The Angiogenic Characterisation of Mesenteric Adipose Tissue in Crohn's Disease

By

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PhD thesis

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Declaration

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

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Mohammad Eddama

Abstract

Introduction

Crohn's disease (CD) has a distinct feature of mesenteric adipose tissue (AT) expansion, the role of which is unclear. This study hypothesises that the angiogenic mechanisms in CD mesenteric AT are dysregulated.

Methods

Mesenteric, subcutaneous and omental AT were harvested from 30 patients who underwent ileocolic resection, including 19 CD and 11 controls. Angiogenic mechanisms were examined by: histology and immunohistochemistry; real time polymerase chain reaction (RT PCR) gene array; and enzyme linked immunosorbent assay (ELISA). ELISA was also used to assess the level of interleukin-6 (IL6) and vascular endothelial growth factor (VEGF) secretion by tissue over an incubation period of 36 hours. Angiogenic capacity was measured by matrigel angiogenic assay.

Results

Microvascular density (MVD) was significantly (p<0.01) higher in CD mesenteric AT (mean=29, SD=20) than control (mean=19, SD=12).

Hypoxia inducible factor-1 (HIF1) staining was higher in CD mesenteric AT (n=22, 67%) than control (*n*=18, 22%) ($X^2_{(2)}$=11.2, *p*<0.01). RT-PCR array confirmed that 47 (56%) of the angiogenic genes were >2-folds down-regulated in CD mesenteric AT. Correlation matrix showed significantly more negative correlations in CD mesenteric AT (*n*=711, 20%) than control (*n*=109, 3%) ($X^2_{(1)}$=501, *p*<0.0001). The mean-*z*-score for negative correlation was significantly (*p*<0.0001) stronger in CD mesenteric AT (mean=0.3, SD=0.2) than control (mean=0.1, SD=0.1). CD mesenteric AT protein expression of IL6 (mean=21 pg/mg, SD=18) and VEGF (mean=34 pg/mg, SD=19) were significantly (*p*<0.05 and *p*<0.01) lower than control (mean=39 pg/mg, SD=43) and (mean=57 pg/mg, SD=43) respectively. In-vitro secretion of IL6 and VEGF was similar in CD and control AT. Vascular sprouting was statistically significantly (*p*<0.01) lower in CD mesenteric AT (mean=3.2, SD=3) than control (mean=5.2, SD=4.1).

Conclusion

CD mesenteric AT demonstrated dysregulated angiogenesis and significantly lower capacity for vascular sprouting in comparison to control. The observed dysregulated angiogenesis may partly explain the role of mesentery in the perpetuation of CD inflammation.

Dedicated to

Mum and Dad

Oya

Ali, Emin and Layla

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List of Abbreviations

ACCESS	Asia-Pacific Crohn's and Colitis Epidemiology
ADGRB1	Brain-specific Angiogenesis Inhibitor 1
Ang	Angiopoietin
ANGPL4	Angiopoietin-like 4
ANGPT2	Angiopoietin 2
ASA	American Society of Anesthesiologists
AT	Adipose Tissue
AUC	Area Under the Curve
bFGF	Basic Fibroblast Growth Factor
BMI	Body Mass Index
CD	Crohn's Disease
CDAI	Crohn's Disease Activity Index
COL18A1	Collagen, Type XVIII, Alpha 1
COL4A3	Collagen, Type IV, Alpha 3 (Goodpasture Antigen)
CRP	C-Reactive Protein
CTGF	Connective Tissue Growth Factor
CXCL1	Chemokine (C-X-C motif) Ligand 1 (Melanoma Growth Stimulating Activity, Alpha)
CXCL10	Chemokine (C-X-C motif) Ligand 10
CXCL5	Chemokine (C-X-C motif) Ligand 5
CXCL6	Chemokine (C-X-C motif) Ligand 6 (Granulocyte Chemotactic Protein 2)

CXCL8	Interleukin 8
CXCL9	Chemokine (C-X-C motif) ligand 9
EC	Endothelial Cells
EDS	Ehlers-Danlos Syndrome
EDTA	Ethylenediaminetetraacetic Acid
EFNA1	Ephrin-A1
EFNB2	Ephrin-B2
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPHB4	EPH receptor B4
EpiCom	Epidemiological Committee
F3	Coagulation Factor III (Thromboplastin, Tissue Factor)
FGF2	Fibroblast Growth Factor 2 (basic)
FGFR3	Fibroblast Growth Factor Receptor 3
FIGF	C-fos Induced Growth Factor (Vascular Endothelial Growth Factor D)
FLT1	Fms-Related Tyrosine Kinase 1 (Vascular Endothelial Growth Factor/Vascular Permeability Factor Feceptor)
HGF	Hepatocyte Growth Factor (Hepapoietin A; Scatter Factor)
HIF	Hypoxia Inducible Factor
HIF1A	Hypoxia Inducible Factor 1, Alpha Subunit (Basic Helix-Loop-Helix Transcription Factor)

HPSE	Heparanase
IBD	Inflammatory Bowel Disease
ID1	Inhibitor of DNA Binding 1, Dominant Negative Helix-Loop-Helix Protein
IFNA1	Interferon, Alpha 1
IFNG	Interferon, Gamma
IGF1	Insulin-like Growth Factor 1 (Somatomedin C)
IL	Interleukin
IL1B	Interleukin 1, Beta
IL6	Interleukin 6 (Interferon, Beta 2)
INF	Interferon
JAG1	Jagged 1
LECT1	Leukocyte Cell Derived Chemotaxin 1
LEP	Leptin
MDK	Midkine (Neurite Growth-Promoting Factor 2)
MDT	Multidisciplinary Meeting
MMP14	Matrix Metallopeptidase 14 (Membrane-Inserted)
MMP2	Matrix Metallopeptidase 2 (Gelatinase A, 72kDa Gelatinase, 72kDa Type IV Collagenase)
MMP9	Matrix Metallopeptidase 9 (Gelatinase B, 92kDa Gelatinase, 92kDa Type IV Collagenase)
MMPs	Matrix Metalloproteinases
MVD	Microvascular Density
NFκB	Nuclear Factor κB

Notch4	Notch4 (Type 1 transmembrane protein)
NPWT	Negative Pressure Wound Therapy
OR	Odds Ratio
PDGF	Platelet-Derived Growth Factor
PECAM1	Platelet/Endothelial Cell Adhesion Molecule
PF4	Platelet Factor 4
PLG	Plasminogen
PlGF	Placental Growth Factor
PML	Progressive Multifocal Leukoencephalopathy
PTGS1	Prostaglandin-Endoperoxide Synthase 1 (Prostaglandin G/H Synthase and Cyclooxygenase)
ROC	Receiver Operating Characteristic
SD	Standard Deviation
S1PR1	Sphingosine-1-Phosphate Receptor 1
SERPINF1	Serpin Peptidase Pnhibitor, Clade F (Alpha-2 Antiplasmin, Pigment Epithelium Derived Factor), member 1
SMA	Superior Mesenteric Artery
TGF	Tumour Growth Factor
TGF-β	Transforming Growth Factor Beta
TGFB1	Transforming Growth Factor, Beta 1
TGFB2	Transforming Growth Factor, Beta 2
TGFBR 1	Transforming Growth Factor, Beta Receptor 1
Th	T helper

THBS1	Thrombospondin 1
TIE1	Sphingosine kinase 1
TIMP1	TIMP Metallopeptidase Inhibitor 1
TIMP3	TIMP Metallopeptidase Inhibitor 3
TNF	Tumour Necrosis Factor
UC	Ulcerative Colitis
UCL	University College London
UCLH	University College London Hospital
VEGF	Vascular Endothelial Growth Factor
VEGFA	Vascular Endothelial Growth Factor A
VEGFC	Vascular Endothelial Growth Factor C
WCC	White Cell Count

Published abstracts

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Oral presentation

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Chapter 1

Introduction and literature review

1.1. Introduction

Crohn's disease (CD) is an inflammatory bowel disease (IBD) that is chronic, progressive and potentially disabling. Indeed, patients with CD can suffer from a wide spectrum of symptoms including: recurrent abdominal pain; diarrhoea; blood in the stool; mouth sores; loss of appetite; weight loss; fatigue; and arthralgia (Mekhjian et al., 1979a). Furthermore, complications from CD including: intestinal structuring, obstruction and perforation; intraabdominal and perianal sepsis; visceral fistulation and damage to other abdominal and pelvic organs, which all can lead to significant morbidity and mortality (Schwartz et al., 2002, Morson and Lockhart-Mummery, 1959). Although the increased risk of mortality in CD has been a controversial finding (Cottone et al., 1996, Farrokhyar et al., 2001), the evidence from older and more recent studies advocates significantly higher risk of mortality in CD patients than the general population (Mayberry et al., 1980, Prior et al., 1981, Persson et al., 1996, Canavan et al., 2007). Specifically, the risk of mortality for those who are diagnosed before the age of 20 years was almost three times greater than in the general population (Canavan et al., 2007). Moreover, a 50-year follow up study showed that approximately 90% of patients' death were either directly or probably related to CD (Weterman et al., 1990). The causes of death were due to postoperative complications; or patients developing malnutrition, amyloidosis, massive hemorrhage, electrolyte disturbance, and colorectal cancer (Weterman et al., 1990). Beyond high risk of mortality, the sufferings from CD can have serious negative

consequences on the quality of life of the patients and their families (Lakatos et al., 2005, Burisch et al., 2014b).

Thus, there are far-reaching socioeconomic implications of CD, for example, the overall annual incidence of hospital admission is estimated to be over 20% (Mekhjian et al., 1979b). In the UK, the overall annual cost to care for any patient with CD was estimated to be approximately £6000. The annual cost for those in remission and relapse was approximately £1800 and £10,000 per patient respectively (Miles et al., 2014). Yet, the overburden of surgical interventions for the treatment of CD is a common recourse. It is estimated that between 70% and 90% of patients with CD would require surgical treatment during their lifetime (Canin-Endres et al., 1999). Beyond the suffering of these patients and their families and the burden on health care provision, CD affects the young generation of patients at the time they are economically and socially most productive. The peak incidence is amongst the youngest members of the society, with age of onset ranging between 10 and 40 years (Mekhjian et al., 1979b, Peyrin-Biroulet et al., 2010). Despite all the compelling facts indicating the need to prevent, and treat CD, the progress in finding a cure is far from grasp. In fact, there is humble progress made over the past three decades, which has not concluded a cause or identified an effective cure. Therefore, further understanding of the aetiopathogenic mechanisms is an urgent necessity.

One of the important pathological features of CD is observed in a neighboring anatomical structure called the mesentery[1]. It is mainly constituted of adipose tissue (AT), attaches the bowel to the abdominal wall and homes its blood supply. Macroscopically, it is remarkably thickened, particularly, at the level of the bowel affected by CD. As a result, the mesenteric bowel angle is obliterated. There is also an associated hypertrophy and migration of the AT to the bowel wall. The affected bowel is subsequently partially or wholly covered with mesentery. For this anatomical portrayal the terms fat wrapping and/or creeping fat were coined (Crohn et al., 1984). In fact, apart from a few cases of bowel tuberculosis, fat wrapping has only been formally reported in CD (Addison, 1983). Thus, fat wrapping is considered a pathognomonic phenomenon to CD. The compelling question is whether the mesentery can be an active contributor to CD pathogenesis? And whether this phenomena plays a role in the development and perpetuation of CD? In order to answer this question, several research studies have been dedicated to characterise CD mesentery (Peyrin-Biroulet et al., 2007, Sheehan et al., 1992, Kredel et al., 2013). Although these studies and others have confirmed the important role of the mesentery in CD pathogenesis (which will be explained in detail in the subsequent section 1.5. of this

[1] Double fold in the peritoneum which attaches the bowel to the posterior abdominal wall

chapter), a decisive understanding of this role remains far from reached.

As described above and demonstrated by other authors (Kredel and Siegmund, 2014), the mesentery in CD is an inflammatory and expanding tissue mass. To survive this expansion, new blood vessels are required to ensure oxygenation, nutrition and disposal of metabolic products. This is achieved by a process called angiogenesis[2]: the development of new blood vessels form pre-existing blood vessels (Folkman, 1971). Angiogenesis is considered a hallmark of inflammation and neoplastic growth (Algire et al., 1950). Unlike inflammation, which has been the focus of investigating the mesentery in CD in several studies, angiogenesis has only been the focus of one (Schaffler et al., 2006). There is a potential role for angiogenesis in the pathogenesis of CD. Further understanding of angiogenic mechanisms may unravel diagnostic, therapeutic and prognostic targets.

The overall aim of this thesis is to characterise the angiogenic mechanisms in CD mesentery. The objectives can be summarised in the following: 1) to describe the angiogenic gene expression profile in CD mesenteric AT; 2) to assess the level of angiogenic protein expression in both mesenteric AT and serum of CD patients; 3) to measure the level of angiogenic factor secretion by the mesenteric AT

[2] The process of creating new blood vessels from pre-existing ones

of CD patients; 4) to evaluate the angiogenic capacity of the mesenteric AT of CD patients.

1.2. Epidemiology of Crohn's disease

An analytical view of the epidemiological data of CD may bring an insight into its potential risks factors, associations, and even causes. Furthermore, awareness of its incidence and prevalence may highlight its socioeconomic impact and puts into perspective the scale at which diagnostic, therapeutic and prognostic health care resources may be deployed.

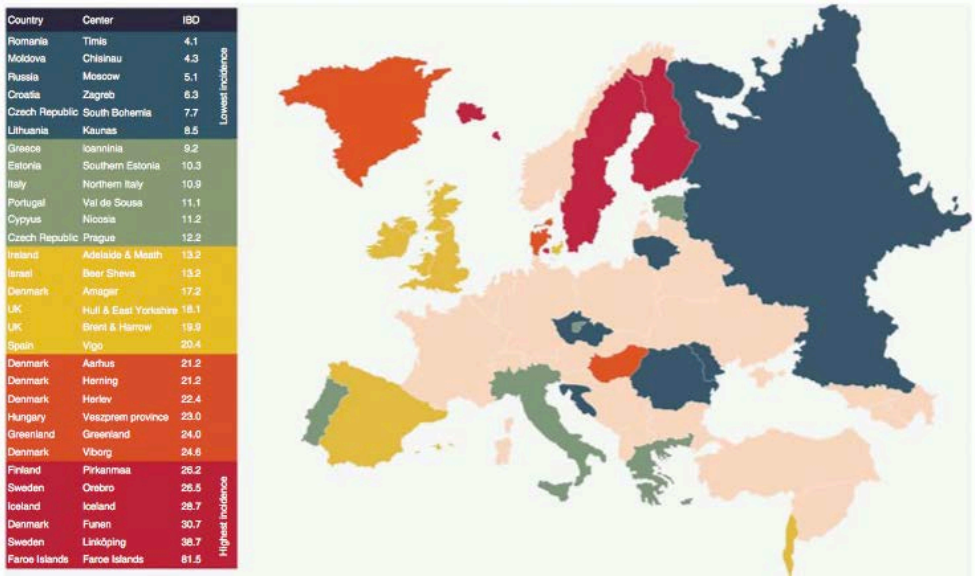
1.2.1. The incidence and prevalence of Crohn's disease

CD has been diagnosed in almost all races regardless of geographic location, but with considerable variations in incidence and prevalence. Industrialised countries have a higher incidence than non-industrialised, the highest being in North Europe (Burisch et al., 2013, Burisch et al., 2014a) and North America (Bernstein et al., 2006, Loftus et al., 2007). Worldwide, the incidence of CD is on the rise, even in regions where incidence use to be low, such as Asia (Thia et al., 2008) and Eastern Europe (Lakatos et al., 2011). This data suggests the possibility of environmental risk factors in attributed to the aetiopathogenesis of CD.

Currently, there are around 115,000 people in the UK suffering from CD (Mayberry et al., 2013). In Europe, the incidence rates for CD ranges from 1.5 to 20.3 cases per 100,000 person-years (Loftus, 2004). The prevalence of CD in Europe ranges from 1.5 (Gheorghe et al., 2004) to 213 (Lapidus, 2006) cases per 100,000 persons. In the united states, the incidence of CD ranges from 0 to 20 per 100,000 person-years (Molodecky et al., 2012) and the prevalence ranges from 26 (Kurata et al., 1992) to 318 (Loftus et al., 2007). Although these variations could be related to potential risk factors, such as diet, life style, sun exposure or even racial origin, one cannot under-estimate the differences derived from the methodological design of these cohort studies informing these data.

Thus, a well-designed methodological study conducted by the Epidemiological Committee (EpiCom) (Burisch et al., 2014a) has included 31 centres from 23 European countries. The EpiCom reported 2:1 West-East gradient of IBD incidence rate in Europe (Figure 1). The overall incidence of CD in all these centres was approximately 5 per 100,000 person-years. Similarly, the Asia-Pacific Crohn's and Colitis Epidemiology (ACCESS) study found the overall incidence of CD in Asian countries to be 0.5 per 100,000 person-years. Interestingly, Australia had the highest incidence of 14 per 100,000 person-years (Ng et al., 2013). The high incidence of CD in the Australian and Western European population considering their common racial origin and a similar life style may support the

hypothesis of a genetic cause, brought about by environmental factors.



Country	Center	IBD	
Romania	Timis	4.1	Lowest incidence
Moldova	Chisinau	4.3	
Russia	Moscow	5.1	
Croatia	Zagreb	6.3	
Czech Republic	South Bohemia	7.7	
Lithuania	Kaunas	8.5	
Greece	Ioannina	9.2	
Estonia	Southern Estonia	10.3	
Italy	Northern Italy	10.9	
Portugal	Val de Sousa	11.1	
Cyprus	Nicosia	11.2	
Czech Republic	Prague	12.2	
Ireland	Adelaide & Meath	13.2	
Israel	Beer Sheva	13.2	
Denmark	Amagier	17.2	
UK	Hull & East Yorkshire	18.1	
UK	Brent & Harrow	19.9	
Spain	Vigo	20.4	
Denmark	Aarhus	21.2	
Denmark	Herning	21.2	
Denmark	Herlev	22.4	
Hungary	Veszprem province	23.0	
Greenland	Greenland	24.0	
Denmark	Viborg	24.6	
Finland	Pirkanmaa	26.2	
Sweden	Orebro	26.5	
Iceland	Iceland	28.7	
Denmark	Funen	30.7	
Sweden	Linköping	38.7	
Faroe Islands	Faroe Islands	81.5	Highest incidence

Figure 1.1. Incidence and prevalence of inflammatory bowel disease in Europe in 2010 (Burisch et al., 2014a).

1.2.2. Crohn's disease demographic data

In terms of age, as described above, CD affects people between the age of 10 and 40 years, with a peak incidence during the early 20s (Peyrin-Biroulet et al., 2010). Some cohort studies indicated another peak of the incidence of CD between 50 and 70 years of age (Calkins and Mendeloff, 1986). However, these late cases might be caused by ischaemic changes in the bowel (Karlinger et al., 2000). Therefore, further investigation in the incidence of CD in older population needs to be verified.

Interestingly, the trend of the onset of the disease among family members appears to affected younger age in consecutive generations of patients suffering from CD (Karlinger et al., 2000). Furthermore, a milder form of the disease “form frustes” may be succeeded by more obvious or even severe forms in later generations (Miest et al., 2016). While these patterns do partly confirm an inherent factor in CD aetiopathogenesis, they may indicate environmental factors that may influence the gene expression leading to a different disease genotype in offspring.

In terms of racial origin, a good example that has been studied extensively in the literature is the increased risk of CD amongst the Jewish population (Yang et al., 1993). Subsequently, several studies have described heterogeneity among the different Jewish historical ethnic groups (Zlotogora et al., 1990, Abu Freha et al., 2015). Specifically, American or European Ashkenazi Jews have a significantly increased incidence and prevalence of CD in comparison to Sephardic or Oriental Jews (Bar-Gil Shitrit et al., 2015, Zvidi et al., 2009). In the context of a North Mediterranean racial origin of Ashkenazi Jews, recently confirmed by mitochondrial DNA studies (Costa et al., 2013), the potential genetic aetiology of CD can not be undermined.

In terms of gender affliction, the results are different depending on the population studied. In an Asian population including, China, Japan,

and Korea there appears to be a male predominance. The male to female ratio ranges from 1.7:1 to 3:1 (Yao et al., 2000, Leong et al., 2004, Yang et al., 2008, Thia et al., 2008). In contrast, studies from a Western population generally reported equal gender distribution, but some showed a slight preponderance in females (Vind et al., 2006, Bernstein et al., 2006). Female predominance was also higher in the Jewish population with a female to male ratio of 1.1:1 (Zvidi et al., 2009). These gender differences appear to be dependent on the population studied, the significance of which is unclear. However, in terms of female preponderancy in a Western population, this could be related to the hypothesis that CD is an autoimmune disease, as females are more likely to be affected.

1.3. Aetiopathogenesis

Although the cause of CD is unknown, the causal mechanisms appear to be mediated by a combination of familial aggregation, genetic susceptibility and environmental factors (Rioux et al., 2007). This may be translated into an abnormal immune response to enteric microflora, in genetically susceptible individuals. The intestinal immune system is shaped by a complex interaction between three factors: host cells; nutrients; and microflora. In terms of microflora, we know now that the intestine is colonised by up to 100 trillion cells of microflora, which are exposed to the host though a mucus-covered surface area of 32 m^2 (Helander and Fändriks, 2014). The intestinal immune system is

responsible for distinguishing the normal microflora from pathogenic bacteria (Bourlioux et al., 2003). This is achieved by an impeccable balanced between the levels of pro and anti-inflammatory cytokines. This balance appears to be impaired in CD patients (Arseneau et al., 2007).

Genetically, the candidate genes found to be implicated in CD are mainly involved in coding for the regulation of the immune and inflammatory processes (Mathew and Lewis, 2004). This is a cause for intolerance to bowel microflora. For example, the susceptible genes linked to CD, such as nucleotide-binding oligomerization domain (NOD) receptors and Toll-like receptors (TLR) are involved in pattern recognition and loss of tolerance to the commensal microflora (Arseneau et al., 2007). However, gene association does not fully explain the aetiology of CD, since NOD genes for example account for only 20% of CD cases (Shanahan, 2002). It is therefore believed that the aetiology of CD is multi-factorial and may be determined by environmental exposures. For example, evidence from a meta-analysis suggested a positive association between smoking and CD with odds ratio (OR) of 1.76 (Mahid et al., 2006). In terms of diet, the association between sugar intake and the development of CD had been inconsistent and subject to methodological limitations (Riordan et al., 1998). Similarly, associations between CD and dietary intake of fatty diet, fish diet rich in vitamin E and C have been subject to inherent recall bias as these studies were retrospective (Probert et al., 1996,

Sakamoto et al., 2005). Other risk factors for developing CD including breastfeeding, domestic hygiene and infective causes remain unconfirmed (Loftus, 2004). Further prospective well designed studies are required to confirm association or causality relationship between environmental factors and the development of CD.

1.3.1. Inflammatory pathways

To identify therapeutic targets, research into CD aimed to describe inflammatory pathways that drive the chronic inflammation. Currently, multiple and possibly independent pathways have been explained. Two main pathways mediated by T-helper (Th) 1 and Th17 cells have been explained. The antigen presenting cells (APC), possibly pathologically reacting to normal flora, activate Th1 and Th17 cells by interleukins (IL) 12 and IL6, IL1β and IL23 respectively (Figure 2). Initially, Th1 cells activated by IL12 were believed to distinctly mediate the pathogenesis in CD. However, the role of Th17 mainly activated by IL 23 dominated the scene. Interestingly, IL12 and IL23 share a common p40 subunit and therefore have an interchangeable effect on activating Th1 and Th17. Further research has subsequently showed that Th17 remains activated in a milieu where L12 and IL23 are blocked. In turn, IL6 and tumor growth factor-β (TGFβ) can activate Th1 and Th17 cells. Indeed, it is now evident that Th17 subset develops from naïve T cells only in the presence of IL-6 and TGFβ

regardless of whether IL23 is present or not (Arseneau et al., 2007).

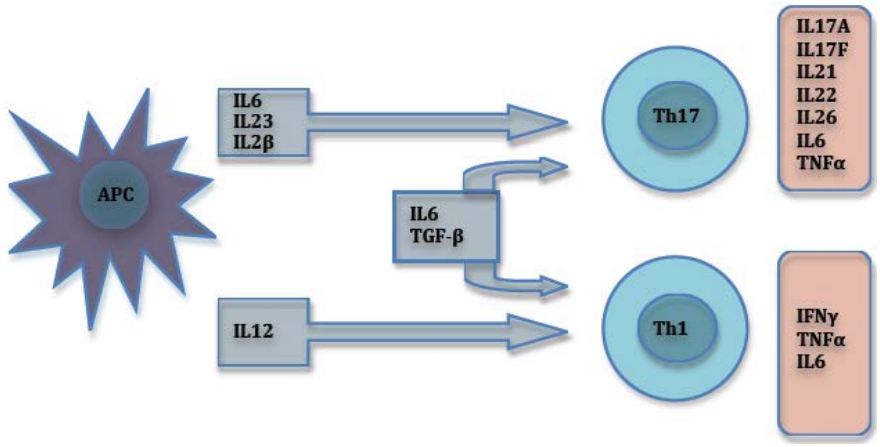


Figure 1.2. The two main inflammatory pathways in CD: 1) T Helper (Th) 17, activated by interleukin (IL) 6, IL23 and IL2β. 2) Th1 cells, mainly activated by IL12. APC: antigen presenting cell, TNFα: tumour necrosis factor alpha, IFNγ: interferon gamma, TGFβ: tumour gowth factor beta

Similar to other autoimmune disorders, CD has a heterogeneous immune response (Cho and Feldman, 2015). It appears that more than a single inflammatory pathway drives the inflammation. This line of thought is reinforced by the different therapeutic response to monoclonal antibodies designed to target distinct levels of inflammatory cytokines, such as TNFα, IL6, IL23, and interferon-γ (INFγ) (Abraham et al., 2017). It is possible that different inflammatory pathways are activated depending on the disease stage. For example, INFγ has been significantly over-produced in CD patients at the time of their first attack, and not in those with long-standing disease (Kugathasan et al., 2007). Zorzi et al demonstrated a significant increase in the level of INFγ in the neo-terminal ileum of

endoscopically normal looking mucosa one-year post-ileocaecal CD resection. In contrast, patients with endoscopic recurrence were found to have a significantly high level of IL17A, a Th17 cytokine (Zorzi et al., 2013). Similarly, TNFα, mainly a Th1 cytokine and a therapeutic target, was also up regulated in the neo-terminal ileum of CD patients, in the presence or absence of endoscopic recurrence, but not in well-established lesions (Zorzi et al., 2013).

Pioneering studies by Desreumaux *et al* in 1997 showed enhanced gene expression of Th2 cytokines (IL4 and IL5) in early CD lesions, subsequent research confirmed the pattern in early and established lesions in CD neo-terminal ileum (Desreumaux et al., 1997). Subsequently, depending on the activated inflammatory pathway, CD pathogenesis may have three different stages: mild (early); moderate (intermediate); and severe (late) (Figure 1.3). For example, although Th1, Th2 and Th17 are active in all the stages of the disease, Th1 activity is predominant in the early stages, while Th17 takes the lead in driving the inflammation in well-established late stage disease. Further understanding of disease mechanisms in each stage may help to tailor the therapy depending on the disease stage.

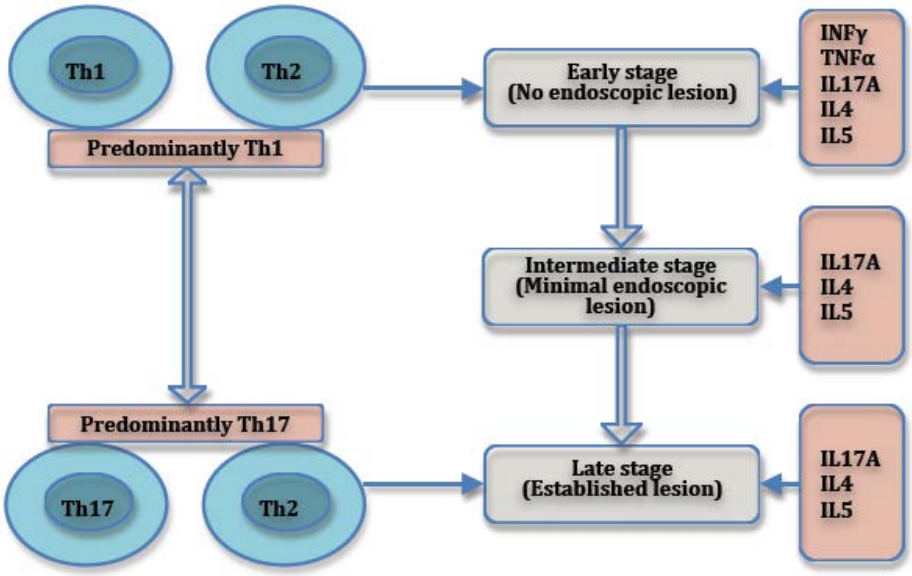


Figure 1.3. Inflammatory pathways in different stages of Crohn's disease.

1.4. Angiogenesis

Angiogenesis is the process at which the interaction of several cell types and mediators creates a specific microenvironment that leads to the formation of new capillaries from pre-existing blood vessels (Conway et al., 2001, Folkman, 1985). Physiologically, angiogenesis is an essential component of organ growth and development (Ribatti et al., 1991). Furthermore, integral healing mechanisms are dependent on a balanced angiogenic response typically intertwined with an appropriate inflammatory response (Gurtner et al., 2008). Wound healing is a prime example: first stage is characterised inflammation (Grose and Werner, 2004); followed by new tissue formation and angiogenesis (Werner and Grose, 2003); and finally remodeling, where all the processes activated after the injury wind down and cease (Lovvorn et al., 1999). These various steps of wound healing are regulated by several factors that activate the appropriate

intracellular signaling pathways of a large diversity of cells. These homeostatic mechanisms are impaired in chronic disease.

One of the hallmarks of chronic disease is impaired angiogenic mechanisms. It is now clear that angiogenesis plays an important role in the development and perpetuation of cancer, rheumatoid arthritis, peptic ulcer, psoriasis, atherosclerosis, Alzheimer's disease and IBD (Danese et al., 2006, Carmeliet, 2005). As inflammation evolves, vessels expand to supply nutrients that sustain the accumulation of activated immune cells. In the chronic phase, local immune cells overproduce endothelial cell growth factors, which subsequently promote the expansion of capillary networks (Carmeliet, 2005). The infiltration of macrophages and lymphocytes in chronic inflammatory disorders and the constant tissue damage and repair occur concurrently resulting in the newly formed vessels to become permanent (Szekanecz and Koch, 2004). The anatomical expansion and increased activation of the remodeled microvascular bed foster further influx of immune cells at which angiogenesis and inflammation become codependent processes.

Although the involvement of vasculature in CD has been known for more than four decades (Bacaner, 1966, Brahme and Lindström, 1970), the angiogenic mechanisms are still poorly understood. In CD, there is a continuous deep ulceration and regeneration, for which angiogenesis is essential to ensure that nutrients and oxygen is

carried to the inflammatory mass and the waste product are disposed. Unlike physiological angiogenesis, in CD the angiogenic mechanisms are driven by the immune response (Pousa et al., 2008b, Chidlow et al., 2007, Jerkic et al., 2010).

The following sections will review the current knowledge of vascular involvement and angiogenic mechanisms in CD.

1.4.1. Microvascular changes in Crohn's disease

The association between microvascular changes and CD has been previously identified at the level of bowel mucosa (Knutson et al., 1968). Histologically, small intramucosal haemorrhagic lesions are seen early in the disease development and before inflammatory changes occur (Sankey et al., 1993). Evidence of early vascular damage was also demonstrated by "summit" lesions, with plumes of fibrin and loss of superficial epithelial cells. Furthermore, disruption of the collagen IV in the basement membrane of mucosal layer capillaries, and extravasation of red blood cells and fibrinogen into the surrounding lamina propria was seen in 17% of a cohort of 35 CD patients (Sankey et al., 1993). In another cohort of 7 CD patients, patchy epithelial necrosis in the absence of acute inflammation was reported as early features of the disease in 2 patients (Dourmashkin et al., 1983). Wakefield *et al* suggested that vascular damage precedes mucosal inflammation in CD patients when they observed occlusion of the arteries supplying the affected bowel (Wakefield et al.,

1989). Although the authors in these studies concluded that these findings are indicative of early vascular changes that are unlikely to be secondary consequence to mucosal inflammation or ulceration, a more recent study suggested that early vascular changes in recurrent disease are concomitant with inflammatory changes (Maunoury et al., 2000). In this latter study fluorescent endoscopy was used to identify characteristic “fluorescent spots”, which histologically relate to vasodilatation. Approximately 80% of the biopsies taken from the fluorescent spots showed vascular involvement as well as inflammatory changes. It is possible that this isolated report has not detected changes early enough and therefore the vast majority of the vascular changes are associated with inflammatory response. Furthermore, a better designed and larger sample size study used magnifying colonoscopy and biopsy of early “red halo appearance” lesions concluded that capillary disruption precedes inflammation (Fujimura et al., 1996). Therefore, it is reasonable to conclude that microvascular changes are present in the early stages of CD, however, further studies are required to conclude that these changes precede or cause inflammation. In depth understanding of these microvascular changes and the mechanisms at which they contribute to CD pathogenesis may clue a novel approach to the disease prevention and therap.

1.4.2. Mucosal capillary microthrombi in Crohn's disease

Bowel ischemia may play a role in the pathogenesis of CD. Although mucosal microthrombi have not been recognised as a widely accepted feature of CD (Knutson et al., 1968, Geller and Cohen, 1983), more recent studies demonstrated the frequent presence of mucosal capillary microthrombi (Dhillon et al., 1992, Wakefield et al., 1991a). Mucosal capillary thrombi are also seen in other forms of colitis including infective colitis and are mainly formed of platelets (Mathan and Mathan, 1985, Donnellan, 1966) and fibrin (Price, 1990) as a lesser component. In the reported cases of CD patients with mucosal capillary thrombi, staining for factor XIIIA was positive (Sankey et al., 1993, Dhillon et al., 1992). Factor XIII is found in plasma and platelets which is activated by thrombin to form factor XIIIA (McDonagh and McDonagh, 1975).

During this process, stabilised fibrin is formed, and that is the final event in the coagulation of blood. Therefore, the presence of XIIIA in the capillary microthrombi of CD suggests that these are true microvascular thrombi. Keeping in mind that mucosal capillary microthrombi are the diagnostic morphological features of ischaemia, disseminated intravascular coagulation, and pseudomembranous colitis (Whitehead, 1971), and that these changes have only occasionally been described in CD, it can only be speculated that ischaemia may play a part in the pathogenesis of CD. The presence

of mucosal microthrombi in CD, UC and other self-limited colitis raises the possibility of common pathogenic mechanisms and may be a base for the design of novel therapies.

