Mesenteric manifestations in Crohn’s disease

A thesis submitted to the University of Limerick in fulfillment of the requirements for the degree of

Doctor of Medicine

By

Dr. Shaheel M. Sahebally

Centre for Interventions in Inflammation, Infection and Immunity (4i), Graduate Entry Medical School, University of Limerick and Department of Colorectal Surgery, University Hospital Limerick

Supervisors: Professor J Calvin Coffey and Professor Colum Dunne
Declaration

I hereby confirm that this thesis is my own and I am the sole author. However, part of the work presented involved other researchers and their contribution is acknowledged.
I authorize University of Limerick to lend and photocopy this thesis to other institutions and individuals for scholarly research.
I declare no conflict of interest in the preparation of this thesis.

_____________________
Shaheel Sahebally, July 2016
Acknowledgements

A number of contributors to the production of this thesis must be acknowledged for their assistance and expertise:

Professor J. Calvin Coffey has been a constant driving force throughout the preparation and production of this thesis. As a Consultant Surgeon and Principal Investigator, he showed my surgical peers and I the importance of researching surgical practice. With such experience, his guidance ensured that the end goals of the project were never far from focus. I am indebted and very grateful that he provided me with the opportunity to carry out my MD.

Prof Colum Dunne facilitated the research and provided constant advice and encouragement. I would like to thank him for this opportunity.

Miranda Kiernan was the research assistant for this project and was a constant source of guidance and support. I would like to thank her for her expertise and patience in teaching me scientific principles.

I am grateful to Dr. Patrick Kiely who welcomed me in his laboratory and ensured that I was made to feel like one of his own students. He also provided constant encouragement and valuable expertise whenever I needed it. I would also like to thank all the science students in Dr. Kiely’s laboratory for their continued patience and support during my time there.

I also have to thank my colleagues John Burke and John Hogan for their assistance with statistical concepts and words of encouragement throughout. I also like to thank Drs. Peter Faul and Vourneen Healy for their help with immunohistochemistry and helping me understand key pathological concepts. I also like to thank Mr Sean Martin from St. Vincent’s University Hospital for his collaboration through the provision of Crohn’s disease patients who were enrolled in the study.
My parents, Fareed and Nargiss, are truly the originators of this work. From my undergraduate years, through to my early surgical training and into research, they relentlessly provided support. They are the persons to whom I am most indebted.
Funding acknowledgements

Graduate Entry Medical School Strategic Funding/University of Limerick Seed Funding
Executive summary

Background
Mesenteric manifestations are of pathobiologic relevance in Crohn’s disease. Clarification of mesenteric morphology provides an opportunity to re-appraise their pathogenic significance. Therefore, we examined the relationship between mesenteric, mucosal and systemic manifestations in Crohn’s disease.

Methods
A multi-institutional study was conducted in which mesenteric disease was quantified (mesenteric disease score) in patients undergoing resection (n=34) for CD. The mesenteric disease score was correlated with a mucosal disease score, and Crohn’s Disease Activity Index (CDAI). The relationship between mesenteric manifestations and surgical recurrence was retrospectively determined in a second cohort (n=94). Local mesenteric and systemic fibrocyte levels were determined using a combination of histology, immunohistochemistry and flow cytometry. Mesenteric and mucosal gene expressions were compared in Crohn’s disease patients, in silico using Chipster©, an R based bio-informatic software.

Results
Mesenteric disease scores correlated with mucosal disease scores (r=0.8, p<0.0001) and CDAI (r=0.7, p<0.0001) and were significantly increased in smokers (p<0.04). Mesenteric disease manifestations independently predicted increased risk of surgical recurrence (HR 4.7, 95% CI: 1.71-13.01, p=0.003) and reduced time to recurrence (p<0.001). Mesenchymal abnormalities included fibrocytosis and adipocyte hyperplasia and were contiguous between mesentery and adjacent intestine. The fibrocyte proportion of circulating monocytes was increased in Crohn’s disease compared with healthy controls (6.4 ± 2.82% vs. 2.0 ± 1.04%, p<0.001). Levels normalized following ileocolic resection (5.7 ± 2.12% vs. 1.7 ± 1.20%, p=0.005), in patients with ileocolic disease. Fibrocyte percentages correlated with mesenteric and mucosal disease scores as well as with the CDAI (r=0.94, p<0.0001). Mesenteric, but not mucosal, gene expression profiles were associated with connective tissue, immunologic and inflammatory disorders.
Conclusions
Mesenteric manifestations are an important part of the pathogenesis of Crohn’s disease; they are associated with smoking and disease severity, and have predictive value for surgical recurrence. Therefore, they should be formally scored and recorded at the time of surgery.
Table Of Contents

Declaration.................................................................................................................. 2

Acknowledgements .................................................................................................. 3

Funding acknowledgements ................................................................................. 5

Executive summary.............................................................................................. 6

Table Of Contents ................................................................................................. 8

Publications from this thesis ............................................................................... 11

Manuscripts submitted ......................................................................................... 12

Abstract publications .......................................................................................... 13

Presentations from this thesis ............................................................................. 15

Awards from this thesis ........................................................................................ 18

List of tables .......................................................................................................... 19

List of abbreviations ............................................................................................ 21

Chapter one: Mesenteric manifestations in Crohn’s disease represent novel opportunities for research............................................................................................................. 23

Crohn's disease....................................................................................................... 24
Management............................................................................................................ 26
Pathobiology............................................................................................................ 26
Disease classification............................................................................................... 27
Assessment of disease activity ............................................................................. 30
Biomarkers.............................................................................................................. 33
Surgery for Crohn’s disease ................................................................................... 33
Mesenteric manifestations in Crohn's disease........................................................ 34
Recent advances in anatomy and histology of the mesentery................................. 36
Opportunities presented........................................................................................ 39
Objectives of thesis............................................................................................... 41
Chapter two: Fat wrapping independently predicts increased risk of surgical recurrence in Crohn’s disease ................................................................. 43

Abstract .................................................................................................................................................................. 44
Introduction ......................................................................................................................................................... 46
Materials and methods .................................................................................................................................. 48
  Patient population ....................................................................................................................................... 48
  Definition of end-points ............................................................................................................................... 48
  Statistical analysis ....................................................................................................................................... 49
Results .............................................................................................................................................................. 50
  Patient population ....................................................................................................................................... 50
  Surgical recurrence following index resection ............................................................................................. 50
  Prevalence of fat wrapping .......................................................................................................................... 51
  Correlation of fat wrapping with distinct clinicopathological features ...................................................... 51
  Analysis of factors associated with surgical recurrence ............................................................................ 51
Discussion ......................................................................................................................................................... 57

Chapter three: Mesenteric manifestations correlate with mucosal and systemic manifestations in Crohn’s disease ......................................................... 59

Abstract ............................................................................................................................................................ 60
Introduction ......................................................................................................................................................... 62
Materials and methods .................................................................................................................................. 64
  Patient cohort ............................................................................................................................................... 64
  The mesenteric disease score ........................................................................................................................ 68
  Mucosal disease score .................................................................................................................................. 72
  Histologic characterisation of mesenteric manifestations .......................................................................... 74
  Functional annotation of mesenteric and mucosal gene expression .......................................................... 74
  Statistical analysis ....................................................................................................................................... 75
Results .............................................................................................................................................................. 76
  Mesenteric manifestations correlated with mucosal disease severity and CDAI ...................................... 76
  Longitudinal distribution of mesenteric and mucosal manifestations correlated ....................................... 82
Discussion ......................................................................................................................................................... 91

Chapter four: The fibrocyte proportion of circulating monocytes is increased in Crohn’s disease ......................................................................................... 94

Abstract ............................................................................................................................................................ 95
Introduction ......................................................................................................................................................... 97
Materials and methods .................................................................................................................................. 99
  Patient cohort ............................................................................................................................................... 99
  Mesenteric and mucosal disease scores and Crohn’s disease activity index ............................................. 101
Flow cytometry of circulating fibrocytes.................................................................102
Histology..................................................................................................................102
Immunohistochemistry ............................................................................................103
Statistical analysis.....................................................................................................104
Results......................................................................................................................105
Percentage fibrocytes in circulating monocytes.........................................................105
Effects of ileostomy formation on the fibrocyte percentage of circulating monocytes...112
Mesenteric and intestinal fibrocytes ..........................................................................112
Discussion...............................................................................................................118

Chapter five: Discussion.........................................................................................120

Discussion...............................................................................................................121
Crohn's disease is a mes-enteropathy .....................................................................124

References.............................................................................................................127
Publications from this thesis

1. **Sahebally SM, Burke JP, Chang KH, Kiernan MG, O’Connell PR, Coffey JC**
Manuscripts submitted

Inclusion of the mesentery in ileocolic resection for Crohn’s disease is associated with reduced surgical recurrence.

Coffey JC, Kiernan MG, Sahebally SM, Jarrar A, Burke JP, Hogan J, Dunne C, Moloney M, Skelly M, Kiely PA, Shen B, Faul PN, Healy V, Martin ST, O’Connell PR, Shanahan F, Dunne C

Submitted to Gut December 2016
Abstract publications

1. ‘Fibroblasts and their precursors can be generated from mesocolic mesothelium-implications for intraperitoneal fibrotic disorders’
   Sahebally SM, Kiernan MG, Burke JP, Dunne C, Walsh SR, Coffey JC
   Ir J Med Sci Jan 2013; 182 (7); S297-357

2. ‘Correlation of fat wrapping with inflammatory changes in Crohn’s intestinal resection specimens- a 10 year experience’
   Sahebally SM, Samaha M, Roddy J, Burke JP, Coffey JC
   Ir J Med Sci Jan 2013; 182 (7); S297-357

3. ‘Circulating fibrocytes in Crohn’s disease- novel biomarker of disease severity’
   Sahebally SM, Kiernan MG, Dunne C, Faul PN, O’Connell PR, Martin ST, Coffey JC
   Ir J Med Sci Jan 2014; 182 (7); S168-257

4. ‘Elevated circulating fibrocyte levels in Crohn’s disease’
   Sahebally SM, Kiernan MG, Burke JP, Dunne C, Walsh SR, Kiely PA, Coffey JC
   Colorectal Disease Dec 2013: 13:S3 (4-39)

5. ‘Fat wrapping independently predicts adverse outcomes following surgery for Crohn’s disease’
   Sahebally SM, Burke JP, Roddy J, Healy V, Waldron D, Condon E, Coffey JC
   Colorectal Disease Jul 2014: 16:S2 (4-39)

6. ‘Mesocolic mesothelium demonstrates pluripotentiality when cultured ex- vivo-implications for gastrointestinal pathologies’
   Sahebally SM, Kiernan MG, Dunne C, Kiely PA, Coffey JC
   Colorectal Disease Jul 2014: 16:S2 (4-39)
7. ‘Identification of the mesocolic mesothelium as a novel source of fibroblasts-implications for Crohn’s disease’
   **Sahebally SM, Kiernan MG, Dunne C, Kiely PA, Coffey JC**
   J of Crohn’s and Colitis Feb 2014; 8:S88

8. ‘The mesenteric organ- a novel source of fibroblasts in benign and malignant colorectal disease’
   **Sahebally SM, Kiernan MG, Dunne C, Kiely PA, Coffey JC**
   Ir J Med Sci Jan 2014; 182 (1); S139-240
Presentations from this thesis

1. Elevated circulating fibrocyte levels in Crohn’s disease
   **Sahebally SM**, Kiernan MG, Burke JP, Dunne C, Walsh SR, Kiely PA, Coffey JC
   Royal Society of Medicine (RSM) Section of Coloproctology Meeting, John of Ardene Medal Session, Nov 2013, London, UK
   Oral Plenary Session

2. Prevalence of mesenteric fat encroachment in Crohn’s disease in our institution
   **Sahebally SM**, Samaha M, Roddy J, Coffey JC
   Irish Association of Coloproctology Meeting, May 2013, Dublin
   Oral Plenary Session

3. Fibroblasts and their precursors can be generated from mesocolic mesothelium-implications for intraperitoneal fibrotic disorders
   **Sahebally SM**, Kiernan MG, Burke JP, Dunne C, Walsh SR, Coffey JC
   XXXVIIIth Sir Peter Freyer Surgical Symposium, Sep 2013, Galway
   Oral Presentation

4. Correlation of fat wrapping with inflammatory changes in Crohn’s intestinal resection specimens- a 10 year experience
   **Sahebally SM**, Samaha M, Roddy J, Burke JP, Coffey JC
   XXXVIIIth Sir Peter Freyer Surgical Symposium, Sep 2013, Galway
   Oral Presentation

5. Identification of a novel and putative biomarker in Crohn’s disease- Circulating fibrocytes
   **Sahebally SM**, Kiernan MG, Burke JP, Dunne C, Kiely PA, Coffey JC
   XXIIIrd Waterford Surgical October Meeting In Conjunction with The Surgical Section of The Royal Academy Of Medicine in Ireland and The Irish Association of Vascular Surgeons, Oct 2013, Waterford

15
Oral Plenary Session

6. Identification of a novel and putative biomarker in Crohn’s disease- circulating fibrocytes
   Sahebally SM, Kiernan MG, Burke JP, Dunne C, Kiely PA, Coffey JC
   Irish Society of Gastroenterology Winter Meeting, Nov 2013, Killarney

Oral Plenary Session

7. The mesenteric organ is a novel source of fibroblasts and fibrocytes- implications for gastrointestinal fibrotic disorders
   Sahebally SM, Kiernan MG, Dunne C, Kiely PA, Coffey JC
   XXIIInd Sylvester O’Halloran Surgical Meeting, Feb 2014, Limerick

Oral Plenary Session

8. Identification of the mesocolic mesothelium as novel source of fibroblasts- implications for Crohn’s disease
   Sahebally SM, Kiernan MG, Dunne C, Kiely PA, Coffey JC
   9th Congress of European Crohn’s and Colitis Organisation (ECCO), Feb 2014, Copenhagen, Denmark

Poster Presentation

9. ‘Fat wrapping independently predicts adverse outcomes following surgery for Crohn’s disease’
   Sahebally SM, Burke JP, Roddy J, Healy V, Waldron D, Condon E, Coffey JC
   RAMI Surgical Section Registrar’s Prize Meeting, April 2014, RCSI

Oral Plenary Session

10. ‘Fat wrapping independently predicts adverse outcomes following surgery for Crohn’s disease’
    Sahebally SM, Burke JP, Roddy J, Healy V, Waldron D, Condon E, Coffey JC
    Tripartite Colorectal Meeting (Combined Meeting of ACPGBI, ASCRS and RSM), June 2014, Birmingham, UK

Oral Presentation
11. ‘Mesocolic mesothelium demonstrates pluripotentiality when cultured *ex-vivo*-implications for gastrointestinal pathologies’
   **Sahebally SM**, Kiernan MG, Dunne C, Kiely PA, Coffey JC
   Tripartite Colorectal Meeting (Combined Meeting of ACPGBI, ASCRS and RSM), June 2014, Birmingham, UK
   Oral Presentation

12. ‘Circulating fibrocytes in Crohn’s disease- novel biomarker of disease severity’
   **Sahebally SM**, Kiernan MG, Dunne C, Faul PN, O’Connell PR, Martin ST, Coffey JC
   XXXIXth Sir Peter Freyer Surgical Symposium, Sep 2014, Galway
   Oral Plenary Session Presentation
Awards from this thesis

1. University of Limerick Young Researcher of the Year Award
   Identification of the visceral peritoneal mesothelium as a novel source of fibroblasts
   **Sahebally SM, Kiernan MG, Dunne C, Kiely PA, Coffey JC**
   University of Limerick Annual Life Sciences Research Day, May 2013, Limerick

2. Oral Presentation Prize Winner (2nd Prize)
   Identification of a novel and putative biomarker in Crohn’s disease- circulating fibrocytes
   **Sahebally SM, Kiernan MG, Dunne C, Burke JP, Walsh SR, Kiely PA, Coffey JC**
   Irish Society of Gastroenterology Winter Meeting, Nov 2013, Killarney

3. Shortlisted by RAMI for best paper in Gastroenterology category with paper entitled ‘Circulating fibrocytes and Crohn’s disease’
   RAMI meeting, Feb 2014, RCSI
List of tables

Chapter one

Table 1: Vienna and Montreal classification of Crohn’s disease.
Table 2: Components of Crohn’s Disease Activity Index.
Table 3: Rutgeerts endoscopic severity scoring system for postoperative recurrent Crohn’s disease.

