UNIVERSITY OF SOUTHAMPTON

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Matrix Metalloproteinase-3 (Stromelysin-1) gene promoter polymorphism in relation to predisposition to inflammatory bowel disease (IBD)

by

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Thesis submitted for the Degree of Medicine

April 2006

ABSTRACT

UNIVERSITY OF SOUTHAMPTON
FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
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MATRIX METALLOPROTEINASE-3 (STROMELYSIN-1) GENE PROMOTER POLYMORPHISM IN RELATION TO PREDISPOSITION TO INFLAMMATORY BOWEL DISEASE (IBD)

by Dr. Joanna Prothero

The two main forms of inflammatory bowel diseases (IBD): Crohn's Disease (CD) and Ulcerative Colitis (UC) are chronic, lifetime, inflammatory disorders. The aetiology and the pathogenesis of IBD remain largely unknown, but evidence has implicated excessive responses to antigens present in the normal bacterial microflora. Matrix metalloproteinases (MMPs) are a group of matrix degrading enzymes which are important in the pathophysiology of IBD. MMP-3 is a key effector molecule in remodelling tissues during intestinal injury and is upregulated in CD and UC compared to control groups. The main cellular source of MMP-3 is subepithelial myofibroblasts. A naturally-occurring polymorphism in the MMP-3 promoter may be important in which one allele has a run of five adenosines (5A) while the other has six adenosines (6A). In atherosclerosis, the 5A allele is associated with conditions of increased extracellular matrix degradation, whereas the 6A allele is associated with conditions of matrix accumulation. We hypothesise that the 5A/6A polymorphism has the ability to regulate the production of MMP-3 in IBD. The aims of this study were to investigate MMP-3 production between individuals with different MMP-3 5A/6A genotypes; and to compare the association of the MMP-3 5A/6A genotype with susceptibility to CD and UC.

Myofibroblast cell lines were isolated from CD, UC patients and control subjects. The phenotype of these cells lines were characterised with immunohistochemistry. Cell lines were stimulated with TNF-α or IL-1β and the production of MMP-3 was measured by western blotting and ELISA. The diseased and control groups were genotyped for the 5A/6A polymorphism using whole blood assay. The production of MMP-3 by myofibroblasts isolated from individuals with different MMP-3 5A/6A genotypes was investigated. A study on MMP-3 5A/6A genotype in German and British families and sporadic cases was setup.

Cell lines from CD, UC and control patients were myofibroblast-enriched populations of cells. Cell lines responded to stimulation with TNF-α by a dose-dependent rise in MMP-3 production. The 5A/6A polymorphism was analysed in 468 German sporadic inflammatory disease trios and 270 British and German multiplex IBD families using the transmission disequilibrium test (TDT). There was an overtransmission of the 5A allele to affected offspring ($p=0.0012$). There was an interaction between the MMP-3 gene 5A/6A polymorphism and the Caspase Activating Recruitment Domain (CARD) 15 gene, a well established gene for CD, such that overtransmission of the 5A allele was a significant in CARD15 carriers ($p=0.0054$) but not in non-carriers. In the CARD15 carriers, overtransmission of the 5A allele was associated with stenosis ($p=0.0027$), fistulising disease ($p=0.0007$), previous surgical resection ($p=0.0023$), disease of the ileum ($p=0.0001$), and disease of the right colon ($p=0.0015$) in CD. The relationship of functional polymorphisms in promoters of the MMP-1,-3, -9 and -12 genes with IBD was also investigated in children and adults, and age-matched controls, described in the Appendices. No significant association was found between these polymorphisms and CD or UC, though the power of these studies was reduced by small sample numbers. In conclusion, the MMP-3 5A/6A polymorphism appears to be a genetic factor in CD.

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ACKNOWLEDGMENTS

I would like to thank the following people for their contributions to this thesis:

My supervisors: Dr. Sylvia Pender, Dr. Shu Ye, and Prof. Tom MacDonald for all their support and guidance;

Peter Croucher, Silvia Mascheretti, Sheila Fisher and Stefan Schreiber, who in addition to my supervisors and myself, were co-authors and collaborators in published work (Pender et al.), the results of which are included in this thesis (chapter 6);

Tory Goddard, Ron Bremner, Judith Holloway and John Gordon: who worked alongside me in the lab and provided friendship, support and advice along the way;

Mark Beattie: for allowing me to invade his clinics and helping me recruit children to the study;

Raj Mehta: for all his kindness and helpfulness with statistical interpretation of data;

Louise Dunleavey: for her extreme patience teaching me DNA extraction and PCR technique;

Endoscopists and endoscopy staff at Royal South Hants Hospital, Southampton, in particular: Roger House, Bernard Stacey, Chris Roseveare and Jo Burden;

All participating subjects: patients of endoscopy unit at RSH and SGH, and Paediatric patients attending Children's Outpatients at Southampton General Hospital;

Staff in Children's Outpatients Department at Southampton General Hospital;

My friends: Sonya, Doug and Rob: for all their IT advice and help with formatting (Sonya, what would I do without you?!);

My parents: without whose love, support and encouragement I would never have been able to get this far;

Lastly I would like to thank the **National Association for Crohn's and Colitis** for funding this research.

Abbreviations

α-SMA	Alpha-smooth muscle actin
Ag	Antigen
CAD	Coronary Artery Disease
CARD	Caspase-Activation Recruitment Domain
CD	Crohn's Disease
CRP	C-reactive protein
ECM	Extracellular matrix
HLA-B27	Human leukocyte antigen-B27
IBD	Inflammatory Bowel Disease
IEL's	Intraepithelial lymphocytes
IFN-γ	Interferon-gamma
IGFBP	Insulin-like growth factor binding protein
IL-	Interleukin-
IL-1β	Interleukin-1 beta
ISEMFs	Intestinal subepithelial myofibroblasts
KGF	Keratinocyte growth factor
LP	Lamina propria
LPMCs	Lamina propria mononuclear cells
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MIP-1α	Macrophage inflammatory protein-1 alpha
MMP	Matrix Metalloproteinase
OCTN	Organic cation transporter gene cluster
PCR	Polymerase chain reaction
PP	Peyer's Patches
SMC	Smooth muscle cell
T-Bet	T-box expressing T cells
TDT	Transmission disequilibrium testing
TGF-β	Transforming growth factor-beta
Th	T helper
TIMP	Tissue inhibitor of Metalloproteinases
TLR-2	Toll-like receptor-2
TNF-α	Tumour necrosis factor-alpha
UC	Ulcerative Colitis

Chapter 1: Introduction

The gastrointestinal tract faces the problem that the tissue is separated from the food antigens, indigenous microbes and infectious agents of the hostile lumenal environment of the hostile luminal environment, by a thin epithelium. The need to defend this vulnerable site is exemplified by the investment in the largest amounts of lymphoid tissue and cells in the body.

Most individuals exist in immune homeostasis, with the foreign antigens in the gut. However, for reasons still poorly understood, some people generate strong T-cell mediated responses to food antigens (gluten in coeliac disease) or IgE antibodies (food allergy).

Food hypersensitivities however, can be treated by dietary exclusion, but inflammatory bowel diseases (IBD) present a more serious health problem in that the antigens triggering the immune response which damage the gut are not clearly defined. In this situation the most productive area of research has been to understand the immunological basis of these diseases and how they cause downstream tissue injury. This thesis is focused on the latter two aspects, namely how the variation in the molecules that damage the gut may be involved in IBD.

1.1 Lymphoid Tissues in the Gut

The mucosal immune system of the gut can be divided into the inductive sites of immunity, such as the Peyer's Patches (PP), the appendix and isolated lymphoid follicles, where antigens stimulate naïve T and B lymphocytes; and the effector sites in the Lamina Propria (LP) and epithelium (Brandtzaeg and Pabst).

Peyer's patches (PP) are areas of organised tissue, found mainly in the ileum of the small intestine, and contain between 200 and 300 lymphoid follicles (Cornes). The PP are sites of antigen presentation and immune recognition, and contain abundant antigen-presenting cells. This is particularly the case below the surface epithelium in the dome region, where antigens are transported by M cells from the gut lumen.

PP contain a large number of T cells and B cells. The B cells respond to luminal antigens and migrate to the LP to become IgA plasma cells (Craig and Cebra). Both $CD4^+$ and $CD8^+$ T cell populations are present, although there are higher numbers of $CD4^+$ cells. It is thought that these T-cells also respond to antigens in the PP and then migrate via the blood to the LP.

The LP is the zone of loose connective tissue between the epithelial lining and the muscularis mucosa, and is separated from the epithelium by the basement membrane. It is maintained by smooth muscle-like cells, myofibroblasts. There is a close association between the myofibroblasts and immune cells within the LP layer.

The LP is densely infiltrated with lymphocytes, approximately 50% of these are T-lymphocytes (MacDonald and Dillon). The majority of T cells are $CD4^+$ (Selby et al.;Selby et al.), and almost all of the T cells in the LP express the α/β T cell receptor (TcR). Their phenotype is indicative of recent activation by antigen (Schreiber et al.;James). LP T cells are capable of producing high amounts of cytokines including IL-2, IL-4, IL-5, and IFN-γ. IgA plasma cells make up the remainder of lymphocytes in the LP. The majority are IgA+, although a small number of IgM+, IgG+ and IgD+ cells can also be found (Tseng). IgA is actively transported by enterocytes from the LP into the gut lumen, and plays an important role in host defence at the mucosal surface (Sandor et al.). In addition to T cells and B cells in the LP, there are also macrophages and dendritic cells (Toner and Ferguson;Spencer, MacDonald, and Isaacson).

Within the gut epithelium lie intraepithelial lymphocytes (IELs). These are found between the epithelial cells covering the crypts and villi, just above the basal lamina. They are heterogeneous in terms of morphology, and size (Ferguson), and are predominantly (Rudzik and Bienenstock;Mayrhofer) $CD8^+$ cells (Guy-Grand, Griscelli, and Vassalli;Selby et al.). 90% of the human IEL express the $\alpha\beta$ TcR heterodimer (Brandtzaeg et al.), with the remaining 10% expressing the γ/δ heterodimer (Jarry et al.). The origin of IELs, and to what extent they are derived from PP remains unclear (Bandeira et al.;London, Cebra, and Rubin). Their exact role is not known, but their primary functions are thought to lie in their cytotoxic activities (Roberts et al.).

1.2 The gut immune response to luminal antigens in healthy individuals

The major stimulus for the abundant T cells in the gut in healthy individuals is control flora (Glaister). Animal models show that in a germ-free environment, lymphoid tissues and lymphoid cells of the PP, LP and epithelium are depleted, but are restored with reintroduction of microbial flora (Umesaki et al.).

For most individuals, ingestion of food antigens does not lead to immune responses resulting in injury and inflammation of the gut mucosa. There are most likely to be number of reasons for this. An intact epithelium is likely to be an important factor. Murine models have shown that, under circumstances of increased intestinal permeability, transmural inflammation develops, mimicking that seen in CD (Hermiston and Gordon).

However, the epithelial barrier is not completely impenetrable, as both bacterial and food antigens are known to permeate through into deeper tissues, and indeed can reach the blood (Husby, Jensenius, and Svehag).Most of the antigens that succeed in penetrating the epithelial barrier, are likely to be phagocytosed by intestinal macrophages that have bactericidal activity, yet are unable to illicit pro-inflammatory responses (Smythies et al.).

Another important factor is oral tolerance, where the ingestion of soluble antigens renders animals systemically tolerant to parenteral immunisation with the same antigen (Wu and Weiner). It is now well known that T cell responsiveness to antigen can follow different functional pathways. The response of activated T-cells depends upon the microenvironment in which T cell activation occurs. $CD4^+$ T cells may be divided functionally into Th1, Th2, Th3 and T regulatory (Tr) subsets, based on their functional capabilities and the cytokines they produce. T helper 1 cells produce pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and tumour necrosis-α (TNF-α), and are responsible for cell-mediated immunity. Th2, Th3 and Tregulatory (Tr) cells, on the other hand, have regulatory functions. Th2 cells produce cytokines such as IL-4 and IL-5, IL-10 and IL-13, and mediate humoral immunity; Th3 cells secrete transforming growth factor (TGF)-β, and Tr cells secrete mainly IL-10. In the gut, the Th2 response plays an important role in immunoregulation. The Th1 and Th2 subsets are derived from common T cell precursors. The type of Ag, dose and route of administration determine the development of

these distinct subsets and their pathways of activation. The cytokine microenvironment under which the Ag binds to the T-cell receptor (TCR) also determines whether the immune response will be type 1 or type 2. In the presence of IL-12 and IFN-γ, ligation of TCR will drive the immune response along a Th1 pathway. By contrast, the presence of IL-4 will drive the immune response towards the Th2 pathway.

Studies have shown that PP and LP $CD4^+$ T cell responses in healthy individuals are very Th1 dominated. A high frequency of cytokine-secreting cells isolated from the LP of healthy individuals, secrete interferon-γ (IF-γ), with negligible production of interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-10 (IL-10) (Hauer et al.). This cytokine-expression profile is indicative of a T helper 1(Th1) response. In addition, normal human PP contain IL-12 (Monteleone et al.), so that the responses to bacterial and food antigens take place in the presence of a Th1 cell polarisation signal. Th1 cells from the PP then migrate to the LP. However, under normal circumstances, particularly following high doses of orally-administered antigen, they undergo apoptosis and so they do not accumulate (Bu et al.). In addition, a generally immunosuppressive environment dampens the inflammatory response in the LP: that is, the presence of IL-10, TGF-β and PGE-2 and functionally poor antigen-presenting cells (MacDonald, Bajaj-Elliott, and Pender). Regulatory T-cells produce these Th2 type cytokines, in response to repeated low doses of antigen (Trentham et al.;Thurau et al.;Chen et al.).

In conclusion, the immune system of the gut of healthy individuals is highly activated in response to antigens of the gut flora, yet most people remain disease free. Responses to food antigens appear to be actively suppressed. Nonetheless, in health, mucosal T cells secrete pro-inflammatory cytokines without causing disease. The gut in healthy individuals can therefore be said to be in a state of controlled or regulated inflammation.

1.3 Inflammatory Bowel Diseases

1.3.1 Definition and Epidemiology

The chronic inflammatory bowel diseases (IBD) comprise Ulcerative Colitis (UC) and Crohn's Disease (CD), and are characterised by extensive inflammation of the gastrointestinal tract, with occasional extraintestinal manifestations (Rubin et al.). They are

common causes of morbidity in Western Europe and North America. Recent studies have shown that the combined prevalence of these diseases in the United Kingdom approaches 400 per 100 000 population (Rubin et al.), and that the incidence of CD has increased over recent years, both in the children and adults (Rubin et al.;Sawczenko et al.). Up to 70% of patients with CD undergo surgery and 30% undergo repeated resections. There is evidence to suggest that the risk of surgical complications has not improved over the last four decades, despite the advances seen in drug interventions. (Rutgeerts, Van Assche, and Vermeire)

Symptoms of both conditions may overlap, and include altered bowel habit, abdominal pain, weight loss and fever. UC and CD may also present with a number of extra-intestinal manifestations, affecting for example, eyes, joints and skin. CD and UC differ in several important aspects, and are most likely two separate conditions. This is supported by epidemiological data. For example, reports show that smoking confers an independent risk of recurrence of CD and influences disease activity after surgery, whereas smoking has a protective effect with in UC (Harries, Baird, and Rhodes;Somerville et al.;Logan et al.).

Discrimination between these diseases is based on a combination of clinical, endoscopic, radiological and histopathological features (see Table1.1).

1.3.2 Characteristics of Crohns Disease

First described by Crohn in 1932, CD is an idiopathic inflammatory bowel disease, which may affect any area of the gut, from mouth to anus, but predominantly affects the terminal ileum and caecum. The clinical course of CD typically includes recurrent relapses, associated with diarrhoea, malabsorption and malaise. The finding of perianal disease such as skin tags, anal fissures, perianal fistulas, is typical of CD rather than UC. More than one area may be affected, with normal bowel tissue intervening, known as skip lesions. Historically, there tends to be sparing of the rectum in CD as opposed to UC (Markowitz et al.). With CD, the intestine is often thickened as a result of oedema and fibrosis, which may lead to stricturing and obstruction. Inflammation in CD is typically transmural.

Microscopically, biopsy specimens of CD mucosa typically show focal or patchy inflammation. The inflammatory infiltrate consists of activated lymphocytes, macrophages

and plasma cells (Schattenfroh, Bartels, and Nagel;Selby et al.). Noncaseating granulomas are often seen in the areas of inflammation, and are highly specific for CD (Seldenrijk et al.;Surawicz et al.) .The epithelium is interrupted with ulcers and deep fissuring. CD is also strongly suggested by segmental crypt atrophy and distortion, segmental mucin depletion, and mucin preservation at the ulcer edge or in the crypts with surrounding neutrophils (Tanaka and Riddell Scand J Gastroenterol 1999).

1.3.3 Characteristics of Ulcerative Colitis

UC was first described in the late nineteenth century. Unlike CD, it is confined to the large bowel, and mainly affects the distal colon and rectal mucosa. The inflammation extends continuously from the rectum to a variable extent of colon (Kirsner and Shorter). On endoscopy, areas of active colitis appear to lose their normal vascular pattern and become granular and hyperaemic. Frank ulceration and spontaneous haemorrhage may be seen in more severe cases.

Microscopically, there is diffuse infiltration of the mucosa with acute and chronic inflammatory cells, with a reduction in mucus-secreting goblet cells and disruption of the regular arrangement of glandular crypts. In active acute inflammation there is neutrophil polymorph infiltration, which leads to crypt abscesses. The combined presence of crypt abscesses and mucous depletion are the main features of UC (Tanaka et al.).

1.3.4 Exaggerated T Lymphocyte response in Inflammatory Bowel Diseases

Although there have been many advances in recent years in unravelling the complex pathogenesis of IBD, the mechanisms by which this end-point is reached are still not fully understood, and early attempts to identify specific pathogens and specific immunological abnormalities in particular have been frustrating and inconclusive. However, there is now epidemiological data to suggest that both environmental and genetic factors are important in the pathogenesis of this group of diseases (Satsangi et al.;Bayless et al.). These observations support a complex immunogenic model for IBD, where genetically susceptible individuals respond adversely to environmental factors.

Table 1.1: A comparison of the histological and clinical characteristics of Ulcerative Colitis and Crohn's Disease

	Ulcerative Colitis	Crohn's Disease
Clinical Features:		
Bloody diarrhoea	Common	Less Common
Abdominal mass	Rare	Common
Perianal disease	Rare	Common
Malabsorption	None	Common (small bowel)
Macroscopic Features:		
Rectal involvement	Invariable	Uncommon
Distribution	Continuous	Discontinuous
Strictures	Rare	Common
Mucosa	Granular, fine ulceration	Deep ulceration
Fistulas	Very rare	May occur
Microscopic Features:		
Distribution	Mucosal	Transmural
Cellular infiltration	Neutrophils, plasma cells, eosinophils	Lymphocytes, plasma cells, macrophages
Glands	Mucin depletion	Gland preservation
Other features	Crypt abcesses	Granulomas, apthoid ulcers

It is now well recognised that LP T cells, particularly $CD4^+$ T-cells, play an important role in mucosal injury, not only in Crohn's Disease, but other diseases, such as coeliac disease, graft-versus-host disease, and intestinal allograft rejection (MacDonald and Spencer;Mullin et al.;Breese et al.;Lundin et al.;Guy-Grand and Vassalli;Cerf-Bensussan et al.). The importance of $CD4^+$ T cells in driving the inflammatory process in CD in particular, is supported by observations that $CD4^+$ antibody has been used with success in the treatment of active CD (Stronkhorst et al.), and that CD undergoes remission in the presence of HIV infection (James).

There is strong evidence that the excessive T-cell response seen in chronic inflammatory diseases is driven by bacterial antigens of the normal gut flora. Firstly, there are clinical observations. For example, CD has a predilection in the small bowel for the terminal ileum, where the normal flora is most abundant. In addition, the antibiotic metronidazole has been shown to be of therapeutic benefit in CD of the distal colon (Rutgeerts et al.); and surgical tactics to divert the faecal stream following resection of diseased bowel in CD

have been found to be efficacious (Rutgeerts et al.). Mucosal T cells from CD patients respond in vitro to antigens derived from their own flora (Duchmann et al.). This theory is further substantiated by evidence from murine models of inflammatory bowel disease. These have shown that under conditions of a germ-free environment, disease is absent or reduced (Sadlack et al.;Kuhn et al.;Davidson et al.;Ehrhardt et al.); Experiments with human leukocyte antigen-B27 (HLA-B27) transgenic rats (Hammer et al.) and IL-10 deficient mice (Kuhn et al.), have demonstrated the importance of the presence of normal flora in the development of colitis. Other models have also shown that $CD4^+$ T cells reactive to antigens of commensal flora can mediate chronic inflammatory bowel disease (Cong et al.). The observations from these models lead to the conclusion that components of the normal bacterial flora are necessary for the induction of inflammation, and that the locus of inflammation is the gut mucosa.

1.3.5 Cytokine Production

$CD4^+$ T cells in CD show a polarized Th1 phenotype and produce high amounts of the pro-inflammatory cytokines, IFN-gamma (IFN-γ) and TNF-α (MacDonald, Monteleone, and Pender). In addition, T-bet (T-box expressing T cells), the recently-identified transcription factor in T cells important in driving the Th1 response, is highly expressed in the mucosa of CD patients (Neurath et al.). The Th1-inducing cytokine, IL-12 is expressed in abundance in the mucosa of active CD, but not UC (MacDonald and Monteleone). This would explain for the granulomatous nature of CD, as both IFN-gamma and IL-12 are required to produce immune granulomata. Animal models have shown that the IL-12/STAT4 signaling pathway is important in the development of Th1-mediated colitis mimicking that of CD (Neurath et al.;Trinchieri, Pflanz, and Kastelein). Other cytokines, such as IL-2, IL-6, and IL-18 are also in abundance in diseased mucosa in CD (Bouma and Strober). IL-18 in particular, is a strong costimulatory factor for IL-12 driven Th-1 responses, and is highly expressed in CD mucosa (Monteleone et al.). The IL-12 induced synthesis of IFN-γ by LP T cells is also enhanced by other cytokines produced in the intestine, including IL-7, IL-15, and IL-21, cytokines that signal through the common γ-chain receptor subunit (Monteleone et al.;Monteleone et al.;Kanai et al.;Monteleone et al.). These cytokines prevent apoptosis of T-cells, thereby causing T-cell accumulation and potentiating the disease process (Boirivant et al.;Sturm et al.). The importance of cytokine-production in the pathogenesis of CD is exemplified by the success of clinical trials, in

which blocking cytokine production such as IL-6 (Ito et al.) and IL-12 (Mannon et al.), have shown to be of therapeutic benefit. One of the most successful advances in the treatment of CD in recent times is infliximab, the anti-TNF antibody. Both infliximab, and the anti-cytokine therapeutic modalities above, are thought to exert their effects through blocking pathways that confer resistance of Th1 cells to apoptotic stimuli (Van Den Brande et al.;Ciccocioppo et al.;MacDonald and Monteleone).

In contrast, in UC the role of T-cells is less well defined. They are not a Th-1 biased (Fuss et al.). This would explain why granulomata are rarely, if ever, seen in this disease group. It has not been proven that UC represents a Th-2 lesion. In vitro studies have shown that when activated with CD3/CD28, LP $CD4^+$ T cells from UC patients produced more IL-5 than similar cells of CD patients or controls. However, IL-4 is not increased in UC patients as might be expected with a Th2 response. Other studies have shown in fact, that IL-4 is decreased in UC (Karttunnen et al.;West et al.). In contrast to CD, there is little production of IL-12 in the colonic mucosa of UC patients.

However, in support of the theory of a Th2 bias, the inflamed bowel in UC contains an abundance of IgG plasma cells and IgG1, which is presumably the result of a T-cell dependent response originating from gut-associated lymphoid tissue (Halstensen, Das, and Brandtzaeg). The association of UC with other autoimmune conditions, such as ankylosing spondilitis, and primary sclerosing cholangitis, would also support this. In addition, a rabbit model in which immune complexes administered intravenously and produced colitis which closely resembled UC (Hodgson et al.), supports the notion the antibody is important.

A recent study has suggested that UC may represent an atypical Th2 response, mediated by nonclassical NKT cells producing IL-13 (Heller et al.;Fuss et al.).

1.3.6 Downstream events following T-cell activation and increased cytokine production

There is limited evidence that cytokines directly damage gut mucosal cells: in vitro studies have shown that direct addition injection of cytokines may have a detrimental effect on the gut epithelial cells (Bajaj-Elliott et al.). While it is recognised that other effector pathways are important in mucosal injury, more recently it has become evident that cytokines are key

players in the regulation of the production of neutral endopeptidases, matrix metalloproteinases (MMPs), which have a principle role in mucosal degradation. Before summarizing the evidence for the contribution of MMPs to tissue injury in the gut, I will first discuss this group of enzymes in more detail; the cellular source of MMPs in IBD; myofibroblasts; MMP substrates; and components of the extracellular matrix (ECM).

1.4 Extracellular Matrix (ECM)

The ECM is a complex structural entity surrounding and supporting cells within tissues. It also has a physiological role in that it plays a part in cell signalling, tissue development and growth. Components of the ECM also act as ligands for cell-surface receptors.

1.4.1 Components of the Extracellular matrix

The ECM is a connective tissue matrix, made up of a mixture of four major types of macromolecules: collagen, glycoproteins, elastin and proteoglycans. The distribution of each class of ECM molecule is tissue-specific, and reflects the importance of the ECM in cell differentiation and development.

The ECM components of the human intestine are the same as the ECM in all tissues, but are present in specific proportions. The basement membrane, directly beneath the epithelial cell layer, is a specialized ECM that separates epithelial and endothelial cells from underlying tissue. It is rich in type IV collagen, laminin, and fibronectin (Basson), as well as entactin and perlecan. The interstitial matrix of the LP contains types I and III collagen, as well as fibronectin and various glycoproteins and proteoglycans, lies deep to the basement membrane, and may be exposed in mucosal injury.

Fibronectin is a 540-kDa glyoprotein dimer of two similar polypeptide chains. Each polypeptide subunit of fibronectin is made up of repeating modular domains, which include two cell-binding domains, in addition to the unique modules interacting with collagen, heparin and fibrin (Raghow;Yamada). The fibrous backbone of ECM is made up of collagens (Vuorio and de Crombrugghe). The collagens are a family of related glycoproteins, and may be represented by fibrillar (types I,II,and III), nonfibrillar (such as type IV), or the so-called FACIT (fibril-associated collagens with interrupted triple helices)

collagens (Shaw and Olsen).Laminin is found predominantly in the basement membrane, and is a 500-kDa glycoprotein composed of three chains designated A(400kDa), B1 (210kDa), and B2 (200 kDa). Laminin is therefore in contact with a variety of epithelial and mesenchymal cells (Yamada and Kleinman).

Proteoglycans represent the most abundant, heterogeneous, and perhaps functionally the most versatile nonfibrillar component of the ECM (Jackson, Busch, and Cardin;Goetinck;Wight, Kinsella, and Qwarnstrom). These complex macromolecules are made up of a core to which heteropolysaccarides, glycosaminoglycan (GAG), are covalently attached either through O-glycosidic linkage to serine or through N-linked aspargine residues.

1.4.2 Functions of the ECM

Historically, the ECM was thought as nothing more than an inert scaffold around cells, but evidence has emerged that this is far from the case. In addition to providing mechanical support to tissue, and in fact, the ECM interacts with the cell population enmeshed within it.

Recent observations suggest that the ECM actively orchestrates the key steps in the program of wound healing and regeneration. For example, during the inflammatory phase of tissue injury, fibrinogen and collagen, abundant in the ECM, activate platelets via binding to integrins present on the platelet surface, leading to degranulation of the platelets, and release of their contents. ECM components also have chemotactic properties, and initiate influx of inflammatory phagocytes. Furthermore, the adhesive interactions among various cells and the surrounding ECM profoundly modify the phenotypic features of mesenchymal and non-mesenchymal cells attracted to the site of injury (Lukashev and Werb). The ECM also has an affect on differentiation of various cells present within the granulation tissue, including endothelial cells, myofibroblasts, and epithelial cells (Lukashev and Werb). During the final stage of issue remodelling, where granulation tissue is replaced with more organised matrix, the composition of the new ECM is determined by interaction of the ECM with the cells that synthesize its components. ECM also acts as binding reservoirs for various cytokines and growth factors that are released once the ECM is degraded. For example, the collagen-associated proteoglycan decorin acts as a depot for

TGF-β, and its degradation by various MMPs makes the otherwise sequestered TGF-β available to carry out its biological functions (Imai et al.).

The process of proteolytic remodelling of the ECM alters matrix-derived signals, resulting in release of modular breakdown products with biological activity. For example, the cleavage of the intact laminin-5 by MMP-2 generates a γ2chain fragment that induces epithelial motility (Giannelli et al.).

Components of the ECM can also have effects on the enzymes that cleave them. For example, type I collagen acts as a ligand for discoidin domain-containing receptor-like tyrosine kinases that induce MMP-1 expression when they are activated by intact collagen and become inactive when they bind MMP-1-cleaved collagen (Vogel et al.;Shrivastava et al.). In addition, through MMP-mediated disruption of matrix structure, cells dependent on matrix for anchorage may undergo apoptosis, and therefore matrix remodelling contributes to normal physiological cell death (Sternlicht and Werb).

1.4.3 ECM in Disease States

A number of conditions are associated with destruction of the ECM. These include: rheumatoid arthritis (Tchetverikov et al.), osteoarthritis (Liu et al.) and peridontitis (Seguier et al.) . Abnormal matrix turnover is also seen in tumour invasion and metastasis (Stetler-Stevenson, Liotta, and Kleiner, Jr.). One outcome of disruption in matrix turnover is increased matrix deposition, as seen as fibrosis and the formation of strictures in chronic inflammatory disease. In situ studies using CD and UC tissue sections show that in both forms of IBD there are significantly increased levels of procollagen mRNA. In CD there is excessive deposition of collagen, especially types V, III, and I, throughout the mucosa, sub-mucosa and serosa (Stallmach et al.), leading to the formation of strictures that is a common complication of CD.

1.5 Myofibroblasts

1.5.1 Morphology

The majority of ECM components of the intestinal wall are secreted by mesenchymal cells: fibroblasts, myofibroblasts, and smooth muscle cells (SMCs). The fibroblast is the major cellular constituent of loose connective tissue, whereas the SMC is confined primarily to the muscularis musosa. There is phenotypic variability among fibroblasts, and some express features of smooth muscle cells (Fries et al.;Komuro;Sappino, Schurch, and Gabbiani). These smooth muscle-like cells, or myofibroblasts, are present in a variety of different tissues throughout the body, and are juxtaposed to epithelial or parenchymal cells.

Myofibroblasts share the functional and morphological characteristics of both fibroblasts and smooth muscle cells, and may transdifferentiate between either one of these cell types (Jester et al.) (Masur et al.). They can be identified by positive immunohistochemical staining for α-smooth muscle actin (α-SMA) and vimentin, and can be distinguished from smooth muscle cells of the muscularis mucosae, which are also α-SMA positive, by absence of staining for desmin (Kaye, Lane, and Pascal) (See table 1.2 below).

Table 1.2: The phenotype of different mesenchymal cells

	α-SMA	vimentin	desmin
Myofibroblasts	+	+	-
Fibroblasts	-	+	-
Smooth muscle cells	+	-	+

Intestinal subepithelial myofibroblasts (ISEMFs) are present throughout the gastrointestinal tract (Guldner, Wolff, and Keyserlingk;Joyce, Haire, and Palade;Kaye, Pascal, and Lane). At a microscopic level, they are found predominantly around the crypts and also beneath the surface epithelium, and are connected by tight junctions and gap junctions (Joyce, Haire, and Palade). They exist as a syncytium that extends throughout the LP of the gut, merging with pericytes of surrounding blood vessels.

1.5.2 Functions of intestinal myofibroblasts

Since their identity 30 years' ago as a distinct cellular entity, myofibroblasts have been shown to be ubiquitous cells which play a significant roles in ontogenesis, homeostasis, and tumorigenesis in a variety of organs. They also have specific functions in the gastrointestinal tract, including the modulation of intestinal motility (Powell et al.) and the regulation of intestinal water and electrolyte transport (Powell et al.).There is strong evidence to show that intestinal myofibroblasts are needed for the development of the gut mucosa and differentiation of epithelial stem cells (Fritsch et al.;Kedinger et al.;Plateroti et al.). They are the main contributors to the basement membrane, and morphogenesis of the extracellular matrix (ECM) (Villaschi and Nicosia;Saunders and D'Amore). They do so through growth factors, and through secretion and formation of interstitial matrix and basement molecules such as collagen, glycosaminoglycans, tenascin, and fibronectin (Birchmeier and Birchmeier;Fries et al.).

Myofibroblasts play a central role in wound healing. They are involved in remodelling of the ECM, as well as proliferation and differentiation of epithelial, vascular and neurogenic elements in the process of repair following injury (Wallace and Granger). Tissue repair is a co-ordinated event, in which there is release of various lipid mediators such as eicosanoids, nitric oxide, cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-2, IL-15, and various other growth factors. Many of these molecules activate myofibroblasts, resulting in myofibroblast motility and the release of ECM proteins and other growth factors. It has also been shown that luminal bacteria or their products, such as lipopolysaccharide (LPS), directly activate myofibroblasts through toll-like receptors (van Tol et al.).

Myofibroblasts take part in the remodelling of intestinal tissue following injury by the secretion of MMPs and other proteases (Daum et al.;MacDonald and Pender), as well as secretion of TGF-α and keratinocyte growth factor (KGF) (Bajaj-Elliott et al.). Healing is also facilitated by the ability of myofibroblasts to contract, which serves to reduce the surface area of denuded wounded tissue (Moore, Carlson, and Madara;Racine-Samson, Rockey, and Bissell).

Myofibroblasts also play a major role in the inflammatory response. They produce chemokines and cytokines, and are capable of augmenting or down regulating the inflammatory response by the secretion of inflammatory mediators (such as TNF-α, IL-1, IL-6, IL-10), and growth, differentiation and immunosuppression factors such as TGF-β, TGF-α, platelet derived growth factor (PDGF), stem cell factor, hepatocyte growth factor and keratinocyte growth factor), and chemotactic factors (such as IL-8 and MIP1-α). Myofibroblasts express adhesion proteins, such as intracellular adhesion molecule 1, and integrins, in response to inflammation. This allows the myofibroblast to interact with other inflammatory cells such as lymphocytes, mast cells and neutrophils as part of an organised inflammatory response (Crowston et al.;Fiocchi;Strong et al.).

Another aspect directly relevant to cell-cell interactions during inflammation is the ability of intestinal fibroblast cells to prolong T-cell survival (Scott, Pandolfi, and Kurnick). This, and the observation from ex-vivo experiments that they can enhance the proliferative responses of T-cells (Roberts, Nadler, and Ebert), would suggest that intestinal myofibroblasts have an important role to play in the duration of the intestinal inflammatory process.

1.5.3 Importance of myofibroblasts in disease States

Myofibroblasts have been implicated in the disease pathogenesis of a number of other conditions including ischaemic heart disease, aortic stenosis, coeliac disease, and periodontitis, through their effects of MMP production, and subsequent matrix turnover. Experiments investigating the role of MMPs in IBD have also observed that fibroblast-like stromal cells are important source of MMP secretion in this group of diseases (Vaalamo et al.). Myofibroblasts contribute to ulceration seen in IBD, through their secretion of MMPs, and also excessive matrix deposition, seen in the fibrosis and stricture-formation that is the picture of chronic IBD, particularly CD. Fibroblasts from strictured segments of bowel affected with CD produce significantly elevated amounts of collagen type III and display heightened response to transforming growth factor -β induction (Stallmach et al.). In vivo experiments also suggest that myofibroblasts and fibroblasts isolated from CD and UC-affected intestinal mucosa have functional characteristics which are distinct from fibroblasts isolated from normal tissue, such as increased capacity for proliferation, and

increased secretion of collagen before and after stimulation with profibrogenic cytokines (Lawrance, Maxwell, and Doe).