1.4.3. Vasculitis in Crohn's disease patients

The association of CD and vasculitis raises the question as to whether these patients have primary vasculitis with CD or CD with secondary vasculitis (Hatemi et al., 2017). The incidence of CD in patients with vasculitis is significantly higher than the general population. In a case series of 44 predominantly Caucasian patients with Takayasu arteritis, in which large-vessels are affected, 9% were suffering from CD (Reny et al., 2003). In comparison, the prevalence of CD in the general population in a similar population is approximately 0.2% (Lapidus, 2006, Hall et al., 1985). A recent systematic review of the literature and a case series confirmed a high incidence of vasculitis in IBD patients (67%, 167/260) (Sy et al., 2016). There were three most common types of vasculitis in IBD including: Takayasu arthritis; cutaneous vasculitis and anti-neutrophilic cytoplasmic antibodies-associated vasculitis. The diagnosis of IBD was made before that of vasculitis in most cases. Furthermore, a hallmark of Takayasu arteritis is granulomatous inflammation, which is also a feature of CD (Wakefield et al., 1991a, Humbert et al., 2015). The demographic of patients suffering from vasculitis and CD are also similar, and so does their response to anti-TNF therapy (Sy et al., 2016).

Another interesting association is found between Behçet's syndrome and CD. Behçet's syndrome has both inflammatory characteristics of CD and features of vasculitis (Dowling et al., 2008). Indeed, the gastrointestinal bleeding and perforation of patients with Behçet's syndrome increases its overall morbidity and mortality (Hatemi et al., 2016). Both conditions share IL23R, IL10 and TLR8 genetic susceptibility (Ortiz-Fernández et al., 2015). These mediators are heavily involved in the regulation of innate immune response (Trinchieri and Sher, 2007, Gautier et al., 2005, Napolitani et al., 2005). Furthermore, the response rate to immunosuppressive therapy and anti-TNF agents in both conditions is similar (Tanida et al., 2015, Hatemi et al., 2015). Fecal calprotectin level is also high in patients with Behçet's syndrome and gastrointestinal involvement similar to what is observed in CD (Özşeker et al., 2016).

Vasculitis is also the hallmark of extraintestinal manifestations of CD. Other organs affected in CD patients, such as: ophthalmic, iritis (Verbraak et al., 2001); neurological, chronic polyneuropathy (Pardi et al., 1998); cereberal vasculitis (Brohee et al., 1997); and necrotizing vasculitis of the skin, penis and lungs (Pagnoux et al., 2005, Sy et al., 2016) are all linked defined as vasculitis. Yet, very little is known about the reason for the association between vasculitis and CD. Further understanding of the mechanisms of disease may indicate some common pathogenesis and unravel significant therapeutic targets.

1.4.4. Angiogenic factors in Crohn's disease

Angiogenesis is mediated by several factors, some of which are proangiogenic and others are antiangiogenic (Bouïs et al., 2006). Impeccable balance of the expression of these factors is required to maintain an appropriate angiogenic response (Kwiatkowski et al., 2013). One of the most important proangiogenic factors in pathological angiogenesis is vascular endothelial growth factor (VEGF) (Pousa et al., 2008b).

There are seven different isoforms of VEGF including: VEGF-A; VEGF-B; VEGF-C; VEGF-D; VEGF-E; VEGF-F; and placental growth factor (PlGF) (Toi et al., 2001). VEGF isoforms exhibit their action on the cells by binding to three cell membrane receptors VEGF-R1, VEGF-R2, and VEGF-R3 (Ferrara et al., 2003). Specifically, VEGF-A promotes angiogenesis by increasing vascular permeability, and inducing endothelial cells (EC) proliferation, migration and differentiation. VEGF-A stimulates the production of proinflammatory cytokines by activating nuclear factor κB (NFκB) and increasing adhesion of leukocytes to vascular endothelium and monocytes (Toi et al., 2001). The level of VEGF was found to be significantly higher in the serum of adult and children with CD, in comparison to controls (Schürer-Maly et al., 1997, Griga et al., 1998, Bousvaros et al., 1999).

The source of VEGF in patients with CD was investigated. Griga *et al* collected mucosal samples during endoscopic examination from CD patients, UC and patients with non-inflammatory conditions. The level of VEGF in the supernatant of cultured mucosal biopsies was significantly higher than controls (Griga et al., 1999b). In a different report the secretion of VEGF by the peripheral blood mononuclear cells was significantly higher in CD patients than controls (Griga et al., 1999a). Further work by the same group demonstrated that VEGF expression is significantly higher in CD patients inflamed endoscopic biopsies in comparison to non-inflamed mucosa of same patients (Griga et al., 2002). More recently, Alkim *et al* confirmed significantly higher expression of VEGF and increased microvascular density (MVD) in CD and UC mucosal biopsies than healthy controls (Alkim et al., 2009, Alkim et al., 2012). One study has disagreed with these results and demonstrated no difference in VEGF expression on bowel tissue of patients with CD, UC, and healthy controls. Nor was there a significant difference in the serum and plasma concentration of VEGF between the groups (Kapsoritakis et al., 2003). Although the latter study has a larger sample size of CD patients (*n*=44), it is unclear whether these patients were treated with immunosuppressive therapy and whether they are demographically different from patients included in other reports. One explanation of this discrepancy in the literature may be due to the samples used. For example, Kapsoritakis *et al* used surgical specimens of CD patients rather than mucosal biopsies, whereas Griga *et al* and Alkim *et al* used endoscopic biopsies. Patients

undergoing surgery for CD are usually at a late stage of the disease, and as has been hypothesised in section 1.3.1., and illustrated in Figure 1.3. of this chapter cytokine expression may differ depending on the disease stage. This is significant as to when developing therapeutic targets.

Platelet-derived growth factor (PDGF) is a potent proangiogenic factor that is secreted in response to hypoxia, thrombin, and other cytokines and growth factors (Alvarez et al., 2006). PDGF is responsible for the recruitment of pericytes and smooth muscle cells to promote tubular vessel formation (Levanon et al., 2006). In CD patients the mRNA and protein expression levels of PDGF-A and PDGF-B and their receptors alpha-R and beta-R were significantly increased in the areas of active inflammation and fibroses of CD patients in comparison to unaffected bowel in the same patient group (Kumagai et al., 2001).

Other angiogenic growth factors were investigated in CD including: basic fibroblast growth factor (bFGF); hepatocyte growth factor (HGF); and transforming growth factor beta (TGF-β). bFGF triggers vessel formation by promoting the growth and differentiation of vascular endothelium (Andres et al., 2009). The serum levels of VEGF and bFGF in CD patients positively correlated with the bowel wall thickness and hypervascularity of the inflamed bowel (Di Sabatino et al., 2004). There was also a positive correlation between the serum level of bFGF and intestinal stricture formation, suggesting a possible involvement of

bFGF in the process of transmural fibrogenesis. HGF is a proangiogenic factor that stimulates endothelial cells mitogenesis, motility and extracellular matrix invasion (Van Belle et al., 1998). There were conflicting reports about the serum level of HGF in CD. In a cohort of 60 patients with CD Srivastava *et al* found a significant elevation in the serum level of HGF in comparison to patients presenting with function and benign bowel disorders (Srivastava et al., 2001). On the other hand Sturm *et al* demonstrated no significant difference in the serum level of HGF in a cohort of 45 CD patients and healthy controls (Sturm et al., 2000). There are two possible reasons for this discrepancy. Firstly, Crohn's disease activity index (CDAI) was only identified in one (2%) patient with severe CD in Strum *et al* study as compared to 12 (20%) patients with severe CD in Srivastava *et al* study. Therefore, the significant elevation found in the latter study may be a reflection of a high CDAI in CD. Secondly, patients' demographics are significantly different in these two studies: the patients in Srivastava *et al* were a pediatrics' population, whereas the population included in Strum *et al* study are adults. It is possible that the difference in patients age is the reason for the discrepancy in the levels of HGF.

TGF-β has three isoforms: TGF-β1; TGF-β2; and TGF-β3. Together they promote angiogenesis by enhancing tubulogenesis (Holifield et al., 2004). However, individually they have been shown to exhibit antiangiogenic effect. Specifically, TGF-β1 inhibits angiogenesis by activating Smad2 pathway (Nakagawa et al., 2004). The level of TGF-

β2 and TGF-β3 were overexpressed in CD endoscopic specimens that also demonstrated active inflammation (Kanazawa et al., 2001). Yet, in a different report TGF-β serum level was similar in adult CD patients when compared to healthy controls (Sturm et al., 2000). Furthermore, the levels of thrombospondins (TSPs), a well-known antiangiogenic factor, in CD patients were inconsistent in the literature. Alkim *et al* reported higher level of TSP-1 expression in mucosal biopsies of a cohort of 14 patients with CD in comparison to 11 healthy controls (Alkim et al., 2012). On the other hand, Wejman *et al* found similar expression of TSP-1 in surgical specimens of 38 CD patients in comparison to controls (Wejman et al., 2013). It is noticeable in a repeated pattern that the tissue expression level of angiogenic factors differ depending on the specimens used for analysis. For example, when surgical specimens obtained from surgical resections, no significant increase in the level of expression is observed. However, when the samples are retrieved from patients endoscopically, significant differences are observed. This has also been seen when the level of VEGF was assessed (see above). Several potential reasons may explain this difference. Firstly, patients who are undergoing surgical resection have usually failed optimal medical therapy and the disease is at its late stage. Whereas endoscopic specimens are usually retrieved from patients at an early stage of the disease. Secondly, surgical specimens are taken from non-living tissue and the selected section for investigation may escape the diseased segment of the bowel. Specimens taken at endoscopy are

visually macroscopically affected either by inflammation or ulceration and therefore, missing a diseased section is less likely. Thirdly, it is unclear whether there is difference in the use of immunosuppressive in patients' population. For example, *mesalamine* and anti-TNF drugs modulates are known to modulate angiogenesis (Deng et al., 2009, Rutella et al., 2011).

After highlighting the most important studies in the literature into angiogenic factors in CD, it appears that the angiogenic mechanisms are barely understood. Yet, angiogenesis is a complex process that involves a variety of cells, molecules and pathways, all of which could be therapeutic targets. Nevertheless, there appears to be discrepancies in the levels of angiogenic factors expression based on the given literature. This may indicate that the angiogenic mechanisms in CD differ depending on the disease stage. The obvious question is whether CD is proangiogenic whereby these factors perpetuate the disease? And therefore could angiogenesis be targeted for therapy? The answer remains unclear; it is difficult to conclude whether CD is characterised by up or down regulation of angiogenesis. Indeed, further in depth understanding of the angiogenic mechanisms is required in order to target angiogenesis for CD therapy.

1.4.5. Angiogenic therapies in Crohn's disease

Angiogenesis remains an essential component of CD pathogenesis and a potential therapeutic target. Hence, the use of angiogenic modulators may be beneficial in the treatment of CD. *Mesalamine*, for example while given as an anti-inflammatory has an angiogenic modulatory role. Deng *et al* shown that *mesalamine* establishes angiogenic balance by reducing *angiostatin* and *endostatin* and induced TNF-α and MMP-9 activity in experimental colitis (Deng et al., 2009). Furthermore, anti-TNF drugs, such as *infliximab*, currently considered an effective therapy for the treatment of CD have been shown to inhibit angiogenesis (Rutella et al., 2011). Similarly, thalidomide, another anti-TNF drug has a strong antiangiogenic effect (Diamanti et al., 2015).

Nevertheless, specific antiangiogenic therapy for CD is yet to be discovered. The use of *Bevacizumab*, a monoclonal antibody against VEGF, for the treatment of CD has been hindered by its reported serious side effect, such as reducing wound healing, anastomotic leak and intestinal perforation (Adenis et al., 2007). Furthermore, lessons have been learnt from the use of tyrosine kinase inhibitors, such as *sunitinib* and *sorafenib*, which inhibit angiogenesis by blocking VEGFR1, VEGFR2 and VEGFR3. When these drugs were used for the treatment of hepatocellular and renal cell carcinoma they have caused relapse of CD (Danese, 2011). They also cause CD relapse in

patients being treated for renal cell carcinoma, even when re-challenged a second relapse occurred (Boers-Sonderen et al., 2014).

Another promising antiangiogenic therapy that was used for the treatment of CD is anti-α4-integrins monoclonal antibodies including: *natalizuma* and *vedolizumab*. α4-integrins were initially discovered on lymphocytes, but have subsequently been reported on angiogenic endothelial cells (Vanderslice et al., 1998). Integrins are the principle adhesion receptors used by endothelial cells to interact with their extracellular environment and play a crucial role in regulating endothelial cell proliferation, migration and survival (Stupack and Cheresh, 2004). By blocking α4-integrins angiogenesis can be inhibited (Calzada et al., 2004, Koch et al., 1995). A recent meta-analysis on the use of *natalizuma* and *vedolizumab* for the treatment of CD including 8 randomised clinical trials concluded that these antiangiogenic and anti-inflammatory drugs are effective in inducing and maintaining remission in CD patients (Chandar et al., 2015). However, the rare but fatal risk of a progressive multifocal leukoencephalopathy (PML) is a major concern (Hunt and Giovannoni, 2012). PML is a fatal neurological disease caused by John Cunningham virus and has been reported in 124 cases of CD patients treated with *natalizumab* between the years of 2008 and 2011 (Hunt and Giovannoni, 2012).

In addition, preclinical studies using antiangiogenic therapy for the treatment of experimental colitis have not been effective. Initially, Knod *et al* used Anti-VEGFR2 monoclonal antibody and placebo to treat a dextran sodium sulfate model of murine colitis. Although there was a favourable weight change in the treatment group, no improvement in inflammation or decrease in MVD was observed (Knod et al., 2013). Subsequently, using the same model, a different strategy was used targeting two angiogenic pathways: VEGFR and PDGF. In this study, VEGFR expression level has significantly decreased, however, neither PDGF nor angiogenic factors, such as Angiopoietin-2 (Ang-2) and epidermal growth factor receptor (EGFR) were significantly different to control. More importantly, this treatment strategy also failed to decrease the desired outcome of MVD or inflammation (Knod et al., 2016b). It appears that additional angiogenic pathways are activated when certain pathways are blocked. These mechanisms require further understanding, in order to design a multiple pathway target strategy that mediates an appropriate angiogenic response in pathological conditions.

1.5. The role of the mesentery in Crohn's disease pathogenesis

The mesentery has a distinctive anatomical and functional structure that should be subject to the investigatory focus as any other organ (Coffey and O'Leary, 2016). Anatomically the mesentery represents a

fold of the peritoneum which attaches the bowel to the posterior abdominal wall (Culligan et al., 2014). It is formed of AT surrounding the major vessels and their branches that carry the blood supply to the bowel (Culligan et al., 2012). In addition, the mesentery contains connective tissue matrix, nerve tissue, lymphatics and immune cells (Frayn et al., 2003, Bertin et al., 2010). The mesentery has a protective role, as part of a phlegmon, it walls off and contains intraperitoneal pathology, preventing a systemic inflammatory response (Kredel and Siegmund, 2014, Siegmund, 2012).

It has been long recognised that the mesentery in CD patients shows fibrosis, inflammation, thickened lymphatic vessels, infiltration of stromal cells, perineuronal chronic inflammation, engorged vessels, and characteristically small-sized adipocytes (Sheehan et al., 1992, Borley et al., 2000). In the following sections, the current knowledge in the literature of the mesenteric AT in CD will be summarised.

1.5.1. The phenomenon of fat wrapping in Crohn's disease

Fat wrapping phenomenon is pathognomonic to CD. It is described as mesenteric AT extension from the mesenteric attachment to partially or wholly covering the small or large bowel, resulting in a loss of the bowel-mesentery angle (Figure 1.4.). Although not all patients with CD suffer from fat wrapping, there is evidence that the vast majority exhibit a degree of mesenteric disease (Weakley and Turnbull, 1971). One of

the first definitive studies that hold a pathological account of the phenomenon and evaluation of the correlation between fat wrapping and other clinic-pathological features of CD identified 80% and 64% rates of fat wrapping in small and large bowel specimens respectively (Sheehan et al., 1992). In fact, in one study fat wrapping was present in all of the CD specimens in 20 consecutive resections (Borley et al., 2000). Out of 225 small bowel resections for various pathologies, fat wrapping was only identified in patients with CD (Sheehan et al., 1992). Thus, the uniqueness of fat wrapping to CD and the high frequency of its occurrence may indicate a substantial role of the mesenteric AT in CD pathogenesis. It is therefore plausible to further understand the mechanisms attributed to this phenomenon.

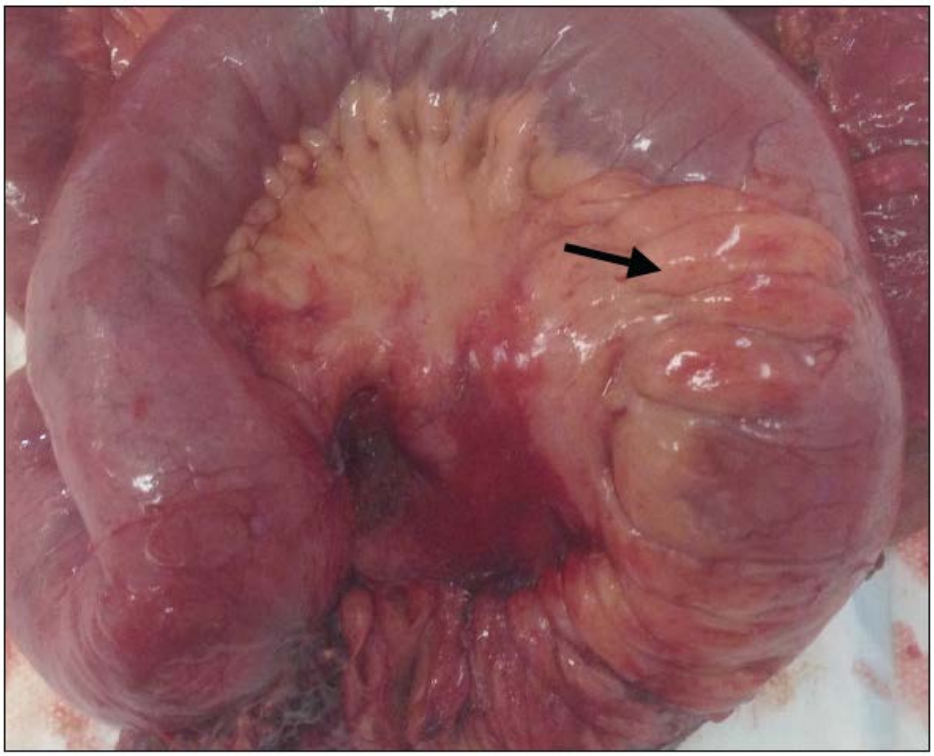


Figure 1.4. Fat wrapping in CD, arrow: note the extension of the mesenteric fat to the bowel wall and the obliteration of the mesenteric bowel angle

Although fat wrapping has been correlated with well-established bowel lesions, particularly transmural inflammation, it is also recognised at early stages of the disease and without apparent bowel lesion. This may suggest that mesenteric AT is implicated in the early aetiopathogenesis of CD. The first description of CD by Crohn in 1932 implies the possibility of early mesenteric involvement (Crohn et al., 1984): "*The mesentery of the terminal ileum was thickened, and containing numerous hyperplastic glands. There were isolated lesions separate from the main pathological mass by normal mucosa. These lesions consisted of oval mucosal ulcerations, about 1cm in diameter, located on the mesenteric border of the small bowel and lying in the long axis, where a groove was naturally formed by the attachment of the mesentery"* (Crohn et al., 1984). Furthermore, using MRI imaging, Desreumaux *et al* (Desreumaux et al., 1999) demonstrated a significantly greater ratio of intra-abdominal fat to total abdominal fat in CD patients in comparison to control. Although Shelley-Fraser *et al* demonstrated a strong correlation between fat wrapping and transmural inflammation, in the form of lymphoid aggregates, using intraoperative endoscopy, Smedh *et al* identified fat wrapping in 14% of CD patients who had macroscopically healthy intestinal mucosa (Smedh et al., 1993). Therefore, the accumulation of fat in the mesentery of patients with CD appears to be an early event and not only an indication of disease severity (Smedh et al., 1993, Desreumaux et al., 1999). These findings provide evidence of early mesenteric involvement in CD.

Thus, the major question here is: “what is distinctive about adipocytes in the mesentery of CD patients?” The current knowledge demonstrates that adipocytes have metabolic and immunologic functions and not only energy storage as was believed previously. Specifically, mesenteric AT in CD patients was found to over express pro- and anti-inflammatory chemokines, adipokines and other cytokines. Increase expression of leptin and adiponectin mRNA as well as their proteins are reported in hypertrophied CD mesenteric tissue (Barbier et al., 2003, Yamamoto et al., 2005). Similarly, pro-inflammatory cytokines such as, interleukin 6 (IL6), tumour necrosis factor (TNF) and monocyte chemoattractant protein-1 (MCP-1) are over expressed and appear to play a role in the inflammatory process in CD (Peyrin-Biroulet et al., 2007). Furthermore, preadipocytes and adipocytes can express various pattern recognition receptors such as TLRs and NOD proteins (Kopp et al., 2010). These cells are also able to differentiate into macrophages with antimicrobial activities (Charriere et al., 2003). These data suggest that the hypertrophied mesenteric tissue in CD is not “*an innocent bystander, but an active player in the intestinal and systemic inflammatory response”* (Drouet et al., 2012). Lack of research focusing on mesenteric AT in CD, may be due to an assumption that it is a result of the transmural inflammation. However, as demonstrated above the literature does not fully support this assumption and complete understanding of the

pathogenic mechanisms surrounding the mesenteric tissue involvement in CD requires further research.

1.5.2. Mesenteric vasculature in Crohn's disease

As described above the mesentery homes the blood vessels that supply the bowel with oxygen and nutrients and dispose of waiste products. Thus, the association of changes mesenteric vasculature and CD is not a surprise. Indeed, patients with CD had reduced vessels diameter, decrease vascular density and diminished blood flow in comparison to controls (Erikson et al., 1970, Brahme, 1966). The dynamic changes in the mesenteric vessels have been a guide for measuring disease activity (Maconi et al., 1996). The velocity of blood flow in the superior mesenteric artery (SMA) was higher in CD than in healthy controls, whereas the resistance index of the SMA was lower in active disease than in control (Bolondi et al., 1992, Kircher et al., 2004). Furthermore, Ludwig *et al* demonstrated a significant correlation between the velocity of blood flow in the SMA, which is an indicator of a hyperdynamic state and CDAI. Lower SMA pulsatility index, a reflection of vascular resistance, was a significant predictor of relapse in CD (Ludwig et al., 1999).

Other indicators of hyperdynamic state in CD is "comb sign", which demarcates prominent pericolic and perienteric vasculature seen on imaging in active CD (Lee et al., 2002). In addition to the evidence of

altered mesenteric vasculature in CD, which is useful for diagnostic purposes, these studies suggest that a potential difference in the angiogenic mechanisms may characterise and play a role in CD pathogenesis.

1.5.3. Angiogenic factors in Crohn's disease mesentery

The ability of AT to secrete important angiogenic factors has been well documented (Rega et al., 2007, Ahima and Flier, 2000a). In terms of proangiogenic cytokines, mesenteric AT in CD expresses VEGF (Schaffler et al., 2006), TNF-α (Desreumaux et al., 1999), IL6 (Zulian et al., 2013) and IL1-β (Hotamisligil et al., 1993) at higher rates in comparison to controls (Table 1.1.). It has been shown that adipocyte mass positively correlates with the level of expression of theses cytokines (Das, 2001). Therefore, the wrapping mesenteric AT in CD is potentially a crucial source of cytokines, and may be responsible for the perpetuation of inflammation (Schäffler and Herfarth, 2005).

Table 1.1. Change in the Expression and/or Levels of Major Angiogenic Factors in mesenteric adipose tissue of Crohn's disease patients

	Angiogenic Effect	Expression/ Levels	References
VEGF	Proangiogenic	Similar	(Schaffler et al., 2006)
TNF-α	Proangiogenic	High	(Desreumaux et al., 1999)
IL6	Proangiogenic	High	(Zulian et al., 2013)
IL1-β	Proangiogenic	High	(Hotamisligil et al., 1993)
Adiponectin	Proangiogenic/ Antiangiogenic	Low	(Rodrigues et al., 2012)
Leptin	Proangiogenic	High	(Jung et al., 2013)

In addition to being highly expressed in the mesenteric AT of CD, TNF-α levels are also elevated in the blood (Murch et al., 1991), mucosa (Breese et al., 1994, MacDonald et al., 1990), and stool (Braegger et al., 1992), suggesting a systemic effect. Although the role of TNF-α in the inflammatory mechanisms of CD have been intensively investigated, its angiogenic effect remains poorly understood. However, in cancer, TNF-α has a strong proangiogenic effect by inducing neovascularization (Tertil et al., 2014). Similarly, the overexpression of IL6 in the mesentery of CD is also associated with elevated levels in the serum (Bertin et al., 2010). Several studies suggested that adipocytes are responsible for approximately 30% of the total level of circulating IL6 (Park et al., 2005). In fact, the serum level of IL6 is a potential marker for the prediction of the disease activity (Reinisch et al., 1999). Patients in remission have low levels in comparison to patients in relapse (Van Kemseke et al., 2000). The angiogenic role of IL6 in CD has not been investigated. However, IL6 is a potent proangiogenic cytokine. It enhances angiogenesis and tubule formation (Hashizume et al., 2009).

Specifically, IL6 up regulates VEGF production. In vitro IL6 treated adipocytes secreted significantly high levels of VEGF (Rega et al., 2007). Only one study to date investigated the level of VEGF secretion in the mesenteric AT in CD patients. Schaffler *et al* incubated mesenteric AT specimens obtained from CD wrapping mesentery or creeping fat, colon cancer and diverticular disease in culture media for

24 hours and compared the concentration of VEGF in the supernatant. They have found similar levels of VEGF secretion between CD and colon cancer patients, but the patients in the diverticular disease group demonstrated significantly lower level of VEGF. Furthermore, mesenteric AT from CD patients who were treated with steroids at the time had significantly lower level of VEGF in comparison to mesenteric AT from patients not on steroids (Schaffler et al., 2006).

As has been explained before the mesentery is mainly made of AT and large number of adipocytes, adipokines have been the focus of previous research. The role of adipokines in the pathogenesis of CD is critical (Schäffler et al., 2005, Schäffler et al., 2006). Two of the most important adipokines namely, adiponectin, and leptin are well known for their important role in inflammation are also strong proangiogenic factors (Adya et al., 2015).

Adiponectin has been known for its anti-inflammatory properties (Trujillo and Scherer, 2005), also shown to induce angiogenesis in endothelial cells via AMP-activated protein kinase-endothelial nitric-oxide synthase (AMPK-eNOS) pathway (Ouchi et al., 2004). Although antiangiogenic potential of adiponectin has been explored in cancer therapy (Man et al., 2010), its role in angiogenesis appears to be critical. For example, low adiponectin level (hypoadiponectinemia) is an independent risk factor for endothelial dysfunction and modulating vessel wall health (Okui et al., 2008). The serum concentration of

adiponectin in CD patients and its mesenteric AT expression have been divergent. Yamamoto *et al* and Paul et al showed significantly higher level of adiponectin level in the mesenteric wrapping fat in CD (Yamamoto et al., 2005, Paul, 2006). Similar results have been shown by another study, in which tissue expression was higher, but serum concertation of adiponectin was similar in comparison to controls (Karmiris et al., 2006). On the other hand, Rodrigues et al showed significantly lower level of adiponectin in the wrapping mesenteric AT and serum of active CD in comparison to controls and CD in remission. The authors contributed these differences to patients' demographics and high variability of adiponectin level identified in the control group (Rodrigues et al., 2012)

Leptin is an adipokine that is involved in appetite regulation, fertility and bone metabolism (Ahima and Flier, 2000b). Yet, one of the important functions of leptin is its role as a proinflammatory factor (Park et al., 2010, Fontana et al., 2007, Jung et al., 2013). Relevant to the immune response in CD leptin modulates the activation of T-cells and promotes its expression of adhesion molecules and production of proinflammatory cytokines (Lord et al., 1998). Specifically leptin stimulates the development of Th1 phenotype, such as IFN-γ, TNF-α, IL6 and IL1 rather than Th2 phenotype (Lord, 2002). In angiogenesis, leptin is a proangiogenic adipokines induces endothelial cell proliferation and stimulates the expression of matrix metalloproteinases (MMPs) (Park et al., 2001). In CD mesenteric AT

leptin is up regulated in the in comparison to colon cancer and diverticular disease (Barbier et al., 2003, Paul et al., 2006b). Interestingly, leptin has a direct effect on mucin production and increase colonic tight junction permeability (El Homsi et al., 2007, Plaisancie et al., 2006, Le Dréan et al., 2014), which contributes to the integrity of the intestinal barrier (Hardwick et al., 2001). Hence leptin deficiency results in an increase bacterial translocation to the blood (Amar et al., 2011). The role of leptin in the angiogenic mechanisms in CD mesentery is yet to be explained.

1.6. The research hypothesis

After explaining the role of the mesentery in the pathogenesis of CD and impact this may have progression, the need to further characterise the mesentery has become apparent. The intimate relationship between angiogenesis and inflammation is also undisputable. For example, major inflammatory cytokines, such as IL6, TNF-α, and IL1-β, known for their profound involvement in CD pathogenesis are also angiogenic factors.

The phenomenon of fat wrapping is shaped by the expansion of mesenteric AT. Like any expanding tissue, the mesentery requires new blood vessels to support the inflammatory mass. It is therefore perceivable to assume an active angiogenic element to the fat wrapping phenomena. To date, there is only one study in the literature

dedicated to assess the level of VEGF secretion by CD mesentery (Schaffler et al., 2006). Considering the complexity of angiogenesis, it is important to understand its mechanisms, in order to ultimately find a cure for CD.

The hypothesis of this work is that angiogenic mechanisms in CD mesentery are dysregulated, leading to the formation of permanent pathological vasculature that perpetuates the inflammatory process.

1.7. Aim and objectives

The aim of this research is to identify angiogenic therapeutic targets for the treatment of CD. The objectives can be summarised as follow:
In tissues from patients:

1) Is there a difference in the morphology of the vasculature between CD mesenteric AT and controls?
2) Is there a difference in the level of angiogenic gene expression of the CD mesenteric AT in comparison to controls?
3) Is there a difference in the level of angiogenic protein expression between CD mesenteric AT and controls?

In in-vitro culture assays:

4) Is there a difference between CD mesenteric AT and controls in their capacity to secret angiogenic factors in vitro?
5) Is there a difference between CD mesenteric AT and controls in their capacity to produce vascular sprouting in vitro?

In blood samples from patients:

6) Is there a difference in level of angiogenic factors in the serum of CD patients in comparison to controls?

Chapter 2

Methods

2.1. Introduction

This chapter will describe how the study was designed and carried out and how the data was analysed. Patient characteristics, recruitment, ethical approval and the design for the five experiments that were carried out to investigate the hypothesis of this research will be explained.

2.2. Study population

2.2.1. Patient recruitment

Patients undergoing bowel resection involving the ileocaecal mesentery for CD (n=23) and control (n=14) were recruited to the study. All patients received treatment at the University College London Hospital during the period from November 2012 until August 2016. The patients were identified at the weekly multidisciplinary meeting (MDT) and the theatre-operation list. Patients were approached prior to surgery and written informed consent to take part in the study was obtained.

2.2.2. Inclusion criteria

1. Age >14 and <85 years

2. Patients with the diagnosis of IBD or any other benign bowel condition, such as diverticular disease and funtioanl bowel disorders
3. Surgical resection includes the ileocaecal region
4. Patients with capacity to consent to take part in the study

2.2.3. Exclusion criteria

1. Age <14 or above 85 years
2. Patients suffering from or having a history of cancer
3. Patients with acute surgical abdomen
4. Patients who are unable to give informed written consent

2.2.4. Ethical approval

Ethical approval was obtained from The National Research Ethics Service Committee (London-Hampstead) (10/H0722/69) and the Joint Research Office at the University College London and University College Hospital. Minor protocol amendment was obtained in March 2013. Good medical practice guidelines were followed during patient recruitment and sample collection, storage and processing.

2.2.5. Blood and tissue collection

A blood sample was taken from the patient by the anaesthetist at the time of inserting a venous cannula before the induction of anaesthesia. Surgical specimens of approximately 1cm cube size were collected at

the time of surgery and included: ileocaecal mesenteric, omental, subcutaneous AT from CD and controls and wrapping mesenteric AT from CD patients. Additionally, full thickness bowel specimen was retrieved. The tissue specimens were collected in three methods: 1) snap frozen in liquid nitrogen immediately after excision; 2) additional AT samples were immersed in a sterile cellgro culture media (Cellgro, Mediatech Manassas, VA); 3) additional AT samples were immersed in formaldehyde for histopathological analysis. All samples were immediately transported to the laboratory.

Blood samples were centrifuged (3000 rpm,15 minutes, 25oC), the serum was collected aliquoted and stored at -80 ^{o}C until analysis. Tissue samples aliquoted immediately upon excision from the patient, labeled and stored at -80 ^{o}C. AT transported in cellgro (Cellgro, Mediatech Manassas, VA) were prepared for tissue culture and processed immediately.

2.3. Histopathology and immunohistochemistry

2.3.1. Haematoxylin and eosin (H&E) staining

Formaldehyde fixed ileocaecal mesenteric, omental and subcutaneous AT from CD and controls; and wrapping mesenteric AT from CD patients were studied after conventional haematoxylin and eosin (H&E) staining. Pieces of 0.3 g AT were fixed in 10% formalin

for 24 hours' room temperature and then transferred to 50% ethanol at 4 ^{o}C prior to being embedded in paraffin. 3 μm sections were deparaffinised and dehydrated in Xylene (Sigma-Aldrich, UK) for 20 min, followed by 100, 90, 80, 70, and 60% ethanol. The slides were then stained by Haematoxylin to define cellular constituents of tissue. This was then differentiated in acid/alcohol to give sharp nuclear stain. Then the slides were plunged into water and counterstained with eosin. AT slides were again dehydrated via various strengths of alcohol and finally washed with xylene.

2.3.2. Immunohistochemistry staining

Blocks were sectioned at a thickness of 4 μm. Buffer was used for antigen retrieval. Several primary antibodies were used to quantify, inflammation, fibrosis, microvascular density and the level of angiogenic factors including; hypoxia inducible factor (HIF)-1α; and vascular endothelial growth factor (VEGF). The primary antibodies that were used are: CD68 (clone PG-M1, Dako), α3 chain of the human collagen VI was used (Abcam, Paris, France); CD31 antibody (DakoCytomation; Glostrup, Denmark); CD34 antibody (Immunotech; Marseilles, France); VEGF antibody (Sigma Chemical, St. Louis, MO); and HIF-1α antibodies (Hi alpha 67 – Novus Biologicals, Littleton, CO). Dilution of the primary antibodies was done as per manufactures' recommendations. Various primary antibodies were diluted in 2.5% goat serum in PBS/T (phosphate buffered saline with 0.05% Tween

20), and incubated overnight. Subsequently, the slides were rinsed in PBS/T, and the respective secondary antibodies were added. A biotinylated goat vs. mouse secondary antibody (Jackson Immuno Research Labs, West Grove PA) was applied for 15 minutes and followed by Elite ABC reagent (Vector Labs, Burlingame, CA) for 20 min. The amplification reagent from the Catalyzed Signal Amplification (CSA) System (Dako) was used next. Streptavidin conjugated to horseradish peroxidase (Zymed/Invitrogen, San Francisco, CA) was used for 20 minutes. This was the last layer. Consequently, in order to visualize the immunoreaction, the slide racks were removed from the machine and the chromogen DAB (Dako) was applied for only 1 minute. All washes between the various steps were done with PBS/T (Tóth et al., 1994).