Chapter two

Table 1: Patient demographics and characteristics at index intestinal resection.
Table 2: Patient clinicopathological characteristics and fat wrapping.
Table 3: Univariate analysis of factors associated with surgical recurrence.
Table 4: Multivariate analysis of factors associated with surgical recurrence.

Chapter three

Table 1: Demographics of all patients undergoing surgical resection for Crohn’s disease (from July 2013 to January 2015).
Table 2: Demographics of the subgroup of patients undergoing ileocolic resection for ileocolic Crohn’s disease (from July 2013 to January 2015).
Table 3A: Mesenteric disease score in Crohn’s disease.
Table 3B: Mucosal disease score in Crohn’s disease.
Table 4A: Univariate analysis to determine association between groups A (< mean expression value), B (> mean expression value) and Crohn’s disease.
Table 4B: Univariate analysis to determine association between groups A (responded to medical therapy), B (did not respond to medical therapy) and incidence of Crohn’s disease.

Chapter four

Table 1: Demographics of all patients undergoing surgical resection for Crohn’s disease (from July 2013 to January 2015).
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming-growth-factor-beta</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming-growth-factor-beta-1</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>Transforming-growth-factor-beta-2</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Transforming-growth-factor-beta-3</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour-necrosis-alpha</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>CD 14+</td>
<td>Cluster of differentiation molecule 14+</td>
</tr>
<tr>
<td>CD 16-</td>
<td>Cluster of differentiation molecule 16-</td>
</tr>
<tr>
<td>CD 34</td>
<td>Cluster of differentiation molecule 34</td>
</tr>
<tr>
<td>CD 45</td>
<td>Cluster of differentiation molecule 45</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha-smooth-muscle-actin</td>
</tr>
<tr>
<td>CCL 21</td>
<td>Chemokine Ligand 21</td>
</tr>
<tr>
<td>CXCL 12</td>
<td>Chemokine Ligand 12</td>
</tr>
<tr>
<td>CCR 2/3/5/7</td>
<td>Chemokine Receptors 2/3/5/7</td>
</tr>
<tr>
<td>CXCR 4</td>
<td>Chemokine Receptor 4</td>
</tr>
<tr>
<td>IL 13</td>
<td>Interleukin 13</td>
</tr>
<tr>
<td>IL 4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>PDGF A</td>
<td>Platelet-Derived-Growth-Factor-Alpha Subunit</td>
</tr>
<tr>
<td>PDGF BB</td>
<td>Platelet-Derived-Growth-Factor-Beta Subunit</td>
</tr>
<tr>
<td>CCR2</td>
<td>Chemokine Receptor 2</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major Histocompatibility Class II</td>
</tr>
<tr>
<td>CD45R0</td>
<td>Isoform of CD45 but lacking exons A, B and C</td>
</tr>
<tr>
<td>25F9</td>
<td>Surface antigen expressed on myeloid cells of the granulocyte-monocyte-histiocyte series during differentiation</td>
</tr>
<tr>
<td>S100A8/A9</td>
<td>Calprotectin</td>
</tr>
<tr>
<td>PM-2K</td>
<td>Antigen expressed only by mature macrophages and absent on fibrocytes</td>
</tr>
<tr>
<td>Pro-col-1</td>
<td>Pro-collagen-1</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic Pulmonary Fibrosis</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PPAR-δ</td>
<td>Peroxisome Proliferator Activated Receptor-gamma</td>
</tr>
<tr>
<td>MMP 9</td>
<td>Matrix Metalloproteinase 9</td>
</tr>
<tr>
<td>ICAM 1</td>
<td>Inter Cellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>b-FGF</td>
<td>basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>YKL 40</td>
<td>40 kDa glycoprotein secreted by macrophages and fibrocytes</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor Tyrosine Kinase Inhibitor Motif</td>
</tr>
<tr>
<td>mTOR-pI3</td>
<td>Mammalian Target Of Rapamycin- Phosphatidyl-Insitol-3-kinase</td>
</tr>
<tr>
<td>ATR 2</td>
<td>Angiotensin Type 2 Receptor</td>
</tr>
<tr>
<td>SAP</td>
<td>Serum Amyloid Protein</td>
</tr>
</tbody>
</table>
Chapter one: Mesenteric manifestations in Crohn’s disease represent novel opportunities for research
**Crohn’s disease**

Crohn’s disease is a chronic, incurable disorder characterized by transmural (occasionally granulomatous) inflammation and prominent mesenchymal abnormalities \(^1, 2\). It can affect any part of the gastrointestinal tract and is thought to arise due to interactions between genetic, environmental and microbial factors \(^3, 4\). The incidence and prevalence of Crohn’s disease in the United States are 5/100,000 and 50/100,000 respectively \(^5\), and are similar in ‘Westernized’ countries.

Crohn’s disease is a phenotypically heterogenous condition with diverse clinical manifestations \(^6\). Patients mostly present with abdominal pain and nocturnal, occasionally bloody diarrhoea, fever and unintentional weight loss \(^3, 7\). Clinical signs may include pallor, cachexia, abdominal tenderness and/or mass, perianal fissures, fistulae or abscesses \(^3\). The natural history is usually progressive \(^6\).

Crohn’s disease can also affect multiple systems leading to extra-intestinal manifestations including ocular (iritis, uveitis, scleritis), oral (aphthous ulcers, stomatitis), dermatological (erythema nodosum, pyoderma gangrenosum), musculoskeletal (sacroilitis, ankylosing spondylitis) and hepatobiliary (primary sclerosing cholangitis, cholestasis) abnormalities \(^3\).

Although it may affect any age group, it most commonly arises in the second and third decades of life \(^3\). The natural history is usually progressive but it can, albeit rarely, present acutely with fulminant colitis. In general, the anatomic site affected remains stable with time. However, disease manifestations generally progress from being inflammatory to involving stricture or fistula formation \(^8\).
The following chapter details the challenges associated with Crohn’s disease. The chapter discusses how these challenges may be met by characterising the cellular basis of mesenteric disease manifestations.
Management

The management of Crohn’s disease continues to be challenging on multiple levels\textsuperscript{9}. The heterogeneity of the disease, coupled with extra-intestinal manifestations and lack of diagnostic biomarkers, make diagnosis difficult\textsuperscript{9,10}. Diagnosis as well as prediction and prognostication rely mainly on exclusion of other conditions and trialling patients with anti-inflammatory, immunomodulatory and biologic agents. Despite considerable advances in the generation of pharmacologic means of reducing symptoms, overall rates of surgical intervention are unchanged\textsuperscript{11}. Following surgery, most patients will develop recurrent disease and a considerable proportion will require repeat surgical intervention\textsuperscript{12-15}. Given the likelihood of patients requiring repeated resections, surgeons have tended to adopt as conservative an approach to resection as possible.

Pathobiology

Challenges in the clinical management of Crohn’s disease are mirrored by deficits in our understanding of its aetiology and pathobiology. This may be considered surprising, as numerous features of Crohn’s disease are characteristic of it, and not seen in other intestinal conditions. For example, it is not known why mesenchymal abnormalities seen in Crohn’s disease are transmural, and not simply confined to the intestinal mucosa or submucosa of the intestine. In addition, it is not known why, following surgical resection and anastomosis, recurrent disease generally occurs immediately proximal to the site of surgical anastomosis. Mesenteric disease manifestations are characteristic of Crohn’s disease\textsuperscript{16}. Notwithstanding this, their aetiology and pathobiology have remained relatively understudied.
Disease classification

In addressing the clinical challenges associated with Crohn’s disease, several classification systems emerged. These aim to assist in prognostication and prediction, but not in diagnosis. Farmer et al. 17 developed the first classification of Crohn’s disease based on anatomic location. They hypothesized that anatomic location directly determines the clinical course and prognosis of the disease. Anatomic locations were divided into ileocolic, small intestinal, colonic and anorectal. Their approach to classification remains in broad use today. Greenstein et al. 18 introduced the concepts of “perforating” and “non-perforating” disease behaviour into classification. They noted the former subtype was more frequently associated with surgical intervention.

Subsequently, the Rome classification 19 was developed by the Working Party of Gastroenterologists in 1991 and adopted the suggestions of Farmer and Greenstein. Additional parameters such as disease extent (i.e. localized or diffuse) and operative history (i.e. primary or recurrent) were also included. The result was a complex classification system with a considerable number of disease subtypes. Moreover, Steinhart et al. 20 examined inter-observer agreement associated with the latter classification system and noted only fair agreement between observers, thus raising concern regarding its reliability.

The Vienna classification 21 (Table 1) was developed in 1998 and incorporated three parameters: age at diagnosis (A1-2), disease location along the intestine (L1-3) and disease behaviour (inflammatory, strictureing or fistulizing (B1-3). Its reproducibility has been assessed by two small studies, yielding somewhat contradictory results. Riis et al. 22 concluded that the Vienna classification was associated overall with a good inter-
observer agreement while another study \(^{23}\) reported an excellent agreement for disease behaviour but only a fair agreement for disease location.

The Montreal classification \(^{24}\) (Table 1) (introduced in 2005) was a slight modification of the Vienna classification. It incorporated an age subgroup for paediatric patients (< 16 years of age), and an additional subgroup for upper gastrointestinal disease (L4). The behaviour category was also amended to include perianal disease (denoted by ‘p’). This classification system has been widely adopted in international collaborative studies examining genotype-phenotype associations \(^{25}\). In an attempt to validate the Montreal classification, 13 expert practitioners from Australia and New Zealand reviewed the data relating to 35 Crohn’s disease patients and concluded that it was associated overall with good inter-observer agreement \(^{26}\). Moreover, Chow \textit{et al.} \(^{27}\) demonstrated superiority of the Montreal classification over the Vienna classification at detecting behavioural phenotypic changes over time and hence predicting the need for surgery.
<table>
<thead>
<tr>
<th>Age at onset</th>
<th>Vienna classification</th>
<th>Montreal classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 &lt; 40 years</td>
<td>≥ 40 years</td>
<td>A1 &lt; 16 years</td>
</tr>
<tr>
<td>A2</td>
<td>A2 17-40 years</td>
<td>A2 17-40 years</td>
</tr>
<tr>
<td></td>
<td>A3 &gt; 40 years</td>
<td>A3 &gt; 40 years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease location</th>
<th>Vienna classification</th>
<th>Montreal classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1 terminal ileum</td>
<td>L1 ileal</td>
<td>L1 ileal</td>
</tr>
<tr>
<td>L2 colon</td>
<td>L2 colonic</td>
<td>L2 colonic</td>
</tr>
<tr>
<td>L3 ileocolon</td>
<td>L3 ileocolonic</td>
<td>L3 ileocolonic</td>
</tr>
<tr>
<td></td>
<td>L4 isolated upper disease*</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease behaviour</th>
<th>Vienna classification</th>
<th>Montreal classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1 inflammatory</td>
<td>B1 non-stricturing, non-penetrating</td>
<td></td>
</tr>
<tr>
<td>B2 stricturing</td>
<td>B2 stricturing</td>
<td></td>
</tr>
<tr>
<td>B3 penetrating</td>
<td>B3 penetrating</td>
<td></td>
</tr>
<tr>
<td></td>
<td>‘p’ perianal disease modifier</td>
<td></td>
</tr>
</tbody>
</table>

*L4 is a disease modifier that can be added to L1-3 when concomitant upper gastrointestinal disease is present. ‘p’ is added to B1-3 when concomitant perianal disease is present.

**Table 1.** Vienna and Montreal classification of Crohn’s disease.
Assessment of disease activity

Several disease activity indices have emerged that aim to assist in the clinical management of Crohn’s disease. The Crohn’s disease activity index (CDAI)\textsuperscript{28} (Table 2) comprises eight parameters. These are summed after adjustment with a weighting factor. Active disease is associated with a CDAI between 150-450. Remission is defined as a CDAI below 150, while a value greater than 450 signifies severe disease. The CDAI is widely used in studying effectiveness of novel pharmacotherapeutic agents in Crohn’s disease. Although intestinal symptoms are included, endoscopic parameters are not. As a result, it cannot be used as a direct score of mucosal disease manifestations.