1.6 MMPs

1.6.1 The MMP family

Interstitial collagenase was the first metalloproteinase to be identified, following experiments showing that diffusible enzymes produced by fragments of involuting tadpole tail could degrade gels made of native fibrillar collagen (GROSS and LAPIERE). Since then a family of related enzymes has been identified which are collectively termed the matrix metalloproteinases (MMPs), because of their dependence on zinc ions for catalytic activity, their potent ability to degrade structural proteins of the extracellular matrix (ECM), and related structures that distinguish them from other closely related metalloproteinases (Stocker et al.).

There are now over 24 recognised members of the MMP gene family (de Coignac et al.), summarized in Table 3 (Sternlicht and Werb). Individual MMPs are referred to by their common names or according to a sequential numeric nomenclature reserved for the vertebrate MMPs. They are now known to be involved in normal remodelling processes such as embryonic development, postpartum involution of the uterus, bone and growth plate remodelling, ovulation and wound healing, as well as important disease processes such as joint destruction in rheumatoid arthritis, tumour invasion, and periodontitis (Birkedal-Hansen), and more recently, necrotizing enterocolitis (Pender et al.) and IBD (Vaalamo et al.;Stallmach et al.). They are secreted not only by myofibroblasts, but also T cells, monocytes, macrophages, neutrophils, keratinocytes and tumour cells.

Members of this family have the following features:

- The catalytic mechanism is zinc-dependent.
- The latent form can be activated by proteinases or by organomecurials. In most cases this occurs extracellularly.
- Activation is accompanied autocatalytic cleavage.
- The cDNA sequences show homology.
- The enzymes cleave one or more components of the ECM, such as collagen, elastin, proteoglycan, fibronectin, laminin and gelatin.
- Activity is inhibited by Tissue inhibitors of metalloproteinases (TIMPs) (Woessner, Jr.)

MMPs are subdivided further into groups, depending on their structure and substrate specificities: collagenases (MMP-1 or intersitial collagenase); stromelysins (MMP-3,-7,-10,-11,-12, and –26); gelatinases (MMP-2 and –9) ; and membrane-type MMPs (MMP-14,-15,-17,-24,and-25), although members such as macrophage metalloelastase (MMP-12), matrilysin (MMP-7), MMP-19 and enamalysin (MMP-20) cannot be classified within these groups (Belaaouaj et al.;Woessner, Jr. and Taplin;Muller et al.;Marti et al.;Pendas et al.;Llano et al.).

There are three members of the collagenase subclass and all cleave fibrillar collagens: interstitial collagenase, neutrophil collagnase and collagenase-3. MMP-1 is also known as collagenase-1, fibroblast collagenase and interstitial collagenase. It is the most widely expressed interstitial collagenase, and is capable of degrading fibrillar collagens (types I,II, and III) (Nagase and Woessner, Jr.). It is almost undetectable in normal resting tissues, although in culture it is produced by a variety of cells including fibroblasts, macrophages, endothelial and epithelial cells. It is usually expressed in physiological and pathological remodelling in vivo, suggesting a broad range role in biology (Nagase and Woessner, Jr.).

Collagens are cleaved by MMPs in their triple helical domain, which leaves the molecules thermally unstable, so they unwind to form gelatin, after which they can be further degraded by other members of the MMP family. Other matrix molecules are also substrates for MMP-1, including aggrecan, versican, perlecan, casein, nidogen, serpins and tenascin-C. Therefore this enzyme plays a pivotal role in ECM remodelling. MMP-1 may also

cleave non-matrix molecules such as antitrypsin, insulin-like growth factor binding protein (IGFBP)-3, IGFBP-5, IL-1β, L-selectin, ovostatin, TNF-α, and stromal derived factor-1 (McCawley and Matrisian).

The stromelysin group includes three members: Stromelysin -1, -2 and –3. The stromelysins were named because of their substrate specificity and relatively broad pH optima (Whitham et al.). Stromelysin-1 is the most extensively researched of this group, and the most abundant. The stromelysins are produced by fibroblasts, but also by macrophages (Birkedal-Hansen et al.) and keratinocytes (Birkedal-Hansen et al.;Saarialho-Kere et al.). Stromelysin-1 is produced after induction by growth factors and cytokines, such as IL-1 and TNF-α (Birkedal-Hansen et al.).

The third group of MMPs are the gelatinases. Two similar but separate gelatinases have been identified: a 72-kDa gelatinase A and a 92-kDa gelatinase B. Gelatinase B was first identified in neutrophils, and later, in monocytes and/or macrophages. Various types of connective tissue cells, including fibroblasts, endothelial cells and oeteoblasts, can secrete gelatinase B, but are poor producers compared to leukocytes (Opdenakker et al.;Meikle et al.). In general, all these types of cell secrete gelatinase A constitutively, whereas the expression of gelatinase B is usually inducible. The gelatinases have specificity for denatured collagens, that is, gelatins, and intact type IV basement membrane.

Macrophage metalloelastase was first identified 30 years' ago as an elastolytic metalloproteinase secreted by macrophages (Werb and Gordon). The major substrate for MMP-12 is elastin. In addition to elastolytic activity, MMP-12 has been shown to be capable of degrading a broad-spectrum of other ECM components, including type IV collagen, fibronectin, laminin, vitronectin, proteoglycans, chondroitin sulphate, and myelin basic protein.

Another group of MMPs are the membrane-type MMPs, which have membrane-anchoring domains (Hernandez-Barrantes et al.). MT1-MMP degrades several ECM components, including collagen types I, II, and III, fibrnectin, laminin, vitronectin, fibrin and aggrecan (Pei and Weiss), and activates gelatinase A.

1.6.2 The domain structure of MMPs

MMPs, although the product of different genes, share structural and functional properties, including optimal activity at neutral pH and the requirement of calcium and zinc for biological activity (Parks and Shapiro). In general, MMP enzymes are composed of three distinct domains: an amino-terminal propeptide domain that is involved in the maintenance of enzyme latency, a catalytic domain that binds zinc and calcium ions; and a hemopexin-like domain at the carboxy terminal. The propeptide domain, which is lost upon activation, consists of about 80 amino acids, and contains the unique PRCGV/NPD sequence. The catalytic domain is about 170 amino acids long, and contains a zinc-binding motif and 2-3 calcium ions which are required for the stability of the enzyme, a five stranded β-sheet, three α-helices, and bridging loops. There is a proline-rich linker peptide that connects the catalytic and hemopexin domains, whose function is not fully understood. With the exception of MMP-7, MMP-23 and MMP-26 (Park et al.), all MMPs have a victronectin/hemopexin-like domain of about 210 amino acids long. When present, the hemopexin domain influences TIMP binding, the binding of certain substrates, membrane activation, and some proteolytic activities. For example, the hemopexin domain is required for collagenases to cleave triple helical interstitial collagens (Bode), although it is the catalytic domain alone that is required for other substrates (Clark and Cawston).
In addition, gelatinases have an additional fibronectin-like domain. This consists of three fibronectin type II repeats and enables binding to gelatin (denatured collagen), laminin and collagen types I and IV (Opdenakker, Van den Steen, and Van Damme). This collagen-V like domain is heavily glycosylated, for reasons that are not fully understood, but may act as a spacer between the zinc-binding domain and the carboxy-terminal hemopexin domain (Mattu et al.).

1.6.3 MMP substrate specificity

MMPs have somewhat overlapping specificities. For example, gelatinase A (MMP-2), gelatinase B (MMP-9), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), matrilysin-1 (MMP-7), macrophage metalloelastase (MMP-12), and collagenase-3 (MMP-13), can all degrade many of the same substrates, such as elastin, fibrillins, and fibronectin. Virtually all of the enzymes cleave type IV and V collagens (Birkedal-Hansen et al.). On the other hand some have more unique properties. For example, only MMP-1 and MMP-8 are able to cleave interstitial collagens (Birkedal-Hansen et al.). But aside from the collagenases,

the set of substrates that overlap among MMPs is more impressive than that of selective substrates. There are two mechanisms that determine substrate selectivity: enzyme affinity and compartmentalization. Kinetic studies have demonstrated that specific enzymes degrade some substrates more efficiently than others .For example, MMP-7 is a more potent proteoglycanase than MMP-3 and MMP-9, and MMP-12 is the most elastolytic enzyme of the MMP family. The location of the tissue environment, and the cells secreting the MMP are also important. For example, ex vivo experiments show that MMP-7 inactivates α1-antiproteinase inhibitor more effectively than MMP-9. However, in the environment of inflamed tissue, MMP-9 selectively cleaves this substrate (Liu et al.). It would seem that cells do not secrete proteases indiscriminately. Rather, MMPs are targeted to specific substrates within the pericellular space (Parks and Shapiro).

It is becoming increasingly clear that substrates of MMPs are not limited to the ECM. MMPs have demonstrated their ability to act fundamentally as proteinases in cleaving a wide variety of proteins. Matrilysin, for example, activates the pro-form of α-defensins (Wilson et al.), and various MMPs can activate the serpin α1-antiproteinase inhibitor (Sires et al.;Pei, Majmudar, and Weiss).

1.6.4 Regulation of MMP expression

Given that MMP substrate specificities overlap, the biologic function of individual MMPs is largely dictated by their differential patterns of expression. The differences in the temporal, special, and inducible expression of the MMPs are often indicative of their unique roles. The regulation of MMPs occurs on three levels: regulation of transcription, activation of latent MMPs, and inhibition of MMP activity.

Table 1.3: The substrate specificity of metalloproteinases

Enzyme	MMP	Latent/ active (KDa)	Preferred substrates
Collagenases	MMP-1	52/43	Collagen types I, II ,III, VII, X ,gelatins, aggrecan, tenascin ,link protein
Collagenase-1 or interstitial collagenase			
Collagenase-2 or Neutrophil collagenase	MMP-8	75/55	Collagen types I,II,III,VIII,X,aggrecan,link protein
Collagenase-3	MMP-13	52/42	Collagen types I,II,III,VII,X,aggrecan,gelatins
Collagenase-4	MMP-18	53/42	
Stromelysins	MMP-3	52/43	Aggrecan,gelatin,fribronectin,laminin,link protein,elastin,collagen typesI,III,IV,V,VIII,IX,X,procollagenase-1(activation),vitronectin,tenascin,decorin
Stromelysin-1			
Stromelysin-2	MMP-10	52/44	Aggrecan,fibronectin,laminin collagen types I,III,VI,VIII,gelatin,elastin,laminin
Stromelysin-3	MMP-11		Casein, laminin, fibrnectin, gelatin, collagen IV, carboxymethylated transferrin
Gelatinases			
Gelatinase A	MMP-2	72/62	Collagens (I,IV,VII,XI and XIV), gelatin,elastin,fibronectin,laminin,galectin-3,versican,proteoglycan,link protein,entactin,osteonectin
Gelatinase B or gelatinase 92 kDa	MMP-9	92/82	Gelatins,collagen types I,II,III,IV,V,VII,X,XI,XIV
		/65	Aggrecan,elastin,vitronectin
Membrane type MMPs			
MT1-MMP	MMP-14	64/54	Pro-MMP-2(activation),collagen typesI,II,dermatan sulphate,laminin,fibronectin,gelatin, vitronectin
MT2-MMP	MMP-15	71/61	Pro-MMP-2(activation)
MT3-MMP	MMP-16	66/56	Pro-MMP-2(activation)
MT4-MMP	MMP-17	62/51	
MT5-MMP	MMP-24	63/45/28	
MT6-MMP	MMP-25	63/58	
Others			
Matrilysin	MMP-7	28/19	Aggrecan,fibronectin,vitronectin,tenascin,laminin,gelatin,collagen type IV,elastin,procollagenase-1,(activation),link protein
Matrilysin-2 , Endometase	MMP-26	28/19	
Macrophage Metalloelastase	MMP-12	52/20	Collagen IV, gelatin,elastin,casein,laminin,proteoglycan monomer,fibronectin,vitronectin,enactin
RASI-1	MMP-19	54/45	
Enamelysin	MMP-20	54/22	Enamel matrix
X-MMP	MMP-21	70/53	
CMMP	MMP-22	52/42	
CA-MMP or MIFR-1	MMP-23	56/65	

(i) Regulation of Transcription

It would appear that the production of most MMPs is regulated almost exclusively at a level of gene transcription, since most MMP genes are expressed only when active tissue remodelling takes place (Matrisian;Fini et al.). A notable exception is MMP-2, which is often constitutively expressed and controlled through unique mechanism of enzyme activation (Strongin et al.) and some degree of post-transcriptional mRNA stabilization (Overall, Wrana, and Sodek). Other exceptions include MMP-7, MMP-26, MMP-25, MMP-24 and MMP-18, which are expressed in low levels in healthy tissues (de Coignac et al.;Cossins et al.;Pei;Pei).

The expression of the various MMPs is regulated by numerous stimulatory and suppressive factors that influence multiple signalling pathways. MMP expression can be up-or down-regulated by phorbol esters, integrin-derived signals, extracellular matrix proteins, cell stress, and changes in cell shape (Sternlicht and Werb). In addition, MMP expression is regulated by several cytokines and growth factors, including interleukins, interferons, tumour necrosis factor -α (TNF-α), interleukin-1α and β (IL-1α ,IL-1β), chemical agents such as prostaglandin E. Factors that inhibit production of MMPs include transforming growth factor -β, (TGF-β) (Fini et al.). Generally pro-inflammatory cytokines increase MMP production.

Many of the MMP genes (including for example MMPs: -1, -3, -9 and –12) have an AP-1 consensus element in their promoter, which interacts with the Fos and Jun family of transcription factors; and one or two copies of the PEA-3 element, which interacts with the Ets family of transcription factors. A number of studies have demonstrated that these cis-elements play an important role in the regulation of MMP transcription, both at basal level and in response to various stimuli, including cytokines and growth factors (Angel et al.;Gaire et al.). Several other regulatory elements have been identified within MMP gene promoters, and shown in functional studies to regulate gene expression, such as β–catenin -regulated LEF/TCF recognition site near the MMP-7 transcription start site (Crawford et al.); and a p-53 binding site in the MMP-2 promoter (Bian and Sun).

(ii) Activation of latent MMPs

Like many other proteolytic enzymes, MMPs are first synthesized as inactive proenzymes or zymogens. It is surprising, perhaps, given the potency of MMPs, that they can be activated by a broad range of agents, including: HOCl, chaotropic agents, and various proteinases, such as trypsin and plasmin. This observation is explained at least in part, by the 'Cysteine switch' model (Springman et al.). In this model, the cysteine atom prevents activation by its association with the zinc atom at the active centre of the molecule instead of a water molecule, which is required for the active form (Vallee and Auld). Reagants may either react with the cysteine molecule to produce a non-binding form, or they may cause the peptide containing the cysteine to fold back, breaking the cysteine-zinc contact. Proteolytic enzymes may also break the cysteine-zinc connection by cleaving peptides around the left of the cysteine. Autolytic cleavage then follows, with loss of the propeptide domain, to reveal the active form. Most MMPs are activated extracellularly, with the exception of MMP-11, MMP-27, and MT-MMPs, which contain an RXK/RR furin-like recognition motif between their propeptide and catalytic domains. This enables them to be activated by intracellular proteinases before they are secreted (Pei and Weiss).

In vivo, most MMPs are activated by other activated MMPs, thereby creating a catalytic cascade (Nagase and Woessner, Jr.). They are also activated by proteinases that can cleave peptide bonds within MMP prodomains such as plasmin (Nagase and Woessner, Jr.;Lijnen), and bacterial exoproteinases (Okamoto et al.). However, MMP-2 is refractory to activation by serine proteinases and is instead activated at the cell surface through a unique multistep pathway involving MT-MMPs and TIMP-2 (Strongin et al.). MMP-3 and MMP-14 in particular, play prominent roles in proactivation of MMPs (Strongin et al.).

(iii) Inhibitors of Metalloproteinases

There are three types of inhibitors of MMP activity: Tissue Inhibitors of MMPs (TIMPs), α2-macroglobulin, and synthetic inhibitors.

TIMPs are the major endogenous regulators of MMPs, and are produced by the same cells that produce MMPs. So far, four have been identified (TIMPs 1 –4) (Gomez et al.). They are a group of proteins that reversibly inhibit MMPs in a 1:1 stochiometric fashion (Gomez

et al.;Edwards). They do this by forming classic noncovalent bimolecular complexes with the active forms of MMPs, and may also block MMP precursor activation (Howard, Bullen, and Banda). They share 37-51% sequence identity, and conserved gene structure. The TIMPs also have in common 6 disulphide bonds, which are mostly very heat stable. The residues around the disulphide bond between Cys1 and Cys70 are the most critical part of the TIMP complex for their inhibition (Gomis-Ruth et al.).

The TIMPs individually have different functions and abilities to inhibit various MMPs (Woessner, Jr. and Nagase). For example, TIMP-2 and TIMP-3 inhibit MMP-14, whereas TIMP-1 does not (Woessner, Jr. and Nagase); TIMP-1 is a potent inhibitor of MMP-3, but a poor inhibitor of MMP-16; and TIMP-3 has greater effects on MMP-9 than the other TIMPs (Sternlicht and Werb). In addition, the TIMPs differ in their gene regulation and tissue-specific patterns of expression (Edwards).

The functions of TIMPs are not confined to inhibition of metalloproteinases. They have also been shown to inhibit cell invasion, tumorigenesis, metastasis and tumour cell growth (Gomez et al.), and in healthy tissues, can have growth-promoting activity, although whether or not this is an effect of their involvement in the regulation of MMP activity, is uncertain.TIMP-1 can also stimulate fibroblasts to produce MMP-1 (Clark et al.). TIMP-3 has been shown to promote apoptosis of cancer cells, and inhibit IL-6 receptor shedding. (Smith et al.;Hargreaves et al.)

Another major endogenous inhibitor of MMPs is α_2-macroglobulin (α2-M) (Sottrup-Jensen and Birkedal-Hansen). It is present in high concentrations in human serum and interstitial fluids (Birkedal-Hansen et al.) and is therefore likely to be the major inhibitor of MMPs in tissue fluids, whereas TIMPs act more locally. Moreover, because α2-M/MMP complexes are removed by scavenger-receptor-mediated endocytosis, α2-M plays an important role in the irreversible clearance of MMPs, whereas the actions of TIMP are reversible (Sternlicht and Werb).

Another recently recognised class of MMP inhibitors, are protein subdomains, which have structural similarity to TIMPs. One example is the NC1 domain of type IV collagen, which has shown to have inhibitory effects upon MMP-2 and MMP-3 (Netzer et al.).

MMP inhibitors were expected to be of benefit in conditions where imbalance in expression of MMP and TIMPs contributes to disease pathogenesis, such as rheumatoid arthritis and cancers. A number of synthetic inhibitors of MMPs have been designed, and have entered clinical trials in humans. The most effective inhibitors have been those that replace a scissile bond for a carboxyl, sulfhydryl, or hydroxamate group, which associates with the zinc atom to inhibit catalysis (Woessner, Jr.). Results of trials using this therapeutic approach in cancer patients have been disappointing so far (Skiles, Gonnella, and Jeng). That said, a number of animal models have shown that MMP inhibitors could be of some benefit in inflammatory diseases such as bacterial meningitis, graft-versus-host disease, emphysema, aortic aneurysm, and atherosclerosis (Brinckerhoff and Matrisian).

1.6.5 Function of MMPs – General Comments

Under normal conditions, most MMPs are produced at almost undetectable levels. However, MMP production is upregulated in repair or remodelling processes, in diseased or inflamed tissue, and in cells grown in culture (Werb;Parks;Saarialho-Kere et al.). For example, Collagenase-1, stromelysin-2, matrilysin (MMP-7), and gelatinase-B, are expressed at increased levels by basal keratinocytes at the wound edge in skin (Okada et al.;Saarialho-Kere et al.;Madlener et al.). In the gut, stromelysin-2 and matrilysin (MMP-7) are expressed by epithelial cells bordering ulcers (Saarialho-Kere et al.;Vaalamo et al.); and collagenase-1 (MMP-1), matrilysin-1 (MMP-7), and stromelysin-2 (MMP-10) are strongly expressed by migrating enterocytes (Salmela et al.). The gelatinases A and B (MMPs –2 and –9), are also necessary for the migration of dermal dendritic cells from skin (Ratzinger et al.). Collagenase-1, stromelysin-2, and matrilysin-1 have been shown to be involved in intestinal re-epithelialization in-vivo, and are upregulated by cytokines important in wound repair (Salmela et al.). Of the many MMPs expressed in wounds, the function of collagenase-1 in the migrating epidermis is the best understood, and many experiments have shown that it is particularly important for keratinocyte migration on native type I collagen (Parks). MMPs also play an important role in wound healing of the skin (Pilcher et al.), airways (Dunsmore et al.;Legrand et al.),and cornea (Sivak and Fini).

MMPs can influence cell behaviour by cleaving cell-cell adhesion molecules, or by cleaving cell surface molecules that transduce signals from the extracellular environment. For example, MMP-3 and MMP-7 both cleave the adherens junction protein E-cadherin,

and the soluble extracellular fragment that is released disrupts cell aggregation and promotes cell invasion in a paracrine manner (Lochter et al.).

MMPs can also release cell surface molecules. For example, MMP-3 can release soluble L-selectin from leukocytes (Preece et al 1996), and active heparin-binding EGF-like growth factor from cell surfaces by cleaving it at a site just outside the cell membrane (Suzuki et al.). MMP-7 can induce Fas-receptor-mediated apoptosis by releasing active soluble Fas ligand from the surface of the same target cells (Powell et al 1999).

1.6.6 MMPs and Immune Response

T cells, macrophages, eosinophils, and neutrophils all produce and secrete MMPs, in cell-specific patterns. Macrophages secrete MMPs –1, -2,-3,-7, –9, and -12 (Birkedal-Hansen et al.;Busiek et al.;Welgus et al.). When T cells are activated by lectins, phorbol ester or anti-CD3 antibody, they can stimulate monocytes and fibroblasts to produce increased amounts of MMP-1 and MMP-9, with little or no increase in TIMP-1 production (Miltenburg et al.). The production of MMPs by macrophages is also down-regulated by IL-4 and IL-10 (Lacraz et al.).

There is evidence to suggest that MMPs may be able to activate pro-inflammatory cytokines. MMP-9 for example, can cleave IL-8, increasing its activity ten-fold (Opdenakker, Van den Steen, and Van Damme). In addition to causing the activation and release of cytokines and growth factors, MMPs can also cleave cell surface receptors. MMP-9, for example, cleaves IL-2 receptor-α (IL-2α) on T cells and significantly decrease T cell proliferation in response to IL-2 in vitro (Sheu et al.).

Other effector functions of activated MMPs expressed by immune cells include the promotion of trans-basement membrane migration by T cells (Leppert et al.), and the migration of T-cells across high endothelial venules and into lymph nodes (Faveeuw, Preece, and Ager). Recent research also suggests that MMP-3 plays a role in the recruitment of $CD4^+$ T cells to the intestinal mucosa (Li et al.).

MMPs are also capable of inhibiting inflammation. For example, MMP-2 is able to cleave monocyte chemoattractant protein-3, thereby inactivating it and generating a chemokine

receptor-binding antagonist that further impedes inflammation (McQuibban et al.). In addition, MMPs can degrade the inflammatory cytokine IL1-β, which is also a potent inducer of MMP expression, but not IL-1α (Ito et al.).

1.6.7 Role of MMPs in Disease states

A number of diseases involve degradative processes of the ECM, but only recently have MMPs been implicated in their pathogenesis.

(i) MMPs in Cancer

The association of matrix proteolysis and cancer has long been recognised, and destruction of the basement membrane in epithelial tumours is a well-established hallmark of malignancy. The role of MMPs in cancer has evolved considerably over the last decade (Brinckerhoff and Matrisian). Activated MMPs are generally present in greater amounts in and around malignant cancers than in normal, benign, or premalignant tissues, with the highest expression taking place in areas of active invasion at the tumour-stroma interface (Sternlicht and Werb). Several MMPs were first cloned as cancer-associated genes (Sternlicht and Werb). Significant correlations have been found between MMP expression and various indicators of a poor prognosis, such as lower overall survival and shorter disease-free time, in virtually all types of cancer (Sternlicht and Werb). There is also compelling evidence that MMPs actively contribute to cancer progression. Several MMPs have been implicated in tumour invasion, metastasis, and angiogenesis, including MMPs -1, -2, -3,-9 and -14 (Sternlicht and Werb). The over-expression of MMPs –1, -3, -9, -13 and MT1-MMP have been demonstrated in colorectal cancers, and there has been noted be a positive correlation of MMP expression with staging of disease and prognosis (Zucker and Vacirca)

The obvious assumption is that the MMPs, by degrading the ECM, enable cancer cells to cross the matrix barriers that would otherwise contain their spread. However, recent data presented show that MMPs are also involved in several aspects of cancer development. For example, MMP-3 can promote epithelial-to-mesenchymal phenotypic changes that are associated with more malignant behaviour (Lochter et al.;Sternlicht et al.).

When considering that MMPs tend to promote cancer development, their inhibitors should counteract these effects. However, this is not necessarily the case, as it has been shown that TIMP-1 expressing tumour cells were capable of metastasis, though not growth at a secondary site (Koop et al.). Recently the interest in MMPs as a therapeutic target for cancer has led to the development of a number of synthetic inhibitors, which have entered clinical trials for their therapeutic use in prevention of cancer progression. In general the results of stage III trials in advanced stage cancers has been so far disappointing (reviewed by Coussens, LM 2002) (Coussens, Fingleton, and Matrisian;Wagenaar-Miller, Gorden, and Matrisian;Pavlaki and Zucker).

(ii) MMPs in Articular Diseases

One of the first links between MMPs and disease came with rheumatoid arthritis (RA), a condition in which the collagen in the cartilage, bone and tendons surrounding the joints is progressively and irreversibly degraded. Although the aetiology is not fully understood, the action of MMPs is believed to play a role in disease pathogenesis, and that of oesteoarthritis (Brinckerhoff and Matrisian;Martel-Pelletier, Welsch, and Pelletier). Recently, knockout animal models have provided interesting insights into disease pathogenesis of articular inflammation, and MMP-2 and MMP-9 have been shown to be important (Itoh J Immunol 2002). In addition, MMP-12 expression by macrophages has been shown to play a pivotal role in arthritis exacerbation in transgenic rabbits (Wang et al.).

(iii) MMPs in Respiratory Diseases

MMPs have been associated with various respiratory conditions. In chronic lung disease of prematurity, MMPs –9 and –2 are detectable in the bronchoalveolar lavage fluid of premature infants, and the MMP/TIMP ratio increases with decreasing gestation of the infant (ie increasing severity of lung disease) (Sweet et al.;Lanchou et al.). MMPs have also been implicated in the lung injury associated with asthma (Prikk et al.). Recent data also point to MMP-12 being important in the development of smoking-induced emphysema (Hautamaki et al.). Experiments with knock-out mice in a model of lung injury have shown that IL-13 mediated expression of MMPs: -2,-9,-13 and-14 is modulated by an MMP-12 dependent pathway in acute lung injury (Lanone et al.).

(iv) MMPs in Cardiovascular Diseases

There has been much data in support of the importance of MMPs in blood vessel matrix remodelling in cardiovascular diseases. There is excessive accumulation of cells and ECM in the intimal layer, such as in atheroscerosis, arterial restenosis following vascular interventions, and processes involving matrix degradation, exemplified by plaque rupture and aneurysm formation.

Several MMPs are expressed at increased levels in atherosclerotic tissue, including MMPs: -1, -2, -3, and-9 (Galis et al.;Henney et al.), and are likely to contribute to vascular remodelling and plaque disruption. In patients with severe coronary stenosis, MMP-9 levels are elevated, and may constitute a predictor of cardiovascular mortality in patients with coronary artery disease (Kalela et al.).

Differences in expression of MMP/TIMPs may determine the evolution of advanced athersclerotic plaques and contribute to their vulnerability. For example, MMP-1 and MMP-3 are highly expressed in human aneurysms but not occlusion plaques, whereas MMP-9 is mainly detected in carotid as compared with femoral arteries (Orbe et al.). Serum levels of MMP-3 and MMP-9 and TIMP-1 are elevated in asymptomatic hyperlipidaemic subjects at high cardiovascular risk, and MMP-3 and TIMP-1 levels are positively associated with carotid lesions (Beaudeux et al.). Murine models have also shown that MMP-3, MMP-9 and MMP-12 deficiency protect against athersclerotic media destruction and subsequent aneurysm formation (Silence et al.;Luttun et al.).

(v) MMPs in Gastrointestinal Diseases

Increased expression of MMPs has been noted in a number of diseases in the gut, although this is still a rather unexplored area. Necrotising enterocolitis (NEC) is a condition of unknown aetiology affecting mainly premature and/or low birth weight infants, in which bacterial infection and hypoxia is thought to play a role. Recent data has confirmed the involvement of MMPs in this disease process (Pender et al.). There is increased expression of MMP-3 and TIMP-1, but not TIMP-2, and gelatinases and collagenases were not expressed. The cellular sourceof MMP-3 has smooth muscle actin positive cells.

In coeliac disease, there is flattening of the villi in the small intestine as a result of T-cell mediated injury driven by gluten. The diseased mucosa of coeliac patients has been shown to express increased levels of mRNA for MMPs-1, -3, and -12, (Daum et al.;Salmela et al.;Ciccocioppo et al.). The expression of MMPs –1 and –3 is localised to subepithelial fibroblasts and macrophages.

The increased expression of MMPs -1, -3 and -7 (Wroblewski et al.;Crawford et al.;Koyama) has been documented in peptic ulcer lesions. Recent studies have also showed that MMP-7 is upregulated in the presence of *Helicobacter pylori*, and it has been suggested that over-expression of this enzyme may confer increased risk of gastric ulceration and metaplasia that is associated with H.pyloris infection (Koyama).

MMPs and activation of the protease cascade have recently been implicated as a contributory factor to mucosal degradation and ulceration in IBD. The first convincing evidence of this was provided by studies using in-situ hybridisation to identify RNA transcripts. Elevated levels of MMP transcripts, in particular, MMP-1 (interstitial collagenase) and MMP-3, were seen in areas of ulceration in individuals with CD or UC (Baugh et al.;Heuschkel et al.;Louis et al.;Bailey et al.;Saarialho-Kere et al.;Vaalamo et al.). It was then also observed that a cellular source of MMP expression in IBD was fibroblast-like stromal cells (Vaalamo et al.). Expression of MMP –1, -3, and -9 protein are increased in the mucosa and fistulae of patients with CD (Kirkegaard et al.). Human macrophage metalloelastase (HME, MMP-12), is highly expressed in macrophages in the vicinity of shedding mucosal epithelium and beneath the necrotic surface of ulcers (Vaalamo et al.). The direct effect of stromelysin-1 on the gut mucosa has been studied, and has been shown to cause rapid, extensive mucosal degradation at very low concentrations (Pender et al.).

The excessive production of MMPs in the gut mucosa of patients with active IBD is coupled with a relatively small increase in production of TIMPs. As a result, there is disturbance of the balance between synthesis and degradation of ECM components, leading to ulceration. For example, studies have shown excessive production of mRNA transcripts of MMPs –1 and –3, in biopsies from patients with IBD, with only marginally increased transcripts of TIMP-1, and unaltered levels of TIMP-2. (Heuschkel et al.;von Lampe et al.). Interstitial collagenase is capable of degrading collagen III, and stromelysin

–1, of degrading collagen IV of the basement membrane, and together they have the potential to destroy the scaffolding of the lamina propria, on which the epithelium lies.

Functionally, the importance of MMPs in degradation of the gut mucosa has been studied in this laboratory. Using a foetal gut explant model, LP T cells were activated by mitogens to produce a strong Th1 biased response, and T cell dependent mucosal degradation (Lionetti et al.). This resulted in degradation of glycosaminoglycans in the LP, which could be inhibited by α-2 macroglobulin (Pender et al.). T-cell mediated injury was associated with markedly increased levels of activated interstital collagenase and stromelysin-1. Furthermore, the direct application of recombinant stromelysin-1 caused mucosal degradation (Pender et al.). In addition, T cell driven injury can be inhibited by synthetic peptidomimetic inhibitors of MMPs, without preventing T-cell activation (Pender et al.;Salmela et al.). Mesenchymal cells derived from the foetal small intestinal mucosa secrete increased amounts of interstitial collagenase, gelatinase A and stromelysin-1 when stimulated by IL-1 and TNF-α, but without a corresponding increase in TIMP-1 or TIMP-2 (Pender et al.;Pender et al.). These results suggest that T-cell activation in the LP results in increased production of MMPs, which then degrade the ECM leading to mucosal injury.

1.7 Role of gene mutations and polymorphisms in IBD

IBD tends to run in families and so in recent years there has been considerable focus on regions of the genome associated with disease.

1.7.1 Genetic Epidemiology of IBD: Genetics versus Environment

There is strong evidence from epidemiological studies that genetic factors play an important role in the aetiology of IBD. For example, CD in particular, has been shown to cluster in families. In population-based studies, the risk to siblings of affected individuals relative to the general population was 15-40 fold for CD, and 8-10 fold for UC (Van Heel et al.). Twin studies have shown a higher concordance rate for monozygotic than dizygotic twins: 33% versus 4% in CD, and 13% versus 2% in UC (Tysk et al.;Orholm et al.;Thompson et al.).

There is also considerable data to support genetic determination of not only susceptibility to disease, but also disease phenotype. Epidemiological studies in multiply-affected kindreds in the United Kingdom have shown high concordance rates for disease type, location, and the presence of extraintestinal manifestations (Satsangi et al.). Data from the United States also suggests that disease behaviour in CD (stenosing, fitsulising, or mixed), may have a familial basis (Bayless et al.).Disease susceptibility has also been noted among certain ethnic groups, such as the Ashkenazi Jewish population, who also tend to have earlier-onset disease.

There are a number of other lines of evidence that compliment the concordance rates in twin pairs and multiply affected families, in providing evidence of genetic susceptibility to IBD. For example, there is a well-recognised association between the inflammatory bowel diseases and other diseases with recognised genetic predisposition, such as primary sclerosing cholangitis (Chapman), and ankylosing spondylitis (Watts and Satsangi).