2.3.3. Microscopic analysis

Histopathological evaluation of all AT for signs for oedema, lymphoid aggregates, granulomas, and macrophage infiltration were examined microscopically. The following criteria was used for the examination of the histopathological sections as described previously (Borley et al., 2000):

1. Lymphoid aggregates were defined as pathological lymphoid cell clusters visible as distinct aggregates and counted for the entire area studied at 40x magnification

2. Granulomata were defined as identifiable macrophage cell-related lesions counted for the entire area studied at 100x magnification
3. Macrophages were quantified using immunohistochemical staining for CD68
4. Fibrosis was quantified using immunohistochemical staining for α3 chain of the human collagen VI

2.3.4. Microvascularity

The proportion between the vascular and non-vascular areas was calculated, and assessment of AT microvascularity was made after CD31 & 34 staining using the Chalkley point method (Salvato, 2001). In brief, three microvessel "hotspots" were chosen in each section and vessel density counted (Chalkley, 1943). Vessel architecture (tortuosity, calibre changes) was also assessed in an ordinal manner as absent, mild, moderate and severe (Thornton and Solomon, 2002). Sections were stained with CD31, and CD34 antibodies to assess vascular density, morphology and vascular alterations. Angiogenic factors including, VEGF and HIF-1α were quantified by immunohistochemical staining.

2.4. Angiogenic gene expression

This section aims to describe the 3 experiments designed to measure angiogenic gene expression. Total RNA was extracted from the AT and used for two PCR experiments: 1) RT^2 profiler PCR angiogenic gene arrays; and 2) The expression of selected angiogenic gene using a Custom RT^2 profiler PCR angiogenic gene arrays.

2.4.1. RNA extraction:

Approximately 0.15 grams of AT was ground in liquid nitrogen using a pestle and mortar. AT was powdered and homogenised in TRIzol reagent (Invitrogen, Life Technologies, UK). Chloroform was added and mixed thoroughly (0.2 ml chloroform per 1ml TRIzol). The mixture was centrifuged (3200 rpm, 15 minutes, 4 ^{o}C), in order to separate the mixture into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The latter contains the total RNA. This was pipetted carefully and transferred into a new eppendorf. Equal volume of 100% isopropanol was added to the RNA solution and mixed gently. The sample was then incubated at -20 ^{o}C for several hours; a minimum of 2 hours was allowed. After another round of centrifugation (3200 rpm, 15 minutes, 4 ^{o}C), the RNA pellet was formed on the bottom of the eppendorf. The pellet was washed twice in 75% ethanol for molecule biology (Sigma-Aldrich, UK); nuclease-free water (Invitrogen, Life Technologies, UK) and dissolved in 40 μl

nuclease-free water (Chomczynski and Sacchi, 1987, Chomczynski and Sacchi, 2006).

The purity and concentration of total RNA were determined spectrophotometrically (NanoDrop-1000 Spectrophotometer, Thermo Scientific, USA). The concentration of RNA was measured as absorbance at 260 nm. The purity was determined by the ratios between 260 nm and 280 nm (260/280) and between 260 nm and 230 nm (260/230). 260/280 and 260/230 ratios within 1.7-2.0 were accepted purity for RNA.

2.4.2. cDNA synthesis

RT^2 First Strand cDNA Synthesis kit was used to prepare the cDNA from 400 ng total RNA (Qiagen Ltd, UK) according to manufacturer's instructions. In brief, genomic DNA elimination mix was prepared (RNA, Buffer GE, and RNas-free water) and gently mixed with RNA samples. The total mixture was incubated for 5 minutes at 42 ^{o}C, then placed immediately on ice for 1 minute. In the mean time, the reverse-transcription mix (5x Buffer BC3, Control P2, RE3 Reverse Transcription Mix and RNas-free water) was prepared. For each reaction, a total of 10 μl Genomic DNA elimination mix was added to 10 μl reverse-transcription mix and incubated at 42 ^{o}C for exactly 15 minutes. Then immediately incubated at 95 ^{o}C for 5 minutes to stop

the reaction. Each reaction was subsequently mixed with 91 μl RNase-free water and placed on ice ready for real-time PCR.

2.4.3. PCR Angiogenesis Array

Human RT^2 Profiler PCR angiogenesis array and Custom RT^2 Profiler PCR array plates were carried out according to the manufacturer's instructions (Qiagen Ltd, UK). In brief, RT^2 SYBR Green Mastermix was centrifuged for 10-15 seconds before being mixed with the cDNA and RNase-free water to create a total volume of 1300 μl for each reaction. A total volume of 10 μl of the PCR components mix was added to each well in the RT^2 Profiler and Custom RT^2 Profiler Arrays plates. Cycling condition for PCR was one cycle at 95 ^{o}C for 10 minutes to activate HotStart DNA Taq Polymerase, followed by 40 cycles at 95 ^{o}C for 15 seconds each, and finally one cycle 60 ^{o}C in ABI Prism 7900HT Sequence Detection System (Applied Biosystems, UK).

Web based data analysis aided by Microsoft excel spreadsheet containing algorithms provided by the manufacturer to calculate the $^{\Delta\Delta}$Ct (Qiagen Ltd, UK). Furthermore, fold-changes were calculated using the same web based analysis as recommended by the manufacture, all analysis were performed using the online on line software (www.SABiosciences.com/pcrarraydataanalysis.php).

2.5. Angiogenic protein expression

The levels of IL6 and VEGF were measured at several levels. Firstly, total protein was extracted from the tissue samples to subsequently measure the tissue expression of IL6 and VEGF. Secondly, the capacity of tissue to secrete these proteins was measured using tissue explant culture assay. Finally, serum levels of IL6 and VEGF were compared.

2.5.1. Protein extraction from tissue samples and quantification

Protein lysate was prepared by using ~150 mg homogenized tissue samples in 500 μl RIPA buffer (Sigma-Aldrich, UK). The total protein concentration was estimated using bicinchoninic acid (BCA) protein Assay (Novagen, EMD Chemicals, CA, USA). Briefly, the standard curve was built by using diluted bovine serum albumin as the standard (Sigma-Aldrich, UK, concentration: 0, 25, 125, 250, 500, and 1000 μg/ml). 25 μl standards and samples were loaded into a 96-well plate followed by 200 μl BCA working reagent (1 portion of 4% Cupric Sulfate plus 50 portions of BCA solution). The plate was incubated at 37 ^{o}C for 30 minutes. Cu^{2+} was reduced to Cu^{1+} by protein in alkaline solution and then reacted with BCA producing a purple colour with absorbance at 562 nm (Smith et al., 1985) in proportion to the protein concentration.

2.5.2. Explant adipose tissue culture

In order to measure AT capacity to secrete proteins, samples were freshly incubated with Cellgro 0.01 g AT minced using micro scissors and incubated in 500 μl Cellgro serum-free media (Mediatech Manassas, VA) with 5% penicillin-streptomycin for a total of 36 hours (37 ^{o}C, 95% O_2, 5% CO_2). Following incubation, the medium was collected and stored at -80 ^{o}C until later assessment of angiogenic protein levels.

2.5.3. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL6 and VEGF was measured by ELISA in three different preparations: 1) total protein extracted from bowel tissue and AT; 2) explant AT culture supernatant; and 3) serum. The concentration of IL-6 was measured using Quantikine Human IL-6 ELISA Kit (R&D Systems, Minneapolis, MN, USA) and VEGF measured using the Quantikine Human VEGF Immunoassay ELISA kit (R&D Systems, Inc. Minneapolis, MN, USA) according to manufacture protocol. Explained here briefly, the standard solutions for standard curve were first prepared by diluting down the standard solution as per protocol. Total protein bowel or AT extraction, supernatant or serum were added to the 96 wells plate with adhesive strip, and was left to incubate for 2 hours at room temperature. After incubation, each well was washed 4 times with wash buffer solution,

ensuring complete removal of liquid between each wash using aspirating and blotting against a clean paper towel. Then conjugate was added to each well, covering with adhesive strip and incubated for one hour at room temperature. After the second incubation, the identical wash technique described before was applied. This was followed by adding substrate solution to each well, which consisted of 1:1 ratio of colour reagents A and B. The plate was again sealed with an adhesive strip and incubated for 30 minutes at room temperature in a draw, protected from light. Stop solution was finally added and colour change from blue to yellow was observed. The optical density of each well was tested using microplate reader (Opsys MR, Dynex) set at the recommended wave length by the manufacture.

2.6. Ex-vivo angiogenesis assay

To assess the angiogenic capacity of mesenteric AT in CD, matrigel assay was used. The ability of AT to sprout capillaries was compared against control tissue. This section will describe the method used to create the matrigel assay, the quantification of vascular sprouting, and the immunofluorescent staining performed to demonstrate that the vascular sprouting is positive for vascular endothelial and smooth muscle markers.

2.6.1. Matrigel assay

Under a sterile environment, ~1mm of each corresponding AT was cut by micro scissors and embedded in an individual well of a 96-well plate (Ibidi, µ-plate 96 well, uncoated, sterile) (Thistle Scientific Ltd, UK) containing 100 µl of growth factor depleted Matrigel (BD Matrigel^{TM}) (VWR International Ltd, UK). Wells were filled with 200µl of endothelial cell basal media (EBM-2) (Lonza, Clonetics® EBM-2®) supplemented with vascular endothelial growth factors and hydrocortisone (VWR International Ltd, UK). The plate is incubated at 37 ^{o}C, 95% O_2, and 5% CO_2. Every alternative day 100µl of the media was changed. Live video imaging was undertaken for 12 days (100X magnification), showing the number of branches forming around the periphery of the tissue (Figure 2.1). Subsequently, by using ImageJ software the ratio of vessels to AT was measured.

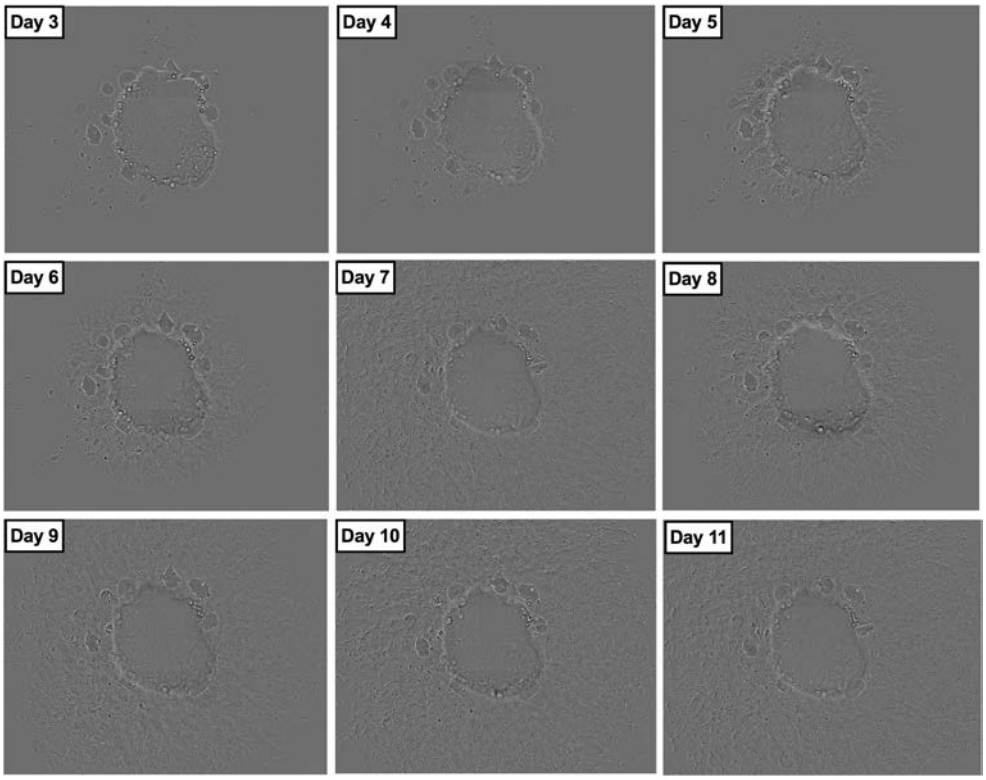


Figure 2.1. Vascular sprouting, live video, progression of growth is shown daily, until day 11

2.6.2. Immunofluorescent staining

On day 12, AT explants embedded in Matrigel were fixed in 4% formaldehyde and stained with primary mouse antihuman antibodies to the following: anti-alpha smooth muscle actin antibody (VWR International Ltd, UK); and endothelial marker anti-eNOS antibody (Sigma-aldrich, UK). In brief, formaldehyde was removed and the matrigel culture was permeabilise with a mixture of PBS, $CaCl_2$, $MgCl_2$ and 0.25% Triton X-100 for 15 minutes at room temperature twice. Block using 1 drop of 2x casein buffer per well was then added for 30 minutes at room temperature. Diluted antibodies according to the

manufacture recommendation was subsequently added and the plate was incubated over night at 4 ^{o}C. On the second day, the wells were washed three times in PBS and 0.1% Triton X-100, with 15 minutes incubation at a room temperature between washes. The secondary antibodies (Abcam, UK) were then added and incubated for 2-3 hours at room temperature.

Following staining with secondary antibodies, pictures were taken using confocal microscopy (Figure 2.2).

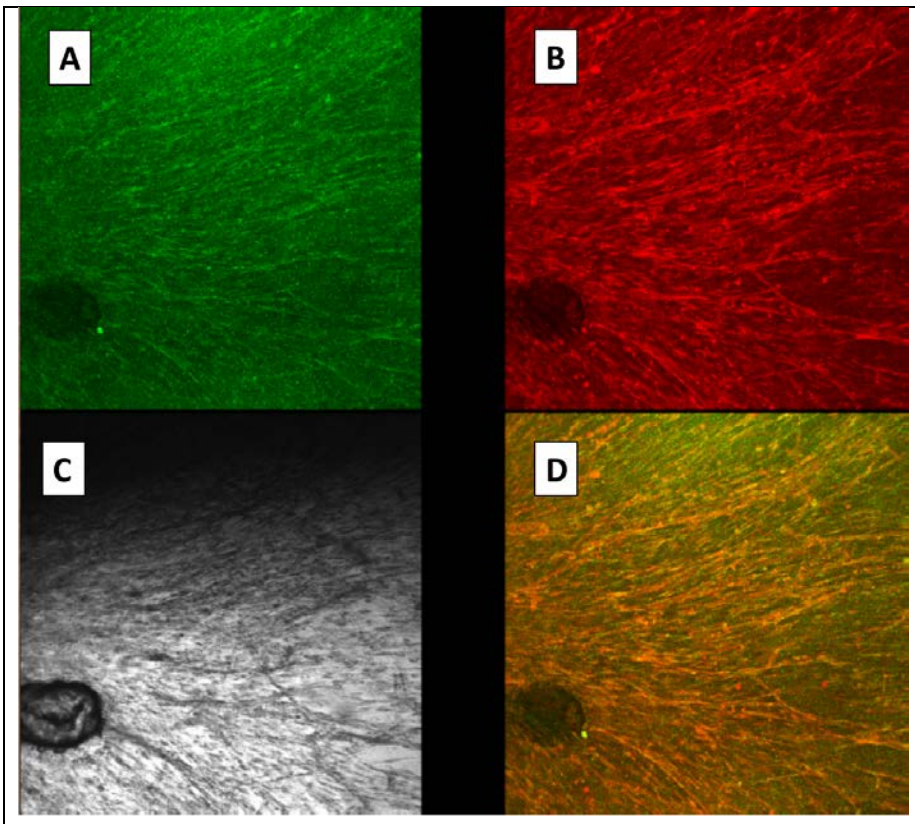


Figure 2.2. Immunofluorescent staining of matrigel assay vascular sprouting: A) anti-eNOS (endothelial marker, green channel); B) anti-alpha smooth muscle antibody (red channel); C) No fluorescence; D) red and green channels.

2.7. Sample size calculation and statistical analysis

Descriptive and inference statistics were applied to demonstrate the most important findings as appropriate. Variables were tested for normality of distribution when applicable using D'Agostino-Pearson omnibus test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. Consequently, means or median were used as appropriate. Post hoc sample size calculated to assess power was used when applicable. For this, ANOVA repeat measures or t-test were used as appropriate. For continuous variables, paired t-test, unpaired t-test, ANOVA, parametric or non parametric tests were used as appropriate. Chi-square and Fisher's exact test were used to analyse categorical data when applicable. Correlation was anlysed using Pearson correlation coefficients for parametric or Spearman correlation for nonparametric data. Furthermore, associations between independent and dependent variables were examined by logistic regression analysis. Statistical Package for the Social Sciences® (IBM SPSS Statistics for Macintosh, version22, Armonk, NY: IBM Corp.) and GraphPadPrism (GraphPad Prism version 6 for MAC OSX, GraphPad Software, SanDiego, CA, www.graphpad.com) were used. The level of statistical significance was set at 5% ($p < 0.05$) for all test procedures.

Chapter 3

Patients' characteristics and postoperative outcome

3.1. Introduction

Despite the effectiveness of medical therapy, and in particular the progress achieved in the potency of biological immunomodulatory drug treatments over the past decade (Cummings et al., 2008), surgical treatment for CD remains an important option (Scarpa et al., 2015). It is estimated that 70-90% of CD patients will still require surgery at some point in their lives notwithstanding medical treatment (Canin-Endres et al., 1999, Mekhjian et al., 1979b). There are several indications for surgery in CD patients, including acute and chronic complications, such as toxic colitis, megacolon, perforation, haemorrhage, fistula, obstruction, stricture, carcinoma and in those who failed medical therapy.

All the patients who participated in this study underwent surgical resection, and excised tissues were used for specific investigations described in this thesis. Due to the diversity of indications for surgical resection in CD and the different diagnoses for controls, there is a potential for clinical and pathological heterogeneity of the study participants. Therefore, it is important to accurately define patients' characteristics. This will allow the identification of confounding factors that may manifest from the nature of their condition, immunosuppressive therapy, systemic inflammatory state and/or their basic characteristics. This is also an opportunity to report the outcome of patients who underwent bowel resection for CD and other benign conditions. Finally, precise pathological characterization may also

inform outcomes of the experimental investigations undertaken in later chapters. To this end, this chapter is dedicated to describes patients' demographics, preoperative, operative and postoperative details. The chapter is divided into three main sections: 1) preoperative parameters; 2) intraoperative details; and 2) postoperative parameters.

3.2. Preoperative parameters

This section addresses: 1) basic characteristics describing age, gender, race, body mass index (BMI), American Society of Anesthesiologist (ASA) score, and preoperative blood results; 2) immunosuppressive therapy; 3) diagnosis; and 4) CD activity.

3.2.1. Basic characteristics

Table 3.1 summarises patients' demographics and preoperative details. The age was similar between CD (mean=34 years, SD=16) and control (mean=39 years, SD=16). There were similar ratios of males and females in the two groups: disease group included 10 (43%) males and 13 (57%) females; and control group included 6 (43%) males and 8 (57%) females. There were more Caucasian patients, 23 (92%) in the disease group, than the controls, 9 (64%). Furthermore, there were more patients from African and Indian origins in the control group, 2 (14%) and 3 (22%) respectively. In comparison,

there were 2 (8%) patients from African origin and none from Indian origin in the disease group. The difference in ratios between the groups was statistically significant ($X^2_{(2)}$=6.5, p<0.05) (Figure 3.1). BMI was similar between CD (mean=24, SD=6) and control (mean=24, SD=5) patients. Patients also had similar ASA score: there were 19 (83%) and 12 (86%) patients with ASA score of II, in the disease and control groups respectively; and 4 (17%) and 2 (14%) patients with ASA score of III, in the disease and control groups respectively.

In terms of biochemical blood markers, only C-reactive protein (CRP) blood levels were significantly (p<0.05) higher in the disease (mean=54 mg/L, SD=66) than control (mean=17 mg/L, SD=25) patients (Figure 3.2). Haemoglobin (mean=13 g/L, SD=2) and WCC (mean=10 x10^9/L, SD=4) levels were identical in both groups. Hematocrit level was also similar in the disease (mean=38%, SD=5) and control (mean=38%, SD=6) patients. Albumen levels were similar in the disease (mean=40 g/L, SD=8) and control (mean=39 g/L, SD=9) patients.

Table 3.1. Patients preoperative characteristics

	Disease (*n*=23)	Control (*n*=14)
Age (SD)	34 (16)	39 (16)
Gender:		
Male	10 (43%)	6 (43%)
Female	13 (57%)	8 (57%)
Race:*		
Caucasian	21 (92%)	9 (64%)
African	2 (8%)	2 (14%)
Indian	0	3 (22%)
BMI (SD)	24 (6)	24 (5)
ASA score:		
II	19 (83%)	12 (86%)
III	4 (17%)	2 (14%)
Haemoglobin: g/L (SD)	13 (2)	13 (2)
Hematocrit: % (SD)	38 (5)	38 (6)
WCC: 10^9/L (SD)	10 (4)	10 (4)
CRP:* mg/L (SD)	54 (66)	17 (25)
Albumen: g/L (SD)	40 (8)	39 (9)
Immunosuppressive therapy:* **		
No	3 (13%)	10 (71%)
Yes	20 (87%)	4 (29%)
Diagnosis	Ileocaecal Crohn's (n=20) Ileocolonic Crohn's (n=1) Colonic Crohn's (n=1) Terminal ileal Crohn's (n=1)	Benign polyp (n=2) Congenital anorectal malformation (n=1) Diverticular disease (n=2) Functional colonic disease (n=5) Ulcerative colitis (n=4)

ASA: American Society of Anesthesiologists. BMI: Body Mass Index. WCC: White Cell Count. CRP: C-reactive protein. SD: Standard Deviation. *p<0.05. ***p<0.001.

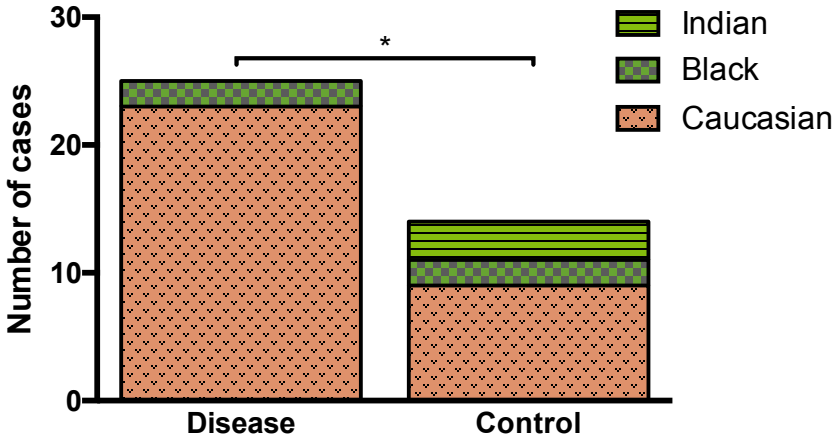


Figure 3.1. Racial origin comparing ratios between disease and control groups. *p<0.05.

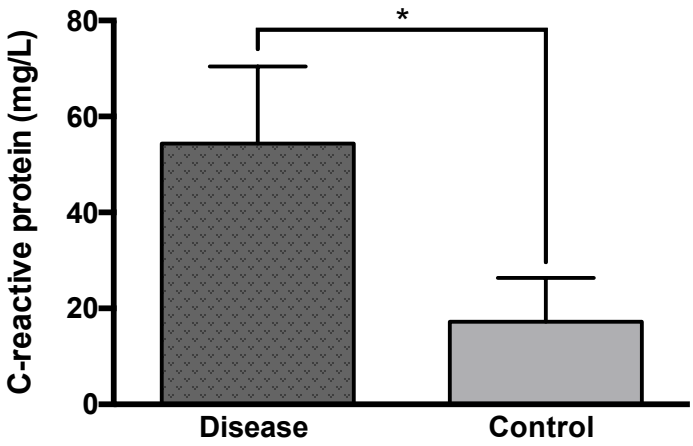


Figure 3.2. C-reactive protein (CRP) level in disease and control patients. *p<0.05.

Table 3.2. Preoperative biochemical markers levels in the blood

	Disease: mean (SD) (*n*=23)	Control: mean (SD) (*n*=14)	*p* value
Urea: (mmol/L)	4.9 (1.7)	4.6 (6)	0.85
Creatinine: (µmol/L)	66 (23)	76 (19)	0.22
EGFR: (mls/min/1.73m^2)	86 (9.8)	79 (12)	0.08
Sodium: (mmol/L)	139 (4)	140 (2.5)	0.38
Potassium: (mmol/L)	4.3 (0.5)	4 (0.4)	0.06
Calcium (total): (mmol/L)	2.3 (0.2)	2.3 (0.2)	-
Albumen: (g/L)	40 (9)	40 (8)	0.7
Bilirubin: (µmol/L)	11 (11)	7.4 (4)	0.3

EGFR: Estimated Glomerular Filtration Rate. SD: Standard Deviation

The levels of other biochemical markers including: creatinine; EGFR; sodium; potassium; calcium; albumen; and bilirubin were similar in CD and control patients. Table 3.2 summarises the means, standard deviations and p values for these variables.

3.2.2. Immunosuppressive therapy

There were significantly more patients on immunosuppressive therapy in the disease group (*n*=20, 87%) than in the control (*n*=4, 29%) ($X^2_{(1)}$=13, p<0.001) (Figure 3.3). Table 3.3 summarises the immunosuppressive therapy in CD and control groups. Of the 20 patients with CD on immunosuppressive therapy 12 (60%) were on combination of two or more immunosuppressive therapy including: adalimumab, azathioprine, budesonide, infliximab, mercaptopurine, mesalazine, methotrexate, and prednisolone. The remainder 8 (40%) patients were on monotherapy: two on adalimumab, two azathioprine, two on budesonide, one on methotrexate, and one on prednisolone. In the control group, one patient with UC out of four patients (25%) on immunosuppressive therapy were on a combination of three drugs: azathioprine, infliximab and methotrexate. The other three patients were on monotherapy (75%): one on mesalazine and two on prednisolone ($X^2_{(1)}$=1.65, p=0.20) (Table 3.3).

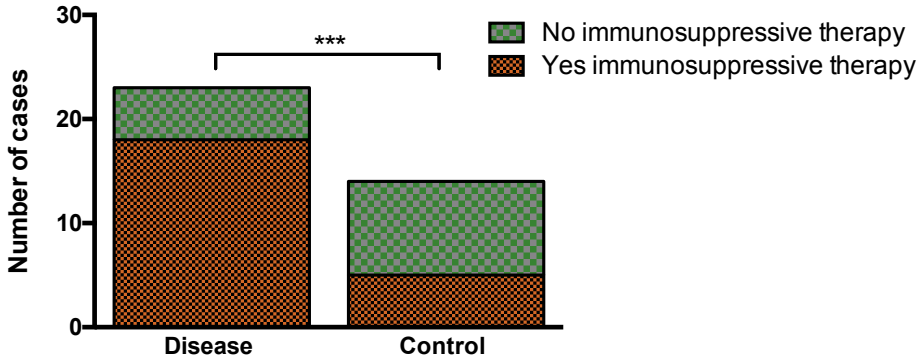


Figure 3.3. The ratios for patients receiving immunosuppressive therapy at the time of surgery in the disease and control groups. ***p<0.001

Table 3.3. Immunosuppressive therapy

	Disease (n=20)		Control (n=4)	
†Multiple therapy	12 (60%)		1 (25%): azathioprine, infliximab, and methotrexate	
Monotherapy	8 (40%)		3 (75%)	
	Drug	Number of patients	Drug	Number of patients
	adalimumab	2	mesalazine	1
	azathioprine	2	prednisolone	2
	budesonide	2		
	methotrexate	1		
	prednisolone	1		

†Two or more of the following drugs: adalimumab, azathioprine, budesonide, infliximab, mercaptopurine, mesalazine, methotrexate, and prednisolone. Crohn's disease patients in grey highlights.

3.2.3. Diagnosis details

The overwhelming majority of CD patients were diagnosed with ileocaecal disease (n=20, 87%). The remaining 3 patients had the disease affecting the ileocolonic; isolated colonic; and isolated terminal ileum respectively (Table 3.1). In the control group, there were 5 patients diagnosed with functional colonic disease and required subtotal colectomy: 3 of which were diagnosed with EDS, and 2 with chronic constipation. Furthermore, there were 4 patients with established diagnosis of UC, 2 with benign colonic polyps, 2 with diverticular disease of the right colon and 1 with congenital anorectal malformation of the colon (Table 3.1).

3.2.3.1. Histopathological diagnosis of Crohn's disease patients

Table 3.4 summarises the histopathological findings of tissue samples in CD patients (*n*=23). Active chronic transmural inflammation was described in 21 (91%) bowel tissue samples. The remainder 2 (9%) of the bowel samples were in remission with no features of active inflammation. There was transmural abscess found in 5 (22%) of the bowel tissue samples. Stricture formation was identified in 10 (43%) patients, and this correlated significantly ($p<0.001$) with the pain score obtained from the CDAI ($r=0.68$, 95% CI [0.38,0.85]) (Figure 3.4). Fistula formation was diagnosed in 7 (30%) of the patients, whereas serosal fibrosis was identified in all cases. The majority 21 (91%) of

the samples featured lymphoid aggregates, and 18 (78%) demonstrated reactive lymph nodes in the mesentery. In 2 cases the resection margin contained inflammation, but these patients had no recurrence after 28 and 24 months follow up. Mesenteric thickening and fat wrapping was confirmed in the specimens of 22 (96%) patients.

There was no significant correlation between the pathological findings reported for the bowel specimens and the blood results. For examples, there was no significant correlation between the active inflammation, intramural puss, stricture formation, WCC, or CRP.

Table 3.4. Histological findings in Crohn's disease tissue (*n*=23)

	Yes	No
Active transmural inflammation	21 (91%)	2 (9%)
Intramural abscess	5 (22%)	18 (78%)
Stricture formation	10 (43%)	13 (57%)
Fistula formation	7 (30%)	16 (70%)
Serosal fibrosis	23 (100%)	-
Lymphoid aggregates	21 (91%)	2 (9%)
Reactive lymph nodes	18 (78%)	5 (22%)
Disease at the resection margin	2 (9%)	21 (91%)
Mesenteric fat wrapping	22 (96%)	1 (4%)

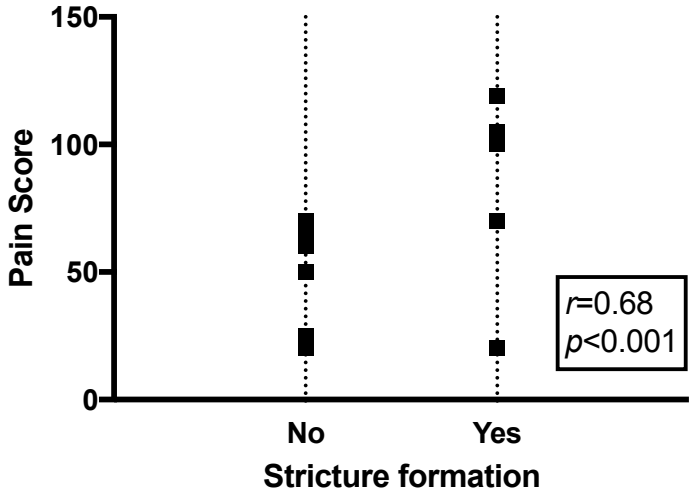


Figure 3.4. There is a significant positive correlation between stricture formation on pathology and the patients' pain score obtained from Crohn's disease activity index (CDAI)

3.2.3.2. Histopathological diagnosis of ulcerative colitis

Out of 14 patients in the control group, 5 (36%) were diagnosed with UC. This is important to characterise as patients with UC can have similar biochemical markers as CD. However, UC is not know to affect the mesentery. The diagnosis of UC, was confirmed on the pathological specimens to be severe active in 3 patients and moderately active in 2 patients. All colonic samples showed focal active inflammation in the form of cryptitis and occasional crypt abscess formation, features diagnostic for UC. No fissuring ulcers, granulomas or dysplasia was demonstrated.

3.2.4. Crohn's disease activity index

Based on CDAI scores, there were 7 (31%), 6 (26%), 9 (39%), and 1 (4%) patients in remission (<150), mildly active (150-219), moderately active (220-450), and severely active (>450) disease, respectively (table 3.4). The level of CRP was significantly positively correlated with CDAI score (r=0.45, 95% CI [0.05,0.73], p<0.05) (Figure 3.5). There was a significant positive correlation between WCC and CDAI score (r=0.48, 95% CI [0.1,0.7], p<0.05) (Figure 3.6, A). Specifically, the neutrophil count was significantly positively correlated with CDAI score (r=0.48, 95% CI [0.08,0.7], p<0.05) (Figure 3.6, B). Furthermore, the CDAI score was significantly negatively correlated with haemoglobin blood levels (r=-0.5, 95% CI [-0.8,-0.1], p<0.05) and haematocrit blood levels (r=-0.5, 95% CI [-0.8,-0.1], p<0.05) (Figure 3.6, C and D).

The pathological specimens of CD patients demonstrated 7 (31%), 6 (26%) and 10 (43%) of features consistent with mildly, moderately and severely active disease respectively. The severity of the disease on the histopathology was significantly positively correlated with the CDAI score (r=0.8, 95% CI [0.6,0.9], p<0.0001) (Figure 3.7).