The Rutgeert’s score\textsuperscript{29} (Table 3) is an endoscopic index that quantitates mucosal disease in the postoperative context. It accurately predicts clinical recurrence\textsuperscript{29}. Eighty to eighty-five percent of patients with a score of i-0 or i-1 are asymptomatic 3 years following surgical resection\textsuperscript{29-31}. The Rutgeert’s score and the Crohn’s Disease Endoscopic Index of Severity (CDEIS)\textsuperscript{32}, are widely used in the endoscopic assessment of Crohn’s disease\textsuperscript{33}.

To date, no scores have been developed that reflect the severity of intestinal disease in surgical specimens.
Clinical or laboratory variable | Weighting factor
--- | ---
Number of liquid or soft stools each day for seven days | X 2
Abdominal pain (graded from 0-3 on severity) each day for seven days | X 5
General well-being, subjectively assessed from 0 (well) to 4 (terrible) each day for seven days | X 7
Presence of complications* | X 20
Taking lomotil or opiates for diarrhoea | X 30
Presence of an abdominal mass (0 as none, 2 as questionable, 3 as definite) | X 10
Haematocrit < 0.47 in men and < 0.42 in women | X 6
Percentage deviation from standard weight | X 1

*One point each is added for each set of complications:
- The presence of joint pains (arthralgia) or frank arthritis
- Inflammation of the iris or uveitis
- Presence of erythema nodosum, pyoderma gangrenosum or aphthous ulcers
- Anal fissures, fistulae or abscesses
- Other fistulae
- Fever during the previous week

Table 2: Crohn’s Disease Activity Index scoring system. Remission: CDAI < 150; Active disease: CDAI 150-450; Severe disease: CDAI > 450.
An endoscopic scoring system for postoperative disease recurrence in Crohn’s disease

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No lesions in distal ileum</td>
</tr>
<tr>
<td>1</td>
<td>( \leq 5 ) aphthous lesions</td>
</tr>
<tr>
<td>2</td>
<td>&gt; 5 aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions, or lesions confined to ileocolonic anastomosis (that is (&lt; 1\text{cm in length})</td>
</tr>
<tr>
<td>3</td>
<td>Diffuse aphthous ileitis with diffusely inflamed mucosa</td>
</tr>
<tr>
<td>4</td>
<td>Diffuse inflammation with already larger ulcers, nodules and/or narrowing</td>
</tr>
</tbody>
</table>

**Table 3:** Rutgeert’s endoscopic scoring system\(^{29}\) for postoperative disease recurrence in Crohn’s disease.
Biomarkers

Accurate molecular biomarkers are lacking in Crohn’s disease. Most molecular markers are sensitive but not specific. Faecal calprotectin is increased in intestinal inflammation and differentiates between irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). Serum calprotectin appears to reflect disease activity in IBD and data suggest it may predict disease relapse following infliximab withdrawal. However, it cannot differentiate Crohn’s disease and ulcerative colitis. C-reactive protein, laminin, procollagen and anti-glycan antibodies are further candidate biomarkers. Their limitations are similar to those identified for serum calprotectin. Accurate cellular markers of disease are similarly lacking.

Surgery for Crohn’s disease

Most patients with Crohn’s disease will require surgery. The type of surgery is predicated on the location and behavioural type of disease. The operation most frequently performed is an ileocolic resection. Other operation types (in order of frequency) are stricturoplasty, colectomy, panproctocolectomy and end ileostomy. Rarely, the disease encountered at operation is too extensive for resection and requires fecal diversion via a defunctioning loop ileostomy.

A significant proportion of patients undergoing surgery will require future reoperation. This reflects the progressive nature of Crohn’s disease. At present, it is not possible to identify those patients who will require reoperation. Given the likelihood of reoperation, surgeons have tended to adopt as conservative an approach as possible to intestinal resection.

The mesentery is frequently diseased in Crohn’s disease and presents particular challenges for the surgeon. In general, the mesentery is thickened and foreshortened.
Its division is associated with significant and sometimes life threatening haemorrhage. As a result, surgeons have tended to adopt non-radical approaches to division of the mesentery. To do this, they cut directly across the mesentery, as flush with the intestine as possible.

Following surgery, recurrence tends to occur at or immediately proximal to the intestinal anastomosis. The reason for this is unknown. Several anastomotic conformations have been assessed with a view to reducing the incidence of postoperative recurrence. An end to side conformation is associated with similar rates of recurrence compared with end-end conformations. There are suggestions that side to side intestinal anastomoses are associated with reduced rates of recurrence.

Mesenteric manifestations in Crohn’s disease

Mesenteric manifestations are characteristic of Crohn’s disease and appear not to occur in any other forms of gastrointestinal disease. They can be classified as (a) fat wrapping (i.e. creeping fat) and (b) mesenteric thickening. The terms “fat wrapping” or “creeping fat” are used interchangeably and refer to the extension of mesenteric fat onto the surface of the gastrointestinal tract. Fat wrapping and mesenteric thickening were recognized by Burrill B. Crohn in his original description with the former described as tubercle-like deposits of “subserosal intestinal fat” and the latter as “shortening” and “fibrotic.” The frequency with which mesenteric derangements occur, and the specific association with Crohn’s disease, point to a disease-specific mechanistic link.

Data indicate that fat wrapping and mesenteric thickening correlate with mucosal and systemic disease manifestations. As per Crohn, mucosal ulceration always occurs on the “mesenteric border” at the “attachment” of the mesentery (i.e. axial polarity). Axial
polarity is characteristic of Crohn’s disease 54, 55 and further points to a pathobiologic relevance. Borley et al. 53 demonstrated a correlation between overlying connective tissue and underlying submucosal changes in ileal Crohn’s disease. Additionally they noted correlations between fat wrapping and composite histopathologic indices of disease activity in acute and chronic disease settings. In Crohn’s disease, fat wrapping always occurs in association with serositis 50. Weakley and Turnbull 50 identified a direct correlation between the longitudinal extent of mesenteric derangements and mucosal manifestations in Crohn’s disease.

The above findings recently led to a characterization of the cellular and molecular basis of mesenteric derangements in Crohn’s disease. Peyrin-Biroulet et al. 56 demonstrated increased mesenteric production of C-reactive protein (CRP) compared with ulcerative colitis and healthy controls. Mesenteric CRP correlated with systemic levels, thus identifying a direct link between systemic and mesenteric events. Zulian et al 57 suggest that mesenteric CRP production is driven by local inflammation consequent upon increased bacterial translocation. Several studies identified increased peroxisome proliferator activated receptor gamma (PPARγ), tumour necrosis alpha (TNFα), macrophage colony stimulating factor (M-CSF), adiponectin, and leptin in areas of diseased mesentery in Crohn’s disease 54, 58. Increasing data support the suggestion that the mesentery exerts an immunologic barrier role protecting the host from systemic bacterial distribution following intestinal translocation 59. Alterations in bacteria clearance (such as might occur in the context of NOD2/CARD15 mutations) are reflected in mesenteric lymphadenopathy, and the longitudinal extent of this in turn correlates with mucosal disease 50.

Whilst fat wrapping and mesenteric thickening have long been recognized, the precise aetiology for this phenomenon remains elusive. Accumulating evidence points to a
direct link between bacterial translocation and development of creeping fat \textsuperscript{60}. Even though bacterial translocation occurs in the normal healthy intestine \textsuperscript{56, 59}, it appears to be exaggerated in the Crohn’s intestine and is believed to result in adipocyte-tissue hyperplasia \textsuperscript{56}. Moreover, nucleotide-binding oligomerization domain (NOD)-2 variants, which are associated with an increased susceptibility to developing Crohn’s disease, were also found to affect bacterial transit through the intestinal barrier as well as adipocyte differentiation \textsuperscript{61}.

Adipocytes in obesity are mainly hypertrophied and harbour altered secretory activity, specifically increased production of pro-inflammatory cytokines and leptin \textsuperscript{62, 63}. In contrast, adipocytes in fat-wrapped areas of Crohn’s disease are smaller in size and up to four times as numerous as those in normal mesenteric fat tissue depots \textsuperscript{64}. They display characteristic patterns of elevated pro- and anti-inflammatory gene expression \textsuperscript{57}. Currently, there are insufficient data to convincingly state whether this mesenteric adiposity in Crohn’s disease potentiates adjacent intestinal inflammation, or indeed attenuates it \textsuperscript{65}.

**Recent advances in anatomy and histology of the mesentery**

Classic teaching depicts the mesentery as a complex and fragmented structure \textsuperscript{66}, with discontinuity between the small intestinal and colonic mesenteries. Recent studies by our group \textsuperscript{67, 68} have shown that not only are they continuous, but that the region around which they are contiguous (i.e. the ileocolic mesenteric confluence) is rich in lymphatics. The recent confirmation of mesenteric anatomy provided us with a novel opportunity to review the microscopic structure. Our group has demonstrated that the mesocolon comprises adipocyte lobules sandwiched between a visceral and parietal mesothelium \textsuperscript{69} (Figure 1). Crucially, a connective tissue layer has been identified
immediately beneath the mesothelium and from which connective tissue septations arose. This mesenteric “lattice” was contiguous with that of the gastrointestinal tract at the mesenteric hilum. The finding is particularly relevant as it may provide an anatomic/histologic platform from which the gastrointestinal, mesenteric and systemic events in Crohn’s disease could be explained.
Figure 1: Digital sculpture demonstrating that the mesocolon is actually composed of a two-layered fold of peritoneum, between which is sandwiched a connective tissue lattice rich in lymphatics (green). This connective tissue framework is contiguous with that of the gastrointestinal tract at the mesenteric hilum.
Opportunities presented

Clarification of mesenteric anatomy and histology presents new opportunities for investigation in Crohn’s disease. These are welcome, given that surgical operation and reoperation rates have remained unchanged.\textsuperscript{11,37}

A better knowledge of mesenteric anatomy facilitates differentiation of separate mesenteric regions (i.e. mesocolon and mesentery) and the differentiation of mesentery from other types of intra-abdominal fat (i.e. omentum, appendices epiploicae, visceral adiposity, retro-peritoneal, and preperitoneal adiposity). This in turn permits a more accurate identification of mesenteric abnormalities such as fat wrapping and mesenteric thickening, and their correlation with outcomes such as surgical recurrence (i.e. requirement for reoperation). As mesenteric disease manifestations are thought to correlate with mucosal disease, it is feasible that they could also correlate with increased rates of re-operation.

Improved ability to identify mesentery and associated abnormalities could permit the generation of an index of mesenteric disease. This would be clinically practicable, as it would enable a numeric correlation between mesenteric, local mucosal and systemic disease indices.

The mesenteric connective tissue lattice represents a further avenue for exploration as it is contiguous with that of the adjacent intestine. Connective tissue contiguity between mesentery and intestine could explain the transmural basis of mesenchymal derangements seen within the intestine.

Mesenteric manifestations in Crohn’s disease are characterised by adipocyte hyperplasia and increased connective tissue deposition.\textsuperscript{64} It is feasible that they are partially explained by increases in a cell type that is capable of differentiation into either adipocyte or fibroblast, i.e. the fibrocyte. Circulating fibrocytes are bone marrow
derived mesenchymal progenitor cells. They are recruited to sites of inflammation/injury and produce a variety of growth factors and chemokines in both physiologic and pathologic settings. Accumulating data suggest they play a key pathobiologic role in fibrotic disorders such as pulmonary, renal and hepatic fibroses. Recent findings by Sazuka et al. demonstrate that they are increased in Crohn’s disease. Future studies should determine their levels in alternative types of intestinal inflammation, as well as within the associated mesentery.
Objectives of thesis

**Objective one:** assess relationship between fat wrapping and rates of reoperation for Crohn’s disease.

Fat wrapping is an important surgical indicator of disease activity and pathologically appears to be a hallmark of Crohn’s disease. However, its relationship with disease recurrence has never been investigated.

An institution-review-board-approved database was retrospectively reviewed to identify all Crohn’s disease patients who underwent surgical resection during a 12-year period. The development of surgical recurrence was examined in relation to clinical and pathological variables.

**Objective two:** develop an index of mesenteric disease and examine the association with local and systemic disease.

Although mesenteric manifestations are frequently seen in Crohn’s disease, no classification system exists to stratify the severity of the pathological features. A novel severity scoring system for mesenteric and mucosal disease was developed and a score ascribed to each Crohn’s disease patient undergoing intestinal resection, following review of the resected pathological specimen. The relationship between mesenteric, mucosal and systemic manifestations in Crohn’s disease was subsequently investigated.

**Objective three:** examine fibrocyte levels in Crohn’s disease and in particular the association with mesenteric disease.
Fibrocytes are bone marrow-derived progenitor cells that can differentiate into either adipocyte or fibroblast and could thus contribute to the mesenchymal abnormalities associated with Crohn’s disease. Based on this the aim was to characterise circulating fibrocyte levels in Crohn’s disease.

Serum fibrocyte levels were determined using flow cytometry in patients undergoing a resection for Crohn’s disease, diverticular disease and colorectal cancer. Levels were correlated with mesenteric and mucosal disease scores as well as with the Crohn’s disease Activity Index. Tissue fibrocytes were evaluated immunohistochemically in both the mesentery and associated intestine in Crohn’s specimens.
Chapter two: Fat wrapping independently predicts increased risk of surgical recurrence in Crohn’s disease
**Abstract**

**Background:** A significant proportion of patients with Crohn’s disease require repeat surgical resection following initial resection. Although fat wrapping is a feature of Crohn’s disease, it is understudied. The aim of this study was to investigate the relationship between fat wrapping and surgical recurrence.

**Methods:** An institution-review-board-approved database was reviewed to identify all Crohn’s disease patients who underwent surgical resection between January 2001 and August 2013. The effects of fat wrapping on surgical recurrence were determined using Fisher’s exact test, Kaplan Meier estimates and Log-Rank comparisons. A p value < 0.05 was considered statistically significant.