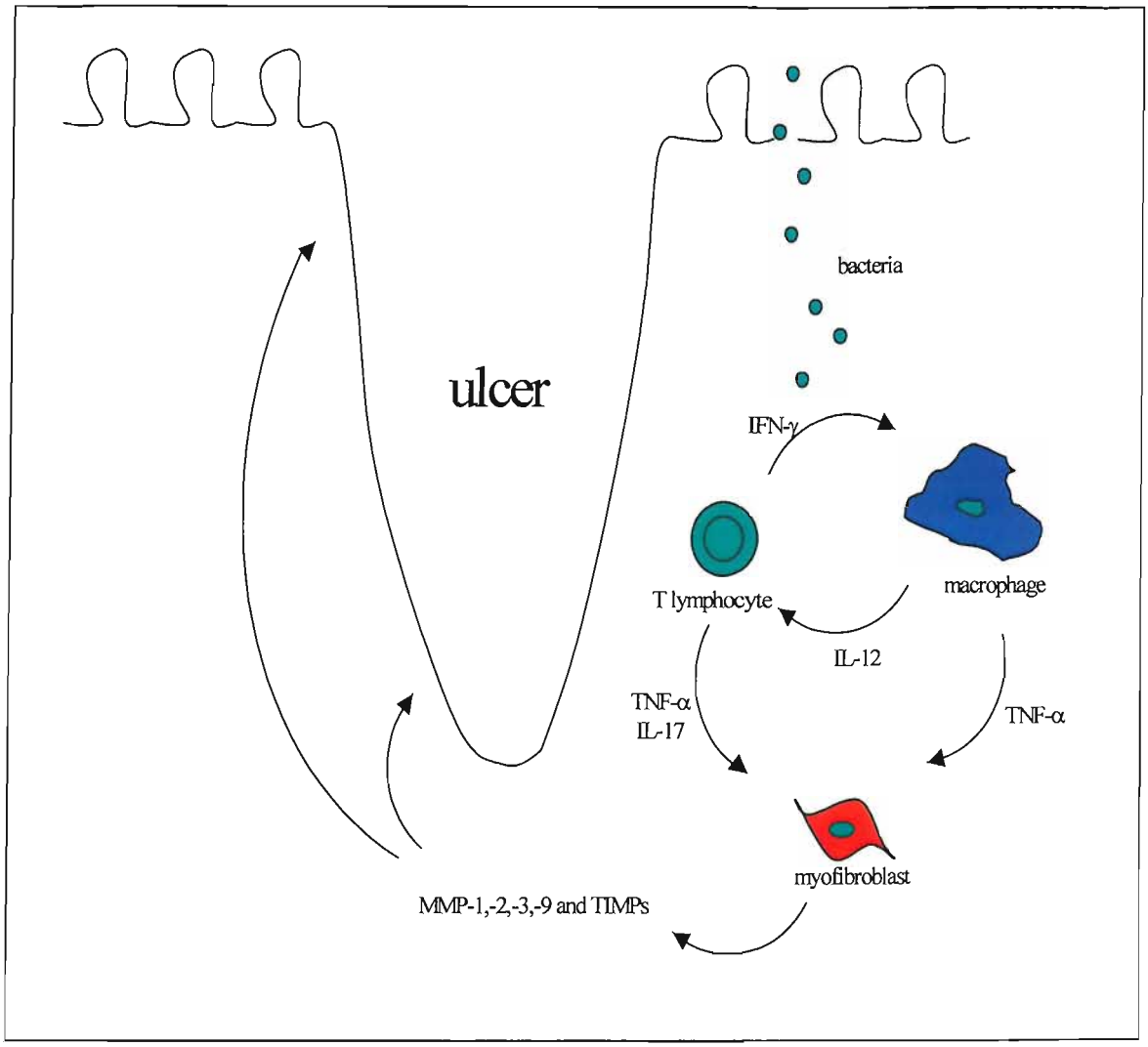

Figure 1.1: Illustration of how TNF-α produced in excess by activated T cells and macrophages can lead to production of MMPs in the gut, causing inflammation and ulceration.

Although there is strong evidence in support of genetic factors being important in IBD, it is also apparent that environmental factors are critical. This is evident, again, looking at the twin studies, where there is less than 100% concordance between monozygotic twins. The twin data also suggest that environmental contribution is relatively stronger in UC than CD. Another clue as to the importance of environment to IBD aetiology, is in the epidemiological data from different populations and migrating populations. It would appear that the incidence and prevalence of IBD varies depending on geographic location. In general, there is an increased risk for developing IBD in urban compared to with rural areas, in cohorts with a higher socio-economic class, as well as in developed rather than less–developed countries (Bonen and Cho). Furthermore, the incidence of the disease increases when populations emigrate from areas of low-risk to higher risk areas (Tsironi et al.;Reddy and Burakoff).

1.7.2 The IBD loci

In addition to epidemiological studies, there is now molecular evidence to suggest that CD and UC are polygenic disorders, and share some but not all susceptibility loci. In the search for genetic determinants of IBD susceptibility, two complementary approaches have been used, that of genetic linkage and candidate association studies. Using these techniques, a number of susceptibility loci have been discovered on chromosomes: 1,5,6,7,12,14,16 and 19 (Hugot et al.;Satsangi et al.;Hampe et al.;Hampe et al.;Rioux et al.;Duerr et al.;Rioux et al.). These clearly indicate the heterogeneity of UC and CD.

(i) The IBD1 locus and CARD15/NOD2 polymorphisms

The best-replicated region is that of the IBD1 locus, in the pericentromeric region of chromosome 16. First identified as a CD locus by Hugot et al (Hugot et al.) in 1996 using genome wide scanning, this observation has been replicated in numerous studies (Ohmen et al.;Brant et al.;Curran et al.;Cavanaugh;Annese et al.;Annese et al.). The evidence for linkage in this region is now known to be largely accounted for by three relatively uncommon amino acid polymorphisms within the NOD2/Caspase-Activation Recruitment Domain (CARD) 15 gene.

NOD2/CARD15 is expressed in peripheral blood monocytes, macrophages (Berrebi et al.), dendritic cells, and gut epithelial cells (Gutierrez et al.) and paneth cells(Ogura et al.). It is related structurally to the well-described R proteins in plants, which mediate host

resistance to microbial pathogens. CARD15 molecules are defined by the presence of three domains: a C terminal leucine-rich repeat (LRR) domain, a nucleotide oligomerization domain, and an N-terminal region that contains a CARD or pyrin-domain (Inohara and Nunez).The N terminus of the gene contains 2 CARD domains, which mediate protein-protein interactions. The LRR domain within the C-terminus is the major structural motif that functions as a pattern-recognition receptor for pathogen-derived products in the cytosol. Specifically, it is muramyl dipeptide (MDP), derived from peptidoglycan, that is being sensed by NOD2 (Inohara et al.;Girardin et al.). Three major coding polymorphisms (Arg702Trp, Gly908Arg, and Leu1007fsinsC) within or near the LRR of NOD2/CARD15 are highly associated with CD (Hugot et al.;Ogura et al.). In particular, the latter polymorphism, involving the insertion of a cytosine nucleotide in exon 10, gives rise to a frameshift mutation at the nucleotide of codon 1007, leading to a premature stop codon, and protein truncation.

Having one copy of the alleles confers a relatively small risk of developing CD (2- to 4-fold), whereas in contrast, having 2 copies of the risk alleles increases the risk for developing the disease 20- to 40- fold, indicating that NOD2/CARD15 functions to a large extent in an autosomal-recessive fashion. Approximately 8 to 17% of CD patients carry 2 copies of the major NOD2/CARD15 risk alleles (compared with less than 1% Caucasian normal populations).

There is a higher preponderance of the NOD2/CARD15 mutations in Caucasian CD patients. Furthermore, genotype-phenotype studies have shown a significant association with ileal disease and the carriage of one or more NOD2/CARD15 risk variant alleles (Cuthbert et al.;Helio et al.).

The functional effect of the frameshift mutation has been investigated, by comparing the ability of wild type and mutant NOD2 to produce NFkB in vitro, in response to exposure to bacterial products (Ogura et al.). It was found that the effect of the mutant NOD2 was to reduce NF-kB activation. This was a surprising finding, since NF-kB, and its targets, the proinflammatory cytokines TNF-α, IL-6 and IL-1β, are raised in the LP of CD patients. Therefore, the question as to why this mutation would increase susceptibility to disease remains unanswered. It has been suggested that one function of NOD2 is to limit the

production of proinflammatory cytokines such as IL-12 and IL-18 by inhibiting the signaling pathway mediated by Toll-like receptor 2 (TLR2) – stimulation (Watanabe et al.). In this model, the mutant form of the NOD2 is unable to inhibit TLR2 signaling. It has also been suggested that individuals with NOD2 mutations may have defective bacterial immunity due to their diminished production of cryptidins, anti-microbial peptides, critical in the innate immune response to bacterial infection (Kobayashi et al.).

(ii) Other IBD loci

Published genome scans have identified other regions with suggestive linkage to IBD susceptibility.

Linkage studies by Satsangi et al revealed a locus spanning 41 centimorgans on the long arm of chromosome 12 (Satsangi et al.). This chromosome 12 linkage has been replicated in a number of studies (Duerr et al.;Curran et al.), although not universally (Brant et al.). Linkage analyses suggest that this may be a susceptibility locus for UC alone. A number of candidate genes have been investigated within this region, such as the beta 7 integrin gene (Van Heel, Carey, and Jewell), and the STAT-6 gene (de Jong et al.), with negative results.

The locus on chromosome 6p has been the focus of much attention in genetic studies in IBD, which encompasses the major histocompatibility complex (MHC). This locus has been implicated in CD and UC. Overall, disease susceptibility for this locus is weak, and there is uncertainty as to whether the allelic associations simply reflect the pronounced linkage disequilibrium within the region, as many other genes juxtaposed to the HLA locus are involved in immunoregulation.
However, strong associations with disease behaviour and allelic variation have been described, particularly in UC. For example, the HLADR B1*0103 allele has been linked with increased severity of UC, surgical intervention, extraintestinal manifestations (Roussomoustakaki et al.), and arthropathy (Orchard et al.).
Another gene susceptibility candidate, within the HLA class III region is the TNF-α gene. As described previously, TNF-α has been shown to play an important role in the pathogenic pathway leading to mucosal ulceration. Japanese studies have reported that an allelic variant in the promoter region of the TNF-α gene confers increased risk of disease onset and progression (Negoro et al.).

A locus on chromosome 14q11 has also been identified as contributing to CD susceptibility in families with early-onset disease (Ma et al.;Duerr et al.). This region contains a number of immunoregulatory cytokines in the pathophysiology of CD, such as the interleukins and the transcription factor, interferon regulatory factor, isoform 1.

Following a genome-wide scan in Canadian families, a region of significant linkage was identified at chromosome 5q31-q33 (Rioux et al.), particularly among families with early-onset disease. Rioux used a linkage approach to show that carriage of the risk alleles increased the risk of developing CD by 2 fold, whereas homozygous carriage increased the risk 6-fold (Rioux et al.). A later study has shown that the IBD5 risk haplotype is associated with CD in British Caucasian subjects, though not in the Japanese population (Negoro et al.). This region contains a number of immunoregulatory cytokines, including interleukins 3,4,5 and 14, as well as other potential candidate genes, such as colony-stimulating factor isoform 2 and the transcription factor, interferon regulatory factor, isoform 1.

Recently, gene loci have been identified within this region, encoding the OCTN cation transporter genes (Peltekova et al.). Mutations within this gene have been shown to affect the transport of amino acids across cell membranes.

A number of other regions have been implicated in many genome scans carried out globally. Although work so far has mainly concentrated on the 1-4 loci, it is becoming increasingly apparent that different loci may be important, and to certain population groups. For example, significant linkage to chromosome 19p13 has been observed in Candian sibling-pair families (Rioux et al.). There are several candidate genes in this region, including intercellular adhesion molecule 1, complement component 3, thromboxane A2 receptor, and leukotriene B4 hydroxylase.

Chromosome 1p has also been a region of interest. The localization of this region was refined using linkage studies (Cho et al.). Interestingly, families with linkage to this region also showed increased linkage to IBD1 (Cho et al.;Edwards), suggesting possible gene-gene interactions between these two loci. Several potential candidate genes are located in this region including the TNF receptor family and caspase 9.

A more recent study has shown an association of CD with a polymorphism in the DLG5 gene located on chromosome 10q23. This single nucleotide polymorphism is thought to

confer increased susceptibility to CD owing to its effect of impairing enzymatic ability of guanylate kinase and function to maintain epithelial polarity (Stoll et al.).

Linkage has also been observed for the locus on chromosome 3p by several research groups (Satsangi et al.;Hampe et al.;Paavola et al.).Genes in this region code for hepatocyte growth factor, epidermal growth factor, and an inhibitory guanine nucleotide-binding protein, GNAI2 (Satsangi et al.). Recently, a polymorphism in the hMLH1 mismatch repair gene in this susceptibility region has been observed to carry an almost 5 times increased of refractory ulcerative colitis, compared to controls (Bagnoli et al.).

1.7.3 MMP Gene Promoter Polymorphisms

MMPs play a central part in the pathogenesis of IBD. Because they are regulated at a transcriptional level, they are worthy of consideration for candidate susceptibility genes in IBD.

Previous studies have identified naturally occurring sequence variants in the promoter regions of a number of MMP genes, which alter the activity of their respective gene promoters in an allele-specific manner (Ye), and have functional consequences for MMP production. These are summarized in the next section.

(i) Gene Polymorphisms

Naturally occurring sequence variants, having a frequency greater than 1% of the population, are termed DNA polymorphisms. They occur at approximately one in every thousand base pairs (Sherry, Ward, and Sirotkin). Ninety percent of DNA polymorphisms are single nucleotide polymorphisms (SNPs) due to single base substitutions (Collins, Brooks, and Chakravarti). Some SNPs result in the generation or removal of a restriction site and thus can be detected and analysed by digesting DNA with an appropriate restriction endonuclease. There are also minisatellite polymorphisms, about 0.1-20 kilobases (kb) long, and microsatellite polymorphisms, which are often less than 0.1kb long, which result from variation in the number of tandem repeats of DNA sequence. The majority of microsatellite polymorphisms occur at di-nucleotide repeat sequences, such as

$(CA)_n$ repeats. Generally, SNPs are bi-allelic whereas microsatellite polymorphisms are multi-allelic.

Although the majority of these DNA polymorphisms are probably functionally neutral, a proportion of them can exert allele-specific effects on the regulation of gene expression, or function of the encoded protein.

Over the last decade, polymorphisms have been identified in the promoters of a number of MMP genes. The promoter polymorphisms of the following genes: MMP-1, -3, -9 and –12, in particular, have been studied in relation to risk from various disease processes, including susceptibility to coronary heart disease, abdominal aortic aneurysm, and cancers.

(ii) MMP-1 (Collagenase-1) Gene Promoter Polymorphism

The MMP-1 gene has 10 exons, and is on chromosome 11q22.2-22.3. It is tightly linked to a cluster of other 8 MMP genes that include MMP–3, -7, -8, -10, -12,-13,-20, and –27. The expression of MMP-1 is partly regulated by promoter sequences upstream of the start of transcription (Buttice et al.). A polymorphism resulting from an insertion/deletion of guanosine, at –1607 bp in the MMP-1 promoter has been reported (Rutter et al.). Two alleles have been detected, one having allele a single guanosine (1G) and the other having two guanosines (2G) at the polymorphic site. The addition of guanosine creates a core binding site for the Ets family of transcription factors. Functional studies showed that the presence of the 2G allele increases MMP-1 transcription in fibroblasts and melanoma cells (Rutter et al.).

The 2G allele is associated with an increased risk of cancer. In a Japanese study of patients with ovarian cancer, there was over-representation of the 2G allele in the cancer group compared to the control group, and expression of MMP-1 was higher among the cancer patients carrying the 2G allele. (Kanamori et al.).The 2G allele has also been shown to be associated with increased lung cancer susceptibility (Zhu et al.), is linked to the progression of malignant melanoma (Noll et al.), endometrial cancers (Nishioka et al.); and to the invasiveness of colorectal cancer (Ghilardi et al.). These findings suggest that the 2G allele is associated with increased expression of MMP-1, increased risk of development of cancer, and increased tumour invasiveness.

This MMP-1 polymorphism has also been investigated for its allele-specific effects on susceptibility to coronary heart disease, but results showed no causal link (Ye, Gale, and Martyn).

(iii) MMP-3 (Stromelysin-1) Gene Promoter Polymorphism

The MMP-3 gene, like that of MMP-1, is located on chromosome 11q22.2-22.3. Expression of stromelysin-1 is regulated at the level of transcription, primarily by proinflammatory cytokines (Quinones et al.).

A polymorphism in the promoter of the MMP-3 gene has been reported, located at position –1612. The polymorphism is due to variation in the length of a track of adenosines at this position, so that one allele has five adenosines (5A), and the other allele has 6 adenosines (6A) (Ye et al.). In vitro studies have shown that the 5A allele has greater promoter activity compared with the 6A allele in fibroblasts and smooth muscle cells (Ye et al.). Transcriptional repressors have been shown to bind to this polymorphic site with a higher affinity for the 6A allele, resulting in decreased activity of the 6A allelic promoter (Ye et al.;Borghaei, Sullivan, and Mochan). Proteins that bind to the promoter element containing this site, upregulate IL-1-induced expression of the MMP-3 gene (Borghaei, Sullivan, and Mochan).

MMP-3, together with other MMPs, is expressed in the atherosclerotic lesion, suggesting that it plays a role in remodelling of the ECM in the formation of athersclerotic plaque (Henney et al.;Galis et al.).The 5A/6A polymorphism has been studied in relation to the risk of coronary heart disease and atherosclerosis. In a study of patients with acute myocardial infarction, the frequency of the 5A allele was higher among patients than controls, and the relative risk of acute myocardial infarction was increased for those carrying the 5A allele than those homozygous for the 6A allele (Terashima et al.). This suggests that individuals carrying the 5A allele are at increased risk of myocardial infarction, which is commonly caused by rupture of atherosclerotic plaque. The 5A allele was also found to be associated with increased susceptibility to abdominal aortic aneurysm (Yoon et al.), and essential hypertension (Beilby et al.).

On the other hand, a number of studies have shown that the 6A allele also confers increased risk of atherosclerotic plaque formation. For example, one study found that individuals homozygous for the 6A allele tended to have increased carotid artery wall thickness (Gnasso et al.). The 6A allele may also be associated with accelerated growth of coronary atheromas (Ye et al.;Humphries et al.;de Maat et al.), and restenosis of coronary vessels (Humphries et al.).

A plausible explanation for these observations is that individuals carrying the 5A allele produce increased amounts of MMP-3, making atherosclerotic plaques in these individuals prone to rupture, whereas insufficient expression of MMP-3 by carriers of the 6A allele leads to accumulation of the vascular matrix, and so build up of atherosclerotic plaque. It should be noted that at the same time others have shown no association with the 5A/6A polymorphism and ischaemic heart disease, although this may have been due to small sample size (McGlinchey et al.).

The MMP-3 allele has also been investigated in other conditions, such as primary sclerosing cholangitis. This is a chronic cholestatic disease characterised by inflammation and fibrosis of the intra-and extrahepatic biliary tree. The 5A allele confers not only increased susceptibility to disease-onset, but also to the development of portal hypertension (Satsangi et al.). The 5A allele has also been found to be associated with increased risk of stroke (Flex et al.), severity of rheumatoid arthritis (Constantin et al.)and metastasis of oesophageal squalmous cell carcinoma (Zhang et al.) but not predisposition to colorectal cancer (Zinzindohoue et al.), ankylosing spondylitis (Jin et al.), renal cell carcinoma (Hirata et al.)or periodontitis (Itagaki et al.).

(iv) MMP-9 (Gelatinase-B) Gene Promoter Polymorphisms

There are two polymorphisms in the MMP-9 gene promoter, which have functional effects on promoter activity. The first is a $(CA)_n$ microsatellite polymorphism at position –90, and the second is an SNP at position -1562 (Shimajiri et al.;Peters et al.).

The miscrosatellite polymorphism of CA repeats is multi-allelic, with a bimodal distribution of allele frequencies. The first peak is at the $(CA)_{14}$ allele, and the second peak is at the $(CA)_{21}$, $(CA)_{22}$ and $(CA)_{23}$ alleles (Shimajiri et al.).It has been shown that alleles

with higher (CA) repeats confer higher transcriptional activities in oesophageal carcinoma cell lines (Shimajiri et al.) and fibroblast cell lines (Peters et al.). One study also reported the positive association of the $(CA)_n$ polymorphism with intracranial aneurysm (St Jean et al.), although this has not been confirmed (Peters et al.;Yoon et al.).

The SNP in the MMP-9 gene at position –1562 is due to a cytosine (C) to thymidine (T) substitution (Zhang et al.). Functional studies have shown that the T allele has higher promoter activity than the C allele, due to preferential binding of a putative transcription repressor protein to the C allelic promoter (Zhang et al.). The T allele has been shown to be associated with increasing severity of atherosclerosis (Zhang et al.), and coronary restenosis after percutaneous coronary catheterisation (Cho et al.). In view of the fact that MMP-9 has been shown to facilitate smooth muscle cell migration (Mason et al.), it may be that individuals carrying the T allele have higher transcriptional activity, and therefore enhanced ability of smooth muscle cells to migrate and proliferate in the formation of atherosclerotic plaque.

(v) MMP-12 (Macrophage Metalloelastase) Gene Promoter Polymorphism

Abnormal MMP-12 expression has been associated with abdominal aortic aneurysm (Curci et al.), atherosclerosis (Matsumoto et al.), and emphysema (Hautamaki et al.). Recently it has also been implicated in articular joint destruction (Wang et al.). Increased expression of MMP-12 has also been noted by macrophages in gastrointestinal ulcerations (Vaalamo et al.).

A polymorphism has been identified in the promoter region of the MMP-12 gene, which is also located on chromosome 11q22.2-22.3. This polymorphism, located at positon –82, is due to an adenosine (A) to guanosine (G) substitution, where the A allele is more prevalent in the normal population, and the G allele was noted to be 0.19 (Jormsjo et al.). This SNP is located immediately adjacent to an AP-1 binding site. In vitro studies have shown that this SNP changes the binding affinity of AP-1, and hence the transcriptional activity of the MMP-12 promoter (Jormsjo et al.). The A allele has been shown confer enhanced activation by AP-1, by increasing the binding of AP-1 to the promoter of MMP-12(Jormsjo et al.). The A allele has also been shown to be over-represented in patients with coronary-artery narrowing compared to controls (Jormsjo et al.).

1.8 Appendix chapters A to D

Additional work has been included in the Appendix sections A to D, which investigate the susceptibility of the MMP-1 polymorphism, the MMP-3 polymorphism, the MMP-9 polymorphism, and the MMP-12 polymorphism in relation to predisposition to IBD.

The additional work in these sections is based on the observation that functional studies have shown that the MMP-1 1G/2G polymorphism, MMP-3 polymorphism, MMP-9 C/T polymorphism, and MMP-12 A/G polymorphism affect susceptibility to certain conditions in which the balance of ECM turnover is altered. In addition, it has been shown that these polymorphisms may affect particular phenotypic subgroups. Therefore the hypothesis was that these functional polymorphisms may affect susceptibility to IBD, and certain subgroups of CD and UC, such as early-onset disease and or in adulthood.

The aims of these studies were to investigate the relationship of the MMP-1, MMP-3, MMP-9 and MMP-12 polymorphsims in relation to CD and UC, in children, adults, and overall in both children and adults. The population-based study of the susceptibility of MMP-3 to CD and UC of Appendix A differs from the TDT analysis of MMP-3 in three cohorts in Chapter 6 in that different populations have been collected (one group including children), and the question of susceptibility to early-onset disease is addressed in Appendix A. The power of these studies was reduced by small sample size, and therefore they have been included as Appendix sections.

1.9 Summary and Hypothesis

The aetiology of IBD remains unknown, but there is emerging evidence that both UC and CD are separate polygenic disorders, but may share some, but not all genetic and environmental susceptibility factors. They both have similar end-points, which involve degradation of the gut mucosa, brought about largely by the over-expression of matrix metalloproteinases, and an imbalance of MMP production compared to their endogenous inhibitors.

Targeting pro-inflammatory cytokines as a therapeutic intervention of IBD has been shown to be effective (Cuzzocrea;Hibi et al.;Ogata and Hibi). Another worthwhile strategy would be to target MMPs as end-effector molecules. Given that MMPs are largely regulated at transcriptional level, investigating the genetic variation in promoter activity of the MMPs would be a logical approach.

Naturally occurring sequence variants have been identified in the promoter regions of the MMP genes: -1, -3, -9 and –12, which alter the activity of these gene promoters in an allele-specific manner. Previous research has shown that these MMP polymorphisms affect susceptibility of an individual to certain diseases, such as coronary heart diseases, aneurysms and certain cancers, whose pathogenesis involves ECM degradation and remodelling.

The MMP-3 gene promoter polymorphism in particular, has been investigated in a number of diseases because of the prominent role of MMP-3 in the pathogenesis of inflammatory conditions. MMP-3 has also been shown to be particularly important as an end-effector molecule in mucosal injury in the gut.

There is functional and epidemiological evidence to suggest that the 5A/6A polymorphism may have allele-specific effects on MMP-3 expression, where those individuals carrying the 5A allele have increased susceptibility to conditions favouring matrix degradation, such as inflammatory bowel disease. Similarly, functional studies have shown that the MMP-1 1G/2G polymorphism, the MMP-9 C/T polymorphism, and the MMP-12 A/G

polymorphism affect susceptibility to certain conditions in which the balance of ECM turnover is altered.

Therefore, the key hypotheses were firstly that myofibroblasts cells cultured from CD and UC subjects produced higher amounts of MMP-3. Studies were also carried out to investigate whether or not the MMP-3 gene promoter polymorphism has allele-specific effects on the expression of MMP-3. Finally, it was also proposed that these naturally occurring variants in the MMP-3 promoter polymorphism confer increased susceptibility to CD and UC.

1.10 Aims

The aims of this study are summarised as follows:

- To compare MMP-3 production by myofibroblasts from CD, UC and control groups.
- To compare MMP-3 production between subjects with different MMP-3 5A/6A genotypes.
- To compare the association of MMP-3 5A/6A genotypes with susceptibility to CD and UC.

Chapter 2: Materials and Methods

2.1 Subjects

The study design was given approval by the Hampshire Research Ethics Committee. Informed consent was obtained from all participating subjects. All those participating were British white subjects. The following details were documented from each patient:

- Age, at the time of participation in the study
- Gender
- Diagnosis

Patients were categorised as having Crohns' Disease and Ulcerative Colitis if they had:

- Endoscopic findings consistent with either UC or CD;
- Histological findings from biopsy sample confirming the diagnosis

98 adult subjects (62% of the 155 subjects originally recruited) participated in the study to compare expression of MMP-3 by myofibroblast cell lines between control, CD and UC subjects, and the study to compare MMP-3 expression by myofibroblast cell lines between different MMP-3 5A/6A genotypes. They included 55 subjects (60.4% of those recruited) in the control group, 23 subjects in the CD group (71.8% of those recruited), and 20 subjects in the UC group (64.5% of those recruited) (see Table 2.1). Samples from subjects were excluded from the study due to the following reasons:

- Failure of samples to grow myofibroblast colonies
- Infection of myofibroblast cell lines
- Diagnosis of subject changed following collection of biopsy sample to either Intermediate colitis or inconclusive

All samples that were successfully grown were genotyped for the MMP-3 5A/6A gene promoter polymorphism. Subjects in the CD and UC groups were further divided into those with active disease and those with disease in remission. The subjects with CD or UC

were considered to have active disease if there were areas of active inflammation on colonoscopy, supported by histological findings from biopsy samples consistent with active inflammation. Of the total of 23 CD subjects, 11 had active disease (aCD), and 12 had disease in remission (rCD). Of the 20 subjects in the UC group, 12 subjects had active disease (aUC), and 8 subjects had disease in remission (rUC).

Cell-lines from 39 subjects were used to characterise the cultured cells: 19 control subjects taken at random from a total of 55, 10 CD subjects taken from a total of 23, and 10 UC subjects taken from a total of 20.

The MMP-3 5A/6A polymorphism was analysed in a collection of trios from Germany (n=468), each consisting of one patient with IBD and their two unaffected parents. In addition, German Muplitplex families (n=218) and British Multiplex families (n=140), were recruited to investigate the association of the MMP-3 gene promoter polymorphism with susceptibility to IBD, by transmission disequilibrium test (TDT) (see Table 2.2). The German trio cohort consisted of only affected individuals and their unaffected parents, with no family history of IBD. Families in the British and German multiplex groups consisted of two or more affected family members with a first degree and/or second-degree relationship to one another. In cases where only one affected offspring was included in the multiplex family cohorts (see Table 2.2), at least either one of the parents was also affected, and/or one of the parents' siblings.

This involved a collaboration of an international group of IBD investigators from the Department of General Internal Medicine at the Christan-Albrechts-Universtitat (Kiel, Germany), St. Mark's, Guy's and King's College Hospitals (London, UK), and University of Southampton, UK. All participants gave informed and written consent to take part. All local ethics committees at the participating centres gave approval for the study.

2.2 Isolation, culture and characterisation of myofibroblasts

2.2.1 Collection of samples

During the colonoscopy procedure, 5 biopsy samples were obtained from each subject. The biopsies were collected from different regions of the large bowel, between (and including)

the rectum and up to (and including) the caecum. A 5 ml sample of peripheral venous blood was also obtained from each subject, either at the time of colonoscopy, or during a follow-up clinic appointment, and collected into a 5 ml EDTA plastic tube. Once collected, the biopsy samples were transferred immediately to culture medium (Eagle's Minimum Essential Medium (MEM) with glutamax and 20% foetal calf serum (FCS)) for transfer to the laboratory. Blood samples were transferred to the laboratory on ice and immediately frozen at -20^{o} C.

2.2.2 Isolation of Myofibroblast cells

To remove epithelial cells, the biopsy samples were washed 3 times' with Hanks' balanced salt solution (HBSS) containing 1mM EDTA, and incubated and stirred in HBSS with 1mM EDTA for 30 minutes at 37°C. This procedure was then repeated twice. The samples were then cut into the very small pieces (less than 0.5 mm), and incubated in a culture flask in Eagle's MEM containing 20% fetal calf serum (FCS), 1% non-essential amino acids, 5mg/ml of penicillin/ streptomycin, 40mg/ml gentamicin, and 5mg/ml amphotericin B.

The culture medium was then changed every 3 days', at the same time replacing the biopsy tissue, until myofibroblast colonies were growing at the bottom of the flasks.

At confluence, the cells were passaged using 0.1% (wt/vol) trypsin-0.2% (wt/vol) EDTA in a 1:2 split ratio. After passaging, the myofibroblast cell lines were cultured in the Eagle's MEM culture medium containing 20% FCS, non-essential amino acids and antibiotics as described above, the culture medium being changed every 3 days. By passage 4, enough cells had been cultured to be able to carry out experiments, and a small portion of cells was stored for future use. Therefore, at passage 4, cells were trypsinized using 0.1% (wt/vol) trypsin-0.2% (wt/vol) EDTA as before, and re-suspended in 1ml MEM solution and counted. For some samples, 50,000 cells were set aside for cell characterisation. The remaining cells were used for cell culture. MMP concentration was measured after stimulation with pro-inflammatory cytokines (see section 2.2.3 below).

In total, ten samples from each group (control, CD and UC) were characterised at passage 4. 5 of the samples in the CD and UC groups were from subjects with active disease, and 5 samples were from subjects with disease in remission. In addition, 3 cell lines from the

control group were characterised simultaneously, at every passage, from passages 1 to 6, to observe any change in phenotype after each passage. This was repeated twice, so that a total of 9 cells lines from control samples were characterised at each passage.

The myofibroblasts that were set aside for immunohistochemistry were cytospinned on glass slides (spun at 700 rpm for 10 minutes), leaving a cell pellet on each slide of 5,000 cells per slide. The slides were allowed to air-dry overnight, then fixed with acetone for 20 minutes, and washed with tris buffered saline (TBS) before immunohistochemistry.

Monoclonal antibodies: vimentin (diluted 1/100), desmin (diluted 1/50), α-SMA (α-smooth muscle actin) (diluted 1/50), PR2D3 (monoclonal goat anti-human undiluted PR2D3 antibody, gift from ICRF), $CD3^+$ (UCHT1 undiluted culture supernatant), anti-cytokeratin (CY-90 diluted 1/100), and CD68 (diluted 1/25), (all from DAKO Ltd., High Wycombe, UK). The specificity of the primary antibodies used are summarised in Table 2.3. PR2D3 is a specific monoclonal antibody that has been shown to stain positively for myofibroblasts but not for fibroblasts (Richman et al.). As a negative control, samples were incubated with buffer.

The slides were incubated with primary antibody for 2 hours' at room temperature in a humidified chamber. The primary antibody was then washed off with TBS and the cell pellets were incubated with the appropriate secondary antibodies (rabbit anti-mouse or rabbit anti-goat conjugated to horseradish peroxidase) for 30 minutes. The slides were developed using diaminobenzidine as a substrate. This substrate, in the presence of horseradish peroxidase, produces a brown precipitate.

For each slide, all positively staining and negatively staining cells were counted in a visual field taken randomly across the slide of cells. A grid over the visual field was used to aid the counting of the cells. The percentage of positively staining cells was calculated as a proportion of all cells in the visual field, and this was repeated a further two times using randomly selected different areas on the slide. An average of the three readings was taken for each slide of each cell line. This process was repeated for each of the 10 cell lines used in each group and for each of the primary antibodies. By taking a mean of the results for each primary antibody of the 10 cell lines, an overall average of positively staining cells

was calculated for each group. The same process was used to count the percentage of positively staining cells for the primary antibodies in 9 control cell lines at 6 different passages.

2.2.3 Stimulation of myofibroblasts with pro-inflammatory cytokines

100,000 cells were added to a 12 well plate and incubated overnight with MEM with full supplementation at 37°C, in order to allow the cells to settle to the bottom of the plate. The cells were washed three times with serum-free MEM and were cultured in supplemented serum-free medium, the presence of pro-inflammatory cytokines (Barro et al.;May, Hooke, and Lees).

TNF-α and IL-1β were used to stimulate the isolated myofibroblasts. Graded doses of TNF-α were added to each well, in increasing concentrations of :0ng/ml, 0.1ng/ml, 0.5ng/ml and 1.0ng/ml. The experiment was repeated in 4 of the remaining wells. Cells were stimulated for 48 hours, at 37°C. The culture supernatant was then collected and stored at –20°C.

2.2.4 Measurement of MMP-3 protein

The concentration of MMP-3 was measured by both western blotting technique and ELISA.

(i) Western Blotting

The frozen supernatants collected as outlined in section 2.1.4, were allowed to thaw on ice. Protein concentration was measured using the Bio-Rad Protein Assay (Biorad Labs, UK). Ready-prepared gels were used (Invitrogen NuPAGE minigels), containing 10% SDS-PAGE. 10 μg of protein were loaded in each lane. The gels were run under reducing conditions. The protein was then transferred to nitrocellulose (Biorad) and incubated with primary antibodies (polyclonal sheep anti-human MMP antibodies, diluted to 1/500 from The Binding site, Birmingham, UK). These antibodies recognise the active and pro-enzyme forms of the MMPs. After washing with 0.1% TWEEN 20 in phosphate buffer saline (PBST), the nitro-cellulose was incubated with secondary antibodies (polyclonal rabbit anti-sheep conjugated to horseradish peroxidase, diluted 1/2500; DAKO Ltd., High

Wycombe, UK) and developed using the ECL plus Western blotting detection system (Amersham Life Science, Amersham, UK). As a positive control, recombinant MMP-3 was used (recombinant MMP-3, Sigma Chemical Co.), and loaded in adjacent lanes. MMP-3 has been shown in previous studies, to be detected as a band of MW 57 kDa in the inactive form (Vincenti;MacDonald, Bajaj-Elliott, and Pender;Pender et al.).

(ii) Quantification of MMP-3 by ELISA

MMP-3 concentrations were measured using commercially available Biotrak ELISA systems (Amersham Pharmacia Biotech, UK). This ELISA kit measured total MMP-3 concentration for each sample, that is, the combined measurement of human active and pro-MMP-3. The sensitivity of the Biotrack ELISA system used, defined as two standard deviations above the mean optical density of 10 zero standard replicates, has been calculated by the manufacturer, as 2.35 ng/ml.

Supernatants were available for analysis from 55 control subjects, 23 CD samples, and 20 UC samples. The standards of varying concentrations were prepared by serial dilutions of a known concentration standard. The microtitre plate wells were pre-coated with anti-MMP-3 antibody. The standards of varying concentrations were added to well plates to provide a reference scale. The microtitre plate wells were pre-coated with anti-MMP-3 antibody. The standards of varying concentrations were added to well plates to provide a reference scale. Supernatant of each sample was added to the wells. In the case of 12 samples, all 4 concentrations of 0,0.1,0.5 and 1.0ng/ml were added to the wells. After incubating at 4°C for two hours, the wells were washed with wash buffer and aspirated and blotted, so that the MMP-3 bound to the precoated wells remained. One hundred microlitres of anti-MMP-3 peroxidase conjugate antibody was added to each well and allowed to incubate at 4°C for 2 hours before washing the wells again. 100 microlitres of TMB substrate was then added to the wells and incubated for 10 minutes at 23°C, and the reaction was stopped by adding 100 microlitres of 1M sulphuric acid to the wells, and quantified by measuring the optical density at 450nm using a Titertek Multiscan Plus Elisa reader (EFLAB, Finland).