Table 3.5. Crohn's Disease Activity Index (CDAI) scores (*n*=23)

Disease in remission, CDAI: <150	7 (31%)
Mildly active disease, CDAI: 150-219	6 (26%)
Moderately active disease, CDAI: 250-450	9 (39%)
Severe actively disease, CDAI: >450	1 (4%)

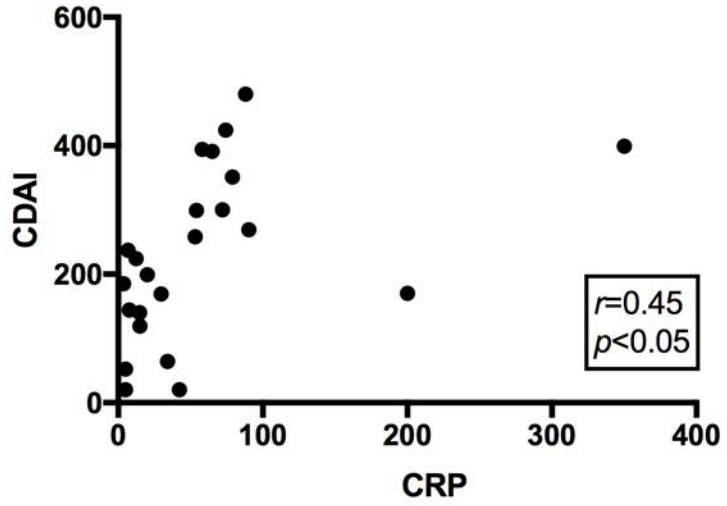


Figure 3.5. Significant positive correlation was found between Crohn's disease activity index (CDAI) and C-reactive protein (CRP)

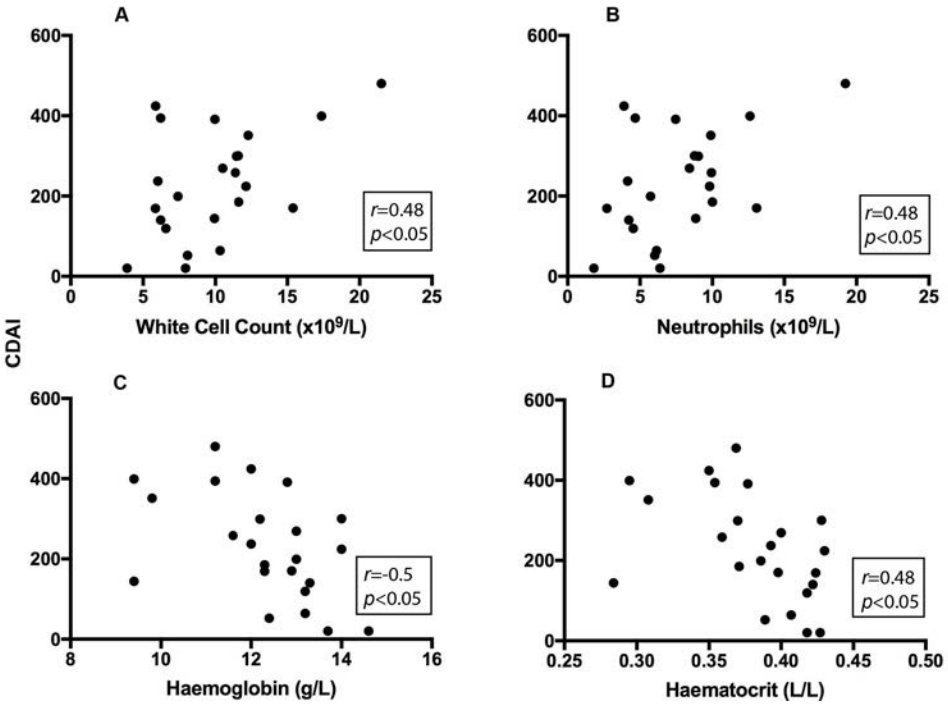


Figure 3.6. Significant correlation between Crohn's Disease Activity Index (CDAI) (*n*=23) and various biochemical markers. White cell count significantly positively correlates with CDAI (A). Neutrophils count significantly positively correlates with CDAI (B). Haemoglobin blood level significantly negatively correlates with CDAI (C). Haematocrit blood level significantly negatively correlates with CDAI (D)

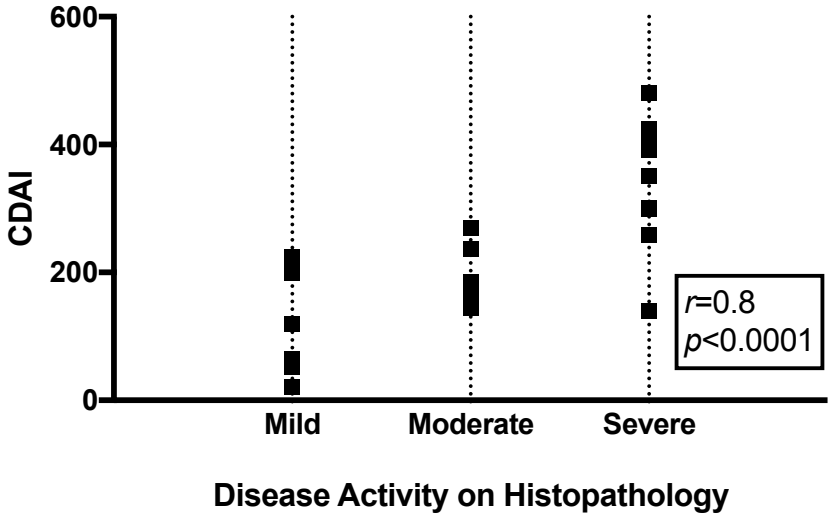


Figure 3.7. Significant positive correlation was found between the disease activity on the histological specimen of the bowel and Crohn's Disease Activity Index (CDAI) score

3.2.5. Logistic regression analysis

Several variables were found to significantly correlate with CDAI as demonstrated above. Therefore, to predict CDAI from other variables can be achieved by logistic regression models. For continuous variables, such as WCC, neutrophils, haemoglobin, haematocrit and CDAI univariate and multivariate linear logistic regression models are appropriate. Furthermore, the severity of bowel inflammation can be predicted by binary ordinal logistic regression models.

3.2.5.1. Predicting Crohn's disease activity index (CDAI) from blood results

Except for CRP, all other variables including WCC, neutrophils, haemoglobin, haematocrit and CDAI were normally distributed when tested for normality using Kolmogorov-Simirnov and Shapiro-Wilk tests. Univariate linear logistic regression analysis was performed on individual independent variables including: WCC, neutrophils, haemoglobin and haematocrit. CDAI was considered the dependent variable. The model produced statistically significant coefficients for all the variables (Table 3.6). This means that a unit increase in WCC ($x10^9$/L) can predict an increase of CDAI by 15.5 points. Similarly, a unit increase in the neutrophils count ($x10^9$/L) or CRP (mg/dL) serum level can predict 16.2 or 0.8 points increase in the CADI score respectively. On the other hand, a decrease in haemoglobin (g/L) and haematocrit (1%) levels can predict an increase in CDAI score of 47.5 and 15.5 respectively (Table 3.6).

Except for the CRP level, multivariate linear regression of WCC, neutrophils, haemoglobin, and haematocrit to predict CDAI produced large multi-collinearity values exceeding 5. Following several variations analyses of these variables for muti-collinearity, CRP and haematocrit were selected for their low values of multi-collinearity and were included in a multivariate analysis. For every unit increase in the CRP (mg/dL) serum level and every unit decrease in the haematocrit (1%), there was an increase in the CDAI score of 0.5 and 11.6 units respectively. However, this was not statistically significant (Table 3.6).

Table 3.6. Univariate and multivariate linear regression analysis to predict the likelihood of CDAI score

	Univariate			**Multivariate**		
	Coefficient B	95% CI	*p* value	Coefficient B	95% CI	*p* value
WCC	15.5	[2.7,28]	0.02	-	-	-
Neutrophils	16.2	[2.5,30]	0.02	-	-	-
CRP	0.8	[0.1,1.5]	0.03	0.5	[-0.2,1.3]	0.15
Haemoglobin	-47.5	[-85,-10]	0.01	-	-	-
Haematocrit	-15.5	[-28,-3]	0.01	-11.6	[-25,1.8]	0.08

CDAI: Crohn's disease activity index. CI: confidence interval. CRP: C-reactive protein. WCC: white cell count

3.2.5.2. Predicting Crohn's disease severity on pathological specimens

CD severity on the pathological specimen was significantly positively correlated with CDAI (Section 3.2.3.1 and Figure 3.7). This section aims to construct a model that can predict the severity of bowel inflammation from preoperative variables. Although the dependent variable of CD pathological severity is ordinal, represented by: 10 (43%) severe; 6 (26%) moderate; 5 (22%) mild; and 2 (9%) in remission, for the model these were classified into two categories. This was to enable a binary logistic regression analysis. The two categories are severe and non-severe. The latter includes: moderate, mild and remission pathological diagnosis. Binary rather than ordinal logistic regression analysis was used for easier interpretation of the results and because patients with severe disease represented the highest ratio, almost half of the CD patients' population.

Univariate analysis of WCC, neutrophils, CRP, haemoglobin, haematocrit, and CDAI was performed to predict the severity of bowel disease on pathology and demonstrated a significant odds ratio for CDAI (OR=1.02, 95% CI [1,1.04], $p<0.05$). This suggests that for every unit increase in the CDAI the likelihood of the patient to have pathologically severe bowel disease increases by 2%. The odds ratio of all the other variables were not statistically significant. Table 3.7 summarises the odds ratio, 95% CI and *p* values of all other variables. Furthermore, in a multivariate binary logistic regression analysis, the

odds ratio for CDAI remain statistically significant (OR=1.03, 95% CI [1,1.05], p<0.05). This suggests that for every one-unit increase in the CDAI, there is 3% increase in the likelihood of the patient having pathologically severe bowel disease. The odds ratio for other variables remain statistically insignificant (Table 3.7). On the univariate CDAI can predict pathologically severe bowel and non-severe bowel disease in 92% and 90% of the patients respectively. Similarly, in the multivariate analysis the model is able to predict severe and non-severe bowel disease in 92% and 80% of the patients respectively.

Table 3.7. Univariate and multivariate binary logistic regression analysis to predict Crohn's disease severity on pathological specimens

	Univariate			Multivariate		
	OR	**95% CI**	***p* value**	**OR**	**95% CI**	***p* value**
WCC	1.18	[1,1.5]	0.17	1.27	[0.1,36]	0.89
Neutrophils	1.18	[1,1.5]	0.19	0.70	[0.02,20]	0.83
CRP	1.02	[0.99,1	0.17	1.00	[1,1.1]	0.99
Haemoglobin	0.55	[0.3,1.1]	0.10	1.38	[0.1,49]	0.86
Haematocrit	0.80	[0.63,1]	0.08	0.85	[0.24,3]	0.81
CDAI	1.02	[1,1.04]	0.01	1.03	[1,1.05]	0.03

CDAI: Crohn's disease activity index. CI: confidence interval. CRP: C-reactive protein. OR: odds ratio. WCC: white cell count

3.3. Intraoperative details

Table 3.8. summarises intraoperative details for the study participants. Open approach[3] was most commonly used for bowel resection in both CD and the control group. Open approach to surgical resection was

[3] A surgical incision into the abdominal cavity, for performing major surgery

performed in 13 (53%) and 10 (71%) of CD and control patients respectively. A total of 5 (22%) patients in the CD group and 3 (21%) patients in the control group had their resection completed laparoscopically[4]. Furthermore, 5 (22%) of CD patients and 1 (7%) of the control patients were converted form laparoscopic to open approach due to operative difficulties.

The majority 15 (65%) of CD patients underwent ileo-colonic resection. In comparison, the majority of control patients 10 (72%) underwent subtotal colectomy. Furthermore, the anastomosis was stapled in the majority of CD patients 15 (65%) and hand sewn in 2 (9%). However, the majority of control patients underwent a stoma formation 10 (72%), due to the nature of their resection being mainly subtotal colectomy.

Table 3.8. Intraoperative details

	Disease (*n*=23)	Control (*n*=14)
Surgical access:		
Laparoscopic	5 (22%)	3 (21%)
Open	13 (56%)	10 (72%)
Laparoscopic converted	5 (22%)	1 (7%)
Surgical resection:		
Ileo-colonic	15 (65%)	-
Right hemicolectomy	3 (13%)	2 (14%)
Subtotal colectomy	3 (13%)	10 (72%)
Panproctocolectomy	2 (9%)	2 (14%)
Anastomosis:		
Sutured	2 (9%)	2 (14%)
Stapled	15 (65%)	2 (14%)
End/loop ileostomy	6 (26%)	10 (72%)

[4] A surgical procedure in which a fibre-optic instrument is inserted through the abdominal wall to view organs and permit the performance of surgery

3.4. Postoperative parameters

Table 3.9 summarises postoperative details including the length of hospital stay, postoperative complications, readmission rate, and CD recurrence rate. The time of follow up ranged from approximately 6 months to 3 years. The median hospital stay for the CD patients was shorter (median=9 days, range: 4, 64) than the control group (median=13 days, range: 3, 36), however the difference was not statistically significant (p=0.67). Two patients from the CD group suffered from post-operative wound infection, one of which required a negative pressure wound therapy (NPWT) (vacuum dressing)[5] for treatment and the other resolved with conservative management. One patient in the control group suffered from wound dehiscence also treated with vac dressing and antibiotics.

Four patients from the CD group were readmitted, one was diagnosed with acute adhesional obstruction, which was treated conservatively. Another patient was readmitted for vac drain related complication. The other 2 were readmitted under the medical team for the treatment of relapse. None of the patients who were readmitted in the disease group required surgery. Two patients from the control group were

[5] Negative-pressure wound therapy (NPWT) is a therapeutic technique using a vacuum dressing to promote healing in acute or chronic wounds and enhance healing

readmitted, one of which had an elective admission for the reversal of functioning ileostomy and another for wound related complication.

The recurrence rate was reported in 5 (22%) of the disease group. Three of which were proven on repeat colposcopy and pathological specimens. The remainder two complained of symptoms of recurrence, such as diarrhoea and abdominal pain. All the patients with recurrence were treated medically.

Table 3.9. Postoperative parameters: (follow up ranges from 6 months to 3 years)

	Disease (*n*=23)	Control (*n*=14)
Length of hospital stay in days: median (range)	9 (4-64)	13 (3-36)
Wound infection or dehiscence:	2 (9%)	1 (7%)
Readmission to hospital:	4 (17%)	2 (14%)
Crohn's recurrence:	5 (22%)	-

3.5. Discussion

To characterise angiogenesis in the mesenteric AT of CD patients, access to mesenteric tissue was essential. The only plausible method to obtain fresh tissue from patients was to recruit patients who were undergoing surgical resection. In this chapter, the aim was to establish the characteristics of patients in the disease and the control groups. The objectives were to identify potential differences between the patients that might have been a cause for biases, such as age, gender,

BMI, levels of biochemical markers, pathological diagnosis and medication.

The main findings of this chapter are that the study participants were comparable in terms of their age, gender, BMI, ASA score, WCC count, haemoglobin, haematocrit, and albumen levels. However, there were significantly more Caucasian patients, higher CRP levels, and more frequent immunosuppressive treatment in the disease group. These differences will be considered when interpreting the results of the subsequent experiments, such as gene and protein expression. The results also confirmed that CDAI, relevant to CD patients, significantly positively correlated with the disease activity on histopathology, WCC, and neutrophils counts. On the other hand, CDAI was significantly negatively correlated with haemoglobin and haematocrit levels. These findings explain the systemic response to CD severity.

Biochemical markers have been investigated for diagnostic and prognostic purposes in CD (Vermeire et al., 2006). In this study, CRP was significantly higher in CD patients. CRP is the most studied, and has been shown to be the best inflammatory marker for differentiating CD from other chronic gastrointestinal conditions. A study from St Bartholomew's Hospital, London (Beattie et al., 1995) found that 26 (100%) of the patients diagnosed with CD had increased CRP levels as compared with those who had polyps or children with otherwise

normal investigations. Similar findings were demonstrated almost a decade before that, by Shine et al at St Mark's hospital, London investigating chronic abdominal pain in 82 patients, out of which all of CD patients had increased CRP as compared with normal CRP levels in all patients with functional symptoms (Shine et al., 1985). Furthermore, CRP levels significantly positively correlate with disease activity (Vermeire et al., 2004), which is consistent with the results of this study.

Another significant finding is that majority of CD patients are from a Caucasian racial origin, which is consistent with previous research. In an epidemiological report including 554 patients with CD, 485 (87%) and 53 (10%) were from Caucasian and African American racial origins respectively (Kanaan et al., 2012). Indeed, population-based studies have demonstrated that the incidence rates and prevalence rates for CD have increased in Western countries since the mid-1970's (Michel et al., 2010). Another fact that may explain this finding is that majority of London citizens are from Caucasian origin. Approximately 60% of the population in the City of London are from a Caucasian racial origin (Census, 2011). This is also reflected in the control group, as relatively high percentage of patients are from a Caucasian racial origin.

As would be expected in the era of advanced medical therapy, the majority of patients with CD were treated with immunosuppressive

therapy. The ratio of patients treated with immunosuppressive medication was significantly higher in the CD patients. Furthermore, multiple immunosuppressive therapy including, azathioprine/mercaptopurine and methotrexate in combination with steroids were used to treat most patients with moderately active localized ileocaecal disease. In the CD group, anti-TNF therapy was used to treat patients with steroid refractory and those who were steroid dependent. This line of treatment is consistent with the current European evidence-based consensus on the diagnosis and management of CD (Dignass et al., 2010). Also, patients in the control group suffering from UC were on optimal medical therapy as recommended by the European evidence-based consensus on the diagnosis and management of UC (Dignass et al., 2012). Immunosuppressive therapy can alter cellular gene expression, therefore the significant increase in the ratio of patients with CD who received immunosuppressive medication will be analysed with vigilance in the subsequent gene and protein expression experiments (Chapters 5 and 6).

This study also demonstrated a significant positive correlation between CDAI and the levels of CRP, WCC and neutrophils count. Several studies in the literature reported a positive correlation between CRP levels and CD activity. Those comparable studies based their correlation on endoscopical, histological, radiological and clinical findings (Takenaka et al., 2015, Solem et al., 2005, Chamouard et al.,

2006). Specifically, Karoui et al, found a significant positive correlation between CDAI and the CRP levels (r=0.3) (Karoui et al., 2007). Correlation that is similar but stronger was demonstrated in this study (r=0.45). The reason for a stronger correlation in this study can be explained by the higher percentage of patients with severe disease, approximately 40%, in comparison to approximately 30% in the previous reports (Cellier et al., 1994, Karoui et al., 2007). Also, raised CRP is strongly correlated with ileocaecal and colonic disease rather than ileal disease (Florin et al., 2006). The vast majority of CD patients analysed in this study suffered from ileocaecal disease. Another group reported a cutoff point, whereby CRP can predict CD activity (Chamouard et al., 2006), using van Hees index: CRP level of 21.6 mg/L predicted that CDAI is ≥ 150 with a statistically significant area under the curve (AUC), receiver operating characteristic (ROC) curve of 0.84. In this study, ROC curve would be limited by the small sample size. The minimal sample size to achieve AUC of 0.725 and a power of 80% would be 19 positives and 38 negatives. In this study, there were 10 patients with severe disease (positive) and 13 with non-severe disease (negative), which is not enough to achieve a powered AUC analysis. However, to predict severity of CD in this study, logistic regression analysis was performed.

Interestingly, independent variables, such as WCC, neutrophil count, CRP, haemoglobin, and haematocrit were significant predictors of CDAI. In a univariate linear regression analysis, these variables can

predict CDAI with significant coefficient values. Previously, similar studies predicted CDAI from a more simplified clinical score, the Harvey-Bradshaw score. This produced a 27 unit increase in the CDAI for every unit increase in Harvey-Bradshaw score (Best, 2006). A more recent prospective clinical trial that employ similar methods as predictors of relapse after cessation of biological therapy in patients with steroid free clinical remission is the STORI trial (Louis et al., 2012). They identified risk factors for CD relapse including: male gender; absence of surgical resection; WCC $>6x10^9$; haemoglobin level ≤145 g/L; CRP level ≥5 mg/L; and calprotectin level ≥300 µg/g. This study suggests that the risk factors for disease severity is high WCC, neutrophils, CRP, and low haemoglobin and haematocrit.

A multivariate model on a logistic equation could be built to predict the CDAI score. For this a multivariate analysis was performed. However, despite negating multicollinearity, the model was not statistically significant. This may be limited by the small sample size used in this study, however further work may be powered and if achieves statistical significance could be a novel method to validate CDAI with more objectivity. However, this concept is not novel, as another group employed similar methods to predict the rate of disabling CD within 5 years of the diagnosis (Loly et al., 2008). This has clinical significance and can guide the treatment choice in CD (Sandborn et al., 2002, de Dombal and Softley, 1987). Yet, this principle of employing statistical methods to predict CDAI by the use of objective measures can have

wider clinical implications, such as predicting the need for monoclonal antibodies or surgical resection. Therefore, further work on this front with larger sample size and a dedicated design may help improve CD management.

Another interesting finding was the significant positive correlation found between CDAI and the severity of CD in the pathological specimen. Other studies have found significant correlation between CDAI and endoscopic findings (Cellier et al., 1994), MRI (Florie et al., 2006), CT enteroclysis (Chiorean et al., 2007), and histopathology (Geboes et al., 2005, Tielbeek et al., 2014). In this study, univariate and multivariate analysis of WCC, neutrophils, CRP, haemoglobin, haematocrit and CDAI were modeled to predict the histopathological disease severity. CDAI was the only significant predictor of the disease severity at the bowel level, emphasising the significant positive correlation between the histological severity of the disease and patients symptoms and signs. Other authors used similar statistical methods to predict the need for biological therapy (Molnar et al., 2013). The ability to establish the likelihood of the final histology of patients with CD can have important therapeutic implications. This study suggests a model using the above-mentioned variables to predict the likelihood of severe bowel disease.

It is known that one of the common complications of CD is anaemia. The incidence of anaemia in CD ranges between 7% to 74%

depending on disease severity (Gasche et al., 1997). Although the haemoglobin level was similar between CD and controls in this study, it is observed that as disease activity increases the level of haemoglobin decreases. This negative correlation was statistically significant showing a correlation coefficient (r=-0.5), similar to what has been reported previously by Bergamaschi *et al* who reported a significant negative correlation (*r*=-0.43) (Bergamaschi et al., 2010). It is also known that anti-TNF-α therapy may cause anaemia through the inhibition of erythroid progenitors and iron metabolism. The latter is characterised by iron retention within macrophages and inhibition of intestinal iron absorption (Johnson et al., 2004, Papadaki et al., 2002, Verma et al., 2002). Although we have not examined the iron level in our group of patients, those who had anaemia, suffered from microscopic, microcytic picture that indicates iron deficiency and were on anti-TNF therapy. The recognition of anaemia and its correlation to disease severity is relevant in this study because anaemia is known to promote HIF1 stabilisation and VEGF production and angiogenesis (Eckard et al., 2010). In the subsequent experiments, the expression of angiogenic factors such as HIF1 and VEGF will be measured and therefore the impact of haemoglobin levels on their expression needs to be explored.

The methods in this section are strengthened by the objectivity and accuracy of the data. The clinical information was anonymised at the time of analysis. Variables, such as biochemical blood levels were

performed in the hospital laboratory for clinical purposes, which is known for its high standard. Subjective measures, such as CDAI are validated and largely used in clinical trials. To ensure that the questionnaire was accurate and the questions were well explained, this was completed by a clinician (ME). Pathological disease activity and diagnosis were interpreted by a qualified consultant pathology consultant (Dr Rodriguez-Justo). The sample size, was powered at 80% for all outcome measures and the statistical methods were appropriately applied to data analysis.

Despite the strengths described above, some limitations were unavoidable. These include: lack of healthy control; potential selection and inclusion biases; and lack of blinding particularly regarding histopathological diagnosis. The control group include patients with UC and other collagen disorders who do not represent healthy control in terms of biochemical findings. Although this study's focus was on mesenteric adipose tissue that is known to be pathologically normal in these diseases, biochemical blood results are not normal. The study included all patients who required bowel resection for CD and could consent for the study, however, these patients do not represent the entire population of CD, such as those who do not require bowel resection. The pathologist could not be blinded to the interpretation and diagnosis for obvious clinical reasons.

In conclusion, CD patients included in this study have similar characteristics to the control group, but are significantly more likely to be from a Caucasian racial background and have higher rate of immunosuppressive therapy. CDAI and the severity of the bowel inflammation can be predicted by using objective variables. Clinically, this can improve the diagnosis and management of CD.

Chapter 4

Histology and immunohistochemistry of mesenteric adipose tissue in Crohn's disease patients

4.1. Introduction

The chronic inflammation in conditions, such as CD is intertwined with angiogenesis, the process of new vessel development from pre-existing vessels (Costa et al., 2007). The inflammatory tissue becomes a proangiogenic stimulus, whereby angiogenic factors such as VEGF and HIF1 are upregulated (Scapini et al., 2002). New blood vessels are required to provide nutrition and oxygen supply to the inflammatory mass. Progressive tissue destruction becomes persistent and the new blood vessels that should undergo apoptosis in normal healing processes become permanent (Greenhalgh, 1998). It is now clear that imbalanced angiogenic mechanisms are involved in the pathogenesis of CD (Fiocchi, 2002). This has been demonstrated at the level of the bowel tissue. However, despite the conclusive evidence of mesenteric thickening and fat wrapping in the vast majority of CD patients (Borley et al., 2000), there are no dedicated studies to characterise angiogenesis in CD mesentery. In this thesis, it was hypothesised that angiogenesis in CD mesentery is dysregulated leading to dysfunctional healing mechanisms and perpetuation of inflammation. Hence, in this study, a set of experiments including, histological and immunohistochemical, methods were employed. The objectives were to firstly demonstrate that CD tissues elicit an increased inflammatory response. Secondly, to assess the vascular morphology in CD mesenteric AT in comparison to controls. Thirdly, to quantify the

expression of angiogenic factors in CD mesenteric AT in comparison to controls.

Therefore, this chapter presents the following results: tissue inflammation, fibrosis, vascular morphology, HIF1 staining, VEGF staining and MVD. The results are presented in two ways: characterisation of the disease tissue versus control tissue.

4.2. Description of tissue comparison

A total number of 30 patients were included in this experiment: 19 with a confirmed diagnosis of CD and 11 controls. The demographics and gross pathological characteristics of these patients are summarised in Chapter 3 (Table 3.1.). There is a discrepancy in the number of patients used in subsequent experiments, reflecting the suitability and availability of tissue samples. Analyses were carried out on disease CD tissues, specifically wrapping AT and ileocaecal mesenteric AT (Tissue considered to be diseased). AT harvested from CD, including: subcutaneous and omental AT were considered control as these AT are not known to be affected by the disease. Therefore, in addition to the ileocaecal mesenteric, subcutaneous and omental AT harvested from non-CD patients, the control group included, subcutaneous and omental AT harvested from CD.

In detail, 90 tissue samples were analysed in total: 33 (37%) of which were classified as disease - [14 (16%) wrapping AT, and 19 (21%) ileocaecal mesenteric disease AT]; 57 (63%) were classified as controls - [10 (11%) ileocaecal mesenteric control AT, 15 (16%) omental disease AT, 15 (16%) subcutaneous disease AT, 9 (10%) omental control AT, and 8 (9%) subcutaneous control AT] (Figure 4.1). Methods used to determine tissue characteristics and associations included: tissue immunohistochemistry, which has been described in chapter 2, section 2.3.; and the corresponding statistical analysis which has been described in chapter 2, section 2.7.

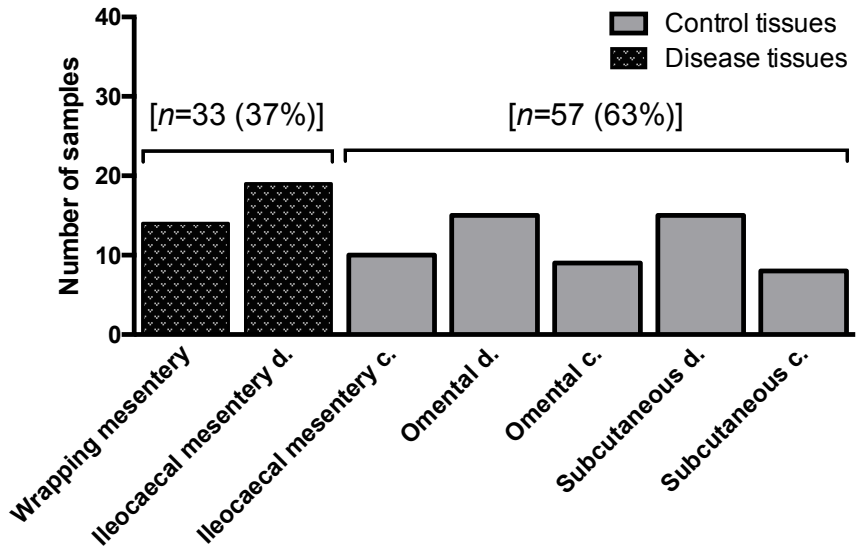


Figure 4.1. Tissue types from Crohn's disease patients included in the disease are "two tissue types in test group" (hatched). Tissue types from Crohn's disease patients (grey, d.) and from control patients (grey, c.) included in the "control" group. These two groups were analysed and compared throughout

4.3. Characterisation of CD wrapping and ileocaecal mesenteric AT

To achieve histological characterisation of the disease tissues, comparison was initially made between all types of AT. Further analysis was made between disease and control tissue types as explained above. Table 4.1 and the following subsections present the summary of these results.

4.3.1. Tissue inflammation

There was significantly higher ratio of tissue inflammation in the disease group compared to control. Mild inflammation was identified in 16 (48%) and 11 (19%) of the disease and control tissues respectively. Moderate inflammation identified in 3 (10%) and 1 (2%) of the disease and control tissues respectively (Table 4.1). This difference was statistically significant ($X^2_{(2)}$=12.7, p<0.01) (Figure 4.2: A, B and C). Furthermore, there was also statistical differences in the presence and degree of inflammation between different types of tissues. In the AT from CD patients, wrapping, ileocaecal mesenteric, omental and subcutaneous AT demonstrated 8 (57%), 8 (42%), 8 (53%) and 2 (13%), mild inflammation respectively. CD patients AT also demonstrated moderate inflammation in wrapping 1 (7%), ileocaecal mesenteric 2 (11%), and omental 1 (7%). There was no moderate significant inflammation in AT harvested from control

(Table 4.2). The difference in inflammation between different tissue types was statistically significant ($X^2_{(12)}$=28.2, p<0.01) (Figure 4.3).

Table 4.1. Summary of the histopathology and immunohistochemistry results. Disease tissue including wrapping and mesenteric adipose tissue. Control tissue includes: subcutaneous, omental adipose tissue from Crohn's disease and control patients and mesenteric adipose tissue from control patients (n=90)

	Disease Tissue (*n*=33)			**Control Tissue (*n*=57)**		
	None	Mild	Moderate	None	Mild	Moderate
Inflammation **	14 (42%)	16 (48%)	3 (10%)	45 (79%)	11 (19%)	1 (2%)
Fibrosis	23 (70%)	6 (18%)	4 (12%)	45 (79%)	11 (19%)	1 (2%)
	Absent		Present	Absent		Present
Vessel tortuosity*	29 (88%)		4 (12%)	56 (98%)		1 (2%)
	Negative	Weak staining	Strong staining	Negative	Weak staining	Strong Staining
HIF1**	11 (33%)	13 (40%)	9 (27%)	39 (68%)	13 (23%)	5 (9%)
	Negative		Positive	Negative		Positive
VEGF	31 (94%)		2 (6%)	55 (96%)		2 (4%)
MVD (Number of vessels/ 0.62mm^2,x200): Mean (SD, SEM)**	29 (20, 3.5)			19 (12, 1.6)		
MVD (Number of vessels/ 0.62mm^2,x200): Median (range)**	23 (11-102)			16 (6-62)		

HIF1: Hypoxia Inducible Factor-1. MVD: Microvascular Density. SD: Standard Deviation, SEM: Standard Error of Mean. VEGF: Vascular Endothelial Growth Factor. Grey highlight is the test group. *p<0.05, **p<0.01 (2 tailed)

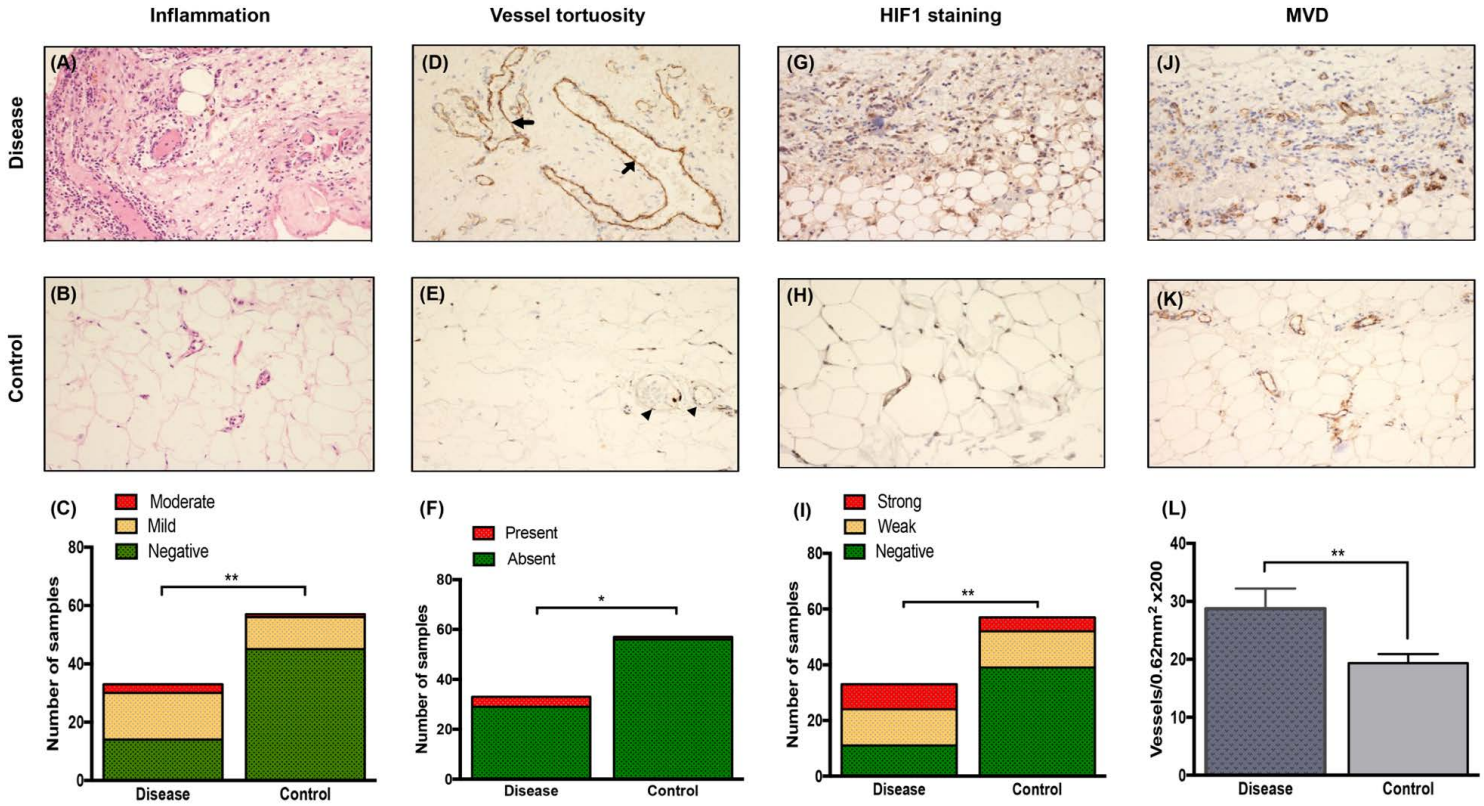


Figure 4.2. Adipose tissue (AT) characteristics: H&E staining for inflammation (A, B, and C); CD31 staining demonstrating vessel tortuosity (D: arrows showing tortuous vessels, E: arrow heads showing normal vessels, and F); hypoxia inducible factor-1 (HIF1) staining (G, H, and I); and microvascular density (MVD) with CD31 staining (J, K and J). Upper panel: representative disease AT. Middle panel: representative control AT. Lower panel: statistical significance (*p<0.05, **p<0.01)

Table 4.2. The presence and degree of inflammation in different type of adipose tissue

	Inflammation**			
	None	Mild	Moderate	Total
Wrapping AT	5 (36%)	8 (57%))	1 (7%)	14
Ileocaecal mesenteric AT d.	9 (47%)	8 (42%)	2 (11%)	19
Ileocaecal mesenteric AT c.	10 (100%)	0	0	10
Omental AT d.	6 (40%)	8 (53%)	1 (7%)	15
Omental AT c.	8 (89%)	1 (11%)	0	9
Subcutaneous AT d.	13 (87%)	2 (13%)	0	15
Subcutaneous AT c.	8 (100%)	0	0	8
Total	59 (66%)	27 (30%)	4 (4%)	90

Adipose tissue (AT). Disease (d.). Control (c.). Grey highlight is the test group. **p<0.01 (2 tailed)

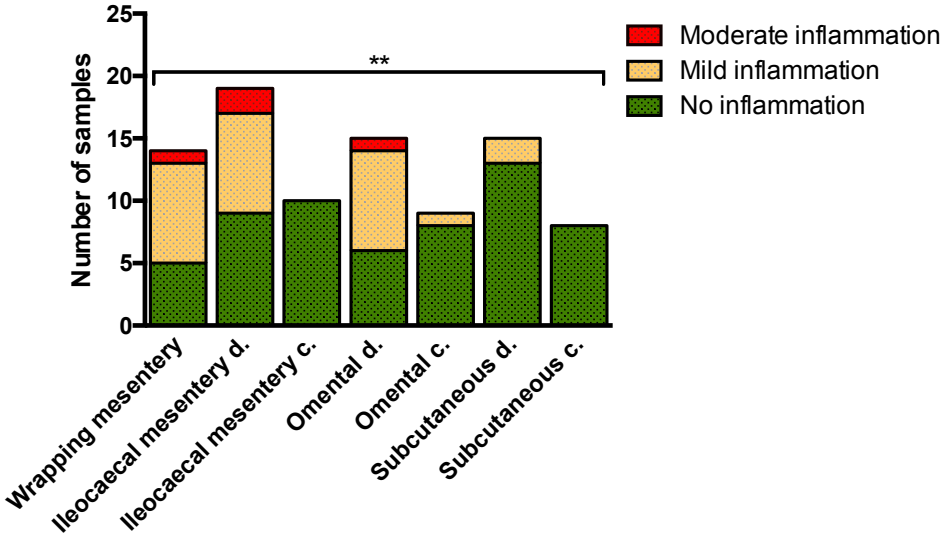


Figure 4.3. The degree of inflammation in different type of AT. The disease group is represented by the wrapping mesentery and the ileocaecal mesentery from Crohn's disease (CD) patients. Other AT are control group, including subcutaneous and omental AT from CD patients. Disease (d.). Control (c.). **p<0.01

4.3.2. Tissue fibrosis

Mild fibrosis was detected in 6 (18%) and 11 (19%) in disease and control tissue respectively. Moderate fibrosis was detected in 4 (12%) and 1 (2%) of the disease and control tissue respectively (Table 4.1).