**Results:** 94 patients (45M, 49F) underwent a total of 119 resections during the 12-year study period. The mean (SD) follow-up was 67.4 +/- 43.6 months. Twenty patients experienced surgical recurrence (21%) after a mean of 43.2 +/- 39.6 months following index resection. Although fat wrapping was associated with stricture formation (p=0.036), there was no association with transmural inflammation (p=1.0), fissuring ulceration (p=0.255), granuloma (p=0.373), body mass index (p=0.314) or fistulae formation (p=0.193). On univariate analysis, penetrating (HR 3.8, 95% CI: 1.3-10.6, P=0.012) and non-penetrating disease behaviour (HR 0.38, 95% CI: 0.15-0.98, P=0.045) and fat wrapping (HR 4.5, 95% CI: 1.77-11.5, P=0.002) were associated with surgical recurrence. On multivariate analysis only fat wrapping was associated with an increased risk of surgical recurrence (HR 4.7, 95% CI: 1.71-13.01, P=0.003). Fat wrapping was associated with a shorter time to recurrence (P<0.001, Log-rank test).
Conclusions: Fat wrapping appears to increase the risk of, and to decrease the time to, surgical recurrence in Crohn’s disease. These findings point to fat wrapping as a novel predictor of disease recurrence requiring reoperation in Crohn’s disease.
Introduction

The management of Crohn’s disease is hampered by several factors. Accurate diagnostic, predictive and prognostic biomarkers are lacking. Despite an increasing medical armamentarium surgical resection rates remain unchanged. Moreover, recurrence rates following resection are also unchanged. Up to 90% of patients have endoscopic evidence of recurrence within a year of surgery. Over 40% become symptomatic and require re-intervention within the first four postoperative years. Given the probability of repeat resection surgeons generally adopt a conservative approach to intestinal resection.

Notwithstanding the prevalence of recurrence requiring re-operation, we remain limited by an inability to identify patients likely to recur and require surgical re-intervention. The single strongest predictor of recurrence mandating surgery remains smoking. Genetic markers have been useful in unraveling disease pathogenesis, but have little predictive utility. As a result there remains a requirement to develop accurate biomarkers that are predictive of surgical recurrence.

Traditional research approaches in Crohn’s disease focused on the gastrointestinal lumen as the primary source of pathology. Mesenteric features of Crohn’s disease are relatively understudied. Recent suggestions indicate that mesenteric events could be of pathobiologic relevance. In particular, attention is being focused on mesenteric-mesenchymal abnormalities including fat wrapping and mesenteric thickening. Fat wrapping (or creeping fat), involves extension of mesenteric fat along the serosal surface of the intestine. Originally described by Burrill Crohn, fat wrapping appears unique to Crohn’s disease. In a review of 225 small bowel resections performed for various pathologies, fat wrapping occurred in 53% of Crohn’s cases, but was largely absent from all other pathologies. To date, the association between fat wrapping at index surgery
with post-operative disease recurrence has not been explored. Based on this, the aim of the present study was to examine the relationship between fat wrapping at index resection and recurrence requiring repeat resection for Crohn’s disease.
Materials and methods

Patient population

All Crohn’s disease patients who underwent intestinal resection between January 2001 and August 2013 at the Department of Colorectal Surgery, University Hospital Limerick were included. Diagnosis of Crohn’s disease was made using clinical, radiological and pathological findings from biopsy specimens or surgical resection specimens. All patients were managed by a dedicated multidisciplinary team of gastroenterologists and colorectal surgeons specializing in the care of inflammatory bowel disease (IBD) patients and all operations were performed by one of six colorectal surgeons. Each case was separately reviewed and treatment individually tailored accordingly.

Data including patient demographics, BMI, indications for surgery, disease recurrence requiring repeat surgical intervention, interval between index and subsequent resections, adjuvant medical therapy, cigarette smoking status, family history, age at diagnosis of Crohn’s disease and age at surgery and clinico-pathological findings were retrieved from a prospectively-maintained, institution-review-board-approved IBD database and supplemented by chart reviews, operation notes, pathology reports and/or pathological archived specimens.

Definition of end-points

Fat wrapping was considered to be present when >50% of the bowel circumference was covered by mesenteric adipose tissue in association with loss of the bowel-mesentery angle, on microscopic histological evaluation. Surgical disease recurrence was defined as recurrence of Crohn’s disease symptoms necessitating repeat surgical intervention (s)
during the study period. Time to surgical recurrence reflected the time interval between the index resection and subsequent surgery.

Statistical analysis

Data were analyzed using SPSS v19 (SPSS Inc., Chicago, IL, USA) and presented as mean ± standard deviation, odds ration (OR) with 95% confidence interval (CI) and ‘n’ represented the number of patients included in the analysis. Fisher’s exact test was used to determine correlation between categorical variables, while continuous variables were assessed using analysis of variance. Kaplan-Meier estimates and logistic regression analysis were performed to determine association between fat wrapping and surgical disease recurrence. A p value < 0.05 was considered statistically significant.
Results

Patient population

The study cohort consisted of 94 patients (45 males, 49 females) having undergone a total of 119 separate intestinal resections during the twelve-year study period (Table 1). The mean age at diagnosis was 30.1±10.4 years, while the mean age at operation was 37.4±10.2 years. The mean follow-up was 67.4±43.6 months, with a median (interquartile range) of 64.5 (73.5) months. The mean duration of disease was 62.4±2.2 months and 42 (44%) patients had ileocolonic disease.

Surgical recurrence following index resection

Seventy-four (78.7%) patients underwent a single intestinal resection. Fifteen (15.9%) patients underwent two resections while five (5.3%) patients underwent three separate resections. The surgical recurrence rate was 21% (20/94), and the mean time to surgical recurrence was 43.2±39.6 months. Twenty-five operations were performed for symptomatic recurrence among a total of 119 resections. Indications for re-operation (s) included symptoms unresponsive to optimal medical therapy (n=10, 40%), discharging entero-cutaneous and/or entero-vesical fistulae (n=7, 28%), recurrent symptomatic stricture formation at the previous anastomotic site (n=7, 28%) and toxic megacolon (n=1, 4%). The commonest operation performed for recurrence was ileocolic resection (n=13, 52%).
Prevalence of fat wrapping

Fat wrapping was identified in 31 resection specimens (i.e. 26% of all resection specimens). Of these cases, two (6.4%) were resected from patients who did not require repeat resection. Eleven of the 31 (35.5%) were excised from patients who subsequently required further surgery for Crohn’s disease. Eighteen of the 31 (58.1%) were resected from patients who were undergoing a second or third resection.

Correlation of fat wrapping with distinct clinicopathological features

Fat wrapping was associated with stricture formation (p=0.036). There was no association with transmural inflammation (p=1.0), fissuring ulceration (p=0.255), granuloma (p=0.373), body mass index (p=0.314), neuronal hyperplasia (p=0.287) or fistulae formation (p=0.193). In addition there was no relationship between fat wrapping and male sex (p=0.678) or age at surgery (p=0.311), (Table 2).

Analysis of factors associated with surgical recurrence

On univariate analysis (Table 3) both penetrating (HR 3.8, 95% CI: 1.3-10.6, P=0.012) and non-penetrating disease behaviour (HR 0.38, 95% CI: 0.15-0.98, P=0.045) as well as fat wrapping (HR 4.5, 95% CI: 1.77-11.5, P=0.002) were associated with surgical recurrence. On multivariate analysis (Table 4) however, only fat wrapping increased the risk of surgical recurrence (HR 4.7, 95% CI: 1.71-13.01, P=0.003). Fat wrapping also shortened the time to recurrence (P<0.001, Log-rank test), as shown in the Kaplan-Meier estimate (Figure 1).
<table>
<thead>
<tr>
<th>Male/Female (%)</th>
<th>45/49 (48/52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis, mean ± SD, years</td>
<td>30.1 ± 10.4</td>
</tr>
<tr>
<td>Age at resection, mean ± SD, years</td>
<td>37.4 ± 13.2</td>
</tr>
<tr>
<td>Duration of disease, mean ± SD, months</td>
<td>62.4 ± 72.2</td>
</tr>
<tr>
<td>Location of disease</td>
<td></td>
</tr>
<tr>
<td>Ileal (%)</td>
<td>34 (36)</td>
</tr>
<tr>
<td>Ileocolonic (%)</td>
<td>42 (44)</td>
</tr>
<tr>
<td>Colonic (%)</td>
<td>18 (19)</td>
</tr>
<tr>
<td>Smoking status at surgery</td>
<td></td>
</tr>
<tr>
<td>Active (%)</td>
<td>40 (42)</td>
</tr>
<tr>
<td>History (%)</td>
<td>16 (17)</td>
</tr>
<tr>
<td>Nonsmoker (%)</td>
<td>38 (40)</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
</tr>
<tr>
<td>Yes (%)</td>
<td>21 (22)</td>
</tr>
<tr>
<td>No (%)</td>
<td>73 (78)</td>
</tr>
<tr>
<td>Preoperative medications</td>
<td></td>
</tr>
<tr>
<td>5-ASA (%)</td>
<td>69 (73)</td>
</tr>
<tr>
<td>Steroids (%)</td>
<td>67 (70)</td>
</tr>
<tr>
<td>6MP/azathioprine (%)</td>
<td>49 (51)</td>
</tr>
<tr>
<td>Anti-TNF-alpha (%)</td>
<td>32 (34)</td>
</tr>
</tbody>
</table>

**Table 1**: Patient demographics and characteristics at index intestinal resection. Data are presented as mean ± standard deviation or n (%) (n=94 patients).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall (n: 119)</th>
<th>Fat wrapping (n: 31)</th>
<th>No fat wrapping (n: 88)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>58 (48.7)</td>
<td>14 (45.2)</td>
<td>44 (50.0)</td>
<td>0.678</td>
</tr>
<tr>
<td>Age at surgery (years)</td>
<td>37.6±1.2</td>
<td>39.6±2.4</td>
<td>36.8±1.4</td>
<td>0.311</td>
</tr>
<tr>
<td>BMI (Kg/M²)</td>
<td>23.5±0.8</td>
<td>23.2±0.7</td>
<td>25.6±4.2</td>
<td>0.314</td>
</tr>
<tr>
<td>Fissuring ulceration</td>
<td>71 (59.7)</td>
<td>16 (51.6)</td>
<td>55 (62.5)</td>
<td>0.255</td>
</tr>
<tr>
<td>Transmural inflammation</td>
<td>82 (68.9)</td>
<td>22 (71.0)</td>
<td>60 (68.2)</td>
<td>1.000</td>
</tr>
<tr>
<td>Stricture</td>
<td>35 (29.4)</td>
<td>14 (45.2)</td>
<td>21 (23.9)</td>
<td><strong>0.036</strong></td>
</tr>
<tr>
<td>Fistulae</td>
<td>14 (11.8)</td>
<td>6 (19.4)</td>
<td>8 (9.1)</td>
<td>0.193</td>
</tr>
<tr>
<td>Granuloma</td>
<td>38 (31.9)</td>
<td>8 (25.8)</td>
<td>30 (34.1)</td>
<td>0.373</td>
</tr>
<tr>
<td>Neuronal hyperplasia</td>
<td>4 (3.4)</td>
<td>2 (6.5)</td>
<td>2 (2.3)</td>
<td>0.287</td>
</tr>
</tbody>
</table>

**Table 2**: Patient clinicopathological characteristics and fat wrapping. Data are presented as mean ± standard deviation or n (%) (n=119 resections).
<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>0.639</td>
<td>0.251-1.566</td>
<td>0.327</td>
</tr>
<tr>
<td>Smoking at the time of surgery</td>
<td>1.667</td>
<td>0.509-5.461</td>
<td>0.399</td>
</tr>
<tr>
<td>Disease behavior:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Non-stricturing/non-penetrating</td>
<td>0.380</td>
<td>0.148-0.977</td>
<td>0.045</td>
</tr>
<tr>
<td>-Stricturing</td>
<td>1.432</td>
<td>0.570-3.598</td>
<td>0.445</td>
</tr>
<tr>
<td>-Penetrating</td>
<td>3.784</td>
<td>1.346-10.636</td>
<td>0.012</td>
</tr>
<tr>
<td>Granulomata in resection specimen</td>
<td>1.091</td>
<td>0.418-2.844</td>
<td>0.859</td>
</tr>
<tr>
<td>Fat wrapping</td>
<td>4.514</td>
<td>1.766-11.535</td>
<td>0.002</td>
</tr>
</tbody>
</table>

**Table 3**: Univariate analysis of factors associated with surgical recurrence. HR: hazard ratio, CI: confidence interval.
<table>
<thead>
<tr>
<th></th>
<th>HR*</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-stricturing/non-penetrating behavior</td>
<td>0.764</td>
<td>0.241-2.428</td>
<td>0.649</td>
</tr>
<tr>
<td>Penetrating behaviour</td>
<td>2.729</td>
<td>0.772-9.649</td>
<td>0.119</td>
</tr>
<tr>
<td>Fat wrapping</td>
<td>4.722</td>
<td>1.713-13.017</td>
<td><strong>0.003</strong></td>
</tr>
</tbody>
</table>

**Table 4**: Multivariate analysis of factors associated with surgical recurrence. HR: hazard ratio, CI: confidence interval, *adjusted for all other factors in the table.
Figure 1: Kaplan-Meier analysis of the effect of the presence of fat wrapping on recurrence of Crohn’s disease requiring surgical resection (P<0.001, Log-rank test).
Discussion

The aim of the study was to investigate the relationship between surgical recurrence and fat wrapping in patients with Crohn’s disease. The presence of fat wrapping at index surgical resection independently predicted increased risk of surgical recurrence as well as a reduced time to recurrence. Fat wrapping was also associated with stricture formation but not with transmural inflammation, non-caseating granuloma, increasing body mass index, disease duration or fissuring ulceration.

The above findings suggest that by documenting and recording the extent of fat present at the time of surgical resection, one might better tailor adjuvant treatment strategies in patients with Crohn’s disease. The “adjuvant treatment paradigm” has significant clinical benefits in the oncologic setting 88, 89, but is less well established in the Crohn’s disease setting. This relates largely to the lack of predictors of disease recurrence in the latter. Given the strength of association between fat wrapping and surgical recurrence observed in the present study, it is reasonable to suggest that patients with these disease manifestations be considered candidates for aggressive postoperative monitoring and prophylactic treatment.

The findings further highlight the pathobiologic relevance of mesenteric fat. Debate continues as to the role of this in inflammatory bowel disease in general 64, 90, 91. Whilst some suggest it contributes 92, others suggest it has a protective role 57. Of note, mesenteric fat is an extra-hepatic source of C-reactive protein (CRP) 56. Mesenteric-derived CRP correlates with systemic levels in patients with Crohn’s disease 56.