The mean optical density of each duplicate standard assay was calculated and plotted against the concentration of the standard to construct a standard curve. See Figure 2.1. From this the optical reading for each sample could be used to determine the concentration

of MMP-3 by interpolation. The final concentration of each sample was determined after adjusting for dilution, and was calculated in ng/ml. The readings that fell outside the optical density of the range of standards were excluded. Samples that had to be excluded were repeated, using less sample.

2.3 Genotyping of MMP- 3 gene promoter polymorphism

Several different methods have been used to genotype the MMP-3 gene 5A/6A polymorphism, including allele-specific oligonucleotide hybridisation (Ye et al.;Terashima et al.), electrophoresis of fluorescence labelled PCR products using ABI sequencers (Humphries et al.), and denaturing polyacrylamide gel electrophoresis of radioisotope labelled PCR products (Yoon et al.). Here we used a rapid, cost-effective technique, based on restriction endonulease digestion (Dunleavey, Beyzade, and Ye).

2.3.1 DNA extraction

The samples of blood were defrosted at 4°C overnight on a rotary mixer. Once thawed, they were relabelled with traceable numbers so that interpretation of genotype could be made in an unbiased way. The samples were washed with electrolyte lysis buffer (ELB). After mixing on ice, the samples were centrifuged at 4°C (at 1200 rpm, for 10 minutes), and the remaining pellet was resuspended in ELB. The washing process was repeated a further two times.

The pellet of each sample was then resuspended in 2 ml nucleic lysis buffer (NLB), 250µl sodium dodecyl sulphate (SDS) and 80µl protease. The samples were then shaken overnight overnight in an incubator, at 37°C. During this process the white blood cells' membranes are denatured, allowing the DNA to release into the solution.

After overnight digestion, the samples were then mixed with 750µl of saturated sodium chloride (approximately 6M), shaken, and centrifuged at 23°C (at 4000rpm for 15 minutes). The clear supernatant of each sample, which contains the DNA, was retained, and to it twice the volume of cold absolute ethanol was added to precipitate the DNA, which is visible as a white precipitate. The white precipitate was removed with each sample, and washed with 70% ethanol. The sample was then allowed to dry in a sterile

plastic tube. The DNA was then resuspended in 500µl autoclaved Tris EDTA (TE) buffer (pH 8.0). The samples were stored at 4°C.

2.3.2 Measurement of DNA Concentration

The DNA concentration of each sample was quantified using PicoGreen assay. Picogreen is an ultra-sensitive fluorescent nucleic acid stain used for the quantification of double-stranded DNA. Using this technique, picogreen, which binds to double-stranded DNA, is added to unknown concentrations of sample DNA, and compared with known concentrations of picogreen standards with a fluroimager. The fluroimager reads the optical density of fluorescence produced by picogreen.

2.3.3 Polymerase chain reaction

Polymerase chain reaction (PCR) is used for amplifying defined target DNA sequences present within a source of DNA. Using this technique, it is possible to amplify a defined target sequence of DNA within a heterogeneous collection of DNA sequences, such as genomic DNA.

Using the 7ng/µl stock concentration of DNA samples, 3 µl (21 ng), of each sample was added to individual wells of a 96 well PCR plate, and denatured by heating at 80°C for 10 minutes.

The method used to genotype MMP-3 has been previously described(Dunleavey, Beyzade, and Ye). The sequences of MMP-3 5A/6A PCR primers were 5'-GATTACAGACATGGGTCACA-3' (forward primer) and 5'-TTTCAATCAGGACAAGACGAAGTTT-3' (reverse primer). PCR was carried out in a total volume of 25 µl for each sample, containing 21 ng genomic DNA, 5 pmol each primer, 200 µM each dATP, dCTP, dGTP and dTTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.05% (v/v) WI (Gibco BRL), 5mM $MgCl_2$, and 3 IU Taq polymerase (Promega). The solution was overlaid with 25µl liquid paraffin and incubated for 2 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 53°C, and 30 seconds at 72°C, and an additional 2 minute extension at the end of the 35 cycles.

2.3.4 Restriction Endonuclease digestion

The recognition sequence of a particular polymorphism can be cleaved by restriction endonuclease digestion in the presence of one of the allelic variants, allowing discrimination of the two alleles following gel electrophoresis. For example, a recognition sequence (5'-GAANNNNTTC-3') created following PCR for MMP-3 as described above using the MMP-3 PCR primers, is cleaved by the restriction endonuclease *Xmn*I, when the DNA template contains the 5A (but not 6As) at the polymorphic site (see Figure 2.2).

A 10 µl aliquot of PCR products of each sample was mixed with a 5µl solution containing a restriction endonuclease (*Xmn* I): 1.5µl 10x NEBuffer 2 (50mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 1.5 µl bovine serum albumin (1 µg/µl), 0.3µl *Xmn*I (20 U/µl), and 1.7 µl sterile deionised H_2O. The solution was overlaid with 15µl of liquid paraffin and incubated at 37°C for 16 hours overnight.

2.3.5 Gel Electrophoresis

A 5µl aliquot of the digests was mixed with 2µl of loading buffer and electrophoresed on a 10% horizontal non-denaturing polyacrylamide gel at 130 volts for 1 hour. The gel was then stained with vistra green (Amersham Life Science, Amersham, UK) and scanned with a fluoroimager. A 50bp marker ladder was used to verify the length of the DNA fragments. The 6A allele is 108 bp, whereas the 5A allele appears as bands at 97bp and 23bp (see Figure 2.3) (Dunleavey, Beyzade, and Ye). Therefore, the MMP-3 genotype of each sample was interpreted based on the position of the bands, and band intensity.

2.4 Transmission disequilibrium testing of MMP-3 gene promoter polymorphism in relation to CD and UC

This part of the study involved a collaboration of an international group of IBD investigators from the Department of General Internal Medicine at the Christan-Albrechts-Universtitat (Kiel, Germany), St. Mark's, Guy's and King's College Hospitals (London, UK), and University of Southampton, UK. Therefore a different technique of DNA extraction and genotyping was used.

2.4.1 Collection of samples

Families were included in the study, if at least one family member was affected with either UC or CD. Where possible, samples of affected family members and both their parents was taken. Clinical, radiological, endoscopic, and histological examinations were required to unequivocally confirm the diagnosis either of CD or UC. Ten ml of peripheral blood was taken from each participant, which was either frozen, or used immediately in the study.

2.4.2 DNA extraction

Genomic DNA was prepared from 10ml of venous blood using the Puregene system (Gentra systems, Minneapolis, MN). The protocol for this system is very similar to the method described previously, but instead of using saturated NaCl, 'Protein Precipitation Solution' (provided by Gentra) is used, and 100% Isopropanol, instead of 100% ethanol, is used to precipitate out the DNA.

10 ml of blood is first washed with RBC Lysis solution (provided by Gentra systems, Minneapolis, MN), and then spun in a centrifuge at 2000 rpm for 5 minutes. The pellet is resuspended in RBC Lysis solution, and 50 µl RNAse A solution (provided by Gentra systems) is added to the cell lysate. The sample is then incubated at 37°C for 15 minutes and then allowed to cool. The cell lysate is mixed with 3.33ml of Protein Precipitation Solution. The solution is spun for 5 minutes at 2000 rpm. The supernatant is retained, and to it, 10ml of 100% isopropanol is added. The sample is mixed by gentle inversion. The DNA precipitate, which appears as a white thread, is retained, washed with 70% ethanol, and spun for 1 minute at 2000rpm, room temperature. The ethanol is poured away and the DNA is allowed to dry, for 10-15 minutes, following which time the DNA is mixed in 600µl DNA Hydration Solution (provided by Gentra systems). The samples were then stored at 4°C.

2.4.3 Genotyping MMP-3 polymorphism: Taqman PCR

Individual DNA samples were arrayed in 96 well microtitre plates. The MMP-3 promoter polymorphism was genotyped by allelic discrimination, Taqman technology (ABI 7700, Applied Biosystems, Foster City, CA) using the following primers and probes (designed on the reverse strand): forward primer 5' GCCACCACTCTGTTCTCCTTGTC 3', reverse

primer 5' CACGGCACCTGGCCTAAAGA 3'; first probe (6A) 5' CAAGACATGGTTTTTTCCCCCCATCA 3', and second probe (5A) 5' CAAGACATGGTTTTTCCCCCCATCA 3'. Primers and probes were designed with Primer Express (Apler, Foster City, Ca) and synthesized through Eurogentec (Liege, Belgium). The amplification reaction, performed in a Biometra, (Gottingen, Germany) thermocycler, involved two pre-PCR steps of 2 minutes at 50°C and 10 minutes at 95°C, followed by 50 cycles including a denaturation step at 95°C for 15 seconds and an annealing step of 1 minute at 64°C in a final volume of 5μl. Final concentrations were 100nM for the probes and 300nM for the primers.

2.5 Statistical Analysis of data

The one-way analysis of variance (ANOVA) performs a comparison of the means of a number of replications of experiments where a single input factor is varied (in this case the different disease groups: control, CD and UC). This method assumes that the errors are normally and equally distributed, with mean zero and constant variance.
ANOVA was used to compare positively-staining cells on immunohistochemistry between control, CD and UC cell lines at passage 4. ANOVA was also used to analyse differences in positively staining cells between 6 passages of 9 normal cell lines, and post-hoc analysis was used to make paired comparisons between passages.
For the investigation of MMP-3 expression by myofibroblasts between CD, UC and control samples, the distribution of concentration of MMP-3 for all samples was observed. If the sample set followed a normal distribution, then samples between the three groups (control, CD and UC) would be compared using ANOVA. If however, the sample set did not follow a normal distribution, then the Kruskall-Wallis test would be used, which is a non-parametric test used when more than two sets of samples are compared.
ANOVA was also used to compare MMP-3 expression between 5A/6A MMP-3 genotypes in the CD, UC and control groups.

In TDT analysis of cohorts of British and German families in the study of the association of the 5A allele of the MMP-3 polymorphism in relation to the predisposition of IBD, genetic analyses were conducted using the standard diagnostic categories CD and UC. The data was managed and checked for mendelian inheritance errors in an integrated database

(Hampe et al.). Families were included whose parental alleles were heterozygous for the 5A/6A allele. Transmission disequilibrium tests (TDT) of the MMP-3 polymorphism in relation to CD and UC, was analysed using the TRANSMIT program, with the robust variance estimator option (Clayton and Jones;Clayton). 'TRANSMIT' is a computer programme that implements the methods described in the report by Clayton et al (Clayton and Jones). The 'robust variance estimator' is an equation used to determine transmission disequilibrium ratio, so that more than one affected offspring per family may be used, even in the presence of linkage.

Possible interaction between the MMP-3 gene and the CARD15 gene was examined by stratifying the patients according to their CARD15 genotype. Individuals carrying one or more copies of the R702W, G908R, or 1007insC mutations (Hugot et al.;Hampe et al.;Ogura et al.) were classified as CARD15+, and individuals not carrying these mutations as CARD15-.

2.5.1 Sample size calculation

When a sample size calculation has been made, it was assumed that the continuous outcome is normally distributed with known standard deviation in the two groups compared. The outcome would be compared using a two-sample t-test. The formula for calculating sample size is given in Appendix E. Nquery was the statistical software used to make the sample size calculation, based on the means, standard deviations of the two groups, at 5% significance level and 80% power of detecting a significant difference.

Table 2.1: Proportions of samples successfully used in each group (CD, UC and Control)

	Attempted	Grown to passage 4	Proportion Successfully grown (%)
Control	92	55	55/92 (60.4)
CD	32	23	23/32 (71.8)
UC	31	20	20/31 (64.5)
Total	155	98	98/155(63.2)

Table 2.2: Patients genotyped for the MMP-3 gene 5A/6A polymorphism in British and German families

	Disease	Number of trios or families	Number of affected subjects	Number of affected offspring per family	Number of trios or families where both parents were genotyped
German sporadic trios	CD	320	320	1	318 (ie, in 99.4% of the collection)
	UC	148	148	1	147 (ie, in 99.3% of the collection)
German multiplex families	CD	135	227	1 in 47 families 2 in 82 families 3 in 6 families	115 (85.2)
	UC	83	122	1 in 44 families 2 in 39 families	70 (84.3)
British multiplex families	CD	91	149	1 in 40 families 2 in 44 families 3 in 7 families	78 (85.7)
	UC	49	74	1 in 28 families 2 in 17 families 3 in 4 families	42 (85.7)

Table 2.3: Antibodies used to characterise myofibroblast cell line

Primary Antibody	Positive for cell type:
Smooth muscle actin (SMA)	Myofibroblasts, fibroblasts, smooth muscle cells
Desmin	Fibroblasts, smooth muscle cells,
Vimentin	Smooth muscle cells, epithelial cells, macrophages
PR2D3	Myofibroblasts, smooth muscle cells
Cytokeratin	Epithelial cells
CD68	Macrophages
$CD3^+$	T-lymphocytes

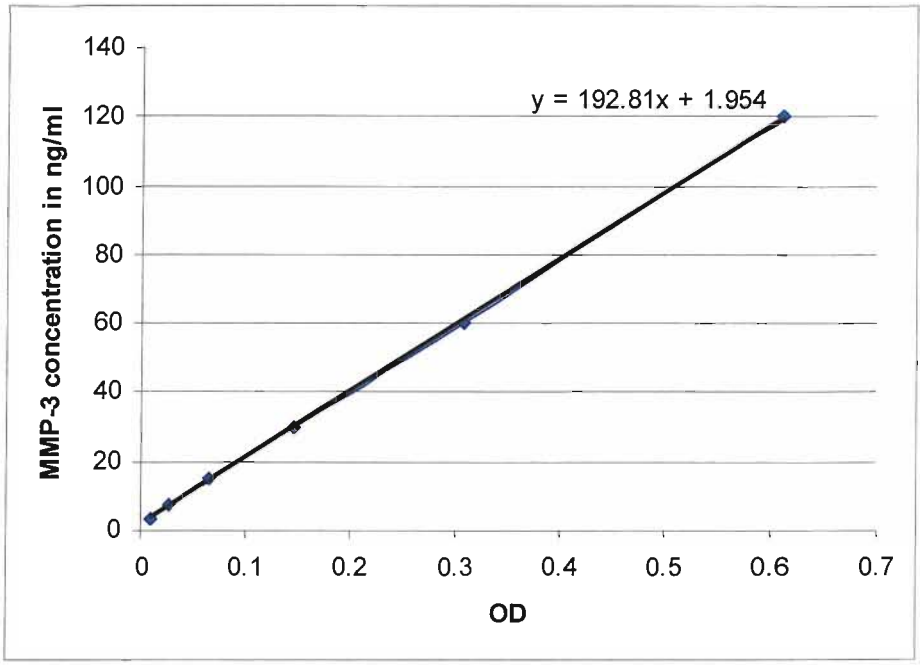

Figure 2.1: Representative standard curve for ELISA MMP-3 experiments. OD is the Optical Density. The equation is the function of the curve, which was used to calculate the MMP-3 concentrations of the samples

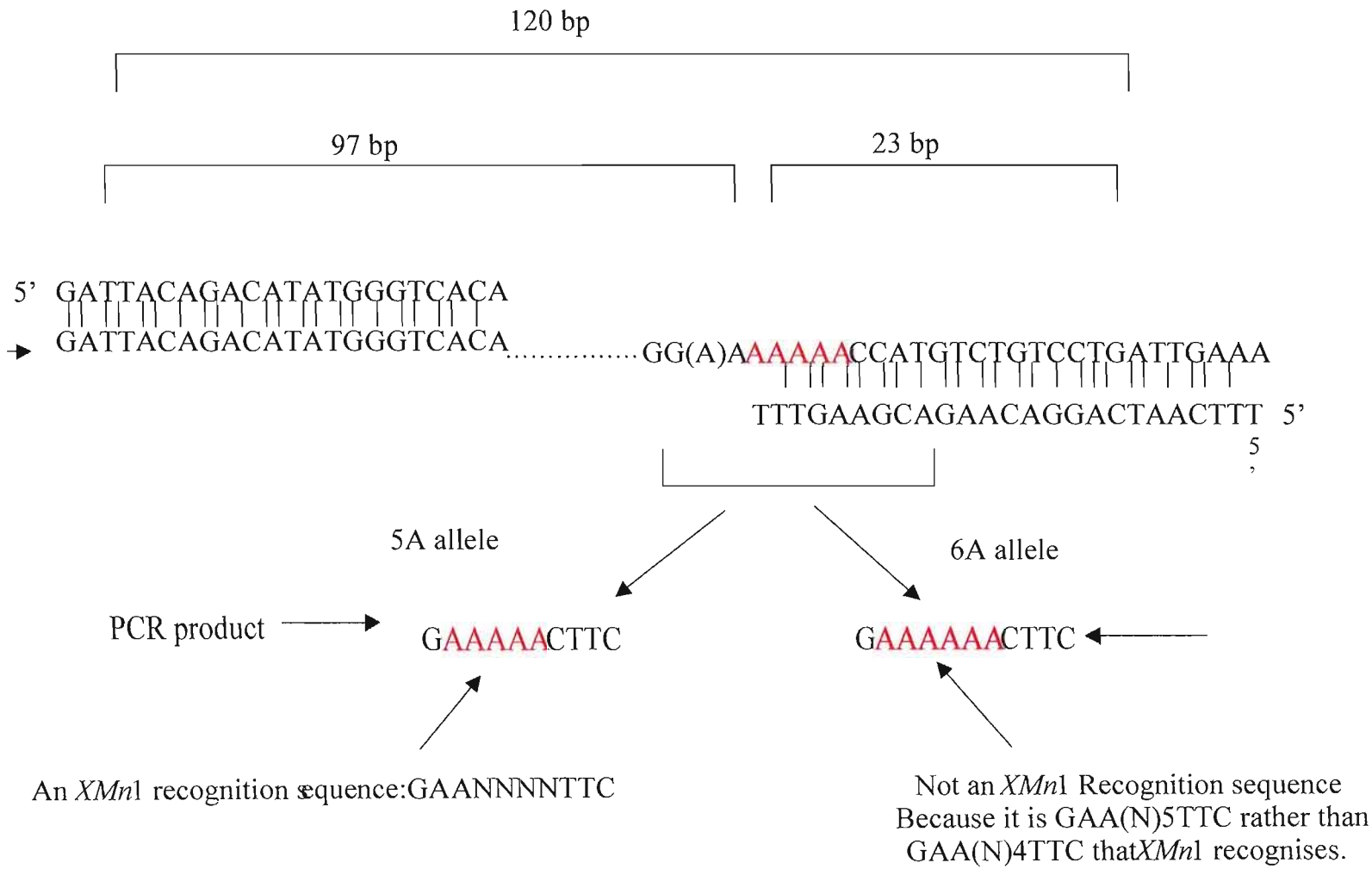

Figure 2.2: Presentation of the XmnI restriction endonuclease digestion based method to detect the 5A/6A polymorphism

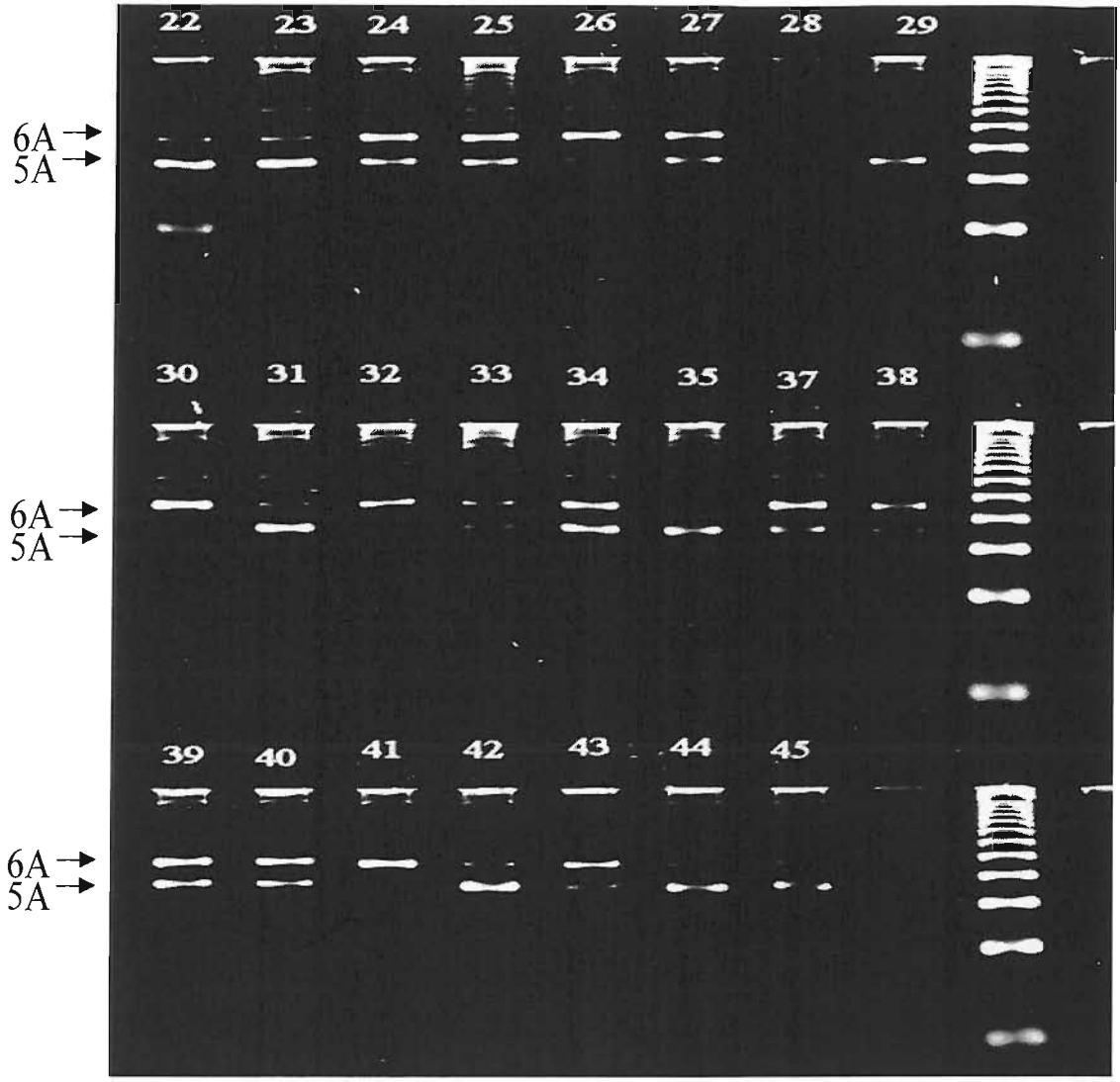

Figure 2.3: Gel electrophoresis run for a group of samples (labelled 22-45), for MMP-3 5A/6A polymorphism after restriction enzyme digest. The markers in the far right lanes are 25 bp ladders. The upper bands in the samples represent the 6A allele at 108bp, and the lower band represents the 5A allele, at 23 and 97 bp (the 23 bp band of the 5A allele is only visible in sample 22). Samples 22,23,29,31,35,42,44 and 45=5A/5A allele; Samples 24,25,27,33,34,37,38,39,40 and 43=5A/6A allele; and samples 26,30,32,41 and 43 =6A/6A

Chapter 3: Isolation, culture and characterisation of intestinal myofibroblast cell lines from control patients and patients with Crohn's disease and Ulcerative Colitis

3.1 Introduction

Intestinal myofibroblasts are a unique population of smooth muscle-like fibroblasts that lie immediately adjacent to the basement membrane in the intestinal mucosa. They exhibit ultrastructural features of both fibroblasts and smooth muscle cells and show positive immunoreactivity for both α-SMA and vimentin (Mahida et al.;Powell et al.;Powell et al.;Sappino, Schurch, and Gabbiani). Myofibroblasts contribute to ECM turnover (Lawrance, Maxwell, and Doe), and also play a critical role in intestinal inflammation through the secretion of chemokines, cytokines, prostaglandins, growth factors, and extracellular matrix components (Powell et al.;Powell et al.). They are the major cellular source of MMP's in IBD (Vaalamo et al.) and in vitro produce high levels of MMP-3 in response to the pro-inflammatory cytokines TNF-α and IL1-β (Pender et al.;Pender et al.). Myofibroblasts therefore play an important role in the healthy and diseased gut. The aims of this part of the study were therefore to culture myofibroblasts from mucosal biopsies of patients with CD, UC, or control subjects.

3.2 Myofibroblast Culture

In total, cell lines were successfully grown from 98 of 155 samples (63.2%): 55 cell lines out of 92 (60.4% of total) from the control group, 23 cell lines from the CD group (71.8% of total), and 20 cell lines from the UC group (64.5%). Of the cell-lines that were not included in the study, 5 were excluded because the diagnosis was later revised to indeterminate colitis. The remainder of unsuccessful samples either became infected with bacteria, or did not grow to confluence in initial culture. There was no difference in number of cell lines that were successfully cultured between the control, CD and UC groups. By dividing subjects in the CD and UC groups further into those with active disease and those with disease in remission, 11 out of the total 23 CD subjects had active disease, and 12 had CD in remission. In the UC group, 12 subjects had active disease, and 8 subjects had disease in remission.

Biopsies were processed as described in the methods section. After 3 to 10 days in culture, isolated myofibroblasts were seen adherent to the bottom of the culture dishes. Single cells were seen at first, but soon grew into colonies of myofibroblasts after 1 to 2 weeks. The colonies increased in size until a monolayer of myofibroblasts formed. The time taken to grow the cell-lines, from collecting the samples, to reaching passage 4 for analysis, was between 4 and 6 weeks.

The myofibroblast cultures were examined by light microscopy after each passage. Cells were elongated and flat in appearance with a flat adherent cytoplasmic outline, typical of fibroblast-like cells (see Figure 3.1) (Lawrance, Maxwell, and Doe).

3.3 Characterisation of Myofibroblasts

Cell lines from 10 random subjects in each group: control, CD and UC, were characterised by immunohistochemistry at passage 4. In the CD and UC groups, 5 of the cell lines were from subjects with active disease, and the remaining 5 cell lines were from subjects with inactive disease. Cells were stained by immunohistochemistry, using the following primary antibodies: smooth muscle actin (SMA), vimentin, desmin, PR2D3, CY90 (cytokeratin), PGM1 (CD68).

In the control samples, the cells isolated were 85.78% α-SMA positive, 95.5% vimentin positive and only 38.76% positive for desmin. The results were similar for CD cell lines (which were 86.02% positive for α-SMA, 94.94% positive for vimentin, and 34.68% positive for desmin), and UC cell lines (which were 84.98% positive for α-SMA, 96.16% positive for vimentin and 33.64% positive for desmin). Relatively few cells stained positively for PR2D3 (28% positive cells in control cell lines, 24% positive cells in CD cell lines and 24% positive cells in UC cell lines).Only a few cells in all samples stained positively for cytokeratin (<7.5%). No cells stained for $CD3^{+}$ or CD68, and there was no staining on any of the negative controls (see Figure 3.2).

3.3.1 Phenotype of myofibroblast lines obtained from control patients and patients with IBD

Cell lines were compared between control, CD and UC groups at passage 4. Using analysis of variance, there was no significant difference in the percentage of positively staining cells for each primary antibody between the three groups: control, CD and UC (see Table 3.1). The percentage of positively staining cells for each primary antibody did not differ significantly between cell lines from active CD patients and inactive CD patients, or between active UC and inactive UC patients.

3.3.2 Characterisation of cells at different passages

Cell-lines from 9 control samples were characterised up to and including passage 6. Cell lines from CD and UC groups were not characterised in this way because there were fewer samples in this group, and therefore they could not be cultured, passaged and characterised at the same time. Cell lines in CD and UC groups were observed to survive to at least 6 passages.

Comparing all passages by analysis of variance, there was no significant difference in positive staining of cells for all primary antibodies over passages 1 to 6, although differences in staining for desmin reached near significant levels ($p=0.08$) (see Figure 3.3, Table 3.2). Using post-hoc analysis, there was a significant decrease in desmin-positive cells between passages 1 and 6 ($p=0.007$), passages 3 and 6 ($p=0.037$), and between passages 4 and 6 ($p=0.022$). There was also significant difference in percentage of positive cells for PR2D3 between passages 1 and 6 ($p=0.028$) and passages 2 and 6 ($p=0.021$).

3.4 Discussion

Sixty-three percent of all samples collected successfully yielded cell lines. The main problems, which led to failure to establish culture, included bacterial infection of samples, and failure of cells to propagate sufficiently. Cell lines could not be cultured to passage 4 in less than 4 weeks', which is consistent with previous observations (Mahida et al.).

The morphological features of the cultured cells were compatible with characteristic features of myofibroblasts reported in the colon and other tissues (Mahida et al.;Powell et al.;Powell et al.;Sappino, Schurch, and Gabbiani;Valentich et al.). There was no difference

in morphological appearance of myofibroblasts between control, CD and UC groups, consistent with previous published findings (Lawrance, Maxwell, and Doe).

The isolation and culture of myofibroblasts provides a useful experimental model for investigating cellular responses associated with mucosal inflammation. This technique of cell isolation and culture has been adapted from previous experimental designs, to achieve the highest yield of cell lines isolated from biopsy samples (Pender et al.;Pender et al.). Cell lines could not be cultured to passage 4 in less than 4 weeks', which is consistent with observations in previous studies (Mahida et al.).

Pericryptal myofibroblasts are α-SMA positive, vimentin positive, and desmin negative cells, and are distinct from smooth muscle cells of the muscularis mucosae, which are vimentin negative and desmin positive (Adegboyega et al.). Therefore the results of this study suggest that the majority of cells in these groups were myofibroblasts, but a small proportion were fibroblasts or smooth muscle cells. However, a low percentage of cells stained positively for PR2D3, a marker for myofibroblasts. It is likely that the observed low percentage of positively staining cells for PR2D3 was due to experimental error, such as problems with staining technique.

When the cells isolated and cultured from the control, CD and UC patients were compared, there was no difference in phenotype between these groups at passage 4, consistent with previous studies, which found no difference in phenotype between myofibroblasts isolated from normal and inflamed mucosa of CD and UC patients (Lawrance, Maxwell, and Doe;McKaig et al.).

The phenotype of the cell line is likely to be determined in part by the technique of cell isolation. Published reports of the isolation of fibroblast-like cells from lamina propria explants by cell out-growth, describe the cells as myofibroblasts with positive α-SMA staining (Mahida et al.;Hinterleitner et al.). A different isolation technique, involving collagenase digestion of the lamina propria and submucosa, produced cells with a fibroblast phenotype (α−SMA negative cells) in another study (Lawrance, Maxwell, and Doe). A consistent technique of cell isolation and culture was used throughout this study, to maintain a consistency of phenotype of cultured cells.

Post-hoc analysis of the differences in staining of cells from control patients for each primary antibody between passages, showed a significant increase in the proportion of cells staining positively for PR2D3 between passages 1 and 6; and a significant decrease in proportion of cells staining positively for desmin between passages 1 and 6 ($p=0.007$) and passages 4 and 6 ($p=0.022$). This would suggest that the proportion of myofibroblast phenotype increased with passage. Smooth muscle cells which stain positively for α-SMA but negatively for desmin, could have been present in higher numbers after initial isolation and culture, but decreased in relative number after passaging. Fibroblasasts could have transdifferentiated after passaging to myofibroblasts, or the population of myofibroblasts increased with passage, relative to other types of mesenchymal cells.

A previous study of adult human myofibroblasts showed that myofibroblasts maintained the same phenotype over 10 passages (Mahida et al.). However, rat myofibroblasts have been observed to transdifferentiate to fibroblasts after repeated passaging and prolonged culture (Zimmermann et al.). In vivo, fibroblasts can transdifferentiate upon activation, to become myofibroblasts (Adegboyega et al.). Various factors including TGF-β, in the microenvironment, may influence α-SMA expression, and account for myofibroblast activation and proliferation (Hautekeete and Geerts;Rockey et al.). It is therefore possible that, under microenvironmental influences in vitro, cultured stromal cells may transdifferentiate to myofibroblasts after prolonged culture.

3.5 Summary

A technique of cell isolation and culture, successfully yielded cell-lines in 63.2% (98 out of 155) of samples. The cells that were isolated, cultured and passaged over 4-6 weeks' contained a high percentage of myofibroblasts. There was no difference in phenotype of cell populations isolated from control, CD and UC patients. When cell-lines from control patients were characterised at different passages, an increase in positively staining cells for PR2D3 and vimentin was observed, with simultaneous decrease in desmin antibody staining. This would suggest that the population of cells had become more myofibroblast-enriched after repeated passaging and prolonged culture.

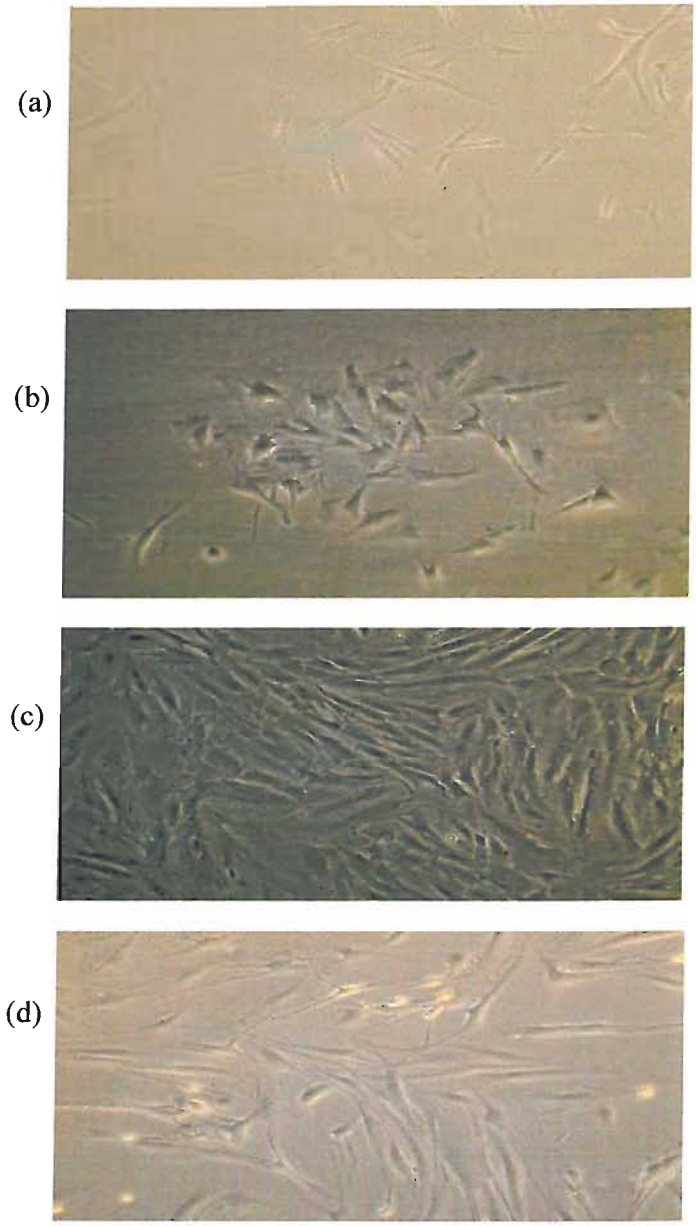

Figure 3.1: (a) Myofibroblasts in primary culture, initially appear as isolated cells at the bottom of the flask, magnification X100; (b) colonies of myofibroblasts grow before passage 1, magnification X100; (c) Myofibroblasts when confluent before passaging, magnification X100; (d) myofibroblasts 24 hours after stimulation with 1.0 ng/ml TNF-α, magnification X250.