There was no statistical difference found in tissue fibrosis between disease and the control groups ($X^2_{(2)}$=4.30, p= 0.11). Furthermore, the level of fibrosis was similar between all types of AT ($X^2_{(12)}$=9.1, p= 0.70) (Table 4.3).

Table 4.3. The presence and degree of fibrosis in different type of adipose tissue

	Fibrosis			
	Normal	Mild	Moderate	Total
Wrapping AT	10 (71%)	3 (22%))	1 (7%)	14
Ileocaecal mesenteric AT d.	13 (68%)	3 (16%)	3 (16%)	19
Ileocaecal mesenteric AT c.	8 (80%)	2 (20%)	0	10
Omental AT d.	11 (73%)	4 (27%)	0	15
Omental AT c.	7 (78%)	2 (22%)	0	9
Subcutaneous AT d.	11 (73%)	3 (20%)	1 (7%)	15
Subcutaneous AT c.	8 (100%)	0	0	8
Total	68 (76%)	17 (19)	5 (5%)	90

Adipose tissue (AT). Disease (d.). Control (c.). Grey highlight is the test group.

4.3.3. Vessel morphology

Vessel tortuosity was present in 4 (12%) and 1 (2%) of the disease and control AT respectively (Table 4.1). This increase in vascular tortuosity was statistically significant ($X^2_{(1)}$=4.3, p<0.05) (Figure 4.2: D, E, and F). However, when comparison was made to different type of depot regardless of the disease state there was no significant difference in vessel tortuosity (Table 4.4).

Table 4.4. The presence or absence of vessel tortuosity in deferent type of adipose tissue

	Vessel tortuosity		
	Absent	Present	Total
Wrapping AT	12 (86%)	2 (14%)	14
Ileocaecal mesenteric AT d.	17 (90%)	2 (10%)	19
Ileocaecal mesenteric AT c.	10 (100%)	0	10
Omental AT d.	14 (93%)	1 (7%)	15
Omental AT c.	9 (100%)	0	9
Subcutaneous AT d.	15 (100)	0	15
Subcutaneous AT c.	8 (100%)	0	8
Total	85 (94%)	5 (6%)	90

Adipose tissue (AT). Disease (d.). Control (c.). Grey highlight is the test group

4.3.4. Hypoxia Inducible Factor-1 (HIF1) staining

HIF1 staining was significantly higher in the disease AT than in controls (Table 4.1). Wrapping and ileocaecal mesenteric AT in CD patients demonstrated 13 (40%) and 9 (27%) weak and strong HIF1 staining respectively, which was significantly higher than controls (Figure 4.2: G, H, and I). Comparing different types of depot regardless of the disease state demonstrated weak staining in 4 (29%) wrapping AT, 9 (47%) ileocaecal mesenteric AT of disease patients, 2 (20%) ileocaecal mesenteric AT of control patients, 3 (20%) omental AT from disease patients, 2 (22%) omental tissue from control patients and 4 (40%) subcutaneous AT from disease patients. Strong HIF1 staining was demonstrated in 7 (50%) wrapping AT, 2 (11%) ileocaecal mesenteric AT from disease patients, and 5 (33%) omental AT from disease patients (Table 4.5). The difference in HIF1 staining between

different type of depots was statistically significant ($X^2_{(12)}$=35.1, p<0.001) (Figure 4.4).

Table 4.5. Hypoxia Inducible Factor-1 (HIF1) of different type of adipose tissue

	HIF1 staining***			
	Negative	Weak	Strong	Total
Wrapping AT	3 (21%)	4 (29%)	7 (50%)	14
Ileocaecal mesenteric AT d.	8 (42%)	9 (47%)	2 (11%)	19
Ileocaecal mesenteric AT c.	8 (80%)	2 (20%)	0	10
Omental AT d.	7 (47%)	3 (20%)	5 (33%)	15
Omental AT c.	7 (78%)	2 (22%)	0	9
Subcutaneous AT d.	9 (60%)	6 (40%)	0	15
Subcutaneous AT c.	8 (100%)	0	0	8
Total	50 (56%)	26 (29%)	14 (15%)	90

Adipose tissue (AT). Disease (d.). Control (c.). Grey highlight is the test group. ***p<0.001 (2 tailed)

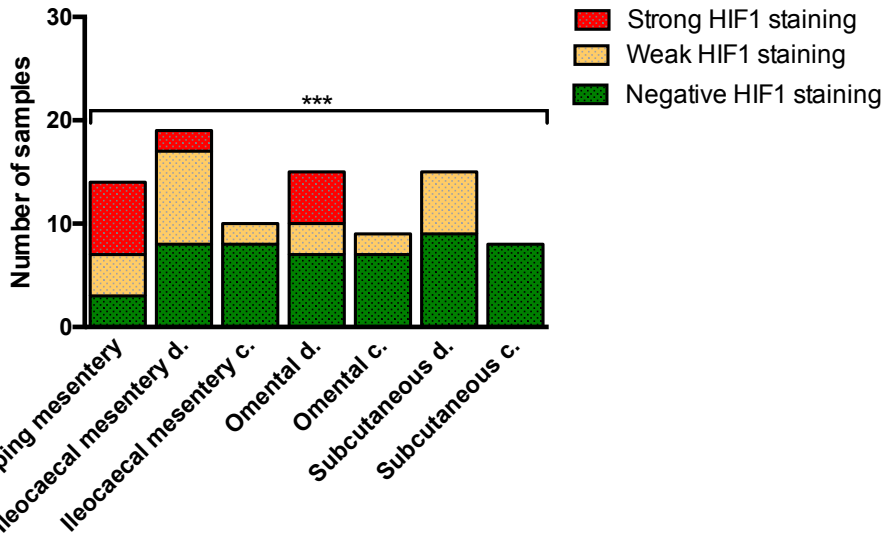


Figure 4.4. Hypoxia Inducible Factor-1 (HIF1) staining in different type of adipose tissue (AT). The disease group is represented by the wrapping mesentery and the ileocaecal mesentery of Crohn's disease (CD) patietns. Other AT are control group, including subcutaneous and omental AT from CD patients. Disease disease (d.) and control (c.) patients. ***p<0.001.

4.3.5. Vascular Endothelial Growth Factor (VEGF) staining

VEGF staining was similar in disease and control tissue: out of 33 and 57 AT, 2 (6%) and 2 (4%) demonstrated positive VEGF in disease and control respectively (Table 4.1) (Figure 4.5). Table 4.6 summarises the ratios for VEGF in all AT types. There was no statistically significant difference in the ratio of VEGF between AT.

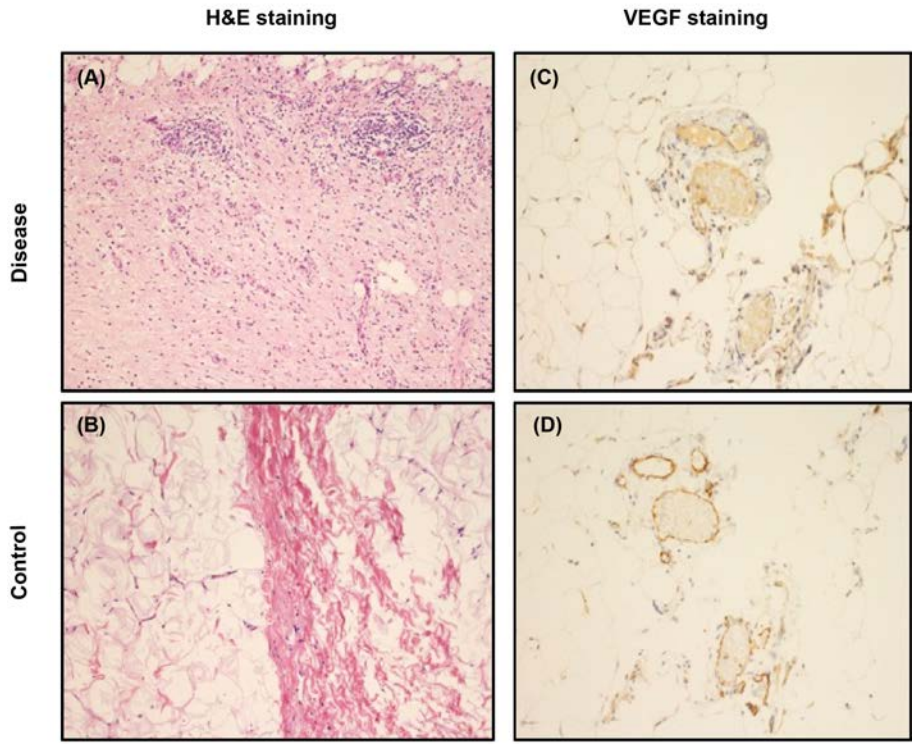


Figure 4.5. Inflammation and fibrosis present in disease mesenteric adipose tissue (AT) (A) but not in control (B). Vascular endothelial growth factor (VEGF) staining is similar in disease (C) and control (D) AT.

Table 4.6. Vascular endothelial growth factor (VEGF) staining in all AT.

	VEGF staining		
	Positive	Negative	Total
Wrapping AT	1(7%)	13 (93%)	14
Ileocaecal mesenteric AT d.	1(5%)	18 (95%)	19
Ileocaecal mesenteric AT c.	0	10 (100%)	10
Omental AT d.	1(7%)	14 (93%)	15
Omental AT c.	1(11%)	8 (89%)	9
Subcutaneous AT d.	0	15 (100%)	15
Subcutaneous AT c.	0	8 (100%)	8
Total	4 (5%)	86 (95%)	90

Adipose tissue (AT). Disease (d.). Control (c.). Grey highlight is the test group.

4.3.6. Microvascular density (MVD)

There was a significant difference in the levels of MVD between disease and control AT (Table 4.1). Disease AT has significantly (p<0.01) higher MVD (mean=29, SD=20) than control AT (mean=19, SD=12) (Figure 4.2: J, K, and L). There was also a significant (p<0.01) difference in the mean MVD demonstrated between all tissue types (Table 4.7) (Figure 4.6).

Table 4.7. The mean, standard deviation (SD), standard error of mean (SEM), median and range of microvascular density (MVD=number of vessels/0.62mm2 x200) are summarised here. ANOVA test demonstrated a significant difference between depot means.**

	Mean (SD, SEM)	Median (range)	Total
Wrapping AT	27.6 (15.3, 4.1)	23.5 (11-62)	14
Ileocaecal mesenteric AT d.	29.6 (23.3, 5.3)	21 (13-102)	19
Ileocaecal mesenteric AT c.	13.3 (5.8, 1.8)	11 (7-26)	10
Omental AT d.	30.4 (15, 3.9)	28 (11-62)	15
Omental AT c.	16.6 (9.2, 3.1)	15 (6-32)	9
Subcutaneous AT d.	17.5 (8.1, 2.1)	16 (7-33)	15
Subcutaneous AT c.	12.5 (5.6, 2)	10.5 (7-23)	8
Total	22.8 (15.9, 1.7)	18 (6-102)	90

Adipose tissue (AT). Disease (d.). Control (c.). **p<0.01 (2 tailed).

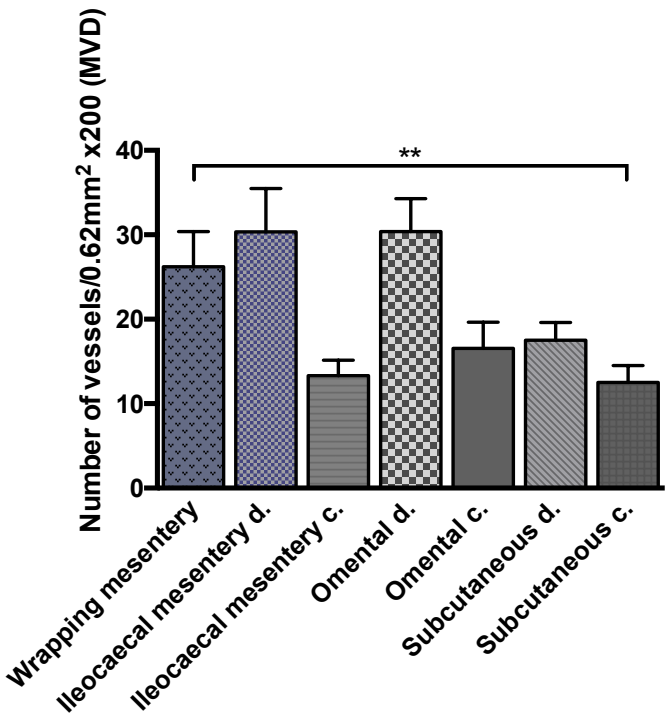


Figure 4.6. The levels of microvascular density (MVD) in all types of adipose tissue. Wrapping mesentery and ileocaecal mesentery d. are the test group. All other AT including subcutaneous and omental AT from Crohn's disease are considered control group. Disease (d.). Control (c.). **p<0.01.

4.4. Correlation between characteristics observed in the adipose tissue

Table 4.8 summarises the Pearson Correlation (*r*) of associations between the histological characteristics of the different types of depot. This included: inflammation, fibrosis, vessel tortuosity, assessment of tissue staining for angiogenic factors (HIF1 and VEGF), and MVD. There was a strong association between these characteristics: particularly MVD was positively correlated with inflammation, fibrosis, vessel tortuosity, HIF1 staining and VEGF staining. The following subsections provide the details and strength of these associations.

Table 4.8. Associations between tissues pathological characteristics. Calculated by Pearson Correlation (*n*=90)

Histopathological variables: *r* (95% CI)	Fibrosis	Vessels tortuosity	HIF1	VEGF	MVD
Inflammation	0.54*** (0.37-0.67)	0.18 (-0.03-0.37)	0.66*** (0.54-0.80)	0.26* (0.05-0.44)	0.65*** (0.51-0.76)
Fibrosis		0.21* (0.01-0.40)	0.26* (0.01-0.42)	0.30** (0.09-0.50)	0.52*** (0.35-0.66)
Vessels tortuosity			0.33** (0.13-0.50)	-0.06 (-0.26-0.15)	0.22* (0.01-0.41)
HIF1				0.33** (0.12-0.50)	0.45*** (0.32-0.64)
VEGF					0.37*** (0.17-0.54)

HIF1: Hypoxia Inducible Factor-1. MVD: Microvascular Density. VEGF: Vascular Endothelial Growth Factor. 95% CI: 95% Confidence Interval. **p*<0.05, ***p*<0.01, ****p*<0.001 (2 tailed)

4.4.1. Tissue inflammation

Inflammation was significantly positively correlated with fibrosis (r=0.53), HIF1 staining (r=0.66), VEGF staining (r=0.26) and MVD

(r=0.65) (Figure 4.7, A). No association was found between inflammation and vessel tortuosity. Furthermore, association of ordinal categories was tested by Pearson Chi-square. This included inflammation and fibrosis, vessel tortuosity, HIF1 staining, and VEGF staining using. Apart from vessel tortuosity, there was a significant association between inflammation and fibrosis ($X^2_{(4)}$=45.9, p<0.001), HIF1 ($X^2_{(4)}$=41.6, p<0.0001), VEGF ($X^2_{(2)}$=15.8, p<0.001).

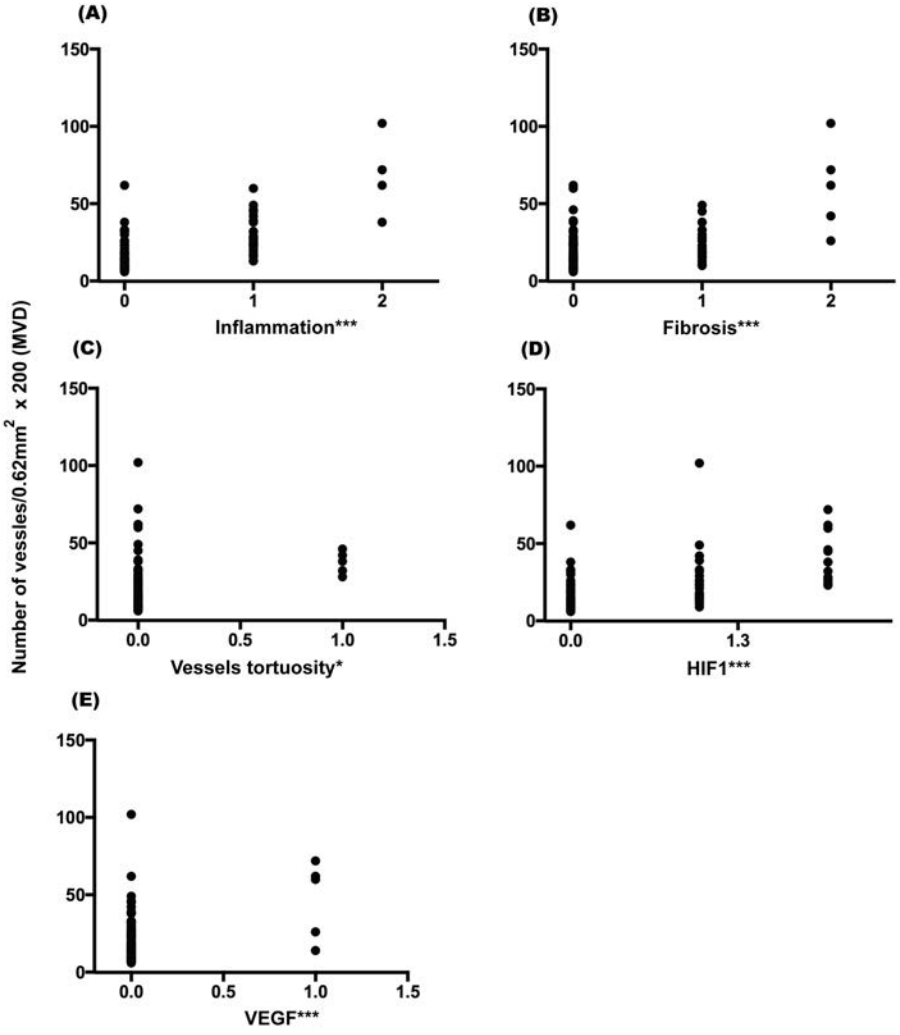


Figure 4.7. Correlation between microvascular density (MVD, Y axis) and inflammation (A), fibrosis (B), vessels tortuosity (C), hypoxia inducible factor-1 (HIF1) (D), and vascular endothelial growth factor (E), in all tissues from CD patients and control patients. *p<0.05. ***p<0.001.

4.4.2. Tissue fibrosis

Fibrosis was significantly positively correlated with vessel tortuosity (r=0.21), HIF1 staining (r=0.26), VEGF staining (r=0.30), and MVD (r=0.53) (Figure 4.7, B). Significant association between fibrosis and vessels tortuosity ($X^2_{(2)}$=6.4, p<0.05), as well as VEGF staining ($X^2_{(2)}$=12.2, p<0.01) was demonstrated when using ordinal Pearson Chi-square test. Using the latter test, there was no significant association between fibrosis and HIF staining ($X^2_{(4)}$=16.1, p=0.19), however, its equivalent McNemar-Bowker test produced a significant association ($X^2_{(3)}$=10.9, p<0.05).

4.4.3. Vessel tortuosity

Vessel tortuosity was significantly positively correlated with HIF1 staining (r=0.33) and MVD (r=0.22) (Figure 4.7, C). There was no significant correlation between vessel tortuosity and VEGF staining (r=-0.05, p=0.58). Furthermore, for ordinal data comparison Pearson Chi-square test demonstrated a significant association between HIF1 staining and vessel tortuosity ($X^2_{(2)}$=9.9, p<0.01).

4.4.4. HIF and VEGF staining

HIF1 staining was significantly positively correlated with VEGF staining (r=0.33) and MVD (r=0.50) (Figure 4.7, D). Similarly, VEGF

staining was significantly positively correlated with MVD (r=0.37) (Figure 4.7, E). Ordinal data Pearson Chi-square test also suggested a significant association between VEGF staining and HIF1 staining ($X^2_{(2)}$=9.9, p<0.01).

4.5. Discussion

CD extends beyond the intestinal wall to affect the adjacent mesentery (Ephgrave, 2007). This thesis has focused on characterising angiogenesis in CD mesenteric AT. The comparison was also made between other AT harvested from CD patients. To avoid confusion the test group is defined as wrapping and ileocaecal mesenteric AT from CD patients. Specifically, this set of experiments was designed to study inflammation, fibrosis, vascular morphology, HIF1 staining, VEGF staining and MVD, in freshly harvested tissues. The tissue was immediately fixed in formaldehyde to preserve its characteristics, aiming to investigate whether the angiogenic features were dysregulated, resulting in a pathological microvasculature. This study and in conjunction with the studies, and the following chapters, characterise the angiogenic features of CD mesenteric AT and their potential role in initiating or perpetuating mesenteric inflammation.

The main findings from this study demonstrated that CD mesenteric AT had significantly more inflammation and vessel tortuosity. The strength of HIF1 staining, a pro-angiogenic factor, was significantly

higher in CD mesenteric AT in comparison to control. MVD was also significantly higher in the test group. Despite the presence of inflammation and the increase in MVD, the level of VEGF staining, a potent pro-angiogenic factor, was not increased. Yet, there was a significant positive correlation between MVD and other characteristics including: inflammation, fibrosis, vessels tortuosity, HIF1 staining, and VEGF staining. This finding suggests that inflammation significantly correlated with increased angiogenesity (Figure 4.7). MVD was significantly positively correlated with an increase in the frequency of vessel tortuosity. Unlike the up-regulated angiogenesis that takes place in physiological healing mechanisms, the up-regulated angiogenesis in CD mesenteric AT is likely to be unbalanced. In wound repair for example, newly formed blood vessels participate in provisional granulation tissue formation and provide nutrition and oxygen to the growing tissues (Li et al., 2003). Thus, when the wound has healed and the tissue is adequately perfused, vessels that are no longer needed die by apoptosis (Greenhalgh, 1998). This did not appear to be the case in CD mesentery, suggesting a dysregulated angiogenic response. Clinically, it is relevant to understand the angiogenic response, particularly when considering anti-angiogenic therapy for the treatment of CD patients (Chandar et al., 2015).

The histopathological changes in the mesentery neighbouring diseased bowel in CD patients have been reported previously. In this study, there was a distinction between the mesenteric AT harvested

from CD and the wrapping mesenteric tissue, which was harvested from the bowel wrapping mesentery. The reason for this was to ensure that the abnormal mesenteric tissue, particularly the wrapping mesentery, is vividly identified and characterised. Furthermore, the ileocaecal mesentery is a common site chosen to adjust for variations in changes within the mesentery when comparing CD mesentery to non-CD mesentery. Also, the terminal ileum and ileocaecal areas are most commonly affected by the disease (Crohn et al., 1984). There was similar inflammation and dysregulated angiogenesis in both sites of the mesentery in CD patients (test group: wrapping and ileocaecal mesenteric AT). This may be explained by the fact that the vast majority of CD patients in this study were diagnosed with ileocaecal Crohn's. The pathological diagnosis of CD specimens including the wrapping mesentery were summarised in chapter 3. The results of the pathological diagnosis stated in chapter 3 and this study are consistent with that described by Crohn's approximately a century ago. The mesentery is thickened, edematous and contains numerous large glands (Crohn et al., 1984). The inflammatory changes in the mesenteric AT in proximity to the diseased bowel have subsequently been confirmed by Borley *et al* (2000). Acute and chronic inflammatory changes have been identified in blocks of mesenteric AT specimens harvested from CD patients (Borley et al., 2000). Depending on the stage of the disease, these changes varied considerably, from mild to severe inflammation. Other reports also described a more severe inflammation in the mesentery that has most proximity to the bowel

(Desreumaux et al., 1999). The findings in this study are unique because they correlated these pathological features to increased MVD and associated abnormal tortuous vessels. This further confirms the intertwined relationship between angiogenesis and inflammation (Deban et al., 2008).

Unlike previous studies, in addition to using non-CD AT as the control group, this cohort included specimens from the omental and subcutaneous AT of CD patients for comparison as well. Initially the comparison was performed between the test group and the control group and subsequently comparison between all tissue type was undertaken. This was to obtain a comprehensive view of the difference between CD and non-CD, as well as differences between different types of AT. Thus, patients with CD demonstrated increased inflammatory changes in both omental and subcutaneous AT. This may partly explain the extra-intestinal manifestations of CD. In a retrospective study of 448 IBD patients, Aghazadeh et al. reported extra-intestinal manifestations in approximately 40% of CD patients involving most of the body systems particularly the skin (Aghazadeh et al., 2005). Inflammatory changes in the subcutaneous AT may explain the skin manifestations of CD including: pyoderma gangrenosum, erythema multiforme, and erythema nodosum (Huang et al., 2012, Marlier et al., 2006, Vavricka et al., 2011).

Fibrosis was also assessed in these tissues. The results demonstrate similar ratio of fibrosis in CD and control. Nevertheles, this study demonstrated that fibrosis in AT was positively significantly correlated with inflammation, vessels tortuosity, HIF1 and VEGF staining, and MVD. This may suggest an interplay between the mechanisms of chronic inflammation and angiogenesis (Kreuger and Phillipson, 2016). A possible explanation to the similar ratio of fibrosis between CD and control is that three patients in the control group were diagnosed with EDS, a collagen disorder associated with increased adipose tissue fibrosis (Burrows et al., 1996). By definition, the term fibrosis refers to the formation of fibrous tissue as a reactive process in the context of chronic tissue disease. Fibrosis has been strongly associated with chronic inflammatory diseases (Ueha et al., 2012). Previous investigation of the mesenteric AT in CD found a significant correlation between bowel mucosal ulceration, transmural inflammation, fibrosis, stricture formation and the phenomena of mesenteric fat wrapping (Sheehan et al., 1992).

Integral mesenteric microvasculature is essential for the regulation of blood flow to the bowel. At this level, exchange of gas, solute and hormones between blood and tissue occurs. Furthermore, the microvasculature entertains the highest regenerative capacity and is consequently an active vascular component. Despite the important role of the microvasculature in the mesentery, little attention has been given to the changes that occur in patients with CD. In early years of

CD description, few reports briefly mentioned vascular changes in bowel and mesenteric tissue (Shnitka, 1964, Saltzstein and Rosenberg, 1963). Others, described vascular lesion, such as chronic phlebitis in small veins and obliteration endoarteritis in all layers of the bowel wall and particularly in the mesentery (Rappaport et al., 1951, Van Patter et al., 1954). Subsequently, obliterative vascular lesions of degenerative rather than inflammatory characteristics gained diagnostic importance (Knutson et al., 1968, Maunoury et al., 2000, Zurawski et al., 2007). This study recognised such vascular changes. There was a significant increase in vessel tortuosity, HIF1 staining and MVD in CD mesenteric AT. Increased vascular tortuosity has been associated with aging, atherosclerosis, hypertension, genetic defects and diabetes mellitus (Han, 2012, Callewaert et al., 2008, Owen et al., 2008, Del Corso et al., 1998). Tortuous microcirculation is an abnormal feature and a disease state that can be attributed to ischaemia (Batra and Rakusan, 1992, Jakob et al., 1996). Also, it has been previously suggested that CD is mediated by multifocal gastrointestinal ischaemia (Wakefield et al., 1989). Vessel tortuosity is also part of systemic diseases (Amemiya and Bhutto, 2001) and it could be that this may be a part of the extra-intestinal manifestation of CD. Particularly, one case of CD was found to have increased vascular tortuosity in the omental AT.

Another important finding is the significant increase in the level of HIF1 in the disease AT. The over expression of HIF1 in obese patients' AT

is well described (Wang et al., 2007, Rausch et al., 2008, Hosogai et al., 2007). When there is tissue hypoxia, most of the cellular oxygen is consumed by the mitochondria (Rolfe and Brown, 1997). As a compensatory mechanism HIF1 becomes activated (Semenza and Wang, 1992). It is now clear that HIF1 accumulation stimulates the transcription of proangiogenic factors including VEGF (Semenza, 2003, Semenza, 2009). In CD bowel specimens HIF1 and not VEGF was overexpressed (Giatromanolaki et al., 2003). This is consistent with the results in this experiment. The lack of VEGF in CD bowel specimens in the context of HIF1 overexpression may be counterintuitive and warrant an explanation. It is possible that HIF1 is inactivated by HIF hydroxylases in this group of patients as they were exposed to several anaesthetic agents shortly before the specimens were collected. Anaesthetic agents such as nitrous oxide can redistribute oxygen toward all compartments outside mitochondria and activate HIF hydroxylase (Hagen et al., 2003). Consequently, hydroxylases do not register hypoxia and HIF1 is inactivated (Taylor, 2008). The other explanation could be that the angiogenic mechanisms in this group of patients are dysregulated and the vessels tortuosity and high MVD is propagated by other angiogenic factors than VEGF. For example, AT angiogenesis can be promoted by Leptin and HIF1 (Wator et al., 2008). Furthermore, similar to AT in obesity, angiogenesis in inflamed wrapping and ileocaecal mesenteric AT of CD patients may fail to maintain normoxia (Hosogai et al., 2007,

Rausch et al., 2008). Subsequently, angiogenesis and inflammation are up-regulated.

Finally, the results presented here show a significant increase in the MVD of disease tissue compared to control. A similar pattern is seen in neoplastic angiogenesis (Yin et al., 2005). Furthermore, the correlation between increased MVD and arteriolar stenosis in CD intestinal tissue has been demonstrated (Wakefield et al., 1991b). Limited tissue flow due to arteriolar stenosis could account for increased MVD in the wrapping and ileocaecal AT in this cohort (Koerselman et al., 2003). It is unclear whether increased MVD in the disease AT samples is related to chronic inflammation or to arteriolar stenosis or ischaemia. However, it is possible to speculate that angiogenesis could be an important perpetuator of AT inflammation in CD AT.

The strength of this study lies mainly in characterising freshly harvested AT from the mesentery of CD patients. Unlike gene and protein expression studies described in the subsequent chapters, in this experiment tissues were fixed immediately in formaldehyde in order to preserve the features of tissues at the time of harvest and negate any bias that may result from sample processing (Carson et al., 1973). The methods are well established and the results were accurately interpreted by two blinded researchers: Dr Manuel Rodriguez-Justo (a consultant pathologist) and Mr Dominic Patel

(biomedical scientist). In addition to the wrapping AT adjacent to the diseased bowel, ileocaecal mesenteric AT from CD and control were harvested and compared so that differences related to mesenteric locality could be adjusted for. Furthermore, subcutaneous and omental AT from CD patients were used for comparison, in order to minimise bias related to patients' characteristics.

Despite the strengths of this study there are some unavoidable limitations. The short duration of the study as well as the small proportion of CD patients requiring surgical resection, restrict sample size. Nevertheless, the significance achieved is powered at 80%. Furthermore, the population investigated in this cohort only represents CD patients at late-stage of the disease and not early- or middle-stage. Although the methods of tissue collection and sample processing were applied meticulously, confounding factors such as processing the samples from collection duration of formaldehyde immersion (ranging from 1 day to 2 weeks) is impractical to adjust for and may produce biases.

In conclusion, wrapping and ileocaecal mesenteric AT from CD patients demonstrate inflammation, and dysregulated angiogenesis characterised by significant increase in MVD and vessels tortuosity.

Chapter 5

Angiogenic gene expression in Crohn's disease mesenteric adipose tissue

5.1. Introduction

The journey of a phenotypic characteristic, whether physiological or pathological, begins at the genome (Johannsen, 2014), starting with the DNA containing genes with specific nucleotide sequences, which are transcribed into mRNA. The latter (Darnell, 1979) travels from the nucleus through the cytoplasm, picked up by the ribosomes, which translate the information code into amino acid sequence that makes up proteins (Palade, 1975, Morisaki et al., 2016). Cells respond to environmental cues and synthesise proteins with precise timing and specific molecular functions (Sonenberg and Hinnebusch, 2009). In particular to this thesis, angiogenic gene expression studies will characterise the mesenteric AT in CD at a molecular level. The hypothesis of a dysregulated angiogenic balance that drives the chronicity of the disease can be further investigated. Furthermore, studying the tissue's gene expression may be the first step to identify therapeutic targets and understand dysfunctional signaling pathways.

The gene expression of several angiogenic factors will be evaluated and summarised in this chapter. Pro- and anti-angiogenic gene expression levels areg compared between disease and control tissue, in order to address the research question of whether angiogenesis is imbalanced in CD mesenteric tissue leading to the development of an unhealthy microvasculature. An array of 84 human angiogenic genes were initially assessed on a small cohort of patients. Subsequently,

the expression of 24 of these genes were further validated on a larger sample size. The chapter has been divided into two sections: 1) angiogenic gene array: the levels of 84 angiogenic genes, investigating the angiogenic balance in the CD mesenteric AT, and 2) angiogenic gene array of selected genes, providing a validation study of gene expression.

5.2. Angiogenic gene expression, RT^2 Profiling^{TM} PCR array

An array of 84 genes involved in human angiogenesis were measured in a total number of 16 patients comprising 8 (50%) with disease and 8 (50%) controls. In terms of tissue samples there were a total of 20 samples processed in a total number of 5 plates. The tissue types in this experiment are as follow. Firstly, the test group: a total number of 4 (20%) wrapping AT and 8 (40%) ileocaecal mesenteric AT from 8 CD patients. Secondly, the control group, a total number of 8 (40%) ileocaecal mesenteric AT from 8 non-CD patients. Patients characteristics are detailed in chapter 3. Table 5.1 summarises the characteristics of the 16 patients included in this study. Briefly, CD and control patients were similar in age, gender, BMI, and ASA score. Out of a total of 8 patients in the CD group, 7 (87%) were treated with immunosuppressive therapy including steroids and anti-TNF monoclonal antibodies. In the control group there were 3 (37%) patients with a diagnosis of UC who were treated with

immunosuppressive therapy at the time of surgery including steroids. The ratios of receiving immunosuppressive therapy or not were statistically significantly different ($X^2_{(1)}$=4.3, p<0.05).

