The role of the renin angiotensin system and its principal receptor AT1 in the pathogenesis of intestinal fibrosis in inflammatory bowel disease

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PhD Research Degree
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Declaration

I, Shuvra Ray confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Acknowledgements

I would like to thank my mentor, Professor Jeremy Sanderson, for all of the many years of guidance, support and training and for igniting my true passion for IBD and research and for encouraging me to always strive to be the very best doctor I can be.

I would like to express my profound gratitude to my primary supervisor, Dr Jude Oben without whom none of this would have been possible. I cannot thank him enough for taking me under his wing, encouraging me to pursue research, providing space in his lab and believing in me when I faltered. Further thanks to my colleagues in the Oben lab in particular Dr Junpei Soeda and Dr Angelina Mouralidarane for patiently introducing me to the bench.

Thanks to Professor Massimo Pinzani for his wisdom, unique insights into the world of fibrosis & for his instrumental role in arranging the transatlantic collaboration which underpins this thesis.

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Countless thanks to the innumerable colleagues and friends who have helped me along the way; I can never repay you.

Finally, I would like to express my profound thanks to my wonderful parents and sister who have always been there to love and support me and encouraged my love of learning which has proved invaluable in completing this thesis; everything I am is yours.
Abstract

Fibrosis is the common pathological end point of many diverse medical conditions and greatly contributes to mortality and morbidity. It can affect any major organ in the human body; it is usually a sequela of chronic inflammation and related to aberrant wound healing processes. Despite major advances in the treatment of recurrent severe gut inflammation characteristic of inflammatory bowel disease (IBD) there has been no tangible progress in reversing gut fibrosis and its complications such as intestinal strictures necessitating surgical resection. This is partly due to our limited understanding of the mechanisms and key molecular participants underlying fibrogenesis. Fibrosis in the gut is difficult to study and thus many advances in this field are initially discovered in other organs. There is now a substantial body of evidence showing that the renin-angiotensin system and its principal receptor AT1 play a central role in fibrosis in multiple organ systems. Furthermore, the oral AT1 receptor antagonist losartan has been shown in multiple studies to reduce fibrogenesis in vitro, in animal studies and in pilot clinical studies.

This thesis makes an original contribution to our knowledge by first exploring the hypothesis that the AT1 receptor is upregulated in human IBD and gut fibrosis. Secondly, it examines the effects of AT1 stimulation and blockade with losartan in vitro on pro-fibrogenic cells. Thirdly, this research describes and validates a novel high fidelity mouse model of small bowel fibrosis. Finally I conclude by using this model to examine the in vivo effects of losartan on fibrosis at different stages of disease before assessing the effects of losartan on fibrosis following suppression of inflammation with steroids. These data provide the necessary evidence to justify a clinical trial of losartan in IBD.
Impact Statement

The work contained with this thesis has had many beneficial effects to me as an individual but I also believe that there will be lasting benefits both within the field of academia but also importantly outside, with potential major benefits to patients.

As an individual, the knowledge and skills I have acquired whilst conducting this research have transformed my outlook and abilities as a clinician scientist. I have a much enhanced understanding of rigorous experimental techniques and complex experimental design which will enable me to lead further studies as an individual but also as a clinical collaborator. I have also had the privilege to present this work locally, regionally and most excitingly to a large international audience as an oral presentation at Digestive Diseases Week in Washington DC.

Within the field of academia, I would expect multiple positive impacts. Firstly, the work contained within this thesis is now in an advanced draft stage to be submitted for publication as a full original research paper in a peer reviewed journal. This work has also generated many new ideas and hypotheses to examine in further work including the role of alternative renin angiotensin pathways in gut inflammation and fibrosis and indeed both my collaborators in Cleveland and in UCL are already planning this. One of the most exciting impacts of this thesis is the description and validation of the SAMP Yit-FC mouse as a high fidelity unique model of small bowel inflammation and fibrosis. The SAMP mouse will enable academics to not only gain novel insights into the pathogenesis of spontaneous gut inflammation and fibrosis, but also the opportunity for it to be used as a platform to test new anti-fibrotic therapies in IBD.

Finally, the results contained within this thesis corroborate earlier evidence of the effects of losartan on gut fibrosis and now provide strong justification for pursuing a clinical trial in patients with Crohn’s disease as outlined in the discussion section. As a consultant gastroenterologist looking after a large number of patients with IBD in a tertiary referral centre, I encounter the terrible deleterious effects of gut fibrosis in IBD on a regular basis. With
this clinical experience in conjunction with the skills I have gained during the course of this PhD, I will design and lead this clinical trial in collaboration with other clinical centres. If this is successful, as losartan is a readily available, licensed, safe, well tolerated and oral medication, the time from bench to bedside can be significantly accelerated with huge putative impact for patients with IBD.
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# Abbreviations

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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>ACEi</td>
<td>Angiotensin Converting Enzyme Inhibitor</td>
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<tr>
<td>AMCA</td>
<td>Anti Mannobioside Carbohydrate Antibodies</td>
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<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
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<td>ASCA</td>
<td>Anti Saccharomyces Cerevisiae Antibodies</td>
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<tr>
<td>ARB</td>
<td>AT1 Receptor Blockers</td>
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<td>Alpha Smooth Muscle Actin</td>
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<td>Angiotensin II receptor Type 1</td>
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<td>CD</td>
<td>Crohn’s Disease</td>
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<td>CTE</td>
<td>Computed Tomography Enterography</td>
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<td>Connective Tissue Growth Factor</td>
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<td>Dexamethasone</td>
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<td>Death Receptor 3</td>
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<tr>
<td>DSS</td>
<td>Dextran Sodium Sulphate</td>
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<tr>
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<td>Extracellular Matrix</td>
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<td>EMT</td>
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<tr>
<td>GWAS</td>
<td>Genome Wide Association Study</td>
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<td>Interleukin 13</td>
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<td>IL-17</td>
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<td>IL-18</td>
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<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>ISEMFF</td>
<td>Intestinal Sub-Epithelial MyoFibroblast</td>
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<tr>
<td>MRE</td>
<td>Magnetic Resonance Enterography</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>RAS</td>
<td>Renin Angiotensin System</td>
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<td>SAMP</td>
<td>SAMP1/YitFc mice</td>
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<tr>
<td>SBFT</td>
<td>Small Bowel Follow Through</td>
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<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
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<tr>
<td>TFS</td>
<td>Total Fibrosis Score</td>
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<tr>
<td>TGF-ß1</td>
<td>Transforming Growth Factor Beta 1</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TH1</td>
<td>T Helper 1</td>
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<td>TH2</td>
<td>T Helper 2</td>
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<td>TH17</td>
<td>T Helper 17</td>
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<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinases</td>
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<td>TIS</td>
<td>Total Inflammation Score</td>
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<tr>
<td>TL1A</td>
<td>TNF-Like cytokine 1A</td>
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<tr>
<td>TNBS</td>
<td>TriNitroBenzene Sulphonic acid</td>
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<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor Alpha</td>
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<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
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Chapter 1. Introduction

Clinical Science

1.1 Fibrosis and human disease

Fibrosis underlies the pathological features of a broad array of disparate medical conditions and contributes to a great deal of morbidity and mortality in humans. Fibrosis is described as the excessive deposition of extracellular matrix (ECM) leading to progressive tissue architectural distortion and organ dysfunction. It is often provoked as part of a perturbed wound healing process in response to recurrent injury and subsequent chronic inflammation. Fibrosis can occur in all organs but most of our understanding of the physiological processes underlying fibrogenesis come from advances made in studying the kidneys, heart and liver [1].

Studying fibrosis in the gut remains a challenging area where progress lags behind other organ systems and subsequently our understanding of the pathological events underlying intestinal fibrogenesis is limited. Complications from intestinal fibrosis are an important area of significant unmet clinical need in patients with inflammatory bowel disease (IBD).

IBD is an idiopathic chronic inflammatory condition of the intestine comprising primarily of the two conditions: ulcerative colitis (UC) and Crohn’s disease (CD) characterised by intestinal inflammation with periods of remission and recurrent relapse. Whilst these two conditions share many similarities, there are important pathophysiological and clinical differences between the two conditions. The cycle of acute inflammation followed by remission seen in IBD will often progress to chronic inflammation and can lead to the development of significant morbidity and complications including those related to gut fibrosis. I will start with a brief overview of UC before focusing the bulk of this introduction on CD, where fibrosis-related
complications are more common and generally lead to more serious and widely recognised sequelae.

1.2 Ulcerative colitis

Ulcerative colitis (UC) is the most common type of IBD with an incidence of 10 per 100,000/year and a prevalence of 243 per 100,000 people [2]. There are estimated to be in excess of 150,000 people living with UC in the UK. The age of onset of UC is bimodal with a peak in young adulthood and then a smaller second peak in later middle age [3].

1.2.1 Clinical features, histopathology & risk factors

UC is characterised by spontaneous mucosal inflammation that starts in the rectum and can progress to the entire length of the colon in a contiguous fashion. It does not affect any other part of the gastrointestinal tract although up to 10% of cases can also involve the terminal ileum in a paracrine manner (backwash ileitis) [4]. In the majority of patients, UC is characterised by periods of activity (or flare) followed either by spontaneous or medically induced remission when mucosal inflammation subsides and epithelial wound healing occurs. During a flare, patients with UC can present with rectal bleeding, diarrhoea, frequency and abdominal pain caused by disruption of normal colonic function due to acute inflammation. Flares cause considerable morbidity and are a leading cause of reduced quality of life in patients with IBD [5]. Many patients also exhibit extraintestinal features of the disease which can involve many organs such as liver, skin, eyes and joints. This is more fully explored in section 1.3.1. Some patients never truly enter remission and experience chronic symptoms.
without mucosal healing (chronic active colitis); in up to a quarter of patients, intractable severe inflammation of the colon can occur (often acutely) resulting in fulminant colitis which can be fatal unless treated, is the leading cause of hospitalisation in patients with UC and is a significant risk factor for the patient requiring an emergency colectomy [6].

Histopathological features of the condition can include crypt architectural distortion, crypt abscesses, infiltration of the lamina propria with inflammatory cells (including plasma cells, eosinophils and lymphocytes), lymphoid aggregates and mucin depletion [7]. Frank ulceration or erosions can also be present but are surprisingly uncommon belying the name “ulcerative” colitis. The inflammation is usually limited in depth to the mucosal layer of the colon and can involve just the rectum (proctitis), the left colon (left sided colitis) or affect the colon proximal to the splenic flexure (pancolitis or extensive colitis). At diagnosis, 30-50% have proctitis or distal colitis, 20-30% have left-sided colitis and approximately 20% have extensive colitis [8].

Risk factors for developing UC are poorly understood; environmental factors which may modestly increase the risk of developing ulcerative colitis include living in a developed country, recent gut infection (especially with Salmonella species) and use of non-steroidal anti-inflammatory drugs [9]. Conversely, smoking tobacco and appendicectomy have been shown to be protective [10]. The clearest risk factor for developing UC is genetic although this plays a far smaller role in disease risk than in CD. Having a first degree relative with UC confers an eight fold increased risk of developing UC [11] with up to 15% of patients diagnosed with UC having a first degree relative with IBD [7]. In the Danish twins study, monozygotic twins had a disease concordance of up to 18% for UC compared to 4.5% for dizygotic twins [12]. The advent of genome wide association studies (GWAS) has led to the discovery of at least 50 susceptibility genes for UC many of which are shared with CD [13]. Of interest, strongly implicated genes such as TNFRSF14 and IL8RA / IL8RB have also been implicated in fibrogenesis in multiple organs [14, 15] although their role in UC fibrosis remains to be determined.
1.2.2 Fibrosis in UC: epidemiological & histopathological features

Until recently, fibrosis was not considered a significant phenomenon in patients with UC; but there is now an increasing recognition that fibrosis occurs in UC colon and contributes to significant organ dysfunction and complications. Strictures are the most easily identified late manifestation of intestinal fibrosis and are seen in between 1.5 - 11% of patients with UC in imaging or endoscopic studies [16]. In addition to strictures, other features of intestinal fibrosis in UC include shortening of the colon, loss of haustral definition (the classic featureless and tube-like colon of longstanding UC), pseudopolyposis, deranged colonic contractility and narrowing of the rectum and subsequent widening of the pre-sacral space [17-19].

In a normal colon, insults to mucosal integrity such as infection, chemical irritation, diverticulitis, ischaemia and ulceration are rapidly healed with full restoration of mucosal integrity. In UC colon, inflammation tends to recur repeatedly resulting in chronic inflammatory changes which lead to progressive tissue damage, intestinal barrier degradation and exposure of the intestinal wall to the gut microbiota. This can trigger a cycle of further inflammation and increasing gut permeability which results in the release of pro-fibrogenic cytokines such as IL-13 [20] and IL-33 [21] which are strongly upregulated in UC. These mediators can activate myofibroblasts and strongly stimulate them to produce ECM whilst inhibiting ECM degradation; a hallmark of fibrogenesis. Relatively few morphological studies have been carried out on intestinal fibrosis in UC but Goulston et al’s study showed that most patients with UC have a thickened muscularis mucosae compared to healthy controls and that this is increased forty fold in areas of benign stricture, suggesting that muscular hypertrophy plays a part in the luminal narrowing seen in strictured areas [22]. Conversely, De Dombal et al noted that strictures had submucosal fibrosis with limited muscular hypertrophy suggesting that strictures in UC are somewhat heterogeneous and compose of varying degrees of muscular hypertrophy and submuscular fibrosis [23].
Due to the larger diameter of the human colon (compared to small bowel in CD fibrosis), the majority of UC patients with benign colonic strictures experience remarkably few symptoms. However, patients with longstanding UC often experience chronic intestinal symptoms such as disordered defaecation, bloating and abdominal pain even when in mucosal remission from inflammation. It is increasingly recognised that progressive intestinal fibrosis leading to loss of function in the colon is likely to underlie many of these symptoms highlighting the importance of expanding our understanding of fibrogenesis in UC to enable us to develop better treatment for this complication.

1.3 Crohn’s Disease

Crohn’s disease is an idiopathic chronic inflammatory bowel condition which is the second most common form of IBD. It can affect any part of the intestinal tract from the mouth to the anus in discontinuous patchy segments but has a particular predilection for the terminal ileum and colon. The incidence of CD has been increasing over the last few decades, and is especially prevalent in the most northern and southern parts of the developed world. The UK (especially parts of Scotland) has one of the highest rates of CD in the world with an incidence of 8.3 per 100,000/year and a prevalence of 145 per 100,000 people with an estimated 115,000 patients suffering from the condition in the UK [2, 24].

1.3.1 Clinical Features, Pathology & Risk Factors

The commonest sites for disease involvement in CD are ileum (45%), colon (32.0%), and ileo-colon (19%) [25]. The symptoms of CD can vary depending on the localisation of the
inflammation but the most common symptoms of uncomplicated CD include abdominal pain, diarrhoea, weight loss, failure to thrive (in children), mouth ulcers, rectal bleeding, fatigue and malaise. Other clinical features of the disease can include vitamin B12, zinc and vitamin D deficiency and iron deficiency anaemia. The symptoms of colonic CD are virtually indistinguishable from those of UC with rectal bleeding, diarrhoea and tenesmus being especially predominant. CD & UC are also associated with a wide array of extra-intestinal manifestations which can affect the eyes (uveitis, iritis), skin (pyoderma gangrenosum, erythema nodosum), joints (type 1 and type 2 enteropathic arthropathy, ankylosing spondylitis) and liver (primary sclerosing cholangitis, autoimmune hepatitis) [26]. Whilst there is a significant amount of overlap between UC and CD, there are important clinical, macroscopic and microscopic pathological differences which are outlined in Table 1.1.

Macroscopically, CD will often be marked by skip lesions: areas of normal bowel interspersed with abnormal bowel in a discontinuous pattern of inflammation. There may also be areas of fat wrapping especially in the small bowel and inflammatory change is transmural. Microscopic changes which are agreed to be more specific for CD include focal chronic inflammation, serositis, transmural lymphoid aggregates and isolated granulomas (excluding those near ruptured crypts) [4].

The development of CD represents a complex interplay between environmental factors including the gut microbiota and genetic susceptibility. When comparing risk factors between UC and CD, there are both similarities and paradoxical effects. Environmental risk factors which increase the risk of developing CD include smoking (especially in early life, both active and passive) [27], appendicectomy [28] and use of NSAIDS [9].

Our understanding of the contribution of genetics to CD susceptibility has been one of the biggest advances in modern genetics. Prior to the era of GWAS, using linkage study techniques, only 2 genes had been identified as risk factors for CD [29]. Since the application of GWAS methods to IBD at the millennium, over 140 susceptibility loci have been identified as being
involved in CD many of which are shared with UC[30]. CD has long been recognised as having a larger underlying genetic component than UC; twin studies in CD have demonstrated a concordance rate of 30% in monozygotic twins compared to 3% in dizygotic twins [31], whereas in UC this rate is 16% (monozygotic) and 4% (dizygotic) [32]. Many of the loci implicated in CD appear to be linked to mucosal barrier function (MUC1, MUC19 and PTGER4), bacterial sensing and autophagy (most famously NOD2) and also changes in adaptive regulatory T-cell responses (IL10, IL2RA, SMAD3) highlighting the importance of the interface of mucosal integrity, gut microbiota and immune responses in the pathogenesis of Crohn’s disease.

**Table 1.1: Comparison of ulcerative colitis with Crohn’s disease**

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<thead>
<tr>
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<th>Ulcerative Colitis</th>
<th>Crohn’s Disease</th>
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<tbody>
<tr>
<td><strong>Disease distribution</strong></td>
<td>Confluent inflammation from rectum up to caecum. No oral, small bowel or perianal disease</td>
<td>Patchy inflammation which occurs in discontinuous segments anywhere within the intestinal tract including perianal and oral</td>
</tr>
<tr>
<td><strong>Endoscopic appearance</strong></td>
<td>Shallow ulceration, friability, intense vascularity, pseudopolyps and loss of haustral definition</td>
<td>Aphthous ulceration, deep penetrating ulcers with cobblestoning</td>
</tr>
<tr>
<td><strong>Depth of inflammation</strong></td>
<td>Mucosal and submucosal</td>
<td>Transmural</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td>Crypt architectural distortion, crypt abscesses common and granulomas rare &amp; always associated with disrupted crypts</td>
<td>Focal chronic inflammation, serositis, transmural lymphoid aggregates &amp; presence of frequent isolated granulomas</td>
</tr>
<tr>
<td><strong>Serological markers</strong></td>
<td>Frequently ASCA positive</td>
<td>Frequently pANCA positive</td>
</tr>
<tr>
<td><strong>Genetics</strong></td>
<td>Lower heritability (10% concordance in monozygotic twins) with a significant association with FCGR2A, 5p15, 2p16 and ORMDL3</td>
<td>Higher heritability (30 - 50% concordance in monozygotic twins) with strong association with variants of NOD2, ATG16L1, IL23R, IRGM</td>
</tr>
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</table>
1.3.2 Natural history of Crohn’s disease: a tale of inflammatory and fibrosis-related complications

The ability to predict the clinical course of CD in a patient has long been a matter of clinical and research interest and numerous attempts have been made to classify the disease and thus enable study of the natural history of the disease and risk stratification. CD is currently widely classified according to age of diagnosis, disease location and behaviour. The Montreal classification is the most commonly used classification system and is a modification of the previously widely used Vienna classification system. Table 1.2 outlines these two classification systems [33].

| Table 1.2: Comparison of the Vienna & Montreal classification systems of CD |
|---------------------------------|-----------------|-----------------|
| **Age at diagnosis**            | Vienna          | Montreal        |
| A1 Below 40 years               | A1 Below 16 years |
| A2 Above 40 years               | A2 Between 17 and 40 years |
| A3 Above 40 years               | A3 Above 40 years |
| **Disease Location**            | Vienna          | Montreal        |
| L1 Ileal                        | L1 Ileal        |
| L2 Colonic                      | L2 Colonic      |
| L3 Ileocolonic                  | L3 Ileocolonic  |
| L4 Upper GI                     | L4 Isolated upper GI |
| **Behaviour**                   | Vienna          | Montreal        |
| B1 Inflammatory disease         | B1 Inflammatory disease |
| B2 Stricturesing disease        | B2 Stricturesing disease |
| B3 Penetrating disease          | B3 Penetrating disease |
| p Perianal disease modifier     |                 |

*Table modified from Satsangi et al. Gut, 2006; 55(6): 749-753 [33]*

According to these classification systems, CD behaves according to three cardinal and separate patterns: inflammatory disease (inflammatory phenotype), stricturing disease (fibrotic phenotype) and penetrating disease (penetrating phenotype). Whilst the sites of disease involvement in CD appear to remain relatively stable over time the same cannot be said regarding disease behaviour. Although approximately 90% of patients with CD have purely inflammatory disease at diagnosis, it is increasingly recognised that complications such as intestinal strictures and fistulisation will occur in the majority of patients over long term follow up [34]. In a large referral centre study, Cosnes et al demonstrated that CD was complicated by
penetrating or fistulising disease in 15% at diagnosis, 50% at 5 years and over 85% at 20 years outlining how the majority change phenotype over time [35].

Estimates of cumulative incidence of stricturing CD behaviour at 20 years vary from 18% to 21% compared to 37% to 70% for fistulising disease [25, 35], but this makes the assumption that penetrating and stricturing disease are separate entities and mutually exclusive. In fact fistulising complications in CD from penetrating disease are actually highly predictive of the presence of a stricture [36] leading to the paradigm that some fistulising disease is actually a variation of stricturing disease [37]. In stricturing CD, wound healing mechanisms, whilst aberrant, have partly activated to successfully contain chronic inflammation by creating a wall of fibroed connective tissue around it within the stricture. In penetrating CD, fistulisation often occurs in a segment of bowel upstream of a stricture in a high pressure environment where transmural inflammation is present and a fibrotic reaction has failed i.e. that the inflammation is inadequately walled off by fibrous tissue causes the inflammatory process to progress transmurally across the bowel wall and fistulate into adjacent loops of bowel or organs.

Many complications of CD result in needing surgical intervention. Within 10 years of diagnosis, 50% of patients will require an operation; unfortunately, remission after surgical resection is only temporary since the rate of endoscopic recurrence after resection approaches 70% of cases after one year and becomes symptomatic within four years [38].

The conclusions we can draw from the natural history of CD is that it is a condition marked by progressive accumulation of complications over time and that many of these complications are related to fibrosis. Whilst disease behaviour is difficult to phenotype according to the Montreal classification due to significant change in behaviour over time and overlap between phenotypes, it is clear that some patients do go on to develop early aggressive disease (often presenting with fibrosis associated complications) with rapid disease recurrence after surgery whilst there are patients who do not develop fibrosis easily despite profound chronic
inflammation. It remains to be seen what factors determine disease progression in these more tightly defined groups.

1.3.3 Clinical, genetic and environmental risk factors for the development of fibrostenotic Crohn’s disease

The clinical risk factors for the development of fibrotic complications of CD are not specific but rather underscore the same risk factor for a more serious disease course. The majority of intestinal strictures in patients with CD occur in the terminal ileum; therefore, it is not surprising that small bowel involvement (especially extensive) is a risk factor for developing intestinal strictures. Interestingly the presence of perianal CD is also a risk factor. Diagnosis of CD before the age of 40 has been shown in multiple studies to be related to more severe disease phenotype and therefore leads to far greater fibrotic complications of CD and subsequent need for surgery [25, 35]. Another clinical marker of disease severity is the need to use corticosteroids at presentation; this also correlates with increased risk of fibrosis [39]. Interestingly, unlike the beneficial effects seen in UC, appendicectomy prior to diagnosis may increase the risk of a fibro-stenotic phenotype in CD [40]. The only confirmed environmental factor which is also the only modifiable risk factor is smoking. Smoking is associated with a generally more severe disease phenotype and increased risk for surgery and postoperative disease recurrence, reiterating the importance of smoking cessation [41, 42]. Physician advice about smoking cessation in CD patients can have a significant impact on success [43] and should be a core part of the clinical management of all smokers with CD.

Genetic markers which increase the risk of fibro-stenotic disease have also been identified. Three variants of the highly studied NOD2/CARD15 gene increase the relative risk of fibro-stenotic disease by a third and are also associated with ileal disease and overall increased
disease severity [44]. Variants in the MMP3 gene and rs1363670 homozygosity are also independently associated with stricturing CD [45]. Whilst currently identified genetic variants are only seen in a small proportion of CD patients limiting their predictive value, they may however provide important future insights into the mechanisms of intestinal fibrosis.

1.3.4 Pathological features of intestinal strictures

On histological assessment, intestinal strictures are characterized by luminal narrowing and bowel wall thickening and may be present with prestenotic dilatation (especially if longstanding and severely narrowed). The strictured area is associated with severe ulceration and often there is circumferential loss of the mucosa in addition to other histological features of CD including granulomas. The complete depth of the bowel wall is thickened with involvement of the submucosa, muscularis propria, subserosa and mesenteric fat. The surface of the serosa will reveal distended blood vessels and may show fibrinous exudate [46]. There is significant excessive ECM deposition with islands of collagen (especially type V), fibroblasts, smooth muscle cells and myofibroblasts in the muscularis mucosa and submucosa and the presence of fibrous septa often projecting from the submucosa through the muscularis mucosa contributing greatly to tissue architectural distortion and luminal narrowing [47]. Another feature which is strongly associated with CD strictures (although occasionally seen in UC strictures) is the phenomenon of fat wrapping. This is when the affected area of intestine is partially surrounded by mesenteric fat and the corresponding section of mesentery itself is usually also thickened and shortened with prominent mesenteric lymph nodes [48]. The role of mesenteric fat in IBD has long been ignored but given its ubiquity in many surgical resection specimens, there is now increasing interest in the role that adipocytes might play in intestinal inflammation and fibrogenesis [49].
1.3.5 Diagnosis of intestinal strictures in CD

Diagnosis of early intestinal fibrosis is fraught with difficulty mostly because in the early stages of fibrosis, there are often few or no symptoms. As fibrosis becomes more severe and the intestinal architecture become distorted, luminal narrowing occurs and may present with obstructive symptoms such as abdominal pain (particularly post-prandially), vomiting, dysphagia, constipation and weight loss dependent on location.

Whilst several experimental serological markers e.g. anti-Saccaromyces cerevisiae antibodies (ASCA), anti-Pseudomonas-associated sequence I2 antibodies, and anti-mannobioside carbohydrate antibodies (AMCA) have been associated with complex CD including fibrostenotic disease [50], there are no currently validated serological markers to diagnose or grade intestinal fibrosis unlike advances in liver disease diagnostics. The development of these is a clear clinical priority but is hampered by the absence of agreement in radiological or histological grading of fibrosis which would be required to robustly validate any serum test.

Ileo-colonoscopy is able to accurately detect mucosal disease and assess the extent of narrowing in the distal parts of the terminal ileum but is limited by our ability to access the lumen with the scope, patient discomfort, risk of perforation and the absence of any information on activity below the mucosal surface or extra-intestinal complications. Balloon enteroscopy can however, improve the ability to view more proximal disease but is time consuming, expensive and extremely invasive. Therefore, imaging remains the primary modality for the diagnosis and monitoring of intestinal fibrosis in the majority of CD patients despite the problems with scoring criteria. An ideal imaging test would be non-invasive, lack radiation, be inexpensive, reliable, replicable and easily accessed. In order to most effectively help guide clinical decision making, such a test would be able to determine the severity of
inflammation and fibrosis and accurately predict those patients who are less likely to benefit from anti-inflammatory therapy and would benefit from early surgery (or in future anti-fibrotic therapy). The search for the ideal imaging modality in CD continues but a great deal of progress has been made in recent years.

The classical imaging technique used to detect small bowel CD and strictures is the small bowel follow through (SBFT). SBFT involves multiple plain radiographs being taken whilst swallowed oral contrast (usually barium) passes through the small bowel. Small bowel enteroclysis is a variation of this technique where contrast is directly injected into the jejunum via a nasojejunal tube but is rarely used now due to being more uncomfortable and limited evidence of benefit over conventional SBFT [51]. Whilst this imaging technique is a longstanding, widely available and inexpensive imaging modality for the diagnosis of intestinal fibrosis, it is hampered by the inability to differentiate between active disease and fibrosis or grade either of these, inter-operator variability, significant radiation exposure and lack of extra-luminal details.

Small bowel ultrasound is an established technique for assessment of CD, particularly in continental Europe where gastroenterologists regularly perform transabdominal ultrasound as a core part of their training. It involves using ultrasound to measure the thickness of small bowel and also look for intra-abdominal complications of CD such as strictures and fistulae. A meta-analysis of the use of ultrasound in evaluating small bowel thickening revealed a sensitivity of 88% and specificity of 93% for the presence of CD when 3mm was used as the upper limit of normal bowel thickness [52]. When evaluating strictures, conventional ultrasound detects between 70 – 79 % of strictures which can be improved up to 90% sensitivity with the use of oral polyethylene glycol as contrast [53, 54]. The advantages of ultrasound include that it is inexpensive, the equipment is widely available, there is no ionising radiation allowing frequent serial scans for monitoring, it is able to give information about extra-luminal pathology and it is non-invasive and well tolerated. Disadvantages of this
technique include limited differentiation between active and inactive disease, poor spacial resolution of extra-intestinal abnormalities, inter-operator variability and poor views in overweight patients. Moreover, gastrointestinal physicians in many countries outside continental Europe (e.g. the USA and UK) do not routinely perform ultrasound making access to small bowel ultrasound more difficult. Nevertheless, this remains a promising technique and further studies are needed to determine the correlation between ultrasound findings and the degree of fibrosis. Novel developments such as intestinal sheer wave ultrasound elastrography may greatly enhance this technique for assessing fibrosis in the near future [55].

Computed Tomography Enterography (CTE) is a technique where detailed cross sectional imaging is performed of the intestine using x-rays, intravenous and intraluminal contrast [56]. This technique gives high resolution, three dimensional images of the bowel and in addition significant information about other intra-abdominal structures and organs. CTE has been found to have a high sensitivity and specificity for the detection of CD when compared to other techniques, in particular, comparable in accuracy to the gold standard of ileocolonoscopy [57]. CTE can also determine the degree of tissue inflammation using radiological correlates such as increased blood flow, mural enhancement and hypervascularity which have been shown to correlate with both endoscopic, serum and histological markers of inflammation [58]. Whilst CTE has been validated in identifying and quantifying CD inflammatory disease activity, little is known about its ability to independently detect and grade intestinal fibrosis. Adler et al conducted a study examining the correlation between CTE findings and histological grade of inflammation and fibrosis [59]. CTE was able to detect inflammation accurately as previously described, but in those patients who had strictures with minimal inflammation on CTE, there was little correlation with the severity of fibrosis. Upstream dilatation was the only CTE finding (only on univariate analysis) that might be independently linked to degree of fibrosis. The authors concluded that the presence of a small bowel stricture without classical CTE findings of tissue inflammation does not necessarily predict the presence of tissue fibrosis. Nevertheless, clinicians can cautiously use CTE findings, combined with evaluation of other parameters, to
estimate the inflammatory burden within a stricture, the presence of significant fibrosis and subsequent likelihood of response to anti-inflammatory treatment. CTE has the advantages of widespread availability, high resolution images, luminal and extraluminal information, quick scanning times and being minimally invasive. The major drawback of CTE is the significant dose of ionising radiation required (precluding recurrent use in the same patient for monitoring).