This is the first study to investigate the relationship between fat wrapping and surgical recurrence and although retrospective in nature, fat wrapping remained an independent predictor on multivariate analysis. Previous reports demonstrate an association between fat wrapping, transmural inflammation and fissuring ulceration 48. A similar relationship
was not observed in the present study and may be explained as follows. Surgical resection is generally reserved for severe Crohn’s refractory to medical treatment and thus advanced in pathobiologic stage. Transmural inflammation and fissuring ulceration are universally present in advanced Crohn’s disease. Thus, the present study was insufficiently powered to identify a potential link between these disease features. A further explanation is also possible. Only cases where fat encroachment exceeded 50% of the intestinal circumference were included. This is at the more severe end of the spectrum of fat wrapping. It is feasible that by including lesser degrees of mesenteric encroachment that further relationships may have emerged.

Future studies should aim to further unravel the relationship between fat wrapping and pathobiology in Crohn’s disease. These could focus on a characterization of the interface between the mesentery and the underlying serosa. Recent data suggest that a connective tissue contiguity occurs between mesentery and the intestine, at this interface. That being the case, this could provide an explanation for the mesenteric and transmural mesenchymal abnormalities observed in Crohn’s disease.

In summary, fat wrapping involving greater than fifty percent of the intestinal surface independently predicted increased risk of recurrence requiring surgical re-intervention, as well as reduced time to recurrence, in patients with Crohn’s disease.
Chapter three: Mesenteric manifestations correlate with mucosal and systemic manifestations in Crohn’s disease
Abstract

Background

Until recently, the mesentery was depicted as a fragmented and discontinuous structure. Recent clarification of mesenteric anatomy disputes this doctrine and provides an opportunity to re-examine the relationship between mesenteric and other manifestations in Crohn’s disease. Given this, the relationship between mesenteric, mucosal and systemic manifestations in Crohn’s disease was investigated.

Methods

Mesenteric manifestations were examined in thirty-four patients undergoing either a complete or total mesocolic excision for medically refractory Crohn’s disease. The severity of mesenteric disease features was assessed and a mesenteric disease score was subsequently developed and correlated with a mucosal disease score and Crohn’s Disease Activity Index (CDAI). Mesenteric mesenchymal abnormalities were characterised using a histologic approach. Mesenteric and mucosal gene expression were determined and compared in silico.

Results

Mesenteric disease scores correlated with mucosal disease scores (r=0.76, p<0.0001) and CDAI (r=0.68, p<0.0001) and were significantly increased in smokers (p<0.05). Mesenteric mesenchymal abnormalities were contiguous between mesentery and adjacent intestine. Mesenteric but not mucosal gene expression was associated with connective tissue and inflammatory disorders.

Conclusions

Mesenteric manifestations correlated with local and systemic manifestations in Crohn’s disease. Mesenteric mesenchymal abnormalities were contiguous between mesentery
and adjacent intestine. Mesenteric gene expression was associated with connective tissue and inflammatory diseases.
Introduction

The recent past has seen several advances in our understanding of mesenteric anatomy. According to classic descriptions, the mesentery is fragmented. However, more recent descriptions demonstrate that the mesentery fans out to span the gut from duodenojejunal flexure to mesorectal level. Accordingly, the small intestinal mesentery continues as the right mesocolon and a substantive mesenteric tissue mass occurs at the ileocaecal junction, with contiguity of connective tissue between mesentery and adjacent intestine. This connective tissue contiguity may serve as a platform through which cellular and molecular signals of intestinal inflammation could be transmitted, thus explaining the transmural nature of Crohn’s disease. In addition, it begs the question as to whether the inflammatory and connective tissue abnormalities pathognomonic of Crohn’s disease arise at the intestinal or mesenteric pole of the connective tissue platform. Therefore improved understanding of the mesenteric organ prompts a re-appraisal of the relationship between mesenteric, mucosal and systemic manifestations of Crohn’s disease.

Although mesenteric manifestations have long been considered unique to Crohn’s disease, they are relatively understudied. Macroscopically they include fat wrapping and mesenteric thickening. At a cellular level they are characterised by neuronal hyperplasia and lymphangiectasia. Crohn et al. noted this association in 1932. Several investigators described an association between mesenteric and mucosal abnormalities. For example, mucosal ulceration occurs at the mesenteric margin of the intestinal circumference, i.e. where the mesentery intersects with adjacent intestine.

In order to preserve as much intestine as possible, surgeons practise a conservative approach to intestinal resection in Crohn’s disease. A similarly non-radical approach is also applied to the mesentery, which, in general, is divided flush with the intestine. This
means the mesentery is retained. Recently, radical mesenterectomy has been associated with improved outcomes in colorectal cancer \(^{98, 99}\). Our institutional approach (to all colorectal resections) as well as that of St. Vincent’s University Hospital involve a similarly radical mesenterectomy, including the mesentery (i.e. mesocolic excision) and is facilitated by the recent advancements in our understanding of mesenteric anatomy. For example, improved knowledge of anatomy enables division of major vascular pedicles without compromising the superior mesenteric pedicle.

The volume of mesentery excised following a mesocolic excision is greater than following a conventional resection. In addition, the mesentery itself is undisturbed and retained intact (except at point of vascular division). As a result it is now possible to better characterise the association between mesenteric and intestinal as well as extra-intestinal abnormalities in Crohn’s disease.

Given the above, the aim of the study was to reappraise mesenteric manifestations in Crohn’s disease and their correlation with local mucosal and systemic manifestations.
Materials and methods

Patient cohort

Following ethical approval and informed consent, 34 patients undergoing resection for Crohn’s disease at University Hospital Limerick or St. Vincent’s University Hospital, Dublin were recruited (from July 2013 to January 2015) (Table 1). Of these, twenty-five patients underwent a complete mesocolic excision as part of treatment of ileocolic Crohn’s disease (Table 2). Five patients underwent a total mesocolic excision as part of treatment of Crohn’s colitis. The remaining patients underwent either a small bowel resection or pouchectomy. Diagnosis of Crohn’s disease was based on a combination of radiological, endoscopic and pathological findings. Patients were managed by a multidisciplinary team of gastroenterologists and colorectal surgeons specializing in the care of inflammatory bowel disease and operations were performed by one of six colorectal surgeons from University Hospital Limerick, or by a single colorectal surgeon from St. Vincent’s University Hospital. Retrieved data included body mass index, indications for surgery, interval between index and subsequent resections, adjuvant medical therapy, cigarette smoking status, family history, age at diagnosis of Crohn’s disease and at surgery and clinico-pathological findings. Patient data were generated by a combination of direct contact, chart reviews, operation and endoscopy notes, pathology reports and/or pathological archived specimens.
<table>
<thead>
<tr>
<th>Variable</th>
<th>N = 34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female (%)</td>
<td>13/21 (38/62)</td>
</tr>
<tr>
<td>Age at diagnosis, mean +/- SD, years</td>
<td>28.7±12</td>
</tr>
<tr>
<td>Age at resection, mean +/- SD, years</td>
<td>34±12</td>
</tr>
<tr>
<td>Duration of disease, mean +/- SD, months</td>
<td>93±65</td>
</tr>
<tr>
<td>Location of disease</td>
<td></td>
</tr>
<tr>
<td>- Ileal (%)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>- Ileocolonic (%)</td>
<td>25 (73)</td>
</tr>
<tr>
<td>- Colonic (%)</td>
<td>5 (14)</td>
</tr>
<tr>
<td>- Pouch excision</td>
<td>2 (6)</td>
</tr>
<tr>
<td>Smoking status at surgery</td>
<td></td>
</tr>
<tr>
<td>- Active (%)</td>
<td>11 (32)</td>
</tr>
<tr>
<td>- History (%)</td>
<td>7 (20)</td>
</tr>
<tr>
<td>- Nonsmoker (%)</td>
<td>15 (44)</td>
</tr>
<tr>
<td>- Unknown</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
</tr>
<tr>
<td>- Yes (%)</td>
<td>8 (23)</td>
</tr>
<tr>
<td>- No (%)</td>
<td>26 (77)</td>
</tr>
<tr>
<td>Preoperative medications</td>
<td></td>
</tr>
<tr>
<td>- 5-ASA (%)</td>
<td>5 (15)</td>
</tr>
<tr>
<td>- Steroids (%)</td>
<td>4 (12)</td>
</tr>
<tr>
<td>- 6MP/azathioprine (%)</td>
<td>5 (15)</td>
</tr>
<tr>
<td>- Anti-TNF-alpha (%)</td>
<td>11 (32)</td>
</tr>
</tbody>
</table>

**Table 1**: Demographics of all patients undergoing surgical resection for Crohn’s disease (during interval from July 2013 to January 2015).
<table>
<thead>
<tr>
<th>Variable</th>
<th>Numbers = 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female (%)</td>
<td>7/18 (28/72)</td>
</tr>
<tr>
<td>Age at diagnosis, mean +/- SD, years</td>
<td>27±12</td>
</tr>
<tr>
<td>Age at resection, mean +/- SD, years</td>
<td>33±10</td>
</tr>
<tr>
<td>Duration of disease, mean +/- SD, months</td>
<td>82±63</td>
</tr>
<tr>
<td>Montreal Classification</td>
<td></td>
</tr>
<tr>
<td>- B1</td>
<td>4 (16)</td>
</tr>
<tr>
<td>- B2</td>
<td>6 (24)</td>
</tr>
<tr>
<td>- B3</td>
<td>15 (60)</td>
</tr>
<tr>
<td>Smoking status at surgery</td>
<td></td>
</tr>
<tr>
<td>- Active (%)</td>
<td>9 (36)</td>
</tr>
<tr>
<td>- History (%)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>- Nonsmoker (%)</td>
<td>12 (48)</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
</tr>
<tr>
<td>- Yes (%)</td>
<td>6 (24)</td>
</tr>
<tr>
<td>- No (%)</td>
<td>19 (76)</td>
</tr>
<tr>
<td>Preoperative medications</td>
<td></td>
</tr>
<tr>
<td>- 5-ASA (%)</td>
<td>3 (12)</td>
</tr>
<tr>
<td>- Steroids (%)</td>
<td>3 (12)</td>
</tr>
<tr>
<td>- 6MP/azathioprine (%)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>- Anti-TNF-alpha (%)</td>
<td>7 (28)</td>
</tr>
</tbody>
</table>

**Table 2:** Demographics of the subgroup of patients undergoing ileocolic resection for ileocolic Crohn’s disease (during interval from July 2013 to January 2015).
Post-resection examination of specimens

All surgical specimens were examined post resection for mesenteric and mucosal features of disease. Mesenteric features were first documented, photographed and recorded in unopened specimens. A score was developed to quantitate mesenteric manifestations (see below). Two trained observers (consisting of a consultant colorectal surgeon and a senior specialist registrar trainee in colorectal surgery) scored mesenteric manifestations independently. A pilot study examining for inter-observer variability demonstrated strong concordance.

Following examination for mesenteric disease, specimens were opened longitudinally along the anti-mesenteric border. They were copiously irrigated to expose the entire mucosal surface. Mucosal features were documented, photographed and recorded. A score was developed to quantitate mucosal manifestations (see below). The two same observers also scored mucosal manifestations independently. A pilot study examining inter-observer variability demonstrated strong concordance.

All mesenteric and mucosal disease scores as well as preoperative Crohn’s disease activity indices (CDAI) were recorded by separate investigators.
The mesenteric disease score

Currently there is no published scoring system to assess the severity of mesenteric disease manifestations in Crohn’s disease. The mesenteric disease score presented here was generated based on fat wrapping and mesenteric thickening. Fat wrapping was graded according to the proportion of intestinal circumference affected (Fig. 1), (Table 3A). Mesenteric thickening was graded based on the appearance of vascular and avascular mesenteric regions (Fig. 1), (Table 3A). In normal mesentery, vascular and avascular regions are readily differentiated. In Crohn’s disease, early mesenteric thickening is confined to vascular pedicles. Intermediate mesenteric thickening extends to adjacent interpedicular regions, but vascular and non-vascular regions can still be differentiated. Advanced mesenteric thickening is pan-mesenteric such that vascular and avascular regions cannot be differentiated.

A score was ascribed according to the degree of mesenteric thickening, and when combined with the grade of fat wrapping, generated an overall mesenteric disease score (Fig. 1), (Table 3A). The highest scoring region of diseased mesentery was taken as the final score for each patient.
Figure 1: (A) Digitally sculpted model of small intestinal mesentery and right mesocolon showing continuity between both. (B) Mesenteric manifestations: digitally
sculpted mesentery and intestinal tract demonstrating fat wrapping and mesenteric thickening. In the normal mesentery, adipovascular pedicle and avascular mesenteric regions can be easily differentiated. In early mesenteric manifestations (far left) thickening is confined to adipovascular regions. Fat wrapping commences at the gastrointestinal margin of the mesentery and is limited. In intermediate mesenteric manifestations (middle), thickening in adipovascular regions is more pronounced but these can still be differentiated from interpedicular avascular regions. Fat wrapping is increased but confined to less than 25% the circumference. In advanced mesenteric manifestations (right), thickening is pan-mesenteric such that adipovascular and avascular mesenteric regions cannot be distinguished. Fat wrapping extends beyond 50% of the circumference.
<table>
<thead>
<tr>
<th>Mesenteric disease score</th>
<th>Severity</th>
<th>Grade</th>
<th>Stage</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW minimal, MT minimal</td>
<td>Early</td>
<td>Mild</td>
<td>One</td>
<td>1</td>
</tr>
<tr>
<td>FW &lt;25%, MT adipovascular pedicle only</td>
<td>Intermediate I</td>
<td>Moderate</td>
<td>Two A</td>
<td>2</td>
</tr>
<tr>
<td>FW &lt;25%, pan-mesenteric MT</td>
<td>Intermediate II</td>
<td>Moderate</td>
<td>Two B</td>
<td>4</td>
</tr>
<tr>
<td>FW &gt;50%, pan-mesenteric MT</td>
<td>Advanced</td>
<td>Severe</td>
<td>Three</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 3A**: Mesenteric disease score in Crohn’s disease (see Figure 1). FW and MT refer to fat wrapping and mesenteric thickening respectively. The specific scores assigned to each of the four categories of mesenteric disease are somewhat arbitrary given the lack of an existing scoring system.
Mucosal disease score

The mucosal disease score was developed based on the presence (in any location) of oedema, pseudopolyps, ulceration (aphthous, linear or confluent), stricture or fistula (Table 3B). Weighted points were arbitrarily allocated for each mucosal feature and the final score was the sum of all points.
Table 3B: Macroscopic scoring system for mucosal disease. For each feature present points were arbitrarily attributed. The final score was the sum of all points accumulated.
Histologic characterisation of mesenteric manifestations

For convenience in the following, the interface between the intestine and mesentery will be referred to as the “hilum”. Based on this, the “gastro-mesenteric axis” was defined as the intestine immediately adjacent the hilum, the hilum itself and mesentery adjacent this. After transfer of resection specimens to pathology, full thickness specimens were taken encompassing each of the above regions. These were formalin fixed and paraffin wax embedded. Paraffin-embedded tissue sections (4 µm-thick) were obtained, deparaffinized and hydrated. Sections were examined by staining for haematoxylin and eosin as previously described. In regions defined above, the thickness of surface mesothelial/connective tissue complex, thickness of connective tissue septations, and adipocyte cell number, were quantitated.