Table 5.1. Characteristics and clinical features of patients studied with the RT^2 Profiler^{TM} PCR Array Human Angiogenesis

	Disease (n=8)	Control (n=8)
Age: (SD)	30.5 (11.51)	44.25 (19.94)
Gender:		
Male	3 (37%)	4 (50%)
Female	5 (63%)	4 (50%)
BMI: (SD)	26.43 (6.79)	26.27 (5.68)
ASA score:		
II	7 (87%)	7 (87%)
III	1 (13%)	1 (13%)
Immunosuppressive therapy:*	7 (87%)	3 (37%)
Diagnosis:	- Ileocaecal CD (n=8)	- Benign polyp (n=1) - Chronic constipation (n=3) - Ulcerative colitis (n=3) - Diverticular disease (n=1)

ASA score: American Society of Anesthesiologist physical status score. BMI: body mass index. CD: Crohn's disease. RT: real time. PCR: polymerase chain reaction. SD: standard deviation. *p<0.05

To demonstrate the difference in gene expression between the groups two methods were used. Firstly, the comparative method was used to generate fold differences of gene expression between test group and controls. This method also allows the generation of a heat map and a dendrogram in order to demonstrate the strength of fold regulation, and group together genes with similar expression patterns . Secondly, the quantitative method using 2^(-Avg.(Delta(CT)) was employed to perform correlations and expression level comparisons. Therefore, this section has been divided into three parts: 1) angiogenic genes fold regulation; and 2) gene expression correlation matrix; and 3) pro- and anti-angiogenic gene expression balance.

5.3. Angiogenic genes fold regulation

The fold regulation of 84 genes involved in human angiogenesis was calculated to identify upregulated and downregulated genes. A total number of 47 (56%) demonstrated two or more-fold decrease in comparison to control (Figure 5.1). The heat map is colour coded and shows the genes' expression fold regulation. For example, gene CXCL10 in row B, column 03 is bright green in colour as it is downregulated by approximately 9-fold. In contrast, NOTC4 in row E, column 06 is dark red in colour as it is upregulated by approximately 1.7 fold (Figure 5.1, A and B). Furthermore, genes were analysed in a hierarchical clustering model to group the genes based on their expression in tissues (Figure 5.1, C). For example, as shown in the

dendrogram, CXCL6 and VEGFC share common gene expression pattern. This pattern is then similar to VEGFA, HIF1A, CTGF and SERPINF1. Both groups are then grouped with FLT1 and so on. The clustergram also groups genes that are similar by colour coding, noted in figure 5.1, C, the upper more upregulated genes are grouped together whereas the downregulated genes are grouped in the lower light green area.

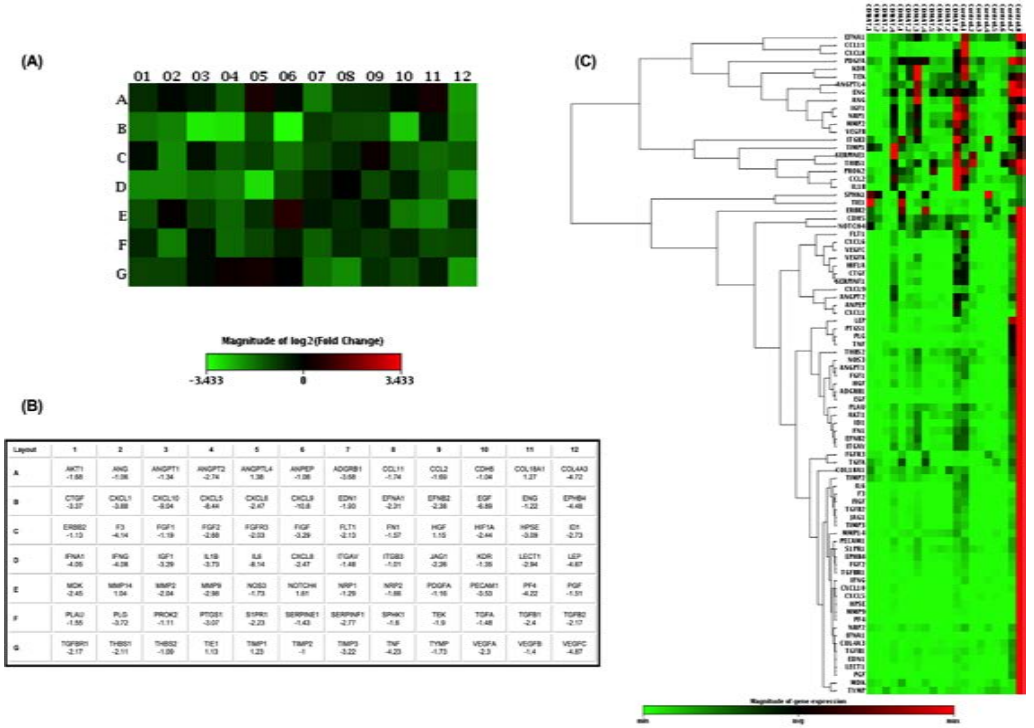


Figure 5.1. Angiogenic gene expression in mesenteric adipose tissue of Crohn's disease patients compared to control (n=8 per group). A) Heat map showing the magnitude of fold change in colour: bright green downregulated and red is upregulated. B) Precise values of fold regulation of angiogenic genes mirroring the heat map above. C) Clustergram and a dendrogram of gene expression pattern grouped in a hierarchal manner, with majority of angiogenic gene showing downregulation light green.

There were no significantly upregulated angiogenic genes in the test group, nor were there genes with a 2 or more-fold increase. However, there were a total of 7 genes with one or more-fold increase: five of these were proangiogenic (ANGPTL4, HGF, MMP14, and TIE1) and two were antiangiogenic (COL18A1, NOTCH4, and TIMP1) (Figure 5.2) (Table 5.2 a summary of genes angiogenic function).

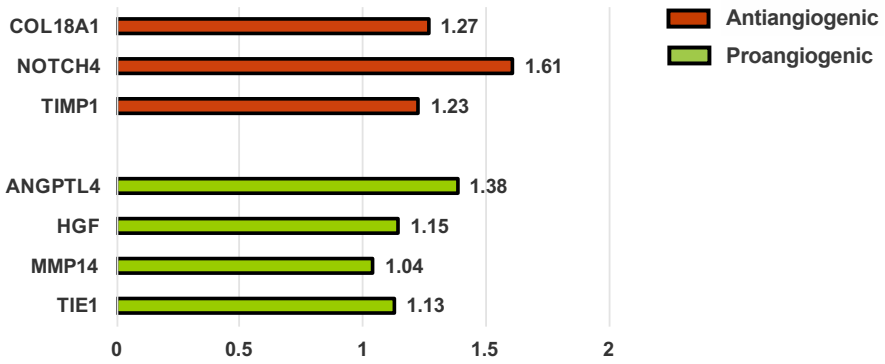


Figure 5.2. Genes upregulated by one, in the test group

5.3.1. Gene expression correlation matrix

The quantification gene expression method was used to calculate the 2^(-Avg.(Delta(Ct)) for each individual gene. This value is dependent on the housekeeping gene for each respective sample. From these values a correlation matrix was created using Spearman Correlation Coefficient (*r*), since the data was non-parametric. The disease tissue was analysed separately to the control tissue, in order to compare the pattern of correlation among genes in the groups. A total number of 6972 correlations were performed; 3486 (50%) for each group correlating all the gene values. A correlation matrix was generated

showing a difference in pattern between the disease and the control group (Figure 5.3, A). In order to demonstrate significant differences in the correlations between the two groups, inference statistics were used to test the difference in proportions of positive and negative correlations. There were significantly more positive correlations in the control group 3377 (96.9%) than in the disease group 2775 (79.6%); and significantly more negative correlations in the disease group 711 (20.4%) than in the control 109 (3.1%) ($X^2_{(1)}$=501, p<0.0001) (Figure 5.3, C).

The strength of correlations, whether positive or negative, was examined by inference statistics. Because, *r* values can only lie between (0 to 1) if positive, and (-1 to 0) if negative, it is not possible to generate confidence intervals and using *t*-test was inappropriate. Instead, Fissure transformation of the *r* values to *z* scores was used to test the average strength of correlations. The mean of the *z* score for the positive correlation demonstrated statistically significantly (p<0.0001) stronger correlation in the control group (mean=1.02, SD: 0.6; median=0.95, range: 0, 3) than in the disease group (mean=0.8, SD: 0.5; median=0.7, range: 0, 2.8) (Figure 5.3, C). Furthermore, the mean of the *z* score for the negative correlation demonstrated statistically significantly (p<0.0001) stronger correlation in the disease group (mean=-0.3, SD: 0.2; median=0.3, range: -.08, -0.007) than in the control group (mean=-0.1, SD: 0.1; median=-0.1, range: -0.7, -0.02) (Figure 5.3, D).

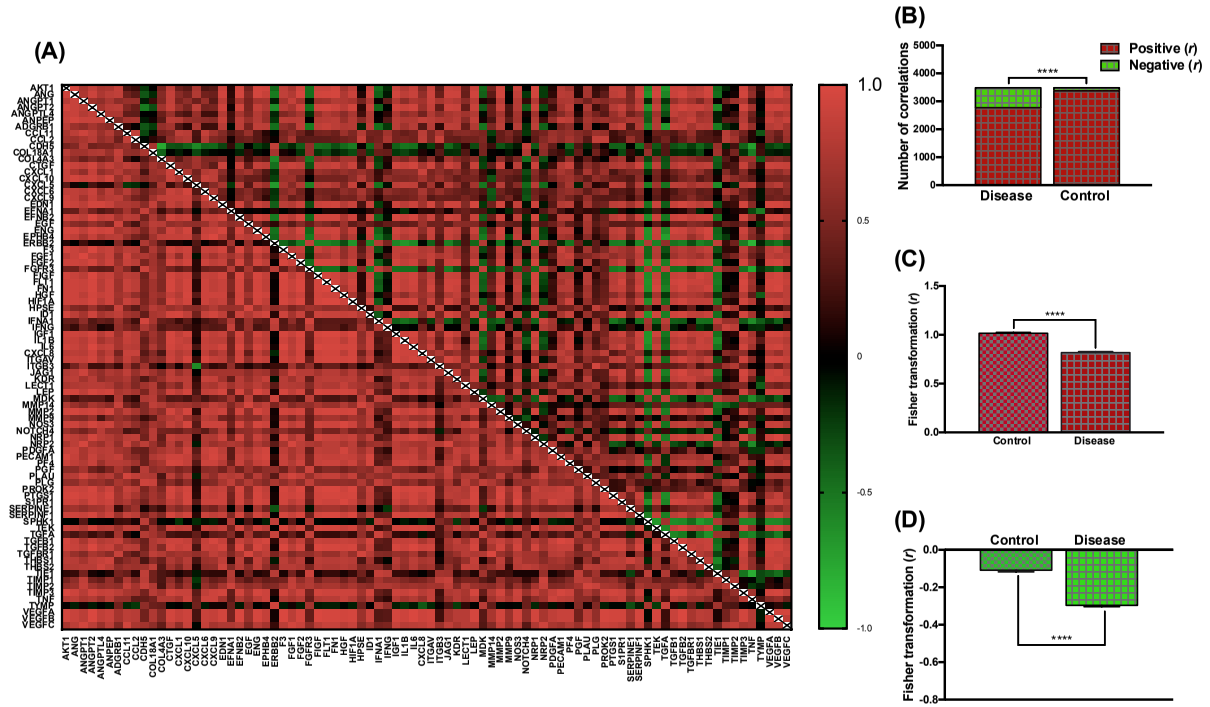


Figure 5.3. Correlation matrix of angiogenic gene expression levels (2^(-Avg.(Delta(CT)). The proportion of negative correlations between angiogenic genes is significantly higher in the disease group, top have of the matrix (A, green small squares), than in the controls, bottom half of the matrix (A). Inference statistics demonstrating significantly different proportions of positive and negative correlations, between the groups (B). In average the Fisher transformation (*z*) of Spearman correlation (*r*) showing statistically stronger positive correlation in the control group than in the disease (C), and stronger negative correlation in the disease group than in control (D). ****p<0.0001.

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
ADGRB1 (--)	Brain-specific angiogenesis inhibitor 1	Inhibits the migration of endothelial cells and supresses angiogenesis.	(Stephenson et al., 2013, Cork et al., 2012, Kaur et al., 2005)
ANGPL4 (++)	Angiopoietin-like 4	Promotes blood vessel formation and stimulates the formation of tubules from endothelial cells. Prevents endothelial cells apoptosis.	(Zhu et al., 2002, Kim et al., 2000)
ANGPT2 (++)	Angiopoietin 2	Regulator of vessel maturation and remodeling.	(Felcht et al., 2012)
COL18A1 (--)	Collagen, type XVIII, alpha 1	Potent inhibitor of endothelial cell proliferation and migration. Induces endothelial cell apoptosis.	(Passos-Bueno et al., 2006, Dhanabal et al., 1999)
COL4A3 (--)	Collagen, type IV, alpha 3 (Goodpasture antigen)	Inhibits bFGF stimulated endothelial cells proliferation, and induces apoptosis.	(Pasco et al., 2005, Maeshima et al., 2000a, Maeshima et al., 2000b)
CTGF (+)	Connective tissue growth factor	Triggers HIF1A-dependent VEGF expression and angiogenesis. Promotes endothelial cells growth,	(Brigstock, 2002, Moussad and Brigstock, 2000, Liu et al., 2014)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
		migration, adhesion and survival.	
CXCL1 (++)	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Stimulates endothelial cell proliferation, migration and tube formation.	(Wang et al., 2006, Strieter et al., 1995)
CXCL10 (--)	Chemokine (C-X-C motif) ligand 10	Interferons' with the proangiogenic activity of IL8 and bFGF. Inhibits endothelial cell differentiation.	(Arenberg et al., 1996, Angiolillo et al., 1995, Luster et al., 1995)
CXCL5 (++)	Chemokine (C-X-C motif) ligand 5	A promoter of neovascularisation.	(Rowland et al., 2014, Walz et al., 1997, McMellen et al., 2010)
CXCL6 (+)	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	Contribute to the neovascularisation in an autocrine way. With IL8 it contributes to endothelial cell proliferation.	(Addison et al., 2000b, Belperio et al., 2000, Gijsbers et al., 2005)
CXCL8 (++)	Interleukin 8	Promotes endothelial cell proliferation.	(Koch et al., 1992)
CXCL9 (--)	Chemokine (C-X-C motif) ligand 9	Suppresses angiogenesis by Increasing the disruption of endothelial cell-cell contact.	(Romagnani et al., 2001, Keeley et al., 2008)
EFNA1 (++)	Ephrin-A1	Endothelial cell migration.	(Song et al., 2013)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
EFNB2 (+)	Ephrin-B2	Regulates the endothelial cells cytoskeletal organisation, motility, invasiveness and adhesion to ensure the efficient growth of blood vessels and lymphatics.	(Wang et al., 2010)
EGF (++)	Epidermal growth factor	Endothelial cell proliferation and antiapoptosis.	(Sasaki et al., 2007, Yazici et al., 2005)
EPHB4 (++)	EPH receptor B4	Promotes endothelial cell migration capillary formation and vascular sprouting.	(Gerety et al., 1999)
F3 (++)	Coagulation factor III (thromboplastin, tissue factor)	Promotes angiogenesis by inhibiting the negative control of protein-activated receptor (PAR)-2.	(Hobbs et al., 2007, Belting et al., 2004)
FGF2 (++)	Fibroblast growth factor 2 (basic)	Triggers vessel formation by promoting the growth and differentiation of vascular endothelium.	(Andres et al., 2009)
FGFR3 (++)	Fibroblast growth factor receptor 3	Stimulates the proliferation of endothelial cells and induces the release of angiogenic factors from other cell types.	(Murakami and Simons, 2008, Presta et al., 2009, Bono et al., 2013)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
FIGF (++)	C-fos induced growth factor (vascular endothelial growth factor D)	Endothelial cell growth.	(Achen et al., 1998, Achen and Stacker, 1998)
FLT1 (++)	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	Activated by VEGF increases vessel permeability and endothelial cell migration.	(Hiratsuka et al., 2005, Shibuya, 2006)
HGF (++)	Hepatocyte growth factor (hepapoietin A; scatter factor)	Stimulates endocellial cell mitogenesis, motility and extracellular matrix invasion.	(Van Belle et al., 1998)
HIF1A (++)	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	Promotes angiogenesis by activation of VEGF.	(Forsythe et al., 1996)
HPSE (+)	Heparanase	Facilitates endothelial cell proliferation and migration. It is upregulates proangiogenic factors such as MMP9, VEGF, HGF in cancer.	(Purushothaman et al., 2008, Folkman et al., 1988, Elkin et al., 2001, Vlodavsky et al., 1996)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
ID1 (++)	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	Endothelial cell growth and differentiation.	(Nair et al., 2014)
IFNA1 (--)	Interferon, alpha 1	Regulates endothelial cell motility and survival with the downregulation of proangiogenic factors such as IL8, MMP9, bFGF.	(Albini et al., 2000, von Marschall et al., 2003)
IFNG (+)	Interferon, gamma	INFG produced by T-helper cells may be antiangiogenic leading to vessels destruction. However, recent research suggested that INFG secreted by natural killer (NK) cell has proangiogenic function by promoting enhanced VEGF expression by macrophages.	(Ibe et al., 2001, Qin et al., 2003, Lee et al., 2014)
IGF1 (++)	Insulin-like growth factor 1 (somatomedin C)	Promotes endothelial cell proliferation and vascular sprouting.	(Hellstrom et al., 2001)
IL1B (++)	Interleukin 1, beta	Induces neovascularisation.	(Voronov et al., 2014, Friesel and Maciag, 1999)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
IL6 (++)	Interleukin 6 (interferon, beta 2)	Enhances angiogenesis and tubule formation through VEGF upregulation.	(Hashizume et al., 2009)
JAG1 (+)	Jagged 1	Angiocrine function, regulation of endothelial branching and vascular maturation.	(Benedito et al., 2009)
LECT1 (--)	Leukocyte cell derived chemotaxin 1	Suppresses endothelial cell proliferation.	(Miura et al., 2010, Oshima et al., 2003)
LEP (++)	Leptin	Induces endothelial cell proliferation and expression of matrix metalloproteinases and inhibition of tissue inhibitors of metalloproteinases (TIMPs).	(Park et al., 2001)
MDK (+)	Midkine (neurite growth-promoting factor 2)	Endothelial cell proliferation.	(Kato et al., 2000)
MMP14 (+)	Matrix metallopeptidase 14 (membrane-inserted)	Regulator of angiogenesis by mediating the degradation of ECM and vascular regression.	(Chang et al., 2016, Zheng et al., 2013, Aplin et al., 2009)
MMP2 (++)	Matrix metallopeptidase 2 (gelatinase A, 72kDa	Extracellular matrix degradation to support	(Doyle and Haas, 2009)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
	gelatinase, 72kDa type IV collagenase)	endothelial cell proliferating and migration.	
MMP9 (++)	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	Extracellular matrix degradation and regulation of endothelial cell migration.	(Funahashi et al., 2011)
Notch4 (--)	Notch4	Downregulates VEGF and VEGFR. Inhibits endothelial cells sprouting.	(MacKenzie et al., 2004, Leong et al., 2002)
PECAM1 (++)	Platelet/endothelial cell adhesion molecule	Endothelial cell-cell adhesion for new vessel formation.	(Tzima et al., 2005)
PF4 (--)	Platelet factor 4	Inhibits endothelial cell proliferation and migration.	(Bikfalvi, 2004, Wang and Huang, 2013)
PLG (+)	Plasminogen	Regulates pericellular proteolytic activity, degradation of extracellular matrix and and endothelial cell migration.	(Kindzelskii et al., 2004, Chen et al., 2013)
PTGS1 (++)	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	Promotes endothelial cell migration.	(Abbas et al., 2014)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
S1PR1 (+)	Sphingosine-1-phosphate receptor 1	Inhibits endothelial cell sprouting and enhances cell-to-cell adhesion.	(Ben Shoham et al., 2012, Gaengel et al., 2012)
SERPINF1 (--)	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	Decreases VEGF expression and and microvascular density.	(Dawson et al., 1999, Becerra and Notario, 2013)
TGFB1 (+)	Transforming growth factor, beta 1	Regulation of angiogenesis by stimulating a complicated intracellular signalling pathway consisting of receptor-activated Smads. Smad3 activation is proangiogenic as it stimulates VEGFA expression, whereas Smad2 has an antiangiogenic effect by mediating TSP1 and sFlt1 expression.	(Nakagawa et al., 2004)
TGFB2 (+)	Transforming growth factor, beta 2	TGFB1 and TGFB2 together enhance tubulogenesis. They may inhibit angiogenesis individually.	(Holifield et al., 2004)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
TGFBR 1 (+)	Transforming growth factor, beta receptor 1	Regulation of vascular morphology and function through Smad activation.	(Holifield et al., 2004, Jakobsson and van Meeteren, 2013, Loeys et al., 2005)
THBS1 (--)	Thrombospondin 1	Disrupts the formation of capillary like structures of endothelial cells and can induce their death.	(Nakagawa et al., 2004, Maroni and Davis, 2011)
TIE1 (+)	Sphingosine kinase 1	TIE1 sustains ANG1/TIE2 signalling in remodelling stalk cells, promoting endothelial cell survival.	(Limaye et al., 2009, Savant et al., 2015)
TIMP1 (--)	TIMP metallopeptidase inhibitor 1	Inhibits endothelial cell proliferation and migration.	(Thorgeirsson et al., 1996, Reed et al., 2003)
TIMP3 (--)	TIMP metallopeptidase inhibitor 3	Inhibits angiogenesis by blockage of VEGF binding to VEGFR2	(Qi et al., 2003)
TNF (++)	Tumour necrosis factor	Induces neovascularisation.	(Tertil et al., 2014)
VEGFA (++)	Vascular endothelial growth factor A	Increase vascular permeability, and stimulates the migration of endothelial cells.	(Ferrara et al., 1992)

Table 5.2. Angiogenic genes with 2 or more decrease in fold regulation, and 1 or more increase in fold regulation. Gene name and a summary of its angiogenic function. Proangiogenic (++). Positively or negative angiogenesis regulator (+). Antiangiogenic (--).

Gene symbol	Name	Function summary	References
VEGFC (++)	Vascular endothelial growth factor C	Promotes and regulates proliferation, migration and differentiation of endothelial cells. Also, increases vascular permeability and contributes to the angiogenesis of lymphatic vasculature.	(Joukov et al., 1997)

5.3.2. Angiogenic gene function

In order to analyse gene expression data, in a way that is reflected on their functional ability to maintain angiogenic balance, a literature search was conducted using PubMed database to establish the angiogenic function of each gene. The search was specifically undertaken on genes with 2 or more-fold decrease expression in the test group and those with one or more increase in gene up-regulation. Based on the outcome from the literature these genes were classified into three categories (Ribatti et al., 2007): 1) proangiogenic group, those which promote angiogenesis; 2) positive or negative angiogenic regulator: those that positively or negatively regulate rather than promote angiogenesis; and 3) antiangiogenic: those with functions that inhibit angiogenesis. Table 5.2, has a summary of the functions of those angiogenic genes.

5.3.3. Fold regulation of angiogenic genes

Out of 47 genes with 2 or more decrease in fold regulation, a total of 10 (21%) were classified as antiangiogenic (Figure 5.4). Genes with antiangiogenic function include: ADGRB1 (3.7), COL4A3 (4.7), CXCL10 (9), CXCL9 (11), IFNA1 (4), LECT1 (3), PF4 (2), SERPINF1 (2.8), THBS1 (2), and TIMP3 (3). Furthermore, 12 (26%) genes were classified as positive or negative angiogenic regulators. These genes include: CTGF (3.4), CXCL6 (2.5), EFNB2 (2.4), HPSE (3.1), IFNG 4.1), JAG1 (2.3), MDK (2.5), PLG (3.7), S1PR1 (2.2), TGFB1 (2.4),

TGFB2 (2), and TGFBR1 (2). Finally, a total of 25 (53%) genes were classified as proangiogenic. Genes with proangiogenic function include: ANGPT2 (2.7), CXCL1 (3.9), CXCL5 (8.4), CXCL8 (2.5), EFNA1 (2.3), EGF (6.9), EPHB4 (4.5), F3 (4), FGF2 (2.7), FGFR3 (2), FIGF (3.3), FLIT1 (2), HIF1A (2.4), ID1 (2.7), IGF1 (3.3), IL1B (3.7), IL6 (8), LEP (4.7), MMP2 (2), MMP9 (3), PECAM1 (3.5), PTGS1 (3.1), TNF (4.2), VEGFA (2.3), VEGFC (4.9).

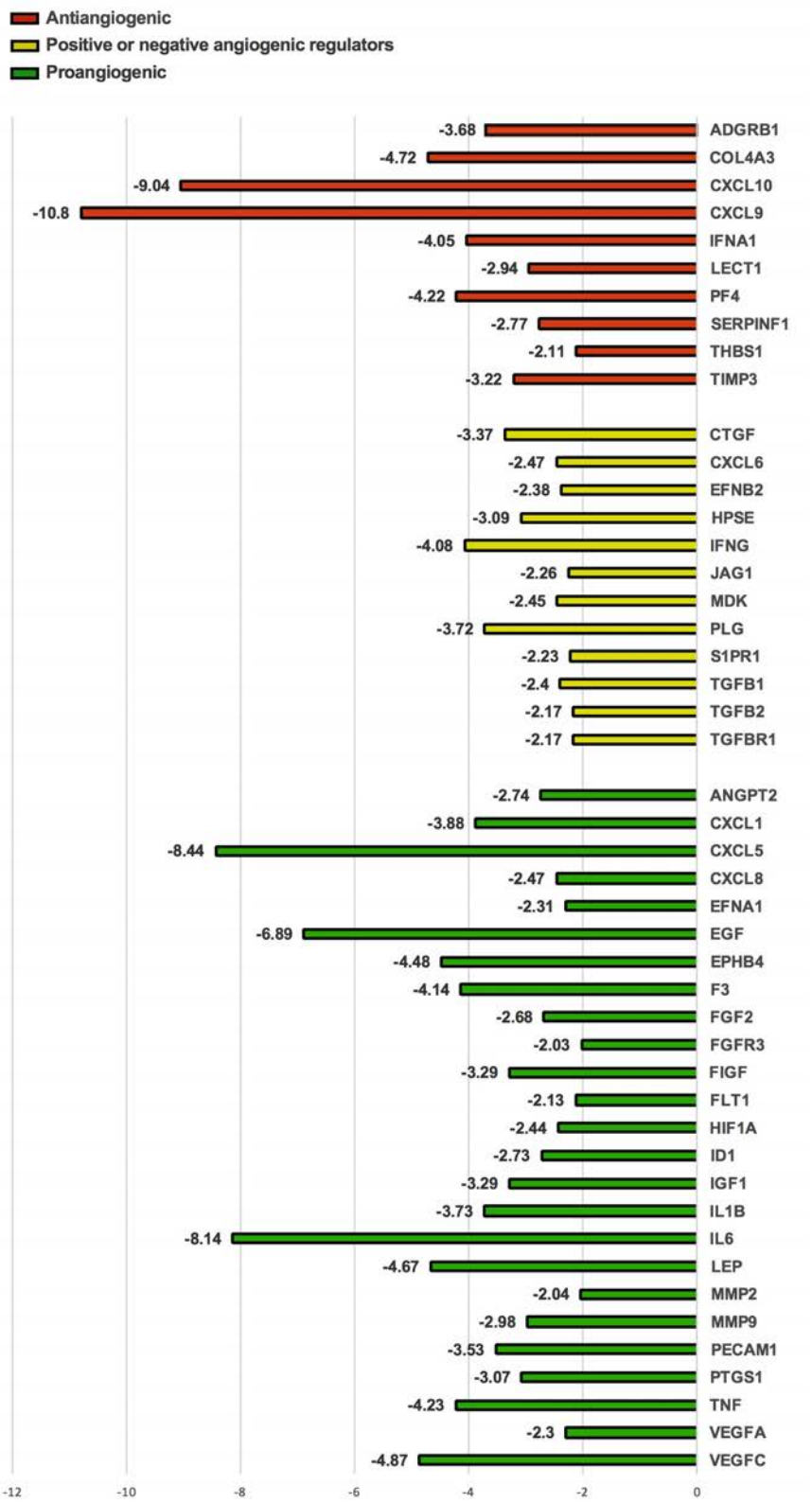


Figure 5.4. Fold regression of antiangiogenic genes (red bars), genes that may positively or negatively regulate angiogenesis (yellow bars) and proangiogenic genes (green bars). Explanation of gene symbols and function is summarised in table 2.

5.3.4. Individual angiogenic genes expression:

Individual gene expression was quantified by calculating the 2^(-Avg.(Delta(Ct)) for levels comparison. Antiangiogenic gene expression was lower in the disease group than controls (Figure 5.4, A). However, the only statistically significant level was observed for CXCL9. The expression of CXCL9 was statistically significantly (p<0.05) lower in the disease tissue (mean=0.05, SD: 0.12; median=0.004, range: 0.0002, 0.4) than in the control tissue (mean=0.21, SD:0.4; median=0.07, range: 0.002, 1.2).

Although not statistically significant, the gene expression levels of those genes positively and negatively regulate angiogenesis, depending on the stimulus, were also downregulated in the disease group (Figure 5.4, B). Furthermore, proangiogenic gene expression in the disease tissue was downregulated in comparison to control (Figure 5.4, C). Specifically, CXCL5 and EFNA1 were downregulated with statistically significant 2^(-Avg.(Delta(Ct)) values. The gene expression level of CXCL5 was statistically significantly (p<0.05) lower in the disease tissue (mean=0.002, SD: 0.003; median=0.0003, range: 0.0001, 0.011) than in the control tissue (mean=0.03, SD: 0.07; median=0.002, range: 0.0003, 0.2). The gene expression level of EFNA1 was statistically significantly lower in the disease tissue (mean=0.008, SD: 0.007; median=0.006, range: 0.001, 0.03) than in the control tissue (mean=0.02, SD: 0.01; median=0.01, range: 0.005, 0.05).

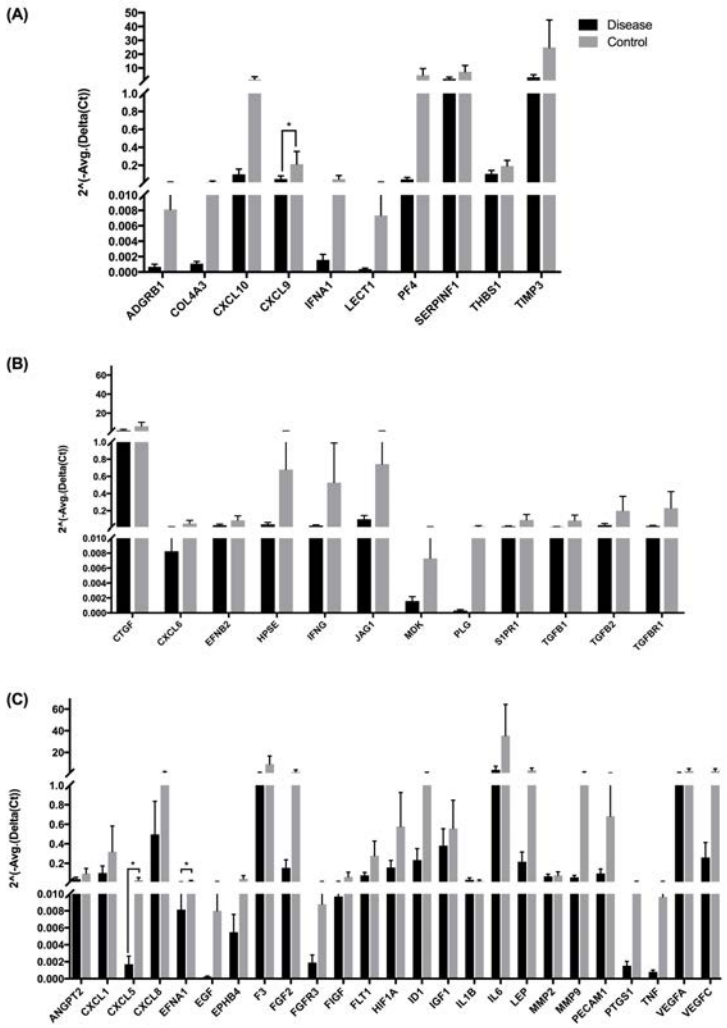


Figure 5.5. Individual gene expression in the disease and control group measured by 2^(-Avg.(Delta(Ct)). A) antiangiogenic genes, B) Angiogenic regulatory genes, C) proangiogenic genes. *p<0.05.

5.3.5. Proangiogenic, antiangiogenic ratio

This section will explain the difference in ratios between pro- and antiangiogenic genes. To measure whether the ratios of gene expression levels differ between disease and control. The gene expression calculated by 2^(-Avg.(Delta(Ct)) for individual proangiogenic and antiangiogenic genes were separated in groups. This would demonstrate deficiency in genes expression in relation to the angiogenic balance.

The means of ratios for VEGFA/CXCL9 and VEGFC/THBS1 were statistically significantly different (p<0.5). VEGFA/CXCL9 was higher in the disease tissue (mean=56.8, SD=62.6) than in the control tissue (mean=9, SD=10.8) (Figure 5.6.).

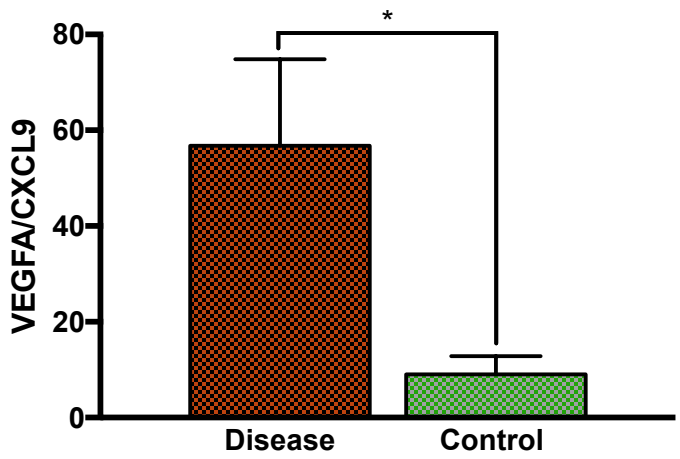


Figure 5.6. The ratio of VEGFA and CXCL9 gene expression in the disease and control tissue. *p<0.05.

The ratio of gene expression of VEGFC and THBS1 was lower in the disease tissue (mean=3.2, SD=3) than in the control tissue (mean=20.8, SD=29) (Figure 5.7).

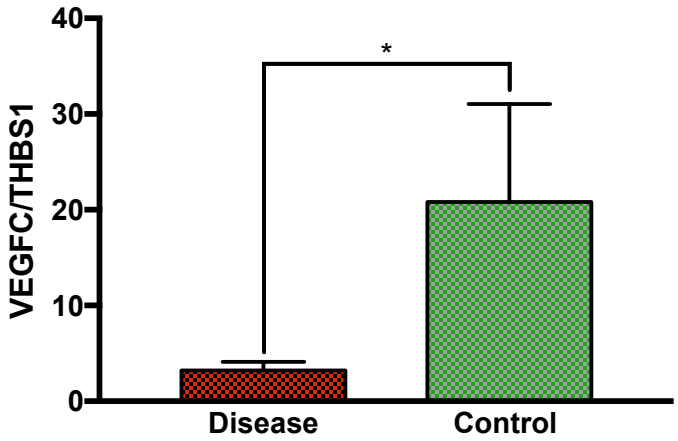


Figure 5.7. The ratio of VEGFC and THBS1 gene expression in the disease and control tissue. *p<0.05.