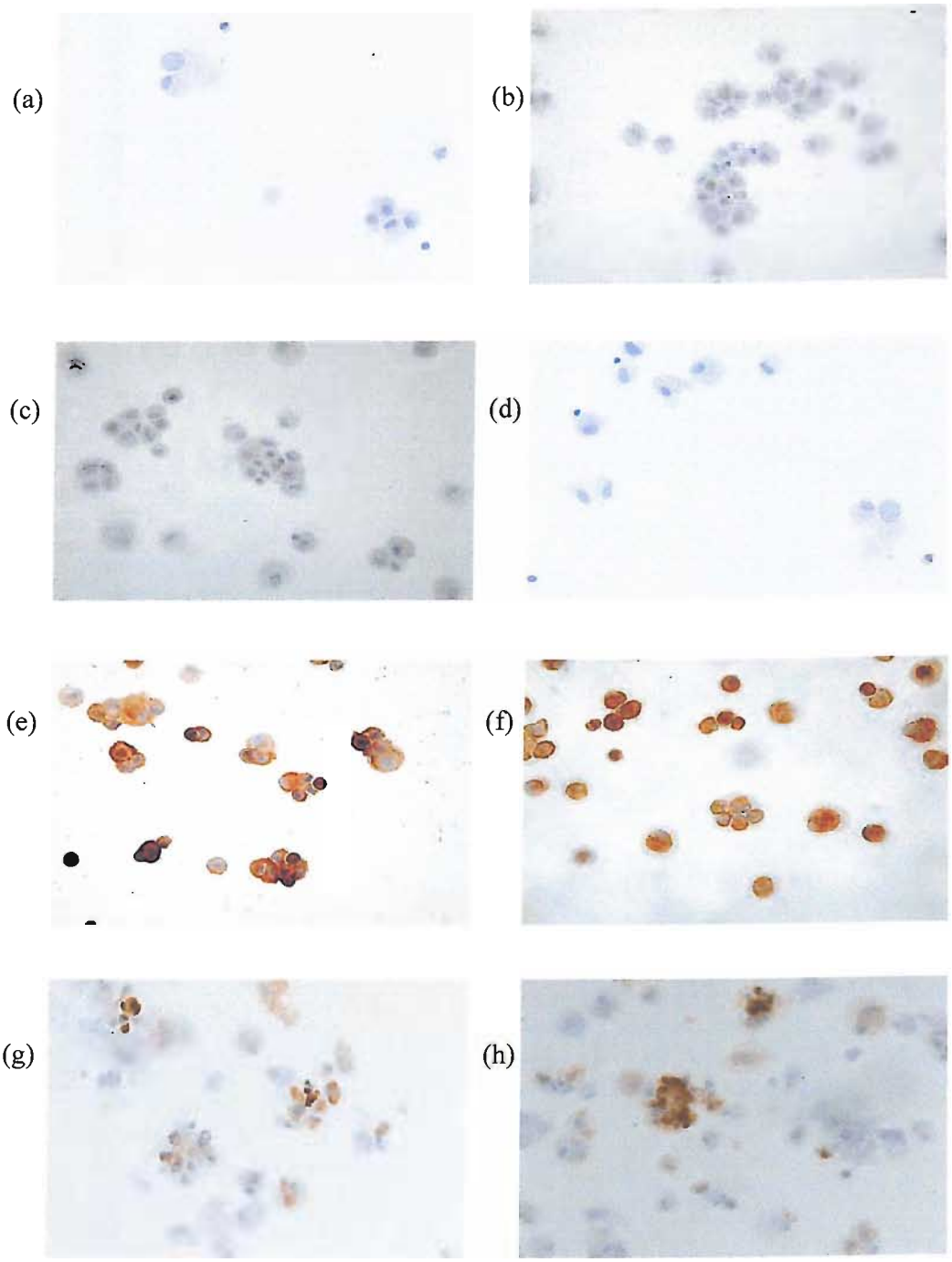

Figure 3.2: Immunohistochemistry of myofibroblasts. Staining for: (a) negative control; (b) cytokeratin, (c) CD3+ (UCHT1), (d) CD68+ (PGM1), (e) SMA, (f) vimentin, (g) desmin, and (h) PR2D3.

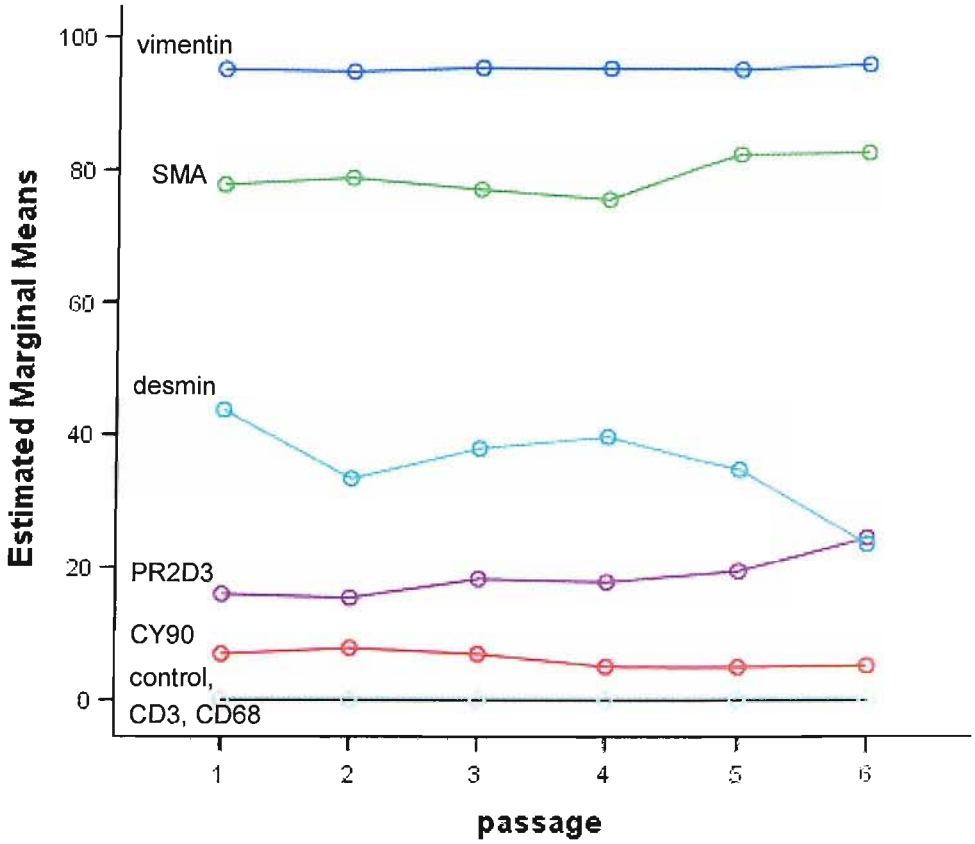

Figure 3.3: Mean of positively staining cells of 9 control cell lines over 6 passages, on immunohistochemistry using primary antibodies: negative control, α-SMA, vimentin, PR2D3, desmin, CY90, CD3, CD68.

Table 3.1: Mean of percentage of positively staining cultured cells of each group: control, CD and UC, at passage 4 (10 samples in each group), on immunohistochemistry, using primary antibodies: no primary antibody (negative control), α-SMA, vimentin, PR2D3, desmin, CY90, UCHT1, and CD68. 'SD' = standard deviation.

	control	SD	CD	SD	UC	SD
negative control	0	0	0	0	0	0
α-SMA	85.7	2.2	86.0	3.2	84.9	5.2
vimentin	95.5	1.8	94.9	1.2	96.1	1.9
PR2D3	28.8	9.9	24.2	8.4	23.6	5.2
desmin	38.7	6.1	34.6	10.7	33.6	13.5
CY90	6.1	2.3	7.0	2.2	7.5	1.9
CD3	0	0	0	0	0	0
CD68	0	0	0	0	0	0

Table 3.2: Mean values of positively staining cells from control cell lines (9 samples), at each passage, on immunohistochemistry, using primary antibodies: negative control, α-SMA, vimentin, PR2D3, desmin, CY90, UCHT1, CD68. 'SD' represents = standard deviation; P1= passage 1; P2=passage 2;P3=passage 3; P4=passage 4; P5=passage 5; P6 = passage 6

	P1	SD	P2	SD	P3	SD	P4	SD	P5	SD	P6	SD
Negative control	0	0	0	0	0	0	0	0	0	0	0	0
α-SMA	77.9	8.4	78.9	1.4	77.2	10.4	75.8	7.3	82.5	8.0	82.8	2.5
vimentin	95.2	1.3	94.8	0.3	95.5	0.4	95.4	.5	95.3	0.6	96.1	0.9
PR2D3	16.0	4.0	15.4	4.3	18.3	2.6	17.8	1.5	19.5	2.0	24.5	7.5
desmin	43.7	12.6	33.4	3.6	37.9	5.3	39.7	5.1	34.8	10.0	23.6	2.2
CY90	6.9	0.5	7.8	2.0	6.9	1.7	5.06	0.6	5.0	0.3	5.2	2.9
CD3	0	0	0	0	0	0	0	0	0	0	0	0
CD68	0	0	0	0	0	0	0	0	0	0	0	0

Chapter 4: MMP-3 expression by patient cell lines from control, CD and UC patients after activation with pro-inflammatory cytokines

4.1 Introduction

An important part of the downstream events that occur in IBD is the activation of protease cascades, and in particular, upregulation of MMPs. MMPs are highly expressed in and around ulcer beds of IBD patients, and the cellular source of MMPs is fibroblast-like stromal cells (Saarialho-Kere et al.;Vaalamo et al.). MMP-3, in particular, is increased in IBD patients, compared to its natural inhibitor, TIMP-1 (Heuschkel et al.). It has a broad range of substrates such as gelatin, aggrecan, elastin, tenascin, decorin and collagen types I, III, IV, V, VIII, IX and X and can therefore destroy basement membranes. MMP-3 has been shown to play an important role in T-cell mediated mucosal injury in the gut: activation of lamina propria T cells in fetal gut explants results in a Th-1 biased response and mucosal degradation (Pender et al.;Pender et al.). Injury is associated with increased production of MMPs (Pender et al.), and MMP-inhibitors prevent injury in this model (Pender et al.). Also, direct addition of MMP-3 to fetal gut explants results in severe tissue injury within 24 hours (Pender et al.;Pender et al.). Stromal cells have been shown to be the major cellular sources of MMP-3 (and MMP-1), and fetal gut stromal cells lines upregulate MMP-3 when incubated with TNF-α and IL1-β (Pender et al.;Pender et al.).

In this study colonic myofibroblast cell lines were used to investigate the responses of myofibroblasts, to pro-inflammatory cytokines, compared to a control group. The materials and methods for this study are outlined in chapter 2. MMP-3 expression in the culture supernatants of myofibroblasts isolated from control (n=55), CD (n=23) and UC (n=20) patients were compared semi-quantitatively by Western blotting, and quantitatively by ELISA.

4.2 Western blotting

In order to investigate spontaneous MMP-3 expression in myofibroblast culture supernatants, western blotting of 5 samples from each group: control, CD and UC were analysed simultaneously. The western blot in Figure 4.1 is representative of 3 separate

experiments. Pro-MMP-3 was detected as bands at 58 and 60 kDa, consistent with previous studies (Pender et al.;Vincenti;MacDonald, Bajaj-Elliott, and Pender). There was great variation in spontaneous production of MMP-3 between myofibroblast cell lines (see Figure 4.2).

(Pender et al.;Bamba et al.)Western blotting analysis was also carried out to analyse MMP-3 production in response to incremental doses of TNF-α and IL-1β (0,0.1,0.5 and 1.0 ng/ml) in 3 representative cell lines from control, CD and UC patient cell lines (ie the experiment was carried out three times in each group). All groups all responded with dose-dependent rise in MMP-3 production in response to cytokine stimulation, as shown in Figure 4.2.

4.3 Measurement of MMP-3 production by ELISA

ELISA was used as a quantitative technique to measure concentration of total MMP-3 concentration in the culture supernatant of myofibroblast cell lines, after stimulation with 0 and 1.0 ng/ml TNF-α. These included samples from 98 subjects: 55 control, 23 CD and 20 UC subjects.

The distribution of concentrations of MMP-3 of samples from the ELISA experiments was analysed in the control, CD and UC groups, without activation with TNF-α and after activation with 1.0ng/ml TNF-α, (Figures 4.3, 4.4 and 4.5). The data sets did not follow a normal distribution. The pattern of distribution was due to some samples producing large amounts of MMP-3. There was a great variability in the production of MMP-3 between different samples, in all groups, at both base-line levels and after 1.0 ng/ml TNFα (controls, CD and UC). Therefore natural logarithmic score was used to normalise the data sets in samples at 0 and 1.0 ng/ml TNF-α in order to compare the three groups (see Figures 4.3, 4.4 and 4.5).

4.3.1 Response to activation with incremental doses of TNF-α in control cell lines

MMP-3 concentration in the culture supernatants of 11 control samples were measured after activation with increasing doses of TNF-α (0,0.1,0.5 and 1.0 ng/ml). MMP-3 production by the cultured myofibroblasts increases in a dose-dependent manner. Overall,

using analysis of variance, there was no significant difference in MMP-3 production between cells that had been stimulated with different incremental doses of TNF-α, ranging from 0 to 1.0 ng/ml (p=0.126) (see Figure 4.6). However, paired comparisons between MMP-3 production after stimulation with different doses of TNF-α, showed that the difference in MMP-3 production between 0 and 0.5 ng/ml TNF-α was almost significant (p=0.066), and the difference between 0 and 1.0 ng/ml TNF-α was significant (p=0.029). These results showed that the cultured myofibroblasts were very sensitive to stimulation with small doses of TNF-α.

4.3.2 Comparison of spontaneous production of MMP-3 between myofibroblast cell lines in control, CD and UC patients

Using analysis of variance, there was no significant difference in spontaneous MMP-3 production by myofibroblast cell lines between control, CD and UC group (p=0.269). The scatter plots demonstrate the great variability in expression of MMP-3 between samples in all groups (see Figure 4.7).

4.3.3 Comparison of MMP-3 production after activation with TNF-α in myofibroblast cell lines from control, CD and UC patients

After stimulation with 1.0 ng/ml TNF-α, mean MMP-3 expression increased in all groups: by paired comparison between MMP-3 production after stimulation with TNF-α and base-line levels, in the control group there was a significant increase in MMP-3 production after stimulation with 1.0 ng/ml TNF-α (p=0.014, paired t-test). There was an increase in production of MMP-3 in the CD group (p=0.173, paired t-test) and the UC group (p=0.167, paired t-test) but not to significant levels.

There were no significant differences in MMP-3 production by myofibroblasts stimulated by 1.0 ng/ml TNF-α between the control, CD or UC groups by ANOVA (p=0.235) see Figure 4.8.

4.3.4 Comparison of MMP-3 production between cell lines from patients with active disease and inactive disease

(i) MMP-3 production by cell lines from patients with active CD, and patients with CD in remission

Eleven of the twenty-three CD subjects had active disease; and the remaining 12 patients had disease in remission. Spontaneous production of MMP-3 was higher in samples from active CD subjects compared with samples from subjects in remission ($p=0.057$, paired t-test). After stimulation with TNF-α, myofibroblasts from subjects in the active CD group also produced higher amounts of MMP-3 compared with myofibroblasts from subjects with disease in remission, to near-significant levels ($p=0.076$ paired t-test). See Figure 4.9.

(ii) MMP-3 production by cell lines from patients with active UC, and patients with UC in remission

Twelve of the twenty UC subjects had active disease, and eight subjects had disease in remission. There was no significant difference between these two groups, although the mean value was higher in the active UC group, by paired t-test. There was also no difference in MMP-3 production after activation with TNF-α (Figure 4.10).

4.4 Discussion

In this study it was shown that MMP-3 is expressed by cultured myofibroblasts before stimulation with a pro-inflammatory cytokine. Both western blotting and ELISA experiments showed that there was a great variation in expression of base-line levels of MMP-3 in cultured myofibroblast supernatants by different samples, in all groups (control, CD and UC). Analysis of MMP-3 expression by ELISA showed that the distribution was skewed before correction by natural logarithm. This is due to occasional isolated high values, which are represented in all groups: control, CD and UC.

Previous studies have shown that MMP-3 expression is very low by cultured myofibroblasts when not stimulated by a pro-inflammatory cytokine, similar to in-vivo expression of MMP-3 by stromal cells that have not been stimulated by cytokine (Pender et al.;Pender et al.;Bamba et al.). However, stromal cells in these studies were cultured from fetal tissue, and not adult colonic myofibroblasts. Variation in MMP-3 expression between

samples in this study may either reflect micro-environmental differences between samples in culture, or may be a true reflection of individual variation in MMP-3 expression between subjects in-vivo. There are a number of phenotypic differences between individuals that may affect differences in MMP-3 production, such as age, gender, and medications. Stratifying for these factors would mean recruiting very large numbers of individuals.

MMP-3 expression was increased when myofibroblasts were stimulated with TNF-α and IL-1β. Previous studies using fetal gut myofibroblasts showed that MMP-3 expression increased in cultured supernatants by 10-fold after 3 days' in culture (Pender et al.).

There may have been a number of reasons for why there was no significant difference in MMP-3 production after stimulation with TNF-α in the CD and UC groups: due to great variability in MMP-3 expression, larger numbers were likely to be required to show a significant difference between spontaneous production of MMP-3 and MMP-3 production after stimulation with TNF-α; it is also possible that there are smaller differences in MMP-3 production after stimulation with TNF-α in these groups, and so larger numbers would be required than in the control group, to demonstrate differences in MMP-3 production by myofibroblasts after stimulation with TNF-α.

Using ELISA technique, it was shown that MMP-3 production increased in a dose dependent manner when stimulated with TNF-α. This is consistent with previous reports, and shows that MMP-3 secretion is tightly regulated by TNF-α and IL-1β (Pender et al.;Bamba et al.;Frisch and Ruley;Quinones et al.).

Comparison of MMP-3 production by cell lines in the control, CD and UC groups showed that there was no overall significant difference in MMP-3 production, either before or after stimulation with 1ng/ml TNF-α. It is possible that observations from this study are not reflective of actual differences in MMP-3 expression by myofibroblasts between the CD, UC and control groups, but owing to small sample numbers and variability in MMP-3 expression between samples, differences were not revealed in this study. It is also possible that differences in MMP-3 expression are only present in phenotypic subgroups of subjects with CD and UC, such as those with active disease at the time of colonoscopy.

Power calculation was used to estimate the sample size of the disease and control groups to observe a significant difference (at the 0.05 significance level). With a power of 80%, 478 CD samples and 817 control samples would be required to be able to detect differences between these two groups; and 359 control samples and 104 UC samples would be required to detect differences between these two groups. Given that cell lines were grown from 71.8% CD samples, 64.5% UC samples, and 60.4% control samples, then 665 CD samples, 1352 control samples and 161 UC samples would need to be collected.

By dividing the disease groups further, into those with active disease and those with disease in remission reduced further statistical power of the study. In the CD group, myofibroblasts from subjects with active disease expressed higher amounts of MMP-3 in cultured supernatants, both at base-line levels, and after stimulation with TNF-α, to near-significant levels, compared with the CD remission group. Mean MMP-3 production was also increased in the active UC group compared to the non-active UC group, at base-line levels and after stimulation with TNF-α, but not significantly. This would suggest that there are functional differences between myofibroblasts of subjects with active CD and UC.

4.5 Summary

MMP-3 was expressed in the culture supernatants of cultured cell lines isolated from CD, UC and control patients. There was a great variation in expression of base-line levels of MMP-3 in supernatants of cultured cell lines in all groups (control, CD and UC).
Cultured adult myofibroblasts were highly sensitive to pro-inflammatory cytokine, and responded to both TNF-α and IL-1β with a dose-dependent increase in MMP-3 protein in cultured supernatants. This was consistent with previous reports, showing that MMP-3 secretion is tightly regulated by TNF-α and IL-1β (Pender et al.;Bamba et al.;Frisch and Ruley;Quinones et al.).
Comparison of MMP-3 production by cultured myofibroblasts in the different groups: control, CD and UC, showed that there was no overall significant difference in MMP-3 production between these groups, either before or after stimulation with TNF-α.

By dividing the disease groups further, into those with active disease and those with disease in remission, statistical power was reduced. Myofibroblasts from subjects with active CD produced higher amounts of MMP-3 compared to myofibroblasts from CD remission subjects at both base-line levels and after stimulation with TNF-α, to near-significant levels. Results from this study suggest that cell lines from patients with active CD and UC produce higher amounts of MMP-3 compared with cell lines from CD and UC patients with inactive disease.

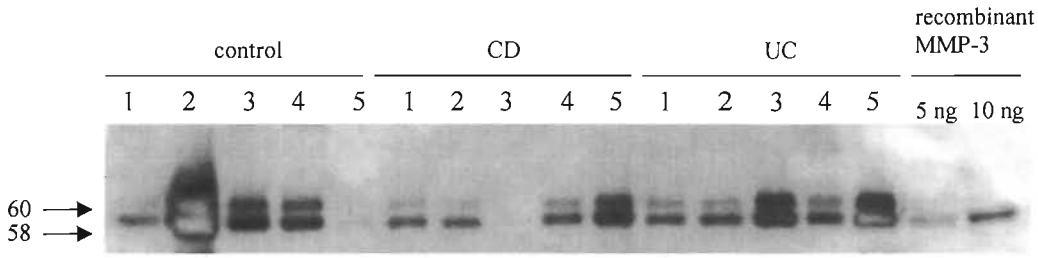

Figure4.1: Western blotting for MMP-3 in culture supernatants, from different myofibroblast cell lines in control, CD and UC patients. Culture supernatants were collected from culture wells containing myofibroblasts (100,000 cells per well). 5 samples from different subjects were taken from each group: normal, CD and UC. Arrows indicate MMP-specific molecular weight detected by polyclonal anti-MMPs. Known amounts (5ng and 10 ng) of recombinant MMP-3, loaded in two adjacent lanes, act as positive controls.

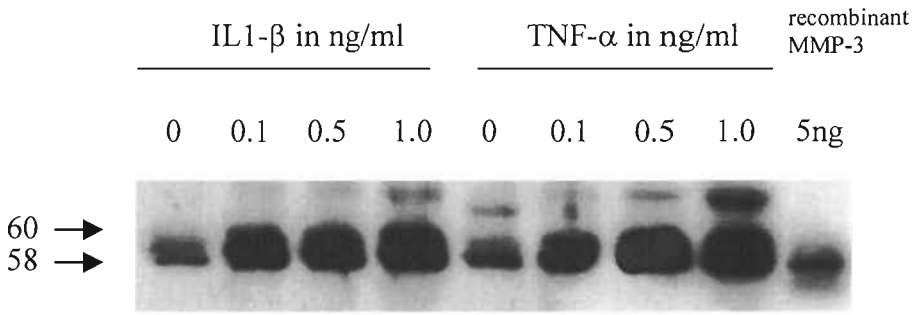

Figure 4.2: Western blotting of MMP-3 in culture supernatants, from a myofibroblast cell line from one subject in the control group, stimulated with incremental doses of TNF-α and IL-1β. Arrows indicate MMP-specific molecular weight detected by polyclonal anti-MMPs. Five nanograms of recombinant MMP-3 was used as a positive control.

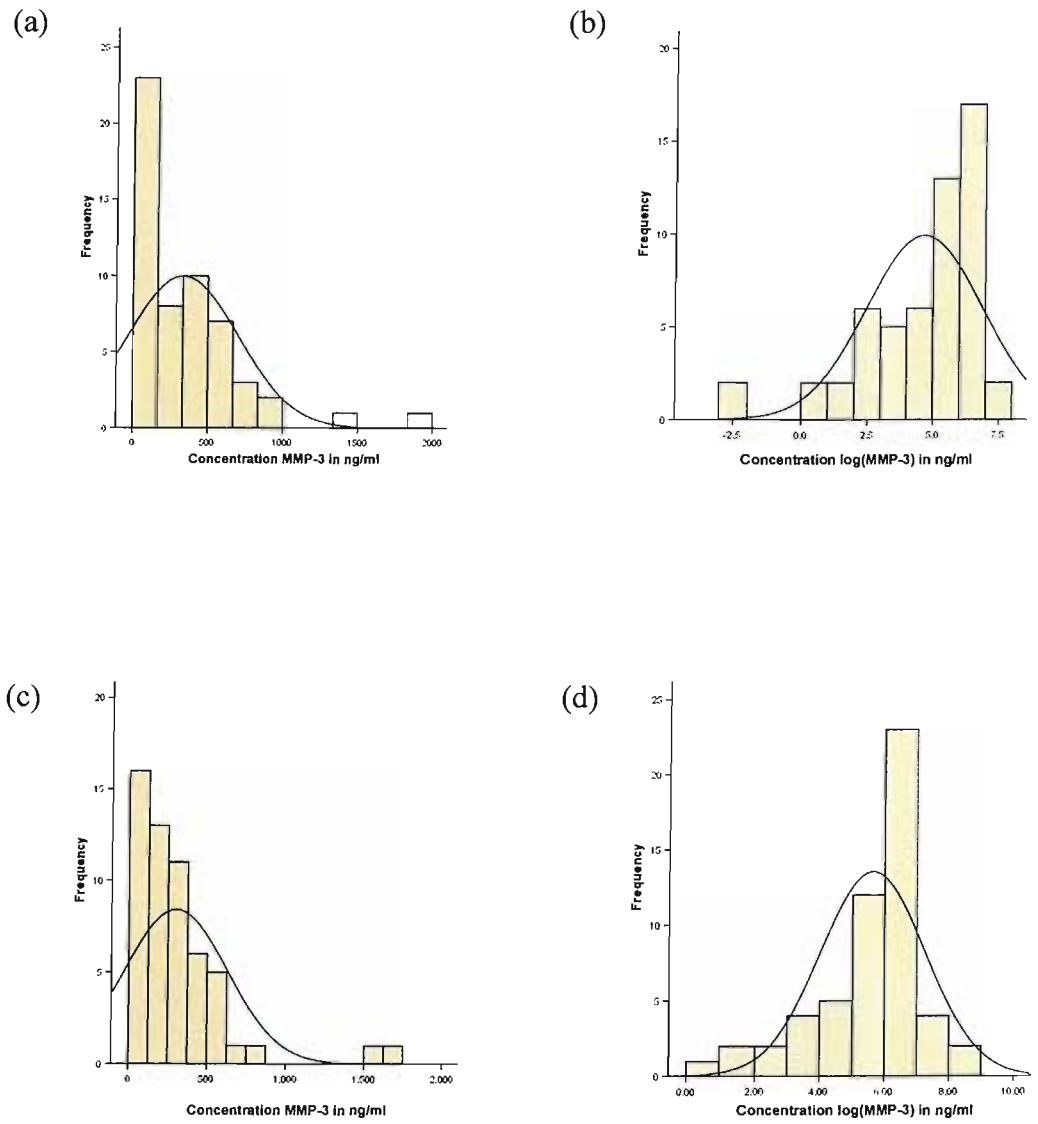

Figure 4.3: Distribution curves of control samples: (a) concentration of MMP-3 without stimulation with TNF-α; (b) log (concentration MMP-3) without stimulation with TNF-α; (c) concentration MMP-3 after stimulation with 1.0 ng/ml TNF-α; (d) log (concentration MMP-3) after stimulation with 1.0 ng/ml TNF-α

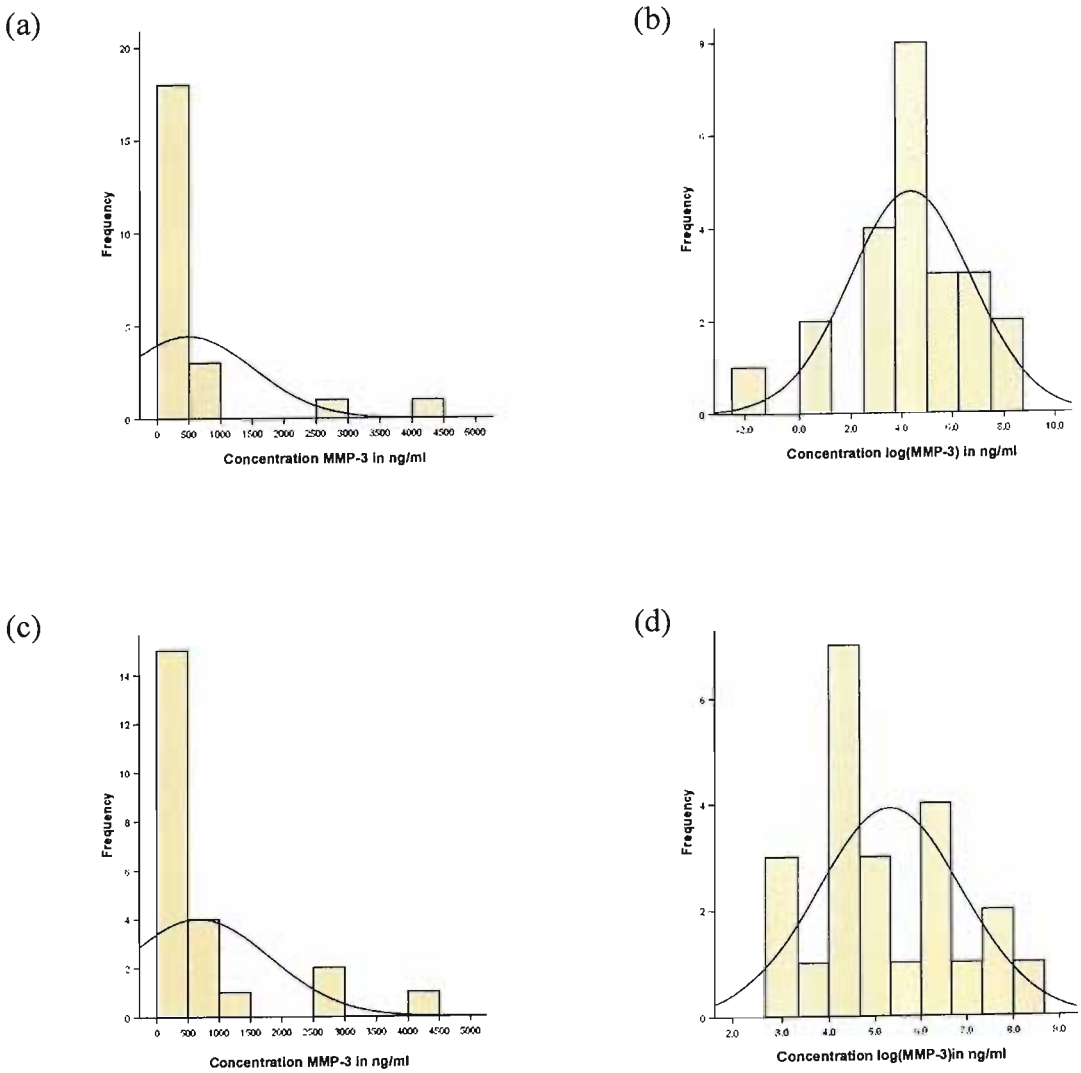

Figure 4.4: Distribution curves of CD samples: (a) concentration of MMP-3 without stimulation with TNF-α; (b) log (concentration MMP-3) without stimulation with TNF-α; (c) concentration MMP-3 after stimulation with 1.0 ng/ml TNF-α; (d) log (concentration MMP-3) after stimulation with 1.0 ng/ml TNF-α

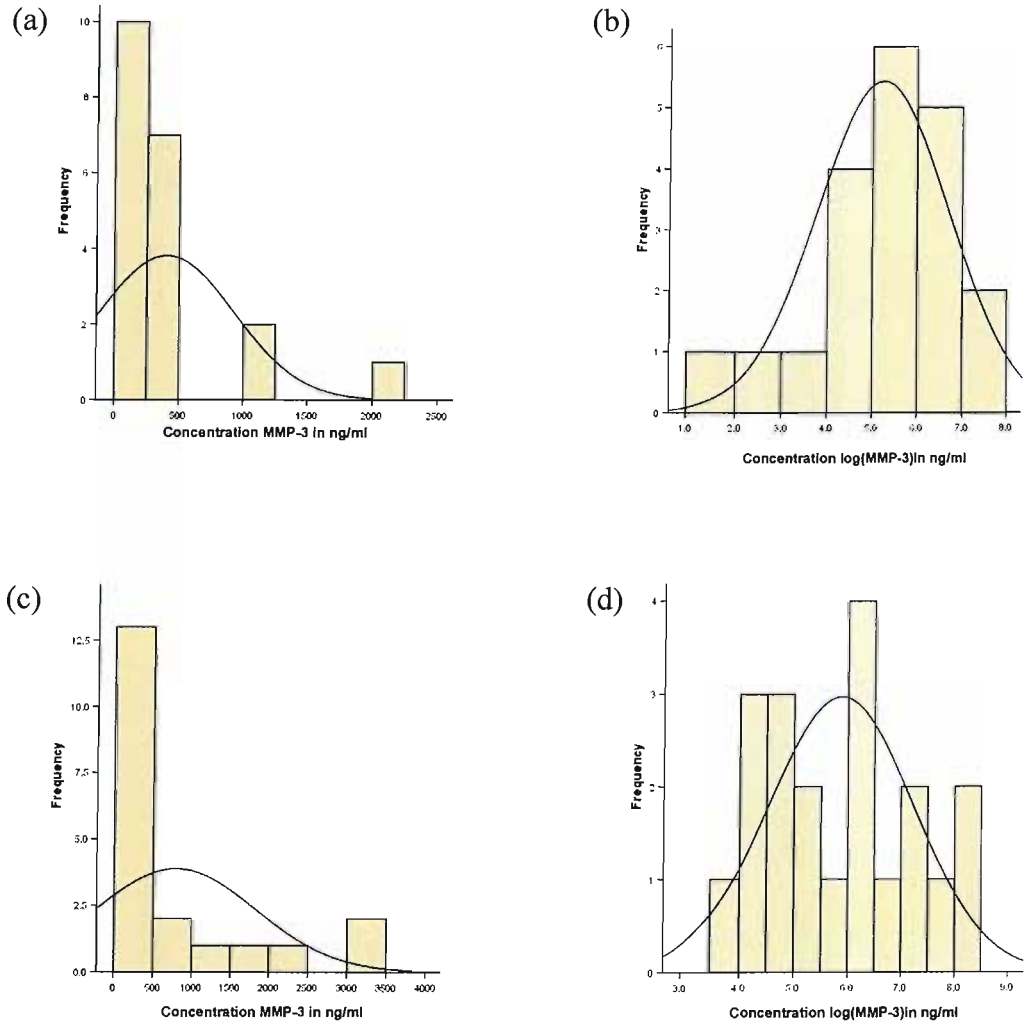

Figure 4.5: Distribution curves of UC samples: (a) concentration of MMP-3 without stimulation with TNF-α; (b) log (concentration MMP-3) without stimulation with TNF-α; (c) concentration MMP-3 after stimulation with 1.0 ng/ml TNF-α; (d) log (concentration MMP-3) after stimulation with 1.0 ng/ml TNF-α

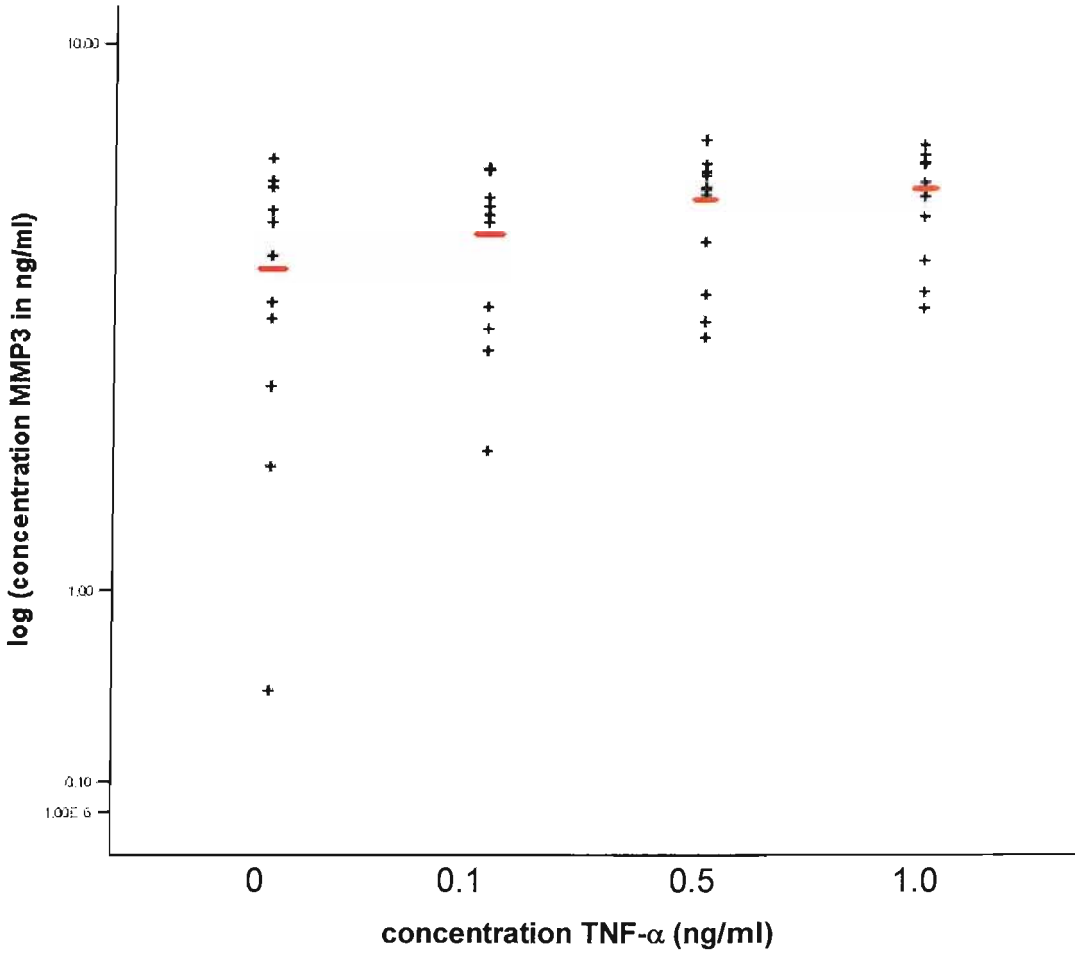

Figure 4.6: MMP-3 production in myofibroblast culture supernatants of control cell lines (n=11) after stimulation with incremental doses of TNF-α. Red lines denote mean values. There was no significant difference overall in MMP-3 production between cells stimulated by different doses of TNF-α by ANOVA (p=0.126).