Magnetic resonance enterography (MRE) is a radiation free alternative to CTE which is rapidly becoming the imaging modality of choice in CD. MRE involves the ingestion of oral contrast (e.g. mannitol) to maintain luminal distension whilst detailed T1 and T2 weighted images are obtained. Intravenous contrast such as gadolinium is used to detect inflammation which shows up as enhancement in T1 weighted images [60]. Numerous studies have clearly shown good correlation between MRI findings and disease activity in CD [61]. In one seminal study, Sempere et al showed that contrast enhancement and bowel wall thickening correlated well with findings of active disease at ileocolonoscopy as well as clinically active disease whilst clinical remission correlated with a significant reduction in contrast enhancement and bowel wall thickening with no significant difference in sections of inactive CD bowel compared to healthy controls [62].

There have also been efforts to investigate the reliability of MRE findings in diagnosing intestinal fibrosis and establish a scoring system with a view to identifying those patients most likely to fail current medical treatment due to a heavy underlying burden of fibrosis. Lawrance et al stratified patients with small bowel CD into four categories of fibrosis and inflammation according to specific MRE findings. These categories correlated with histological differences at surgery and they were also able to demonstrate differences in response to treatment according to grouping; in particular the presence of intestinal narrowing and prestenotic dilatation predicted poor response to treatment [63]. Fornasa et al investigated the utility of a formative MRE activity score comprising of subscores of both T2 signal intensity and post-gadolinium T1 enhancement. They showed that this scoring system had a 95.8% sensitivity and
100% specificity for the detection of fibrosis on MRI, although this study is crucially limited by the fact only patients with low activity scores underwent surgical resection allowing histological assessment of fibrosis whereas almost all patients with MRE active disease did not have histological evaluation for the presence or absence of fibrosis. The Barcelona group overcame some of these limitations by prospectively assessing 42 patients undergoing surgery allowing full comparison between MRE findings and histology which was graded according to degree of fibrosis [64]. They found a good association between certain MRE findings (T2 hypersignal, mucosal enhancement, ulceration and blurred margins) and degree of inflammation. They also found that the degree of fibrosis correlated well with the percentage of enhancement gain, pattern of enhancement at 7 minutes and the presence of stenosis. Using MRE, they were able to demonstrate a sensitivity of 94% and specificity of 89% for the presence of intestinal fibrosis.

The advantages of MRE include the minimally invasive nature of the scan, lack of any ionising radiation, high sensitivity and specificity for detecting inflammation and now fibrosis with correlation to clinical outcomes to medical therapy, the availability of extra-intestinal views and high resolution soft tissue planes giving accurate assessment of complications such as fistulae. The drawbacks of MRE include slower scanning times and subsequent susceptibility to movement artefact, high cost, lower accessibility and lower resolution images of extra-intestinal complications such as abscess formation compared to CTE. Nevertheless, MRE has grown to become the gold standard imaging modality in the majority of IBD centres as it is clear that MRE is the safest, most accurate and replicable tool for diagnosing advanced fibrosis as well as inflammation in small bowel CD. Initial animal and human data from further imaging enhancements in MRE such as the technique of magnetisation transfer may herald unprecedented accuracy in non-invasively grading fibrosis in CD from early to severe, allowing high quality longitudinal studies for evaluating and assessing the process of fibrogenesis in CD patients and the effects of novel anti-fibrotic therapies on fibrosis progression [65-67].
1.3.6 Medical management of fibro-stenotic CD

There is currently no specific anti-fibrotic therapy to treat fibrosis in CD and many patients eventually require surgical intervention. Despite significant advances in the management of inflammation in CD, there has been little change in the rates of surgery in patients with CD. There are some tentative signs that early, prolonged, aggressive medical therapy optimised to achieve mucosal healing may be beginning to change this situation and lead to reduced rates of surgery [68]. Nevertheless, it is clear that there are a substantial number of patients who will fail medical therapy and develop progressive recurrent fibro-stenotic complications of CD despite good control of inflammation. This corroborates the paradigm that whilst inflammation is required for the initiation of fibrogenesis, separate fibrogenic pathways become activated and continue to progress fibrosis even after inflammation is controlled [69]. This highlights the importance of earlier diagnosis and grading of fibrosis and the clinical need for specific anti-fibrotic pharmacotherapy. However, in the absence of a perfect anti-fibrotic therapy, management of fibro-stenotic CD focuses on lifestyle changes, anti-inflammatory therapy and the dilatation or surgical removal of strictures when this fails.

Lifestyle changes can play a part in managing CD fibrosis. As described earlier, smoking is a key reversible driver of fibrogenesis in CD and smoking cessation advice and support is an important step in the management of all patients with CD but particularly salient in those with fibrosis. Recent evidence has confirmed that smokers have more frequent relapses and that smokers that quit smoking quickly normalise their relapse rate to that of non-smokers [70]. We can sensibly extrapolate from this and advise patients that smoking cessation is likely to lead to lower rates of fibrosis and subsequent surgery.
A low residue diet is also frequently recommended in the management of fibro-stenotic CD. A low residue diet consists of soft food which is low in fibre and easily digested; the theory being that soft non fibrous food is less likely to get stuck in narrowed areas of strictured bowel and cause intestinal obstruction. Surprisingly, whilst empirically we appear to see some benefits from this strategy in our clinical practice, there is no robust data supporting this and limited historical data from Italy suggested it made little or no difference to rates of hospitalisation or obstruction in CD [71].

Once intestinal narrowing has been detected, current initial medical pharmacotherapy largely focuses on the aggressive control of inflammation. Inflammation is invariably present alongside fibrosis in intestinal strictures and bowel wall oedema certainly worsens luminal narrowing. A relatively small decrease in bowel wall thickness and oedema will have a dramatic effect on luminal area (Figure 1.1) [37, 72].

**Figure 1.1:** *A modest 17% decrease in bowel wall thickness (e.g. through medical treatment) leads to a 625% increase in luminal area*

*Figure adapted from Yaffe et al, Journal of Clinical Gastroenterology, 1983, Jun;5(3)211-5 [72]*
Corticosteroids are drugs that are used in a myriad of inflammatory conditions from asthma to arthritis and are the oldest group of drugs used in CD. Steroids have good efficacy in rapidly reducing symptoms of CD but have limited ability to induce mucosal healing; a key outcome in preventing the deleterious complications of CD such as fibrosis. Systemic steroids such as prednisolone still play a role in quickly reducing the symptoms of active CD in many patients. Whilst widely used, they are not recommended for frequent use or for maintenance of remission due to multiple serious side effects including osteoporosis, weight gain, diabetes, hypertension, cataracts and adrenal suppression [73]. In an effort to reduce systemic effects of steroids, relatively locally acting steroids have been developed such as Entocort® which contains budesonide in capsules designed to delay release avoiding absorption in the stomach and proximal small bowel and allowing the release of steroid directly in the ileum. Entocort® has efficacy in short term use, is better than placebo at inducing remission and has far fewer systemic side effects than prednisolone but is less effective and systemic effects do accumulate with prolonged use[74]. Clinical benefit beyond 3 months of therapy has not been demonstrated and mucosal healing rates remain well below conventional immunosuppressants [75].

The effects of corticosteroids on fibrogenesis are uncertain with paradoxical evidence. Corticosteroids have been shown to be beneficial in reducing fibrosis and disease progression in several different fibrotic conditions affecting disparate organ systems such as systemic sclerosis [76], pulmonary fibrosis [77] and retroperitoneal fibrosis [78]. Conversely, recent studies have shown that intra-lesional steroid injection for intestinal strictures has no significant impact on the need for subsequent surgery [79, 80], and in vitro studies of steroids on human fibroblasts show that at low doses collagen synthesis is not altered whilst higher doses actually increase markers of fibrosis [81]. Furthermore, regardless of any putative
benefits of steroids at reducing fibrosis in CD, the multitude of adverse effects of steroid therapy would preclude their long term clinical use.

Thiopurines such as azathioprine play a central role in the medical management of Crohn’s disease. Azathioprine is an immunomodulator that works by disrupting DNA synthesis through purine inhibition in fast growing cells and consequently suppresses the immune system and reduces inflammation in a wide array of chronic inflammatory conditions such as IBD. In CD, azathioprine has been shown to induce remission in over 50% of patients and maintain clinical remission in over two thirds [82]. It has also been shown to be beneficial in other fibrotic conditions such as systemic sclerosis, idiopathic pulmonary fibrosis and retroperitoneal fibrosis [83, 84]. Whilst azathioprine may delay the need for repeat surgery in fibro-stenotic CD [85], there is insufficient evidence that it has any significant anti-fibrotic activity and it does not appear to alter overall rates of surgery [86].

Tumour necrosis factor alpha (TNF-α) is a central mediating cytokine in the pathogenesis of several chronic inflammatory including IBD and medications which target TNF-α have been found to have powerful anti-inflammatory actions. Anti-TNF-α therapies such as infliximab and adalimumab (especially in combination with thiopurines) have revolutionised the management of IBD potentiating rapid clinical response in the majority of patients, clinical remission in over half of patients and higher rates of mucosal healing than azathioprine alone [87, 88].

Due to its potent efficacy, the first anti-TNF therapy to be licensed in CD, infliximab rapidly found widespread use in the management of CD, shortly followed by the introduction and widespread use of adalimumab. Whilst having a dramatic effect on inflammation in CD, early case reports suggested that infliximab might conversely worsen intestinal stricturing by putatively promoting over-rapid uncoordinated intestinal healing leading to excess ECM deposition in the bowel wall and subsequent luminal obstruction [89, 90]. This was supported by in vitro data which suggested that infliximab (as well as other anti-TNF agents) stimulates myofibroblast migration and upregulates production of tissue inhibitor of metalloproteinase-1
(TIMP-1) in myofibroblasts isolated from CD patients in a dose dependent manner [91]. TIMP-1 is an important pro-fibrogenic mediator which acts to inhibit enzymes which usually degrade ECM and consequently promotes ECM stabilisation, expansion and thence fibrosis. However, as our experience with anti-TNF therapy in CD has increased, this early putative complication has not borne out to be the case in real world clinical use. In fact, anti-TNF appears to reduce hospitalisation and the rate of major abdominal surgery in those CD patients with sustained clinical response and has even been demonstrated to be an effective therapy in treating symptomatic intestinal strictures [92, 93].

Vedolizumab is the most recent biological agent to be licensed for the treatment of IBD. It is a monoclonal antibody which binds to the α4β7 integrin subunit interfering with trafficking of lymphocytes to the intestine and thus having anti-inflammatory actions. The Gemini II study demonstrated clear benefit of vedolizumab over placebo in the induction and maintenance of remission and clinical response in CD [94]. There is as yet no data on its effects on intestinal fibrosis and surgical rates and very limited data on mucosal healing in CD (which would likely parallel efficacy in reducing future surgery rates) although current clinical studies are ongoing specifically looking into this [95].

Whilst it is anticipated that ongoing aggressive and early medical management of active CD using a combination of immunomodulators and biologics will have some impact on future surgical rates, it is clear that a substantial proportion of patients will continue to fail medical treatment and develop intestinal strictures. In these patients, there are options for endoscopic treatment and different forms of surgical intervention.
1.3.7 **Endoscopic management of fibro-stenotic CD**

Dilatation of strictures at endoscopy has become an established, widely available, safe and minimally invasive treatment for the management of obstructive strictures in patients with CD [37]. This procedure first relies on being able to navigate through the bowel to the area of the stricture with an endoscope. The commonest site of stricturing in CD is in the terminal ileum and dilatation here is undertaken during colonoscopy, whilst oesophageal and duodenal strictures can be treated during gastroscopy. Double balloon enteroscopy has also been used to successfully treat deeper small bowel strictures but is not a wide used technique in dilatation [96]. Once an appropriate stricture is endoscopically visualised, dilatation is performed in stages using a pneumatically inflated through the scope balloon, often with the assistance of fluoroscopy. There have been many studies examining the utility of endoscopic dilatation in treating CD strictures but there is a lot of heterogeneity in the protocols used and follow up periods. In an attempt to address this, a meta-analysis was conducted by Hassan et al in 2007 [97]. They discovered that the majority of studies were conducted in post-surgical anastomotic strictures which appear particularly amenable to endoscopic dilatation. Overall, technical successful dilatation was performed in 86% of cases with the majority of the remaining requiring surgery. Of those who were successfully dilated, 58% remained surgery free over a very variable period of follow up. Factors associated with higher efficacy in multivariate analysis included having a short stricture (length of less than 4 cm). There was a trend that both efficacy and technical success were reduced in patient with active CD at the time of dilatation. The major complication rate overall was low (2%), with no mortality reported in any of the studies and was not related to the size of balloon used. A more detailed recent meta-analysis of endoscopic dilatation in fibrotic CD included 25 studies with 1089 patients and 2664 dilatations [98]. They reported that technical success was achieved in 92.6% of cases but with a clinical success (symptom resolution) in 70.2% of patients. Major
complications appeared to be a higher at 6.4% (but with a perforation rate of 2.4% where reported) without any cases of mortality recorded. However, within 5 years, 75% of patients eventually required surgery despite dilatation (sometimes serial and repeated). What we can conclude from this is that endoscopic dilatation is technically feasible and successful in the short term in many patients. However, the long-term success of endoscopic dilatation as an alternative to surgery appears to be limited with the majority of patients requiring surgical treatment eventually although it remains to be seen if technical refinements such as stents or intra-lesional steroids may help improve outcomes in the future.

1.3.8 Surgical management of fibro-stenotic CD

The majority of patients with CD who have symptomatic strictures or who have developed penetrating complications secondary to fibro-stenotic disease will usually require surgery. There are also some patients with limited ileal disease with contraindications to medical therapy who may elect for surgery. There are two main surgical options to consider: resection and strictureplasty. The most common operation performed in CD is resection of the ileocaecal region correlating with this being the most common site of stricturing disease. Surgical resection of a stricture involves removal of the affected segment of bowel either through an open operation or increasingly laparoscopically. The surgeon may form a primary anastomosis, an anastomosis with a diverting stoma or a permanent end stoma depending on the region being resected and patient parameters such as nutritional status, use of steroids and disease activity [99]. There are significant disadvantages of intestinal resection as a treatment for fibro-stenotic CD. Whilst the morbidity and complications of surgical resection are low, complications can include devastating ones such as anastomotic leakage. Surgery is also not a cure for CD; remission after surgical resection is only temporary since the rate of endoscopic recurrence after resection occurs in 70% of cases after one year and becomes symptomatic
within four years [38]. Many patients will therefore require repeated surgery with reoperation rates of up to 32% at five years, 44% at 10 years and 55% at 20 years [100]. Numerous attempts have been made to reduce recurrence after surgical resection with variation in techniques such as adjusting the anastomosis (hand sewn versus stapled, side to side versus end to end) but significant changes in outcome remain elusive [101]. Enhanced medical therapy such as the use of postoperative metronidazole and azathioprine, or early use of infliximab or adalimumab, have also shown promise in small scale studies [102-104]. However, despite these innovations, the overall re-operation for fibro-stenotic CD remains stubbornly high [105]. In addition to all the excess morbidity caused by recurrence and re-operation, repeated operations reduce intestinal length and therefore function. The majority of patients following ileal resection have vitamin B12 deficiency and as recurrent operations occur, some may develop devastating intestinal insufficiency known as short gut syndrome [106]. This has led to the development of surgical techniques that conserve bowel length, primarily strictureplasty.

Strictureplasty is a surgical technique which involves surgically opening a stricture and resuturing in a different configuration to preserve luminal diameter and reduce pressure stress from luminal narrowing. A variety of surgical approaches have been employed but the two commonest techniques are the Heinecke-Miculicz strictureplasty (81% of cases) and the Finney strictureplasty (10%) [107]. In the Heinecke-Miculicz procedure, a longitudinal incision is made across the strictured portion of bowel and it is then closed transversely thus making it only suitable for relatively short strictures not exceeding 10cm in length. The Finney procedure is more suitable for longer strictures and involves longitudinal incision of the stricture, formation of a loop and side to side closure [108]. The risk of recurrence has always been of concern due to retention of diseased intestine after strictureplasty. In one large study, surgery-free survival after strictureplasty was 70.7% at 5 years and 26.6% at 10 years, with young patients doing particularly badly [109]. Another study showed that strictureplasty appears to have similar complication rates to resection but again demonstrates a higher recurrence rate (odds ratio
1.36) and shorter recurrence free survival time (hazard ratio 1.08) vs surgical resection [110]. Nonetheless, strictureplasty in expert hands remains a very important tool in the surgical management of CD fibrosis allowing for preservation of bowel length and therefore particularly important for patients with long and multiple small bowel strictures or limited small bowel following previous resections.

It is clear that neither current medical, endoscopic or surgical approaches to intestinal fibrosis have robust long-term efficacy in the majority of patient with fibro-stenotic CD. The pressing clinical need for the development of specific anti-fibrotic agents to treat and prevent intestinal fibrosis underlines the critical importance of understanding the pathogenic processes underlying fibrogenesis and I will therefore now review our current understanding of the basic science underlying gut fibrosis.
Basic Science

1.4 Pathogenesis of intestinal fibrogenesis

Gut fibrosis is defined as the excessive deposition of ECM in the intestinal wall resulting in progressive tissue architectural distortion, loss of function and luminal narrowing. The key triggering stimulus in fibrogenesis is inflammation. In acute diseases of the intestine such as diverticulitis and peptic ulcer disease, there is short lived inflammation and epithelial layer damage. In these conditions, there is usually full restoration of mucosal integrity and normal intestinal function. However, recurrent bouts of disease leading to a more chronic recurrent phenotype, can lead to fibrotic complications in these conditions such as peptic and diverticular strictures. In CD, the intestine is exposed to recurrent injury due to aberrant interactions of microbiota, environmental stimuli, adaptive and innate immunity. This state of chronic inflammation eventually leads to trafficking and transformation of profibrogenic cells and activation of fibrotic pathways which results in intestinal fibrogenesis and eventually stricture formation as outlined in Figure 1.2 below. In this section I briefly review some of the fundamental cells and pathways involved in this process before focussing on emerging evidence of novel interconnecting pathways which are greatly changing our understanding of intestinal fibrogenesis and provide exciting new therapeutic targets.
Figure 1.2: The interplay of key cells involved in intestinal fibrosis and the growing evolution of novel networks of profibrogenic molecular mediators expanding the existing TH2 paradigm

1.4.1 Central cells involved in intestinal fibrosis

Three groups of mesenchymal cells appear to be especially important in gut fibrosis: fibroblasts, smooth muscle cells (SMCs) and intestinal sub-epithelial myofibroblasts (ISEMFS). There is increasing recognition that the interplay and transition between these three cell types appears to play a central role in fibrogenesis in the intestine.

Fibroblasts are ubiquitous ECM producing cells which play an important part in maintaining structural integrity in the intestinal tract and usually stain positive for vimentin but are usually alpha smooth actin (αSMA) negative [112]. Fibroblasts are mesenchymal cells that are derived from the embryonic mesoderm and are not terminally differentiated often becoming myofibroblasts during fibrogenesis [113]. Chemokines and cytokines produced in the inflamed...
gut such as TNF-α, Insulin-like growth factor-1 (IGF-1), Platelet derived growth factor (PDGF) and interleukin 6 (IL-6) have been shown to increase the proliferation of these cells [114]. Fibroblasts are attracted to the site of inflammation by both autocrine and paracrine mediators, the most powerful of which is fibronectin: a primarily autocrine chemoattractant in gut fibrosis [115]. Whilst there is a large expansion of fibroblasts and increase in their chemotaxis, their role appears to be secondary in gut fibrogenesis compared to ISEMFs [116].

SMCs are contractile cells containing myosin and actin within the intestinal tract which are classically described as having a central role in gut contraction and peristalsis. They also appear to have important roles in IBD: in CD, SMCs contribute to luminal narrowing by contributing to thickening of the bowel wall by directly multiplying and infiltrating the bowel wall and by promoting ECM deposition and also by its contractile properties which cause narrowing and aid stricture formation by increasing tissue stiffness which in itself promotes fibroblast and ISEMF activation [117]. Interestingly, SMCs also secrete IL-6 which is an important mediator of leukocyte trafficking in inflammatory conditions as well as playing a role in fibrogenesis in chronic inflammation by promoting ISEMF proliferation and migration [118, 119]. In addition to these actions, SMCs are themselves able to transdifferentiate into ISEMFs which probably play the most central role in fibrogenesis in CD [120].

ISEMFs are key ECM producing mesenchymal cells involved in intestinal fibrosis that are modulated by a large array of molecular mediators, cytokines and other cell types. ISEMFs have features of both fibroblasts and SMCs and can be transdifferentiated from either of these cells, recruited from adjacent tissues by mesenchymal trafficking or produced from mesenchymal stem cells/precursors. ISEMFs may also be produced at the site of fibrosis by epithelial to mesenchymal transition (EMT). EMT is a complex process through which epithelial cells lose their innate characteristics (such as cell to cell adhesion) and become migratory invasive mesenchymal cells such as ISEMFs. The role of EMT in CD pathogenesis is controversial and evidence is not yet conclusive although emerging evidence suggests that
EMT is a possible underlying process in CD fibrosis. The presence of EMT-associated molecules have been demonstrated in the fibrotic lesions of CD patients [121].

1.4.2 Interleukin 13

The fundamental principle underlying fibrosis is ECM deposition overtaking degradation; this can be caused by overstimulation of myofibroblast ECM deposition and perturbation of the activity of ECM degradation by matrix metalloproteinases (MMPs) and its regulator, tissue inhibitors of matrix metalloproteinases (TIMPs), by pro-fibrogenic mediators. One such mediator is Interleukin 13 (IL-13). IL-13 is a T-helper cell type 2 (TH2) cytokine and is implicated in fibrogenesis in the setting of TH2 inflammatory responses such as asthma [122]. It is upregulated in the intestinal strictures of patients with CD via IL-13 producing lymphoid cells which inhibit fibroblast MMP synthesis leading to excess ECM deposition and fibrosis [123]. Contractility of intestinal smooth muscle is enhanced by pre-treatment with IL-13 which is considered a crucial step in the mechanism of stricture formation in CD intestine [124]. Furthermore, the use of silencing RNA against IL-13 receptors leads to reduction in inflammation related fibrosis in murine models of colitis [125]. IL-13 is a potent stimulator and activator of transforming growth factor beta 1 (TGF-ß1) and is believed to enacts most of its pro-fibrogenic actions through the TGF-ß1 pathway [126], one of the most critical mediators involved in fibrogenesis [127].

1.4.3 TGF-ß

TGF-ß is a crucial player in the extremely complex nexus of intracellular and extracellular pathways promoting fibrosis by activating mesenchymal cells leading to ECM deposition and
fibrosis. TGF-β and its receptors are upregulated in fibrostenotic CD tissue [128]. The canonical intracellular TGF-β signalling pathway is mediated by multiple smad proteins which become phosphorylated and migrate to the nucleus activating important target genes. The TGF-β/smads pathway is directly responsible for activation of intestinal myofibroblasts and increases TIMPs and connective tissue growth factor (CTGF) expression whilst inhibiting MMP expression resulting in increased ECM deposition and decreased degradation, the hallmark central process of fibrogenesis [116]. CTGF appears to be an important co-effector molecule of the TGF-β/smads pathway and is also highly expressed in all fibrotic conditions [129]. Moreover, CTGF and a multitude of other pro-fibrogenic molecules including Angiotensin II (Ang II) can directly interact with and activate TGF-β/smads signalling [116]. Disruption of the TGF-β/smads pathway leads to reduced fibrosis in multiple organs in experimental models of fibrosis including liver [130] and intestinal fibrosis [131]. Whilst undoubtedly playing a key role in fibrogenesis, TGF-β also has multiple pleiotropic actions in inflammation, cell apoptosis and tumorigenesis with extremely complex, often contradictory actions depending on the cell type secreting it and the timing of secretion, unfortunately making it an inappropriate target for direct inhibition as an anti-fibrotic strategy. In conjunction with the existing dogma highlighting the centrality of the TH2/IL-13 axis acting through the TGF-β/smads pathway in fibrosis, an emerging paradigm links T-helper 17 (TH17) cells, interleukin 17 (IL-17) and associated molecules with fibrogenesis in IBD [111].

1.4.4 IL-17

IL-17 is a family of cytokines produced mainly by CD4 (Th1 and Th17) cells which have been implicated in fibrosis in multiple organs [132]. Early evidence of a pro-inflammatory role for IL-17A include reports that IL-23 acting via IL-17A is a key enactor of inflammation in a T cell mediated model of colitis [133], IL-17A is upregulated in the inflamed mucosa of CD patients
increased in the faeces of patients with active CD and there are increased numbers of IL-17A producing cells in the lamina propria of patients with active CD [135]. Conversely, IL-17A appears to be protective against inflammation in the CD45RBhi model of colitis where adopted transfer of T-cells from IL17A−/− mice greatly worsened disease severity [136]. Dige et al showed that whilst the number of circulating IL-17A producing CD45RO+CD4+ T-cells are significantly increased in CD patients compared to normal controls, patients who were treated with adalimumab had a 2 – 3 fold increase in IL-17A cells suggesting a possible protective role [137]. Moreover, a recent clinical trial [138] of the anti IL-17A antibody, secukinumab, in active CD not only failed to show benefit but actually showed a deterioration in symptoms in marked contrast to successful phase II trials in other inflammatory conditions [139-141]. The researchers speculated that this could be due to inhibition of the protective effects of IL-17A in the intestine as seen in earlier animal studies. Colombel et al have alternatively speculated that this could be due to a role for IL-17A in countering yeast infections (a longstanding suspected provocateur in CD inflammation) and that the clinical deterioration seen could therefore be due to overgrowth of *Candida* species leading to a disease flare [142].

Whilst the complex roles of IL-17 in CD inflammation remain unresolved, the role of IL-17A in fibrosis has continued to evolve. IL-17A promotes activation of hepatic stellate cells [143] and proliferation of cardiac fibroblasts [144]. IL-17A−/− mice also experience reduced skin fibrosis in dermatological models of fibrosis [145] whilst IL-17A is essential in fibrogenesis in animal models of pulmonary fibrosis [146]. Biancheri et al have recently investigated the role of IL-17 in CD fibrosis in humans [147]. They showed that IL-17 is overexpressed in CD strictures, myofibroblasts in these strictures possess IL-17A receptors and have reduced migratory ability and produce more collagen and TIMP1 in response to IL-17A stimulation. IL-17A is important in promoting intestinal fibrosis but given the complex biology of IL-17 and the deleterious outcomes seen with secukinumab, patient selection in any clinical study inhibiting IL-17A to treat fibrosis would need to be carefully considered. Targeted treatment of cohorts of patients
with fibrosis with limited inflammatory activity, as judged by MRI, serum markers of inflammation and endoscopic appearances, may be a safer strategy. Recent evidence suggests that only a novel specific subpopulation of IL-17A producing TH17 cells with transient C-KIT expression and stable MDR1 activity are pro-inflammatory in CD patients [148]. It remains to be seen if this subpopulation plays an important role in CD fibrosis. Specific inhibitory therapy against these cells may be a better and safer therapeutic aim than unselective inhibition of IL-17A.

1.4.5 TL1A

An emerging mediator, the TNF superfamily member, TNF-like cytokine 1A (TL1A), also known as TNF superfamily member 15, is associated with IL-17 and fibrosis. TL1A is a type II transmembrane protein predominantly expressed in endothelial cells and its expression is inducible by TNF-α and IL-1α. TL1A binds with high affinity to death receptor 3 (DR3), now known as TNFRSF25. Depending on the cell context, ligation of DR3 by TL1A can trigger one of two signalling pathways: activation of the transcription factor NF-κB, or activation of caspases and apoptosis [149]. Subgroup analysis from the secukinumab anti IL-17A study has suggested a link between lack of response and the absence of a polymorphism in the TL1A gene believed to increase TL1A activity and suggests a link between IL-17A and TL1A-driven inflammation [138]. Using the T cell transfer colitis model, Wallace and colleagues investigated this further [150]. They showed that transfer of T-cells from IL-17−/− mice to RAG−/− mice reiterated the results of the secukinumab study by worsening intestinal inflammation. Importantly, RAG−/− mice lack the recombination-activating genes RAG1 and RAG2 and as such lack the proteins necessary for immunoglobulin and T-cell receptor gene recombination [151]. T-cell transfer from mice overexpressing TL1A (TL1A transgenic mice) to RAG null mice produced even more severe colitis whilst transfer of T-cells from transgenic TL1A mice that were also IL-17
deficient (IL-17-/-) showed an improvement in inflammation compared to transfer from TL1A Tg mice alone. This is supported by earlier work in murine experimental encephalitis where TL1A was found to be crucial in TH17 differentiation and proliferation and disease severity [152]. This corroborates the concept that the TH17/IL-17 axis is involved in TL1A-driven inflammation. Conversely, recent evidence suggests that in acute inflammation, the TL1A/DR3 axis may also have some protective roles. Jia et al demonstrated that TL1A knockout mice were highly susceptible to acute dextran sodium sulphate (DSS) colitis whilst DR3 knockout mice were even more sensitive with higher mortality and inflammation scores and upregulated TH17 pathways [153].

TL1A is the only recognized ligand for its functional receptor, DR3. Previous studies report its importance in the pathogenesis of IBD: in active CD and UC, DR3 is overexpressed in macrophages, dendritic cells, and lymphocytes [154, 155]. In IBD GWAS studies, they have identified and confirmed the existence of protective and at-risk polymorphisms in the tnfsf15 (TL1A) and tnfrsf6b (DcR3) genes in patients [156-158]. Global overexpression of TL1A, results in increased collagen deposition in both the small intestine and colon [159]. Increased expression of TL1A and DR3 has been reported in the adopted transfer and trinitrobenzene sulphonic acid (TNBS) animal models of colitis, and treatment with TL1A neutralizing antibodies were shown to ameliorate this [160]. Furthermore, specific TL1A over-expression in myeloid, lymphoid or both lineages induces collagen deposition and mild fibrosis in the colon of TL1A Tg mice, with increased TGF-β and collagen α2 mRNA expression [161]. Induction of colitis by DSS administration or T-cell transfer in these mice further exacerbates inflammation and also causes small and large intestinal fibrotic disease associated with elevated IL-17 [162]. Shih et al. demonstrated that fibrosis can be prevented in this model by treatment with a TL1A neutralizing antibody: interestingly whilst fibrosis was prevented, colonic inflammation was reduced but not eliminated [163], suggesting a possible direct anti-fibrotic effect that is at least partially independent of the resolution of inflammation. They furthermore demonstrated that intestinal fibroblasts express DR3 and responded to TL1A by increased expression of
COL1-α2 and IL-31Rα in a dose dependent manner. Anti TL1A neutralising antibody treatment also reduced the total number of fibroblasts and myofibroblasts in the intestine [163]. We can therefore conclude that TL1A is a convincing central player in intestinal fibrogenesis acting in concert with IL-17. It remains to be seen how it interacts with more conventional established fibrogenic pathways. However its role in CD inflammation like that of IL-17 is contradictory with data both supporting reduction in inflammation [153] and worsened inflammation by this axis [162]. Until we fully understand the dichotomous roles of TL1A and DR3 in CD inflammation, we should apply great caution in targeting this pathway as a treatment for CD as unintended paradoxical effects may occur as seen in the secukinumab study [138].

