide association studies use a case-control method to investigate association between disease and thousands of SNPs throughout the genome. Technological progress through the human genome project and the development of the HapMap has enabled this technique. The HapMap is a schematic of the human genome in which a minimal number of SNPs can be used to tag common haplotype blocks, rather than using every known SNP throughout the genome. The technique can use either SNPs evenly spaced across the entire genome or use the HapMap to tag common haplotypes. Allele frequencies are then compared between cases and controls. Large sample size and well matched ethnic and geographical controls are needed due to the multiple
testing employed and the modest increase in disease risk conferred by any variant. Statistical methods have had to be developed to enable interpretation of the large number of results produced.
Chapter 7 Conclusions

Crohn’s disease is a common chronic disease with an incidence in Northern Europe of 3.9-7 per 100,000 person years (Shivananda, Lennard-Jones et al. 1996). It is a cause of increased morbidity and mortality in predominantly people of working age with a significant cost to the overall population (Hellier, Sanderson et al. 2006). Crohn’s disease is a relapsing and remitting condition with a wide spectrum of severity. There are currently no reliable, objective predictors of disease progression at presentation. It would be useful to be able to stratify patients according to risk of severe disease in order to target more aggressive treatment to the high risk groups.

The majority of people with Crohn’s disease will require surgery at some point in the disease course and many people require multiple operations. Carriage of a CARD15 (NOD2) mutation is reliably associated with ileal disease as demonstrated in a recent meta-analysis (Economou, Trikalinos et al. 2004). The purpose of this investigation was to establish whether CARD15 (NOD2) carriage was associated with shorter time to re-operation in terminal ileal Crohn’s disease.

This is a large investigation of the time to second operation in people who have undergone terminal ileal resections for Crohn’s disease. The influence of gender, age at diagnosis, time to first surgery, disease behaviour, smoking habit and azathioprine/6-mercaptopurine use was investigated. The influence of CARD15 (NOD2) genotype and 5q31 haplotype carriage on time to second operation in people with Crohn’s disease was explored.

No difference was found in survival to second operation and gender or age at diagnosis. This study demonstrates that CARD15 (NOD2) genotype does not significantly influence time to second operation in terminal ileal Crohn’s disease. There was a trend to longer median survival to second operation in patients carrying two CARD15 (NOD2) mutations. Carriage of the 5q31 haplotype was not associated with shorter survival to second operation.

Smoking significantly reduced the median time to second operation from 173 months to 113 months, a clinically important difference of 5 years. Those who had never smoked had a median time to second operation of 288 months (24 years) compared to 113 months (9.4 years) in current smokers. Thiopurine use was associated with a
shorter time to second operation, 184 months in those never exposed, 103 months in those currently on thiopurine and 113 months in those who had ever had thiopurines.

Previous investigations showing a positive association of \textit{CARD15 (NOD2)} mutation with shorter time to second operation had small number of patients who had undergone more than one operation for Crohn’s disease (Brant, Picco et al. 2003). By taking an unselected cohort of patients with Crohn’s disease, there will be a bias of patients with ileal disease having \textit{CARD15 (NOD2)} mutations. These patients are also more likely to have surgery for Crohn’s disease. Therefore there is bias towards a positive association with \textit{CARD15 (NOD2)}. By taking a cohort of patients all of whom have had resections for Crohn’s disease, this bias is reduced and the influence of \textit{CARD15 (NOD2)} can be more accurately investigated.

The effect of smoking on time to second operation is not unexpected. It has previously been reported that cessation of smoking improves outcome (Cosnes, Beaugerie et al. 2001). This demonstrates that our cohort can detect differences in outcome and therefore acts as a positive control.

5q31, IBD5, is a haplotype with an established association with Crohn’s disease (Ma, Ohmen et al. 1999; Duerr, Barmada et al. 2000). We investigated whether 5q31 haplotype carriage was a risk factor for reduced time to second operation in terminal ileal Crohn’s disease. In our cohort of patients with terminal ileal Crohn’s disease, there was no significant effect found with carriage of 5q31 haplotype and time to second operation. This may because this study does not have the power to detect a weak effect of this haplotype. However that weak effect is unlikely to be of clinical significance. Smoking reduced time to second operation in all patients, whether they carried one or two of the disease associated haplotypes.