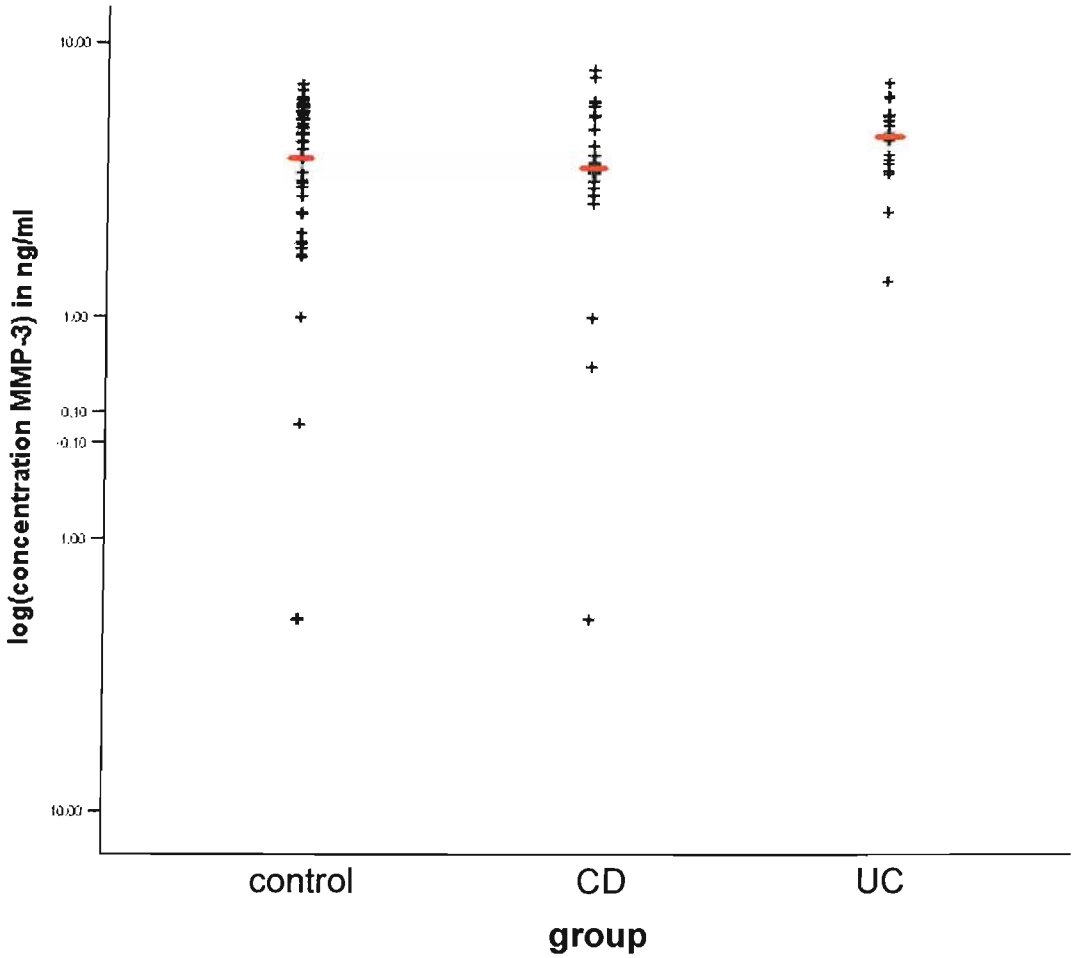

Figure 4.7: Scatter plots of spontaneous production of MMP-3 in control, CD and UC patients. Red lines denote mean values. There was no significant difference in spontaneous production of MMP-3 between control, CD and UC groups by ANOVA (p=0.269).

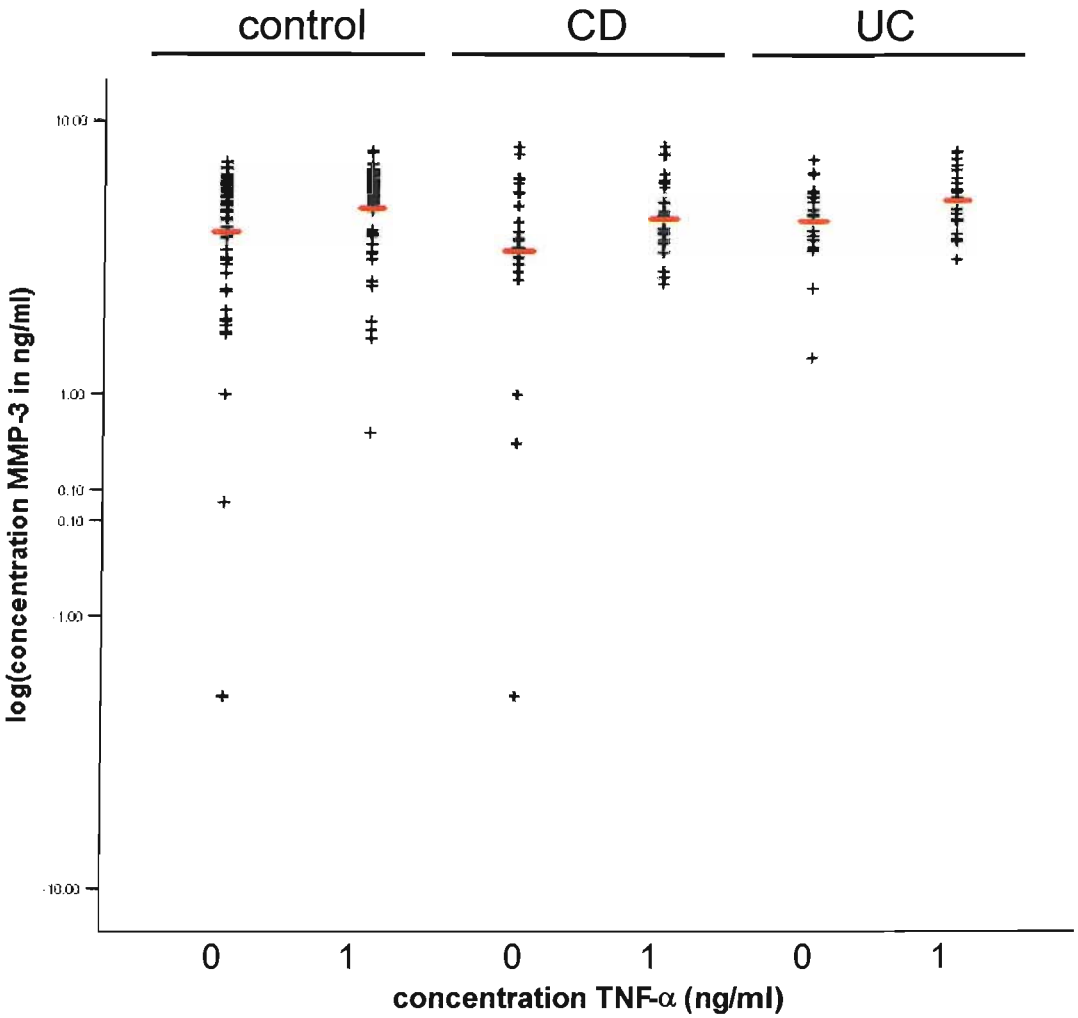

Figure 4.8: Scatter plots of MMP-3 concentration of samples from control, CD and UC groups, at spontaneous levels of MMP-3 production (0 ng/ml TNF-α) and after stimulation with 1.0 ng/ml TNF. Red lines denote mean values. Paired comparisons between spontaneous MMP-3 production and MMP-3 production after stimulation with TNF-α showed that there was a significant increase in MMP-3 production in the control group (p=0.014, paired t-test), but not in the CD group (p=0.173, paired t-test), or the UC group (p=0.167, paired t-test).

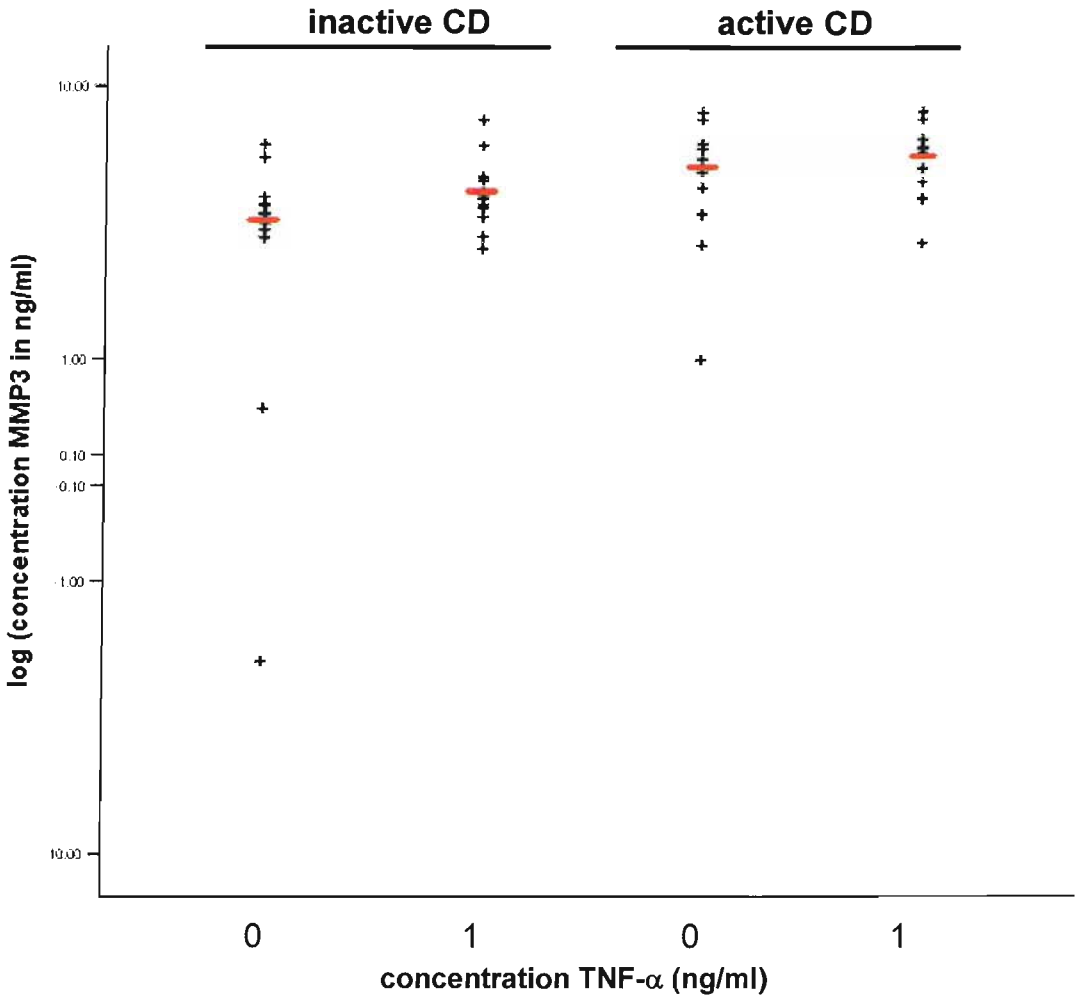

Figure 4.9: MMP-3 concentration of inactive CD group (rCD) and active CD group (aCD), at spontaneous levels of MMP-3 production (0 ng/ml TNF-α) and after stimulation with 1.0 ng/ml TNF-α. Red lines denote mean values. Spontaneous production of MMP-3 was higher in the active CD group than the CD group in remission by paired t-test (p=0.057); and MMP-3 production was also higher in the active CD group than the CD group in remission by paired t-test (p=0.076)

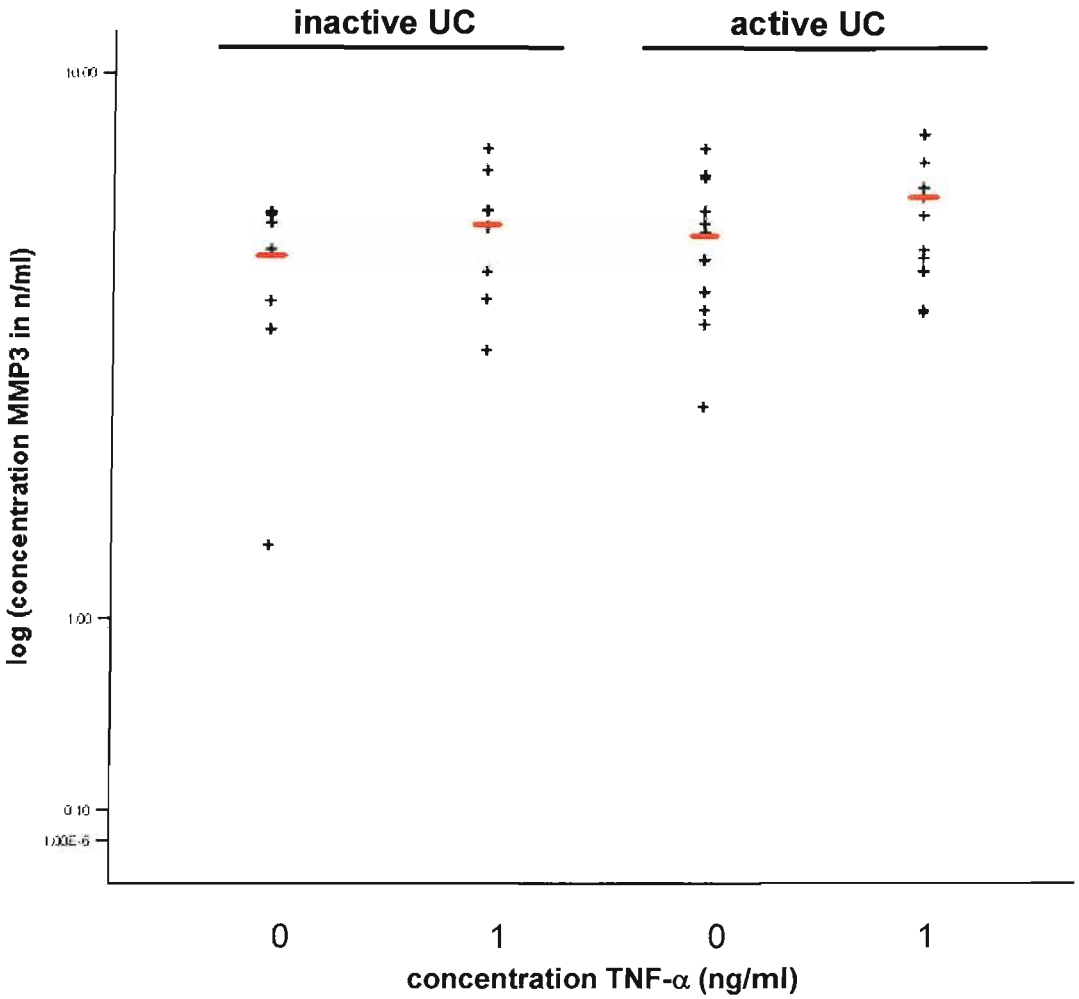

Figure 4.10: MMP-3 concentration of inactive UC group (rUC) and active UC group (aUC), at spontaneous levels of MMP-3 production (0 ng/ml TNF-α) and after stimulation with 1.0 ng/ml TNF-α. Red lines denote mean values. There was no difference between the active UC and inactive UC groups, either at spontaneous levels of MMP-3 production or after activation with TNF-α, by paired t-test.

Chapter 5: Comparison of MMP-3 expression by patient cell lines and MMP-3 5A/6A genotype

5.1 Introduction

MMP-3 is expressed at low levels in the gastrointestinal tract under control conditions, and it's expression is increased in the inflamed mucosa of CD and UC patients (von Lampe et al.;Heuschkel et al.;Kirkegaard et al.). *In vitro* experiments have identified the importance of MMP-3 in tissue destruction in T-cell mediated mucosal injury in the gut (Pender et al.). The MMP-3 gene is located on chromosome 11q22 (Formstone et al.). An MMP-3 SNP polymorphism has been identified in the promoter region of the gene, at position –1612, where either a run of 5 adenosines (5A allele) or 6 adenosines (6A allele) are present (Ye et al.). The 5A allele, as opposed to the 6A allele is related to matrix degradation and tissue damage, whereas the 6A allele is related to matrix accumulation (Ye et al.). No previous studies have investigated the functional effect of the MMP-3 5A/6A polymorphism on MMP-3 expression in the gut. The hypothesis was that individuals homozygous for the 5A genotype are able to produce higher amounts of MMP-3. The aim of this study was therefore to compare the expression of MMP-3 of cell lines that had been stimulated with a pro-inflammatory cytokine, between the different genotypes: homozygous 5A allele, heterozygous 5A/6A allele, and homozygous 6A allele.

The materials and methods are described in chapter 2. In brief, 5 biopsy samples were taken at colonoscopy from adult subjects, and at the same time, 5 mls of blood was taken. 98 subjects participated in this study, which included control (n=55), CD (n=23) and UC (n=20) subjects. Myofibroblasts were isolated and cultured according to the method described previously. At passage 4, the myofibroblasts were seeded onto 24 well-plates, and stimulated with 0 and 1.0 ng/ml TNF-α for 48 hours. The production of MMP-3 was measured by ELISA. Genomic DNA was extracted from peripheral blood leukocytes. Standard PCR was carried out using oligonucleotide primers to amplify the flanking regions of the MMP-3 polymorphisms. The PCR products were cut by restriction endonuclease digestion and then separated on a polyacrylamide gel under reducing conditions. The digest products of the cut (5A) allele were 90 and 28 bp length, whereas the uncut (6A) allele was measured at 118 bp, consistent with previous studies which have

investigated the MMP-3 5A/6A polymorphism by this method (see Figure 5.1) (Dunleavey, Beyzade, and Ye).

5.2 Frequencies of MMP-3 5A and 6A allele in control, CD and UC patients

The genotype frequencies of the 5A allele in the control, CD and UC groups are shown in Table 5.1. They correlate with the genotype frequencies previously published for the MMP-3 5A/6A polymorphism (Ghilardi et al.;Satsangi et al.;Wiencke et al.). There was no significant difference in allele frequency for the 5A and 6A alleles, between the control, CD and UC groups.

5.3 Comparison of MMP-3 production by cell lines between 5A/5A, 5A/6A and 6A/6A patient genotypes in control patients

The number of samples in the CD and UC groups was too small to be able to investigate differences between the MMP-3 genotypes, in MMP-3 expression by cultured myofibroblasts in these groups. However, differences in MMP-3 expression between genotypes was studied in the control group (n=55).

There were 11 5A/5A patients, 33 5A/6A patients, and 11 6A/6A patients in the control group. Mean values of MMP-3 production by 5A/6A MMP-3 genotypes in the control group are shown in Table 5.2. There was no significant difference in MMP-3 production between these groups by ANOVA, either at base-line levels, or after stimulation with 1.0ng/ml TNF-α (see Figure 5.1).

5.4 Discussion

In this study, the effect of the MMP-3 promoter 5A/6A polymorphism was investigated on MMP-3 expression in myofibroblasts isolated from CD, UC and control subjects. No association was found between the MMP-3 5A allele or 6A allele and the altered expression of MMP-3 by gut myofibroblasts among patients in the control group.

Since the MMP-3 5A/6A polymorphism was identified, a number of studies have shown an association of the 5A allele with pathological conditions involving excessive matrix degradation such as myocardial infarction; whereas 6A homozygotes have greater atherosclerotic plaque growth, a process associated with matrix accumulation. The MMP-3 5A/6A gene promoter polymorphism has also been shown to have a functional effects in vitro: in transient expression experiments, cultured fibroblasts and vascular smooth muscle cells transfected with the constructs containing the 6A polymorphism expressed approximately 2 fold lower amount of reporter gene product as compared with the transfectants of the constructs containing the 5A polymorphism (Ye et al.). In addition, another study showed that MMP-3 mRNA expression in dermal biopsies was 4-fold higher in subjects homozygous for the 5A allele than heterozygotes, and 2-fold lower in 6A homozygotes compared to heterozygotes (Medley et al.). The greater transcriptional activity of the 5A allele compared to the 6A allele appears to be due to preferential binding of a transcriptional repressor to the latter (Ye et al.;Ye et al.). Taken together, these observations suggest that the 5A/6A MMP-3 promoter polymorphism has a functional effect on MMP-3 expression, such that the 5A allele confers imbalance of extracellular matrix favouring matrix degradation, and the 6A allele confers an imbalance of extracellular matrix favouring matrix accumulation.

Investigation of the effects of the MMP-3 polymorphism on MMP-3 expression by myofibroblasts in the disease groups was not possible owing to small sample numbers in these groups. Fewer numbers in the CD and UC groups were attained due to fewer subjects being available for colonoscopy than in the control groups. In addition, myofibroblast cell lines were isolated and grown from only a proportion (79.3% of CD samples and 60.6% of UC samples) of all samples taken in these groups (see Table 2.1, chapter 2). In addition, the variability in MMP-3 expression between samples allowed for large margins of error,

and this unpredicted factor meant that even larger numbers of samples would need to be attained to draw a meaningful conclusion from these results.

If the MMP-3 promoter polymorphism has functional effects on MMP-3 expression by myofibroblasts, then this could be an independent factor, and therefore would be expected to have allele-specific effects on MMP-3 expression, regardless of disease group. However, investigation of the control group did not yield significant results.

By investigating the functional effects of the MMP-3 polymorphism on MMP-3 expression by cell lines in all samples overall, an assumption was made that there is no difference in MMP-3 expression between these groups following studies on MMP-3 expression alone (see chapter 4), and that the frequency of the 5A allele was similar in all groups. In this limited study the frequency of the 5A allele was similar, but is not necessarily representative of the prevalence of the 5A allele in larger populations of CD and UC subjects. Nevertheless, there were no apparent allele-specific effects of the MMP-3 polymorphism on MMP-3 expression and therefore this study did not support the hypothesis that the MMP-3 polymorphism has allele-specific effects on MMP-3 expression by stromal cells in the gut.

Based on the allele frequencies in control groups, the sample size in this part of the study that would allow detecting a significant difference between the homozygous 5A patients and homozygous 6A patients would be 188 patients with a power of 80% at the 0.05 significance level. This would mean that 311 control samples would need to be collected, given that 60.4% of control cell lines were successfully grown. Based on the observed mean values of log (MMP-3 concentration), and with a power of 80% at the 0.05 significance level, 256 CD patients, and 24 UC patients would be required to allow detection of differences between the homozygous 5A and 6A genotypes in these groups. This would mean the collection of 357 CD samples (given that 71.8% cell lines were successfully cultured in the CD group), and 37 samples in the UC group (given that 64.5% cell lines were successfully cultured in the UC group).

5.5 Summary

There were no apparent allele-specific effects of the MMP-3 5A/6A polymorphism on MMP-3 expression by gut myofibroblasts of control patients, therefore not supporting the hypothesis that the MMP-3 promoter polymorphism has a functional affect on MMP-3 expression in stromal cells in the gut. Sample numbers were too small to investigate the functional effects of this MMP-3 polymorphism in the CD and UC groups.

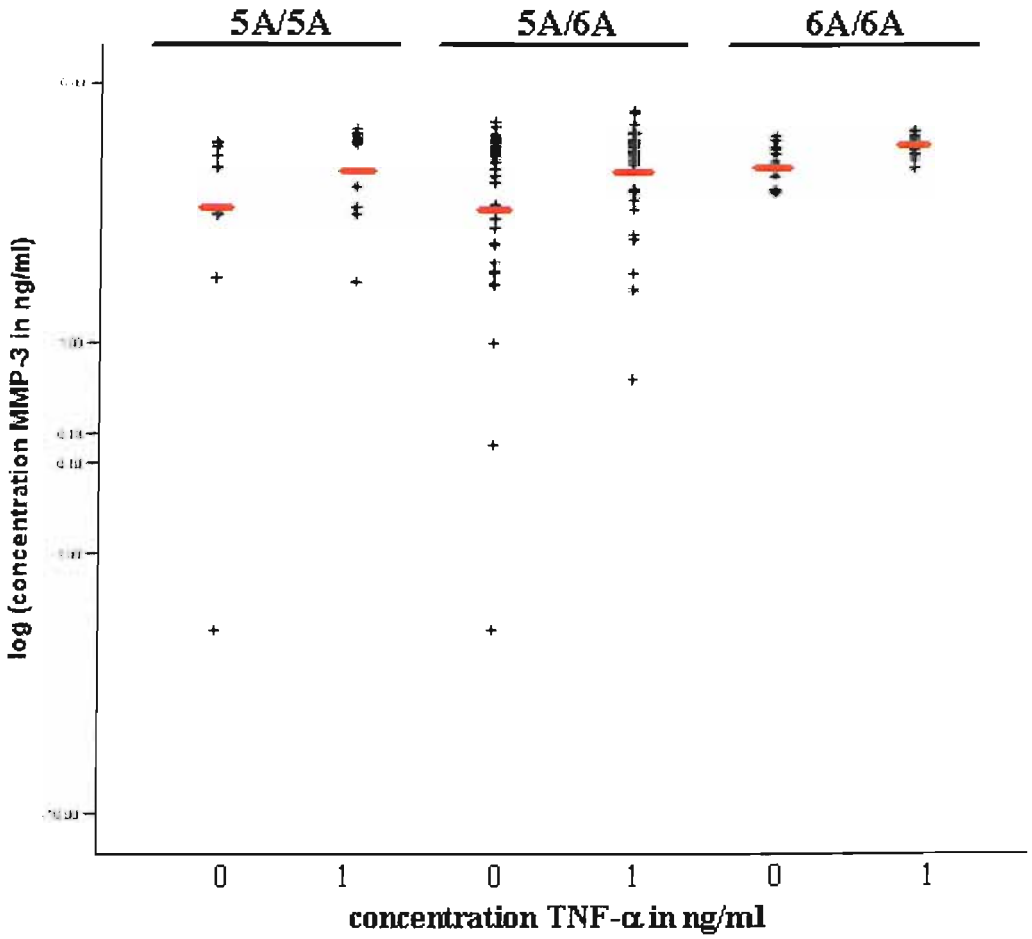

Figure 5.1: Scatter plots of MMP-3 concentration in cell line culture supernatants from control patients according to MMP-3 5A/6A genotypes. The red lines denote mean values. There was no significant difference in spontaneous production of MMP-3 between the control, CD and UC groups, and in production of MMP-3 after activation with TNF-α between control, CD and UC groups, by ANOVA.

Table 5.1: Number of frequencies of MMP-3 5A/6A genotypes in each group: control, CD and UC

group	number	5A/5A(%)	5A/6A(%)	6A/6A(%)	5A frequency	6A frequency
Control	55	11 (20)	33 (60)	11 (20)	0.5	0.5
CD	23	1 (4.3)	18 (78.3)	4 (17.4)	0.43	0.57
UC	20	3 (15)	9 (45)	8 (40)	0.375	0.625
Total	98	15 (15.3)	60 (61.2)	23 (23.5)	0.409	0.591

Table 5.2: Mean values of MMP-3 expression for each 5A/6A MMP-3 genotype in control group: at base-line levels (0 ng/ml TNF-α) and after stimulation with 1.0 ng/ml TNF-α. SD = standard deviation

Genotype	Number	0 ng/ml TNF-α		1.0 ng/ml TNF-α	
		Mean	SD	Mean	SD
5A/5A	11	4.44	2.65	5.52	1.72
5A/6A	33	4.46	2.34	5.46	1.79
6A/6A	11	5.50	0.88	6.26	0.58

Chapter 6: Transmission disequilibrium testing of MMP-3 gene promoter polymorphism in relation to CD and UC

6.1 Introduction

MMP-3 is a key effector molecule in mucosal injury following T cell activation in the gut (Pender et al.). Both mRNA transcripts and protein levels of MMP-3 are elevated in patients with active inflammatory bowel disease (Heuschkel et al.). The MMP-3 gene, located on chromosome 11q23, is therefore a good candidate gene that may modify susceptibility to the inflammatory bowel diseases, CD and UC. A common polymorphism in the MMP-3 gene, located at position 1612 upstream of the start of transcription, has been identified, in which one allele has a cluster of 5 adenosines (5A allele), and the other allele has a cluster of 6 adenosines (6A allele) (Ye et al.). In vitro studies have shown a functional affect of the 5A allele, in that it confers increased transcriptional activity due to differential binding of a transcriptional repressor to the 6A allele (Ye et al.;de Maat et al.;Medley et al.). The hypothesis was therefore that this functional affect of the 5A allele influences disease susceptibility to CD or UC, and that there is an over-representation of the 5A allele in CD and UC groups.

The materials and methods are described in chapter 2. In brief, three groups of patients were recruited to the study: German sporadic trios (affected offspring and unaffected parents) (CD trios n=320, UC trios n=148); German multiplex families (CD families n=135, UC families n=83) and British multiplex families (CD families n=91, UC families n=49). DNA was extracted from blood samples of these subjects, and the 5A/6A polymorphism was genotypes using Taqman PCR. Transmission disequilibrium tests (TDT) of the MMP-3 5A/6A polymorphism was carried out on these families, using the TRANSMIT program with robust estimator option (Clayton and Jones;Clayton). Significances were verified using 10,000 bootstrap replicates.

6.2 TDT analysis of the 5A/6A MMP-3 polymorphism in German and British families

The results of the TDT in the British and German families are shown in Table 6.1. In both the British and German families where one or more family member was affected with CD, the observed number of transmitted 5A alleles between parent and affected offspring was not significantly different to the expected number of transmitted 5A alleles (British families: ratio of observed/expected: 151/152.69, p=0.76; German families: ratio of observed to expected: 252/247.51, p=0.5133). Similarly, in the UC families, the observed to expected transmission ratio of 5A alleles from parent to affected offspring was not significant (British families: ratio of observed /expected transmission of 5A alleles: 62/64.46, p=0.46; German families: ratio of observed/expected 124/119.91, p=0.4331). The CARD15 frequency was not significantly different between the British and German multiplex families and the German sporadic trios cohort.

6.3 TDT analysis of the MMP-3 5A/6A polymorphism in German sporadic IBD trios

There was a highly significant association between the CD and over-transmission of the 5A allele (observed/expected 5A allele transmission ratio: 279/250.63, p=0.0012, Table 6.1). There was no significant association between UC and the 5A/6A polymorphism.

There was an interaction of the MMP-3 polymorphism with the CARD15 mutations, such that over-transmission of the 5A allele to affected offspring was only significant in CARD15 gene variant carriers (p=0.0054) but not in non-carriers (p=0.67), see Table 6.2. Furthermore, among the CARD15 carriers, over-transmission of the 5A allele was associated with phenotypic subgroups: stenosis (p=0.0027), fistulising disease (p=0.0007), disease of the ileum (p=0.0001), surgical resection (p=0.0023), disease of the right colon (p=0.0115), but not the left colon (see Table 6.2).

6.4 Discussion

There is epidemiological evidence to show that genetic factors are important in susceptibility to CD and UC. Although the MMP-3 gene located on chromosome 11q23 is

not located within the particular chromosomal regions (19p13, 16q12, 16p, 14q11-q12, 12p13.12-q24.1,6p,5q31 and 1p36) that have been shown to be in linkage with the IBD, it is a strong functional candidate gene. Given that MMP-3 plays an important role in tissue injury in the gut, and that previous studies have shown an allele specific effect on MMP-3 expression, individuals carrying the 5A allele may have increased susceptibility to CD and UC.

This study showed an association of the MMP-3 5A/6A polymorphism with susceptibility to CD in a cohort of German sporadic CD trios. This is likely to reflect increased expression of MMP-3 in carriers of the 5A allele, and is consistent with previous functional studies which have shown that the 5A allele confers higher transcriptional activity than the 6A allele (Ye et al.; Medley et al.).

This study also found that there is an interaction between the MMP-3 gene and CARD15 gene in determining susceptibility to CD. Reasons for this are unclear, although it may be that the relatively modest effects of the 5A allele are unmasked in carriers of CARD15 mutations, and non-genetic factors are more influential in the development of CD in non-carriers of CARD15 mutations.

This study found no association between the inheritance of the 5A allele of the common MMP-3 promoter polymorphism, and susceptibility to CD or UC, in British and German multiplex families. There are a number of possible explanations for the finding of an association between the MMP-3 polymorphism and susceptibility to CD in the sporadic cases, but not in the cohorts with familial disease. The observation that there is an association between the MMP-3 polymorphism and susceptibility to CD in the sporadic cases, but not in the cohorts with familial disease, may be explained in part by reduced statistical power in the familial studies, owing to smaller numbers than in the sporadic group. In addition, in 13 out of 91 CD families, and 7 out of 49 UC families (15% of families in both cases), only one parental genotype was available for TDT analysis, reducing the statistical power of this study. Another explanation could be that differences in results between the familial cases and sporadic cases were due to variation in phenotypic subgroups between the cohorts. Further analysis showed that over-transmission of the 5A allele in CARD15 carriers of the sporadic cohort was associated with ileal disease, fistulae-formation, stenosis and surgical interventions. If these subgroups were relatively under-

represented in the multiplex CD cases, this could lead to varying results from the sporadic cohort. On the other hand, the difference in results between these cohorts may represent aetiological differences in onset of CD between sporadic and familial cases. It is possible that as yet unknown genetic factors are more influential in disease onset in familial Crohn's Disease than in sporadic cases, and therefore the modest effect of the MMP-3 polymorphism is more easily detectable in the latter.

The association of the 5A/6A polymorphism with susceptibility to CD and UC was investigated with the use of Transmission disequilibrium testing (TDT). TDT is an improved alternative to population based studies, and uses both their parents and nontransmitted alleles as family-based controls. The advantage of this methodology is that it avoids false positive results due to population admixture. This study used the TRANSMIT program with robust variance estimator option, which enables analysis to be carried out with optimal accuracy if data is unavailable. Despite this, statistical power of such studies can be limited if a high proportion of cases are used in which one of the parental genotypes is unknown.