1.5 Animal models of intestinal fibrosis

1.5.1 Conventional animal models

Advances in our understanding of intestinal fibrosis continue to lag behind progress made in other organs. One of the key difficulties in progressing our understanding of intestinal fibrosis is the relatively late presentation of clinical signs and symptoms. By the time patients become symptomatic, fibrosis is generally well advanced and severely limits our ability to investigate crucial early changes that drive fibrogenesis. Moreover, serial sampling of fibrotic tissue over time to better understand the process of fibrogenesis in CD is not possible due to the relative inaccessibility of many strictures. Therefore, representative animal models of fibrosis are a key requirement in expanding our understanding [164]. Table 1.3 outlines conventional animal models of IBD which have varying degrees of gut fibrosis often bearing little resemblance to the pattern of inflammation and fibrosis seen in CD. Nevertheless, these models have allowed us to discover many of the advances I have reviewed in this thesis and it is hoped that ongoing refinement of animal models of gut fibrosis will lead to a rapid increase in our knowledge of intestinal fibrogenesis.
<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>MODELS</th>
<th>Administration/Outcome</th>
</tr>
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<tbody>
<tr>
<td>Chemically Induced</td>
<td><strong>DSS</strong> <em>(dextran sulphate sodium)</em></td>
<td>Administered in drinking water causes permeabilisation of the mucosa and subsequent inflammatory infiltrates and fibrosis</td>
</tr>
<tr>
<td></td>
<td><strong>TNBS</strong> <em>(2,4,6-trinitro benzene sulfonic acid)</em></td>
<td>Enema administration, causes T cell-dependent transmural inflammation</td>
</tr>
<tr>
<td>Microbial</td>
<td><strong>PGPS</strong> <em>(peptidoglycan polysaccharide)</em></td>
<td>Injection into the caecal or small bowel wall induces transmural enterocolitis with severe fibrosis</td>
</tr>
<tr>
<td></td>
<td><strong>Faecal Injection</strong></td>
<td>Injection of fecal suspension into the bowel wall of colon causes aggressive colitis and transmural fibrosis</td>
</tr>
<tr>
<td></td>
<td><strong>Chronic Salmonella Infection</strong></td>
<td>Oral administration after pretreatment with streptomycin causes mucosal and transmural inflammation in the colon with extensive transmural fibrosis</td>
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<td></td>
<td><strong>AIEC</strong> <em>(adherent invasive Escherichia coli)</em></td>
<td>Gavage administration of NRG857 after pretreatment with streptomycin causes ileocolic inflammation (Th-1, Th-17-mediated) and fibrosis</td>
</tr>
<tr>
<td>Gene Knockout and Transgenic</td>
<td><strong>TGF-81 TG</strong> <strong>TBR1Δk-fib TG</strong></td>
<td>Enema administration of adenoviral vector leads to acute and chronic inflammation, ECM deposition and thickening of muscularis layers</td>
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<tr>
<td></td>
<td><strong>TGF-81 KO</strong> <strong>TGF-B/Smad KO</strong></td>
<td>Gene targeting and cloning techniques. High mortality, no significant fibrosis</td>
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<td></td>
<td><strong>MCP-1</strong> <em>(monocyte chemoattractant protein 1)</em></td>
<td>Intramural injection of adenoviral vector carrying MCP-1 in the rectum leads to transmural inflammation, collagen deposition and fibrosis</td>
</tr>
<tr>
<td></td>
<td><strong>IL-10 KO</strong> <em>(interleukin-10 knockout)</em></td>
<td>CD4⁺ T cell-mediated transmural lesions, crypt abscesses, bowel wall thickening with increased susceptibility to developing fibrosis in small intestine after ileo-caecal resection</td>
</tr>
<tr>
<td>Immune-Mediated</td>
<td><strong>T cell Transfer</strong></td>
<td>Injection of CD4⁺/CD45RB⁺ into immunodeficient mice results in a wasting disease, colitis and mild fibrosis</td>
</tr>
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Adapted from Ray et al, Current Opinion in Gastroenterology, 2014, Nov;30(6):531-8 [111]
1.5.2 SAMP1/YitFC: a spontaneous high fidelity mouse model of experimental ileitis and intestinal fibrosis

Having reviewed the broad array of induced animal models of intestinal fibrosis, we now review a key new and spontaneous murine model of intestinal fibrosis. Spontaneous models of IBD are rare and provide a unique opportunity to investigate fibrogenesis. The SAMP1/YitFc (SAMP) mouse is an animal model that develops acute and then chronic ileitis with the subsequent development of transmural ileal fibrosis [165]. A significant advantage of these mice is the ability for the first time to investigate the natural course of small bowel disease over a stable time course: prior to the onset of ileitis, during acute and chronic mucosal inflammation without fibrosis, and during the formation and progression of intestinal fibrosis and fibro-stenotic lesions. Although SAMP mice are known to possess a clear genetic predisposition to this ileitis phenotype, one disadvantage of this spontaneous model is that the precise aetiologic factors leading to the development of ileitis and ileal fibrosis is unknown. This disadvantage is also shared with the problems we encounter when studying patients with IBD and can therefore be considered as its greatest advantage, acting as an important platform for discovering novel and hitherto unconsidered fibrogenic pathways.

SAMP (senescence accelerated mouse program) mice were derived in Japan following selective breeding from the parental strain AKR/J in an effort to develop a model to study ageing [166]. A subgroup of these SAMP mice were discovered to spontaneously develop mild ileitis at 30 – 40 weeks of age and were named SAMP-Yit mice. Following transfer to the USA, a substrain developed with early onset ileitis (10 weeks), severe chronic inflammation and the development of fibrosis by 20 weeks with a high incidence of strictures by 40 weeks of age. This new strain was named SAMP1/YitFC. SAMP mice represents a model of Th1/Th2-driven chronic enteritis, with TH1 events predominating early and increasing as disease severity progresses, whilst TH2 immune responses are observed later when chronic gut inflammation is
established [167, 168]. Disease in SAMP mice is localized to the ileum, occurs spontaneously without chemical, genetic or immunological manipulation, and shares common histological features and response to therapy with human CD [165]. Hypertrophy of the ileal muscularis propria, extensive collagen deposition, and the formation of frank strictures have been reported in SAMP mice [168]; however, the progression and potential mechanisms of intestinal fibrosis in these mice has never been fully characterized.

1.6 Renin angiotensin system: classical, local and alternate pathways

1.6.1 Classical Renin-Angiotensin System

The classical view of the renin angiotensin system (RAS) until relatively recently, has been that it is primarily an endocrine system where peptides cleaved by enzymes are released into the circulation and enact important regulatory effects on blood pressure and fluid homeostasis through actions on distant target organs [169]. Under this classical view, the precursor peptide angiotensinogen is produced in the liver and released into the circulation. The enzyme renin in the kidneys is released into the circulation which then cleaves angiotensinogen to angiotensin I which in turn is cleaved to its active form Angiotensin II (Ang II) by the angiotensin converting enzyme (ACE) in the pulmonary circulation. The octapeptide Ang II then enters the circulation and acting via its main receptor, angiotensin receptor 1 (AT1), causes vasoconstriction leading to a rise in blood pressure due to increased cardiac resistance before being degraded into metabolites. Ang II also acts on the adrenal glands to promote the release of the hormone aldosterone which acts on distal tubule cells in the kidney to promote sodium and fluid retention, further raising blood pressure. Ang II also increases thirst via actions on the central
nervous system and provokes release of vasopressin which further increases blood pressure and reduces fluid loss in the kidneys. These carefully choreographed actions lead to a global rise in blood pressure and reduction in urine output and were no doubt evolutionarily essential to survival. Considerable research has focused on disruption of this pathway as a treatment for essential hypertension which led to the development of ACE inhibitors (ACEi) and then AT1 Receptor Blockers (ARBs) which are highly effective and well tolerated oral therapies for the management of hypertension. However, clinicians have long noted significant benefits of these medications well beyond their direct anti-hypertensive actions in different organs (especially heart and kidney) which alongside enormous advances in our understanding of RAS biology have led to a new paradigm of ever more complex interconnecting systemic and local networks of RAS involving multiple active peptides extending to every organ system and with wide ramifications in many disease processes including inflammation, fibrogenesis and tumorigenesis [170, 171].

1.6.2 Local and alternative RAS pathways

In addition to the classical components of RAS, there is increasing interest in other components of the pathway outlined in the simplified schematic below (Figure 1.3). In addition to the widely expressed AT1 receptor, the more sparsely expressed angiotensin II receptor 2 (AT2) long thought to be only involved in foetal development has been identified as having broader counter-regulatory and protective effects compared to AT1 [172]. A homolog of ACE, the ACE2 enzyme has been discovered to be involved in degradation of Ang II to its metabolites. Many of these previously ignored metabolites are being discovered to have active roles, in particular the main metabolite produced by ACE2 known as Angiotensin 1-7 (Ang (1-7)). Ang (1-7) acts on its receptor mas and has been discovered to have anti-proliferative, anti-
inflammatory and anti-fibrotic activities in multiple tissues and it is postulated that some of the beneficial effects of ARBs may be due to diversion of Ang II to Ang (1-7) and its actions on mas [173]. Moreover, AT2 receptors are also responsive to Ang (1-7) and other peptides of Ang II metabolism providing another possible axis for the beneficial effects of ARBs.

**Figure 1.3:** Simplified schematic showing multiple active RAS metabolites, the receptors which they act on and their opposing actions

![Angiotensinogen](image)


There is considerable evidence that in addition to the systemic circulation of Ang II from the lungs, many other organs have active local RAS networks which are involved in regional blood flow regulation, inflammation and fibrosis and fluid and electrolyte transport [173].
The heart expresses angiotensinogen, renin and AT1 & AT2 receptors and this local system has critical roles in cardiac remodelling. The kidney is also able to locally produce all the components of RAS which are able to act independently of systemic Ang II levels and play an important role in a wide variety of chronic kidney diseases. The liver is also able to express functional components of RAS and multiple subcomponents are raised in liver disease [174]. Local RAS has also been investigated in the normal gastrointestinal tract, particular the small bowel. Angiotensinogen is expressed in the epithelial and muscularis layers in rat intestine, ACE and ACE2 is expressed in high quantities in the terminal ileum and colon [175] and finally AT1 (and to a lesser extent AT2) receptors are widely expressed confirming that all the components of a fully functional local RAS are expressed in the intestine. This is of particular relevance when we consider the role of RAS in fibrosis and inflammation.

1.6.3 RAS in inflammation and fibrosis outside the gut

Most of our understanding of the role of RAS in inflammation and fibrosis comes from studies in other organs systems looking at the main effector peptide Ang II. Ang II increases reactive oxygen species and consequent tissue damage in the heart [176] and kidney [177]. Ang II is also involved in promoting chemotaxis of inflammatory cells, an effect abrogated by the ARB losartan [178]. The pro-inflammatory actions of Ang II are also partially enacted through dendritic cells (DC) which have enhanced migration, maturation and antigen presenting ability when stimulated by Ang II, a phenomenon that is reversed by ARBs [179, 180]. Ang II also appears to modulate T-cell responses as demonstrated in a murine model of experimental autoimmune encephalitis where CD4 T-cells were found to have increased levels of Ang II and increased secretion of pro-inflammatory cytokines such as IFN-γ and IL-17 and that this effect was reversed by ARBs or ACEIs resulting in improvement in the inflammatory scores [181].
In addition to being involved in inflammation that could then lead to fibrosis, increasing evidence links Ang II directly to specific fibrotic pathways. Ang II promotes renal fibrosis and cardiac fibrosis through induction of TGF-β, leading to the activation of core fibrotic pathways [69, 182]. In the lung, Ang II has been shown to increase pro-fibrogenic markers, an effect reversed by the ARB candesartan [183]. In the liver, Ang II promotes hepatic stellate cell proliferation and promotes TGF-β production and is in turn produced locally by hepatic stellate cells [184, 185]. It is upregulated in cirrhotic human liver tissue and ARBs have been shown to attenuate fibrosis in animal models [186, 187], and pilot human liver disease studies [188]. Perhaps the most definitive evidence of the deleterious effects of Ang II in many diseases comes from our clinical experience in humans. Pharmacotherapy which suppress RAS such as ACE inhibitors and ARBs decrease disease progression, reduce fibrosis, morbidity and mortality and have thus become central to the clinical management of chronic kidney and cardiac disease [189, 190].

1.6.4 Emerging role of RAS in IBD

There is emerging body of evidence corroborating a role for RAS in IBD and the pathogenesis of intestinal fibrosis. All components of the RAS pathway are expressed in the gastrointestinal tract [191] and mucosal levels of Ang II are found to be raised in the inflamed mucosa of colonic CD compared to ulcerative colitis [192]. Concentrations of ACE and ACE2 mRNA in the terminal ileum and colon are more highly expressed than in many other human tissues mirroring the commonest sites of involvement in CD highlighting a possible role in IBD pathogenesis [175]. The presence of the Angiotensinogen-6 AA genotype (which results in higher production of the Ang II precursor angiotensinogen) is significantly associated with the development of CD [193]. Serum levels of RAS subcomponents are found to be significantly altered in IBD [194].
Several murine studies support an anti-inflammatory role for ACEi/ARBs in experimental colitis. Transanal delivery of the minimally systemically absorbed ARB, deschloro-losartan, at high doses, significantly reduced markers of inflammation in the DSS mouse model [195]. The ACEi enaliprilat, when given by enema, reduces inflammation in the IL-10 knockout model of colitis [196]. Administration of the ARB valsartan in drinking water, reduced colitis severity in both the DSS and TNBS models of colitis and significantly reduced TGF-β levels in the mucosa of the TNBS mice [197]. In the only published study looking at fibrosis, Wengrower et al recently investigated the effects of losartan in the chronic TNBS colitis model [198]. Oral administration of losartan by gavage, significantly reduced fibrosis scores on histology and dramatically decreased mucosal TGF-β levels in the colon of TNBS mice. Whilst the disease distribution and pathological processes underlying this animal model have limited relevance to human IBD (TNBS mice generally develop mild fibrosis, restricted to the distal colon and limited to the mucosa without transmural involvement or strictures), the significant reduction of fibrosis and TGF-β in this study suggests that targeting of RAS using ARBs is an important putative anti-fibrotic strategy that merits further investigation in a more representative model of CD.

### 1.7 General hypothesis

Fibrosis is amongst the most serious complications that occur in a broad array of inflammatory diseases in humans. Treatment options are limited by our lack of understanding of the processes underlying fibrogenesis. Fibrosis is increasingly recognised to be a separate process from inflammation that can continue in even the absence of the triggering inflammatory stimulus through the activation of fibrotic pathways. Significant advances have been made in our understanding of these pathways in other organs but our knowledge of the key pathways underlying intestinal fibrogenesis remains limited. It is clear that there are similarities between
fibrogenic pathways in different organs and that this can guide us in understanding and treating intestinal fibrosis. As our knowledge of the complexity and ubiquity of local and systemic RAS pathways increases, I can hypothesise that RAS is likely to play a role in human CD pathogenesis as it does in liver, kidney and cardiac disease. Furthermore, I hypothesise that disruption of RAS acting through its AT1 receptor using ARBs is likely to ameliorate intestinal fibrosis.

1.8 Aims of the thesis

- The specific aims of this thesis are to investigate the following:

- Differences in Ang II receptor expression in ileal fibrosis in human CD using angiotensin receptor immunohistochemistry on archival resection specimens.

- The presence of Ang II receptors in human intestinal myofibroblasts and the effects of their stimulation with Ang II on profibrogenic gene expression.

- The progression of fibrosis in SAMP mice over a time course and the relevance of RAS subcomponents.

- The effect of the oral ARB losartan on fibrosis and inflammation in SAMP mice ileum when administered at different ages and durations of treatment.

- The development of a remission relapse model using dexamethasone in SAMP mice and the effects of co-administration of losartan on ileal fibrosis and pathways involved.
Chapter 2. Materials and Methods

2.1 Human Immunohistochemistry study

2.1.1 Ethical approval & patient selection protocol

A research protocol was designed, along with appropriate consent forms and patient information leaflets and a full ethics application was made to the National Research Ethics Service using the IRAS online application procedure. The application was heard by the Research & Ethics committee at the Leeds Central REC and approval was granted in March 2012 (REC reference 12/YH/0130). The final approved version of the patient information leaflet, consent form and research protocol are included in the Appendix.

Formal fixed paraffin embedded specimens were obtained from the archives of the Department of Pathology, Guy’s and St Thomas’ Hospitals. Possible samples were identified (in conjunction with a consultant histopathologist with a specialist expertise in IBD) from the pathology database of patients who had undergone ileal resection (or colectomy with attached ileum) from March 2005 to August 2006. Medical notes and histology reports were carefully examined for all possible cases. Any patients taking ACE inhibitors or ARBs were automatically excluded. I selected three cohorts of patient samples:

(a) Histologically normal ileum of patients who underwent right hemicolecction for colon cancer.

(b) Histologically normal ileum of patients who underwent pancolectomy for ulcerative colitis (excluding any with backwash ileitis).

(c) Diseased ileum from patients undergoing ileocecal resection due to stricturing Crohn’s disease.
2.1.2 Cutting and mounting of slides

Sections were obtained and slides prepared from the blocks following a standard protocol:

(1) Paraffin blocks were chilled on a cooling plate

(2) The block was sectioned using a Leica microtome to obtain a ribbon of sections at 5 microns thickness.

(3) Sections were floated on warm distilled water and separated using brushes

Sections were mounted onto polysine coated histology slides and then left to dry for 48 hours prior to immunohistochemistry.

2.1.3 Immunohistochemistry protocol

Immunohistochemistry for AT1 and AT2 receptors was performed on archival formalin fixed paraffin embedded ileum of patients who had undergone resection for stricturing CD (n=22), histologically normal ileum in patients undergoing colectomy for UC (n=9) or right hemicolecetomy for colon cancer (n=10). I used commercial antibodies for AT1 (SC1173) and AT2 (SC9040) from Santa Cruz Biotechnologies and the secondary antibody (Vector ImmPRESS Anti-Rabbit Ig (peroxidase) Polymer Detection Kit) under optimised conditions. Activity was demonstrated using ImmPact DAB (diaminobenzidine based) peroxidise substrate kit (Vector Laboratories) as detailed below.

1. Deparaffinisation & dehydration
   a. Deparaffinisation was conducted in xylene using three changes for 5 minutes each
b. 100% ethanol twice 3 min each.

c. 95% ethanol once - 5 min

d. 70% ethanol once - 5 min

e. Washed in tap water - 1 min

2. Antigen retrieval

   a. Antigen unmasking reagent (Vector) was prepared (concentrate stock 7.5ml + distilled water 800ml).

   b. The slides were placed in a 500ml glass beaker and immersed in 600ml of unmasking reagent

   c. The beaker was microwaved at full power for 20 - 25 min (15-20min boiling).

   d. The slides were cooled for at least 20 min

   e. The slides were then washed with distilled water three times for 5 min each.

3. Blocking

   a. Shake and wipe off excess water

   b. 75 to 100ul of horse serum blocking agent (Vector) was added and incubated 1h at room temperature.

4. Primary antibody

   a. I drain the blocking buffer off the slide and wiped around the section

   b. I added 50ul to 100ul of diluted primary antibody (diluted in 1% Bovine Serum Albumin 1:200 for SC1173 and 1:400 for SC9040), and incubated overnight at 4°C in a humidified chamber.

   c. I removed the liquid and washed with TBS three times for 5 mins each.

5. Secondary reagent & colour development.

   a. I wiped off excess TBS

   b. I then added 50µl to 100µl of diluted secondary antibody (in 1% BSA) and incubated for 1h at room temperature.

   c. The slide was washed with TBS 3 times for 5 min each.
d. Incubated with 75 – 100µl of diluted DAB chromogen (ImmPACT Dab, Vector labs) for 1-2 mins.

e. Wash with TBS. Counterstained with haemotoxylin for 1 minute.

f. Washed with running tap water for 1 minute

g. Dehydrated with rising concentrations of ethanol and clarified with xylene.

6. Cover slip

A small amount of VectaMount medium was pipetted on to the section and a glass cover slip was applied. Slides were left to dry for 7 days prior to microscopy.

2.2 CCD18Co cell studies

2.2.1 CCD18Co cell culture

I used the normal human colonic myofibroblast cell line CCD18Co cells from the ATCC, USA at passage 9.

1. Cells were rapidly defrosted in their cryovial under running warm water and resuspended in myofibroblast culture medium. This culture medium is composed of high glucose Dulbecco’s Modified Eagle Medium with added 10% Fetal Bovine Serum and antibiotics (penicillin and streptomycin) all obtained from Gibco, Life Technologies.

2. The cells were centrifuged at 180 rcf for 5 minutes.

3. The supernatant was discarded and the cells re-suspended in fresh warmed myofibroblast culture medium.

4. The cell suspension was then placed in a vented T75 culture flask and placed in a cell incubator at 37°C and 5% carbon dioxide.
5. Cells were checked daily and when reaching 75% confluence they were considered ready to passage.

6. To passage the cells, the culture medium was aspirated and the cells briefly washed in warmed phosphate buffered saline (PBS).

7. The PBS was aspirated and 2 mls of trypsin-EDTA was added and agitated to cover the T75 flask.

8. This was incubated for 1 minute at 37°C and checked under an inverted microscope to ensure optimal cell detachment.

9. The trypsinised cells were resuspended in culture medium and split 1:3. The average time between passages was 72 hours. Culture medium was changed every 48 hours.

2.2.2 Cell RNA isolation

Cell lysis

a) CCD18Co cells prior to passage 20 were selected.

b) After removal of culture medium, the cells were trypsinised and centrifuged at 185 x g for 5 minutes and the supernatant discarded.

c) Trizol reagent (Life Technologies) was added directly to the cells and the tube vigorously vortex twice for 30 seconds. Cells were further disrupted by passing through a blunt 20G RNAse free needle to lyse the cell membrane and free RNA.

Phase Separation
a) The homogenised samples were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes.

b) 0.2ml of chloroform was added to the homogenised cells.

c) The samples were vortexed for 15 seconds prior to incubation at room temperature for 2-3 minutes.

d) The samples were then centrifuged at 11,600 x g for 10 minutes at 4°C. Following centrifugation, the mixture separated into a lower red phenol chloroform phase, an interphase and an upper aqueous phase. The RNA remained exclusively in the upper aqueous phase.

RNA Precipitation

a) 0.5 ml of isopropyl alcohol was added to precipitate RNA from the aqueous phase.

b) The samples were incubated at room temperature for 10 minutes followed by centrifugation at 11,600 x g for 10 minutes at 4°C. The RNA precipitate formed a pellet on the bottom of the tube.

RNA Wash

a) The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol.

b) The sample was vortexed and centrifuged at 7,500 x g for 5 minutes at 4°C.

Re-dissolution of RNA

a) RNA pellet was briefly air-dried for 5-10 minutes, taking care not to completely dry the pellet and reduce its solubility.

b) RNA pellet was re-dissolved in RNase-free water by passing the solution a few times through a pipette tip prior to incubation at 60°C for 10 minutes.
c) The concentration of RNA in ng/μL was determined using a NanoDrop ND-1000 Spectrometer. A “260/280” ratio was also recorded to ensure adequate re-dissolution of RNA and ascertain RNA purity. To ensure accurate loading of RNA during the polymerase chain reactions (PCRs), RNA concentrations were standardised to 100ng/μL by diluting samples with RNase free H2O.

2.2.3 Determination of RNA integrity by denatured gel electrophoresis

Whilst spectrophotometry was able to determine purity and lack of salt contamination of RNA, it is not able to accurately determine RNA integrity. To confirm that any RNA used for cDNA manufacture and subsequent qPCR was not degraded, a simple bleach denatured gel electrophoresis technique was used as outlined by Aranda et al [199].

1. 0.5 g of agarose (Thermofisher, USA) was added to 50 mls of rtase free TAE buffer (Thermofisher, USA).
2. 500 μl of Clorox bleach was added to the solution and it was microwaved until the solution was boiling and the agarose had dissolved.
3. The solution was cooled and then ethidium bromide (Sigma) was added to a final concentration of 0.5 μg/ml.
4. The solution was poured into a mould with combs inserted to make the wells.
5. 500 ng of RNA (dissolved in DNA loading buffer (Invitrogen)) was loaded into each well and the first well was used for a DNA ladder (Hyperladder I, Bioline).
6. The gel was immersed in TAE buffer and electrophoresed for 30 minutes at 90V, 60mA. Subsequent to electrophoresis, the bands were visualised under an ultraviolet transilluminator (UVP Laboratory Products, EPI Chemi II Darkroom). High quality RNA was determined by an intense clear band at 4.8 Kbp (representing 28S ribosomal RNA),
and a clear band which is approximately half as intense as the top one at 2 Kbp (representing 18S ribosomal RNA).

### 2.2.4 Manufacture of cDNA and genomic DNA elimination

CDNA was manufactured using the Quantitect Reverse Transcription Kit (Qiagen). This kit not only synthesises cDNA but also effectively eliminates genomic DNA. The Quantitect Reverse Transcription procedure comprises of 2 main steps.

1. **Elimination of Genomic DNA**
   
   Incubation of purified and quality screened RNA samples with the supplied gDNA wipeout buffer eliminates genomic DNA. The RNA samples were incubated at 42°C for 2 minutes in a thermal cycler.

2. **Reverse Transcription**

   The total product was then used in reverse transcription. I added Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix, and vortexed. The tubes were then incubated at 42°C for 15 minutes and then incubated at 95°C for 3 minutes to inactivate the reverse transcriptase.

### 2.2.5 Primer Design

Gene specific primers were designed as below and manufactured by Qiagen and diluted to an appropriate concentration in TE buffer.

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AGTR1</strong></td>
<td>GGCTATTGTTCAACCAATGAAGT</td>
</tr>
<tr>
<td><strong>AGTR2</strong></td>
<td>GTACCATGGTCATCTACCCCT</td>
</tr>
</tbody>
</table>
Nucleotide base sequences were obtained from GenBank and processed using Primer-BLAST for design of gene-specific primers. Primers were designed to anneal to exon-intron-exon or exon/exon boundaries. It was also verified that the primers were not self-complementary or complementary to each other at the 3’ ends.

2.2.6 Reverse transcription polymerase chain reaction

PCR was performed using the Taq PCR Mastermix kit from Qiagen which contains a ready prepared mix of Taq DNA polymerase, dNTPs and optimised PCR buffer to reduce pipetting and contamination. CDNA manufactured from the CCD18Co cells was added to the mastermix alongside the primers for AGTR1 (AT1 receptor) and AGTR2 (AT2 receptor) in 200 µl RNAse free eppendorf PCR tubes and then placed in a thermal cycler. Initial denaturation was conducted at 94°C for 3 minutes followed by 30 cycles of amplification as per manufacturer’s instruction at an annealing temperature of 55°C.

2.2.7 DNA electrophoresis and imaging

Amplification products of the RT-PCR were mixed with loading dye and then loaded onto 1.5% agarose gel containing ethidium bromide alongside a DNA ladder (Hyperladder IV, Bioline) and electrophoresed for 1 hour at 90V, 60mA. Subsequent to electrophoresis, the bands were visualised under an ultraviolet transilluminator (UVP Laboratory Products, EPI Chemi II Darkroom).
2.2.8 Western blotting

1. CCD18Co cells that had been loosened from their flasks using trypsin/EDTA solution were washed in PBS and pelleted.

2. The cell pellets were homogenised in ice cold RIPA buffer (150nM sodium chloride, 1% Triton X 100, 0.5% sodium deoxycholate, 50mM Tris, pH 8.0 and 0.1% SDS).

3. The cell lysate protein concentration was determined using the copper/bicinchoninic assay (Sigma), and standardised to 2 mg/ml by dilution into Laemmli buffer.

4. 20μg total protein was loaded into a cassette to undergo SDS-PAGE (Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis).

5. After electrophoresis, Separated proteins were transferred onto a PVDF Immobilon P membrane (Millipore, Billerica, MA).

6. The membrane was incubated in blocking buffer for 1 hr (0.5% non-fat dehydrated milk, 0.5% TBS Tween).

7. The blocked membrane was then washed three times using wash buffer (Tris buffered saline with 0.5% Tween).

8. The membranes were then incubated at 4°C overnight in primary antibody solution (the antibodies used for the CCD18CO Western blot experiments were sc-1173 for AT1 (Santa Cruz) used at 1:400 concentration, sc-9040 for AT2 (Santa Cruz) used at 1:400 and sc-47778 for β-actin (Santa Cruz) used at 1:600.)

9. After overnight incubation, the membrane was thoroughly washed three times in TBS-Tween solution.

10. The membrane was then incubated in secondary antibody solution (goat anti-rabbit antibody (Cell Signalling) diluted in TBS-T containing 5% BSA) for 1 hour at room temperature

11. The membrane was once again thrice washed with washing buffer.
12. The washed membrane was then prepared for enhanced chemoluminescence using the Pierce™ ECL Western Blotting kit and imaged on Kodak film in a dark room.

13. Densitometry software (ImageJ 1.50i, National Institutes of Health, USA) was used to analyse the differences in protein expression levels adjusted for levels of expression for β-actin to correct for small differences in protein loading between samples of CCD18CO cells.

2.1 SAMP mice experiments

2.1.1 SAMP and AKR/J breeding and handling protocol

SAMP and AKR/J mice for these experiments were obtained from them main mice colonies kept in Case Western Reserve University’s animal facilities which are certified specific pathogen free. SAMP mice are poor breeders and therefore require specialised breeding protocols as outlined below and these same protocols are used to raise the AKR/J colonies

1. The mice were placed in microisolator cages with a filtered lid and housed in ventilated racks which provide HEPA filtered air to the cages.

2. Breeding cages were set up in trio with two female mice (each 6 weeks of age) and one male mouse (more than 8 weeks of age).

3. Food pellets were placed in a grid above the mice with a water bottle for drinking.

4. A square of cotton was provided in each breeding cage to provide nesting material.

5. Feed and water were replaced at least twice weekly and the cage changed weekly.

6. All mouse handling was carried out in strict aseptic conditions inside a HEPA filtered laminar flow hood.
7. Mice were monitored daily for signs of pregnancy and litter. Once a litter of pups was delivered, the cages were not disturbed for a minimum of seven days as SAMP are very prone to cannibalise their young if touched during this period.

8. Pups were weaned at 3 weeks of age and housed into single sex groups of five male or five female mice until the correct age for the experiments was reached.

2.1.2 Euthanasia protocol and tissue harvesting & preparation of tissue for histology

All experimental protocols using animals were fully approved by the IACUC (The Institutional Animal Care and Use Committee, Case Western Reserve University).

At the conclusion of each experimental protocol, the mice were euthanised and tissue prepared in the following method:

1. Mice were individually restrained and injected with 1ml of 2.5% of tribromoethanol in PBS. This deliberate overdose of tribromoethanol quickly leads to induction of deep anaesthesia within one minute.

2. The anaesthetised mice were then stimulated to confirm adequate anaesthesia using pedal pinch and loss of righting reflex. These mice were then euthanised by cervical dislocation.

3. Midline laparotomy was rapidly performed following euthanasia and the ileum dissected out. The ileum was flushed with copious volumes of ice cold PBS before longitudinally opened using scissors. The cut open and cleansed ileum was then laid on a cork board from proximal to distal and pinned in position.
4. Using a clean scalpel, a small ribbon of tissue was removed from one edge of the ileum for RNA extraction. This was immediately placed in RNALater solution (Invitrogen, USA) and stored at -20°C until ready for RNA isolation.