The strengths of this study are its large size, robust diagnostic criteria and length of follow-up data. The study includes 346 people with terminal ileal resections for Crohn’s disease, all of whom have a robust histological diagnosis of Crohn’s disease. The mean length of follow up was 213 months, range 4 to 663 months. The mean length of follow up from first operation was 168 months, range 0 to 663 months. The mean time to first operation was 45 months, range 0 to 481 months. The mean time to second operation was 93 months.
Previous reports have suggested that Paneth cells from people with Crohn’s disease and associated \textit{CARD15 (NOD2)} mutations have reduced defensins (Wehkamp, Harder et al. 2004; Wehkamp, Salzman et al. 2005). In order to investigate a possible link between Paneth cells, \textit{CARD15 (NOD2)} and Crohn’s disease \textit{in situ} hybridization technique was used to estimate the quantity of mRNA for the antimicrobial peptides in Paneth cells of normal terminal ileum and that of patients with Crohn’s disease.

This investigation has confirmed that Paneth cell secretory granules contain mRNA for antimicrobial peptides; lysozyme, sPLA\(_2\) and human \(\alpha\)-defensins 5 and 6. The expression of mRNA can be detected in archival material preserved in paraffin wax, however tissue of 30 years old does not have reliable detection. TNF-\(\alpha\) mRNA can be demonstrated in Paneth cells, although not in secretory granules.

No difference in the expression of defensin mRNA was demonstrated between sections from people with Crohn’s disease and \textit{CARD15 (NOD2)} mutations. There was no difference when a correction was made for the area of density measured, thereby reducing error due to Paneth cell numbers. This is consistent with a recent report in \textit{Gut} using quantitative real-time PCR that did not show a difference in defensin expression in \textit{CARD15 (NOD2)} mutations (Simms, Doecke et al. 2008). The reduction previously reported is likely to be due to inflammation rather than a defensin deficiency as a result of \textit{CARD15 (NOD2)} mutation. This study used sections with minimal inflammation in order to maximise yield from the \textit{in situ} and therefore this provides further evidence that the previously reported reduction in defensin gene expression is a function of Paneth cell number rather than an effect of \textit{CARD15 (NOD2)} mutations.

TNF-\(\alpha\) is a proinflammatory cytokine that is found in Paneth cells (Keshav, Lawson et al. 1990). It is implicated in the pathogenesis of Crohn’s disease. Treatment with anti-TNF antibody is known to be of benefit in Crohn’s disease. The expression of TNF-\(\alpha\) in Paneth cells was investigated. TNF-\(\alpha\) mRNA expression was demonstrated in Paneth cells. There was an increase in TNF-\(\alpha\) expression in sections from patients with terminal ileal Crohn’s disease compared to controls. No difference was seen between \textit{CARD15 (NOD2)} genotypes. Of interest sections from three different patients with terminal ileal Crohn’s disease did not express TNF-\(\alpha\). This represents 10\% of the total number of Crohn patients. None of the patients had received treatment with anti-
TNF-α antibody. The finding of a difference in TNF-α mRNA expression acts as a positive control for this technique.

In contrast to this investigation it has been shown by quantitative real-time PCR techniques that expression of α-defensin is reduced in biopsies from human ileum with Crohn’s disease, and reduced still further in patients who carry two mutations in the CARD15 (NOD2) gene (Wehkamp, Harder et al. 2004; Wehkamp, Salzman et al. 2005). Simms et al found no difference in defensin expression (Simms, Doecke et al. 2008).

An advantage of this technique was that it enabled use of archival tissue and thereby increased the pool of available tissue from patients carrying CARD15 (NOD2) mutations. All patients used in this study had confirmed Crohn’s disease. As each section was examined by hand, areas of minimal inflammation were identified and used for the in situ process. This therefore reduces the confounder of inflammation and suggests any difference found would be an underlying defect rather than a function of inflammation, and therefore Paneth cell number. The sections were grouped therefore minimizing intra-experimental variation.

In situ hybridization is a relatively insensitive method for detection of differential expression of mRNA, however it would be expected to demonstrate a significant deficiency in defensin expression that was suggested by the Wehkamp group. This present study demonstrates differential TNF-α expression. The difference in results seen may be accounted for by the fact that the method of in situ hybridization is highlighting defensin mRNA expression within secretory granules and therefore may be a marker of propeptide formation. Defensins undergo post-translational processing on release from the secretory granules to the active peptide form. There may still be a defect in this process leading to a deficiency in functional peptide formation that would not be shown by this technique.