6.5 Summary

This is the first study to show an association of the MMP-3 5A/6A polymorphism with susceptibility to Crohn's Disease (Pender et al.). Using TDT analysis, over-transmission of the 5A allele was observed to affected offspring in individuals with sporadic CD in a German cohort ($p=0.0012$), though there was no association with susceptibility to CD in German multiplex families ($p=0.995$) and British multiplex families ($p=0.4331$). Over-transmission of the 5A allele in the German trios was significant in CARD15 carriers ($p=0.0054$) but not in non-carriers. In the CARD15 carriers, over-transmission of the 5A allele was associated with stenosis ($p=0.0027$), fistulising disease ($p=0.0007$), disease of the ileum ($p=0.0001$), surgical intervention ($p=0.0023$) and disease of the right colon ($p=0.0115$). These results suggest an association of the MMP-3 5A/6A polymorphism with susceptibility to CD and phenotypic variants of this condition.

Table 6.1: Results of Transmission Disequilibrium Test in German and British families

	CD		UC	
	O/E	p value*	O/E	p value*
German sporadic IBD trios	279/250.63	0.0012	129/128.98	0.9955
German multiplex IBD families	252/247.51	0.5133	124/119.91	0.4331
British multiplex IBD families	151/152.69	0.7600	62/64.46	0.4642

* After 10,000 bootstrap replicates
IBD = inflammatory bowel disease; O/E = observed/expected 5A allele transmission ratio

Table 6.2: Results of Transmission Disequilibrium Test in German sporadic inflammatory bowel disease trios stratified for CARD15 genotype

	CARD15	O/E	p value*
CD	+	166/147.49	0.0054
	-	170/167.04	0.6718
Stenosis	+	116/98.56	0.0027
	-	100/100.89	0.871
Fistulae	+	103/84.60	0.007
	-	90/90.39	0.942
Resection	+	106/89.87	0.0023
	-	147/124.78	9.8284
Ileal disease	+	124/124.84	0.0001
	-	124/109.3	0.891
Right colon	+	124/109.3	0.0115
	-	114/114.84	0.8805
Left colon	+	57/52.19	0.2165
	-	113/113.56	0.9206

* after 10 000 bootstrap replicates.
CARD15 mutation, + indicates carriers of the R702W, G908R, or 1007insCmutation; - indicates non-c
O/E, observed/expected 5A allele transmission ratio.

Chapter 7: Discussion

7.1 Isolation and Culture of Colonic myofibroblasts

7.1.1 Myofibroblasts as an *ex-vivo* model

Chronic mucosal inflammation in the gut is characterised by infiltration of inflammatory cells and epithelial cell restitution, accompanied by increased turnover of extracellular matrix components in the subepithelial region (Riddell;Stenson). Myofibroblasts are a cellular source of MMPs and TIMPs, thereby contributing to extracellular matrix remodelling. The precise functions of these cells remain unclear due to lack of experimental models.

A number of studies have used primary cultures of gut myofibroblasts as an *ex-vivo* model in order to investigate the role of these cells in normal physiological and pathological processes (Stallmach et al.;Strong et al.;van Tol et al.;Pender et al.;Pender et al.;Pender et al.;Pender et al.). The method of cell isolation and culture used in this study has also been used in previous studies, in which cells are isolated from biopsy samples by cell-outgrowth, and are described as myofibroblasts with positive α-SMA staining(Hinterleitner et al.) (Mahida et al.). Myofibroblast cell-lines have previously been used in this laboratory, isolated from human fetal gut explants. It has been shown that activation of lamina propria T cells by mitogen in explant cultures of second trimester human small intestine results in increased production of MMPs and degradation of the extracellular matrix (Pender et al.;Pender et al.). These experiments underscore the importance and relevance of myofibroblasts in the process of chronic inflammation in the gut. This *ex-vivo* system is useful to delineate the biochemical basis for T cell-mediated injury in the gut, as it does not contain any blood-borne inflammatory cells, B cells, mast cells, or eosinophils. However, there are a number of limitations using this *ex-vivo* model. For example, myofibroblasts take approximately four to six weeks to culture to passage four that was used in these experiments. There is potential during this period for cell-lines to differentiate.

The study of myofibroblasts in culture allows us to make observations about mesenchymal cells, their function under normal physiological conditions, and their functional phenotype

in IBD subjects, which may provide further insights into the pathogenesis of the inflammatory bowel diseases.

7.1.2 Phenotype of myofibroblast cell lines

It is increasingly evident that intestinal mesenchymal cells are more heterogeneous than previously suspected. We have shown that cells isolated by a consistent method, are largely α-SMA positive and vimentin-positive cells, indicative of a mesenchymal myofibroblast phenotype. The remaining cells were likely to represent other mesenchymal cells such as fibroblasts. Heterogeneity of mesenchymal cells has been demonstrated in previous studies. For example, enteric smooth muscle cells with mature or immature phenotypes differ in levels of α-SMA and γ-SMA expression (Dvorak and Dickersin). In normal intestine, subepithelial myofibroblasts and fibroblasts are found in the submucosa, serosa, and intermuscular connective tissue, and are the primary sites of expression of collagen mRNA and protein (Matthes et al.;Pucilowska et al.). The origin of the myofibroblasts is still controversial, and they might have multiple origins (Ronnov-Jessen et al.). In this study it has been shown that populations of cell lines isolated from adult biopsies of colonic mucosa, have the capacity over repeated passaging to become more myofibroblast-enriched cells. This has been demonstrated in other *in-vitro* culture experiments (De Wever and Mareel). It is therefore possible that subepithelial myofibroblasts are derived from fibroblast trans-differentiation, an event that can be modulated by environmental factors: cytokines and growth factors such as TGF-β, and platelet-derived growth factor (PDGF)(De Wever and Mareel).

7.1.3 Production of MMPs upon stimulation of pro-inflammatory cytokines

Our studies showed that incubates myofibroblasts with pro-inflammatory cytokines TNF-α or IL-1β induced a dose-dependent MMP-3 secretion. This suggests that MMP-3 secretion is tightly regulated by IL-1β and TNF-α, consistent with previous reports(Frisch and Ruley;Quinones et al.;Pender et al.;MacNaul et al.).

Myofibroblasts also have the ability to dose-dependently proliferate in response to IL-1β, IL-6, and TNF-α, and to modulate dose-dependent expression of the stimulatory

cytokine(Strong et al.). These experiments therefore show that myofibroblasts can also act as active regulators of intestinal immunity under normal and inflammatory conditions.

A number of functional differences have been observed between myofibroblast phenotypes of CD, UC, and control subjects. For example, myofibroblasts isolated from inflamed gut of UC and CD patients proliferated at higher rates when stimulated by growth factors compared to those that isolated from controls(Lawrance, Maxwell, and Doe;McKaig et al.). Myofibroblasts of CD and UC patients also produced more collagen (Lawrance, Maxwell, and Doe) and total protein at base-line levels than control groups(Lawrance, Maxwell, and Doe;McKaig et al.). Differences have also been observed in adult human mesenchymal cells of IBD subjects in response to stimulation with cytokines. Myofibroblasts isolated from strictured bowel of CD patients, have been shown to produce increased collagen III when exposed to bFGF, PDGF, TGF-β1, and IGF-1, compared with myofibroblasts from non-strictured but inflamed tissue (Stallmach et al.).

In this study, when MMP-3 expression in myofibroblasts was investigated following stimulation with pro-inflammatory cytokines, no differences were observed overall between cell lines from CD, UC and control individuals, either at base-line levels or after stimulation with TNF-α. However cell lines from CD patients with active disease tended to produce higher amounts of MMP-3 than CD patients in remission, and cell lines from UC patients with active disease tended to produce higher amounts of MMP-3 than UC patients with disease in remission, but in neither case to significant levels. Overall, these findings support the existence of a functionally distinct mesenchymal cell subpopulation within inflamed mucosa that is independent of the disease type, and that their altered phenotype may contribute to the pathogenic changes seen in CD and UC. More detailed studies of cultured cells following exposure to cytokines, immune cells, or ECM are required to provide a useful definition of an activated phenotype of mesenchymal cells.

7.2 Contribution of MMP-3 5A/6A polymorphism studies to our understanding of IBD genetics

The past few years have witnessed dramatic improvements in our understanding of the pathogenesis of the inflammatory bowel diseases. Some of the key developments that have led to this include a significant increase in our understanding of the relationship between

the host and commensal bacteria, and several epidemiological observations that underpin the importance of environmental factors in IBD(MacDonald and Monteleone). Confirmed IBD susceptibility regions include 16p12-q13 (IBD1), 12p13.2-q24.1 (IBD2), the major histocompatibility complex region on chromosome 6 (IBD3), 14q11-12 (IBD4), 5q31 (IBD5) and chromosome 10(Duerr et al.;Hampe et al.;Hugot et al.;Ma et al.;Ohmen et al.;Yang et al.;Peltekova et al.). Linkage studies have identified different susceptibility loci for CD and UC(Lawrance, Fiocchi, and Chakravarti), confirming that CD and UC are two related yet different forms of chronic intestinal inflammation resulting from the interaction of multiple gene products.

The aetiology of CD remains to be precisely defined but current favoured hypothesis describes a genetically susceptible individual who encounters a single or series of environmental triggers to manifest the disease(Hume and Radford-Smith;Fowler et al.). Identification of the association between the NOD2/CARD15 gene and risk of CD represented a landmark observation(Hugot et al.;Ogura et al.).Although variants of the CARD15 gene contribute to only a minority of cases of CD, the identification of the first susceptibility gene heralds the identification of many other genes associated with CD and UC. However, the ultimate importance of identifying genes associated with CD the inflammatory bowel diseases lies in the ability of these observations to improve the understanding of the underlying aetiology and pathogenesis of CD and UC. Associations of IBD with IL-1 receptor antagonist, ICAM-1 and TNF-α genes have been described, but the strength of these associations is variable and weak(Mansfield et al.;Yang et al.;Plevy et al.;Bouma et al.).

Despite the fact that the locus of the MMP-3 gene, located on chromosome 11q23, has not been identified as a genetic locus on linkage analyses for CD or UC, it is a strong candidate gene for susceptibility to IBD, given the important role of MMP-3 in pathogenesis of chronic inflammation in the gut. This study has provided the first link between the MMP-3 5A/6A gene promoter polymorphism and susceptibility to CD (Pender et al.).

There are several observations from this study. An association was only found between susceptibility to CD (not UC) in German sporadic trios, but not in German and British cohorts of familial disease, and over-transmission of the 5A allele of the MMP-3

polymorphism was only associated with susceptibility to CD in carriers of the CARD15 mutation, a well established gene for CD (Hugot et al.;Ogura et al.). This would suggest that CARD15 mutation interacts with the MMP-3 polymorphism, with the effect of unmasking the relatively modest effects of the latter, in predisposing an individual to CD. This observation supports the concept that multiple genetic variants act to influence complex diseases such as CD and UC. Other studies have investigated the interaction of other polymorphisms with the CARD15 variants. For example, there are conflicting reports in the literature as to whether or not there is interaction of the IBD5 locus at chromosome 5q31 with CARD15 to increase susceptibility to CD (Armuzzi et al.;Negoro et al.;Giallourakis et al.;McGovern et al.;Mirza et al.). Another study has described an association between IBD and a TNF promoter polymorphism in a British Caucasian population(Van Heel et al.).

We are now beginning to learn of the complexity of interactions of independent genetic factors in determining susceptibility to IBD. There are a number of ways in which these interactions may occur, not just in an additive manner (genetic heterogeneity), exemplified by the interaction of the CARD15 gene mutations with the MMP-3 polymorphism, but also in a non-additive manner, referred to as epistasis. In humans, epistatic interactions between genetic variants has been identified in Alzheimer's disease (Zubenko, Hughes, III, and Stiffler), breast cancer (Ritchie et al.), Hirshsprung's disease (Carrasquillo et al.), and sickle cell anaemia (Nelson et al.). In IBD, a genome-wide search for colitis susceptibility loci in the interleukin-10 deficient mouse demonstrated complex epistatic interactions between loci (Farmer et al.). Furthermore, preliminary evidence for epistasis has already been reported between 1p36 and IBD1 loci (Cho et al.). Different interactions may be present in other loci, and it is likely that both epistasis and genetic heterogeneity coexist in human IBD.

The other important observation in our study was that over-transmission of the 5A allele was associated with phenotypic subgroups, but only among carriers of one of the CARD15 mutations. Phenotypic subgroups including ileal disease(Cuthbert et al.), surgical resection(Kugathasan et al.), and stenosis(Cuthbert et al.)are also independently associated with the CARD15 mutations. However, NOD2 mutations are not very specific for ileal disease. In previous studies, NOD2 mutations have been observed in unaffected control patients. Likewise, some CD patients with NOD2 mutations have isolated colonic disease.

This would suggest that, although NOD2 mutations seem to predispose to ileal CD, other factors contribute to disease phenotype, including among others, the MMP-3 promoter polymorphism.

When we investigated affects of the MMP-3 polymorphism on MMP-3 production in gut myofibroblasts, we could not detect any differences in MMP-3 production between myofibroblasts of different 5A/6A genotypes. This does not necessarily mean that the MMP-3 polymorphism does not have a functional affect on MMP-3 expression by gut myofibroblasts. The negative results could have been either because the study was too small to discriminate any modest effect of the MMP-3 polymorphism on MMP-3 production, or these results were not reflective of its effects *in-vivo*. Previous studies have demonstrated that the 5A allele has higher transcriptional activities than the 6A allele, with preferential binding of a transcriptional repressor to the latter in atherosclerosis (Ye et al.;Ye et al.;Ye et al.). In addition, the functional affect of the MMP-3 5A/6A polymorphism has been shown in vascular smooth muscle cells and skin cells (Medley et al.;Ye et al.). It is therefore likely, given that our work implicates the 5A allele with susceptibility to CD that the MMP-3 polymorphism does have a functional affect on MMP-3 expression in the gut that has not been elucidated in this study.

7.3 Functional polymorphisms: clinical implications for IBD subjects

7.3.1 Functional polymorphisms as potential biomarkers in IBD

Biomarkers are indices that can be used for interpretation of disease activity or severity, and can be used to predict relapse in those patients in remission. Genetic variants have been used successfully as biomarkers in other diseases, such as lymphomas and leukaemias (Haferlach et al.;Stilgenbauer and Dohner). In these conditions, molecular genetic methods and cytogenetics are used alongside a comprehensive panel including cytomorphology, cytochemistry, and mulitparameter flow cytometry (MFC) to make an accurate diagnosis in acute leukaemias (Haferlach et al.). The results of these parameters serve as a mandatory prerequisite for individual treatment strategies and for evaluation of response to treatment.

The inflammatory bowel diseases lend themselves to a similar approach, given their genetic heterogeneity and divergent number of phenotypic variations. The use of biomarkers has already been explored in the clinical setting of management of IBD. For example, C-reactive protein (CRP) is a serological marker that when raised, is a reliable indicator of active disease. A raised CRP in patients with IBD predicts with a high degree of certainty the likelihood of surgical intervention(Rutgeerts, Van Assche, and Vermeire). In addition, trials involving treatment of IBD patients with anti-TNF and anti-adhesion molecules have shown that a high CRP predicts better response to these drugs (Rutgeerts, Van Assche, and Vermeire). Another biomarker that has recently received much attention, is faecal calprotectin(Pardi and Sandborn). Calprotectin represents 50-60% of neutrophilic cytosolic protein, is stable in faeces for several days after excretion, and correlates well with leukocyte excretion (Roseth, Schmidt, and Fagerhol). It appears to be a relatively sensitive marker for relapse in UC but not in CD (Pardi and Sandborn). Tissue inhibitor of Metalloproteinases –1 (TIMP-1), has also been suggested as a predictor of disease activity in IBD, since it has recently been demonstrated that TIMP-1 levels reveal significant positive correlations with scored endoscopic degree of mucosal injury, disease activity index and clinical activity index values, as well as CRP concentrations in UC patients (Wiercinska-Drapalo et al.). However, both CRP and TIMP-1 are non-specific, since both indices are raised in conditions of chronic inflammation, such as chronic hepatitis (Flisiak et al.). The serological markers, anti-neutrophil cytoplasmic antibody (ANCA) and anti-Saccharomyces cytoplasmic antibodies (ASCA), are well-established markers in IBD, the former having high specificity for diagnosis of UC and the latter for diagnosis of CD (Gupta, Derbes, and Sellin). However, the clinical impact of ANCA/ASCA testing in clinical practice has not been fully established. Putting these observations together, there is a potential for multiple parameters, of which MMP-3 polymorphism and CARD15 mutations are two examples, to be used in diagnostic, predictive and therapeutic management of the Inflammatory Bowel Diseases.

Much attention has focused recently on the potential use of genetic polymorphisms as predictors of drug response in IBD. Knowledge about the influence of allelic variants on drug response can be used to identify, through pre-treatment genetic screening, the patients with the best chance of responding to a specific medication and those at greater risk of experiencing an adverse event, thereby enabling treatment protocols to be individually tailored to the patient's needs. The most extensive pharmacogenetic research has been

aimed at identifying genetic variants accountable for the haematopoietic toxicity of azathioprine/6-mercaptopurine (AZA/6-MP) seen in certain individuals (Hanauer and Present). This has been shown to be due to single nucleotide polymorphisms in the thiopurine S-methyltransferase enzyme (Mascheretti, Croucher, and Schreiber). Preliminary genetic screening allows identification of individuals homozygous for the causative TPMT allele and who are therefore at risk of toxicity.

Studies have also investigated likely gene targets to predict the response to glucocorticoids and infliximab in IBD, but without any positive results in either case (Mascheretti, Croucher, and Schreiber).

7.3.2 MMP gene promoter polymorphisms

Results from this work suggest that MMP-3 polymorphism is a genetic factor in susceptibility to CD. But given the modest effects of the 5A/6A MMP-3 polymorphism on susceptibility to CD and that both 5A and 6A alleles have almost equal frequency in the population, molecular testing for the MMP-3 polymorphism alone would not be of clinical benefit. On the other hand, the CARD15 variants and MMP-3 polymorphism interact to increase risk of developing CD, an observation that suggests that the multifactorial genetic factors may be informative in the prediction of establishing the development and outcome phenotypes of IBD.

7.3.3 NOD2/CARD15 gene mutations

The three main mutations in CARD15 (R702W, G908R, and 1007fs) represent 80% of CARD15 mutations (Lesage et al.), and they are easy to genotype in laboratories involved in molecular diagnosis, properties that make the CARD15 gene favourable to clinical genetic analyses. But would information regarding a patient's CARD15 genotype alone be relevant and useful to clinical practise?

The risk of developing CD in individuals carrying one of the CARD15 mutations is estimated to be approximately 2-3 fold that of the general population, and 20-40 fold in people carrying two mutations (Hugot et al.;Ogura et al.;Hampe et al.). Using these statistics, and that the prevalence of CD is 1/1000 inhabitants in Western countries, Hugot et al (Hugot, Zouali, and Lesage) calculated that the probability of developing CD is no higher than 0.04 for the group at highest risk of carrying two mutations. In addition,

Hampe et al. found that only 6.5% of CD patients were homozygous for the CARD15 mutations, and they estimated that the CARD15 mutations only accounted for 18% of genetic risk for CD in the population (Hampe et al.). Given this statistical data, and that no prophylactic therapy or effective screening strategy in non-symptomatic individuals is available, would make genetic testing in at risk individuals unfeasible.

If genetic screening for the CARD15 mutations is not a worthwhile strategy, then could molecular testing in patients with a clinical diagnosis of CD be useful in terms of defining their phenotypic subgroup and predicting disease outcome? A number of studies have looked into the relationship between CARD15 genotype and phenotype in order to determine whether or not the CARD15 mutations could predict phenotypic subgroups of CD. These groups have shown that CARD15 mutations are not limited to sporadic or familial disease. The c-insertion mutation in particular is associated with stenosis (Abreu et al.;Radlmayr et al.;Abreu et al.). But yet there is lack of specificity in this subgroup, in other words, CD patients with fibrostenosis are more likely not to be carriers of a CARD15 mutation. Moreover, most clinical presentations of CD are represented in the CARD15 negative, CARD15 heterozygote, and CARD15 homozygote groups, as genetic heterogeneity does not account for variability in clinical presentation (Linde et al.). That said, homozygosity for the CARD15 mutations appears to be much more predictive of phenotype. For example, it has been noted that homozygotes for the CARD15 mutations have younger age of onset (Lesage et al.). In fact, homozygote CARD15 mutation carriers account for a third of patients who have been diagnosed before the age of 10 years (as opposed to 3% of patients with an age onset of greater than 40 years)(Hugot, Zouali, and Lesage). In addition, homozygotes are much more likely to have ileal disease, much less likely to have colonic disease, and are 3 times' more likely to have strictures(Lesage et al.). Also a UC-like phenotype is very uncommon among those carrying two CARD15 mutations(Lesage et al.).

Few studies have explored the possible relationship between CARD15 genotype and response to treatment or likelihood of side effects. Two studies have concluded that there is no association between CARD15 genotype and response to infliximab, an anti-TNF agent (Vermeire et al.;Mascheretti et al.). Further larger studies are required in order to gather more data and to determine the significance of different genotypes with respect to

phenotype, screening, prophylactic measures and intervention measures for established disease.

Genetic screening for CARD15 mutations would also not be practicable in trying to confirm or exclude the diagnosis of CD in a patient with established symptomatology, given that a negative test would not rule out CD, and a positive test would not confirm it's presence.This information, however, is unlikely to have clinical impact on disease management, as for example, even with the knowledge of a person's increased risk of stricture-formation, there are at present no prophylactic measures that could be offered to these individuals.

Putting this information together, it is not impossible to imagine that the future may herald the use of combined measurements of such genetic markers as the CARD15 variants, the MMP-3 5A/6A polymorphism, and other functional polymorphisms together with other biological markers, for the purpose of mapping of an IBD patient's prognosis and individually tailored treatment regimens.

7.4 Gene association studies

Gene association studies are studies that investigate specific alleles at a marker locus that are more frequent in affected individuals than in the unaffected population (Clayton and Jones). Genome wide scans have identified a large number of candidate genes in IBD, on a number of susceptibility loci (Satsangi et al.). There has been a deluge of association studies in recent years, investigating the association of genetic variants with susceptibility to disease, disease phenotype, prognosis and treatment response in a variety of heterogeneous conditions, including in addition to those already mentioned: periodontal disease (Shapira, Wilensky, and Kinane), rheumatoid arthritis (Yamamoto and Yamada), obesity (Rosmond), and mood disorders (Serretti and Artioli). The advantages of the association method include its relative robustness to genetic heterogeneity and the ability to detect much smaller effect sizes than would be detectable using feasible sample sizes in linkage studies. However, the literature investigating genetic variants of the same condition is well stocked in reports in associations that cannot be replicated in other studies. The

study of genetic determinants, including gene promoter polymorphisms of MMPs, is a prime example of this (Ye).

Transmission Disequiliubrium Testing (TDT) is as an alternative to population-based association studies. This method is based on cases and family-based controls. An attractive feature of the use of TDT is that it permits study of transmission of haplotypes that extend over adjacent markers. Descriptive analyses have shown that such data may produce more convincing evidence of association, by identifying ancestral haplotypes. A disadvantage of this methodology is that one of the parental samples may not be available. In this situation, genotype of the parent unavailable to genotype may be inferred from offspring genotypes, however, this has been shown to lead to bias, and therefore the number of family trios in which one of the parental samples is not available should be kept to a minimum.

TDT also has similar limitations to population-based association studies. Confounding factors which may account for inconsistencies in results between studies include: selection bias favouring positive association studies (or negative association studies); a heterogeneous background of the populations studied; different enrolment criteria (for example, differing diagnostic criteria, or comparing prospective versus retrospective studies); variable study population (such as differing proportions of gender, different ages); differences in baseline characteristics and study design; sample-size bias; differences in drug therapy; selection bias; and differences in methodology (Samani et al.;Petrovic and Peterlin). For example, in the part of this work investigating the association between the 5A/6A MMP-3 polymorphism and susceptibility to IBD by TDT analysis, there was observed to be an over-transmission of the 5A allele in a cohort of German sporadic trios, but not in German multiplex families or British multiplex families. The differing results may have been due to differences in phenotypes between these groups: the different cohorts were selected from different geographical populations; and the muliplex families represented familial disease, whereas the cohort of German trios represented sporadic disease. In addition, sample numbers in the German and British multiplex families were substantially smaller than those in the trio collection thereby reducing statistical power in the former groups.

Case-control association studies are also prone to type II error, that is, failing to reject the null hypothesis that there is no difference in allelic distributions between the two groups.

When it is false, one way to tackle this problem is to increase the study sample size. Longitudinal studies are likely to prove essential in improving our understanding of genetic aetiologies of complex polygenic conditions such as the inflammatory bowel diseases, in which environmental factors are likely to interact with genetic determinants in disease susceptibility.

7.5 Suggestions for Future Research

In this study, TDT was used to investigate the association of the MMP-3 polymorphism with susceptibility to CD and UC. A replication of this study would be required, and with larger sample numbers to improve the validity of the significance of these findings. Further TDT studies in IBD patients could include larger scale investigation of the MMP-1, -9 and –12 polymorphisms. Given the relatively low frequency of the MMP-9 and MMP-12 polymorphisms in particular, it may only be possible to achieve this through collection of samples in collaborative studies.

Results from this work and other studies (Mendoza et al.)suggest that susceptibility to CD and UC is determined at least in part by the presence of more than one genetic variant. Different genetic variants may also be risk factors for certain subsets of IBD but not others (for example those with fistula disease, stricture-formation, etc), and may be useful in terms of predicting disease outcome and treatment response. More than 20 susceptibility loci have been identified from individual studies of genome-wide scans(Van Heel et al.), and candidate genes investigated with patchy success. Investigation into the interaction of these candidate genes is warranted. However, this would require large multi-centre studies. A recent genome-scan meta-analysis, using genome-scan data from 10 separate studies identified loci for IBD at 2q, 3q, 5q, 7q and 16 (CARD15 region); loci for CD at 2q, 3q, 6p 16 (CARD15 region), 17q and 19p; and a locus at 2q for UC (Van Heel et al.). Such large scale meta-analyses will enable researchers to prioritise these genomic regions for further evaluation of potential candidate genes.

7.6 Concluding Comments

The results from this work suggest that the MMP-3 5A/6A polymorphism represents a genetic factor in Crohn's Disease. The association of this MMP-3 polymorphism with CD further implicates the role of MMP-3 in the pathogenesis of chronic inflammation in the gut, and contributes to our further understanding of the underlying genetic aetiologies of the IBD. Results from this work also support the idea that more than one genetic variant contribute to the development of IBD, and that these genetics factors may vary between individuals and different populations and hence results in different phenotype of the disease under different environment. Sample numbers may limit studies investigating the association of genetic variants with susceptibility to polygenic conditions such as the IBD, and larger longitudinal studies in this field are required. Recent studies of genetic variants in relation to outcome of treatment in a number of conditions have indicated the possibility of devising testing including the combination of genetic variants and other markers to predict diagnosis, outcome of treatment response and prognosis. MMP polymorphisms in conjunction with other biomarkers such as CARD15 could therefore be used to aid the clinician in management of IBD patients. Further studies in this important field are necessary.

Summary

Cell lines were grown from colonoscopic biopsies of CD, UC and control subjects. The cell lines that grew over 4-6 weeks were myofibroblast-enriched cells. There was no significant difference in cell markers we tested (vimentin, α-SMA, vimentin, PR2D3, desmin, CY90, CD3, CD68) . Cell lines from control subjects became more myofibroblast-enriched after maintaining over 6 passages.

MMP-3 was constitutively expressed in the culture supernatants of cell lines isolated from CD, UC and control individuals. Cell lines in patients with active CD produced higher amounts of MMP-3 than those isolated from CD patients with disease in remission; and isolated myofibroblasts from active UC patients produced higher amounts of MMP-3 than those from UC patients in remission. But these differences were not to significant levels. Cell lines isolated from diseased or control subjects responded to stimulation with pro-inflammatory cytokines TNF-α and IL-1β with a dose-dependent rise in MMP-3 protein.

When investigating whether the production of MMP-3 is governed by the predisposition of MMP-3 polymorphism 5A/6A genotype, there was no significant difference in MMP-3 production between MMP-3 5A/6A SNP in cell lines isolated from control.

Using TDT analysis, over-transmission of the 5A allele was observed to affected offspring in individuals with sporadic CD in a German cohort (p=0.0012), though there was no association with susceptibility to CD in German multiplex families (p=0.995) and British multiplex families (p=0.4331).

Over-transmission of the 5A allele in the German trios was significant in CARD15 carriers (p=0.0054) but not in non-carriers. In the CARD15 carriers, over-transmission of the 5A allele was associated with stenosis (p=0.0027), fistulising disease (p=0.0007), disease of the ileum (p=0.0001), surgical intervention (p=0.0023) and disease of the right colon (p=0.0115). These results suggest an association of the MMP-3 5A/6A polymorphism with susceptibility to CD and phenotypic variants of this condition.

Appendix A: population-based study to investigate the prevalence of 5A/6A MMP-3 promoter polymorphism in children and adults.

Introduction

A naturally occurring and common polymorphism has been shown to affect the MMP-3 promoter, where either 5 or 6 consecutive adenines (5A/6A) alter transcription binding and affect MMP-3 promoter activity (Ye et al.). The 5A allele has been shown to have greater promoter activity in cell culture experiments and in human skin (Ye et al.;Medley et al.). The 5A allele has been linked with susceptibility to diseases such as myocardial infarction (Terashima et al.) and increased severity of rheumatoid arthritis (Constantin et al.), conditions where there is increased extracellular matrix degradation, while the 6A allele has been associated with susceptibility towards conditions of increased extracellular matrix deposition such as atherosclerosis. MMP-3 has been shown to be elevated in diseased tissue of IBD patients, and is an important end-effector molecule in mucosal damage and matrix degradation. In a collaborative study, the MMP-3 5A/6A polymorphism was investigated in relation to susceptibility to CD and UC in both German and British families, and in sporadic disease in German subjects, using Transmission disequilibrium testing (Pender et al.). There was no association with onset of CD or UC and the 5A allele in the German or British families, but there was over-transmission of the 5A allele in German sporadic subjects, when at least one of the CARD15 mutations was also present. In the CARD15 carriers, over-transmission of the 5A allele was associated with stenosis, fistulising disease, previous surgical resection, and disease of the right colon(Pender et al.). Individuals with early-onset disease (disease onset in childhood) represent another phenotypic subgroup of CD and UC. Prevalence of the 5A allele among individuals with early-onset IBD has not previously been investigated. In this study, the hypothesis was that the 5A allele is more prevalent among those diagnosed with CD and UC in childhood. The aim of the study was therefore to investigate the prevalence of the 5A allele among adults and children with CD and UC.

Materials and Methods

The materials and methods used in this part of the study were outlined in chapter 5. In brief, 5ml blood samples were taken from 273 subjects in total. These included 73 subjects with CD (48 children and 25 adults), 50 subjects with UC (19 children and 31 adults), and 150 subjects in the control group (59 children and 91 adults). All adults with CD or UC were diagnosed with the condition after 16 years of age. All participating subjects were white British ethnicity. DNA was extracted using the 'salting out' method, and standard PCR was carried out. A restriction enzyme was mixed with the PCR products, so that if the 5A allele was present the PCR products were cut. The products were then run on a horizontal polyacrylamide gel under reducing conditions. The gels were then stained with vistragreen and then scanned using a fluoroimager.

Prevalence of the 5A allele in CD and UC subjects with early-onset disease and adults

The digest products of the cut (5A) allele were 90 and 28 bp length, whereas the uncut (6A) allele was measured at 118 bp. These results are consistent with previous studies which have investigated the MMP-3 5A/6A polymorphism by this method (see Figure 5.1) (Dunleavey, Beyzade, and Ye). The genotype frequencies of the 5A allele in the control, CD and UC groups are shown in Table 5.1. They correlate with the genotype frequencies previously published for the MMP-3 5A/6A polymorphism (Ghilardi et al.;Satsangi et al.;Wiencke et al.).

When both children and adult groups were combined, there was no overall significant difference between prevalence of the 5A allele in the CD and UC groups, and the control group, using chi squared test.

The results of the prevalence of the 5A allele in children with CD and UC compared to the control group, are shown in Table A.1. Using chi-square test, there was no significant difference in prevalence of the 5A allele of the MMP-3 promoter polymorphism between the CD or UC groups and control group in children. The results of the prevalence of the 5A

allele in adults, are shown in Table A.2. There was no association of the 5A allele with susceptibility to CD or UC, compared with the control group (see Table A.3).

Discussion

The expression of MMPs is regulated at a number of levels, including transcription, activation of the pro-enzyme, and inhibition of MMP activity by tissue inhibitors of metalloproteinases (TIMPs). It would seem however, that most MMPs are largely regulated at the transcriptional level, since most MMP genes are expressed only when tissue remodelling is taking place (Matrisian;Fini et al.).

The promoters of a number of MMP genes (including MMP-1,-3,-9,-10,-12, and –13) encompass an AP-1 consensus element at position approximately –70 and one or two copies of the PEA-3 element at position –140 and –200; the former interacts with the Fos and Jun families and the latter interacts with the Ets family of transcription factors. A large number of studies have demonstrated that these two cis-elements play an important role in the regulation of MMP gene expression both at the basal level and in response to various stimuli including phorbol ester, cyokines and growth factors (Angel et al.;Gaire et al.). There is growing evidence that naturally occurring sequence variation in the promoters of MMP genes may result in differential binding of transcription factors to the MMP promoters, and thus differential expression of MMPs in different individuals.

This study found no association of the 5A allele of the MMP-3 promoter polymorphism with susceptibility to early-onset disease in CD or UC patients, or in susceptibility overall to CD or UC. The sample size in this study was too small to discriminate any modest effects that the 5A allele may have on susceptibility to CD or UC. Results of a study of British subjects with familial CD and UC found no over-transmission of the 5A allele to affected offspring by their parents, using transmission disequilibrium testing (TDT) (Pender et al.).

The association between the MMP-3 5A/6A polymorphism and CD was not detected in this study, but was detected in the TDT study of a German cohort where transmission of the 5A allele was investigated from unaffected parents to offspring in sporadic disease families (see chapter 6). There are several explanations for this observation. Firstly, the

sample numbers in this study were smaller and therefore lacked statistical power compared to the previous study. This was a population based association study, whereas a different statistical method of analysis was used in the previous study, to investigate the association of the 5A allele with susceptibility to CD in the German cohort. Also, the two groups of subjects were of European Caucasian ancestry, but from different geographical areas and therefore represented distinct populations. It is possible that the phenotypes associated particularly with over-transmission of the 5A allele (for example, carriers of the CARD15 mutations, subjects with ileal disease, stenosis and fistulising disease), were relatively under-represented in this study, but were better represented in the German cohort group of the previous study. It was not possible to investigate the association of the 5A/6A MMP-3 polymorphism with phenotypic subgroups in this study, owing to small sample numbers. Based on the allele frequencies in the disease and control groups, the sample size in this part of the study would allow detecting a relative risk by allele of 3.0 in the children's group, 2.2 in the adult group, and 1.85 in the combined groups, with a power of 70% at the 0.05 significance level.

Summary

This study did not support the hypothesis that the 5A allele is associated with early-onset of CD or CD, or increased susceptibility to CD or UC overall in both children and adult groups. However the sample size in this study was too small to discriminate any modest effects that the 5A allele may have on susceptibility to CD or UC.

Table A.1: Distribution of genotypes and alleles of MMP-3 5A/6A polymorphism in CD, UC and control groups, **in children**.