5. The cork board and attached ileum were then placed in a 50 ml tube and immersed in Bouin’s solution (Picric Acid-Formalin-Acetic Acid Mixture from Ricca Chemical, Texas, USA).

6. After 24 hours of fixation the sample was copiously washed 3 times with 70% ethanol and then immersed in 70% ethanol for another 24 hours.

7. The ileum was then embedded in paraffin and sectioned and mounted on slides as outlined in section 2.1.2.

2.1.3 Haematoxylin & eosin staining

1. Sections were brought to water by first immersing the slides in xylene to deparaffinise them (2 x 10 minutes).

2. Sections were then immersed in 100% ethanol x 2 for 5 minutes each.

3. Sections were then immersed in 90% ethanol x 2 for 5 minutes each.

4. Sections were then immersed in 70% ethanol x 2 for 5 minutes each.

5. Sections were then washed in distilled water x 3 for 5 minutes each.

6. Sections were immersed in filtered Harris Haematoxylin for 60 seconds.

7. Sections were rinsed with water until the water remained free of dye.

8. Sections were then immersed in Eosin stain for 2 minutes.

9. Subsequent to washing, slides were dehydrated in ascending alcohol solutions – 50%, 70%, 80%, 90% (x2) and 100% (x2).

10. To remove impurities, stained slides were soaked in xylene.

11. Slides were coverslipped with VectaMount gel as an adhesive.
2.1.4 Masson’s trichrome staining

1. Sections were brought to water with xylene and alcohol as outlined in section 2.1.3. I used the Polysciences Masson’s Trichrome Kit for all the reagents and instructions.

2. Sections were then mordanted in Bouin’s solution at 60°C for 1 hour

3. Sections were then wash in running tap water to remove the picric acid for 5 minutes.

4. Sections were then stained in Weigert’s iron hematoxylin working solution for 10 minutes. (To prepare Weigert’s iron hematoxylin working solution mix Weigert’s Hematoxylin A and Weigert’s Hematoxylin B at a 1:1 ratio).

5. Sections were washed in running tap water for 5 minutes and then rinse repeatedly in distilled water.

6. Biebrich Scarlet - Acid Fuchsin Solution for 5 minutes.

7. Sections were then rinses in distilled water.

8. Phosphotungstic/phosphomolybdic acid for 10 minutes, discard solution.

9. The slide was drained and then transferred to Aniline Blue for 5 minutes.

10. Sections were then rinse in distilled water (3 changes).

11. Sections were immersed in 1% Acetic acid for 1 minute, discard solution

12. Sections were then rinsed in distilled water.

13. Dehydrate in 95% ethanol, then 100% ethanol for 1-2 minutes each.

14. Clarification was performed in xylene for 1-2 minutes.

15. Slides were coverslipped with VectaMount (Vector Labs, USA) as an adhesive.
2.1.5 Total inflammatory score template

The following scoring template (Table 2.1) was used by an expert GI histopathologist to score the TIS for every sample. They were blinded to the group and experimental conditions.

<table>
<thead>
<tr>
<th>Slide #</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>L</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Villus distortion (0-3)</td>
<td>% cross sectional area (0-4)</td>
<td>Villus distortion index (A*B)</td>
<td>Active inflam. (0-3)</td>
<td>% cross sectional area (0-4)</td>
<td>Active inflam. index (D+E)</td>
<td>Mononuclear inflammation (0-3)</td>
<td>% cross sectional area (0-4)</td>
<td>Mononuclear inflammation index (C<em>D</em>E)</td>
<td>Chronic inflam. (0-3)</td>
<td>% cross sectional area (0-4)</td>
<td>Chronic inflam. index (F*K)</td>
<td>Total score (C+F<em>G</em>I+H)</td>
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<td>2</td>
<td>1</td>
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<td>1</td>
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<td>1</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

*Table 2.1: Example histology scoring sheet for calculating total inflammatory score*

2.1.6 Total fibrosis score template

The following table (Table 2.2) shows an example scoring template that was used by an expert GI histopathologist to score the TFS for every sample. They were blinded to the group and experimental conditions.

<table>
<thead>
<tr>
<th>Slide #</th>
<th>Depth of fibrosis (1-4)</th>
<th>Area involved (1-4)</th>
<th>Density of fibrosis (1-4)</th>
<th>Score (C<em>D</em>E)</th>
<th>Description</th>
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<tr>
<td>23915</td>
<td>3</td>
<td>2.5</td>
<td>2</td>
<td>15</td>
<td>MODERATE</td>
</tr>
<tr>
<td>24015</td>
<td>3</td>
<td>3</td>
<td>1.5</td>
<td>13.5</td>
<td>MODERATE</td>
</tr>
<tr>
<td>24115</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>MODERATE</td>
</tr>
<tr>
<td>24215</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>MODERATE</td>
</tr>
</tbody>
</table>

*Table 2.2: Example histology scoring sheet for calculating total fibrosis score*
2.1.7 Isolation of RNA from tissue

1. RNA was isolated from tissue using the Qiagen RNEasy Mini Kit (Qiagen, Netherlands).
2. Prepare buffer RLT by adding 10 µl of β-mercaptoethanol (Gibco, USA) to each ml of RLT buffer. This can be kept at room temperature for up to 1 month.
3. Weight tissue (no more than 30µg). Place in homogeniser tubes containing FastPrep® Lysis Beads (MD Bio, USA).
4. Directly pipette 400 µl of RLT to each homogeniser tube.
5. Homogenise tissue for 30 seconds. Centrifuge the lysate at maximum speed for 3 mins.
6. Remove supernatant and place in RNAse free Eppendorf tube.
7. Add 350 µl of 70% ethanol to the samples and mix by pipetting.
8. Transfer 700 µl of each sample to a RNeasy Mini column in a 2 ml tube.
9. Centrifuge for 15 seconds at >8000 x g. Discard flow through.
10. Prepare DNase I by injecting 550 µl supplied buffer into the vial. Do not vortex. Mix by gentle inversion. Aliquot to samples and store at -20°C for up to 9 months.
11. Add 350 µl of Buffer RW1 to each column. Close lid and centrifuge at >8000 x g for 15 seconds. Discard flow through.
12. Add 10 µl of DNase solution to 70 µl of buffer RDD. Mix by inversion and spin down briefly. Add 80 µl of this solution to each column.
13. Incubate at room temperature for 15 minutes.
14. Add 350 µl Buffer RW1 to each column. Close lids and centrifuge at >8000 x g for 15 seconds. Discard flow through.
15. Add 500 µl Buffer RPE to each column. Close lids and spin at >8000 x g for 15 seconds.
16. Add 500 µl buffer RPE to each column. Close lids and spin at >8000 x g for 2 minutes.
17. Discard flowthrough. Respin columns to dry for one minute.
18. Transfer columns to 1.5 ml tube (included). Add 30 µl RNAse free water to each column. Centrifuge at 8000 x g for 1 minute to elute RNA.

19. Test RNA on nanodrop. If yield is > 30 ug – repeat step 16 one more time to get even more RNA out.

20. Check RNA integrity on a bleach gel as outlined in section 2.2.3

21. CDNA produced for use in realtime PCR as outlined in section 2.2.4.

2.1.8 Quantitative Real Time PCR Protocol

Real time PCR was performed in a Roche lightcycler machine using Power SYBR™ Green PCR Master Mix (Applied Biosystems, USA) The formulation contains highly purified AmpliTaq Gold® DNA Polymerase, LD to offer greater sensitivity than classic SYBR® Green PCR master mix. The mix includes a proprietary version of ROX™ dye, an internal passive reference, to normalize non-PCR–related fluorescence fluctuations to minimize well-to-well variability that result from a variety of causes, such as pipetting error and sample evaporation. Mixing error is further reduced by using one mixture containing all the reagents for PCR excluding the template cDNA and primers.

PCR was performed as per manufacturer’s instructions. In brief:

1. cDNA, reverse and forward primers and PCR mastermix were added to PCR plates at the recommended concentrations in triplicate.

2. Beta-actin was selected as the housekeeping gene in all our experiments after initial experiments examining the appropriateness and stability of expression of five different housekeeping genes showed it was the most suitable choice.

3. qPCR was performed in a Lightcycler 480 instrument with annealing temperatures set according to the recommended temperature for the primers.
4. qPCR data was analysed using the ΔΔCt algorithm. The housekeeping gene, beta-actin, was assumed to be uniformly and constantly expressed in all samples and compared to relative changes in target gene expression. This was then transformed to fold change in expression compared to control.

2.1.9 GeneChip™ Mouse Gene 2.0 ST Micro Array

2 representative samples (DXM vs DXM + losartan) were sent to the Case Western Gene Expression and Genotyping facility to run on a microarray. The whole process was carried out by the core facility but here follows in brief the protocols used. Firstly the RNA quality was rechecked by running on a bleach denatured gel as outline in main methods. The best 2 samples were then diluted to 50 ng/µl and taken to the core facility where they underwent further quality testing including analysis on the Agilent Tape Station for RNA integrity analysis. Qualified samples were then converted to labelled cRNA using NuGEN Ovation Standard labelling protocol and quality checks performed to confirm accurate labelling. Samples were then fragmented and added to a hybridization cocktail. The cocktail was then run on the Affymetrix Genechip Mouse 2.0 ST microarray (Thermofisher, USA)

Bioinformatics analysis: data from the microarray was extracted into a CEL file as raw data. This was analysed by a bioinformatician from the Case Western Gene Expression and Genotyping facility using Affymetric software.

2.1.10 Statistics

All statistical analysis in this thesis were conducted using the statistics module within the Graphpad Prism software.
For the majority of the experiments, two groups were being directly compared. For this I used the unpaired student’s t test to compare the mean differences between 2 independent but identically distributed data sets. Although randomisation is not a prerequisite for this test, mice/cells were still randomly assigned to either the control or treatment arm. The paired student’s t test was considered unsuitable for this thesis as repeated measures from the same sample, were not being analysed except for the change in weight experiments where this was employed.

A two-way ANOVA was used to study the interaction effects between two or more variable factors across the data sets. $P < 0.05$ was considered statistically significant.
Chapter 3. Investigating the presence of Ang II receptors on intestinal myofibroblasts and the effects of their modulation on profibrogenic gene expression

3.1 Introduction

The exact cellular mechanisms underlying intestinal fibrosis are complex and incompletely understood. There is however increasing appreciation that myofibroblasts play a central role in fibrogenesis. Myofibroblasts are mesenchymal cells in the intestine which normally reside in the submucosa and muscularis of the intestine. In fibrosis, there is a significant increase in their number and infiltration into the epithelial layers of the intestine.

There are two principal varieties of intestinal myofibroblast with greatly differing functions: interstitial cells of Cajal (ICCs) and ISEMFS. ICCs were described more than 100 years ago as stellate shaped cells present as networks located in a layers of the muscularis propria in gastrointestinal tissues including colon and ileum [200]. Since their discovery, three principal overlapping functions have been attributed to ICCs including mediating enteric neurotransmission, acting as intestinal pacemakers and helping to propagate electrical activity within the enteric muscles [170, 199, 201]. No direct role for ICCs has been discovered in intestinal fibrogenesis although they are shown to be reduced in CD intestine compared to normal controls [202]. It is an intriguing unexplored possibility that loss of ICCs or dysfunction in their networks could conceivably play a role in the regional dysmotility, intestinal muscle constriction and consequent stricture formation seen in fibro-stenotic CD.
As our understanding of gut fibrosis has gradually increased, ISEMFs have taken centre place and are considered one of the most important cells participating in intestinal fibrogenesis although there are many significant gaps remaining in our knowledge of their biology and function. Therefore in this chapter, I briefly review our knowledge of the location, function and roles of these cells before investigating whether Ang II acting via its receptors plays any role in their functionality.

ISEMFs are actually found throughout the subepithelial layers of the normal gastrointestinal tract from the oesophagus to the anus, but most of our knowledge about them comes from studies in the colon and small intestine [203]. ISEMFS found in the region of crypts are morphologically oval and scaphoid whereas those found in the ileal villi and colonic crypts are more stellate [204, 205].

ISEMFS share characteristics with both fibroblasts and smooth muscle cells and, as alluded to in the previous chapter, it is likely that there is plasticity between these three cell types allowing ISEMFs to be derived from them both by transdifferentiation through EMT and/or endoMT in the right profibrogenic environments [206]. Consequently, differentiating ISEMFs from SMCs and fibroblasts requires a panel of markers unless ultrastructural details can be studied using electron microscopy. ISEMFs stain positively for αSMA like SMCs whilst also staining positively for vimentin like other intestinal fibroblasts [207]. ISEMFs stain negatively for desmin (unlike SMCs and fibroblasts) except in exceptional circumstances of intestinal injury where they may be weakly positive [208]. Therefore ISEMFs are classified as αSMA positive, vimentin positive and desmin negative cells as summarised below in Table 3.1.
<table>
<thead>
<tr>
<th></th>
<th>αSMA</th>
<th>Vimentin</th>
<th>Desmin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SMCs</strong></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Fibroblasts</strong></td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Myofibroblasts</strong></td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**Table 3.1:** Antibody marker reactivity for common intestinal mesenchymal cells in normal intestinal tissue

In strictured CD tissue (as seen in the histology from the previous chapter), there is a marked increase in the number of ISEMFS, SMCs and fibroblasts in all layers of the intestine but especially within the muscularis with surrounding increased deposition of collagen and other ECM materials and a massive increase in the thickness of this layer [37]. Histological and immunostaining studies show that there is an increase in type III collagen whilst in situ hybridisation studies show an increase in procollagen transcripts such as procollagen I and III within this ECM [209].

During intestinal fibrogenesis, it is unclear if the characteristic increase in matrix production seen in is as a result of activated fibroblasts, smooth muscle cells, or myofibroblasts or a combination of all three. Whilst established markers for myofibroblasts in normal intestine, as outlined in Table 3.1, are widely accepted, the markedly abnormal tissues of stricturing CD creates problems differentiating myofibroblasts from SMCs. Nevertheless, due to the presence in these cells of more prominent stress fibres, and extensively, dilated endoplasmic reticulum (cardinal features of myofibroblasts), these cells are very likely to be ISEMFS rather than SMCs [203, 210]. It is possible that the occasional desmin positivity comes about because some of those cells may have transdifferentiated from other cells to myofibroblasts e.g. from SMCs and kept their previous markers.

The activation and regulation of ISEMFS in fibrosis and their activity is very poorly understood. Recent studies have demonstrated that central factors known to be important in the
pathogenesis of CD inflammation and subsequent fibrosis such as TNF-α and IL-17 can also activate ISEMFs and stimulate ECM production [210, 211]. Furthermore, emerging molecules likely to be involved in fibrogenesis such as TL1a have recently been shown to be intricately involved in regulation and activity of ISEMFs [212]. There has never been any investigation of the presence of RAS and Ang II receptors and their effects on ISEMFs within the GI tract.

Aims:

- To evaluate the presence of the principle RAS receptors AT1 and AT2 in an ISEMF cell line using PCR and Western blotting
- Evaluate the effect of exogenous Ang II on transcription of key profibrogenic genes in quiescent ISEMFs and review if they are activated by them and the effects of the antagonist losartan on this.
- Evaluate the effect of exogenous Ang II on transcription of key profibrogenic genes in pre-activated ISEMFs and the effects of the AT1 antagonist losartan on this.

My hypothesis is that AT1 receptors are present on ISEMFs and that they will stimulate cell activation and profibrogenic gene expression. AT2 receptors are likely to be absent or expressed in very low quantities.
3.2 Methods

3.2.1 Cell culture

CCD18Co cells are a well-established ISEMF cell line originally obtained from a biopsy taken from a 2 month old infant’s colon [213]. They were cultured as outlined in section 2.2.1. In brief, CCD18Co cells were obtained from ATCC (Manassas, VA, USA) and cultured in T75 vented flasks in an incubator set at 37°C and 5% CO$_2$. They were cultured in myofibroblast culture medium as per manufacturer’s recommendations; this culture medium is composed of high glucose Dulbecco’s Modified Eagle Medium with added 10% Fetal Bovine Serum and the antibiotics penicillin and streptomycin (all obtained from Gibco, Life Technologies). Cells were passaged every 2 – 3 days 1:3 and used in experiments between passages 9 and 15. Half the cells were pre-stimulated with TGF-β1 at a concentration of 1ng/ml for 24 hours prior to harvest. This concentration was picked based on a review of the literature and in particular experiments conducted by Simmons et al, showing this concentration to be the optimal dose for maximal effects in CCD18Co cells [114].

3.2.2 Cell RNA extraction, quality control and cDNA manufacture

CCD18Co cells from 2 separate culture flasks of each condition (unstimulated and prestimulated) were trypsinised, washed and lysed and underwent RNA extraction using Trizol as fully explained in section 2.2.2. RNA integrity was checked by electrophoresis on a bleach denatured RNA. This RNA was then quantified and standardised to 100ng/μl. I then used 1μg of this RNA to produce cDNA using the Qiagen Quantitect Reverse Transcription kit with integrated genomic DNA elimination as per manufacturer’s guidelines.
3.2.3 Primer design, Conventional PCR and Gel Electrophoresis

Gene specific primers were designed as below and manufactured by Qiagen and diluted to an appropriate concentration in TE buffer.

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tr>
<td><strong>AGTR1</strong></td>
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</tr>
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<td>GGCTATTGTCACCCAATGAAGT</td>
<td>TGGGACTCATAATGGAAGCAC</td>
</tr>
<tr>
<td>GTACCAATCTGTACCATCTACCCCT</td>
<td>CAGGCCCCACACACAAAGG</td>
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</table>

Nucleotide base sequences were obtained from GenBank and processed using Primer-BLAST for design of gene-specific primers. Primers were designed to anneal to exon-intron-exon or exon/exon boundaries and cover all main transcript variants. It was also verified that the primers were not self-complementary or complementary to each other at the 3’ ends.

Conventional PCR was conducted as outlined in main materials and methods (see section 2.2.6) but specifically, samples were prepared using the Quantitect mastermix, the appropriate primers for AGTR1 (AT1 receptor) or AGTR2 (AT2 receptor) and the previously manufactured CCD18Co cDNA. Samples were placed inside a thermal cycler (Applied Biosystems). Initial denaturation was conducted at 94°C for 3 minutes followed by 30 cycles of amplification as per manufacturer’s instruction at an annealing temperature of 55°C. The samples underwent gel electrophoresis for 1 hours on a 1.5% agarose gel and the DNA visualised using ethidium bromide under a UV lamp.
3.2.4 Western Blotting Protocol

Samples of CCD18Co cells from 2 separate culture flasks were prepared in RIPA buffer and underwent Western blotting as fully outlined in main methods (section 2.2.8). Specifically the antibodies used for the Western blot were sc-1173 for AT1 (Santa Cruz), sc-9040 for AT2 (Santa Cruz) and sc-47778 for β-actin (Santa Cruz). Densitometry software (ImageJ 1.50i, National Institutes of Health, USA) was used to analyse the differences in protein expression levels adjusted for levels of expression for β-actin to correct for small difference in protein loading between samples. Beta actin was chosen as the housekeeping gene as this is readily available, extensively used in our lab with established protocols and is also widely used in the published intestinal fibrosis literature [147, 205, 206].

3.2.5 Naïve CCD18Co cell stimulation/inhibition study with Ang II and losartan protocol

CCD18Co cells cultured to 70% confluence (between passages 9 – 15) were trypsinised, washed and resuspended as previously outlined in main methods but this time in incomplete myofibroblast culture medium (serum free). The cell suspensions were then counted manually using a haemocytometer and adjusted to give a final concentration of 500,000 cells per ml. The cells were carefully pipetted into 6 wells plate giving a final cell count of 1,000,000 cells in each well. The plates were covered and placed in the incubator for 24 hours to allow the cells to adhere. Each well was checked carefully under a microscope to confirm even distribution and good attachment. No or negligible dead/detached cells were detected during any of these experiments. The culture medium was removed carefully without disturbing the cells and replaced with control serum (standard serum free DMEM with antibiotics), low dose
angiotensin II (Ang II from Sigma diluted in culture medium to a concentration of $1 \times 10^{-9}$ mol/l = 1 nanogram/l), high dose ang II (Ang II from Sigma diluted in culture medium to a concentration of $1 \times 10^{-7}$ mmol/l = 100 nanogram/l, losartan alone ($10^{-7}$ mol/L) and the last group containing high dose ang II and losartan together (Ang II $10^{-7}$ mol/L + losartan $10^{-7}$ mol/L). These concentrations were picked from previous studies of Ang II on hepatic stellate cells [184]. All cell conditions were repeated in duplicate and the cells harvested for RNA after 24 hours of stimulation. qPCR was performed for a panel of profibrogenic genes.

3.2.6 TGF-β1 Preactivation and CCD18Co cell stimulation/inhibition study with Ang II and losartan protocol

This experiment essentially repeated the experiment from section 3.2.5 above with some alterations in the set up. Cells were plated as above in 6 well plates but the serum free medium was spiked with TGF-β1 at a concentration of 1ng/ml (obtained from a previous study where this was shown to be optimal for activating CCD18Co cells [114]). After 24 hours, the culture medium was removed and the cells washed and checked. New serum free culture medium was added containing the following conditions: serum free alone (control), high dose Ang II ($10^{-7}$ mol/L), Losartan alone ($10^{-7}$ mol/L) and the last group containing high dose ang II and losartan together (Ang II $10^{-7}$ mol/L + Losartan $10^{-7}$ mol/L). [184]. All cell conditions were repeated in triplicate and the cells harvested for RNA after 24 hours of stimulation. qPCR was performed for a panel of profibrogenic genes.
3.2.7 Quantitative PCR

Quantitative PCR was performed on all the cell stimulation samples as described in the main methods section. Specifically, the primers used for the profibrogenic panel were commercially validated and manufactured human primer pairs from Qiagen (RT² Primer assays for human tgfβ1, il13, igf1, col1a1, col3a1).
3.3 Results

3.3.1 ISEMFs in culture express AGTR1 (AT1) but not AGTR2 (AT2) as demonstrated by RT-PCR

After initially validating the primers by using known positive and non-template controls for the Ang II receptors agtr1 (AT1) and agtr2 (AT2), I was then able to confidently investigate Ang II receptors expression in ISEMFs. Figure 3.1 demonstrates that there is significant agtr1 expression in both unstimulated and stimulated CCD18Co ISEMFs and there is a visible trend towards increased agtr1 mRNA transcription in cells that have been stimulated compared to unstimulated cells. I have also demonstrated the absence of any detectable AGTR2 mRNA in unstimulated CCD18co ISEMFs and that prestimulation with TGF-β1 does not lead to induction of any detectable AGTR2 mRNA expression. Having demonstrated difference in mRNA expression I decided to proceed to Western blotting to review protein expression.
Figure 3.1: RT-PCR of AGTR1 and AGTR2 in controls, unstimulated and stimulated CCD18Co cells. Panel A shows a gel electrophoresis with +ve controls for AGTR1 and AGTR2 (HepG2 cDNA) and non template controls. Panel B shows the differential gene expression of agtr1 (AT1) and agtr2 (AT2) in CCD18Co cells. CCD18Co cells were incubated for 24 hours in 6 well plates in serum free medium (unstimulated, n=2) or serum free medium spiked with 1ng/ml TGF-β (stimulated, n=2) prior to being lysed and mRNA extracted. This was then used to produce cDNA and run on an 1.5% agarose gel and visualised in a UV lamp using ethidium bromide. The gel shows the strong presence of AGTR1 in both unstimulated and stimulated cells and little to no AGTR2 in both unstimulated and stimulated cells.
3.3.2 **Intestinal myofibroblasts in culture possess AT1 receptors but not AT2 receptors as demonstrated by Western blotting**

Our gene expression analysis using RT-PCR for Ang II receptors showed differences in AT1 and AT2 expression and also between stimulated and unstimulated cells and so I decided to further assess this by conducting Western blotting and then using densitometry analysis to appraise differences between naïve cells and TGF-β1 pretreated cells. **Figure 3.2** shows the results of Western blotting of protein lysates from CCD18Co cells. Once again, the results show strong expression of AT1 receptors in both stimulated & unstimulated CCD18Co cells. There was an absence of AT2 receptors in the unstimulated cells & stimulation with TGF-β1 did not alter this.

**Figure 3.2**: Western blotting to determine protein expression of AT1 and AT2 in controls, stimulated and unstimulated CCD18CO cells. *This Western blot shows differential gene expression of agtr1 (AT1) and agtr2 (AT2) in CCD18Co cells. CCD18Co cells were incubated for 24 hours in 6 well plates in serum free medium (unstimulated, n=2) or serum free medium spiked with 1ng/ml TGF-β (stimulated, n=2) prior to being lysed and protein extracted. A non-template control (antibody only without protein n=2) and positive control (HEPG2 cell lysate) were also loaded onto a separate blot but added to this figure for comparison (acknowledging that ideally they should have been run simultaneously on the same gel as the other samples).*

**Figure 3.3** demonstrates the results of analysing the Western blot using densitometry software and then using ANOVA (with Bonferroni correction for multiple comparisons) for statistical
analysis. This shows that TGF-β1 stimulated CCD18Co cells have higher levels of AT1 expression compared to naïve CCD18Co cells (p<0.01); an expression pattern supporting the trend seen in Figure 3.1.

**Figure 3.3: Densitometric comparison of AT1 and AT2 expression in unstimulated vs stimulated CCD18Co cells.** The western blot in the previous figure was analysed using densitometric software. The results were statistically analysed using ANOVA (with Bonferroni correction for multiple comparisons). This demonstrates a statistically significant increase in AT1 expression in TGF-β stimulated cells vs unstimulated cells and no significant AT2 expression regardless of stimulation.
3.3.3 Exogenous administration of Ang II in isolation to ISEMFs is insufficient to activate the cell and doesn't affect the expression of profibrogenic genes.

Having conclusively demonstrated the presence of AT1 receptors in CCD18CO ISEMFs, I proceeded to study the effects of stimulating and antagonising these receptors. Figure 3.4 shows the results of our initial cell studies using naïve CCD18Co cells. I used qPCR and ANOVA for statistical analysis (conducted using Graphpad Prism 5.03) which surprisingly showed no statistically significant differences in the expression of the profibrogenic genes (tgfβ1, il13, igf1, col1a1 and col3a1) in the control cells and cells treated with low dose Ang II, high dose Ang II, losartan alone and losartan combined with high dose Ang II.
Figure 3.4: The effects of Ang II and losartan stimulation in quiescent CCD18CO cells using real time PCR to determine key profibrogenic gene expression. CCD18Co cells were plated in 6 well plates in serum free medium overnight. The cells were then washed and the medium replaced with serum free medium (control, n=3), low dose ang II (n=3), high dose ang II (n=3), losartan (n=3), losartan and ang II (n=3). The cells were then lysed after 24 hours and the mRNA used in qPCR. This experiment was repeated once more and the results pooled. The results were analysed by ANOVA and showed no statistically significant difference in profibrogenic gene expression between the different groups and are presented here as fold change compared to the control.
3.3.4 CCD18Co cells pre-treated and activated by TGF-Beta are responsive to Ang II with a significant upregulation of collagen gene transcription; an effect which is reversed by the AT1 antagonist losartan

In the previous results section 3.3.3 I demonstrated that not even high dose Ang II had any effect on the expression of genes important in CD fibrosis using naïve CCD18Co cells indicating that Ang II alone does not activate CCD18Co and/or has no effect on profibrogenic gene expression. I therefore decided to investigate if pre-activating the cells using a potent profibrogenic agent (TGF-β1) would make the cells sensitive to Ang II stimulation.

Figure 3.5 demonstrates that there was significantly reduced TGFβ1, IGF1 and COL1A1 in cells that had not been prestimulated with tgfβ. There was no significant difference in the expression of TGFβ1, IL13 and IGF1 between control activated cells compared to all the other conditions. However when comparing genes responsible for collagen production in CD fibrosis: col1a1 and col3a1, these activated ISEMfs did have a significantly different response to Ang II stimulation compared. Whilst low dose Ang II had no effect on these genes, high dose Ang II caused a significant increase in col1a1 (p<0.001) and col3a1 (p<0.01) compared to controls. Furthermore, this effect was entirely reversed by the addition of the AT1 receptor antagonist losartan to high dose Ang II.
Figure 3.5: The effects of Ang II and losartan stimulation in control and tgf-β activated CCD18CO cells using real time PCR to determine key profibrogenic gene expression. CCD18CO cells were plated in 6 well plates in serum free medium spiked with 1ng/ml of TGF-β overnight. The cells were then washed and the medium replaced with serum free medium (control, n=3), low dose Ang II (n=3), high dose Ang II (n=3), losartan (n=3), losartan and Ang II (n=3). The cells were then lysed after 24 hours and the mRNA used in qPCR. This experiment was repeated twice and the results pooled. Data was analysed using ANOVA and are presented here as fold change compared to the control. Whilst low dose Ang II had no effect on gene expression, high dose Ang II significantly increased col1a1(p<0.0001) and col3a1(p<0.001 expression; an effect reversed by losartan. Unstimulated cells expressed significantly less TGFB1 (p<0.05), IGF1 (p<0.05) and COL1A1 (p<0.0001).
3.4 Discussion

Summary of main findings

- ISEMFS express AT1 receptors and these are upregulated by stimulation with TGF-β1
- ISEMFS do not express AT2 receptors and this is not changed by stimulation with TGF-β1
- Ang II alone does not activate ISEMFS and increase the profibrogenic mediators tgfβ1, il13, igf1.
- Ang II does not activate or increase collagen gene expression in quiescent ISEMFS
- Ang II does not increasing expression of conventional profibogrenic mediators tgfβ1, il13, igf1 in activated ISEMFS.
- Ang II increases collagen gene expression in ISEMFS preactivated with TGF-β1 and this effect is reversed by losartan.

In this chapter I initially demonstrated for the first time that ISEMFS, key cells in intestinal fibrogenesis, possess AT1 receptors without possessing AT2 receptors. This mirrors the complete absence of AT2 receptors seen in the previous chapter in CD patients and both muscularis and epithelial layers. As stated previously, many of the cells infiltrating the epithelium and muscularis in CD ileum were morphologically consistent with ISEMFS and our findings support the premise that the strong AT1 staining seen in the epithelial layer is partially due to infiltration of ISEMFS and other mesenchymal cells into this layer during fibrogenesis. The total absence of AT2 in ISEMFS is mirrored by their complete absence in all our CD samples. The AT2 axis is assumed to have many counter-regulatory actions during fibrosis as demonstrated in the cardiac and renal literature but unlike these organs, AT2 seems to be completely absent in CD patients. This may simply be a feature of the very low levels of AT2 expression seen in healthy intestinal tissues but it is intriguing to postulate whether a failure to activate the AT2 axis may be playing a role in intestinal fibrogenesis by failing to alleviate the
unopposed actions of Ang II on the abundant AT1 receptors. This finding is supported by previous work done looking at hepatic stellate cells (HSCs). The HSC is the central cell in liver fibrogenesis and is akin to the ISEMF although our understanding of these cells is far greater than our knowledge of ISEMFs. Bataller et al demonstrated that activated HSCs possess angiotensin receptors of only the AT1 subtype and that stimulation with exogenous Ang II stimulated cell proliferation, DNA synthesis and cell contraction; an effect entirely reversed by the AT1 antagonist losartan [184].