Functional annotation of mesenteric and mucosal gene expression

The transcriptional profile of Crohn’s mesentery was determined in a comparative study as follows. Two data sets were imported into Chipster© (R-based bio-informatic software). GSE 20881 (raw data generated in the Bioinformatics department, Genentech Inc. San Francisco, CA, USA, available at http://www.ncbi.nlm.nih.gov/geo/) comprises gene expression data from 65 mucosal samples (17 normal, 48 Crohn’s disease) generated on the Agilent-012391 Whole Human Genome Microarray (GPL1708). GSE 46754 comprises expression data derived from the mesentery of patients (n=4) with Crohn’s disease. Data were generated using the Illumina HT-12v3.0 human expression beadchip (Illumina, Essex, England; GPL 6947), at the Instituto Auxologico Italiano and is available at http://www.ncbi.nlm.nih.gov/geo/. The bioinformatics pipeline used to compare data sets is described in the “Statistical analysis”.

74
**Statistical analysis**

Pearson’s correlation coefficient was used to determine correlations between mesenteric, mucosal and systemic parameters in SPSSv22 (SPSS Inc., Chicago, USA). A two-tailed t-test was used to compare parametric variables whilst a Mann-Whitney U test was utilized for non-parametric comparisons.

*In silico* analysis of gene expression was performed in Chipster©, where each data set was filtered to identify the top 147 genes that differed significantly from the overall mean. Two datasets were generated (one for each microarray data set), which were imported to Ingenuity© for functional annotation. Each identifier was mapped to a corresponding object in Ingenuity’s Knowledge Base and outputs (termed network eligible molecules) were overlaid onto a global molecular network. A “core analysis” was conducted on all data sets establishing the topmost associated diseases, molecular functions, canonical pathways and transcription factors. The strength of associations was established using a right-tailed Fisher’s exact test to calculate a P-value which determined the probability that each parameter (e.g. biological function and/or disease) was associated due to chance alone (Ingenuity® Systems 2011). Canonical pathway analysis was conducted on the data sets. The significance of associations between data sets and canonical pathways was measured in two manners: (1) a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed, (2) Fisher’s exact test was used to calculate a P-value determining the probability that the association between the genes and the canonical pathway is explained by chance alone.
Results

Mesenteric manifestations correlated with mucosal disease severity and CDAI

Mesenteric disease scores correlated with mucosal disease scores ($r=0.76$, $p<0.0001$) and CDAI ($r=0.68$, $p<0.0001$) (Fig. 3B, A). Mucosal disease scores and CDAI also correlated ($r=0.68$, $p=0.001$, Fig. 3C). Smoking was associated with increasing mesenteric disease scores (4.1±1.66 vs. 2.7±1.50, $p=0.05$, Mann-Whitney U test) for smokers and non-smokers respectively (Fig. 4A-C). These observations held in the subgroup analysis of patients undergoing ileocolic resection (Fig. 5A-C). There was no difference in mesenteric disease score, mucosal disease score and CDAI between groups receiving immunosuppressant or immunomodulatory biologic drugs and patients not receiving these, at the time of surgery (Fig. 4D-F, 5D-F).
Figure 3: (A-C) Mesenteric disease scores, mucosal disease scores and Crohn’s Disease Activity Index (CDAI) for all resection types (N=34). (D-F) Mesenteric disease
score, mucosal disease score and CDAI in subgroup with ileocolic disease, and undergoing an ileocolic resection (N=25). The Pearson correlation coefficient and associated trend lines are included for each comparison.
Figure 4: (A-C) The effects of smoking on mesenteric disease score, mucosal disease score and Crohn’s disease activity index (CDAI) when all patients in the prospective
study were included in the analysis. (D-F) The effects of preoperative medication on mesenteric disease score, mucosal disease score and CDAI in all Crohn’s patients.
Figure 5: (A-C) The effects of smoking on mesenteric disease score, mucosal disease scores and Crohn’s disease activity index (CDAI) when analysis was limited to patients with ileocolic Crohn’s disease. (D-F) The effects of preoperative medication on mesenteric disease score, mucosal disease score and CDAI in the same subgroup.
Longitudinal distribution of mesenteric and mucosal manifestations correlated

The distribution of macroscopic mesenteric and mucosal abnormalities correlated precisely in all cases. Where the mesentery was normal in appearance, adjacent mucosa was also normal. In regions of early mesenteric manifestations, mucosal abnormalities were limited to scattered aphthous ulceration. Ulcers were confined to the mesenteric border. In regions of intermediate mesenteric disease, ulceration was confluent, but again confined to the mesenteric border of the mucosa. In regions with advanced mesenteric disease, confluent mucosal ulceration, stricture and fistulae were observed.

A short transition was apparent in every case between regions of normal and diseased mesentery. At the mesenteric transition zone, mesenteric abnormalities progressed from early to advanced (Fig. 2). At the mucosal transition zone, mucosal abnormalities progressed from early to advanced, in tandem with the severity of mesenteric disease (Fig. 2). The start of the gastromesenteric transition zone served as a landmark to guide division of the intestine and adjacent mesentery.
Figure 2: Case 1 (ileocolic Crohn’s disease): Intraoperative specimen following resection and longitudinal opening of the specimen. The patient had ileocolic Crohn’s disease and underwent a complete mesocolectomy. The mesenteric transition zone occurred where the mesentery changed from normal to early, intermediate and advanced mesenteric disease. A mucosal transition zone occurred adjacent this where mucosal disease changed from normal to, early, intermediate and advanced mucosal disease. The line of resection was just proximal to start of the mesenteric transition zone.
Mesenteric and gastrointestinal mesenchymal abnormalities were contiguous

Non-diseased mesentery comprised a surface mesothelium and an underlying submesothelial connective tissue layer. Connective tissue septae arose from the latter and separated adipocyte compartments of the mesentery (Fig. 6A, B). In regions where the mesentery was normal, the thickness of surface mesothelium/connective tissue complex was 24±13µm (Fig. 6B). The surface complex was significantly thickened in early (62±16µm, p<0.001, t-test), intermediate (215±70µm, p<0.001, t-test) and advanced mesenteric manifestations (408±3µm, p<0.001, t-test) (Fig. 6C). The thickness of mesenteric connective tissue septations followed a similar pattern ranging from 16±7µm (in normal mesentery), to 53±17µm (p<0.001, t-test), 101±21µm (p<0.001, t-test) and 245±100µm (p<0.001, t-test) in early, intermediate and advanced mesenteric manifestations (Fig. 6C). Within adipocyte compartments, adipocyte numbers were 23±6 per high power field (HPF) in normal mesentery (Fig. 6B). A graded increase in adipocyte number occurred in early (28±4/HPF, p=0.02, t-test), intermediate (37±7/HPF, p<0.001, t-test) and advanced (60±7/HPF, p<0.001, t-test) mesenteric disease (Fig. 6C).

The region of intersection between mesentery and adjacent intestine was examined in all cases. In non-diseased mesentery, a connective tissue band occurred between mesentery and the outer muscle layer (Fig. 6D, Fig. 7). Both mesenteric and intestinal connective tissue were contiguous with the connective tissue band, on either side. Hence a connective tissue contiguity was apparent between mesentery and intestine (Fig. 6D, E, Fig. 7). The connective tissue abnormalities identified in abnormal mesentery (i.e. thickening of septae) also occurred at the intestinal surface and within the outer longitudinal muscle layer (Fig. 6E-I, Fig. 7).
Figure 6: (A) (Left) Digital sculpture demonstrating the junction between the small intestinal mesentery and the right mesocolon. The small intestinal mesentery is contiguous with the right mesocolon. (Right) Digital sculpture of histologic structure of mesentery showing mesothelium on both sides, and the connective tissue lattice (in grey). Lymphatic channels and nodes are conceptually inserted (green). Adipocytes occupy the cavities within the mesocolon but have been conceptually removed in this instance to illustrate the connective tissue structure. (B) Photomicrograph demonstrating normal mesentery, a thin surface mesothelium (single arrow) and underlying connective tissue. A connective tissue septation (double arrows) arises from the submesothelial connective tissue layer and subdivides the mesentery. (C)
Photomicrograph demonstrating mesentery in Crohn’s disease. Surface mesothelium and submesothelial connective tissue (single arrow) are thickened in comparison to that seen in normal mesentery. Connective tissue septations are also thickened in comparison with normal mesenteric septations (multiple arrows). (D) Photomicrograph (haematoxylin and eosin – H&E) demonstrating interface between normal mesentery and intestinal outer wall (arbitrarily referred to as the mesenteric hilum). A connective tissue layer (arrows) is apparent at the interface between the mesentery and the outer surface of the longitudinal muscle layer. This layer is contiguous with the connective tissue lattice of the mesentery. Septations arise from this connective tissue layer and penetrate the outer longitudinal layer (asterisk). (E) Photomicrograph (H&E) demonstrating the normal inner circular muscle layer of the intestine. (F) Photomicrograph (H&E) demonstrating increased connective tissue at the interface between the mesentery and intestine (i.e. the mesenteric hilum) in fat wrapping in Crohn’s disease. Adipocyte hyperplasia is evident. (G) Photomicrograph (H&E) demonstrating higher magnification view of mesenteric/intestinal interface in region of fat wrapping in Crohn’s disease. The increased connective tissue deposition extended to involve connective tissue septations in the outer longitudinal muscle layer. (H) Photomicrograph (H&E) demonstrating mesentery, outer and inner muscle layers in a region of fat wrapping in Crohn’s disease. (I) Schematic diagram of connective septations (blue lines) in (H) and along which cells appeared to infiltrate from adjacent mesentery. The yellow coloured region corresponds to the connective tissue lattice of the adjacent mesentery.
Figure 7: (A) Digital sculpture demonstrating the histologic contiguity between mesentery and adjacent intestinal tract (arrow). (B) Digital sculpture derived from (A) in which all histologic structures have been removed with exception of the connective tissue. The resultant model demonstrates connective tissue contiguity between mesentery and intestinal tract (arrow).
Mesenteric (but not mucosal) gene expression was associated with connective tissue and inflammatory disorders

The Ingenuity® platform was used to identify, within published data, disorders associated with mesenteric (Table 4A) and mucosal (Table 4B) gene expression profiles. Mesenteric gene expression profiles were significantly associated with literature on connective tissue disorders, inflammatory disease, cancer and canonical pathways, including interleukin-17 (IL-17) signaling in fibroblasts. Mucosal gene expression profiles were associated with literature on cardiovascular, hematologic, metabolic and lipid related diseases in addition to canonical pathways such as IL-17 signaling in psoriasis and chronic obstructive airway disease.
Table 4A: Two groups were generated in relation to each gene, A) < mean and B) > mean expression value. A univariate analysis (chi square test) was employed to determine the association between groups A/B and Crohn’s disease.
Table 4B: Two groups were generated in relation to each gene, A) < mean and B) > mean expression value and whether they responded to medical therapy or not. A univariate analysis (chi square test) was employed to determine the association between groups A/B and the incidence of Crohn’s disease.
Discussion

Recent advancements in our understanding of mesenteric anatomy \(^{97, 101, 102}\) enable surgeons conduct a mesocolic excision as part of surgical management of Crohn’s disease. This in turn provides an opportunity to reappraise the relationship between mesenteric, mucosal and systemic manifestations in Crohn’s disease. Based on fat wrapping and mesenteric thickening, a score was developed that permitted quantitation of mesenteric disease. This in turn enabled a correlation of mesenteric, mucosal and systemic disease manifestations. Both mucosal disease score, and the Crohn’s disease activity index strongly correlated with mesenteric disease. In addition, mesenteric and mucosal abnormalities occurred in tandem. When examined histologically, a connective tissue contiguity was apparent between mesentery and adjacent intestine. Mesenteric and intestinal mesenchymal abnormalities were contiguous across this platform.

The mesenteric disease score generated in this study requires formal validation. Notwithstanding this, it is derived from established indices of mesenteric disease, i.e. fat wrapping and mesenteric thickening. This could explain the strong correlation observed with mucosal abnormalities and also with the Crohn’s disease activity index. The latter is widely used in evaluating the severity of disease as well as response to treatment. Of note, the mesenteric disease score (but not the mucosal score or CDAI) worsened with smoking in both cohorts examined. Although smoking is a strong determinant of disease progression and relapse in Crohn’s disease \(^{103, 104}\), the reason for this is unknown. The relationship between smoking and mesenteric manifestations could provide a valuable insight into the pathobiology of Crohn’s disease in general.

A connective tissue contiguity was apparent between the intestine and the adjacent mesentery (Figure 7). In addition, mesenteric and intestinal mesenchymal abnormalities
were contiguous across this platform. These observations may explain the transmural basis of Crohn’s disease. It is feasible that cellular and molecular signals could be transmitted through the connective tissue platform. This observation also prompts the question as to the whether the inflammatory and connective tissue abnormalities that are the hallmarks of Crohn’s disease, commence at the intestinal or the mesenteric pole of the connective tissue platform. In an attempt to address this question, mesenteric and mucosal gene expressions were compared in silico. The investigator conducting the analysis was blind to the nature of the groups under investigation. Mesenteric, but not mucosal gene expression, was associated with connective tissue and inflammatory disorders.

The identification of a mesenteric and mucosal transition zone is highly relevant from a surgical perspective. At this zone, both mesenteric and mucosal manifestations progressively worsened and in tandem with each other. Proximal to the zone, the intestine was always normal. This held for confluent ileocolic disease, as well as skip lesions. The transition zone thus serves as a pathologic hallmark to guide surgeons in making the proximal intestinal and mesenteric incision.