		CD		UC		Normal		p value (x^2 test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	5A/5A	16	0.333	7	0.368	13	0.220	0.261
	5A/6A	17	0.354	8	0.421	33	0.559	
	6A/6A	15	0.312	4	0.210	13	0.220	
allele	Total	48		19		59		0.692
	5A	49	0.510	22	0.578	59	0.5	
	6A	47	0.489	16	0.421	59	0.5	
	Total	96		38		118		

Table A.2: Distribution of genotypes and alleles of MMP-3 5A/6A polymorphism in CD, UC and control groups, **in adults**

		CD		UC		Normal		p value (x^2 test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	5A/5A	5	0.2	4	0.129	19	0.208	0.284
	5A/6A	16	0.64	16	0.516	43	0.472	
	6A/6A	4	0.16	11	0.354	29	0.318	
allele	Total	25		31		91		0.372
	5A	26	0.52	24	0.387	81	0.445	
	6A	24	0.48	38	0.612	101	0.554	
	Total	50		62		182		

Table A.3: Distribution of genotypes and alleles of MMP-3 5A/6A polymorphism in CD, UC and control groups **overall in both children and adults**

		CD		UC		Normal		p value (x^2 test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	5A/5A	21	0.28	11	0.22	32	0.21	0.68
	5A/6A	33	0.45	24	0.48	76	0.50	
	6A/6A	19	0.26	15	0.3	42	0.28	
allele	Total	73		50		150		0.371
	5A	75	0.51	46	0.46	140	0.46	
	6A	71	0.48	54	0.54	160	0.53	
	Total	146		100		300		

Appendix B: Functional polymorphism in MMP-1 gene promoter in relation to predisposition to Crohn's disease and Ulcerative Colitis in children and adults.

Introduction

MMP-1 (interstitial collagenase) degrades fibrillar collagens, the most abundant class of extracellular matrix proteins in interstitial connective tissue. Under normal physiological conditions, MMP-1 is expressed at very low levels in the gut. But under certain conditions such as wound healing and tissue inflammation, expression of MMP-1 can be induced to very high levels by stimuli such as growth factors and cytokines (Aho et al.;Vincenti). In vivo studies have shown that MMP-1 transcripts are increased in mucosal tissue of CD subjects with active disease (Bailey et al.;Stallmach et al.;von Lampe et al.), and there is also increased expression of MMP-1 in gastrointestinal ulcers (Saarialho-Kere et al.). Therefore, MMP-1 gene, located on chromosome 11q22.2-11q22.3, would be a good candidate gene in the study of genetic susceptibility to CD and UC. MMP-1 expression is regulated by a larger extent, at the transcriptional level. A common polymorphism has been identified in which there is either an insertion (2G allele) or deletion (1G allele) of a guanine nucleotide in the promoter region of the MMP-1 gene (Rutter et al.). The presence of the 2G allele creates a core binding site for transcription factor Ets, resulting in higher promoter activity (Rutter et al.). Functional studies have shown that endometrial and ovarian tumour tissues in subjects homozygous for the 2G allele, express higher levels of MMP-1 (Nishioka et al.;Kanamori et al.). Furthermore, the MMP-1 1G/2G polymorphism has been shown to affect susceptibility to lung cancer (Zhu et al.), prognosis in colorectal cancer (Zinzindohoue et al.)and disease severity in rheumatoid arthritis (though not disease susceptibility) (Lee et al.).

MMP-1 is the most abundant interstitial collagenase capable of degrading fibrillar collagens (Zhu et al.). It is highly expressed in the inflamed colon of subjects with CD and UC (von Lampe et al.;Stallmach et al.) and in gastrointestinal ulcers (Saarialho-Kere et al.). Binding sites for members of the Ets family of transcription factors are present within MMP promoters and are potent positive regulators (Buttice et al.).

A common polymorphism has been identified at –1607bp within the MMP-1 promoter that can then modify the level of MMP-1 expression (Rutter et al.). The 2G polymorphism displays significantly higher transcriptional activity than the 1G allele (Rutter et al.). In addition, ovarian and endometrial tumour tissues in individuals homozygous for the 2G genotype express increased levels of MMP-1 (Kanamori et al.;Nishioka et al.). Therefore it was proposed that the 2G allele is associated with increased susceptibility to IBD. The aim of this part of the study was therefore to investigate the relationship of the 2G allele with susceptibility to CD and UC in children and adult subjects.

MMP-1 promoter polymorphism was analysed in a sample of 73 subjects with CD (48 children and 25 adults), 50 subjects with UC (19 children and 31 adults), and 150 subjects in the control group (59 children and 91 adults). All adults with CD or UC were diagnosed with the condition after 16 years of age. All participating subjects were white British ethnicity.

Materials and Methods

DNA extraction and measurement of DNA concentration was carried out as described in Section 2.3.1. MMP-1 was genotyped as previously described (Dunleavey, Beyzade, and Ye). The sequences of primer used were 5'-TCGTGAGAATGTCTTCCCATT-3' (forward primer) and 5'- TCTTGGATTGATTTGAGATAAGTGAAATC-3' (reverse primer). PCR was carried out in a total volume of 25 µl, containing 50 ng DNA, 10 pmol each primer (see table 2.7), 200mM each dATP, dCTP, dGTP, and dTTP, 20 mM Tris-HCl (pH 8.4), 50 Mm kcL, 0.05%(v/.v) W1 (Gibco BRL), 1.5 mM $MgCl_2$, and 1 unit Taq polymerase (Promega). The solution was overlaid with 25 µl of liquid paraffin and incubated for 1 minute at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C.

The amplicon was digested by a restriction enzyme (*Xmn1*) that cleaves only the 1G allele. A 10 µl aliquot of PCR products of each sample was mixed with a 5µl solution containing *Xmn* I: 2µl 10x NEBuffer 2 (50mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.2 µl bovine serum albumin (1 µg/µl), 0.3µl *Xmn*I (20 U/µl), and 2.5 µl sterile deionised H_2O. The solution was overlaid with 15µl of liquid paraffin and

incubated at 37°C for 16 hours overnight. The gels were then run on a polyacrylamide gel under reducing conditions and visualised by vistra green staining.

Frequency of the 2G allele in children and adults with CD and UC

The digest products of the cut (1G) allele were 90 and 28 bp length, whereas the uncut (2G) allele was measured at 118 bp. These results are consistent with those previously documented using this method of genotyping with endonuclease digestion (see Figure B.1) (Dunleavey, Beyzade, and Ye).

The MMP-1 genotype distribution for children and adults with CD and UC, and the control group, are shown in Tables B.1 and B.2. There was no significant difference in frequency of the 2G allele between the CD, UC and control groups, using chi-squared test.

Children and adult groups were combined to increase sample numbers and to investigate the overall association of the MMP-1 1G/2G polymorphism with susceptibility to CD and UC. There was no overall significant difference in frequency of the 2G allele between the combined CD and UC groups, and the control group (see Table B.3).

The frequency of the 1G/2G polymorphism shown in CD, UC and control groups, is similar to the reported frequency of this polymorphism in a normal European Caucasian population (Zhu et al.;Zhang et al.).

Discussion

This study sought to investigate a relationship with the MMP-1 1G/2G polymorphism, and susceptibility to CD and UC, both in children (ie early-onset disease), and in adults, and in combination. This study did not support the hypothesis that there is an association with the MMP-1 promoter polymorphism and susceptibility to CD or UC. The statistical power of the study is too low to be able to demonstrate modest affects that this polymorphism may have on disease susceptibility, or possible affects on phenotypic subgroups. Larger numbers of participating subjects would be needed to identify smaller risk in these populations. For example, based on the allele frequencies in the disease and control groups, the sample size in this part of the study would allow detecting a relative risk by allele of

2.8 in the children's group, 2.2 in the adult group, and 1.85 in the combined groups, with a power of 70% at the 0.05 significance level.

Summary

This study did not support the hypothesis that MMP-1 1G/2G polymorphism influences susceptibility to CD or UC, in children or adults.
The sample number of this study would need to be increased to identify possible modest affects of this polymorphism on disease susceptibility, or susceptibility of certain phenotypic subgroups of CD and UC.

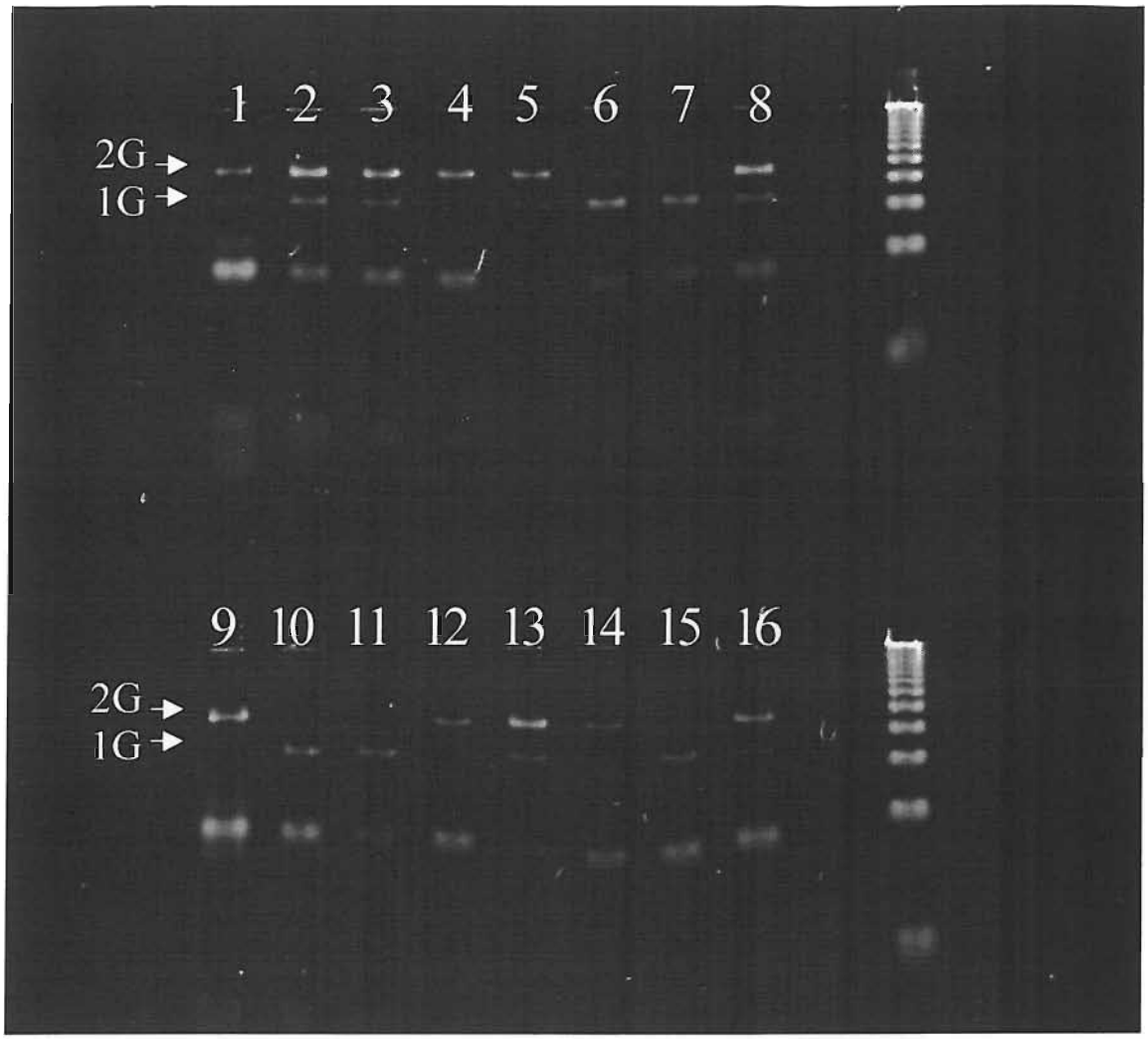

Figure B1: *gel electrophoresis run for a group of samples (labelled 1-16), for MMP-1 1G/2 polymorphism following restriction enzyme digest. The markers in the far right lanes are 25 bp ladders. The upper bands in the samples represent the 2G allele at 118bp, and the lower band represents the 1G allele, at 90 bp. Inferred genotypes: samples 6,7,10,11,15 = 1G/1G; samples 2,3,8,13,and 16 = 1G/2G; samples 1,4,5,9,12,14 = 2G/2G.*

Table B.1: Distribution of genotypes and alleles of MMP-1 1G/2G polymorphism in CD, UC and control groups, **in children**.

		CD		UC		Normal		p value (x^2 test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	1G/1G	13	0.27	5	0.26	10	0.16	0.682
	1G/2G	22	0.45	8	0.42	27	0.45	
	2G/2G	13	0.27	6	0.31	22	0.37	
allele	Total	48		19		59		0.312
	1G	48	0.5	18	0.47	47	0.39	
	2G	48	0.5	20	0.52	71	0.60	
	Total	96		38		118		

Table B.2: Distribution of genotypes and alleles of MMP-1 1G/2G polymorphism in CD, UC and control groups, **in adults**

		CD		UC		Normal		p value (x^2 test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	1G/1G	5	0.20	4	0.129	19	0.208	0.602
	1G/2G	12	0.48	13	0.419	45	0.494	
	2G/2G	8	0.32	14	0.451	27	0.296	
allele	Total	25		31		91		0.268
	1G	22	0.44	21	0.338	83	0.456	
	2G	28	0.56	41	0.661	99	0.543	
	Total	50		62		182		

Table B.3: Distribution of genotypes and alleles of MMP-1 1G/2G polymorphism in CD, UC and control groups overall **in both children and adults**

		CD		UC		Normal		p value (x^2 test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	1G/1G	18	0.246	9	0.180	29	0.193	0.68
	1G/2G	34	0.465	21	0.420	72	0.480	
	2G/2G	21	0.287	20	0.400	49	0.326	
allele	Total	73		50		150		0.598
	1G	70	0.479	39	0.390	130	0.433	
	2G	76	0.520	61	0.610	170	0.566	
	Total	146		100		300		

Appendix C: Functional polymorphism in the MMP-9 promoter in relation to predisposition to Crohn's Disease and Ulcerative Colitis in children and adults

Introduction

MMP-9, or gelatinase-B, is particularly active against denatured collagens (gelatins) and type IV collagen (Opdenakker, Van den Steen, and Van Damme). It therefore plays an important role in extracellular matrix turnover. Both MMP-9 transcripts and protein are highly expressed in granulocytes and fistulae of patients with CD (Kirkegaard et al.). MMP-9 is produced by neutrophils, and antigen-presenting cells, such as monocytes, fibroblasts, dendritic cells and lymphocytes (Opdenakker, Van den Steen, and Van Damme). It is unregulated in a fetal gut explant model following T-cell activation (Salmela et al.).

An SNP has been identified in the MMP-9 gene at position -1562 (Peters et al.;Shimajiri et al.;Zhang et al.). The SNP is due to a single C to T substitution. In-vitro studies have shown that the C to T substitution results in increased transcriptional activity in macrophages, due to loss of binding of a transcriptional repressor in this region (Zhang et al.). Furthermore, individuals carrying the T allele have increased risk from coronary artery disease, and restenosis (Zhang et al.;Cho et al.). Given the modulating effect of this polymorphism on MMP-9 expression, and the importance of MMP-9 as an end-effector molecule in mucosal inflammation in the gut, this polymorphism would be a good candidate as a genetic determinant in IBD. Therefore the hypothesis was that this C-1562T polymorphism modulates susceptibility to IBD. An allele-specific effect of this polymorphism on childhood-onset disease was also investigated.

Materials and Methods

Five ml blood samples were taken from 273 subjects in total. These included 73 subjects with CD (48 children and 25 adults), 50 subjects with UC (19 children and 31 adults), and 150 subjects in the control group (59 children and 91 adults). All adults with CD or UC were diagnosed with the condition after 16 years of age. All participating subjects were white British ethnicity.

DNA was extracted using the 'salting out' method, and standard PCR was carried out as described in Section 2.3.1. MMP-9 was genotyped as previously described (Zhang et al.). The sequences of primer used were 5'- GCCTGGCACATAGTAGGCCC -3' (forward primer) and 5'- CTTCCTAGCCAGCCGGCATC -3' (reverse primer). PCR was carried out in a total volume of 25 µl, containing 50 ng DNA, 10 pmol each primer (see table 2.7), 200mM each dATP, dCTP, dGTP, and dTTP, 20 mM Tris-HCl (pH 8.4), 50 Mm kcL, 0.05% (v/.v) W1 (Gibco BRL), 1.5 mM $MgCl_2$, and 1 unit Taq polymerase (Promega). The solution was overlaid with 25 µl of liquid paraffin and incubated for 1 minute at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C.

The amplicon was digested by a restriction enzyme (*SpH* I) that cleaves only the 1G allele. A 10 µl aliquot of PCR products of each sample was mixed with a 5µl solution containing *SpH* I: 1.5µl 10x NEBuffer 2 (50mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.4µl *SpH*I (20 U/µl), and 8.1 µl sterile deionised H_2O. The solution was overlaid with 15µl of liquid paraffin and incubated at 37°C for 16 hours overnight.

Frequency of the T allele among children and adults with CD and UC

The digest products of the cut T allele were 240 and 192 bp length, whereas the uncut C allele was measured at 432 bp (see Figure C.1). These results are consistent with previous studies that have investigated the MMP-9 C-1562T polymorphism by this method (Zhang et al.;Zhang et al.). The genotype frequencies of the T and C alleles in the control, CD and UC groups in children and adults, are shown in Tables 9.1 and 9.2. The frequency of the less common T allele in the normal adult population of 0.203 is similar to that previously reported (Zhang et al.).

The results of the prevalence of the T allele in children with CD and UC compared to the control group, are shown in Table C.1. Using Fisher's exact test, there was no significant difference in prevalence of the 5A allele of the MMP-3 promoter polymorphism between the CD or UC groups and control group in children.

The results of the prevalence of the 5A allele in adults, are shown in Table C.2. There was no association of the T allele of the MMP-9 C-1562T polymorphism in CD or UC, compared with the control group. When both children and adult groups were combined, there was no overall significant difference between prevalence of the T allele in the CD and UC groups, and the control group (see Table C.3).

Discussion

Results from this study do not support the hypothesis that the MMP-9 C-1652T polymorphism has allelic effects on susceptibility to CD or UC, in children or adults. Given the relatively low frequency of this polymorphism, a larger cohort would be needed to provide increase the statistical power to discriminate effects of this polymorphism on susceptibility to IBD or phenotypic subgroups in CD and UC.

Based on the allele frequencies in the disease and control groups, the sample size in this part of the study would allow detecting a relative risk by allele of 3.4 in the children's group, 2.4 in the adult group, and 2.1 in the combined groups, with a power of 70% at the 0.05 significance level.

Summary

This study did not support the hypothesis that MMP-9 C-1652T polymorphism is associated with risk from CD or UC, either in childhood-onset disease or in adult-onset disease. Sample numbers in this study were small given the relative low frequency of the T allele of this polymorphism, and therefore limit the interpretation of these results.

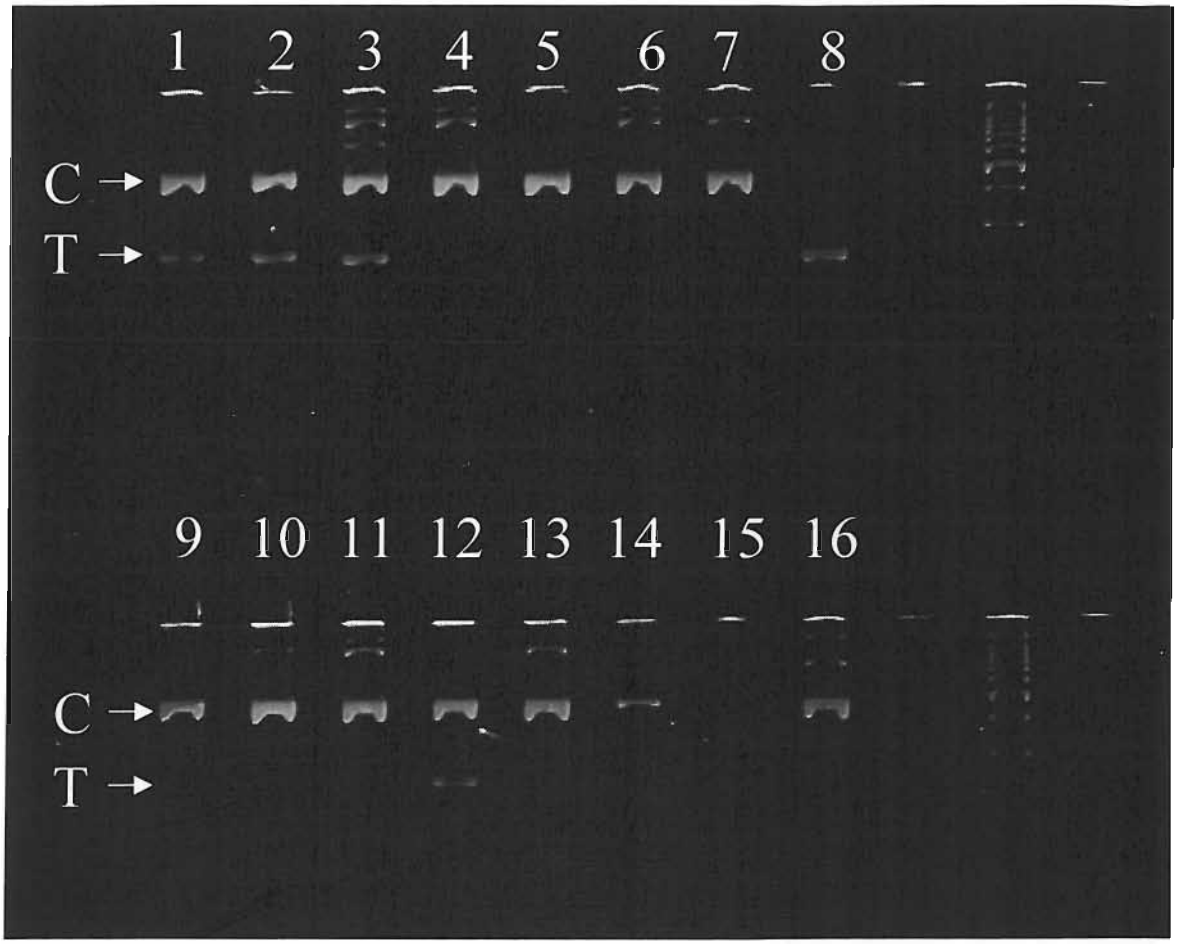

Figure C.1: gel electrophoresis run for a group of samples (labelled 1-16), for MMP-9 C-1652T polymorphism following restriction enzyme digest. The markers in the far right lanes are 100 bp ladders. The upper bands in the samples represent the C allele (432 bp) and the lower bands represent the T allele (240 and 192 bp). Inferred genotypes: samples 4,5,6,7,9,10,11,13,14 and 16= C/C; samples 1,2,3 and 12 = C/T; sample 8 = T/T.

Table C.1: Distribution of genotypes and alleles of MMP-9 C-1652T polymorphism in CD, UC and control groups, **in children**

		CD		UC		Normal		p value (Fisher's exact test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	C/C	33	0.687	15	0.789	43	0.728	0.704
	C/T	15	0.312	4	0.210	14	0.237	
	T/T	0	0	0	0	2	0.033	
allele	Total	48		19		59		0.732
	C	81	0.843	34	0.894	100	0.847	
	T	15	0.156	4	0.105	18	0.152	
	Total	96		38		118		

Table C.2: Distribution of genotypes and alleles of MMP-9 C-1652T polymorphism in CD, UC and control groups, **in adults**

		CD		UC		Normal		p value (Fisher's exact test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	C/C	17	0.68	20	0.645	57	0.626	0.963
	C/T	8	0.32	11	0.354	31	0.340	
	T/T	0	0	0	0	3	0.032	
allele	Total	25		31		91		0.754
	C	42	0.84	51	0.822	145	0.796	
	T	8	0.16	11	0.177	37	0.203	
	Total	50		62		182		

Table C.3: Distribution of genotypes and alleles of MMP-9 C-1652T polymorphism in CD, UC and control groups overall **in both children and adults**

		CD		UC		Normal		p value (Fisher's exact test)
		No.	frequenc y	No.	frequenc y	No.	frequenc y	
genotype	C/C	50	0.684	35	0.7	100	0.666	0.575
	C/T	23	0.315	15	0.3	45	0.3	
	T/T	0	0	0	0	5	0.033	
allele	Total	73		50		150		0.663
	C	123	0.842	85	0.85	245	0.816	
	T	23	0.157	15	0.15	55	0.183	
	Total	146		100		300		

Appendix D: Functional polymorphism in MMP-12 promoter in relation to predisposition to Crohn's Disease and Ulcerative Colitis in children and adults

Introduction

MMP-12 (macrophage metalloelastase) is an elastolytic metalloproteinase secreted and activated by macrophages. Besides elastase activity, it can cleave a number of extracellular matrix enzymes, including fibronectin, laminin, vitronectin and type IV collagen. MMP-12 is abundantly expressed in the vicinity of shedding mucosal epithelium and beneath the necrotic surface of ulcers in subjects with IBD (Vaalamo et al.). A common polymorphism has been identified immediately adjacent to the consensus element of the transcription factor activator protein (AP-1) site in the MMP-12 gene (Jormsjo et al.). This polymorphism is due to an adenosine [A]-to-guanosine [G] substitution at position –82, the A allele being more prevalent. The A allele has been shown to confer increased transcriptional activity of the MMP-12 gene promoter than the G allele (Jormsjo et al.). The aim of this study was therefore to investigate whether or not the MMP-12 gene polymorphism was associated with predisposition to CD and UC in children (ie those diagnosed under the age of 16) and adults.

Materials and Methods

Five ml blood samples were taken from 273 subjects in total. These included 73 subjects with CD (48 children and 25 adults), 50 subjects with UC (19 children and 31 adults), and 150 subjects in the control group (59 children and 91 adults). All adults with CD or UC were diagnosed with the condition after 16 years of age. All participating subjects were white British ethnicity. DNA was extracted using the 'salting out' method.

The method to genotype MMP-12 A-82G polymorphism has been described and used previously (Jormsjo et al.;Jormsjo et al.;Zhang et al.). PCR volume was carried out using 25 μl volume, containing 10 pmol each primer, 200 μM each DATP, dCTP, cGTP and dTTP, 20mM Tris-HCl (pH 8.4), 4mM $MgCl_2$, and 3IU Taq Polymerase (Promega). The sequences of primer used were 5'- GTCAAGGGATGATATCAGCT -3' (forward primer) and 5'- CTTCTAAACGGATCAATTCAG -3' (reverse primer). The solution was overlaid

with paraffin oil and incubated at 95°C for 2 minutes, then 35 cycles of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds, followed by a 2 minute extension at 72°C. The DNA sequence containing the polymorphic site was amplified by PCR, and the amplicon was digested with a restriction enzyme (*PvuII*) that only cleaves the G allele of the MMP-12 polymorphism. A 10 µl aliquot of PCR products of each sample was mixed with a 5µl solution containing *PvuII*: 1µl 10x NEBuffer 2 (50mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9), 0.2µl *PvuII* (20 U/µl), 1µl bovine serum albumin (1 µg/µl), and 2.2 µl sterile deionised H_2O. The solution was overlaid with 15µl of liquid paraffin and incubated at 37°C for 16 hours overnight. The gels were then run on a polyacrylamide gel under reducing conditions and visualised by vistra green staining.

Frequency of the T allele among children and adults with CD and UC

The digest products of the cut G allele were 140 bp in length, whereas the uncut A allele was measured at 127 bp (see Figure 10.1). These results are consistent with previous study that has investigated the MMP-12 A/G polymorphism by this method (Zhang et al.). The frequency of the G allele was 0.15 in the normal adult group similar to those previously published (Jormsjo et al.;Zhang et al.).

The results of the prevalence of the G allele in children with CD and UC compared to the control group, are shown in Table D.1. Using Fisher's exact test, there was no significant difference in prevalence of the 5A allele of the MMP-3 promoter polymorphism between the CD or UC groups and control group in children.

The results of the prevalence of the G allele in adults, are shown in Table D.2. There was no association of the G allele of the MMP-12 polymorphism in CD or UC, compared with the control group. When both children and adult groups were combined, there was no overall significant difference between prevalence of the G allele in the CD and UC groups, and the control group (see Table D.3).

Discussion

The transcription factor activator protein-1 (AP-1) is located at approximately 70 bp upstream of the start of transcription in a number of MMP genes, and plays an important role in the regulation of transcription (Borden and Heller). It has previously been shown that the AP-1 consensus element has a functional effect in the MMP-12 promoter (Monet-Kuntz et al.;Jormsjo et al.). AP-1 has higher binding affinity to the A allele of the MMP-12 polymorphism, resulting in higher MMP-12 promoter activity. Given this observation, and that MMP-12 is highly expressed in ulcerated mucosa of IBD subjects, the MMP-12 polymorphism would be a good candidate gene in susceptibility to CD and UC.

This study did not show an association of the MMP-12 polymorphism with susceptibility to CD or UC, in children or adults, in the population studied. However, particularly given the low frequency of the G allele in the normal population, further studies would need to increase sample numbers in order to achieve power to detect more modest effects of the MMP-12 polymorphism on disease susceptibility or disease phenotype. Based on the allele frequencies in the disease and control groups, the sample size in this part of the study would allow detecting a relative risk by allele of 3.9 in the children's group, 2.6 in the adult group, and 2.2 in the combined groups, with a power of 70% at the 0.05 significance level.

Summary

This study did not support the hypothesis that the MMP-12 A/G promoter polymorphism is associated with susceptibility to CD or UC, in children or adults, in the population studied. Sample numbers in this study were small given the relative low frequency of the T allele of this polymorphism, and therefore limit the interpretation of these results.

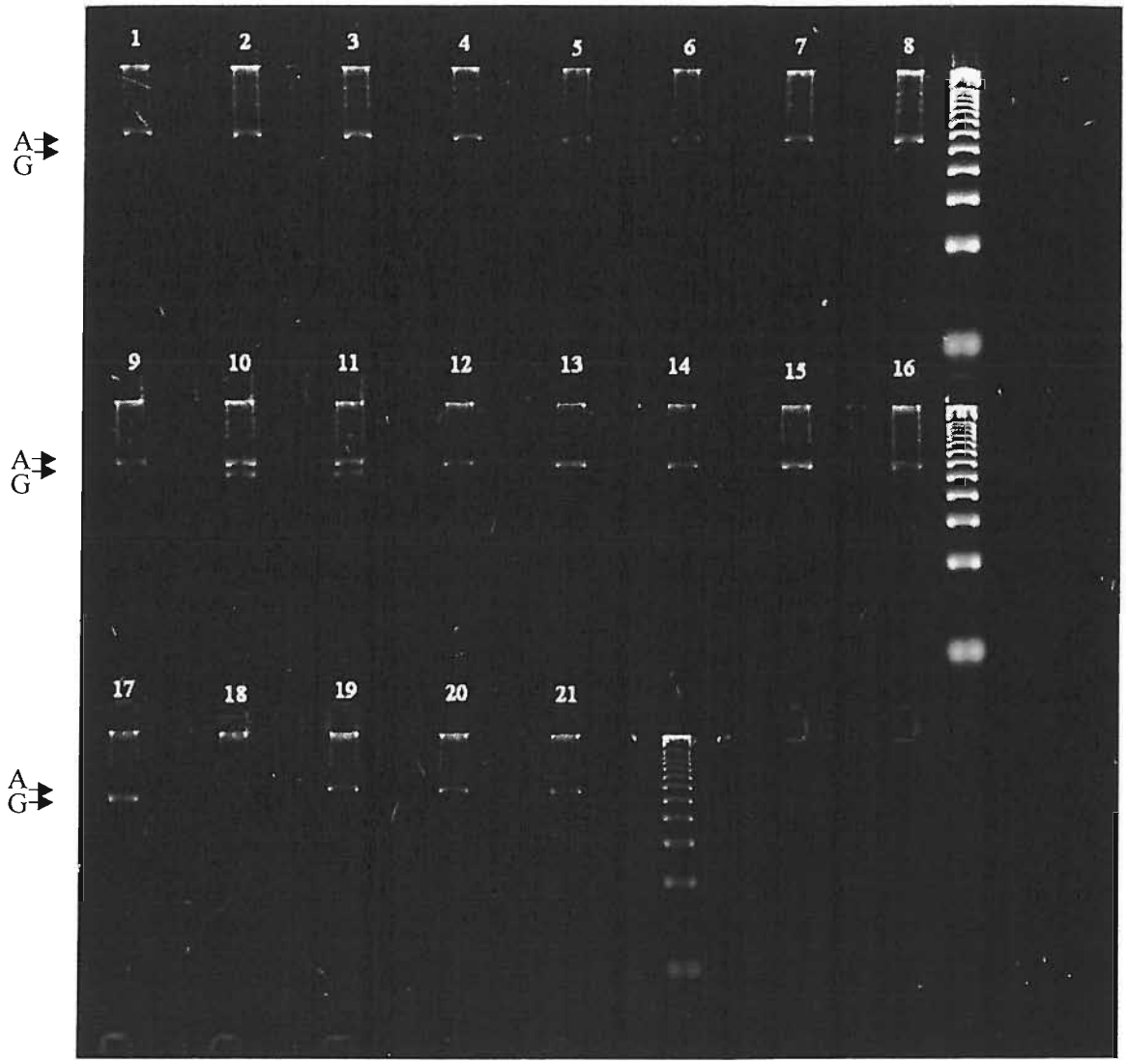

Figure D.1: *Gel electrophoresis run for a group of samples (labelled 1-21), for MMP-12 A/G polymorphism following restriction enzyme digest. The markers in the far right lanes are 25 bp ladders. The upper bands in the samples represent the A allele and the lower bands represent the G allele. Inferred genotypes: samples 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,19,20 and 21= A/A; samples 10 and 11 = A/G; sample 17 = G/G.*

Table D.1: Distribution of genotypes and alleles of MMP-12 polymorphism in CD, UC and control groups, **in children**.

		CD		UC		Normal		p value (Fisher's exact test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	A/A	40	0.833	14	0.736	48	0.813559	0.139
	A/G	5	0.104	5	0.263	11	0.186441	
	G/G	3	0.062	0	0	0	0	
allele	Total	48		19		59		0.766
	A	85	0.885	33	0.868	107	0.90678	
	G	11	0.114	5	0.131	11	0.09322	
	Total	96		38		118		

Table D.2: Distribution of genotypes and alleles of MMP-12 polymorphism in CD, UC and control groups, **in adults**

		CD		UC		Normal		p value (Fisher's exact test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	A/A	19	0.76	23	0.741	64	0.703	0.937
	A/G	6	0.24	8	0.258	26	0.285	
	G/G	0	0	0	0	1	0.010	
allele	Total	25		31		91		0.829
	A	44	0.88	54	0.870	154	0.846	
	G	6	0.12	8	0.129	28	0.153	
	Total	50		62		182		

Table D.3: Distribution of genotypes and alleles of MMP-12 polymorphism in CD, UC and control groups overall **in both children and adults**

		CD		UC		Normal		p value (Fisher's exact test)
		No.	frequency	No.	frequency	No.	frequency	
genotype	A/A	59	0.808	37	0.74	112	0.746	0.142
	A/G	11	0.150	13	0.26	37	0.246	
	G/G	3	0.041	0	0	1	0.006	
allele	Total	73		50		150		0.616
	A	129	0.883	67	0.837	261	0.87	
	G	17	0.116	13	0.162	39	0.13	
	Total	146		80		300		

Appendix E: Sample size calculation formula

The number required to participate in each group is given in the equation below:

$$n = 2\left\{\frac{\left(z_{\alpha}+z_{\chi}\right)\sigma}{\delta}\right\}^{2}$$

or

$$n = 2\left\{\frac{f(\alpha,\beta)\sigma^{2}}{\delta^{2}}\right\}$$

where:

α: the level of significance test used for detecting a difference (often set $\alpha = 0.05$).

$1-\beta$: the degree of certainty that the difference if present would be detected (set $1-\beta = 0.80$)

σ was the mean standard deviation of the two groups;

δ was the observed mean difference between the two groups being compared.

Nquery was the statistical software used to make the sample size calculation and power calculations.

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