It was hoped that a histological phenotype would be found associated with carriage of CARD15 (NOD2) mutations. CARD15 (NOD2) is thought to be an intracellular receptor for muramyl-dipeptide (MDP), a component of the bacterial cell wall. Functional mutations in the CARD15 (NOD2) gene associated with Crohn’s disease are likely to affect CARD15 (NOD2) function, however the precise effect on the receptor cascade had not been established (Fritz, Ferrero et al. 2006). As mutations in CARD15
(NOD2) are thought to affect bacterial handling, the hypothesis was that there may be more severe inflammation and a different histological phenotype associated with CARD15 (NOD2) genotype.

No differences in lymphoid aggregates, granulomas and overall inflammation score was seen in CARD15 (NOD2) genotypes.

This investigation found a novel association between pyloric metaplasia and CARD15 (NOD2) mutations. Pyloric metaplasia is readily recognised with Haematoxylin and eosin staining (Wong, Stamp et al. 2000). A follow-up study using histopathology reports did not replicate this finding. This may be because pyloric metaplasia is not conventionally seen as pathognomonic of Crohn’s disease or required to make a diagnosis. The initial finding of an association warrants further study.

This investigation found an association of an increased number of Paneth cells and Crohn’s disease. No association was found between CARD15 (NOD2) genotype and Paneth cell number. A significant association was found between Crohn’s disease and the height of Paneth cells within the crypt. This may well be a function of the increased number of Paneth cells. Paneth cells may play an integral part in innate immune response. They are found in greater numbers in inflammatory conditions, as demonstrated in this investigation and in Kelly (Kelly, Feakins et al. 2004). This may be as a result of a functional deficiency leading to an increase in Paneth cell proliferation, or this may be a part of an “immune overdrive” response to commensal flora.

The novel finding of an association between CARD15 (NOD2) genotype and the presence of pyloric metaplasia needs to be replicated in an independent cohort. Future investigations should include a more detailed cell populations found in lamina propria in Crohn’s disease. This could involve immunophenotyping of cells within granulomas and lymphoid aggregates. The recent finding of an association between Crohn’s disease and mutations in genes involved in autophagy; ATG16L1 and IRGM warrant investigation. It would be interesting to look for manifestations of defective autophagy; and whether this is manifest as granuloma formation. The patterns of expression ATG16L1 and IRGM, IL23 within tissue sections could be investigated.

In a number of sections “budding” of the crypts was noted. This may be a sign of abnormal crypt proliferation and warrants further investigation. It was observed in too
few sections to be useful statistically. Further studies using cell proliferation markers such as MIB and Ki67 may be useful to identify the site of origin of the budding.

Osteoporosis is an important complication of Crohn’s disease as it is potentially preventable and is a cause of morbidity and mortality. A positive association between IL-6 genotype and bone mineral density was found. This was a statistically significant finding, however is probably not clinically significant. IL-6 genotype may be a surrogate marker for increased IL-6 expression and thereby increased intestinal inflammation.

There was no significant association with bone mineral density and VDR or COL1α1 genotype.

To date, genetic studies have not been helpful in the prediction of disease progression in individuals or populations. However, genetic findings have been extremely useful in highlighting potential pathogenic pathways that were previously not associated with Crohn’s disease. Up until recently there was only one gene reliably associated with Crohn’s disease; CARD15 (NOD2). This is a PRR for MDP and is thought to be a cytosolic receptor involved in a proinflammatory cascade with activation of NFκB.

This study was carried out between August 2004 and March 2006. Since then there have been many significant genetic findings in Crohn’s disease, see Chapter 6 Genetics. As part of this project, DNA and phenotype data was collected on a large cohort of patients. This significantly contributed to published studies in the genetics of inflammatory bowel disease. The most recent published genetic studies (Parkes, Barrett et al. 2007; Prescott, Fisher et al. 2007; Tremelling, Cummings et al. 2007; WTCCC 2007; Barrett, Hansoul et al. 2008; Fisher, Tremelling et al. 2008; Anderson, Massey et al. 2009), to which the DNA collection from Newcastle as part of this project has significantly contributed, have highlighted a role for the IL12/IL23 pathway and for primitive immune defence of autophagy.