I also demonstrated that quiescent naïve ISEMFs do not become activated by Ang II stimulation and therefore do not increase profibrogenic gene expression. The CCD18Co cell line is initially quiescent and becomes activated with stellate morphology and with classical stress fibres after stimulation either through prolonged culture and high passage number, or being in a profibrogenic environment and stimulation with mediators including TGF-β1 and forskolin [114, 213]. After pre-treatment with TGF-β1, ISEMFs become activated and thus when these cells were subsequently challenged with Ang II, I demonstrated a significant difference in COL1A1 and COL3A1 expression, which are key collagen genes involved in CD fibrosis and that this was reversed by blockade of AT1 by losartan. This is supported by previous findings showing collagen upregulation in vascular and renal tissue in response to Ang II [214]. Interestingly I did not see any increase in TGF-β1 expression which differs from the findings of Yoshiji et al who showed that Ang II treatment of rat HSCs provoked upregulation of tgfβ1, an effect which was completely reversed by the ARB candesartan [215]. This may be a masking effect due to our use of TGF-β1 as pretreatment to stimulate the CCD18Co cells, and it would be interesting to see what the effects of using another pre-stimulating factor would be.

Our results suggest that ISEMFs, despite possessing AT1 receptors, cannot be activated by Ang II alone but instead requires initial stimulation by other key profibrogenic mediators. RAS and Ang II carry out many vital functions throughout the body in addition to their emerging profibrogenic roles and it may be that this requirement for preactivation of these cells by other
separate central molecules in fibrogenesis acts as a brake on inappropriate stimulation of these cells by these every day actions of RAS outside the setting of intestinal injury or fibrosis. These findings are supported by findings in the liver where it has been shown that RAS components are very poorly expressed in quiescent HSCs. Bataller et al went on to show that activation of HSCs then leads to a large increase in these RAS subcomponents activating the local RAS in the liver [185]. There is then local production of Ang II by HSCs which allow a positive feedback loop via further paracrine stimulation of these AT1 receptor-bearing HSCs which leads to their increased proliferation and subsequent collagen production. This is a useful initial part of the body’s wound healing and mucosal restoration efforts but triggering this self-sustaining fibrotic reaction may be one of the reasons why despite elimination of inflammation, we continue to see progression of fibrosis in many different diseases.

In conclusion, our in vitro studies using human ISEMFs (CCD18Co cells) supported my hypothesis that they possessed AT1 receptors whilst they did not possess AT2 receptors. I also showed that AT1 receptors are increased by stimulation and activation by the key profibrogenic mediator TGF-β1. Interesting Ang II has no significant effect on collagen gene transcription in inactive ISEMFs and is unable to activate these cells alone. Ang II provokes upregulation of collagen gene transcription in cell prestimulated and activated by TGF-β1 and that this effect is reversed by the AT1 antagonist losartan. One of the significant weaknesses of this study was that due to insurmountable procurement issues I was disappointingly unable to source and extract ISEMFs from freshly resected fibrotic CD ileum and conduct these experiments on them as Battaler did with HSCs from cirrhotic livers showing increased RAS expression and activity [185]. However, CCD18Co cells are widely considered as an excellent model for studying fibrosis in the gut and given the overall pattern of present AT1 expression and absence of AT2 matched our human CD immunohistochemistry findings, the results still provide important insights and it is reasonable to extrapolate these findings to human CD. These in vitro data strongly support investigating the role of RAS and the effects of AT1 blockade on ileal fibrosis in a high fidelity in vivo model of Crohn’s disease.
Chapter 4. An investigation of the relevance of RAS receptors in human CD of the ileum using angiotensin receptor immunohistochemistry

4.1 Introduction

The octapeptide Ang II is the central effector peptide in RAS, and acting through its principal receptor AT1, is critically involved in the pathogenesis of multiple cardiovascular diseases such as atherosclerosis, hypertension and heart failure [216]. RAS is also highly expressed in and central to the progression of many disparate chronic kidney diseases [217]. Whilst it is increasingly recognised that RAS is overactive in a multitude of other chronic inflammatory and fibrotic diseases involving multiple organ systems including gut, liver, lung and skin, the largest body of work still comes from the cardiac and renal literature leaving key gaps in our understanding in other organs.

The key end effector pathway that has been most studied in RAS is Ang II and its widely expressed principal receptor AT1. Ang II acting through AT1 is known to enact the majority of the best recognised actions of RAS including vasoconstriction [218], cell proliferation [184], inflammation [219], reactive oxygen species production and apoptosis [220, 221]. The AT1 receptor is a seven transmembrane G-protein coupled receptor which is ubiquitously expressed in a wide myriad of tissues. The AT1 receptor is activated by Ang II and then interacts with an array of heterotrimeric G-proteins, including $G_{q/11}$, $G_i$, $G_{12}$ and $G_{13}$ which provokes the stimulation of secondary messengers, such as inositol trisphosphate, diacylglycerol and reactive oxygen species [9]. Various intracellular protein kinases are also activated which lead to the many pleiotropic effects of Ang II.
Relatively little was known about the enigmatic AT2 receptor until it was cloned in 1990s when it was shown that AT2 only shares approximately 34% homology with AT1 [222]. Studies have shown that AT2 receptors are highly expressed in foetal tissues and organs, but with the exception of the central nervous system and female reproductive organs, AT2 receptors are very sparsely expressed in normal adult tissues.

The expression of angiotensin receptors in other organs, in both health and fibrotic disease states has been widely studied compared to limited investigation of their role in the gastrointestinal tract. AT1 is highly expressed in fibrosing diseases in the kidney and heart such as glomerulosclerosis and cardiac failure[201]. In the liver, AT1 receptors are markedly present in vascular smooth muscle cells as well as activated hepatic stellate cells and liver parenchymal cells and AT1 expressing cells are significantly increased in the fibrous septa seen in the livers of cirrhotic patients compared to normal liver tissue [223]. The role of AT2 receptors in disease states is far more enigmatic. As previously stated, AT2 receptors are sparsely and infrequently expressed in the majority of normal adult tissues. However, it appears that in disease states, this pattern can alter. Whilst AT2 is minimally expressed in normal adult kidney after adolescence, AT2 receptor expression is significantly upregulated during kidney disease [224]. Similarly, in normal myocardial tissues, AT2 is expressed at low levels; this is subsequently significantly upregulated in disease states such as cardiac failure [225]. This upregulation is poorly studied or understood but evidence in the cardiac and renal literature appears to suggest that this represent a counter-regulatory Ang II/AT2 centred axis to protect and balance against some of the deleterious actions of the Ang II/AT1 receptor pathway [222].

Despite the body of evidence expanding the role and influence of RAS on inflammation and fibrosis in disease processes in many organs, there has been limited exploration of angiotensin receptor expression in the gastrointestinal tract. Ewert et al studied small bowel preparations from rats and humans using Western Blotting and demonstrated the presence of AT1 receptors in the small bowel (duodenum, jejunum and ileum) [226]. AT2 receptors were not
detected in human intestinal samples at all whilst being sparsely present in rat small bowel. This was confirmed in a later study using Western Blotting showing AT1 being ubiquitously expressed in human jejunum whilst AT2 was infrequently and seldom expressed; furthermore they demonstrated localisation using immunohistochemistry, revealing strong staining for AT1 in the muscularis without AT2 staining and strong AT1 and sparsely positive but distinct staining of AT2 receptors in the myenteric plexus [227]. Furthermore, studies in the jejunum and colon have demonstrated AT1 receptors in the epithelial layer and crypt bases with inconsistent and weaker staining for AT2 in these compartments [191, 228].

There has only been one study published exploring changes in RAS receptor and pathway subcomponent in IBD patients. Suekane et al studied the accumulation and phenotypic change of SMCs in colonic strictures in Japanese CD patients [120]. They demonstrated that AT1 receptors were present in all layers of the colon in normal specimens and relatively increased in the submucosa of CD patients. They also confirmed that there is proliferation and subsequent migration of moderately differentiated intestinal smooth muscle cells from the muscular layers of the colon. Furthermore, they confirmed that many of these SMCs are AT1 positive and there is any accompanying increase in RAS subcomponents (such as mast cell chymase) in accumulating mesenchymal cells thereby implicating RAS in intestinal fibrogenesis. Whilst this initial study provides intriguing insights into a possible role of AT1 receptors in CD fibrogenesis there are also areas which require further investigation: the study looked at colonic strictures instead of ileal strictures (which are far more common and problematic in CD clinical practice), AT2 receptor expression was not examined, and only Japanese patients have been investigated whose CD is already known to be genetically different to Western IBD [229].
Aim of this chapter

To investigate the presence and staining pattern of AT1 and AT2 receptors using immunohistochemistry in full thickness ileal tissues of patient who have undergone terminal ileal resection for colon cancer, UC (2 different control types) and stricturing CD and comparing and contrasting the muscularis and epithelial/mucosal layers.

4.2 Methods

4.2.1 Patient identification, selection and assessment protocol

Following the ethics protocol mentioned in the general methods section, patients were initially selected by interrogating the Guy’s & St Thomas’ Hospitals electronic pathology database. Initially searched for all pathology reports from January 2005 until September 2006 which contained the search terms “ileum”, “right hemicolecotomy” or ileocaecal resection excluding biopsies. I then filtered this list to produce three groups: (a) normal ileum from patients who underwent surgery for right sided colonic tumours (some terminal ileum is usually removed during right hemicolecotomy), (b) normal ileum from patients who underwent pancolectomy for severe ulcerative colitis and (c) strictured ileum from CD patients. All pathology reports for these groups were analysed, the diagnosis confirmed and only patients with completely histologically normal ileum or strictured fibrotic ileum were selected. I then reviewed the medical record for all of these patients including the original operation record and medication history. Any patients where there was clinical ambiguity about the diagnosis or those who were taking ACEIs or ARBs were immediately excluded.

4.2.2 Brief Experimental Protocol

Paraffin embedded tissue blocks (chosen to correspond with the location of normal ileum or strictured ileum as described in the pathology report) were obtained from the pathology archives. These were cut to 4μm thickness using a Leica microtome, floated on a warm water bath containing distilled water and then mounted on to polysine coated slides (Fisher UK).
Immunohistochemistry was performed using AT1 and AT2 antibodies using the technique described in main methods (see section 2.1.3). In brief, slides were deparaffinised and rehydrated using xylene and alcohol. The slides then underwent antigen retrieval by immersing in antigen unmasking solution and microwaved. After cooling and washing, the slides were blocked using horse serum blocking agent. Primary antibody was then added at a concentration of 1:200 for SC1173 and 1:400 for SC9040 diluted in 1% BSA (optimal concentrations which I reviewed in the literature and also optimised myself by performing serial dilutions on human kidney tissue slides to obtain clear staining with minimal background). The slides were incubated overnight in a humidified chamber at 4°C. After washing, secondary antibody was added (Vector ImmPRESS Anti-Rabbit Ig (peroxidase) Polymer Detection Kit). Then slides were then incubated with DAB chromogen before being counterstained with haemotoxyline. After mounting slides were dried for 24 hours before being double read by myself and another blinded reader. Both readers noted the pattern of staining within the epithelial and muscularis and graded the intensity on a simple scale (- absent, +/- minimal and inconsistent, + present, ++ strong staining, +++ intense staining).

4.3 Results

4.3.1 AT1 receptors are abundantly present in the muscle layer of normal ilea and upregulated in the muscularis of strictured CD ileum

Figure 4.1 shows that staining for AT1 in the muscularis of normal ileum was consistent across all samples and particularly strongly stained smooth muscle cells, the walls of blood vessels and occasional spindle shaped cells morphologically like myofibroblasts. There was no difference in staining intensity and pattern between ileum from UC patients compared to cancer patients with both scoring an average of strong staining (++) . A similar pattern of
widespread staining was also seen in CD tissue but staining was generally more intense confirming upregulation in the muscular layer of diseased fibrotic ileum scoring an average of intense staining (+++).

Figure 4.1: Low and high magnification views of the muscle layer in CCa, UC and CD ileum after AT1 IHC. This figure shows widespread presence of AT1 receptors in the muscular layer of representative sections of terminal ileum from patients with CD, UC and colon cancer treated overnight at 4℃ with the AT1 antibody SC1173 (at a concentration of 1:200) prior to treatment with secondary antibody and DAB chromogen followed by counterstaining with H&E. 20 CD sections were examined vs 10 UC and 10 colon cancer.
4.3.2 **AT1 receptors are strongly expressed in the epithelium of CD ileum compared to minimal expression in normal ileal epithelium**

Having shown that there is AT1 expression in the muscular layer of both normal and diseased terminal ileum, I decided to review more closely the expression of AT1 in the epithelial layer. **Figure 4.2** demonstrates through immunohistochemistry that in normal ileum, there is no or minimal staining of AT1 receptors within the epithelial layer with occasional weak and inconsistent staining of AT1 in the submucosa of both UC and colon cancer controls. This was consistently seen across all samples from UC and cancer patients and is a novel finding as it is somewhat different to previous findings from the ileum in mice which show consistent AT1 staining in the epithelial layer [228]. It also partially parallels previous findings from the colon in humans where AT1 is very inconsistently expressed in the submucosa but is generally present in the epithelial layer [120].

There was a difference in AT1 staining in the CD ileum where there was strong staining (++) throughout the epithelium and submucosa which was consistently seen across all samples with the caveat that some of the staining was of relatively poor quality with some likely non specific and non nuclear binding.
Figure 4.2: Low and high magnification views of the mucosal layer in CCa, UC and CD ileum after AT1 IHC. This figure shows the presence of AT1 receptors in the mucosal layer of representative sections of terminal ileum from patients with CD and no staining in UC and colon cancer patients treated overnight at 4°C with the AT1 antibody SC1173 (at a concentration of 1:200) prior to treatment with secondary antibody and DAB chromogen followed by counterstaining with H&E. 20 CD sections were examined vs 10 UC and 10 colon cancer.
4.3.3 AT2 receptors are sparsely and inconsistently present in normal ileum and completely absent in CD ileum

Having demonstrated that the AT1 receptor appears to be highly expressed in the muscular layer of both normal and diseased ileum whereas it is generally absent in the epithelial layer in normal tissues and expressed in diseased CD terminal ileum I decided to investigate the expression of AT2 receptors in these tissues in health and disease. Figure 4.3 and Figure 4.4 shows generally weak staining for AT2 receptors across all samples in all layers of the ileum. In the normal control tissues, there was no difference between the colon cancer and UC samples which generally showed absence of staining in the majority of samples in all ileal layers. There were a few samples in both UC and colon cancer which demonstrated weak poor quality staining in the epithelial layer (+/-). This is consistent with the literature where previous studies have shown absent or generally weak and inconsistent staining of AT2 receptors in different intestinal tissues from adult humans and rodents [191, 226]. Our novel finding was the fact that there was no AT2 receptor staining in any layer of the intestine in CD samples with remarkable consistency across all samples and between both readers suggesting a possible genuine downregulation of an already sparsely present AT2 receptor in fibrotic CD.
Figure 4.3: Low and high magnification views of the muscle layer in CCa, UC and CD ileum after AT2 IHC. This figure shows the absence of AT2 receptors in the muscular layer of representative sections of terminal ileum from patients with CD treated overnight at 4°C with the AT2 antibody SC9040 (at a concentration of 1:400) prior to treatment with secondary antibody and DAB chromogen followed by counterstaining with H&E. Sections from normal UC and colon cancer ileum showed a similar pattern with very occasional sparse and inconsistent staining for AT2. 20 CD sections were examined vs 10 UC and 10 colon cancer.
4.3.4 Summary of comparative immunohistochemical differences in AT1 and AT2 expression in CD, UC and colon cancer ilea

Whilst it is difficult to precisely measure staining in IHC, we did approximately assess the percentage of tissues stained in the muscular and epithelial layers by AT1 and AT2 antibodies in an effort to quantify the above findings. Figure 4.5 shows the stratification of individual slides into groups based on intensity/area of staining (divided into 0-25%, 25-50% and 50-75%
and 75-100%). This demonstrates significantly higher staining of AT1 in CD muscle and CD mucosa compared to controls (p<0.0001), reduced mucosal staining of AT2 in CD mucosa vs controls (P<0.05) and no significant difference in AT2 in the muscular layer of CD compared to controls.

Figure 4.5: Comparison of AT1 and AT2 IHC staining intensity/extent in the muscular and mucosal layer of CCa, UC and CD ileum. These simplified scatter plots compare AT1 and AT2 IHC percentage of cell staining in CD vs control ileal muscular layers and mucosal layers. Panel A & B shows significantly increased AT1 staining in the muscle and mucosal layers of CD vs controls (CD n=20 vs CCa n=10 and UC n=10, p>0.0001); Panel C shows significantly lower AT2 staining in the mucosal layer in CD vs controls (CD n=20 vs CCa n=10 and UC n=10, p>0.05) and no staining in the muscle layer of CD or controls.
4.4 Discussion

Summary of main findings

- Staining overall was of a poor quality but general trends could be seen despite this limitation.
- AT1 receptors are strongly expressed in the muscularis propria and submucosa of normal human terminal ileum.
- AT1 receptors are not normally expressed in the epithelial layer of normal human terminal ileum.
- There is no discernible difference in expression of Ang II receptors in histologically normal ileum from colon cancer patients compared to UC patients.
- AT1 receptors are upregulated and highly expressed in the epithelial layer, submucosa and muscularis propria of strictured CD terminal ileum.
- AT2 receptors are generally poorly expressed in the human terminal ileum with occasional epithelial expression in normal mucosa and no expression in CD terminal ileum.

The role of RAS in fibrosis and inflammation in multiple organ systems is becoming widely accepted based on manifold evidence as reviewed earlier. The role of RAS in the pathogenesis of CD is less certain but early evidence from animal studies is supportive. However, this evidence is limited to findings in poorly representative animal models where fibrosis manifests poorly or with no correlation to the pathology or characteristics of human CD. Moreover, since the finding of raised Ang II levels in CD colon more than 20 years ago [192], there has been remarkably slow progress in examining the role of RAS in CD. Furthermore, there has also been very little progress in understanding its role and expression in the ileum, the commonest site of fibrosis and subsequent stricture formation in CD patients. The limited studies available
looking at receptor expression have either only examined the colon or looked at the small bowel of normal rodents. It could be argued that the terminal ileum is narrower than the colon and therefore it is not surprising that subsequent strictures are more common here and also cause a more frequent and significant clinical problem. However, this underplays the fact that this same region is by far the most commonly affected area for inflammatory Crohn’s disease suggesting that the terminal ileum as the watershed region between ileum and colon has unique properties e.g. bacterial milieu that make it a key area to study in CD fibrosis. Therefore our aims were to investigate a role for RAS in human CD through RAS receptor immunohistochemistry of the terminal ileum. I report for the first time on changes in RAS receptor expression in the terminal ileum in both fibrotic diseased tissue as well as normal ileum.

Our experiments were undermined by generally poor staining quality and microscope images but there were suggestive trends seen which demonstrated that AT1 receptors are indeed present in human terminal ileum and that they are highly expressed throughout the submucosal layers. In particular they are highly expressed in the walls of blood vessels, a finding which is consistent with previous reported findings in the human colon [191]. As a key novel finding, I have demonstrated differences in the expression of AT1 receptors in the ileal epithelium of patients with fibrotic compared to control ilea from cancer or UC controls. I have shown that this key effector receptor is highly expressed in CD ileum and in particular is expressed in the epithelial layer and mucosa of CD patients whereas it is consistently not seen in the ileal epithelial layer of control patients. This is a significant and consistently replicated finding despite the limitation of the staining quality and were seen across all samples that may reflect the fact that in the normal undiseased bowel, there are few mesenchymal cells in the epithelial layer (which is predominantly derived from ectoderm). Mesenchymal fibrogenic cells ordinarily reside in the submucosal and muscularis layers of the intestine. Activated mesenchymal cells migrate to the mucosa during intestinal injury, aberrant wound healing and subsequent fibrosis. It is probable that throughout this migration they continue to express AT1
receptors and therefore this migration of a new population of AT1 positive cells may explain some of the differences I noted in AT1 expression at the ileal mucosal epithelium in disease vs normal states. Furthermore during fibrogenesis, the mesenchymal compartment of pro-fibrogenic cells can be expanded by EMT or endothelial to mesenchymal cell transition (EndMT) [209, 213]. In future experiements this could be clarified by using confocal microscopy and fluorescent antibodies against ang II receptors as well as mesenchymal cells to see if there is colocalisation. All of these morphological changes could lead to increased AT1 expression or even be driven by Ang II via its AT1 receptors as seen in the kidney [230]. A variety of complex and incompletely understood processes plays a role in the migration of fibrotic cells to the epithelium in intestinal fibrosis. Another possible source of new cells in the epithelium are bone marrow derived fibrocytes which have features of fibrogenic ECM producing cells such as αSMA and collagen 1 expression. These cells circulate in the blood and are attracted to sites of inflammation and injury and undergo trans-differentiation to fibroblasts and myofibroblasts [203]. Fibrocytes in the kidney are known to possess AT1 receptors and their profibrogenic actions are regulated by RAS [200]. With RAS and AT1 playing a role in so many of these cell types and migratory processes, it is unsurprising that there would be an increase in epithelial AT1 receptor expression in the diseased ileum as demonstrated by our results.

In this study, the much more sparsely expressed AT2 receptor was completely absent in all CD patient samples whilst seen only intermittently and weakly in the epithelial layer of the control ilea. It is difficult to draw a firm conclusion about AT2 given that the difference between control ilea and CD ilea was small due to the general paucity and inconsistency of AT2 expression and the poor quality of the staining. However, the complete absence of AT2 in all the CD patients, if verified, is suggestive. As previously reviewed, the AT2 receptor has been shown to have some counter-regulatory roles to AT1’s pro-fibrogenic and pro-inflammatory roles and its complete absence in CD ileum could be contributing to the upregulation of AT1 and inflammatory and fibrogenic pathways.
AT1 is expressed on so many different cells types in our study that it is impossible from this work to identify what the involved mechanisms might be or what downstream effects receptor activation might be having but given the significant and consistent differences seen between and diseased and healthy ileum, it is not difficult to conclude that AT1 and RAS are likely playing an important role in human CD associated intestinal fibrosis.

In conclusion the evidence from these experiments help support the hypothesis that RAS acting through its AT1 receptor plays a salient and important role in fibrogenesis in human CD. The role of the enigmatic AT2 receptor is less clear, but its likely total absence in CD ileum may suggest that failure or downregulation of the counter-regulatory AT2 pathway may exacerbate RAS-AT1 driven fibrogenesis.
Chapter 5. Investigating the timeline of fibrosis and the relevance of RAS pathway subcomponents in a high fidelity murine model of experimental Crohn’s disease-like ileitis

5.1 Introduction

There has been mounting evidence from animal data that RAS is involved in gut fibrosis as reviewed in the introduction to this thesis. Building on the foundation of early human data showing raised Ang II levels in human CD colon [192], in the previous two chapters, I have for the first time provided compelling evidence that there is a role for RAS in human fibro-stenotic CD and that this seems to be principally enacted through Ang II and its main receptor AT1. Having then demonstrated important profibrogenic activity in vitro I wanted to investigate the effects of modulating this receptor on the progression of intestinal fibrosis in vivo. One of the major problems with studying fibrosis and the effects of any therapy in the human gut is the inability to sample tissue serially over time in order to monitor progression or regression of fibrosis. Intestinal fibrosis in CD is a transmural process and we cannot easily obtain transmural tissue except in the end stages of fibrosis when patients undergo intestinal resection. This means that early changes in fibrogenesis are extremely poorly understood. This difficulty in studying human gut fibrosis is exacerbated by a lack of a histological fibrosis scoring system (analogous to widely used and clinically validated system such as the METAVIR score in liver cirrhosis [231]).
In recent developments, despite these problems, progress is finally being made in using abdominal MRI imaging to grade and assess intestinal fibrosis in CD [64, 232]. Further validation and consensus needs to be reached before we can routinely start to use this in clinical studies. Therefore as a prelude to a human study, I decided that I needed to study RAS in a high fidelity animal model of IBD and intestinal fibrosis. In section 1.5.1 I reviewed the major conventional animal models of IBD which are induced by chemical, genetic or immunological manipulation of the animals. Despite being widely available and reproducible and having been used to provide valuable initial data about RAS and gut fibrosis, I did not feel that any of these models provided a suitable platform for investigating intestinal fibrosis. The majority of these models do not affect the ileum and the fibrosis they produce lacks many of the histological features and patterns seen in human CD fibrosis [164]. Moreover the mechanisms through which they achieve intestinal inflammation and then fibrosis bears little relation to the inflammatory and fibrogenic processes seen in CD [111].

Having ruled out working with conventional animal models of CD, I decided to investigate the SAMP mouse. The SAMP is a murine model of spontaneous ileitis which develops without genetic, immunological or chemical modulation and shares remarkable fidelity with human CD, affecting the ileum and causing transmural inflammation with similar responses to therapy as seen in human CD [165]. The development of inflammation in the ileum occurs with nearly 100% penetrance by 10 weeks of age and there is then subsequent development of features of intestinal fibrosis including frank stricture formation which has never been fully explored or validated [168]. I therefore decided to further explore ileal fibrosis within the SAMP mouse over a timecourse and investigate any role for RAS subcomponents.
Aims

- Describe the macroscopic and microscopic features of transmural ileitis within the SAMP mouse
- Explore the development of fibrosis over a time scale in the SAMP mouse
- Compare and contrast differences in RAS subcomponent expression in the ileum of pre-inflamed non-fibrotic SAMP and control mice
- Compare and contrast differences in RAS subcomponent expression in the ileum of SAMP mice with significant inflammation and fibrosis and age matched controls.

5.2 Methods

The details of the methods used are outlined in the main methods section but this provides an overview of the processes used.

5.2.1 AKR/SAMP husbandry, euthanasia and tissue harvesting and preparation

AKR or SAMP mice in breeding cages (trio breeding protocol) from our main colonies were observed daily for pregnancy and new-born pups to garner an accurate age for the offspring. Pups were weaned at 3 weeks of age and placed into cages of 5 mice of the same age and sex. Mice were then used for experiments as appropriate according to their age. Mice were euthanised by cervical dislocation and the ileum immediately harvested as per protocol. A strip of terminal ileum was removed and placed into RNAlater® for RNA isolation.
5.2.2 Preparation of histology slides and initial haematoxylin and eosin (H&E) staining

The remaining ileum was prepared for histology as per main methods including fixation in Bouin’s for 24 hours, cleansing and rinsing in 70% ethanol before being paraffin embedded and cut to 4µM using a microtome and mounted on coated glass slides (Superfrost plus slides, Thermo Fisher). After 24 hours of drying and resting, slides were deparaffinised and rehydrated before undergoing H&E staining as per main methods before alcohol dehydration and clarification with xylene before gluing on a cover slip.

5.2.3 Masson’s trichrome staining

Additional slides were deparaffinised and rehydrated and then serially stained as per protocol (see section 2.1.4) to achieve Masson’s trichrome staining. Slides then underwent alcohol dehydration and clarification with xylene before gluing on a cover slip.

5.2.4 Histological scoring systems for inflammation and fibrosis

Histological samples were read by a GI pathologist blinded to the experimental conditions who used a validated scoring sheet to assess and calculate TIS (H&E slides) and TFS (Masson’s trichrome slides) as outlined in main methods.
5.2.5 RNA isolation and cDNA manufacture

RNA was isolated using the Qiagen RNeasy Mini Kit as per manufacturer’s instructions from 10 µg of ileal tissue. RNA concentration and purity was determined using the Nanodrop spectrophotometer, and integrity confirmed using an RNA gel as outline in main methods. This RNA concentration was then standardised to 100ng/µl. I then used 1µg of this RNA to produce cDNA using the Qiagen Quantitect Reverse Transcription kit with integrated genomic DNA elimination as per manufacturer’s guidelines.

5.2.6 Quantitative PCR and analysis

QPCR was performed using Applied Biosystem’s Power SYBR® Green PCR Master Mix as per manufacturer’s instruction in a Roche Lightcycler 480 II instrument. Pre-validated primers from Qiagen (as shown in Table 5.1) were used at the manufacturer’s recommended concentration and annealing temperature. After testing a panel of housekeeping genes, I decided to use Actb (β-actin) during these experiments as it displayed the best consistency.

<table>
<thead>
<tr>
<th>Mouse Genes</th>
<th>Catalogue Number</th>
</tr>
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<tbody>
<tr>
<td>Actb</td>
<td>PPM02945B-200</td>
</tr>
<tr>
<td>Col1a1</td>
<td>PPM03845F</td>
</tr>
<tr>
<td>Col3a1</td>
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<tr>
<td>Ctgf</td>
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<td>PPM03021B</td>
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<tr>
<td>TGF-β1</td>
<td>PPM02991B</td>
</tr>
</tbody>
</table>

Table 5.1: List of utilised commercially validated premixed primers for qPCR from Qiagen
5.3 Results

5.3.1 Histological findings in inflamed SAMP ileum vs age matched AKR mice

Although previously reported in the literature, I wanted to start this experiment by repeating and reviewing the inflammatory changes seen in SAMP ileitis which have been previously reported as occurring after 4-6 weeks of age, with 100% penetrance at 10 weeks of age and reaching maximal severity by 20 weeks of age [165]. Therefore I used 20 week old mice to compare the findings in the ileum between SAMP mice and their parental control the AKR/J mouse. Figure 5.1 shows classically normal ileum in the AKR with undisturbed crypt architecture, healthy villi and lack of inflammatory cell infiltrate. In comparison the SAMP ileum shows crypt elongation, villous blunting and destruction with infiltrates of active and chronic inflammatory cells in the lamina propria and submucosa consisting of mainly polymorphonuclear and mononuclear cells. In other sections (not shown), occasional granulomas and crypt abscesses were seen as well. This pattern of transmural inflammation is strikingly similar to that seen in human CD.
Figure 5.1: This figure compares representative sections stained with H&E from the terminal ileum of 20 week old AKR/J mice (control) and SAMP mice (disease) demonstrating intense inflammatory changes in SAMP vs normal ileum in AKR/J.

5.3.2 SAMP mice develop progressive severe transmural fibrosis with age which has a consistent and predictable time course reaching maximal fibrosis at 30 weeks

Figure 5.2 demonstrates Masson’s trichrome staining of ilea of age and sex matched AKR and SAMP mice at different time points. This special technique stains collagen (the main component of ECM) blue, keratin and muscle fibres red and nuclei black. I demonstrated the evolution of fibrosis over a time scale in SAMP mice. At the age of 4 weeks, there was no evidence of any inflammation, fibrosis or muscular hypertrophy in either SAMP or AKR ilea. By the age of 20 weeks, in the SAMP ilea, there were intense inflammatory infiltrates throughout the bowel wall with significant accumulation of collagen at the submucosal interface, with transmural infiltration into the muscularis leading to a significant increase in gut wall thickness as well as mucosal collagen accumulation. The AKR ilea did not display any pathological abnormalities.
Using the fibrosis scoring system, TFS was calculated for all of the samples. TFS was negligible in all the AKR mice (<2). In SAMP mice, TFS was also negligible (1.8 ± 0.4) at 4 weeks, 11.4 ± 0.9 at 20 weeks, 14.1 ± 0.6, at 30 weeks and 14.8 ± 1.3 at 40 weeks (Figure 5.3). Using one way ANOVA with Bonferroni correction, I was able to demonstrate a highly statistically significant differences between 4 week, 20 week and 30 week old mice (p<0.0001). There was no difference between 30 week and 40 week mice (p=0.68). I therefore demonstrated that whilst TFS was negligible in young mice, in SAMP mice, TFS significantly increased in severity with age and that the severity of fibrosis peaked at 30 weeks of age.