5.4. Validation of angiogenic gene expression

The expression of selected angiogenic genes from the group of genes that had 2 or more fold regulation were assessed in order to validate the above presented gene array experiment. Identical quantification methods used for the RT^2 Profiling^{TM} PCR array were adopted, but with less genes and larger sample size. Furthermore, the approach to the analysis was similar to the above including: comparative and quantitative methods for gene expression.

A total number of 59 AT were examined, out of which 29 (49%) were considered as the disease group and 30 (51%) controls. The disease tissue were wrapping AT (*n*=11) and ileocacal mesenteric AT (*n*=18) from CD patients. The control tissue were ileocaecal mesenteric AT (*n*=13), omental AT (*n*=3), and subcutaneous AT (*n*=4) from control patients and omental AT (*n*=4), and subcutaneous AT (*n*=6) from CD patients (Figure 5.8).

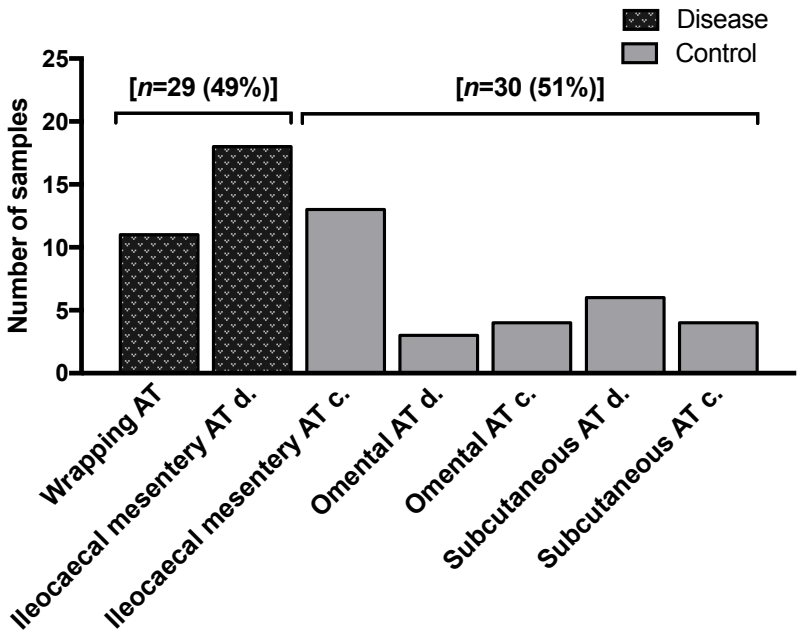


Figure 5.8. Adipose tissue (AT) type and number harvested from disease (d.) and control (c.) patients and used to validate the angiogenic genes expression.

In this section the results are presented as fold regulation as well as difference in expression level based on 2^(-Avg.(Delta(Ct)).

5.4.1. Fold regulation

In the disease tissue, COL3A3 and IL6 genes expression was decreased 1 and 1.6 folds respectively, in comparison to control tissue. On the other hand, CXCL9, MMP2, SERPINF1 and VEGFA genes expression was increased 1.4, 1.3, 1.6 and 1 fold respectively, in comparison to control tissue (Figure 5.9.)

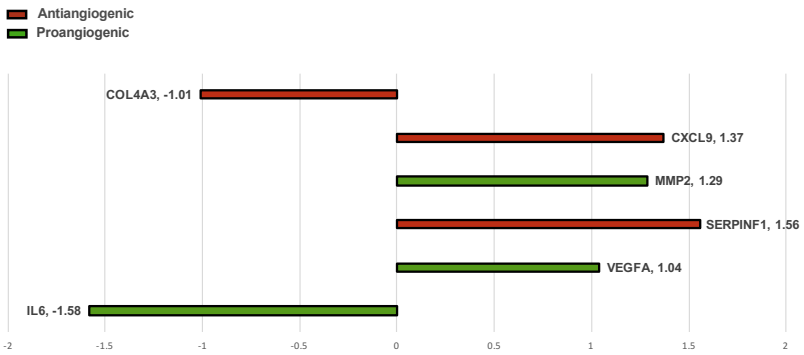


Figure 5.9. Antiangiogenic and proangiogenic fold change.

5.4.2. Angiogenic genes expression levels and ratios of pro and antiangiogenic genes

Although IL6 gene expression were higher in the control group, this did not reach statistical significance. The mean for IL6 level in the test group (mean=7, SD=26) was lower than the controls (mean=31, SD= 147) (Figure 5.10). All other angiogenic genes that were validated were similar between the groups. The means and SDs for the other genes are summarised in table 5.3.

Table 5.3. Summary of the levels of angiogenic gene expression measured by 2^(-Avg.(Delta(Ct)), in the validation experiment.

	Disease Tissue (*n*=29)	Control Tissue (*n*=30)	
	Mean (SD)	Mean (SD)	*p* value
COL4A3	4.4 (8)	2.6 (6)	0.43
CXCL9	1.2 (5.7)	0.1 (0.4)	0.67
IL6	7 (27)	31 (147)	0.76
MMP2	0.3 (0.7)	0.1 (0.1)	0.4
SERPINF1	4.4 (8)	2.6 (6)	0.42
VEGFA	1.3 (2.5)	1.1 (2)	0.49

Note. Please find summary of genes names and function in table 5.2.

In order to assess the difference in angiogenic balance between disease and control, the ratios of pro- and antiangiogenic factors were assessed and compared. Table 5.4 summarises the means and SD for these ratios. These levels were also similar (Figure 5.10).

Table 5.4. Summary of the pro-/antiangiogenic ratios of gene expression levels.

	Disease Tissue (*n*=29)	Control Tissue (*n*=30)	
	Mean (SD)	Mean (SD)	*p* value
IL6/COL4A3	404 (716)	1154 (2468)	0.3
IL6/CXCL9	69 (114)	169 (416)	0.15
IL6/SERPINF1	1 (1.6)	11 (37)	0.13
MMP2/COL4A3	136 (174)	303 (874)	0.85
MMP2/CXCL9	19 (30)	21 (35)	0.94
MMP2/SERPINF1	0.4 (0.4)	0.8 (1.5)	0.61
VEGFA/COL4A3	211 (246)	384 (563)	0.73
VEGFA/CXCL9	35 (52)	61 (117)	0.87
VEGFA/SERPINF1	0.6 (0.6)	2.6 (9)	0.17

Note. Please find summary of genes names and function in table 5.2

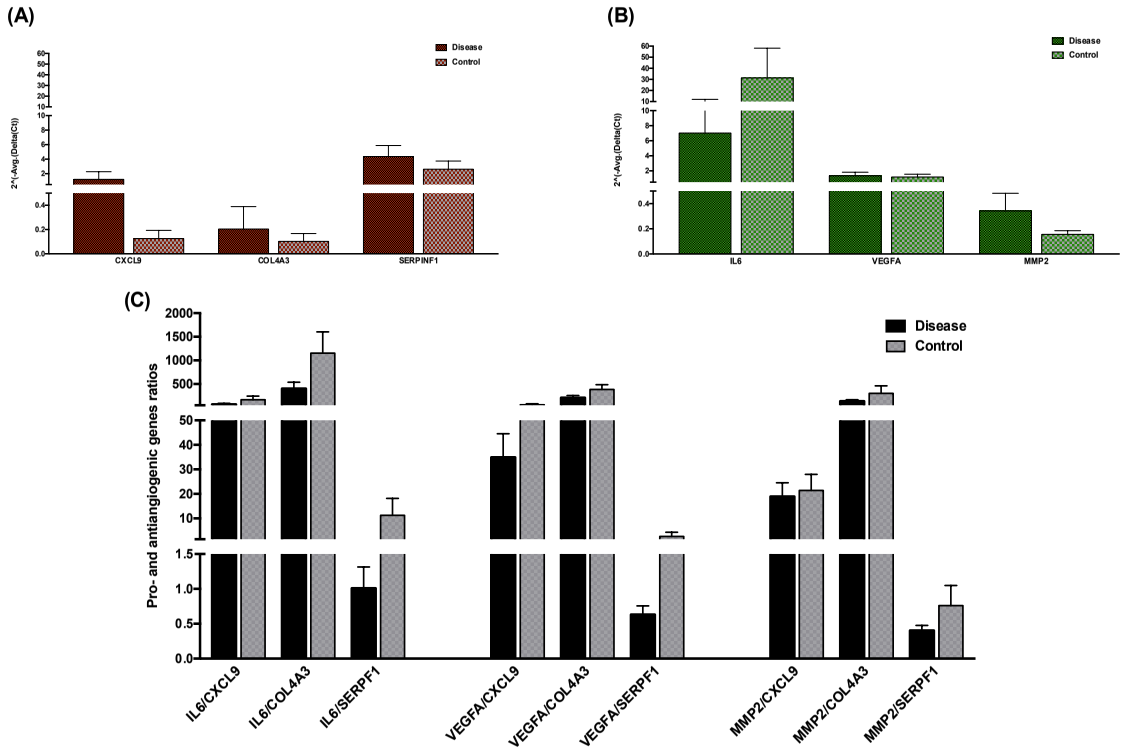


Figure 5.10. Pro- and antiangiogenic gene expression levels measured by 2^(-Avg.(Delta(Ct)). Antiangioenic genes including CXCL9, COL4A3, and SERPINF1 comparison between disease and control groups (A). Proangioenic genes including IL6, VEGFA, and MMP2 comparison between disease and control (B). The ratio of pro- and antiangiogenic genes comparing disease and control patients (C).

5.5. Discussion

Angiogenesis is a complex process that is controlled by a milieu of angiogenic factors, acting together to achieve a balanced response in physiological and pathological processes, such as healing of injured tissue and inflammation (Jackson et al., 1997). The balance required to achieve an intact angiogenic response is tightly controlled by factors that promote angiogenesis, for example VEGF and others that inhibit angiogenesis, for example INFA. Following from experiments reported previously in this thesis, where it was demonstrated that the mesenteric AT in CD patients have higher MVD and more vessel tortuosity, this set of experiments aims to discover whether the angiogenic balance in CD patients is different to control. By assessing the expression of genes involved in human angiogenesis the objectives were to assess their levels, correlations and ratios in relation to their angiogenic function. This would bring evidence to support or reject the hypothesis of this study that angiogenesis in CD mesenteric AT is dysregulated.

The main findings here demonstrated that angiogenic genes in CD are downregulated in comparison to control. The correlations between angiogenic genes were significantly different between the disease and control tissue gene expression. Angiogenic genes correlation are functionally relevant. For example, angiogenic genes, such as HIF1

and IL6 promote the expression of VEGFA (Hashizume et al., 2009, Semenza, 2003, Semenza, 2009). Impaired correlation between genes may potentially result in angiogenic dysregulation. In fact, this is an important indicator that angiogenic mechanisms in CD mesentery may differ from control. Furthermore, in the angiogenic gene array experiment, the proportion of proangiogenic to antiangiogenic gene expression were significantly different. In the validation gene expression experiment, although antiangiogenic factors were relatively higher in the disease patients, these genes were lower when the ratio of pro- and antiangiogenic genes was performed. However, it is difficult to conclude these results as the validation study failed to detect significant differences in the gene expression of important angiogenic genes including, VEGF and IL6.

Although abnormal angiogenesis has been suggested to be a feature of CD (Carmeliet, 2003), detailed information about the angiogenic mechanisms in the inflamed bowel and specifically in the mesentery is essentially non-existent (Laroux and Grisham, 2001, Hatoum and Binion, 2005). Currently, there are no published studies dedicated to characterise angiogenic gene expression in the mesenteric AT in CD. However, few studies have demonstrated an association between angiogenesis and CD in the bowel specimens of CD patients. Recently, Knod *et al*. demonstrated a significant increase in VEGFB and CD bowel specimen (Knod et al., 2016a). The levels for other angiogenic genes including PDGFB, ANG1 and ANG2 was also

elevated but this increase did not reach statistical significance. They also demonstrated a significant increase in MVD in bowel specimens of CD patients.

In this study, the MVD was increased in the mesentery of CD patients (Chapter 4, section 4.3.5). However, the angiogenic gene expression including VEGF and IL6 were downregulated. The apparent disagreement in gene regulation may be accountable as follows. Firstly, angiogenic gene expression in the mesentery is different to that presented in bowel specimens. Secondly, the multiple immunosuppressive drugs used to treat the patients in this study, may have altered the gene expression profile. Keeping in mind that the population investigated by Knod *et al*. are paediatirc patients with CD, who may have a different gene expression profile to adult patients, and are less likely to be chronically treated with multiple immunosuppressive therapy. Lastly, the disease activity in the cohort of patients recruited to this study have less CDAI in comparison to the patients investigated by Knod *et al (Knod et al., 2013, Knod et al., 2016a)*.

The finding of downregulated angiogenesis in this study is also inconsistent with previous research investigating angiogenic factors in CD bowel specimens. Danese *et al*. described upregulated angiogenic factors including VEGF, TNF, bFGF, and IL8 in the intestinal microvascular human endothelial cells cultured from CD and UC patients in comparison to control (Danese et al., 2006). The reason for

the disagreement could be due to the different samples used, as the cells studied in the previous report are cultured vascular endothelium that may have changed their gene expression profile due to their proliferative state. In comparison, this study looked at mesenteric AT samples, that were snap frozen. Therefore, there are two obvious explanations that can be counted for this difference. Firstly, mesenteric AT may have a different angiogenic profile in comparison to the bowel epithelium. Secondly, fresh frozen tissue may have a different angiogenic profile to cells in culture (vitro environment), as explained above. It could be argued, the findings in this study are more accurate as freshly harvested, snap frozen tissue are a more genuine representation of gene expression profile.

One of the most interesting findings in this study is the significant difference in angiogenic gene correlations found between CD and control. In CD mesenteric AT (test group) there were significantly less positive correlations between angiogenic genes and on average weaker correlation coefficients (*r*). Furthermore, the CD mesenteric AT angiogenic gene expression were significantly more negatively correlated than the control. This may suggest that angiogenic genes in the disease tissue have a distinctly different pattern of expression. Functionally, the exhibited angiogenic response may be dysregulated, supporting this study's main hypothesis. For example, VEGF and VEGF receptors, are positively correlated and upregulated in response to hypoxic conditions (Tuder et al., 1995, Dvorak, 2005). Thus, for

VEGF to achieve its function as an inducer of endothelial cell differentiation, migration, survival and permeability control (Ferrara et al., 2003), it typically binds to tyrosin kinase receptors (VEGFR1 and VEGFR2) (Takahashi and Shibuya, 2005, Olsson et al., 2006). In this study both VEGFR1 and VEGFR2 are significantly positively correlated with VEGFA gene expression. Similarly, the expression of VEGF is significantly positively correlated with the expression of MMP2 and MMP9 in human glioblastomas (Munaut et al., 2003). In this study, although the level of MMP2 was positively correlated with VEGF in disease and control tissue, MMP9 was only positively correlated with VEGF in the control tissue and not in the disease tissue. Other angiogenic factors such as FGFR, TGFA, and ERBB2 were significantly strongly correlated with the level of VEGFA in the control tissue, but negatively correlated in the disease tissue. Although more detailed studies are required to highlight the functional implications of these differences, this study suggests that the gene expression levels in CD are dysregulated in comparison to control.

To shed a light on the angiogenic function of the gene array analysed in this experiment, angiogenic genes were classified into three categories: 1) those that promote angiogenesis (proangiogenic); 2) those with either positive or negative angiogenic regulatory function; and 3) those that inhibit angiogenesis (antiangiogenic). A literature review was conducted to confirm the current concluded function of each gene. In order to investigate the angiogenic balance between

pro- and antiangiogenic gene expression, ratios of individual pro- and antiangiogenic genes were calculated and compared. Statistically significant differences were found in the ratios of VEGFA to CXCL9, indicating that although CXCL9 gene expression was down regulated in the disease tissue, relative to VEGF the expression is actually lower. CXCL9 is a potent antiangiogenic chemokine (Addison et al., 2000a) and belongs to the CXC, Glu-Leu-Arg ("ELR" motif) negative family. Specifically, CXCL9 displays antiproliferative and antimigratory effects on VEGF-stimulated endothelial cells by reducing VEGFR2 (KDA), phospholipase Cγ (PLCγ), and exracellular signal-regulated kinase (ERK) phosphorylation (Sulpice et al., 2002). Furthermore, systematic administration of CXCL9 and drugs that promote its action leads to attenuation of neoangiogenesis[6] in experimental liver fibrosis models (Mejias et al., 2009, Tugues et al., 2007) and in pulmonary fibrosis (Keane et al., 1999). The antiangiogenic and antfibrotic function of CXCL9 is confirmed to be mainly through reducing endothelial cell activation rather than anti-inflammatory (Sahin et al., 2012). The angiogenic balance with relatively higher proangiogenic function, may explain the increase MVD reported in the immunohistochemistry in chapter 4 (Section 4.3.5). Functionally, these findings support the hypothesis of this thesis that angiogenesis in CD mesenteric AT is dysregulated, resulting in more vessels, but these vessels are abnormal anatomically with increased tortuosity.

[6] Neoangiogenesis: is the mechanism that permits the creation of new blood vessels to supply tumours and ensure their growth.

On the other hand, although the expression of the antiangiogenic factor THBS1 (Tolsma et al., 1993) was lower in CD mesenteric AT in comparison to control, the ratio of VEGFC (a strong proangiogenic factor) to THBS1 demonstrated a relatively higher expression of THBS1 in the disease tissue. Although not statistically significant, THBS1 expression level is also higher in the disease tissue relative to VEGFA. The mechanisms for which THBS1, also known as thrombospondin 1, in relation to VEGF are mediated by interacting with CD36 on the endothelial cell membrane (Dawson et al., 1997). This appears to inhibit the migration and apoptosis of endothelial cells (Iruela-Arispe et al., 1999). In view of the gene correlation findings, stated above, VEGFA and VEGFB were positively and significantly correlated with THBS1 with Spearman correlation coefficient (r) values of 0.83, 0.62 respectively. However, the correlation was different in the disease tissue: VEGFA correlation with THBS1 was weaker (r=0.16) and VEGFC was stronger (r=0.84). This may indicate that the disease tissue has different angiogenic mechanisms in comparison to control. Furthermore, the angiogenic balance in the disease tissue is different, not necessarily promoted or inhibited, but dysregulated angiogenic response.

This study has strong and well-established methods; the RNA and the PCR quality controls were optimal. The data were analysed in two different validated methods: quantitative and relative. The construction of a correlation matrix to demonstrate differences between multiple

angiogenic genes and illustrate their relationship to each other may explain the dysregulated function in angiogenesis. Comparison between disease and control in the correlation matrix showed significant differences. Furthermore, the comparison based on the angiogenic gene functions and the ratio between proangiogenic and antiangiogenic genes, made clear that the angiogenic balance is deviant in CD mesenteric AT in comparison to control.

Despite these strengths in study design, some unavoidable limitations exist regarding for example less optimal sample size, restricted by the duration of this project. A post hoc sample size calculation showed that to establish statistical power of 80%, this study requires at least 120 samples. This is due to the variability of the standard deviation in gene expression levels. Furthermore, for many genes, the expression levels change dramatically from cell to cell or during various experimental conditions. However, this is not unique to this experiment but a general weakness which applies to gene expression studies (Ren et al., 2007).

Further studies investigating individual genes with larger samples size and exploring the mechanismal pathways of gene function are required to build up and validate these findings. On a larger sample size, structural equation modeling to correlate structure analysis of angiogneic genes in CD, including bowel tissues' gene expression, may aid understanding their functional relationships and improve our understanding of angiogenesis in CD. In addition, angiogenic protein

expression is equally important and requires evaluation. This will be the focus of the following two chapters (Chapter 6 and 7).

In conclusion, angiogenic gene expression in CD mesenteric AT has a dysregulated pattern and a different balance of pro- and angiogenic factors. This may result in the formation of abnormal vessels and the perpetuation of a chronic inflammatory response.

Chapter 6

Angiogenic proteins expression in Crohn's disease mesenteric adipose tissue

6.1. Introduction

Angiogenic cytokine level analysis may provide additional information to the mechanisms of angiogenesis in the mesenteric AT of CD patients. In particular, this experiment will focus on two potent proantiogneic cytokines: IL6 and VEGFA. These two cytokines have been selected because they were down regulated and positively significantly correlated in the disease tissue in the gene expression experiments. Although their signaling pathway will not be the focus of this chapter, it is important to demonstrate their similarity in mechanism of action through STAT and ERK pathways. This is to enable the understanding of their crucial role in the angiogenic mechanisms. While IL6 is principally associated with the inflammatory response, it is also known for its proangiogenic potential (Tzeng et al., 2013, Sainson et al., 2008). Specifically, IL6 binds to the IL6 receptor gp80, a non-signal-tranducing receptor. Subsequently, the signal-transducing receptor gp130 is activated. The IL-6 receptor gp80, and gp130 form a binding complex, which activates downstream signaling molecules, such as STAT3 and ERK1/2 (Zhang et al., 2006, Yang et al., 2003). The activation of ERK1/2 and STAT3 pathways activate endothelial cell proliferation, migration and microvascular tube formation (Yahata et al., 2003, Deo et al., 2002).

Similarly, VEGFA isoforms bind with high-affinity to VEGFR1 (Flt-1) (de Vries et al., 1992, Seetharam et al., 1995) and VEGFR2 (KDR)

(Terman et al., 1991, Terman et al., 1992). VEGFR2 mediates cell proliferation and survival (Vaisman et al., 1990, Fong et al., 1995, Shalaby et al., 1997). VEGFR2 phosphorylation induces the activation of ERK1/2 pathway (Mazure et al., 1997). Specifically, ERK pathway is involved in the regulation of endothelial cell proliferation apoptosis and differentiation (Zhuge et al., 2005, Wang et al., 2016). Furthermore, VEGFR2 mediates the activation of STAT3 pathway (Bartoli et al., 2000, Bartoli et al., 2003). Therefore, deficiency in either cytokines, IL6 or VEGF may in varying degrees impact on the regulation of angiogenesis. The objectives in this experiment are to assess IL6 and VEGF concentrations at the tissue level as well as in the patients' serum.

Subsequently, this chapter has been classified into two main sections: 1) IL6 and VEGF cytokines tissue level, and 2) IL6 and VEGF serum level.

6.2. Tissue expression of IL6 and VEGF

A total number of 69 ATs were analysed for the levels of IL6 and VEGF, out of which 29 (42%) were from CD: 11 (16%) were wrapping mesentery and 18 (26%) were ileocaecal mesentery. The following tissues from control patients were considered control: 12 (17%) ileocaecal mesentery; 6 (9%) omental; and 6 (9%) subcutaneous AT. Furthermore, AT from CD patients other than the mesentery were

classified as control. This includes: 5 (7%) omental and 11 (16%) subcutaneous AT (Figure 6.1).

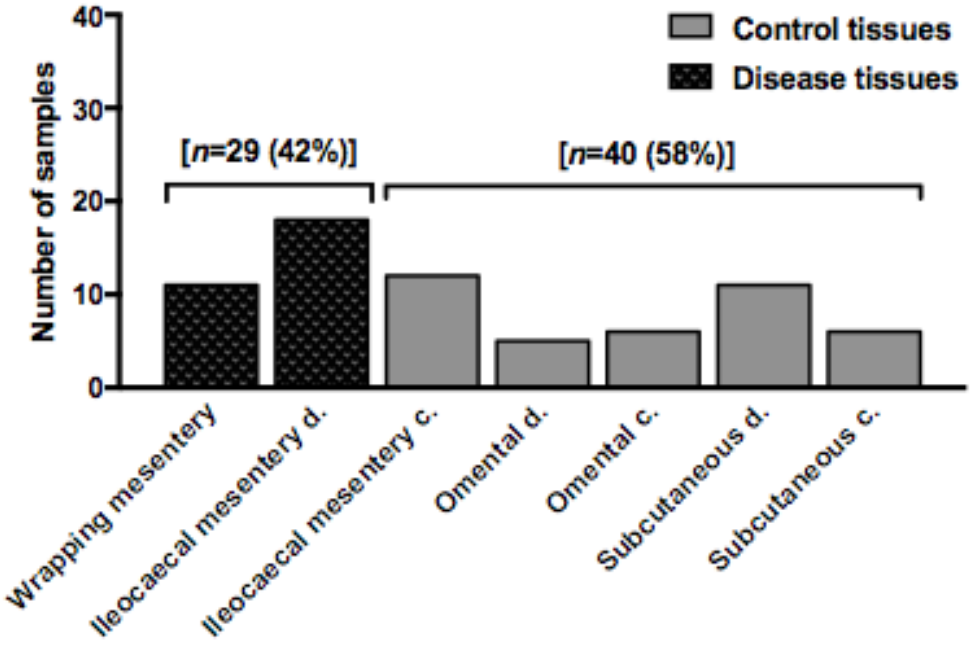


Figure 6.1. Adipose tissue types used in the analysis of Interleukin 6 (IL6) and Vascular Endothelial Growth Factor (VEGF) levels. Disease (d.). Control (c.).

6.2.1. Tissue expression of interleukin 6 (IL6)

The expression of IL6 was statistically significantly (p<0.05) lower in CD wrapping and ileocaecal mesenteric AT (mean=21 pg/mg, SD=18) than in control (mean=39 pg/mg, SD=43) (Figure 6.2). Comparing all tissue type also demonstrated statistically significantly (p<0.05) lower level of IL6 in CD wrapping AT (mean=17 pg/mg, SD=9) in comparison to subcutaneous AT (mean=41 pg/mg, SD=35). There were no other statistically significant levels of IL6 expression in multiple comparison ANOVA.

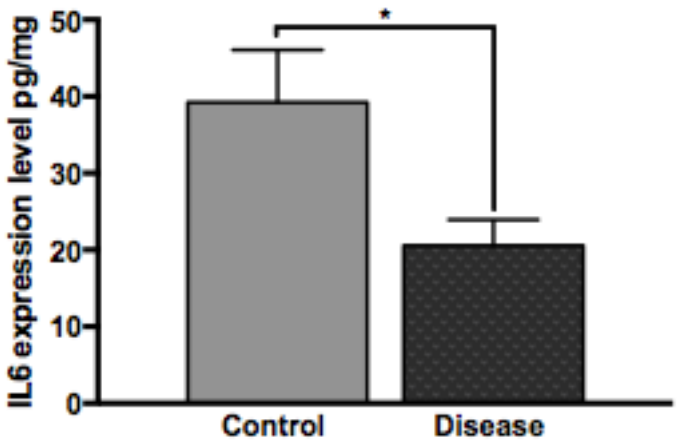


Figure 6.2. Interleukin 6 (IL6) expression in Crohn's disease (test group) and control adipose tissue. *p<0.05.

Paired values from CD patients IL6 expression demonstrated statistically significantly (p<0.05) lower expression of IL6 in CD mesenteric AT (mean=23 pg/mg, SD=24) in comparison to subcutaneous AT (mean=41 pg/mg, SD=35) (Figure 6.3) (Figure 6.3,

B). On the other hand, paired values of IL6 expression in the control patients demonstrated higher mean in the mesenteric AT (mean=22, SD=13) than in subcutaneous AT (mean=20, SD=6), however, this difference was not statistically significant (Figure 6.3, A).

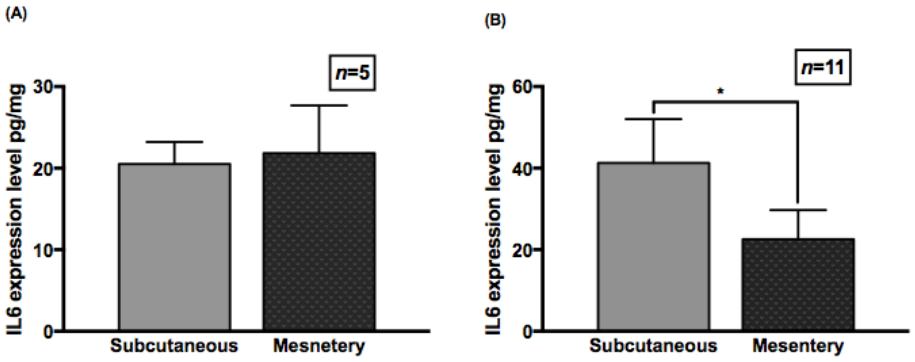


Figure 6.3. Paired interleukin 6 (IL6) expression levels in Crohn's disease (test group) (A) and in control (B). *p<0.05.

6.2.2. Tissue expression of vascular endothelial growth factor (VEGF)

VEGF expression level was significantly (p<0.01) lower in CD wrapping and ileocaecal mesentery (mean=34 pg/mg, SD=19) than in the control AT (mean=57 pg/mg, SD=43) (Figure 6.4).

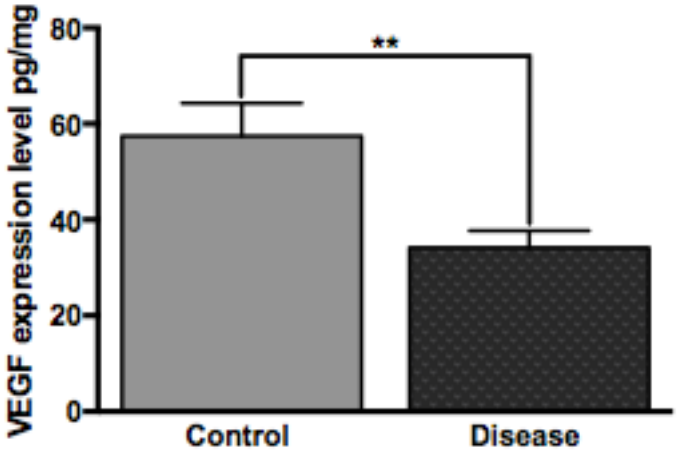


Figure 6.4. Adipose tissue level of vascular endothelial growth factor (VEGF) level expression. **p<0.01.

The means for VEGF expression of all AT types were compared by ANOVA test and showed statistically significant ($p<0.05$) difference between tissue. The means for VEGF expression were 37 (SD=24), 33 (SD=15), 41 (SD=30), 51 (SD=36), 68 (SD=73), 68 (SD=51), 64 (SD=15) for wrapping mesentery, ileocaecal mesentery from CD, ileocaecal mesentery from control, omental from CD, omental from control, subcutaneous from CD and subcutaneous from control respectively (Figure 6.5).

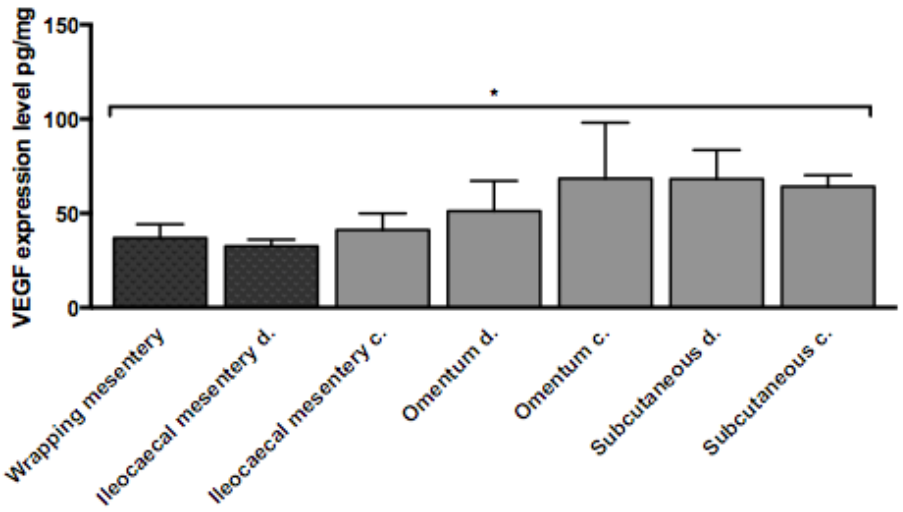


Figure 6.5. Vascular endothelial growth factor (VEGF) expression level across all adipose tissue types. ANOVA test *p<0.05.

Statistically significant (p<0.05) difference was also found in paired values of VEGF expression level in CD patients. The VEGF expression level in the mesenteric AT (mean=36 pg/mg, SD=27) was lower than the subcutaneous AT (mean=68 pg/mg, SD=51) (Figure 6.6, B). There was no statistically significant difference in the paired values of VEGF expression in control patients (Figure 6.6, A).

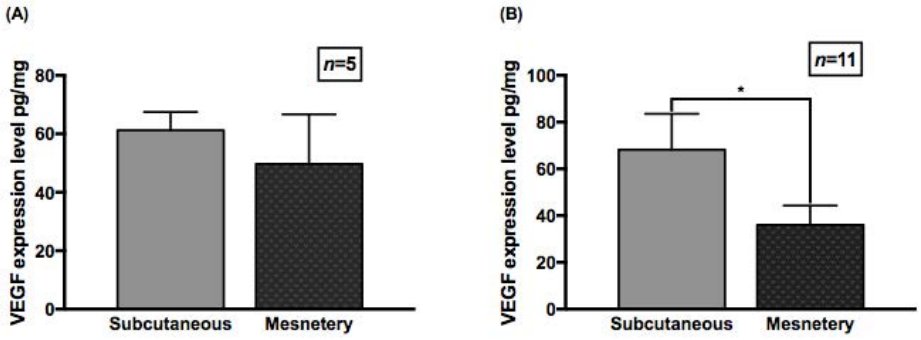


Figure 6.6. Vascular endothelial growth factor (VEGF) expression level in Crohn's disease (A) and control (B) comparing paired values from mesenteric and subcutaneous adipose tissue. *p<0.05.

6.3. Vascular endothelial growth factor (VEGF) correlation with interleukin 6 (IL6):

A strong and statistically significant Spearman correlation was found between VEGF and IL6 tissue expression in CD (r=0.5; 95% CI: 0.2, 0.8; p<0.01) and the control AT (r=0.7; 95% CI: 0.4, 0.8; p<0.0001) (Figure 6.7, B and A).

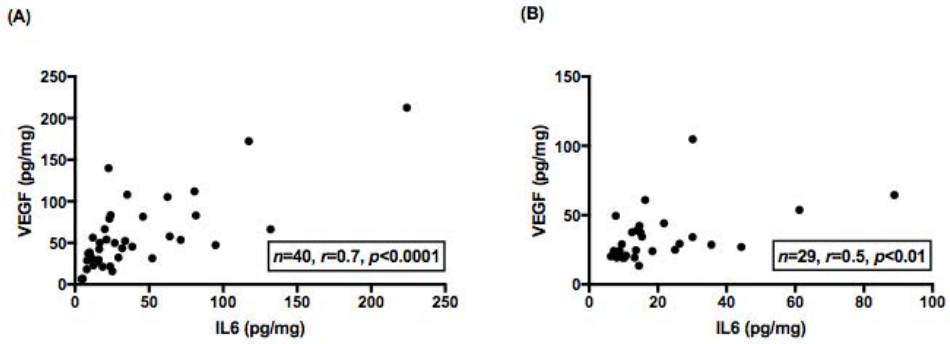


Figure 6.7. Vascular endothelial growth factor (VEGF) and interleukin 6 (IL6) tissue expression strongly correlate in CD (B) and controls (A). *r*: Spearman correlation coefficient.