The IL12/IL23 pathway involves activation of naïve CD4+ T cells into producing IL17 and can be termed Th17. It has been shown that blocking the IL12 pathway prevents intestinal inflammation in murine models of colitis (Neurath, Fuss et al. 1996; Davidson, Hudak et al. 1998). Further investigation however found that many of the effects on intestinal inflammation previously thought to be due to IL12 were secondary to IL23 (Yen, Cheung et al. 2006). IL 23 and IL12 are heterodimers sharing a common
subunit p40. IL12 is made up of p40 and p35 units and IL23 p40 and p19 subunits (McGovern and Powrie 2007). Activation of IL23 receptor leads to increased inflammatory cytokines including TNF-α, IL6, interferon-γ and IL17. The IL23 receptor is expressed by activated T cells and cells of the myeloid lineage including macrophages and dendritic cells. Induction of CD4+ T cells by IL23 leads to the generation of Th17, activated T cells are thought to play an important role in intestinal inflammation. Most recently SNPs in IL23 have been shown to be associated with Crohn’s disease (Duerr, Taylor et al. 2006; Libioulle, Louis et al. 2007; Tremelling, Cummings et al. 2007; Yamazaki, Onouchi et al. 2007).

Autophagy is the major intracellular degradation system delivering cytoplasmic components to lysozyme and is the method by which most long lived proteins and organelles are degraded. A double membrane structure is formed around the cytoplasmic components or bacteria. This then fuses with lysozymes and the contents of the vacuole are then disrupted and recycled. ATG16L1 is a gene whose product is involved in the process of autophagy. Recent studies have highlighted its role in Paneth cells and the pathogenesis of Crohn’s disease (Cadwell, Liu et al. 2008).

This is translational research which aims to link the molecular genetic research with clinical work. This project attempted to do this. The ultimate aim is of the direct clinical application of individual genotypes e.g. in prediction of prognosis has been looked for in this project and importantly is still not reached. The information and structure from this project now exists to examine for the clinical implication with the new genetic discoveries. The commonest genetic variant CARD15 (NOD2) has been found to be associated with ileal disease, otherwise it has been unhelpful in the clinical setting. Other variants are rare, even if they were found to be useful in disease classification, unless significant difference they are too rare to be of use in testing patients.

The genetic collection developed during this project has been a core resource used in major publications of genetics of inflammatory bowel disease in the last three years. With more genes this is being expanded to meet the needs of the next project. The resection based collection is unique and allows integration for histological features that other collections cannot achieve.
It was hoped that genetic variants would be useful in predicting disease progression and would be found to influence disease phenotype and aid in classification of disease. However to date, this has not been the case. Genetics has helped direct research around complex immune pathways, it suggests that many defects in a complex immune pathway rather than single defect results in Crohn’s disease.

Ultimately genetics have not been particularly helpful in predicting disease progression or phenotype. However it has directed investigation into the pathogenesis and common pathways for disease development. While genetic research started out by suggesting that Crohn’s disease was many separate genetic diseases is now likely that it is a more homogenous disease process of defective innate immune response to commensal flora.
8 Appendix

8.1 Patient forms
8.1.1 **Immunohistochemistry**

Poly-l-lyscine slides

Xylene

100% ethanol

<100% ethanol solutions made up using milli-Q filtered water

0.3% and 3% hydrogen peroxide in methanol made up from 30% H$_2$O$_2$ stock.

PBS, phosphate buffered saline

Bovine albumin

Goat serum

Rabbit Anti lysozyme antibody

Biotinylated goat anti rabbit antibody

ABC reagent

DAB-H$_2$O$_2$

Haematoxylin

DPX

Horse serum
8.2 Publications arising from this paper

The Newcastle cohort of 250 patients contributed to the 1748 strong Crohn’s disease cohort in the Wellcome Trust Case Control Consortium genome-wide association study (WTCCC 2007). Patients were recruited from the inflammatory bowel disease register and through clinic attendance at the Freeman Hospital and the Royal Victoria Infirmary, Newcastle upon Tyne, and the Queen Elizabeth Hospital, Gateshead. All patients had a diagnosis of Crohn’s disease and were under the care of either a surgical or medical gastroenterologists and were diagnosed by a combination of radiology, endoscopy and biopsy. Family and smoking history were taken by direct questioning where at all possible. Phenotyping data was collected by review of hospital notes and recorded on standardised forms. All patients gave written informed consent to participate. Within the small team involved in Newcastle my role was to identify patients, perform phlebotomy with informed consent and collect detailed phenotype information. At the completion of my MD thesis we had collected 1800 patients.