Figure 5.2: Masson’s trichrome stain of sections of terminal ileum showing the development of spontaneous severe transmural fibrosis over time in SAMP mice compared to age matched AKR mice. Terminal ilea of AKR/J (control) and SAMP (disease) were collected after sacrifice of mice over a time course at the age of 4 weeks, 20 weeks, 30 weeks and 40 weeks. These ilea underwent Masson’s trichrome staining which shows no fibrosis in either group at 4 weeks, increasing fibrosis with collagen deposition particularly in the submucosa infiltrating transmurally into the muscularis and a dense inflammatory infiltrate in the SAMP mice at 20 weeks peaking at 30 weeks plateauing at 40 weeks with no fibrosis in the AKR/J at any point.
Figure 5.3: TFS significantly increases with age in SAMP mice, reaching a maximum plateau at 30 weeks of age with no further significant increase at 40 weeks of age. This figure shows the comparison of fibrosis in the ilea of SAMP mice over a time course. Ilea from SAMP mice aged 4 weeks (n=20), 20 weeks (n=20), 30 weeks (n=20) and 40 weeks (n=20) were stained with Masson’s trichrome and then blindly scored by a GI pathologist using a standardised fibrosis scoring template to calculate a total fibrosis score (TFS). The age groups were compared using a one way ANOVA with Bonferroni correction and this shows a statistically significant increase in TFS between 4 week and 20 weeks (p<0.001), and 20 and 30 weeks (p<0.01) with no further statistically significant change in TFS from 30 weeks to 40 weeks age.

5.3.3 Select RAS subcomponents are differentially expressed in the ilea of 4 week old SAMP compared to AKR/J controls

Figure 5.4 compares the gene expression of key subcomponents of the renin angiotensin system (see RAS pathway schematic in Figure 1.3) in the terminal ilea of young uninflamed AKR and SAMP mice using qPCR. I demonstrated increased expression of Agt and Ren mRNA in young SAMP compared to AKR that was highly statistically significant (p<0.0001). Conversely, there was no difference in expression of Ace, Ace2, Agtr1 and Agtr2 mRNA between young SAMP and AKR.
Figure 5.4: A comparison of RAS subcomponent mRNA expression in the ilea of 4 week old AKR and SAMP showing significantly higher Agt and Ren expression in SAMP vs AKR and no significant difference in the expression of other genes: this figure compares pre-diseased ileum at 4 weeks ages in AKR/J vs SAMP. Strips of ilea from 4 week AKR/J and SAMP mice terminal ileum (n=10 each) were homogenised and RNA extracted before conversion to cDNA. This cDNA was then used in qPCR with commercially purchased and validated primers for a panel of important RAS subcomponents. Each PCR was run in triplicate with each run including non-template controls and with β-actin as the housekeeping gene. The data was then analysed using the ΔΔCT method and each gene compared using unpaired T Test. Despite being pre-diseased, there were significant increase in the expression of Agt and Ren mRNA in SAMP compared to AKR/J (p<0.0001). No statistically significant difference was seen in Ace, Ace2, Agtr1 or Agtr2.
5.3.4 RAS subcomponents are differentially expressed in 30 week old SAMP compared to 30 week AKR controls

Having demonstrated maximal fibrosis occurring at 30 weeks of age in section 5.3.2, in this experiment I subsequently compared the relative gene expression of key RAS subcomponents in the terminal ileum of 30 week old AKR and SAMP mice. The AKR ilea were histologically completely normal whereas the SAMP ilea displayed severe transmural inflammation and fibrosis as previously outlined in Figure 5.2. There were skip lesions and macroscopically visible strictures entirely consistent with the changes seen in our time course analysis. There were minimal variation in TFS within each age matched group.

Figure 5.5 shows significant differences in the expression of nearly every RAS subcomponent were noted. Agt (p<0.001), Ace (p=0.0221), Ace2 (p=0.0098) and Agtr1 (p<0.0001) were significantly downregulated in the old SAMP mice compared to the AKR. Ren (p=0.0016) was significantly upregulated in old SAMP vs AKR (whereas Agtr2 whilst higher in SAMP vs AKR did not reach statistical significance (p=0.1799).
Figure 5.5: A comparison of RAS subcomponent mRNA expression in the ilea of 30 week old AKR and SAMP showing significantly lower Agt, Ace, Ace2, and Agtr1 and higher Ren expression in SAMP vs AKR and no significant difference in the expression of Agtr2: this figure compares diseased ileum at 20 weeks ages in AKR/J vs SAMP. Strips of ileum from 20 week AKR/J and SAMP mice terminal ileum (n=10 each) were homogenised and RNA extracted before conversion to cDNA. This cDNA was then used in qPCR with commercially purchased and validated primers for a panel of important RAS subcomponents. Each PCR was run in triplicate with each run including non-template controls and with β-actin as the housekeeping gene. The data was then analysed using the ∆∆CT method and each gene compared using unpaired T Test. There were significant decreases in Agt (p<0.0001), Ace (p<0.01), Ace2 (p<0.001) and Agtr1 (p<0.0001) in SAMP compared to AKR/J. Conversely Ren (p<0.001) was significantly increased in SAMP compared to AKI. Agtr2 was not statistically different between the two groups although a possible trend towards being higher in SAMP was noted.
**5.3.5 RAS subcomponent expression is similar in young vs old AKR mice but differs significantly between young and old SAMP mice**

Having demonstrated subtle but significant differences in RAS gene expression between young AKR and SAMP mice and then subsequently shown major differences in RAS expression in old SAMP vs AKR, I decided to subanalyse our data and compare differences in RAS subcomponent in young vs old AKR (i.e. normal ageing) and analysing changes in RAS expression in young vs old SAMP (ageing in a model with fibrosis). **Figure 5.6** shows that expression of RAS subcomponents did not significantly change with age in AKR mice apart from *Agt* which significantly increased with age (*p*<0.0001). However, a significant pattern was seen in young vs old SAMP where *Agt* (*p*<0.0001) and *Agtr1* (*p*<0.005) were significantly downregulated whilst *Ren* (*p*<0.05), *Ace2* (*p*<0.005), *Agtr2* (*p*<0.05) were significantly upregulated. There were no significant changes in *Ace* expression between old and young SAMP.
Figure 5.6: A comparison of RAS subcomponent mRNA expression in the ileum of 4 week and 30 week old AKR and SAMP showing minimal change in expression in AKR and significantly lower Agt and Agtr1 and higher Ren, Ace2 and Agtr2 in old vs young SAMP. This figure compares RAS subcomponent mRNA expression in young AKR (control) and SAMP (prediseased) ileum at 4 weeks ages to old AKR (control) and (diseased) SAMP at 20 weeks age. Strips of ileum from old and young AKR/J and SAMP mice terminal ileum (n=10 each) were homogenised and RNA extracted before conversion to cDNA. This cDNA was then used in qPCR with commercially purchased and validated primers for a panel of important RAS subcomponents. Each PCR was run in triplicate with each run including non-template controls and with β-actin as the housekeeping gene. The data was then analysed using the ΔΔCT method and each gene compared using unpaired T Test. Young AKR/J had minimal differences in RAS subcomponent expression with no statistically significant changes in Ren, Ace, Ace2, Agtr1 and Agtr2. Agt alone was significantly increase in old vs young AKR/J (p<0.0001). Conversely Agt (p<0.0001) and Agtr1 (p<0.001) were significantly decreased in old vs young SAMP, whilst Ren (p<0.01), Ace2 (p<0.001) and Agtr2 (p<0.01) were all increased in old vs young SAMP mice. Ace remained unchanged in old vs young SAMP.
5.4 Discussion

Summary of main findings

- SAMP mice in our colony develop severe transmural inflammation with closely related features to human CD as elsewhere reported in the literature.
- SAMP mice develop stable progressive ileal fibrosis from 10 weeks of age until reaching a maximum at 30 weeks of age.
- Some RAS subcomponents are significantly differentially expressed in the ileum of young pre-inflamed non-fibrotic mice compared to AKR controls.
- There are paradoxical but highly significantly different patterns of RAS subcomponent expression in aged SAMP mice with severe fibrosis compared to parental controls with a suggestion that protective anti-fibrotic pathways may be upregulated.
- In normal ageing, there is little change in RAS pathway subcomponent expression whereas in the aged fibrotic SAMP, there is again paradoxical downregulation of the active fibrotic parts of the pathway and some upregulation of protective ACE2/AT2 parts of the RAS.

As mentioned in the introduction to this chapter, progress in our understanding of intestinal fibrosis significantly lags behind that of fibrosis in other organs such as the liver. The reasons for this slow progress are due to a series of interconnected and multifactorial issues. There has been a historical lack of agreement in defining the severity of fibrosis histologically which is why our laboratory in conjunction with other groups developed a standardised histological scoring system which correlates with the degree of fibrosis. A second problem is the inability to serially sample intestinal tissue undergoing fibrosis which does not afford us the ability to assess early fibrogenic changes and their evolution. There is also a lack standardised validated non-invasive tests to detect and monitor the progression of intestinal fibrosis analogous to the
serum ELF test or transient elastography (Fibroscan) in liver cirrhosis [233, 234]. In conjunction with these human related issues there has also been no representative, predictable and easily reproducible animal model of intestinal fibrosis.

In this chapter I have explored in more detail the time course of the intense transmural ileal inflammation and then intestinal fibrosis that occurs spontaneously in the SAMP model. For the first time, I demonstrated a stable and predictable time course for this disease and with normal ileum at 4 weeks of age, avid inflammation with minimal fibrosis at 10 weeks, and then progressive severe fibrosis which peaks at 30 weeks of age. The macroscopic appearances of skip lesions and fibrotic strictures and the histological changes seen bear remarkable similarity to human CD [168]. This is in stark contrast to the most popular animal models of fibrosis currently used: the DSS and TNBS colitis models. In comparison to the SAMP’s progressive spontaneous ileal disease, these models rely on chemical agents to trigger inflammation and subsequent modest fibrosis, do not involve the ileum, occur over a very rapid and transient timescale, do not cause formation of dense fibrosis nor strictures. DSS colitis does not even require the presence of B cells or T cells being inducible in immunodeficient mice highlighting its limitations as the adaptive immune system plays an important role in human CD [235]. The data I present here establishes the SAMP as a high fidelity model of intestinal fibrosis with a timeline consisting of a histologically normal period (0 to 4 weeks), a predominantly acute inflammatory period (4-10 weeks) and a subsequent chronic inflammatory and progressively fibrotic period (20 weeks and peaking at 30 weeks).

Having investigated the time course of fibrosis in the SAMP mouse I decided to investigate if the expression of the main components of the RAS pathway were altered in the SAMP mice compared to their parental control the AKR/J mouse. Initially I compared young pre-inflamed mice to see if any early changes occurred even in the absence of inflammation or fibrosis. This demonstrated that the majority of the downstream parts of the pathway were similarly expressed but that a key precursor to Ang II, Agt and the enzyme Ren that principally cleaves it
were highly significantly upregulated in 4 week old SAMP vs AKR. This likely reflects the early upregulation of the RAS pathway which then likely plays an active role in inflammation and subsequently fibrosis in the ileum as seen in other inflammatory diseases in other organs. I then compared old SAMP with severe fibrosis to their controls. The results show that nearly every subcomponent of the RAS pathway was differentially expressed between aged SAMP and AKR and in a surprising pattern. Agt, Ace, Ace2 and Agtr1 were all downregulated whilst Ren remained upregulated. Expression of AT2 did not significantly change but this receptor is largely absent in CD as demonstrated in Chapter 4. This finding is a surprise as it looks like most components of RAS were downregulated in fibrotic mice vs normal controls. This may reflect a negative feedback control whereby the SAMP in a situation of severe fibrosis is downregulating RAS in an ultimately futile attempt to prevent further fibrogenesis. We must also note that this experiment is looking at whole tissue extracts of the ileum rather than specifically pro-fibrogenic cells and looking at mRNA rather than protein expression. When cirrhotic liver is examined, AT1 for example, is substantially downregulated in whole liver tissue whereas it is upregulated in specific areas of active fibrogenesis and more specifically to a subpopulation of cells within the fibrous band [236, 237].

It is widely reported in the renal literature that the RAS is downregulated in the senescent kidney although I do not have any information on this in the human intestine [238]. I therefore decided to subanalyse our data to look at the effects of ageing in this model. When I compared young and old AKR and also young and old SAMP I were able to compare what happened to RAS expression in the ileum “normal” ageing compared to the pathological diseased state found in SAMP. This showed that in normal ageing in the control AKR mouse without inflammation very few RAS subcomponents are altered suggesting that RAS pathways are not significantly altered by ageing in the normal mouse. This comparison further highlights the stark differences with the SAMP mice where in old SAMP Agt and Agtr1 were downregulated and Ren, Ace2 and Agtr2 were all upregulated. This reinforces that RAS expression in the SAMP model is behaving very differently and again there seems to be a suggestion that the
protective anti-fibrotic ACE2 and AT2 pathway are upregulated which may highlight a regulatory negative feedback loop to suppress inflammation and fibrotic pathways.

In conclusion, our results demonstrate for the first time a high fidelity model of intestinal fibrosis that bears remarkable similarity to human CD with both macroscopic and microscopic features consistent with this condition. I have explored the timeline of the development of fibrosis showing maximal fibrosis at 30 weeks of age. These findings establish the SAMP mouse as a useful model for investigating the changes in early and late fibrosis and also its utility as a platform for the effects of investigational medications on fibrogenesis. Our experiments subsequently demonstrate that subcomponents of RAS are significantly differentially expressed in AKR and SAMP mice and that some of these changes occurs even before detectable inflammation or fibrosis are noted. We see some paradoxical changes in RAS subcomponents in old SAMP with severe fibrosis which may provide interesting insights into the compensatory mechanisms employed in conditions of extreme inflammation and fibrosis in the ileum. Having demonstrated a distinct period of inflammation and then fibrosis and the importance of RAS in these changes, I will be using SAMP mice in the next chapter to investigate the effects of ARBs on intestinal fibrosis compared to placebo.
Chapter 6. Investigating the effects of losartan administered in drinking water ad libitum at different time intervals in the SAMP murine model of experimental Crohn’s disease-like ileitis

6.1 Introduction

Losartan is the first commercially available AT1 antagonist which was approved for the treatment of hypertension in UK and USA in 1995 and marketed under the brand name Cozaar®. This was subsequently joined by other competitors such as candesartan, telmisartan and irbesartan. It is an orally administered tablet medication which is well absorbed and tolerated and beyond hypertension has subsequently been shown to improve clinical outcomes in chronic cardiac and kidney disease [239, 240]. The pleiotropic effects of angiotensin receptor blockade in the treatment of inflammatory and fibrotic disease in other organs has also rapidly evolved in recent years. Following on from copious supportive data from animal models, pilot studies of losartan in patients with pulmonary fibrosis, liver fibrosis and chronic pancreatitis have shown significant promise with no problems with drug tolerability and importantly minimal effects on blood pressure in normotensive patients [1, 38, 41].

With regard to gut fibrosis and RAS, I demonstrated earlier in this thesis that RAS acting through its AT1 receptor is involved in fibrosis in human CD. Furthermore, RAS subcomponents were found to be significantly altered when comparing SAMP and control mice supporting a role for RAS in this model of intestinal fibrosis. Initial animal data on losartan and gut fibrosis
was reviewed in section 1.6.4. These studies have shown promising results and would suggest that it would be appropriate to start planning human pilots. However there are a few issues with the existing animal data. None of these studies have observed the efficacy of orally administered losartan in reducing fibrosis. They have also all administered losartan before the induction of inflammation making any specific anti-fibrotic effects difficult to differentiate from purely anti-inflammatory effects. Finally, all the previous studies have used animal models (DSS colitis and TNBS colitis) which have limited fibrosis, is poorly representative of human IBD with short duration “acute” experiments which moreover do not affect the ileum.

In the previous chapter I reviewed and further characterised the evolution and progression of ileal fibrosis following acute inflammation in the SAMP mouse which bears remarkable similarity to the inflammation to fibrosis sequence seen in human CD. Further, the stable disease timeline in SAMP allows for intervention to occur in different phases: prior to onset of any disease (from 4 weeks age), during avid acute inflammation without significant fibrosis (10 weeks of age), and during severe chronic inflammation with progressive fibrosis (20 weeks of age). This makes the SAMP an ideal platform for investigating the effect of orally administered losartan on ileal fibrosis as precursor to finalising human studies.

Aims

- Observe the effects of losartan in drinking water on water intake and weight change in SAMP mice
- Assess the effects of losartan in drinking water on inflammation and fibrosis scores in SAMP mice ileal when administered at different disease phases: (a) from 4 weeks of age until 30 weeks of age (longterm study starting before inflammation and ending at an age when very severe fibrosis is expected), (b) from 20 weeks of age until 30 weeks of age (starting during the phase of chronic inflammation with less severe fibrosis and ending when very severe fibrosis is expected) and (c) from 10 weeks of age until 20
weeks of age (starting during the acutely inflammatory period with limited fibrosis and ending when chronic inflammation is established and fibrosis is moderately severe).

- In mice where fibrosis score is reduced, investigating the effects on key profibrogenic gene expression.
6.2 Methods

The details of the methods used are outlined in the main methods section but this provides an overview of the processes used.

6.2.1 Animal husbandry, set up of experimental groups and administration protocol for losartan treatment

SAMP mice were obtained from the main mouse breeding colony and placed in cages of 5 mice matched for gender and age. In the first experiment test cages (of mice aged 20 weeks) were initially set up to examine if losartan might affect the taste and therefore the palatability and subsequent quantity of water consumed compared to placebo. Losartan (Sigma, St Louis, MO) was dissolved in water, sterile filtered (using the END Millipore Stericup 0.22 micron vacuum assisted filtration system) and used to fill the cage water bottle. Placebo was prepared identically using the same filtration method. Three groups were initially set up for the ingestion experiments: placebo, losartan low dose (0.2g/L) and losartan high dose (0.6g/L). The cage water bottle was weighed twice a week to calculate consumption. The mice were also weighed twice a week to examine if losartan differentially affected body weight. The mice were also closely observed for any unusual findings and mortality. The experiment was concluded after 10 weeks.

Dosing was subsequently optimised to 0.6 g/L for all future experiments based on no adverse changes seen, stable weight and water intake and best approximation to human dose calculated using the principals outlined by Sharma et al [240]. This gave a dose of approximately 70mg/Kg weight; this is higher than the standard human dose but accounts for
metabolic differences and is well below the human toxic dose and comparable to other groups [239].

<table>
<thead>
<tr>
<th>Stages of disease</th>
<th>No of SAMP</th>
<th>Starting Age</th>
<th>Age at sacrifice</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe fibrosis with chronic inflammation stage</td>
<td>10 placebo (5F+5M)</td>
<td>20 weeks</td>
<td>30 weeks</td>
<td>10 weeks</td>
</tr>
<tr>
<td>Preinflamed to severe fibrosis stage</td>
<td>10 placebo (5F+5M)</td>
<td>4 weeks</td>
<td>30 weeks</td>
<td>26 weeks</td>
</tr>
<tr>
<td>Severe inflammation with evolving fibrosis stage</td>
<td>10 placebo (5F+5M)</td>
<td>10 weeks</td>
<td>20 weeks</td>
<td>10 weeks</td>
</tr>
</tbody>
</table>

Table 6.1: Details of the setup of experimental groups covering different disease periods comparing placebo to losartan

For the main experiments, three experimental groups were set up as outlined in Table 6.1. These conditions were set up to allow assessment of the effects of losartan on different stages of disease in the SAMP. At 4 weeks SAMP do not have any detectable inflammation which usually develops from 6 weeks and is present in all mice by 10 weeks [165]. From 10 to 20 weeks there is avid acute inflammation followed by chronic inflammation with progressive
Fibrosis is already severe by 20 weeks but worsens to its peak at 30 weeks as outlined previously in Figure 5.3.

Animals were checked daily and the drinking solution changed a minimum of two times a week. The mice were also weighed twice a week.

6.2.2 Preparation of histology slides and initial haematoxylin and eosin (H&E) staining

Mice were euthanised by cervical dislocation and the ileum immediately harvested as per protocol. A strip of terminal ileum was removed and placed into RNAlater® for RNA isolation. The remaining ileum was prepared for histology as per main methods including fixation in Bouin’s for 24 hours, cleansing and rinsing in 70% ethanol before being paraffin embedded and cut to 4µM using a microtome and mounted on coated glass slides (Superfrost plus slides, Thermo Fisher). After 24 hours of drying and resting, slides were deparaffinised and rehydrated before undergoing H&E staining as per main methods before alcohol dehydration and clarification with xylene before gluing on a cover slip.

6.2.3 Masson’s trichrome staining

Additional slides were deparaffinised and rehydrated and then serially stained as per protocol to achieve Masson’s trichrome staining. Slides then underwent alcohol dehydration and clarification with xylene before gluing on a cover slip.
6.2.4  **Histological scoring systems for inflammation and fibrosis**

Histological samples were read by a GI pathologist blinded to the experimental conditions who used a validated scoring sheet to assess and calculate TIS (H&E slides) and TFS (Masson’s trichrome slides) as outlined in main methods.

6.2.5  **RNA isolation and cDNA manufacture**

RNA was isolated using the Qiagen RNeasy Mini Kit as per manufacturer’s instructions from 10 µg of ileal tissue. RNA concentration and purity was determined using the Nanodrop spectrophotometer, and integrity confirmed using an RNA gel as outline in main methods. This RNA concentration was then standardised to 100ng/µl. I then used 1µg of this RNA to produce cDNA using the Qiagen Quantitect Reverse Transcription kit with integrated genomic DNA elimination as per manufacturer’s guidelines.

6.2.6  **Quantitative PCR and analysis**

QPCR was performed using Applied Biosystem’s Power SYBR® Green PCR Master Mix as per manufacturer’s instruction in a Roche Lightcycler 480 II instrument. Pre-validated primers from Qiagen (as shown in Table 6.2) were used at the manufacturer’s recommended concentration and annealing temperature. Actb was used as a housekeeping gene as per previous experiments.
Qiagen RT² qPCR Primer Assay

<table>
<thead>
<tr>
<th>Mouse Genes</th>
<th>Catalogue Number</th>
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</thead>
<tbody>
<tr>
<td>Actb</td>
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</tr>
<tr>
<td>Col1a1</td>
<td>PPM03845F</td>
</tr>
<tr>
<td>Col3a1</td>
<td>PPM04784B</td>
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<td>Ctgf</td>
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<td>Igf-1</td>
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<tr>
<td>Il-33</td>
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<td>Mmp-9</td>
<td>PPM03661C</td>
</tr>
<tr>
<td>Il-13</td>
<td>PPM03021B</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>PPM02991B</td>
</tr>
</tbody>
</table>

*Table 6.2: List of commercially validated premixed primers for qPCR from Qiagen used in this chapter*
6.3 Results

6.3.1 Losartan in drinking water is well tolerated by SAMP mice with no effect on mortality, weight change or quantity of water consumed

All previous investigations of losartan in gut fibrosis have involved administering losartan by intraperitoneal injection or gavage. I ruled out using the intraperitoneal route in favour of oral methods to more closely replicate the route of administration in humans. Moreover, given the length of the experiments and the dangers of daily gavage, I opted for losartan in drinking water. Losartan has been successfully administered this way previously without deleterious consequences or altering water consumption in an experiment looking at the effects of losartan on sarcopenia [241]. In these early experiments using losartan in drinking water I wanted to firstly confirm that losartan would not affect the palatability of the water and allow for stable dosing to be given. Figure 6.1 shows that there was no significant difference in water intake between mice given placebo water, water containing low dose losartan or water containing high dose losartan. Moreover, I monitored water intake in the fortnight before starting this experiment and each mouse drank on average 3.8 mls per day; a value which did not change with the start of the experiments (data not shown).

There was no discernible effect on the health of the animals and I had no mortality during the course of these experiments. Figure 6.2 shows that incidentally at the setup of the first experiment animals given losartan were slightly heavier on average than the placebo group. This difference was maintained throughout the experimental period but the change in weight by the end of the experiment was not statistically different between losartan and placebo treated mice. Therefore losartan did not have any effect on weight gain/loss.
Figure 6.1: This figure compares average water intake in SAMP (measured by daily weighing of the bottle and standardised to an average 30g mouse over a 10 week period) which were given ad libitum access to either placebo water (n=10), water containing 0.2 g/L of losartan (n=10) or 0.6 g/L of losartan (n=10). Results were compared using one-way ANOVA and no statistically significant difference in water consumption was seen between the different groups.
Figure 6.2: (A) Average weight and (B) Average change in weight when treated with placebo or losartan 0.6g/L in drinking water over time (from 20 to 30 weeks of age): this figure shows that SAMP mice aged 20 weeks given placebo drinking water (n=10) vs losartan 0.6 g/L spiked drinking (n=10) had (A) no difference in total weight over a 10 week period when compared using one way ANOVA and (B) no significant difference in change in weight when compared using one way ANOVA.
6.3.2 Losartan has no significant effect on inflammation and fibrosis in SAMP mice when administered between 4 – 30 weeks of age.

In this experiment, I was attempting to see if long duration therapy with losartan might alter the natural history of disease in SAMP mice. Therefore treatment was started at 4 weeks (uninflamed period), continued through the acute and chronic inflammatory period and concluded at the time point of maximal fibrosis. Figure 6.3 shows that long duration treatment with 0.6 g/L of losartan in drinking water had no effect at all on inflammatory scores or fibrosis scores in the ileum of SAMP. Severe inflammation and fibrosis were seen in both groups as clearly seen in Figure 6.4 & Figure 6.5.

![Figure 6.3: A comparison of inflammatory and fibrosis scores in the ileum of SAMP mice treated with placebo vs losartan from 4 weeks of age until 30 weeks of age: SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 4 weeks to sacrifice at 30 weeks of age. Ileae were stained using H&E and Masson’s trichrome and a pathologist calculated TIS and TFS on each slide. This figure shows that using a T test there was no statistically significant difference in TIS or TFS between placebo and losartan treated mice.](image)
Figure 6.4: H&E ileal sections showing no difference in TIS in placebo vs losartan treated SAMP mice from 4 to 30 weeks: this figure shows no difference in histological appearances of inflammation in representative sections of ileum in placebo vs losartan treated mice. These SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 4 weeks to sacrifice at 30 weeks of age and the harvested ilea stained with H&E.

Figure 6.5: Masson’s trichrome stained ileum showing no difference in TFS in placebo vs losartan treated SAMP mice from 4 to 30 weeks: this figure shows no difference in histological appearances of fibrosis in representative sections of ileum in placebo vs losartan treated mice. These SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 4 weeks to sacrifice at 30 weeks of age and the harvested ilea stained with Masson’s trichrome stain to highlight fibrosis.
6.3.3 **Losartan has no significant effect on inflammation and fibrosis in SAMP mice when administered between 20 – 30 weeks of age**

In this experiment I wanted to see if losartan might have an effect when used during a period of chronic inflammation with extremely severe and progressive transmural fibrosis having endured the acute inflammatory period without any intervention. **Figure 6.6** shows that there is no significant difference in TIS and TFS between placebo treated mice and those given losartan 0.6g/L in drinking water. We can clearly see similarly severe chronic inflammation in the two groups as shown in the H&E slides of **Figure 6.7**; the Masson’s trichrome stain in **Figure 6.8** shows the same pattern of severe gut fibrosis in both groups.

![Bar chart showing comparison of total inflammation score and fibrosis score between placebo and losartan treated mice](image)

**Figure 6.6**: A comparison of inflammatory and fibrosis scores in the ileum of SAMP mice treated with placebo vs losartan from 20 weeks until 30 weeks of age: SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 20 weeks to sacrifice at 30 weeks of age. Ilea were stained using H&E and Masson’s trichrome and a pathologist calculated TIS and TFS on each slide. This figure shows that using a T test there was no statistically significant difference in TIS or TFS between placebo and losartan treated mice.
Figure 6.7: H&E ileal sections showing no difference in TIS in placebo vs losartan treated SAMP mice from 20 to 30 weeks: this figure shows no difference in histological appearances of inflammation in representative sections of ileum in placebo vs losartan treated mice. These SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 20 weeks to sacrifice at 30 weeks of age and the harvested ilea stained with H&E.

Figure 6.8: Masson’s trichrome stained ileum showing no difference in TFS in placebo vs losartan treated SAMP mice from 20 to 30 weeks: this figure shows no difference in histological appearances of fibrosis in representative sections of ileum in placebo vs losartan treated mice. These SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 20 weeks to sacrifice at 30 weeks and the ilea stained with Masson’s trichrome to highlight fibrosis.
6.3.4 **Losartan significantly reduces inflammation and fibrosis in SAMP mice and downregulates key profibrogenic genes when administered between 10 – 20 weeks of age.**

In this experiment, I wanted to see if the introduction of losartan during the most acute inflammatory period would have any effect on ileal fibrosis and inflammation. I also ended the experiment at an earlier point to see if there might be an initial effect of losartan on acute inflammation and fibrosis that perhaps wanes as severe chronic inflammation progresses.

*Figure 6.9* shows a significant (p<0.05) reduction in TIS in the losartan treated mice compared to placebo. This is mirrored by a clearly significant albeit modest drop in TFS (p<0.005). *Figure 6.10:* shows the differences in TIS in H&E section whilst *Figure 6.11* shows the difference in TFS using Masson’s trichrome stain. Having noted these marked differences, I wanted to see if these histological changes were associated with an alteration in the expression of a panel of key profibrogenic genes which have previously been shown to be important in the SAMP as well as in human CD [37, 242]. In *Figure 6.12* I show that there is significant downregulation of *Col1a1, Igf1, Mmp9, Il13, Tgfβ1* expression in the ileum of losartan treated mice compared to controls (p<0.05) whilst there was a non-significant trend in reduction in *Il33* and no change in *Col3a1* and *Ctgf*. 
Figure 6.9 A comparison of inflammatory and fibrosis scores in the ileum of SAMP mice treated with placebo vs losartan from 10 weeks until 20 weeks of age: SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 10 weeks to sacrifice at 20 weeks of age. Ileae were stained using H&E and Masson’s trichrome and a pathologist calculated TIS and TFS on each slide. Using a T-test, there was statistically significantly lower TIS in losartan vs placebo mice (p<0.01) and lower TFS in losartan vs placebo mice (p<0.001).