Several avenues of research emerge from this study. Firstly, the mesenteric disease score must be validated in a prospective manner. This could facilitate development of a more accurate score, whilst also determining predictive and prognostic properties. Importantly, the above findings also prompt the development of non-invasive means of assessing mesenteric disease activity. Options include radiologic or serologic markers of mesenteric disease. In the recent past the fibrocyte has emerged as a candidate biomarker of Crohn’s disease.  

78
In summary, mesenteric manifestations correlate with local mucosal and systemic manifestations in Crohn’s disease. They worsen with smoking. The mesenteric transition zone provides a landmark to guide surgeons in deciding on proximal resection margins.
Chapter four: The fibrocyte proportion of circulating monocytes is increased in Crohn’s disease
Abstract

Background

Although fibrosis in Crohn’s disease is mediated by fibroblasts, their exact source is unknown. Fibrocytes are bone marrow-derived progenitor cells that are recruited to sites of inflammation where they can differentiate into fibroblasts and could thus contribute to the mesenchymal abnormalities associated with Crohn’s disease. Based on this the aim was to characterise circulating fibrocyte levels in Crohn’s disease.

Methods

Following ethical approval and informed consent circulating fibrocyte levels were determined using flow cytometry in patients undergoing a resection for Crohn’s disease, diverticular disease and colorectal cancer. Levels were correlated with mesenteric and mucosal disease scores as well as with the Crohn’s disease Activity Index (CDAI). Tissue fibrocytes were evaluated immunohistochemically in both the mesentery and associated intestine in Crohn’s specimens. P<0.05 was considered statistically significant.

Results

Fibrocyte percentages were increased in Crohn’s disease compared with healthy controls (6.4±2.82% vs. 2.0±1.04%, p<0.001). Levels normalized following ileocolic resection (5.7±2.12% vs. 1.7±1.20%, p=0.005, t-test), in patients with ileocolic Crohn’s disease. Levels also significantly reduced following fecal diversion via loop ileostomy (12±9% versus 6.5±8%, p=0.06). Fibrocyte percentages correlated with mesenteric and mucosal disease scores as well as with the CDAI (r=0.94, p<0.0001).
Conclusions

The fibrocyte proportion of circulating monocytes was increased in Crohn’s disease and correlated with indices of mesenteric, mucosal and systemic disease activity. Therefore, fibrocytes could represent a novel therapeutic target in Crohn’s disease and anti-fibrocyte regimens could help dampen Crohn’s associated fibrosis and improve patient outcomes.
Introduction

The management of Crohn’s disease is hampered by a lack of accurate diagnostic, prognostic and predictive biomarkers. Recent findings have identified mesenteric inflammation as an important event in the pathobiology of Crohn’s disease. The development of a biomarker that reflects mesenteric inflammation could greatly aid in tailoring treatment regimens at the individual level in patients with this condition.

Recent findings have implicated circulating fibrocytes in multiple conditions characterised by an exaggerated generation of mesenchyme, i.e. idiopathic pulmonary fibrosis, myocardial infarction and renal scarring. These cells are thought to derive from circulating monocytes and are recruited to sites of inflammation. They are highly plastic and can differentiate into multiple cells types including fibroblasts and adipocytes. In Crohn’s disease, mesenteric mesenchymal derangements are characterised by excessive adipocyte proliferation and connective tissue deposition. It is feasible, given the pluripotentiality of fibrocytes, that they may play a pathobiologic role in the mesenteric and intestinal mesenchymal abnormalities seen in Crohn’s disease.

Surprisingly little is known regarding fibrocytes in Crohn’s disease. Sazuka et al. found the ratio of circulating fibrocytes/leukocyte to be increased in Crohn’s disease when compared to healthy controls. Tissue-based fibrocytes were also increased in active lesions but not in regions of established fibrosis.

Based on the above the aim of the current study was to characterise circulating fibrocytes in Crohn’s disease and compare these with other pathologies. A further aim
was to assess the relationship between circulating fibrocyte levels and established indices of mesenteric, mucosal and systemic activity in Crohn’s disease.
Materials and methods

Patient cohort

Following ethical approval and informed consent, 34 patients undergoing resection for Crohn’s disease at University Hospital Limerick or St. Vincent’s University Hospital, Dublin were recruited (from July 2013 to January 2015) (Table 1). Of these, twenty-five patients underwent a complete mesocolic excision as part of treatment of ileocolic Crohn’s disease. Five patients underwent a total mesocolic excision as part of treatment of Crohn’s colitis. The remaining patients underwent either a small bowel resection or pouchectomy. A separate cohort of three patients underwent a laparotomy and were found to have extensive mesenteric disease. They underwent defunctioning ileostomy rather than definitive resection.

Diagnosis of Crohn’s disease was based on a combination of radiological, endoscopic and pathological findings. Patients were managed by a multidisciplinary team of gastroenterologists and colorectal surgeons specializing in the care of inflammatory bowel disease and operations were performed by one of six colorectal surgeons. Retrieved data included body mass index, indications for surgery, interval between index and subsequent resections, adjuvant medical therapy, cigarette smoking status, family history, age at diagnosis of Crohn’s disease and at surgery and clinico-pathological findings. Patient data were generated by a combination of direct contact, chart reviews, operation and endoscopy notes, pathology reports and/or pathological archived specimens.
### Table 1: Demographics of all patients undergoing surgical resection for Crohn’s disease during interval from July 2013 to January 2015.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N = 34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female (%)</td>
<td>13/21  (38/62)</td>
</tr>
<tr>
<td>Age at diagnosis, mean +/- SD, years</td>
<td>28.7±12</td>
</tr>
<tr>
<td>Age at resection, mean +/- SD, years</td>
<td>34±12</td>
</tr>
<tr>
<td>Duration of disease, mean +/- SD, months</td>
<td>93±65</td>
</tr>
<tr>
<td>Location of disease</td>
<td></td>
</tr>
<tr>
<td>- Ileal (%)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>- Ileocolonic (%)</td>
<td>25 (73)</td>
</tr>
<tr>
<td>- Colonic (%)</td>
<td>5 (14)</td>
</tr>
<tr>
<td>- Pouch excision</td>
<td>2 (6)</td>
</tr>
<tr>
<td>Smoking status at surgery</td>
<td></td>
</tr>
<tr>
<td>- Active (%)</td>
<td>11 (32)</td>
</tr>
<tr>
<td>- History (%)</td>
<td>7 (20)</td>
</tr>
<tr>
<td>- Nonsmoker (%)</td>
<td>15 (44)</td>
</tr>
<tr>
<td>- Unknown</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
</tr>
<tr>
<td>- Yes (%)</td>
<td>8 (23)</td>
</tr>
<tr>
<td>- No (%)</td>
<td>26 (77)</td>
</tr>
<tr>
<td>Preoperative medications</td>
<td></td>
</tr>
<tr>
<td>- 5-ASA (%)</td>
<td>5 (15)</td>
</tr>
<tr>
<td>- Steroids (%)</td>
<td>4 (12)</td>
</tr>
<tr>
<td>- 6MP/azathioprine (%)</td>
<td>5 (15)</td>
</tr>
<tr>
<td>- Anti-TNF-alpha (%)</td>
<td>11 (32)</td>
</tr>
</tbody>
</table>
Mesenteric and mucosal disease scores and Crohn’s disease activity index

Both scores and the Crohn’s disease activity index were determined and recorded as previously described (refer to Chapter 3). In brief, all surgical specimens were examined post resection for mesenteric and mucosal features of disease. Mesenteric features were first documented, photographed and recorded in unopened specimens. Following examination for mesenteric disease, specimens were opened longitudinally along the anti-mesenteric border. They were copiously irrigated to expose the entire mucosal surface. Mucosal features were documented, photographed and recorded. Mesenteric and mucosal disease scores as well as preoperative Crohn’s disease activity index (CDAI) were recorded by separate investigators.

Serum sample collection, preparation and processing

Prior to intestinal resection (or in healthy controls), a single 10 mL sample of heparinized venous blood was collected via peripheral, upper extremity venipuncture. This was repeated at 4 weeks postoperatively in all Crohn’s disease patients who underwent surgical resection. Samples were collected in sodium heparin (EDTA) vacutainer tubes and transferred to the laboratory within 3 hours. Samples were then processed to isolate the buffy coat layer using density gradient centrifugation (Histopaque, Sigma-Aldrich, Wicklow, Ireland). The resulting peripheral blood mononuclear cells were subsequently washed in Phosphate Buffered Saline (PBS) and resuspended in freezing medium (50% foetal bovine serum, 40% RPMI medium and 10% dimethyl sulfoxide) prior to transfer to cryogenic vials in 1-ml aliquots. Finally, samples were cooled in a cryogenic temperature control rate container to -80 °C until processing for flow cytometry.
**Flow cytometry of circulating fibrocytes**

For each patient undergoing resection for Crohn’s disease, a blood sample was obtained both preoperatively and postoperatively (at 4 weeks post surgery). Similar samples were also obtained from healthy volunteers (n=10) and patients undergoing resection for diverticular disease (n=4) or colon cancer (n=5). Following white blood cell isolation using density gradient centrifugation, $1 \times 10^6$ cells were re-suspended in flow cytometry buffer (RPMI medium supplemented with 10% horse serum, 0.1% sodium azide and 25 mM HEPES). Cells were fixed and permeabilised using BD Cytofix/Cytoperm solution (BD Biosciences, Oxford, England) prior to intracellular staining of Collagen-I with mouse anti-human Collagen-I antibody (Millipore, Cork, Ireland) which was subsequently stained with Alexa-Fluor 488 goat anti-mouse secondary antibody (Jackson ImmunoResearch Europe, Suffolk, England). Cells were then stained for cell surface antigen CD45 using PerCP anti-human CD45 (Biolegend, London, England). Finally, cells were re-suspended in phosphate buffered saline (PBS; pH 7.4 for subsequent analysis on a Guava EasyCyte HT (Millipore). Data were analysed using GuavaSoft 2.7 (Millipore) and results were displayed as a percentage of the total white blood cell population. A suitable protocol was established to detect fibrocytes (i.e. positive for CD45 and Collagen-1) by running relevant controls. These markers have been used by several groups to identify fibrocytes 73, 78, 116, 117.

**Histology**

After transfer of resection specimens to pathology, full thickness specimens were taken encompassing the intestine immediately adjacent the hilum, the hilum itself and mesentery adjacent this. These were formalin fixed and paraffin wax embedded. Paraffin-embedded tissue sections (4 μm-thick) were obtained, deparaffinized and
hydrated. Sections were examined by staining for haematoxylin and eosin as previously described. In regions defined above, the thickness of surface mesothelial/connective tissue complex, thickness of connective tissue septations, and adipocyte cell number, were quantitated.

**Immunohistochemistry**

Serial sections were stained immunohistochemically as follows. Slides were immersed in pre-heated EnVision™ FLEX Target Retrieval Solution (Dako Diagnostics Ltd, Ireland) and incubated for 30 minutes at 97°C. Sections were left cool to 65 °C then washed in EnVision™ FLEX Wash Buffer (Dako Diagnostics) for 5 minutes at room temperature. Slides were placed on a Dako AutoStainer Link instrument and EnVision™ DuoFLEX Dual Endogenous Enzyme Block (Dako Diagnostics) added. Following endogenous enzyme blockade, samples were incubated for 30 minutes at room temperature with primary monoclonal mouse anti-human CD45 (1:450, Dako Diagnostics) and monoclonal mouse anti-human αSMA (1:250, Dako Diagnostics) followed by incubation with EnVision™ DuoFLEX/HRP (horseradish peroxidase; Dako Diagnostics) and EnVision™ DuoFLEX/AP (alkaline phosphatase; Dako Diagnostics). The reaction was visualized by EnVision™ DuoFLEX DAB+ Chromogen for mouse antibody and EnVision™ DuoFLEX Permanent Red for rabbit antibody. Slides were counterstained with 20% Mayer’s haematoxylin for 30 seconds. Finally, slides were dehydrated, cleared and cover slipped using an aqueous mounting medium (Pertex; Medite GmbH, Burgdorf, Switzerland). Sections of human tonsil were used as positive and negative controls. Slide review and interpretation was conducted via consensus by a consultant pathologist and the principal investigator.
To concurrently demonstrate CD45 and αSMA staining, digital images were imported to Microsoft Office 2010 in which hue and saturation parameters were altered. The effect was to reduce the intensity of the CD45 chromogen (i.e. DAB). This enabled visualisation of the permanent red chromogen, which in turn permitted identification of dual stained CD45⁺αSMA⁺ cells. CD45⁺αSMA⁺ cells were examined in normal mesentery and in mesentery with early, intermediate and advanced disease manifestations. In each analysis, CD45⁺αSMA⁺ cells were examined in surface mesothelium, connective tissue septations and adipocyte lobules. All reviews were conducted by a pathologist and the principal investigator. These markers have been previously used to identify myofibrocytes in tissue. ¹¹⁸⁻¹²⁰

Statistical analysis

Pearson’s correlation coefficient was used to determine correlations between mesenteric, mucosal and systemic parameters in SPSSv22 (SPSS Inc., Chicago, USA). A two-tailed t-test was used to compare parametric variables whilst a Mann-Whitney U test was utilised for non-parametric comparisons.
Results

Percentage fibrocytes in circulating monocytes

The fibrocyte percentage of circulating monocytes was determined. The fibrocyte percentage was significantly increased in Crohn’s disease compared with healthy control subjects (6.4±2.82% vs. 2.0±1.04%, p<0.001) (Fig. 1). Levels were similar when all Crohn’s subtypes were grouped together and compared with ileocolic Crohn’s disease alone. Although the fibrocyte percentage of circulating monocytes was greater in Crohn’s disease compared with diverticular disease, this was not significant (6.4±2.82% vs. 3.9±2.75%, p=0.1, t-test) (Fig. 2A). The fibrocyte percentage in Crohn’s disease trended towards being greater than that seen in colorectal cancer (6.4±2.82% vs. 3.3±1.59%, p=0.08, t-test) (Fig. 2A).

Postoperative fibrocyte percentages were significantly reduced relative to preoperative levels, in patients undergoing ileocolic resection for Crohn’s disease (5.7 ± 2.12% vs. 1.7 ± 1.20%, p=0.005, t-test) (Fig. 2B). Following surgery, fibrocyte proportions decreased to levels observed in healthy controls.