10ml venous blood was obtained from each patient into commercial plastic tubes containing EDTA using standard precautions. Blood samples were then labelled with a unique code and frozen at –20°C until DNA extraction. Blood samples were kept frozen while transported to the Genetics Department, Guys, Kings and St. Thomas’ School of Medicine, London.

The Newcastle cohort was included in a metanalysis of 3 genome-wide association studies (Barrett, Hansoul et al. 2008). 3230 Crohn patients and 4829 controls were combined. This gave the advantage of power. The combined sample gave a 74% power to detect an odds ratio of 1.2 enabling alleles with a small effect to be identified. 32 areas have now been identified of interest in Crohn’s disease.

The following papers have included the Newcastle cohort:

8.3 Summary of recent gene findings

NOD2 (16q21) is part of the NOD1/Apaf (Ogura, Inohara et al. 2001) family and encodes a protein with 2 caspase recruitment domains (CARD) and 6 leucine rich repeats (LRR). It is expressed in peripheral blood leucocytes and Paneth cells of the small intestine (Lala, Ogura et al. 2003; Ogura, Lala et al. 2003). NOD2 recognises MDP derived from LPS (Inohara, Ogura et al. 2003) and leads to NFκB activation (Morré, Ouberg et al. 2004; Maeda, Hsu et al. 2005). Functional mutations in NOD2 are associated with Blau syndrome (Miceli-Richard, Lesage et al. 2001), an aseptic site granulomatous disorder.

DLG5 (10q23) (Discs, large homolog 5) is one of a subset of the membrane-associated guanylate kinase (MAGUK) superfamily. It is composed of a catalytically inactive guanylate kinase domain. It is thought to act as a scaffolding molecule at sites of cell-cell contact. The protein product localizes to the plasma membranes and cytoplasm (Nakamura, Sudo et al. 1998; Wakabayashi, Ito et al. 2003).

TNFSF15 (9q32) (Tumour necrosis factor ligand superfamily, member 15) is expressed in endothelial cells

IL23R (1p31.3) is a subunit of the receptor for IL23A/IL23. It pairs with the receptor molecule IL12RB1/IL12Rβ1. Both are required for IL23A signalling. The protein associates constitutively with JAK2 and binds to the transcription activator STAT3. The IL23A α subunit p19 is located on chromosome 12q13.2

ATG16L1 (2q37.1) (Autophagy related, 16-like 1) is a component of a large protein complex essential for autophagy.

PHOX2b (4p12) (paired-like homeobox 2b) is a DNA associated protein localized to the cell nucleus. It is a transcription factor involved in the development of several major noradrenergic neurone populations and the determination of neurotransmitter phenotype.

FAM92B (16q24.1) (family with sequence similarity 92, member B) hypothetical protein.

NCF4 (22q13.1) (neutrophil cytosolic factor 4) is a cytosolic regulatory component of the superoxide-producing phagocyte NADPH-oxidase. NCF4 is preferentially expressed in cells of the myeloid lineage.
IRGM (5q33.1) (immunity-related GTPase family, M) probably plays a role in autophagy and control of intracellular mycobacteria (Singh, Davis et al. 2006).

NKX2-3 (10q24.2) (NK2 transcription factor related, locus 2) transcription factor

PTPN2 (18p11.3-p11.2) (protein tyrosine phosphotase, non-receptor type 2) is part of the PTP family whose signal molecules regulate cell processes.

NELL1 (11p15.2-p15.1) is a cytoplasmic protein that contains epidermal growth factor like repeats that may be involved in cell growth regulation and differentiation.

CCR6 (6q27) (chemokine c-c motive receptor 6) part of the β chemokine receptor family expressed in immature dendritic cells and memory T cells.

IL12B (5q31.1-q33.1) (subunit p40) encodes subunit p40 that forms a heterodimer with p35 (common to IL23). IL12 is expressed in activate macrophages and is an inducer of Th1 cell development.

STAT3 (17q21.31) (signal transducer and activator of transcription 3) is involved in cellular processes-cell growth and apoptosis. It is activated through phosphorylation in response to cytokines and growth factors; IFNs, EGF, IL5, IL6, LIF and BMP2, STAT3 then translocates to the nucleus where it activates transcription.

LRRK2 (12q12) (leucine rich repeat kinase 2) is a cytoplasmic protein associated with the mitochondrial outer membrane.

CDKAL1 (6p22.3) CDK regulatory associated subunit associated protein like 1

ICOSLG (21q22.3) inducible T-cell co-stimulator ligand

ITLN1 (1q22-q23.5) intelectin 1
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