Figure 6.10: H&E ileal sections showing a significantly lower TIS in losartan vs placebo treated SAMP mice from 10 to 20 weeks: this figure shows a reduction in the histological appearances of inflammation in representative sections of ileum in losartan treated mice compared to placebo. These SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 10 weeks to sacrifice at 20 weeks of age and the harvested ilea stained with H&E.
Figure 6.11: Masson’s trichrome stained ilea showing reduced fibrosis in losartan vs placebo: this figure shows a reduction in the histological appearances of fibrosis in representative sections of ileum in losartan treated mice compared to placebo. These SAMP mice were treated with placebo water (n=10) or losartan 0.6 g/L spiked water (n=10) ad libitum from age 10 weeks to sacrifice at 20 weeks of age and the harvested ilea stained with Masson’s trichrome to highlight fibrosis.
Figure 6.12: qPCR of ileal tissue genes showing reduced expression of multiple profibrogenic genes in losartan vs placebo treated mice: this figure compares differential gene expression of a panel of profibrogenic genes in the whole ileum of SAMP mice treated with placebo (n=10) or losartan 0.6g/l in drinking water ad libitum from 10 weeks to 20 weeks of age: Strips of ilea were homogenised and RNA extracted before conversion to cDNA. This cDNA was then used in qPCR with commercially purchased and validated primers. Each PCR was run in triplicate with each run including non-template controls and with β-actin as the housekeeping gene. The data was then analysed using the ΔΔCT method and each gene compared using unpaired T Test. There was a modest but statistically significant decrease in the expression of Col1a1 (p<0.01), Igf1 (p<0.01), Mmp9 (p<0.01) and Il13 (p<0.01) in the losartan treated group vs placebo. There was no statistically significant difference in Ctgf, col3a1, il33 and tgfβ1 between the two groups.
6.4 Discussion

Summary of main findings

- Losartan in drinking water is well tolerated by the mice and does not alter the volume of water intake nor affects changes in body weight.
- Losartan 0.6 g/L has no effect on TIS or TFS compared to placebo in the ilea of SAMP mice treated from 4 weeks until 30 weeks of age.
- Losartan 0.6 g/L has no effect on TIS or TFS compared to placebo in the ilea of SAMP mice treated from 20 weeks until 30 weeks of age.
- Losartan 0.6 g/L reduces both TIS and TFS compared to placebo in the ilea of SAMP mice treated from 10 until 20 weeks of age. Losartan significantly downregulates the expression of the majority of key profibrogenic genes in these mice.

The results from this chapter were somewhat mixed and at times surprising when compared to previous animal studies and the experience in human cardiac and renal disease. This highlights the value of our decision to conduct these experiments in a high fidelity model of CD inflammation and fibrosis. Previous studies looking at RAS modulation in IBD have been conducted in poorly representative short lasting models of fibrosis always involving the colon only; a site which is far less clinically relevant in gut fibrosis than the ileum.

Another important difference in our study, apart from the long duration of the experiments and the high fidelity model, was the administration of losartan in a way which is far more realistic to how we would use it in humans. I therefore did not wish to use the intraperitoneal route or infusion pumps as used in other hypertension type experiments. I explored three options for how to administer losartan orally. I declined the use of daily gavage which would
allow precise dosing and the use of the oral route due to multiple disadvantages: the length of the experiments leading to excessive risk to the mice of perforation, high levels of animal stress and intensive laboratory labour demands (over 100 mice were used in these experiments). I explored the option of mixing losartan into chow but this was found to be more difficult, labour intensive and hard to closely measure intake. Therefore I decided to dissolve the losartan in drinking water. I firstly confirmed from the FDA environmental reports on Cozaar® that losartan was highly water soluble and stable for up to 30 days in water and then noted from initial animal reports that suggested it did not seem to upset or harm the mice in any way [241, 243].

In our initial pilot study (Figure 6.1), I demonstrated that adding losartan to drinking water does not affect the quantity of water consumed and one could therefore reasonably conclude did not affect the palatability. I continued to intensively monitor this for all the subsequent experiments and no differences were seen in any of the experiments (data not shown). I also reviewed the consistency of water intake over time and found that mice were very consistent in the amount of water per gram body weight that they consumed (data not shown). This meant that in the various experiments, I would not need to adjust the concentration of losartan given to the mice; using 0.6g/L of losartan in drinking water, I was able to stably dose mice at 70 mg/Kg body weight; a value which is somewhat higher than in humans (as would be expected in murine experiments) but well below the toxic dose and similar to other studies. In addition more detailed pharmacokinetic and blood pressures studies have been previously conducted by other groups in the literature allowing us to choose this appropriate dose [195, 244]. I established in Figure 6.2 that losartan in SAMP did not materially affect the change in body weight which removed another potential difficulty in maintaining steady dosing. Body weight was closely monitored for all subsequent experiments and no differences between losartan and controls was seen.
Having established a suitable stable dose for the mice, I was able to then examine the effects of losartan at different stages of the SAMP’s disease. In the long term experiment which started at 4 weeks (pre-inflamed) and concluded at 30 weeks of age (the time of maximal fibrosis) I did not see any reduction in inflammation or fibrosis. Similarly, I did not see any change in inflammatory and fibrosis scores in those mice started on treatment at 20 weeks (chronic inflammatory period) and concluding at the same age. Interestingly, when the experiment was started at 10 weeks (peak of acute inflammation) and concluded earlier at 20 weeks, I was able to show subtle but significant reductions in inflammation and subsequently fibrosis scores in the SAMP treated with losartan compared to placebo. Investigating this further using RTPCR, I showed significant reductions in the expression of Col1a1, Igf1, Mmp9, Il13, Tgfβ1 and a non-significant diminution of Il33 and no reduction in Col3a1 expression. These results show that losartan was able to reduce fibrosis in SAMP by downregulating key profibrogenic genes in mice aged between 10 – 20 weeks.

There are several possible explanations for the lack of effect of losartan in mice sacrificed at 30 weeks. It is possible that losartan has no anti-fibrotic activity in SAMP mice or when administered orally. This theory goes against the changes seen in the 10 to 20 weeks mice and the downregulation of key genes involved in fibrosis and the significant differences I showed in Figure 5.5 in RAS expression in SAMP compared to AKR/J. It also contradicts the literature reviewed earlier in this thesis reviewing the extensive anti-fibrotic effects of ARBs in multiple organs including the limited studies previously carried out in the gut [195, 198]. It could be the case that losartan is a medication that has weak anti-inflammatory and anti-fibrotic abilities and any differences it makes in TIS and TFS, is overwhelmed by the sheer severity of the inflammatory burden and subsequent fibrosis that accrues in old SAMP (30 weeks). It is also possible that tolerance to losartan develops which is why we see a difference at 20 weeks and no difference by 30 weeks. Alternatively, it could be possible that losartan has greater efficacy in reducing acute inflammation and then as the more TH2 primed chronic inflammatory phase emerges, this is less affected by losartan leading to no significant difference in TIS or TFS when
the mice are taken out to 30 weeks. Finally, at the time these experiments were conducted, we used whole ileal tissue for analysis. This carries major disadvantages as we are working with heterogenous cells and tissue layers which may have divergent responses to the treatment protocol thus muting the signal from key fibrogenic cells. Furthermore, the inflammation and fibrosis seen in SAMP is very similar to human CD with skips lesion and intervening normal ileum which would contribute to attenuating any positive signal from the diseased tissues themselves. If I were to repeat these experiments there a number of changes which I could make to potentially overcome the limitations of whole tissue analysis in our study. Our own institution has investigated the use of stereomicroscopy to more accurately identify and excise islands of diseased ileum away from normal tissue which could be a useful technique to improve our protocol [208]. An alternative technique could be laser capture microdissection which would allow microscopically targeted tissue acquisition further improving the specificity of the tissue being analysed [207]. There have also been major developments in the development of single cell RNAseq which allow highly focused analysis of specific fibrogenic cells of interest but require extremely careful sample preparation and separation either using high targeted laser capture microdissection (which requires fixation), FACS sorting, microaspiration or microcolumns [210]. With the use of these sophisticated techniques, it would be interesting to see how the results are affected.

In order to focus on the putative anti-fibrotic actions of losartan, in the next chapter I will investigate mitigating the avid inflammation in SAMP by creating a remission relapse model of IBD and seeing if this alters fibrosis in older mice and then investigating the additive effects of losartan in drinking water on TFS.
Chapter 7. The development of a novel remission relapse model of inflammation related fibrosis and investigating the effects of losartan on maintenance of remission and downstaging fibrosis via novel pathways

7.1 Introduction

In the previous chapter, I demonstrated somewhat mixed results for losartan. Whilst I showed that oral losartan was well tolerated and did not alter weight changes or water consumption, the effects on inflammation and fibrosis were somewhat modest and inconsistent. In mice treated from 10 weeks up to 20 weeks I showed a significant reduction in TIS and TFS. However in older mice, I was unable to show a sustained effect. This is likely related to the very extreme and severe inflammation and fibrosis seen in the SAMP model particularly beyond 20 weeks. I would postulate that losartan as an agent on its own may have relatively weak anti-inflammatory effects and thus is a weak agent for inducing remission. In the clinical management of IBD, it is common to combine agents which have fast acting and potent anti-inflammatory activity with a second medication to maintain remission. This is commonly seen with azathioprine which whilst able to induce remission usually takes 3 months upwards to enact clinical benefit. Thus we often concurrently use an induction agent such as a limited course of steroids (commonly prednisolone) as a bridge to control and abate inflammation and symptoms during this pre-therapeutic window. Steroids are effective at inducing clinical
remission but are undesirable long term due to manifold side effects, high relapse rates on stopping and have poor rates of mucosal healing [41-43].

In addition to the broad and serious undesirable effects of steroids which limit their use, there is also contradictory evidence of their effects on intestinal fibrosis. Therapeutically, corticosteroids have long been used with clinical benefit in a variety of fibrotic conditions from systemic sclerosis to retroperitoneal fibrosis [44]. However results with use of topical injections of steroid in intestinal strictures have been contradictory and in vitro studies involving intestinal fibroblasts show no suppression of collagen production following treatment with dexamethasone [86, 245, 246]. In view of this contradictory evidence I wanted to explore the effects of a short period of steroid therapy alone in the SAMP mouse and then whether this could be utilised as a remission relapse platform on which I could observe the anti-fibrotic effects of losartan. Importantly it also brings a degree of clinical realism to our experiments mirroring current therapeutic practice as drugs are increasingly used in combination in IBD.

Aims

- Investigate the effects of intraperitoneal (IP) dexamethasone in 20 week SAMP on inflammation and fibrosis over a timescale.
- Investigate if losartan can maintain remission from inflammation and/or fibrosis after induction with steroids in the SAMP mouse
- Investigate conventional and novel pathways through which losartan might be enacting its therapeutic benefits in the SAMP mouse after steroid induction
7.2 Methods

The details of the methods used are outlined in the main methods section but this provides an overview of the processes used.

7.2.1 Set up of experimental groups and administration protocol for dexamethasone or vehicle

SAMP mice were obtained from the main mouse breeding colony and raised in cages of 5 mice matched for gender and age until 20 weeks of age (chronic inflammation with moderate to severe fibrosis stage).

As outlined in Figure 7.1, in the first experiment, these 20 week old SAMP aged 20 weeks old were weighed and then injected intraperitoneally (IP) once daily with aseptically prepared vehicle (PBS) or DXM 1mg/Kg (DXM dissolved in PBS). Mice were sacrificed at the end of the week of DXM, after a further 2 weeks (3 weeks total) and after a further 3 weeks (6 weeks total).

In the second experiment, 20 week old SAMP were similarly weighed and then injected intraperitoneally (IP) once daily with aseptically prepared vehicle (PBS) or dexamethasone 1mg/Kg (DXM dissolved in PBS). They were then treated for either a further 2 or 5 weeks with...
placebo drinking water vs losartan 0.6g/L being sacrificed at 3 weeks and 6 weeks respectively from the start of the experiment as outlined in Figure 7.2 below.

**Figure 7.2**: Simple schematic outlining timings of IP therapy, oral therapy in drinking water and sacrifice of mice

7.2.2 Preparation of histology slides and initial haematoxylin and eosin (H&E) staining

All ileum from the sacrificed animals had a small strip removed for RNA isolation. The remaining ileum was prepared for histology as per main methods including fixation in Bouin’s for 24 hours, cleansing and rinsing in 70% ethanol before being paraffin embedded and cut to 4µM using a microtome and mounted on coated glass slides (Superfrost plus slides, Thermo Fisher). After 24 hours of drying and resting, slides were deparaffinised and rehydrated before undergoing H&E staining as per main methods before alcohol dehydration and clarification with xylene before gluing on a cover slip.
7.2.3 Masson’s trichrome staining

Additional slides were deparaffinised and rehydrated and then serially stained as per protocol to achieve Masson’s trichrome staining. Slides then underwent alcohol dehydration and clarification with xylene before gluing on a cover slip.

7.2.4 Histological scoring systems for inflammation and fibrosis

Histological samples were read by a GI pathologist blinded to the experimental conditions who used a validated scoring sheet to assess and calculate TIS (H&E slides) and TFS (Masson’s trichrome slides) as outlined in main methods.

7.2.5 RNA isolation and cDNA manufacture

RNA was isolated using the Qiagen RNeasy Mini Kit as per manufacturer’s instructions from 10 µg of ileal tissue. RNA concentration and purity was determined using the Nanodrop spectrophotometer, and integrity confirmed using an RNA gel as outline in main methods. This RNA concentration was then standardised to 100ng/µl. I then used 1µg of this RNA to produce cDNA using the Qiagen Quantitect Reverse Transcription kit with integrated genomic DNA elimination as per manufacturer’s guidelines.
### 7.2.6 Quantitative PCR and analysis

QPCR was performed using Applied Biosystem’s Power SYBR® Green PCR Master Mix as per manufacturer’s instruction in a Roche Lightcycler 480 II instrument. Pre-validated primers from Qiagen were used as outlined in Table 7.1 at the manufacturer’s recommended annealing temperatures.

#### Qiagen RT² qPCR Primer Assay

<table>
<thead>
<tr>
<th>Mouse Genes</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>PPM02945B-200</td>
</tr>
<tr>
<td>Col1a1</td>
<td>PPM03845F</td>
</tr>
<tr>
<td>Col3a1</td>
<td>PPM04784B</td>
</tr>
<tr>
<td>Ctgf</td>
<td>PPM03798B</td>
</tr>
<tr>
<td>Igf-1</td>
<td>PPM03387F</td>
</tr>
<tr>
<td>Il-33</td>
<td>PPM32527A</td>
</tr>
<tr>
<td>Mmp-9</td>
<td>PPM03661C</td>
</tr>
<tr>
<td>Il-13</td>
<td>PPM03021B</td>
</tr>
<tr>
<td>TGF-61</td>
<td>PPM02991B</td>
</tr>
</tbody>
</table>

*Table 7.1: List of commercially validated premixed primers for qPCR from Qiagen used in this chapter*

### 7.2.7 GeneChip™ Mouse Gene 2.0 ST Micro Array

Two pooled samples (DXM vs DXM + losartan) were sent to the Case Western Gene Expression and Genotyping facility to run on a microarray. The whole process was carried out by the core facility but here follows in brief the protocols used. Firstly the RNA quality was rechecked by running on a bleach denatured gel as outline in main methods. The best 2 samples were then diluted to 50 ng/µl and taken to the core facility where they underwent further quality testing including analysis on the Agilent Tape Station for RNA integrity analysis. Qualified samples were then converted to labelled cRNA using NuGEN Ovation Standard labelling protocol and
quality checks performed to confirm accurate labelling. Samples were then fragmented and added to a hybridization cocktail. The cocktail was then run on the Affymetrix Genechip Mouse 2.0 ST microarray (Thermofisher, USA)

7.2.8 Bioinformatics analysis

Data from the microarray was extracted into a CEL file as raw data. This was analysed by a bioinformatitian from the Case Western Gene Expression and Genotyping facility using Affymetrix software.
7.3 Results

7.3.1 Dexamethasone treatment reduces inflammation and fibrosis which rapidly recrudesces in SAMP mice resulting in a remission relapse model of CD inflammation and fibrosis.

Figure 7.3 & Figure 7.4 demonstrate that at the end of one week of IP therapy, there is a significant and substantial reduction in the ileal TIS in the dxm treated group compared to vehicle (p<0.05). At week 3 there is rapid recurrence of inflammation with no significant difference in TIS between the DXM and vehicle groups. This continues at the end of the experiment at week 6 where there was still no significant difference in the TIS of between DXM and vehicle treated mice.

The effects on the development of ileal fibrosis closely mirrored the findings from TIS over time (see Figure 7.5 & Figure 7.6.). There was a highly significantly lower TFS in DXM treated mice compared to controls at the end of the first week (p<0.005). Similarly to TIS, this normalised by week 3 where no significant difference remained; this continued at week 6 where the differences in TFS between DXM and control mice remained insignificant.
Figure 7.3: Comparison of total inflammatory scores in the ilea of SAMP treated with one week of IP vehicle or dexamethasone at 1 week, 3 weeks and 6 weeks: SAMP mice were treated with IP 1mg/kg dxm or vehicle for one week only. A group was sacrificed immediately (1 week, 10 vehicle, 10 dxm. A second group was sacrificed after a further fortnight (3 weeks, 10 vehicle 10 dxm) and a final group were sacrificed after a further 3 weeks (6 weeks group, 10 vehicle, 10 dxm). All ilea were prepared and stained for H&E and a blinded pathologist scored them for TIS. The score was compared between vehicle and dxm groups using the unpaired t test. In the one week group, dxm significantly reduced TIS compared o vehicle (p<0.01). This difference was not seen in the 3 weeks or 6 weeks groups.
Figure 7.4: A comparison of H&E stained ilea of SAMP treated with one week of IP vehicle or dexamethasone at 1 week, 3 weeks and 6 weeks: SAMP mice were treated with IP 1mg/kg dxm or vehicle for one week only. A group was sacrificed immediately (1 week, 10 vehicle, 10 dxm). A second group was sacrificed after a further fortnight (3 weeks, 10 vehicle 10 dxm) and a final group were sacrificed after a further 3 weeks (6 weeks group, 10 vehicle, 10 dxm). All ilea were prepared and stained for H&E. In this figure, representative sections demonstrate reduced inflammation in the one week dxm group compared to vehicle with no differences seen at the other time points between the two treatment groups.
Figure 7.5: Comparison of total fibrosis scores in the ilea of SAMP treated with one week of IP vehicle or dexamethasone at 1 week, 3 weeks and 6 weeks: SAMP mice were treated with IP 1mg/kg dxm or vehicle for one week only. A group was sacrificed immediately (1 week, 10 vehicle, 10 dxm). A second group was sacrificed after a further fortnight (3 weeks, 10 vehicle 10 dxm) and a final group were sacrificed after a further 3 weeks (6 weeks group, 10 vehicle, 10 dxm). All ilea were prepared and stained for Masson’s trichrome and a blinded pathologist scored them for TFS. The score was compared between vehicle and dxm groups using the unpaired t test. In the one week group, there was significantly increased TFS in the vehicle vs dxm. There was no significant difference in TFS in the other groups.
Figure 7.6: Comparison of Masson’s trichrome stained ilea of SAMP treated with one week of IP vehicle or dexamethasone at 1 week, 3 weeks and 6 week: SAMP mice were treated with IP 1mg/kg dxm or vehicle for one week only. A group was sacrificed immediately (1 week, 10 vehicle, 10 dxm). A second group was sacrificed after a further fortnight (3 weeks, 10 vehicle 10 dxm) and a final group were sacrificed after a further 3 weeks (6 weeks group, 10 vehicle, 10 dxm). All ilea were prepared and stained for Masson’s trichrome. In this figure, representative sections demonstrate reduced fibrosis in the one week dxm group compared to vehicle with no differences seen at the other time points between the two treatment groups.
7.3.2 Losartan reduces fibrosis but not inflammation in dexamethasone treated SAMP mice

In this experiment, I investigated the ability of losartan to maintain the therapeutic benefits of steroid induction. As depicted in Figure 7.7, the ilea of DXM + losartan treated mice showed a small but significant increase in total inflammation score at the 3 week mark when compared to DXM + placebo treated mice (p<0.05). At 6 weeks, whilst a slight trend of higher TIS in losartan treated mice compared to controls remained, this was no longer statistically significant. Figure 7.8 displays representative sections from control and losartan mice ileum at the 6 week mark (stained with H&E) showing severe chronic inflammation in both groups.

Conversely the effects of losartan on fibrosis (outlined in Figure 7.9) are very different. There is a substantial and highly significant (p<0.005) reduction in fibrosis in the losartan + DXM group compared to the DXM alone at both the 3 week and 6 week mark. This is visually obvious on the Masson's trichrome stained representative sections shown in Figure 7.10. There is markedly reduced basement membrane thickening, infiltration of the ileal wall with collagen, architectural distortion and villous atrophy in the losartan group ileum compared to the control group.
Figure 7.7: Comparison of total inflammatory scores in the ilea of dexamethasone treated SAMP subsequently treated with placebo vs losartan at 3 weeks and 6 weeks: SAMP mice were treated with IP 1mg/kg dxm for one week. Half the mice were then continued on placebo water whilst half were given losartan 0.6 g/L spiked water ad libitum. 10 placebo and 10 losartan mice were sacrificed after another 2 weeks (3w group) and 4 weeks (6 w group). All ilea were prepared and stained for H&E and a blinded pathologist scored them for TIS. Using the unpaired t test, there was a small increase in TIS in the 3 week group in the losartan vs placebo group (p<0.01). In the 6 week group there was no significant difference in TIS.
Figure 7.8: Comparison of H&E sections of the ilea of dexamethasone treated SAMP subsequently treated with placebo vs losartan at 6 weeks: SAMP mice were treated with IP 1mg/kg dxm for one week. Half the mice were then continued on placebo water whilst half were given losartan 0.6 g/L spiked water ad libitum. 10 placebo and 10 losartan mice were sacrificed after another 4 weeks (6 w group). All ilea were prepared and stained for H&E. This figure shows representative H&E sections from the losartan and placebo groups at 6 weeks showing no significant differences in inflammation.

Figure 7.9: Comparison of total fibrosis scores in the ilea of dexamethasone treated SAMP subsequently treated with placebo vs losartan at 3 weeks and 6 weeks: SAMP mice were treated with IP 1mg/kg dxm for one week. Half the mice were then continued on placebo water whilst half were given losartan 0.6 g/L spiked water ad libitum. 10 placebo and 10 losartan mice were sacrificed after another 2 weeks (3w group) and 4 weeks (6 w group). All ilea were prepared and stained for Masson’s trichrome and a blinded pathologist scored them for TFS. The scores were compared using the unpaired t test. In both the 3 weeks and 6 weeks groups there was a highly significant and major reduction in TFS in the losartan treated group compared to placebo (p<0.001).
Figure 7.10: Comparison of Masson’s trichrome stained ilea of dexamethasone treated SAMP subsequently treated with placebo vs losartan at 6 weeks: SAMP mice were treated with IP 1mg/kg dxm for one week. Half the mice were then continued on placebo water whilst half were given losartan 0.6 g/L spiked water ad libitum. 10 placebo and 10 losartan mice were sacrificed after another 4 weeks (6 w group). The ileum was stained with Masson’s trichrome and this figure shows representative sections highlighting the reduction in fibrosis in the losartan group compared to placebo.
7.3.3 Losartan reduces fibrosis without modulating conventional profibrogenic genes and may be modulating novel pathways in dexamethasone treated SAMP mice

Having demonstrated a marked reduction in fibrosis in the losartan treated group, I was surprised to see that this was not enacted through downregulation of conventional profibrogenic genes as seen in the previous chapter. In Figure 7.11 we can see no significant difference between losartan and controls in the expression of tgfβ1, col1a1, col3a1, ctgf, il13 and mmp9. Moreover, there was a slight but significant increase in igf1 and il33 at 6 weeks in losartan group compared to the controls.

In order to investigate this further, a microarray was conducted. The heatmap generated in Figure 7.12 shows marked differences in gene expression with large numbers of genes differentially upregulated and downregulated. Figure 7.13 (A) shows the results of detailed pathway analysis showing the top 25 pathways modulated by losartan compared to control. After reviewing the most highly significantly modulated genes with the bioinformatician, I was able to highlight a short selection of highly modulated genes which have previously been linked to fibrosis and/or IBD (Figure 7.13(B)).
Figure 7.11: A comparison of the relative expression of key pro-fibrogenic genes in the ileum of SAMP mice treated with dxm alone vs dxm followed by losartan: SAMP mice were treated with IP 1mg/kg dxm for one week. Half the mice were then continued on placebo water whilst half were given losartan 0.6 g/L spiked water ad libitum. 10 placebo and 10 losartan mice were sacrificed after another 2 weeks (3w group) and 4 weeks (6w group). Strips of ileum were homogenised and RNA extracted before conversion to cDNA. This cDNA was then used in qPCR with commercially purchased and validated primers. Each PCR was run in triplicate with each run including non-template controls and with β-actin as the housekeeping gene. The data was then analysed using the ΔΔCT method and each gene compared using unpaired T Test. No significant difference in profibrogenic gene expression was seen in any groups apart from a small increase in IL-33 expression in the losartan group at 6 weeks compared to placebo (p<0.01)
Figure 7.12: A heatmap showing significant differences in gene expression of dxm + placebo treated SAMP ileum vs dxm + losartan treated SAMP ileum on a microarray: SAMP mice were treated with IP 1mg/kg dxm for one week. Half the mice were then continued on placebo water whilst half were given losartan 0.6 g/L spiked water ad libitum. 10 placebo and 10 losartan mice were sacrificed after another 4 weeks (6 w group). Strips of ilea were homogenised and RNA extracted. 5 samples with the best quality RNA were taken from each group and pooled. A microarray was then run comparing pooled placebo to pooled losartan at this 6 week mark. This heatmap shows a very brief snapshot of differential expression of genes in this comparison.
Figure 7.13: (A) Pathway analysis showing differential upregulation and downregulation in the ileum of losartan treated DXM mice compared to the DXM treated controls and (B) highlighted selection of novel interesting genes significantly downregulated in the losartan group compared to controls: SAMP mice were treated with IP 1mg/kg dxm for one week. Half the mice were then continued on placebo water whilst half were given losartan 0.6 g/L spiked water ad libitum. 10 placebo and 10 losartan mice were sacrificed after another 4 weeks (6 w group). Strips of ilea were homogenised and RNA extracted. 5 samples with the best quality RNA were taken from each group and pooled. A microarray was then run comparing pooled placebo to pooled losartan at this 6 week mark. (A) Pathway analysis showing differential upregulation and downregulation in the ileum of losartan treated DXM mice compared to the DXM treated controls and (B) highlighted selection of novel interesting genes significantly downregulated in the losartan group compared to controls.
7.4 Discussion

Summary of findings

In this final data chapter I demonstrated for the first time:

- The development of a remission relapse model of IBD in SAMP mice using a short IP course of DXM which shows rapid temporary reduction and subsequent recrudescence of ileal inflammation and fibrosis.
- Losartan greatly reduces recrudescence of fibrosis in the DXM-SAMP model but does so without improving inflammation.
- Losartan in the DXM-SAMP model does not significantly alter conventional profibrogenic genes which have previously been modulated by losartan therapy in SAMP treated from 10 weeks to 20 weeks of age.
- Losartan in the DXM-SAMP model differentially modulated many novel genes and pathways compared to placebo which require further investigation and validation.
- Losartan particularly downregulated the IL-18, the Reg family and Vanin-1 providing exciting new insights into the mechanisms of Ang II mediated gut fibrosis.

In the previous chapter I experimented with losartan treatment during different phases of the disease (which correlate closely with age in the SAMP [247]). Whilst I was able to show that losartan reduces gut inflammation and also fibrosis in younger mice with moderately severe inflammation and fibrosis, in older mice, losartan loses efficacy and is unable to significantly reduce either inflammation or fibrosis. I concluded that monotherapy with losartan might be useful in mild- moderate disease but appears to lose efficacy in the setting of more severe inflammation.

In the management of IBD, it is increasingly common to combine drugs with different mechanisms of action to enhance and prolong the efficacy of CD treatments. A classic example
of this is the use of potent oral steroids as a temporary bridging therapy whilst concurrently introducing azathioprine or vedolizumab with an aim to withdraw steroids [248, 249].

In order to test the effects of dual therapy with losartan (anti-inflammatory therapy followed by losartan anti-fibrotic therapy) I have demonstrated the development of a steroid induced remission-relapse model of IBD. In the first part of this experiment I examined the effect of giving a short course of IP DXM on the evolution of inflammation and fibrosis in the SAMP mouse. At 20 weeks, the SAMP mouse has established severe ileal chronic inflammation and moderate to severe intestinal fibrosis. Unsurprisingly, after a week of DXM therapy, I showed that TIS was significantly lower than in the vehicle group. This correlates with the known effects of corticosteroids on human CD whereby there is temporary reduction in inflammation and up to 30% mucosal healing [43].

Interestingly, there is also a significant reduction in the TFS. This could be due to partial reversal of intestinal fibrosis; a phenomenon which was previously considered irreversible and now recognised to have the capacity to regress in other organs which is explored in further detail in the general discussion. Alternatively, another explanation for this modest but significant decrease could be the fact that certain subscores which we use to calculate the TFS are related to wall thickness and architectural distortion which would also be improved by resolution of intestinal inflammation thus modestly decreasing the overall TFS.

After the cessation of steroid therapy (akin to the limited steroid courses we use in the clinical management of IBD albeit these are usually tapered) there was rapid recrudescence of both inflammation and fibrosis. This was sustained out to 26 weeks with only a small diminution of overall TIS and TFS confirming that DXM pre-treatment in SAMP had created a novel research opportunity. The DXM-SAMP model is now shown to be a unique model of remission and relapse of IBD providing us with a useful platform to study the efficacy of losartan in a setting where inflammation has been controlled by another agent. The utility of this model also extend beyond this work allowing us to test the efficacy of putative drug candidates for
treating CD inflammation and fibrosis, not just in their ability to induce remission but also in the maintenance of remission.

Having established previously that losartan treatment in drinking water was able to reduce inflammation and fibrosis in younger SAMP, I also showed that it was unable to do so for older mice, which have progressively worsening inflammation and fibrosis. Consequently in the second part of this experiment, I utilised the DXM-SAMP model to evaluate the efficacy of oral losartan in these mice to determine if it could keep inflammation and fibrosis from recurring in these mice in temporary remission. The results were perhaps the most surprising and exciting of this entire experiment. There was no reduction in the severity of ileal inflammation seen at either 3 weeks or 6 weeks. Conversely, there was a big and sustained decrease in TFS in these mice as early as 3 weeks and also at the end of the experiment. Thus I demonstrate for the first time in intestinal fibrosis, a large reduction in fibrosis without any corresponding reduction in inflammation suggesting that in this experiment, losartan is acting directly on anti-fibrotic pathways without significantly influencing inflammatory ones. Furthermore, when I performed qPCR on a panel of conventional pro-fibrotic genes, surprisingly these were not significantly modulated by losartan. This could suggest that losartan is not acting directly on conventional anti-fibrotic pathways but may be acting on novel pathways. However as I was looking at gene expression analysis rather than protein, an alternative explanation is that whilst gene expression was no longer being altered by losartan, downstream protein production and post-translational modifications were being downregulated. If the experiment were to be repeated, we could investigate this by looking for changes in profibrogenic protein expression through techniques such as western blotting or through immunostaining and confocal microscopy.

Although left with very little RNA and constrained by resources, as my final experiment we were able to perform a simple microarray. Given the fact only two samples were analysed, it is difficult to draw any firm conclusions from this data. Nevertheless this small exploratory
experiment was able to demonstrate interesting differences in many unconventional genes and pathways including selected genes which have previously been associated with IBD and/or fibrosis and below I briefly explore three genes with the most significant downregulation ($p < 0.0000001$).