A direct correlation occurred between circulating fibrocytes and CDAI in both the main cohort (r=0.94, p<0.0001) and in the group including ileocolic Crohn’s disease alone (r=0.96, p<0.0001) (Fig. 3). A direct correlation also occurred between circulating fibrocyte percentages and mesenteric and mucosal disease scores in both Crohn’s disease cohorts (Fig. 4).
Figure 1: (A) Panel demonstrating fibrocytes (upper outer quadrant) on flow cytometry (left). In normal controls fibrocytes constitute 1.9% of the circulating monocyte
population. The fibrocyte proportion of monocytes increased significantly in patients with Crohn’s disease (right). (B) Bar chart demonstrating the fibrocyte component of circulating monocytes (expressed as a percentage) in healthy controls, in the full cohort with Crohn’s disease (i.e. ileocolic, colonic and perianal Crohn’s included), and in patients with ileocolic Crohn’s disease. The fibrocyte proportion increased significantly in Crohn’s disease compared with healthy controls.
Figure 2: (A) Bar chart demonstrating the fibrocyte component of circulating monocytes (expressed as a percentage) in the full cohort with Crohn’s disease (i.e.
ileocolic, colonic and perianal Crohns included), in patients with ileocolic Crohn’s disease, in diverticular disease and in colorectal cancer. The fibrocyte proportion of circulating monocytes was significantly increased in Crohn’s disease versus that seen in patients with colorectal cancer. The fibrocyte proportion was also increased in Crohn’s versus diverticular disease although not significantly so. **(B)** Bar chart demonstrating the fibrocyte component of circulating monocytes (expressed as a percentage) pre and post ileocolic resection for Crohn’s disease. There was a significant reduction in the fibrocyte proportion of circulating monocytes following ileocolic resection.
Figure 3: (A) Association between the fibrocyte percentage of monocytes and Crohn’s disease activity index in the full cohort (i.e. all Crohn’s subtypes included). (B) Association between the fibrocyte percentage of monocytes and Crohn’s disease activity index in patients with ileocolic Crohn’s disease.
Figure 4: Association between the fibrocyte percentage of monocytes and mesenteric (A) and mucosal (B) disease scores in the full cohort (i.e. all Crohn’s subtypes included). Association between the fibrocyte percentage of monocytes and mesenteric (C) and mucosal (D) disease scores in patients with ileocolic Crohn’s disease.
Effects of ileostomy formation on the fibrocyte percentage of circulating monocytes

Three patients had extensive Crohn’s disease that was not amenable to resection at the first laparotomy. These patients underwent loop ileostomy diversion rather than resection. Post-defunctioning, fibrocyte percentages were reduced relative to preoperative levels (12±9% versus 6.5±8%, P=0.06).

Mesenteric and intestinal fibrocytes

CD45$^{+}\alpha$SMA$^{+}$ myofibrocytes were not detectable in normal mesentery and intestine. In contrast they were too numerous to accurately quantify in patients with Crohn’s disease. Within the mesentery itself they clustered near vessels (Fig. 5,6). They were diffusely distributed throughout the connective tissue of the mesentery. Clusters of CD45$^{+}\alpha$SMA$^{+}$ myofibrocytes were observed at the intersection between the mesentery and the intestine (Fig. 6). They were also diffusely distributed within the connective tissue of the outer and inner intestinal muscle layers.
Figure 5: (A) Digital sculpture demonstrating the mesentery and associated intestine. (B) Photomicrograph demonstrating fibrocytes within the mesentery. Here they are clustered nearby blood vessels. (C) Higher magnification photomicrograph demonstrating fibrocytes in close proximity to blood vessels within the mesentery.
Figure 6: (A) Digital sculpture demonstrating the mesentery and associated intestine. (B) Photomicrograph demonstrating mesenteric fibrocytes clustered at the surface of the intestine. Inset, corresponding serial section stained with haematoxylin and eosin. (C) Photomicrograph demonstrating mesenteric fibrocytes clustered at the surface of the
intestine (arrow). They are also seen within the subjacent circular smooth muscle layer.

Inset demonstrates fibrocytes within the circular smooth muscle layer.
Figure 7 (A) Photomicrograph demonstrating dual stained CD45$^+$αSMA$^+$ cells (i.e. fibrocytes) within the mesentery in a patient with ileocolic Crohn’s disease. Using
standard imaging the DAB chromogen was prominent and the permanent red chromogen could not be visualised. (B) By changing the thus and saturation settings the DAB and permanent red chromogen became more and less prominent respectively. This approach enabled identification of dual stained CD45$^+$ αSMA$^+$ cells.
**Discussion**

The aim of this study was to investigate fibrocyte levels in Crohn’s disease. Fibrocytes are a proportion of circulating monocytes and thus their levels must be described in as a percentage of circulating monocytes \(^{75, 121}\). Fibrocyte percentages were significantly increased in Crohn’s disease and normalized following surgical resection. Percentages also reduced significantly following fecal diversion via loop ileostomy. Fibrocyte percentages correlated with the Crohn’s disease activity index as well as with mesenteric and mucosal disease scores. Histologically, fibrocytes were diffusely distributed throughout the connective tissue platform shared by mesentery and adjacent intestine. They clustered at the serosal surface of the intestine.

Increases in the fibrocyte proportion of circulating monocytes may explain several of the mesenchymal abnormalities that are hallmarks of Crohn’s disease. Fat wrapping and mesenteric thickening are strongly associated with Crohn’s disease and are underpinned by adipocyte hyperplasia and connective tissue thickening \(^{16, 54}\). Given that fibrocytes may differentiate into either adipocyte or fibroblast \(^{122, 123}\), their increases in Crohn’s disease could provide a cellular explanation of both fat wrapping and mesenteric thickening.

The management of Crohn’s disease continues to be hampered at diagnostic, prognostic and predictive levels due to lack of accurate biomarkers \(^{34, 124}\). Circulating fibrocyte percentages could help address this deficit. In this study, the mean percentage was increased in Crohn’s disease compared with that observed in diverticular disease and colorectal cancer. Fibrocyte percentages correlated with the Crohn’s disease activity index and mucosal disease scores. Circulating fibrocyte percentages could thus provide...
a diagnostic biomarker for Crohn’s disease. Larger scale studies comparing fibrocyte percentages across a range of pathologies are required to investigate this possibility.

The mean fibrocyte percentage was also increased in diverticular disease requiring surgical excision. This is significant as diverticular disease is also associated with pronounced mesenchymal thickening\textsuperscript{125}. Diverticular disease is not associated with fat wrapping unless complicated by a perforation. In addition, fibrocyte percentages correlated with mesenteric disease scores in Crohn’s disease. It is feasible that mesenteric inflammation leads to a systemic fibrocytosis and that this could provide a serologic biomarker of mesenteric inflammation. Given the importance of mesenteric inflammation in Crohn’s disease, circulating fibrocyte levels may then be utilized to better tailor individual patient treatment regimens.

In summary, the percentage of fibrocytes in circulating monocyte populations was increased in Crohn’s disease, and returned to normal levels following either surgical resection, or fecal diversion via loop ileostomy. The biomarker properties of fibrocytes warrant further investigation in Crohn’s disease in general.
Chapter five: Discussion
Discussion

The management of CD continues to be hampered at multiple levels 79, 124. Lack of accurate diagnostic biomarkers means patients undergo extensive laboratory and radiologic investigations that aim to exclude other conditions (i.e. appendicitis, terminal ileitis and ulcerative colitis) 126. Diagnosis requires exclusion of other pathologies and medical treatment aims to ameliorate the consequences rather than the cause of its pathobiology 127. Many of the features that are characteristic (i.e. transmural mesenchymal and mesenteric abnormalities) remain unexplained. In terms of treatment, rates of surgery and reoperation remain unchanged despite significant advancements in pharmacologic agents 11, 15, 79.

The classic approach to CD suggests that it arises from a complex interplay of environmental, genetic and immunologic factors played out at the mucosal level 3, 128, 129. Inflammation then spreads to involve all bowel layers and persistence is followed by transmural mesenchymal abnormalities. In turn, mesenteric inflammation occurs and gradually worsens.

Certain features are consistently associated with the disease. Taken together with recent advancements in our understanding of mesenteric anatomy they represent avenues for research that could help in diagnosis and prognostication in Crohn’s disease. For example, mesenteric manifestations of disease are largely confined to Crohn’s disease and out with the setting of complicated diverticular disease, are not observed in other intestinal conditions. Mesenteric manifestations include fat wrapping and mesenteric thickening and although there has been increasing focus on these, they are thought to represent an epiphenomenon, and thus are relatively understudied.
In the first component of this research the relationship between fat wrapping and surgical recurrence rates was determined. In a multivariate analysis fat wrapping was the only pathobiologic feature of Crohn’s disease that predicted increased surgical recurrence as well as reduced time to recurrence. The data indicate that levels of fat wrapping should be recorded at the time of surgery, with a view to tailoring postoperative surveillance investigations and adjuvant treatment in a more targeted manner. Surgical recurrence is a proxy index of disease severity as it generally means that patients are refractory to medical treatment. The relationship observed between fat wrapping and recurrence points to a pathobiologic link between mesenteric encroachment on the intestinal surface, and disease progression.

As part of the second component of the research described, a novel scoring system was generated to quantitate mesenteric disease manifestations. The mesenteric disease score correlated with a similar mucosal disease score and with the Crohn’s disease activity index. The score also worsened significantly with cigarette smoking. The change with smoking was greater than that associated with either the mucosal disease score or CDAI. Whilst it is universally recognised that smoking is prognostic in Crohn’s disease and harbours an increased risk of disease progression, surgery as well as postoperative recurrence, the exact mechanism is still unclear. It is postulated that cigarette smoking may influence colonic mucus production and cause endothelial dysfunction and that it may selectively modulate the T-helper cell 1 (Th1) pathway, hence promoting intestinal inflammation. Another study showed that smokers with active Crohn’s disease harbour a clinically relevant intestinal dysbiosis, which could potentially affect the disease prognosis.
The anatomic distribution of mucosal and mesenteric abnormalities corresponded precisely. Mesenteric and mucosal transition zones (where mesentery and mucosa changed rapidly from entirely normal to severely diseased) occurred in parallel. Where mesenteric manifestations were minimal, mucosal disease was similarly limited. Where mesenteric disease was advanced, mucosal and transmural disease was at its most severe.

The findings have a number of clinical implications. They strengthen the suggestion that mesenteric manifestations should be recorded at the time of surgery. Mesenteric and mucosal disease scores may be combined when considering patients for postoperative surveillance investigation and adjuvant treatment. The concept of mesenteric and mucosal resection transition zones is surgically relevant. The mesenteric transition zone provides the surgeon with a proximal margin at which to commence resection. More importantly, we believe that all Crohn’s disease patients requiring intestinal resection should undergo a complete mesocolic excision such that the diseased mesentery is removed undisturbed together with the affected intestine as this may be associated with reduced surgical recurrence. A further study comparing conventional surgical resection with complete mesocolic excision (i.e. inclusion of the diseased mesentery in the specimen) on cumulative reoperation rates is warranted to investigate this possibility.

These findings suggest that mesenteric-based mesenchymal abnormalities are of primary pathobiologic relevance in Crohn’s disease and prompt the question as to which arises earliest in the chronology of the disease. To address this question, mesenteric and mucosal patterns of gene expression were determined and, using a network-linkage based approach, correlated with published literature. Mesenteric, but not mucosal, gene
expression was strongly associated with connective tissue and inflammatory disorders in particular. This suggests that mesenchymal events originate in the mesentery and progress towards the adjacent intestine. In support of this, mesenteric mesenchymal derangements (including adipocyte hyperplasia and connective tissue thickening) were contiguous between mesentery and outer layers of adjacent intestine.

The final component of the study examined the relationship between the percentage of fibrocytes in circulating monocytes, with mucosal and mesenteric disease manifestations. Given that fibrocytes may differentiate into either adipocyte or fibroblast they could provide a cellular explanation for mesenteric mesenchymal abnormalities. The circulating percentage of fibrocytes was significantly increased in Crohn’s disease compared with healthy controls. Sazuka et al. 78 had similar observations. In addition, the fibrocyte percentage was increased in Crohn’s disease compared with diverticular disease and colorectal cancer. The fibrocyte percentage decreased to normal levels following either ileocolic resection or faecal diversion via a loop ileostomy.

Crohn’s disease is a mes-enteropathy

The following paradigm is based on the findings described above and may help to explain the transmural and mesenteric features of Crohn’s disease.

Fibrocytes are concurrently recruited to the intestine and mesentery. The recruitment process is influenced by an environmental factor (s) present in ileal effluent. Mesenteric fibrocytes are recruited along the mesenteric connective tissue lattice to the intestinal surface. There they contribute to fat wrapping and exploit the connective tissue
platform to infiltrate outer layers of the intestinal tract. A bipolar progression of fibrocyte-driven mesenchymal processes then occurs. At the intestinal pole this results in transmural mesenchymal abnormalities. At the mesenteric pole it leads to fat wrapping and mesenteric thickening. Continued progression at the intestinal pole leads to stricture formation and/or perforation/fistulation, whilst continued progression at the mesenteric pole leads to advanced mesenteric manifestations.
Conclusions

Mesenteric manifestations of Crohn’s disease such as fat wrapping, predict increased recurrence and reduced time to recurrence. Mesenteric manifestations correlate with local and systemic disease manifestations. The percentage of fibrocytes in circulating monocytes is increased in Crohn’s disease, correlates with mesenteric disease scores and returns to normal following surgical resection or faecal diversion.
References


49. Fazio VW, Jones IT. Clinical Surgery International- Surgery of Inflammatory Bowel Disorders. 1987: (SpringerLink).


134


80. Lee SM, Han EC, Ryoo SB, Oh HK, Choe EK, Moon SH, Kim JS, Jung HC, Park KJ. Long-term Outcomes and Risk Factors for Reoperation After Surgical Treatment


88. Baird JR, Friedman D, Cottam B, Dubensky TW, Jr., Kanne DB, Bambina S, Bahjat K, Crittenden MR, Gough MJ. Radiation therapy combined with novel STING-


121. LaPar DJ, Burdick MD, Emaminia A, Harris DA, Strieter BA, Liu L, et al. Circulating fibrocytes correlate with bronchiolitis obliterans syndrome