Interleukin 18 (IL-18) was found to be greatly reduced in the DXM + losartan mice ileum on the microarray. IL-18 is a member of the IL-1 superfamily and has significant inflammatory roles in many organs and diseases. In CD, IL-18 has previously been shown to be upregulated compared to normal tissue and UC controls [129]. Moreover IL-18 levels are significantly raised in the serum of CD patients and appear to be produced by inflammatory macrophages and stimulate CD lymphocytes [130]. T-cells from CD patients are also stimulated by IL-18 to produce pro-inflammatory cytokines and IL-18 blockade reduces inflammation in an acute model of colitis [110]. Whilst IL-18 has not been specifically studied in CD intestinal fibrosis, it has been shown to be involved in renal, pulmonary and liver fibrosis [131, 174, 228].

The Reg3 family of proteins were also highly downregulated and are a group of proteins involved in the control of epithelial cell proliferation and differentiation particularly in including pancreas and intestine and are important in mucosal immunity and have been previously shown to be increased in the serum of patients with IBD and decreased by anti-inflammatory therapy[172]. They have also been implicated in liver and pancreatic fibrosis but remain wholly unexplored in IBD [169, 175].

Vanin-1 has been shown to be involved in animal models of IBD as vnn1-deficient mice have significantly reduced inflammation, is upregulated in human IBD and polymorphism appear to increase IBD severity making it a candidate biomarker for IBD activity [250]. Vanin-1 has not been studied in CD fibrosis but is strongly implicated in the pathogenesis of skin fibrosis in systemic sclerosis and also important in the activation of hepatic stellate cell (a key mediator of liver fibrosis) and therefore important in liver fibrogenesis [251, 252].
A full exploration of the genes differentially regulated in the microarray is well beyond this remit of this thesis but part of our future work will be exploring and validating more of these candidates and the novel pathways which may elucidate novel pathways and mechanisms which may lead to novel drug targets to achieve our ultimate clinical goal of stopping and reversing intestinal fibrosis in IBD.
Chapter 8. General Discussion

In their landmark paper published in 1932, Crohn et al, working at the Mount Sinai Hospital in New York City, described the pathophysiological features of what is now known as Crohn’s disease, stating that “the ulceration of the mucosa is accompanied by a disproportionate connective tissue reaction of the remaining walls of the involved intestine, a process which frequently leads to stenosis of the lumen of the intestine”[253]. Since that seminal publication, whilst our understanding of CD has expanded exponentially, the basic fate awaiting many of our patients remains largely as described in that paper, with CD driven gut inflammation frequently leading to fibrogenesis, fibro-stenotic disease and high subsequent needs for surgical resection.

Until recently, scientists have expounded the paradigm that organ fibrosis occurs purely due to uncontrolled inflammation and is considered irreversible. This dogma has hindered fibrosis research as most groups have purely focused on inflammation, rejecting fibrosis research as a lost cause, especially within the world of IBD research. We have long known that in many organs, once fibrotic pathways become activated, they can continue to act despite resolution of inflammation and lead to independent progression of fibrosis [254-256]. A landmark paper in 1979 first demonstrated the concept of reversing fibrosis in the liver [257], a theory that has taken nearly a further quarter of a century to be fully accepted. It is therefore no surprise that we have virtually no licensed anti-fibrotic medications in any organ or disease with the exception of the very modestly efficacious anti-fibrotic pirfenidone in interstitial lung disease [258]. In view of this pressing clinical need, this project has focussed on growing the evidence surrounding RAS and IBD, hoping to rapidly build a path from the laboratory bench to the bedside by repurposing an already licensed drug, losartan.
8.1 Aims encompassed by this thesis

The overarching theme of this PhD thesis was to examine the relevance and role of RAS in the pathogenesis of fibrosis in IBD and subsequently to investigate the effects of the readily available, safe and well tolerated oral ARB losartan on gut fibrogenesis as a prelude to planning a future clinical trial in human CD. Specifically, this thesis addresses the following aims:

a) Determine the relevance of Ang II and its main receptors AT1 and AT2 on intestinal fibrosis using immunohistochemistry – this was achieved comparing archival specimens of ileal resections of CD patients vs controls.

b) Determine the effect of Ang II stimulation and blockade on profibrogenic gene expression in quiescent and pre-activated human ISEMFs – this was achieved by culturing commercially available ISEMFs and conducting repeated stimulation and blockade experiments on them prior to harvesting the cells and extracting RNA for PCR.

c) Investigate the evolution of fibrosis over time in the SAMP mouse model of small bowel fibrosis and determine if RAS subcomponents might play a role in order to establish this as a platform for testing anti-fibrotic drugs – this was achieved by breeding and euthanizing dozens of SAMP mice of different ages to study the timeline and pattern of inflammation and fibrosis in the ileum.

d) Investigate the effect of oral administration of the ARB losartan on TIS and TFS in SAMP mice at different timepoints and stages of disease severity – this was achieved by conducting lengthy experiments at different ages and time points before euthanizing, assessing histology and where appropriate PCR of profibrogenic genes.

e) Establish a model of remission and relapse of ileal inflammation and fibrosis using dexamethasone and then test the ability of losartan to maintain remission following steroid induction – this was achieved by using DXM in SAMP mice and comparing the
effects of this over a timecourse and then using this model to study the effects of losartan.

f) Determine novel pathways through which losartan might be affecting fibrosis in the ileum using a microarray – this was achieved by pooling samples and using the powerful Affymetrix gene array and specialist bioinformatics software and assistance of an experience bioinformatician.

8.2 Future research

8.2.1 Investigation and validation of novel pathways identified from the microarray experiment

Perhaps one of the most surprising and exciting discoveries made during the course of this work was the effect of losartan in the DXM-SAMP model. At both 3 weeks and 6 weeks, I saw a massive drop in fibrosis scores (reducing by >50% at 6 weeks p<0.0001) with no corresponding decrease in inflammation. To compound this surprising result, I saw minimal change in conventional profibrogenic gene expression. When a microarray was performed comparing a representative mouse treated with DXM vs DXM + losartan, I saw massive differential changes in gene expression. In view of these exciting findings, I think it would be crucial to improve and repeat the experiments. There are significant shortcomings in the study. This experiment with DXM and losartan has only been carried out once (admittedly on a sufficiently large sample size) and would benefit from being repeated. The microarray was performed with only two pooled samples due to cost, paucity of left over RNA and time and is therefore of limited value. I would propose to repeat the entire DXM study and then conduct a larger microarray to strengthen and validate the findings from our original experiment. I would then wish to
validate a small selection of highly differentially expressed and biologically relevant novel genes (such as those described in the previous chapter), by performing direct qPCR for these to precisely define their expression in the ileum of these mice. It would also be helpful to obtain direct protein estimation of these novel genes by Western blotting to confirm they match the qPCR data. In order to discover further novel and unexpected pathways involved in fibrosis in IBD, RNAseq technology could be used instead of microarrays to obtain more sensitive and broader amplitude analysis of the expression of both coding and non-coding genes, microRNAs and transcript variants.

8.2.2 Investigating the mechanism of pathogenesis by RAS of fibrosis in experimental ileitis

In this thesis, I confirmed the major differences in Ang II receptor expression in CD compared to controls and then demonstrated the pro-fibrotic effects of Ang II on ISEMFs in vitro; an effect which was reversed by the ARB losartan. Subsequently I demonstrated the benefits of losartan in vivo by treating SAMP mice which develop intense ileitis without genetic, immunological or chemical manipulation. Having seen these important effects, I would propose further experiments to gain a deeper understanding of how this occurs. Our laboratory is able to create SAMP with genetically modified characteristics through a process of generational breeding involving repeated backcrossing between SAMP mice and conventional knockout or transgenic mice (on a non SAMP background e.g. C57BL/6) as most recently demonstrated with NOD2 knockout mice [259]. AT1 receptor knockout mice are commercially available and after 8 generations of crossbreeding (breeding pure SAMP with the offspring of the previous generation), I will have SAMP with the AT1 receptor knocked out. I would study the timeline, severity and pattern of inflammation and fibrosis in SAMP/AT1KO mice compared to native SAMP and would hypothesise a significant reduction in the
SAMP/AT1KO. Subsequently, I would want to investigate the mechanism through which this was occurring. Does the diminution of inflammation and fibrosis occur due to an alteration in function of immune cells derived from the haemopoietic compartment or due to changes in cells derived from non-haemopoietic sources such as epithelial cells lining the intestine? To investigate this, I would generate bone marrow chimeras using irradiated SAMP and SAMP/AT1KO mice and then reciprocal bone marrow transplantation from SAMP and SAMP/AT1KO mice as previously conducted in our other studies [260]. This experiment would help us answer if the deleterious effects of Ang II are occurring due to effects on the immune system, interactions with non-immune cells (as shown in vitro with ISEMFs) or a mixture of both. All of these experiments could also be repeated using the DXM model to investigate the different way in which losartan appears to behave once inflammation has been treated.

8.2.3 Investigating the role of the ACE2-Ang(1-7)-mas axis in gut fibrogenesis

This thesis has focussed on the centrality of the Ang II-AT1 axis in fibrogenesis and explored the benefits of disrupting this by using the oral ARB losartan. There are however many other components to the RAS as outlined in Section 1.6 and Figure 1.3. One of our most intriguing findings was the significant upregulation of Ace2 and Agtr2 in the ilea of fibrosed older SAMP compared to AKR/J or young uninflamed SAMP. Whilst our knowledge of the role of the main classical RAS axis of Ang II and its principal receptor AT1 is well developed and continues to grow, there has been less clarity of the role of AT2 and one of its ligands Ang (1-7), a metabolite of Ang II produced by its degradation by the enzyme ACE2. Ang (1-7) also primarily stimulates its own receptor mas. There is a growing interest in these alternative RAS pathways
as they appear to have counterregulatory and consequently anti-inflammatory and anti-
fibrotic effects. Moreover, ACE2 and Ang (1-7) levels have been shown to be increased in the
serum of IBD patients [194]. Furthermore, AT2 and \textit{mas} appear to have similar and strikingly
similar functionality involving tissue regeneration, anti-fibrotic and anti-inflammatory activity
[261]. Recent studies have also shown that \textit{mas} agonists have blocked the action of AT2
receptor antagonists and vice versa [262]. This has led to the theory that AT2 and \textit{mas} directly
interact with each other and heterodimerise. Indeed, this has recently been published in an
elegant study using brain astrocytes with the authors concluding that AT2 receptors and \textit{mas}
not only form heterodimers but appear to functionally depend on each other. In my opinion it
is likely that the role of AT2 and \textit{mas} will differ depending on tissue type and the status and
function of the rest of the local RAS network and remains a huge area of evolving interest.

I would propose to investigate the role this alternative axis has in the gut. Although I was
unable to show a significant presence of AT2 receptors in human ileum, it would be useful to
expand the immunohistochemistry study to include Mas and ACE2 examining their presence
and distribution in the ileum in disease compared to healthy controls. Subsequently, paralleling
a study conducted in the kidney, I would use CCD18-Co cells and exam the effect of the AT2
specific agonist CGP42112A and/or \textit{mas} agonists on profibrogenic gene expression [35].
Mirroring our earlier experiments, I would establish whether any effects of CGP42112A were
abolished by the AT2 specific antagonists PD123319 and PD123177 in the same way that I
showed losartan blocked the profibrogenic changes of Ang II on CCD18-Co cells. These effects
could then be studied \textit{in vivo} by investigating the possible deleterious effect on fibrosis and
inflammation of orally administering AT2/\textit{mas} inhibitors in the SAMP and SAMP-DXM models
of experimental ileitis and gut fibrosis. Bruce et al have shown the putative therapeutic
benefits of AT2 agonists in a model of pulmonary hypertension and cardiopulmonary fibrosis
[262]. I could also explore the putative therapeutic benefits of AT2/\textit{mas} agonists on fibrosis in
our SAMP and SAMP-DXM models of gut fibrosis, similar to the losartan studies.
8.2.4 Investigating the effects of losartan on CD: designing a human pilot study

As a clinician treating many patients with complex fibro-stenotic CD, the ultimate aim of this thesis was to lay the groundwork for a clinical study. I believe that my work combined with other important studies has underlined the importance of RAS in gut fibrosis and highlighted the potential for ARBs as an anti-fibrotic therapy. Losartan is a widely used anti-hypertensive which is fully licensed for use in humans, safe, well tolerated, cheaply available and orally administered. Moreover, it has been previously used in anti-fibrotic studies with no significant tolerability or safety issues including in non-hypertensive patients [36, 38, 188].

There are formidable challenges to designing a trial looking at the effects of pharmacotherapy on fibrosis in CD. Currently there is no serum, urine or stool biomarker which can detect and track early fibrogenesis in CD. It is not feasible to serially sample transmural tissue from the ileum of CD patients and the hugely variable timeline for the development of gut fibrosis could make a study very long and expensive. There have however been some major advances since I started this work including closer agreement in histological grading leading to the development of scoring systems (including our own system used in this study) and the development and validation of an MRI scoring system to grade the severity of fibrosis in CD[263]. With these tools now available, it has become more feasible to design a study to look at the effects of losartan in human CD.

I propose a relatively simple pilot study to assess the effect of losartan on CD in postoperative patients where there is already a standardised treatment protocol in place in our institutions. Currently all patients with CD who undergo surgical resection for a terminal ileal stricture have their standard CD medication restarted shortly after surgery and are also given three months
of oral metronidazole to reduce postoperative recurrence [264]. At one year, patients undergo surveillance for recurrence comprising ileocolonoscopy and MRI small bowel. The surgical anastomosis is graded according to the Rutgeerts score which has been shown to predict postoperative recurrence and need for further surgery [126].

**Aims**

To investigate the addition of losartan compared to standard postoperative care following terminal ileal resection for fibro-stenotic CD

**Study Design**

This will be double blinded placebo controlled study whereby patients undergoing ileocolic resection and/or anastomosis formation will be administered either placebo or losartan 50 mg OD in addition to standard care as previously described. All patients will undergo MRI small bowel, blood tests and ileocolonoscopy with biopsies of the anastomosis at one year and also regular clinical disease activity scores.

**Major inclusion criteria**

- Patients aged ≥ 18 years undergoing ileocolic resection and anastomosis formation for fibrotic strictures secondary to confirmed CD.
- Patients must be able and willing to give written informed consent and to comply with the requirements of this protocol.
- Patients must be stable on existing CD medication with no changes in the three months prior to undergoing surgery and willing and or suitable to restart them postoperatively as per protocol.
- Patients must be willing to undergo ileocolonoscopy, blood tests and MRI scanning

**Major Exclusion criteria**
• Previous or current use of ACE inhibitors or ARBs
• Patients with a contraindication or allergy to losartan or other ARBs
• Pregnancy or breastfeeding
• Patients with alteration in drug therapy (e.g. new introduction of CD medication such as anti-TNF agents)
• Patients without a primary ileocolic anastomosis i.e. permanent stoma formation or diverting loop ileostomy
• Patients with symptomatic hypotension

**Primary objective**

Determine the effect of losartan on the Rutgeerts score (particularly presence of strictures) at the ileocolic anastomosis following CD surgery.

**Secondary objectives**

Determine the effect of losartan on:

• MRI scores for fibrosis
• Surrogate profibrogenic markers in ileal biopsies as determined by qPCR
• Clinical disease severity scores
• Serum inflammatory markers and faecal calprotectin

8.2.5 **Concluding remarks**

A substantial body of evidence has now accumulated supporting the role of RAS in intestinal fibrosis in CD. Losartan and other ARBs are already part of standard care in the management of chronic kidney and cardiac diseases. This thesis has drawn together evidence from human CD, human *in vitro* work and experiments *in vivo* in an exciting high fidelity murine model of...
experimental ileitis driven fibrosis. As with many pieces of research, this thesis raises almost more questions than it answers; there are many new frontiers to investigate in the Byzantine overlapping nexus of local RAS networks and subcomponents and novel anti-fibrotic pathways to evaluate. Nevertheless, the central message of this thesis is clear; it corroborates previous evidence that RAS is important in fibrosis and that the ARB losartan is a promising anti-fibrotic in CD. Given the tolerability, safety and availability of losartan, I firmly believe, there is now sufficient data to support investigating the putative effects of losartan on gut fibrosis in a human study.
Chapter 9. References

45. Meijer, M.J., et al., *Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene


Appendix
Research Ethics Committee

Research Protocol
PROTOCOL TITLE:

THE ROLE OF ANGIOTENSIN & FUTURE BIOMARKERS IN INTESTINAL FIBROSIS IN INFLAMMATORY BOWEL DISEASE

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1. Background & Rationale

Both Crohn’s disease & ulcerative colitis are conditions characterised by chronic intestinal inflammation. One of the key challenges facing doctors treating inflammatory bowel disease is the development of gut fibrosis and intestinal stenosis and strictures. This is especially prevalent in Crohn’s disease. Despite all the advances made in recent years in our understanding and management of intestinal inflammation, the incidence of patients requiring surgical resection have remain largely unchanged at around 2/3 of all Crohn’s disease patients. Furthermore, once intestinal fibrosis and stenosis have occurred, there are no current effective medical treatments to reverse this or to prevent progression.

Fibrosis in the liver, kidney and heart has been widely studied and many of the mechanisms involved have been elucidated and found to be related. In comparison, study of intestinal fibrosis is still in its infancy and little is known about the intricate pathways and triggers for gut fibrosis. The study of this field is hindered by the lack of good animal models of gut fibrosis and the difficulty in detecting and grading intestinal fibrosis in both animal and human subjects.

Recent advances in imaging, putative biomarkers of fibrosis and the discovery of better animal models have started to fuel interest in this field.

As stated earlier, the mechanism underlying tissue fibrosis in different organ systems carry many similarities. An area of promise is the renin-angiotensin system which is widespread throughout most of the tissues of the body and plays a variety of roles including salt and water homeostasis. Earlier work done on renal and cardiac fibrosis have shown that angiotensin also plays a key role in fibrogenesis and that reduction of angiotensin II production with the use of Angiotensin Converting Enzyme inhibitors and drugs that blockade angiotensin II (AT1a receptor antagonists) are successful in reducing and preventing progression of fibrosis in patients. They have now become key drugs used in the treatment of chronic cardiac and kidney diseases. More recently, AT1a receptors have been found to be upregulated in the liver of humans with liver fibrosis. In numerous mouse models of liver fibrosis, AT1a blockade has been shown to significantly reduce the severity of liver fibrosis. AT1a knockout mice also have significantly abrogated liver fibrosis in these models than the wild type. Pilot studies in humans have also been promising with a reduction in fibrosis scores in both a small hepatitis C cohort and non-alcoholic fatty liver disease. Further large scale clinical studies are now being conducted.

Bearing in mind the similarities between pathways of fibrosis in multiple disparate organ systems, it has been suggested that Angiotensin II may play an important role in gut fibrosis in Crohn’s disease. Initial animal studies of both ACE inhibitors and AT1a antagonists have indeed shown reduced markers of inflammation and fibrosis in animal models of colitis. Assessment of human colonic strictures in IBD have demonstrated upregulation of AT1a receptors suggesting this is an attractive target to study.

In this study we wish to investigate further the role of angiotensin II in fibrogenesis in Crohn’s disease. We wish to examine this in historical archival specimens and in cells cultured from fresh surgical resections in patients with Crohn’s, ulcerative colitis and non-IBD controls. Additionally we wish to take blood samples to examine the renin-angiotensin system and putative biomarkers
in patients with Crohn’s disease and controls.

2. Study Objectives and Design

2.1. Study Objectives

In this study we wish to ask:

1. Is there a difference in angiotensin receptor II expression in the intestine of patients with fibrostenotic Crohn’s versus controls or other non-fibrostenotic Crohn’s patients?

2. Do intestinal myofibroblasts grown from gut tissue taken from patients with fibrostenotic disease express AT1a receptors and does stimulation of these receptors provoke enhanced extracellular matrix deposition and fibrosis versus controls. Does blockade of these receptors negate this effect.

3. Are there differences in the serum renin-angiotensin system in patients with Crohn’s disease versus controls?

4. Can we identify a putative serum biomarker for gut fibrosis?

Major Outcomes:
The proposed study will provide us with a clearer understanding of the putative role of angiotensin II in Crohn’s disease fibrosis and whether it is a viable and attractive target for therapeutic intervention in future human studies

Secondary Outcome:
We hope to detect novel serum biomarkers for the assessment of fibrosis including components of the renin-angiotensin system.

2.2. Study Design

1. Is there a difference in angiotensin receptor II expression in the intestine of patients with fibrostenotic Crohn’s versus controls or other non-fibrostenotic Crohn’s patients?

Methodology: Experimentally this will initially require study of archival paraffin embedded tissues of ileal and colonic resections of patients with Crohn’s disease and controls (e.g. cancer resections). Samples will be processed using different techniques such as immunohistochemistry, quantitative real time PCR, Western Blot and gel electrophoresis to assess the presence of AT1a receptors.

2. Do intestinal myofibroblasts grown from tissue taken from patients with fibrostenotic disease express AT1a receptors and does stimulation of these receptors provoke enhanced extracellular matrix deposition and fibrosis versus controls?

Methodology: This involves culturing myofibroblast cells from fresh resection specimens of patients undergoing surgery for Crohns disease and controls (e.g. cancer, diverticular disease etc). Fresh tissue will be obtained from the resected gut by a pathologist. This will then be processed and myofibroblasts extracted using various techniques including centrifugation and columnisation. The extracted cells will then be cultured in vitro and once sufficiently mature, used for experimentation including assessment of the presence of angiotensin receptors.
and then provocation experiments with exogenous angiotensin II, blockade with ARBs and analysis using Western blot and qRT-PCR to assess markers of fibrosis and elements of extracellular matrix formation.


Additional serum will be taken from patients undergoing routine bloods tests or cannulation as part of their routine therapy in gastroenterology outpatients, endoscopy and inpatient wards. Samples will be taken from patients with IBD with known fibrosis, patients with IBD with no evidence of gut fibrosis and healthy controls. The serum will be stored and analysed for renin and angiotensin levels and putative fibrosis markers such as the ELF test (currently used for liver fibrosis).

2.3. Sample Size

1. **Archival paraffin embedded samples:** intestine resected from patients with IBD (Crohn’s & UC) and controls (e.g. cancer cases) will be obtained (n=20 of each of Crohn’s and UC, n=10 for controls; total 50).

2. **Freshly resected bowel tissue:** samples of the intestine of patients undergoing resection for Crohn’s, UC and control (e.g. colon cancer/diverticular disease) will be obtained and cells cultured from this (n=10 for CD/UC and n=5 controls; total 25). The quantity and location of bowel tissue donated will be carefully decided by the pathologist on a case by case basis so that it has no effect whatsoever on the tissue needed in diagnosis. We will not use tissues in cases where this is not the case.

3. **Serum:** We will invite up to a total of 300 patients to donate 50 mls of serum for analysis whilst undergoing venepuncture/cannulation for their own clinical need (n=300 Crohn’s & n=150 controls)

2.4. Analysis and Statistics

It is difficult to make the final call in terms of how data is analysed until we see the characteristics of the data (whether or not it is normally distributed). For example non-parametric continuous variables will be compared with a Mann-Whitney U test. Categorical data will be compared using a Chi-squared test. Additional analyses will be performed as appropriate.

Statistical advice for the study has been provided by the Kings College NIHR Biomedical Research Centre Research Consulting Service in statistical analysis.

3. Selection and Withdrawal of Subjects

3.1. Inclusion Criteria
Some of the proposed study involves the analysis of tissue sections taken from paraffin blocks from the Department of Histopathology, St Thomas’ Hospital. Blocks will be used from archived material stored before 2006. Blocks of tissue put into storage after 2006 will be excluded from our study unless specific consent can be obtained on a case by case basis.

For the analysis of fresh tissues/serum:
Patients will be included in the study
- If they understand English sufficiently to be able to understand the patient information sheet and consent forms.
- If they are 18 or over.
- If they consent & have capacity

3.2 Exclusion Criteria

Paraffin blocks will be excluded if they were from operations that took place after September 2006 unless we have secured specific consent for their use from the patient.

For fresh tissues, patients will be excluded:
- If they do not understand English sufficiently to be able to understand the patient information sheet and consent forms.
- If they are not over 18.
- If they have infectious disease.
- If they do not wish to consent or lack capacity to do so.

3.3. Withdrawal of Subjects

If subjects withdraw, samples specifically collected for this project will be removed from storage and disposed of according to the HTA guidelines for disposal of tissues. Identifiable data will be removed from the study.

4. Assessment of Safety

This study will be conducted in compliance with the rules in the gastroenterology Department. Of particular importance; human tissues will be handled in class II facilities.

5. Ethics & Regulatory Approvals

This study will be conducted in compliance with the rules of the local REC committee.

6. Data Quality
The study will be continuously reviewed through regular lab meetings to support this joint clinical/laboratory project. Findings will be reported at scientific meetings and through scientific journals.

7. Signature

__________________________________________________  6th February 2012
Chief Investigator
Jeremy Sanderson

Date
Consent Form
CONSENT FORM: Version 1. 6th February 2012

STUDY: THE ROLE OF ANGIOTENSIN & FUTURE BIOMARKERS IN INTESTINAL FIBROSIS IN INFLAMMATORY BOWEL DISEASE

PATIENT ID NUMBER FOR THIS STUDY: DATE:
Project Ethics Approval Number:
Name of Researcher: Jeremy Sanderson/Shuvra Ray/Jude Oben

Please initial the boxes below to confirm that you have understood and agree with the statements

1. I confirm that I have read and understood the patient information sheet dated 6th Feb 2012 (version 1) for the above study. I have had opportunity to consider the information, ask questions and have had these answered satisfactorily. □

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. □

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to me taking part in this research. I give permission for these individuals to have access to my records. □

4. I agree to take part in this study by donating tissue that is not needed for my diagnosis or treatment. □

5. I agree to take part in this study by donating a blood sample. □
6. I understand that my donation is a gift and I agree to the use of my sample in future studies provided they are studies approved by a research ethics committee.

Name of Patient……………………  Date………………………
Signature……………………………………

Name of person
Taking consent ..........................Date............................
Signature……………………………………

Researcher ..............................
Date..........................Signature……………………………………

When completed: 1 copy for patient, 1 for research site file, and 1 (original) to be kept in medical notes.
STUDY: THE ROLE OF ANGIOTENSIN & FUTURE BIOMARKERS IN INTESTINAL FIBROSIS IN INFLAMMATORY BOWEL DISEASE

You are being invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

5. Part 1 tells you the purpose of the study and what will happen to you if you take part.
6. Part 2 gives you more detailed information about the conduct of the study.

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1 – STUDY INFORMATION

Background

Inflammatory bowel disease is a condition of the gut characterised by chronic inflammation. Chronically inflamed tissues can eventually become scarred in a process known as fibrosis. This results in loss of function and narrowing sometimes leading to intestinal blockage. This can result in bloating, abdominal pain and vomiting. Currently there is no effective medical therapy to treat or prevent the development of intestinal fibrosis and many patients will undergo surgical removal. Furthermore there is no easy way to monitor the development of fibrosis in the gut. Recent evidence has suggested a role for a protein called angiotensin II in the development of fibrosis in other organs of the body and we intend to investigate this in the gut.

What is the purpose of the study?
The objectives of this study are to:

- Identify the presence of angiotensin II receptors in the cells responsible for fibrosis which we will extract from gut tissue taken at the time of surgery for the bowel.
• Investigate how stimulation of these cells with different products affects fibrosis.
• Investigate if blocking these receptors with specific medications may reduce or prevent this.
• Analysing blood samples from participants for levels of angiotensin and other novel markers of fibrosis to help determine a new non-invasive means of detecting intestinal fibrosis.

Why have you been chosen?
• You have been chosen because you have been either been recommended by your doctor to undergo bowel surgery or endoscopy or you are having routine blood tests.
• To carry out our studies we need samples from the gut from individuals who do not have inflammatory bowel disease (controls) and individuals who have inflammatory bowel problems such as Crohn's disease.

Do you have to take part?
• It's up to you whether or not to take part.
• If you do you will be given this sheet to keep and asked to sign a consent form.
• A decision to withdraw at any time or a decision not to take part will not affect the standard of care you receive.

What will happen if you take part?
• If you decide to take part in the study there will be very little difference to what normally happens in your planned procedure.
• As part of your clinical care your doctor may have recommended blood tests. You may also be about to undergo bowel surgery.
• You will be asked to give an additional blood sample. This will be around half a teacupful (50ml). This will allow us to assess blood angiotensin activity and other markers of fibrosis. The blood sample will either be taken from the needle already placed in your arm to give you medicine as part of your test or taken at the same time as other samples. There is no additional discomfort when the sample is taken.
• If you are having a bowel surgery, the pathologist will take a small part of the removed bowel for this study. There are no additional risks to donating resected bowel tissue for research.
• You may be asked whether you are willing to donate a further blood sample in future visits to the clinic, but you will be approached afresh with a new patient information sheet and consent form.
• We may also ask your permission to be able to look at any samples that are taken by hospital staff during your clinical investigation and treatment and that are left over after your diagnosis has been made.

What are the potential hazards of taking part?
There are no additional risks to donating bloods for research.
There are no additional risks to donating resected bowel tissue for research

What are the benefits of taking part?
There is no clear benefit to you but improved understanding of intestinal fibrosis (scarring) may help improve treatment for future patients.

What happens if there is a problem?
• Please contact the researchers on the numbers below if there are any problems with the research.
- If you are unhappy at any point about anything to do with your care and would prefer not to deal with researchers then complaints can be made to the hospital just as you would during your routine care.
- The contact number for the Patient Liaison Service (PALS) is 020 7188 8801.

Will my taking part in the study be confidential?
- As soon as your sample is taken it will be given a code that links it to the other samples you donate, but not to you. The samples will be completely anonymised.
- The only people with access to information that can identify you will be the lead researcher who is a practicing gastroenterology doctor. This information will be kept as confidential as your hospital notes.

PART 2. ADDITIONAL INFORMATION

What happens to the samples I give?
- The samples will be anonymised and stored in a locked freezer in a secure laboratory at either King’s College London or University College London.
- Once they have been used in the proposed experiments any cells left or tissue left over will be stored by the research team and used for future research into intestinal fibrosis, provided such studies are approved by the Research Ethics Committees.

Will my GP be informed?
As this study is only experimental data collection and our study does not involve any new treatments your GP will not routinely be informed, unless you would like us to do so.

What will happen to the results of the study?
The final results will be sent for publication in a peer-reviewed medical journal in the future. The data from your samples may be part of this but will be completely anonymous.

Who has reviewed this research?
This study has been reviewed by the Research Ethics Proportionate Review Sub-Committee.
QPCR

Amplification & Efficiency

Graphs
Actb

\[ y = -3.5257x + 40.14 \]

\[ R^2 = 0.9973 \]

PCR efficiency = 92.1%
il13

\[ y = -2.9x + 42.133 \]

\[ R^2 = 0.9763 \]

PCR efficiency = 121.2%

igf1

\[ y = -3.2771x + 40.72 \]

\[ R^2 = 0.997 \]

PCR efficiency = 101.9%

Melting Peaks

[Graph showing melting peaks for il13 and igf1]
ctgf

\[ y = -3.5514x + 40.147 \]

\[ R^2 = 0.9987 \]

PCR efficiency = 91.2%

il33

\[ y = -3.6143x + 41.5 \]

\[ R^2 = 0.9982 \]

PCR efficiency = 89.1%

Melting Peaks

ctgf  actinb  il33