6.4. Serum levels of interleukin 6 (IL6) and vascular endothelial growth factor (VEGF):

Unlike the analysis of the AT samples, where UC is not known to affect the mesentery and therefore can be considered a control, for the measurement of serum levels of IL6 and VEGF patients with UC were placed on a third category other than the controls. Serum samples of a total number of 36 patients were analysed for the levels of VEGF and IL6, 22 (61%) of which have undergone surgery for CD, 5 (14%) for UC and 9 (25%) controls. The serum of CD (mean=58 pg/ml, SD=138) patients and UC (mean=50 pg/ml, SD=104) had higher IL6 levels than the control (mean=19 pg/ml, SD=39) (Figure 6.8, A). However, this difference in mean was not statistically significant. The mean of VEGF was statistically significantly ($p<0.05$) higher in the CD patients (mean=373 pg/ml, SD=333) and in UC (mean=297 pg/ml, SD=101) than controls (mean=165 pg/ml, SD=109) (Figure 6.8, B).

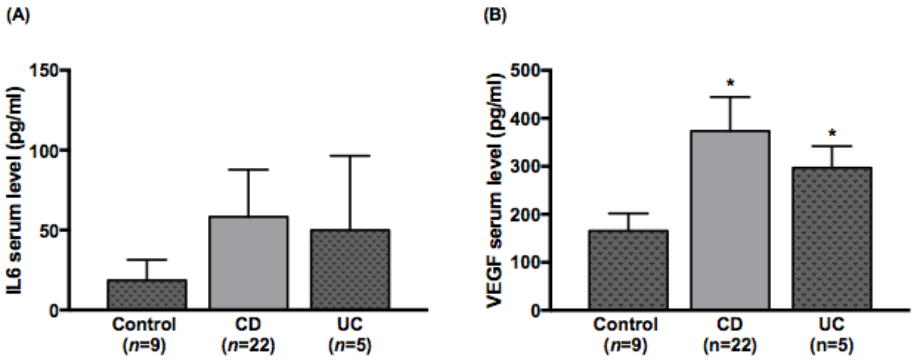


Figure 6.8. Serum level of vascular endothelial growth factor (VEGF) (A) and interleukin 6 (IL6) (B) in Crohn's disease (CD), ulcerative colitis (UC) and control patients.*p<0.05.

Furthermore, there was a significant negative correlation between the levels of haemoglobin and the VEGF in the disease patients (r=-0.5; 95% CI: -0.8, -0.1; p<0.05). In a correlation matrix, there was no significant correlation between the serum levels of IL6, VEGF, CRP, WCC, neutrophils count, haemoglobin count, CDAI, pathological severity, and stricture formation. Finally, there was no significant correlation between the tissue expression of IL6 and VEGF and serum level.

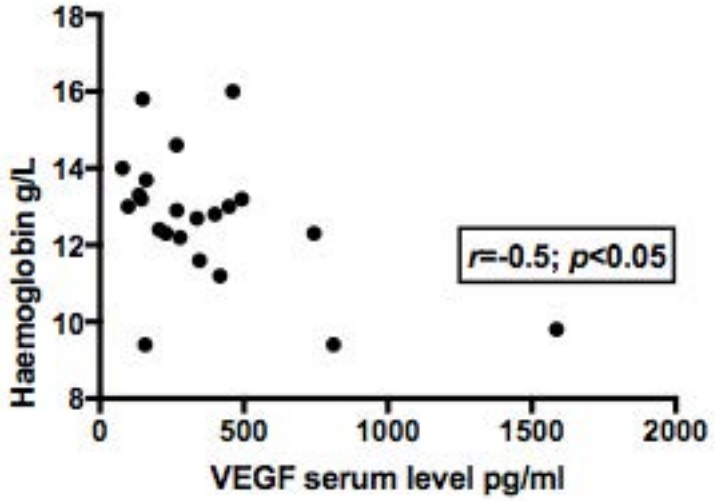


Figure 6.9. Significant negative correlation between vascular endothelial growth factor (VEGF) serum level and haemoglobin blood level.

6.5. Discussion

The interaction between IL6 and VEGF is an example of the interplay between inflammation and angiogenesis (Mahnke et al., 2000, Su et al., 2005, Tzeng et al., 2013). They are both recognised as potent proinflammatory and proangiogenic cytokines and play an important role in the pathogenesis of CD (de Souza and Fiocchi, 2016). The overall aim of this thesis was to characterise angiogenesis in CD mesenteric adipose tissue. In the gene expression experiments, both IL6 and VEGF were down regulated at the level of mRNA. To validate this finding at the protein level this experiment was designed to evaluate the tissue levels of IL6 and VEGF cytokines expression (Maier et al., 2009, Vogel and Marcotte, 2012). Furthermore, the serum levels of IL6 and VEGF were evaluated in CD and control patients.

The main finding in this experiment indicates that the expression of IL6 and VEGF are statistically significantly deficient in the mesenteric AT in this cohort of CD patients in comparison to controls. On the other hand, the serum level of IL6 and VEGF were both higher in CD patients than in control, however, statistical significance was only observed for VEGF. This may suggest that the source of serum increase levels of IL6 and VEGF in CD patients serum is unlikely to be from the wrapping mesenteric AT, but other inflammatory sites, most likely being the bowel in this context. The increase expression of IL6 in CD patients' serum and the cultured bowel mucosa have been previously reported (Mitsuyama et al., 1995, Hosokawa et al., 1999). The only study that investigated the IL6 protein expression level at the mesenteric AT of CD patients, demonstrated similar means with the control tissue (Yamamoto et al., 2005). Another study measured the level of IL6 secreted from mesenteric AT of CD, colonic cancer and diverticular disease, demonstrated similar levels (Paul et al., 2006a). However, the weakness of these studies may be the use of patients undergoing bowel resection for colonic carcinoma as the control group, which may affect the expression level of IL6. For example, IL6 protein and mRNA levels are up regulated in the serum and tissue of patients with colon and other type of cancers (Heikkila et al., 2008). In this experiment however, the control patients are of benign conditions. The explanation of the lower level of IL6 in the mesenteric AT in this cohort of CD patients may be due to the immunosuppressive therapy

(Nawata et al., 1989, Amano et al., 1993). What goes against this speculation however, is the significantly low level of IL6 in the paired values of mesenteric and subcutaneous AT in CD patients. Supported by the similar level found in similarly paired AT from controls, one may conclude that the mesenteric AT in this cohort of CD patients is IL6 deficient. Given the significantly increased inflammation in the mesenteric AT of these patients in the histopathology experiment, this may suggest that the inflammatory process in the mesenteric AT in CD patients is mediated by other proinflammatory cytokines than IL6. An example may be IL23/Th17 pathway, which has been described as a mediator of the chronic inflammation in CD (Duerr et al., 2006, Hue et al., 2006) and is a potential therapeutic target (Massey and Parkes, 2007, Toussirot, 2012).

Similarly, VEGF was up regulated in the bowel mucosa, plasma and serum of patients with CD (Kapsoritakis et al., 2003, Pousa et al., 2008a, Scaldaferri et al., 2009). However, the expression of VEGF from the mesenteric AT of CD has not been investigated before. One study compared the secretion of VEGF from mesenteric AT of patients undergoing bowel resection for CD, bowel cancer and diverticular disease patients. The capacity of CD mesenteric adipose tissue to secrete VEGF in CD patient was significantly higher than controls (Schäffler et al., 2006). Among CD patients, the same study demonstrated a significantly lower level of VEGF secretion in patients treated with steroids than those who were not receiving steroids

(Schaffler et al., 2006). In this study, the expression level of VEGF was significantly lower CD patients than in controls. This may be explained by the immunosuppressive therapy received by the patients included in this cohort. Alternatively, the deficiency of VEGF in CD mesenteric AT may be a validated finding explaining the increase in vessel tortuosity found in the histological analysis (Chapter 4). This is supported by the significant lower level of VEGF mesenteric expression in paired samples of CD patients in comparison to subcutaneous AT, a finding that was not demonstrated in the control group.

Similar results were reported by Kapsoritakis *et al*. investigating the expression of VEGF in the bowel specimens. Although the serum level of VEGF was significantly higher in CD patients than controls. The bowel specimens of CD patients failed to stain for VEGF in all intestinal layers as well as the inflammatory components including lymphocytes and macrophages (Kapsoritakis et al., 2003). Beside the latter study, the increased expression of VEGF in the bowel mucosa of CD patients has been confirmed by several reports (Danese et al., 2006, Tsiolakidou et al., 2008, Alkim et al., 2009, Alkim et al., 2012). It is reasonable to conclude that VEGF is up regulated in CD mucosa and the protein level is higher in the serum and plasma, but further validation needs to be confirm its expression level in the mesentery.

Another interesting finding in this experiment is the positive correlation found between the expression of IL6 and VEGF in both CD and control tissues. This positive correlation has been reported before in malignant conditions, such as pituitary (Borg et al., 2005), breast (Benoy et al., 2002), gastric cancers (Huang et al., 2002), and melanoma (Yang et al., 2009). IL6 and VEGF signaling pathways are highly intertwined. For example, IL6 induces signal transduction of VEGF and regulates VEGF promoter activity (Loeffler et al., 2005). They both activate common pathways, such as STAT3 and ERK1/2 (Bartoli et al., 2003, Yang et al., 2003). In terms of angiogenesis, they both promote endothelial cell proliferation, migration and apoptosis (Zhang et al., 2006). In this cohort of CD patients and control however, the correlation was stronger and more significant in the control group than in CD. Although, the angiogenic mechanisms mediated by IL6 and VEGF appear to correlate positively, in CD mesentery, the deficiency in IL6 and VEGF at this local level may support the hypothesis of this project that the angiogenic mechanisms in the mesentery are dysregulated and may be mediated by other angiogenic factors. Further assessment of these mechanisms is required to draw conclusions from this finding.

This study is distinctive in two main aspects: firstly, for investigating the expression level of two important angiogenic proteins at the level of the mesentery in CD patients; and secondly, for the method used to measure these levels. The majority of the studies conducted in this

regard either used immunohistochemistry of formalin-fixed and paraffin-embedded tissue, or tissue culture to assess the level of protein secreted in the supernatant. However, in this experiment the total protein was extracted from the AT freshly collected and snap frozen to preserve the proteins from degradation. Subsequently, well established ELISA quantification methods were used to precisely quantify the level of protein expression by the tissue. The use of immunohistochemistry to quantify the level of VEGF and IL6 expression may be the reason of conflicting reports of expression levels as described above. Furthermore, in the immunohistochemistry experiment reported in chapter 4, there was no significant difference in the staining for VEGF between CD and control AT, and IL6 staining failed after several attempts. This may be due to the very low expression that was subsequently observed in this experiment. The total protein levels were assessed and the observed level of IL6 and VEGF was divided by the total protein level to ensure accuracy. To prevent protein degradation, protease and peptidase inhibitors were added to the AT sample, which were kept constantly on ice through out the experiment. Furthermore, paired analysis of mesenteric and subcutaneous AT from CD patients was performed to ensure that variables between subjects, such as medication, age, BMI are all adjusted for. Finally, the use of ethylenediaminetetraacetic acid (EDTA) plasma to collect blood samples was avoided to prevent the activated platelets from secreting more VEGF in the ex-vivo stage

(Wynendaele et al., 1999). Instead, serum was immediately isolated and stored in -80 ^{0}C, in order to preserve accurate levels.

Despite the strengths of this experiment, there were unavoidable limitations, including: low sample size and control group who were suffering from pathological conditions, such as diverticular disease, benign polyps and UC. This project had limited funds and time-frame therefore more samples could not be obtained. Also, access to mesenteric tissue biopsy is not feasible from healthy individuals, as patients must have been undergoing bowel resection.

A way to improve this study would be to recruit healthy matched individuals to analyse the serum level of these cytokines. Also, the deficiency of protein expression of IL6 and VEGF in the mesentery could be validated in a larger sample size and potential pathway impairment needs to be explored. As deficiency of these cytokines may be a reason for an abnormal perpetuated chronic inflammation in the mesentery leading to fat wrapping phenomena.

Chapter 7

Crohn's disease mesenteric adipose tissue secretion of angiogenic proteins

7.1. Introduction

It is now known that AT is not only an energy storage organ, but acts as an endocrine organ secreting a variety of cytokines, including: IL-6 (Ahima and Flier, 2000a) and VEGF (Rega et al., 2007). In conditions such as obesity, whereby there is an expansion of AT mass, increase vasculature is observed (Rupnick et al., 2002). Similarly, in CD patients the phenomena of fat wrapping is characterised by expansion of the mesenteric AT to cover the inflamed bowel. In the current project the histological specimens of CD mesentery demonstrated a significant increase in MVD. In other studies related to obesity, differentiation of preadipocytes into adipocytes seemed to be dependent on new blood vessel formation (Fukumura et al., 2003), and the inhibition of angiogenesis appeared to result in reversible weight reduction and AT loss (Dallabrida et al., 2003, Brakenhielm et al., 2004). The effect of antiangiogenic factors on CD mesenteric AT expansion to date is unclear.

On a molecular level, VEGF plays a major role in the AT angiogenesis (Zhang et al., 1997). It is expressed and secreted by AT, preadipocytes and adipocytes (Miyazawa-Hoshimoto et al., 2005). Moreover, up regulated expression of VEGF mRNA is associated with increased AT mass during weight gain (Hattori et al., 2004). In previous chapters (5 and 6) studying VEGF mRNA expression in CD mesentery and subsequently protein expression and serum levels,

appeared that in this cohort of CD patients VEGF was lower than the control group at the mesenteric AT level, and so were the levels of IL6. However, VEGF levels in the serum of CD patients were higher than controls. In this chapter, the ability of CD mesenteric AT to secrete these proteins was tested, by an in vitro assay. This was performed with two objectives in mind: 1) is to ameliorate the possible effect of patients' characteristics such as medications on the AT behaviour; and 2) is to determine the ability of CD mesenteric AT to secrete rather than express IL6 and VEGF.

This chapter is divided into three sections: 1) description of AT used for analysis; 2) assessment of IL6 concentration in the tissue culture supernatant and 2) assessment of VEGF in the tissue culture supernatant.

7.2. Descriptive statistics of adipose tissue used to investigate angiogenic protein secretion levels

In order to assess the level of IL6 and VEGF secretion by the AT, 0.01 g of freshly harvested AT was minced into smaller pieces and incubated in culture media for 36 hours (chapter 2, section 2.5.2.). After which the concentration of the IL6 and VEGF was measured by ELISA (chapter 2, section 2.5.3.). A total number of 69 ATs from CD and non-CD patients were analysed, out of which 28 (41%) were from

CD patients and classified as the disease AT: 14 (20%) were wrapping mesentery and 14 (20%) were ileocaecal mesentery. The following AT from control patients were considered control: 6 (9%) ileocaecal mesentery; 5 (7%) omental; and 5 (7%) subcutaneous AT. Furthermore, AT from CD patients other than the mesentery were classified as controls. This included: 13 (19%) omental and 12 (17%) subcutaneous AT (Figure 7.1).

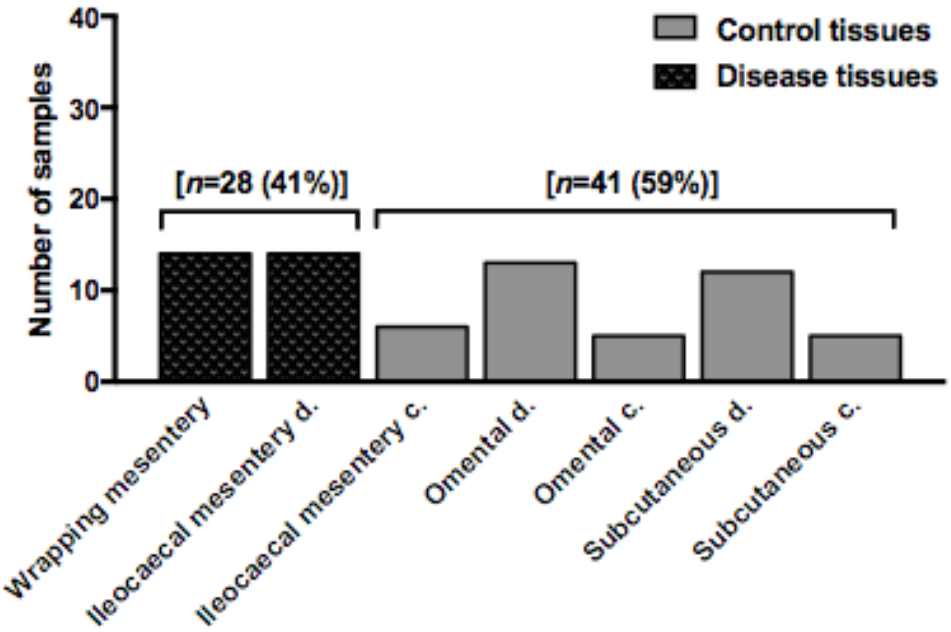


Figure 7.1. Adipose tissue types used in the analysis of Interleukin 6 (IL6) and Vascular Endothelial Growth Factor (VEGF) levels in the supernatant of tissue culture. Disease (d.). Control (c.).

7.3. Adipose tissue's secretion of interleukin 6 (IL6)

The level of IL6 secreted by the test group (wrapping and ileocaecal mesentery from CD) (mean=264 pg/ml, SD=75) was identical to that

secreted by the controls (mean=264 pg/ml, SD=70) (Figure 7.2, A). In order to compare the test group to non-CD AT, the analysis was performed by excluding the subcutaneous and omental AT harvested from CD patients from the control group. This demonstrated similar concentrations of IL6 in the non-CD controls (mean=237 pg/ml, SD90) and the test group (Figure 7.2, B). Furthermore, ANOVA of difference in means between all types of AT showed similar concentrations.

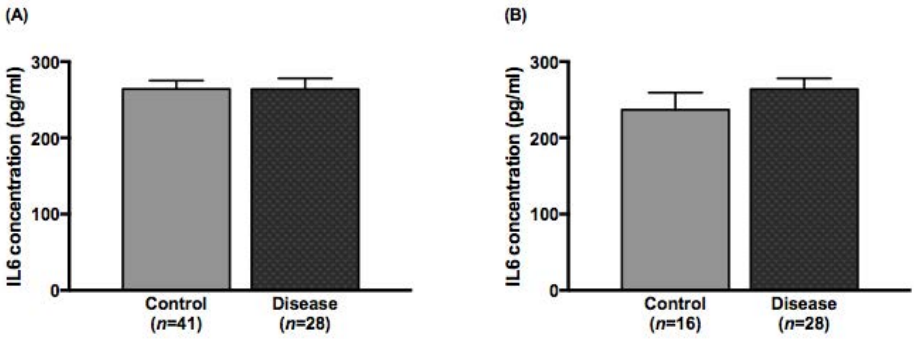


Figure 7.2. Interleukin 6 (IL6) concentration in the supernatant of adipose tissue (AT) in Crohn's disease (CD) and controls (A) and the controls excluding omental and subcutaneous AT from CD patients.

Investigations were also applied to paired samples from the same patients of CD and control. Paired IL6 concentrations in the supernatant from 203 CD patients wrapping (mean=236 pg/ml, SD=105), ileocaecal (mean=294 pg/ml, SD=18), omental (mean=286 pg/ml, SD=52), and subcutaneous (mean=277 pg/ml, SD=50) AT were similar (Figure 7.3, A). Similarly, paired levels of IL6 concentration in the ileocaecal mesentery (mean=258 pg/ml, SD=79), omentum (mean=279 pg/ml, SD=44), and subcutaneous (mean=174 pg/ml, SD=119) AT in the control patients were also similar (Figure 7.3, B).

Repeated measure ANOVA comparing these means demonstrated no significant difference.

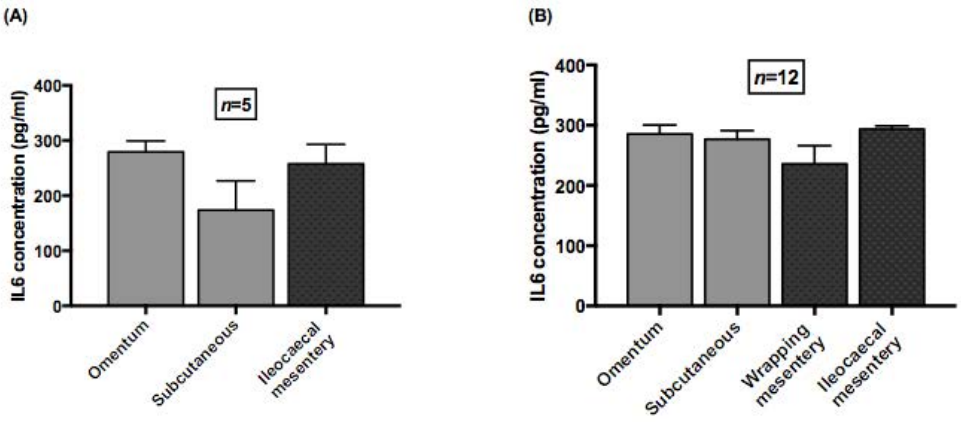


Figure 7.3. Interleukin 6 (IL6) concentration in paired adipose tissue in control patients (A) and Crohn's disease patients (B).

7.4. Adipose tissues' secretion of vascular endothelial growth factor (VEGF)

VEGF concentration in the supernatant of the test group (wrapping and ileocaecal mesenteric AT from CD patients) (mean=90 pg/ml, SD=159) and controls (mean=81 pg/ml, SD=181) were similar (Figure 7.4, A). To examine the difference between CD and non-CD AT, the subcutaneous and omental AT harvested from CD patietns was excluded. Thus, the following comparison is between the test group and the non-CD AT including ileocaecal mesentery, omental and subcutaneous AT. In this comparison, there was statistically significant (p<0.01) higher concentration of VEGF in the test group than control (mean=20 pg/ml, SD=23) (Figure 7.4, B).

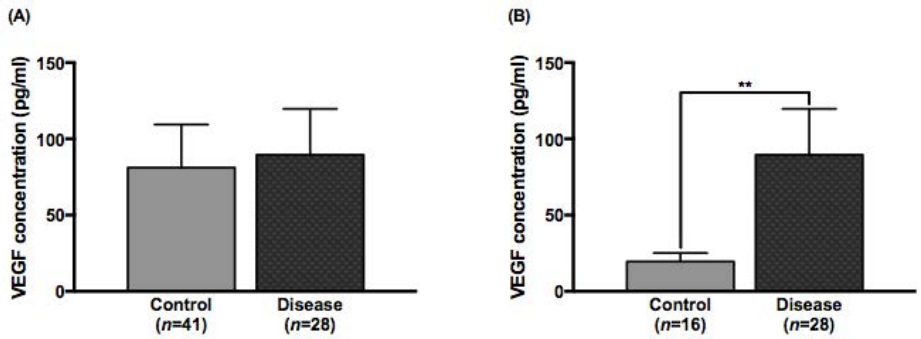


Figure 7.4. Vascular endothelial growth factor (VEGF) concentration in the supernatant of adipose tissue for Crohn's disease and controls (A) and controls excluding omental and subcutaneous tissue of Crohn's disease patients (B). **p<0.01.

Analysis of VEGF concentration in different AT types demonstrated statistically significantly (p<0.05) lower concentrations of VEGF level in the mesenteric (mean=22.3 pg/ml, SD=33), omental (mean=19 pg/ml, SD=17) and subcutaneous (mean=17 pg/ml, SD=16) AT of control patients in comparison to wrapping mesentery (mean=88 pg/ml, SD=122) from CD patients. Furthermore, the VEGF concentration in the supernatant of subcutaneous AT from CD patient (AT that belongs to the control group) was significantly (p<0.05) lower than wrapping mesentery (test group) (Figure 7.5).

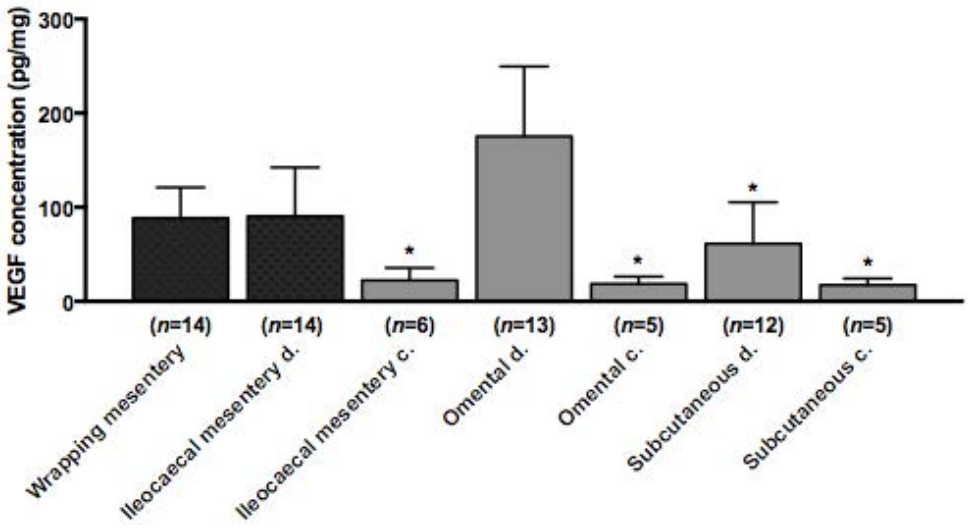


Figure 7.5. Vascular endothelial growth factor (VEGF) concentration in different types of adipose tissue from Crohn's disease (d.) and control (c.) patients. *p<0.05.

The levels of VEGF concentration were also investigated within patients and were similar in 5 paired samples of control patients' ileocaecal mesentery (mean=25 pg/ml, SD=36), subcutaneous (mean=17 pg/ml, SD=16), and omental (mean=19 pg/ml, SD=17) AT (Figure 7.6, A). However, the concentration of VEGF in 12 paired samples of CD patients were significantly (p<0.01) lower in the subcutaneous (mean=61 pg/ml, SD=152) than in the omental (mean=188 pg/ml, SD=276) AT (Figure 7.6, B). The concentration of VEGF in the supernatant of the two groups comprising the "test group", the wrapping mesentery (mean=101, SD=128) and ileocaecal mesentery (mean=104, SD=207), were higher than that in the subcutaneous AT and lower than the omental AT, however, these differences in means were not statistically significant (Figure 7.6, B).

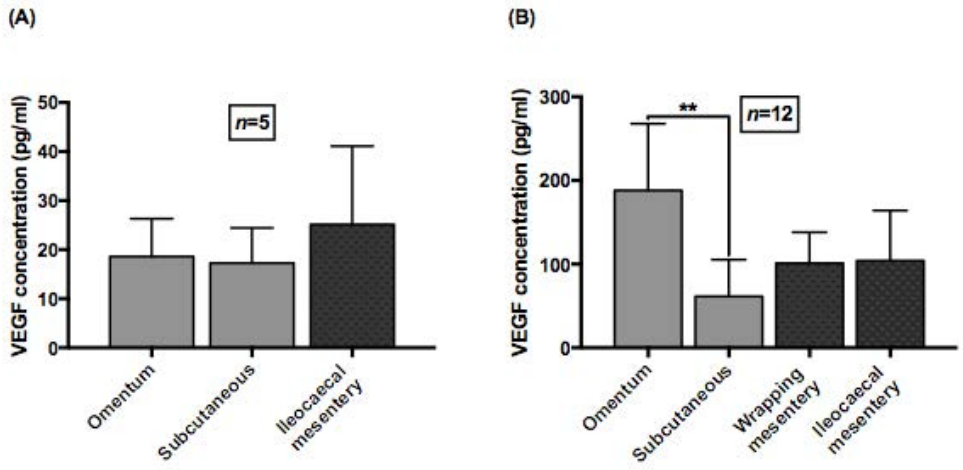


Figure 7.6. Vascular endothelial growth factor (VEGF) concentration in paired adipose tissue of control (A) and Crohn's disease (B). **p<0.01.

7.5. Discussion

The vasculature of the mesentery in CD patients is characterised by functional and structural abnormality (Wakefield et al., 1989). Histological features of vascular injury, focal arteritis, fibrin deposition, arterial occlusion, granulomatous vasculitis, and altered vascular endothelium are observed in the segments of resected specimens from CD patients and support the role of vasculature in the disease pathogenesis (Wakefield et al., 1991b, Danese, 2011). Furthermore, correlation between microvascular dysfunction and the pathological features of CD have been described (McLaren et al., 2002). However, the underlying molecular mechanisms of dysregulated angiogenesis are still unclear. In this thesis, increased MVD (chapter 4) and dysregulated angiogenic gene expression (chapter 5) have been demonstrated, supporting the hypothesis that angiogenesis in CD mesenteric AT is dysregulated. IL6 and VEGF, two of the most

important angiogenic factors were deficient at the mRNA and protein expression at tissue level. This experiment had the objective of assessing the level of secretion of these two cytokines by the mesenteric AT of CD patients.

The main findings in this experiment demonstrated that the level of VEGF secreted by CD mesenteric AT is significantly higher than the level secreted by AT from control patients. However, in paired comparison of different AT types from CD patients the level of VEGF secreted by the omental AT was higher than that secreted by the mesentery and statistically significantly higher than that secreted by subcutaneous AT. Earlier studies demonstrated that VEGF is elevated in the serum of CD patients (Bousvaros et al., 1999, Kanazawa et al., 2001, Kapsoritakis et al., 2003). The serum level of VEGF of this cohort is described in chapter 6. Briefly, the level was significantly higher than controls. However, at the intestinal mucosal level the expression of VEGF was enhanced in patients with active disease and in patients at remission (Danese et al., 2006). Similarly, the expression of VEGF in the AT is up regulated only during adipose tissue differentiation, during hypoxia or tissue stimulation with norepinephrine or insulin (Claffey et al., 1992, Zhang et al., 1997, Fain and Madan, 2005). On the other hand, the level of VEGF expression is reduced during the medical therapy of CD with infliximab (Rutella et al., 2011, Algaba et al., 2014). Indeed, the level of VEGF has been used to predict the response to treatment in CD (Algaba et al., 2014).

The majority of this study's CD participants were treated with infliximab at the time of surgery, which may explain the down regulation of VEGF, described in the gene and protein expression results chapters 5 and 6. However, in this ex vivo experiment, whereby AT are not subject to immunosuppressive therapy VEGF levels secreted by the tissue is in fact higher in CD mesenteric and omental AT than controls AT and subcutaneous AT of CD patients. Therefore, it is possible to speculate that CD mesentery is able to secrete angiogenic factors and not endogenously deficient in VEGF.

There was no difference in the ATs' ability to secrete IL6 in this ex vivo experiment, suggesting that the IL6 deficiency observed in the gene and protein expression experiments (chapters 5 and 6) is more likely to be a patients systemic effect and not attributed to the AT endogenously, but influenced by the patients immunosuppressive therapy.

There are several strengths attributed to this experiment: firstly, it is done under ex vivo controlled conditions whereby patient related effects, such as immunosuppressive therapy are circumvented; secondly, the tissue is minced into small pieces during incubation to avoid hypoxia; thirdly, the ELISA method used to quantify IL6 and VEGF is well established and accurate.

There are some unavoidable limitations, such as variabilities in the processing, for example although mincing the tissue into smaller pieces was done very carefully, there can still be small sub variations. Furthermore, although the IL6 levels were similar in the CD disease and controls some variabilities exist and a larger samples size to power the study further would add strength to the findings.

Further analysis of other angiogenic proteins to assess the angiogenic profile of the mesenteric AT in CD may improve the understanding of the angiogenic mechanisms and explain its connection to fat wrapping phenomena. Functional angiogenic assay results have been performed and will be discussed in the next chapter to shed light on the angiogenic capacity of mesentery AT in CD patients.

Chapter 8

Crohn's disease mesenteric adipose tissue's angiogenic capacity

8.1. Introduction

The plasticity of AT is considered to be the highest among all multicellular organs (Cinti, 2005). The remarkable ability of AT to rapidly expand and regress is well demonstrated when individuals repetitively diet, as they can lose and gain dozens of kilograms over short periods of time, and change their fat store by more than 50% (Lemoine et al., 2013). Yet, it is estimated that approximately 20% of the average adult weight consists of AT (Bosy-Westphal et al., 2013). The expansion of AT requires an extensive capillary network to: firstly, support oxygen and nutritional demand of differentiating adipocyte and infiltrating inflammatory cells; and secondly, to remove waste products (Powell, 2007). Furthermore, circulating progenitor cells are able to differentiate with varying degrees into vascular endothelial cells and adipocytes, suggesting that AT is highly vascular (Tang et al., 2008). During embryonic development, the vasculature and ECM precede the differentiation of adipocytes (Hausman and Richardson, 2004). However, during later development, adipocytes appear to drive and maintain their blood vessel supply (Mandrup et al., 1997, Mandrup and Lane, 1997). Thus, it is reasonable to conclude that AT may be one of the most angiogenic tissues in the body.

The dynamic nature of AT growth and regression is essential for survival and requires tightly balanced angiogenic and inflammatory responses. In some living organisms, the expansion of fat mass is

crucial for their existence, for example, hibernating mammals require a large fat store to survive the cold (Humphries et al., 2003, Molnar et al., 2011). In humans, we know now that AT is not an organ that only stores energy as triglycerides to be released as free fatty acid, but a dynamic organ with endocrine, paracrine and autocrine functions (Ahima and Flier, 2000a). To maintain angiogenic and inflammatory balanced responses, AT produces high levels of proangiogenic, antiangiogenic, proinflammatory and anti-inflammatory cytokines (Fasshauer et al., 2002, Kralisch et al., 2005). Specifically, VEGF is expressed and secreted by adipocytes (Zhang et al., 1997) and this thesis demonstrates that several pro-and antiangiogenic genes are expressed in different types of AT including the mesentery. Regarding CD mesentery, these characteristics may provide the conceptual background behind the expansion of the mesentery conferring the phenomena of fat wrapping. The milieu of inflammatory cytokines at the site of the inflamed bowel, and the plastic endogenous nature of AT are substantive reasons that support the observation of fat wrapping. The results in Chapter 5 described the antigenic gene expression in CD mesenteric AT as dysregulated and the correlation matrix between gene expression levels was inconsistent with that of the controls. Subsequently, VEGF gene and protein expression at the tissue level was also down regulated (Chapter 6) suggesting possible angiogenic dysfunction. This was supported by the increased vessel tortuosity seen in the histological studies (Chapter 4).

the objective here is to demonstrate the functional response of the gene and protein expression profile. The chapter reports the results of angiogenic capacity of the mesenteric AT in CD patients cultured in the matrigel assay; hypothesising thatis the angiogenic capacity of CD patients mesenteric AT is impaired in comparison to control. For the purposed of demonstration, this chapter has been divided into four sections: 1) descriptive statistics of the AT used in this experiment; 2) ratio comparison of AT vascular sprouting versus no vascular sprouting; 3) the extent of vascular sprouting at day 10 of tissue culture; and 4) the speed of vascular sprouting in different types of AT.

8.2. Description of the adipose tissue used for comparison in the Crohn's disease and control groups

Matrigel assay was performed using tissues from 5 patients: 3 CD and 2 controls, one of which underwent subtotal colectomy for UC and another underwent colectomy for constipation. A total number of 234 duplicates of tissue culture were performed including: 1) 44 (19%) wrapping mesenteric, 50 (21%) ileocaecal mesenteric, 30 (13%) omental and 30 (13%) subcutaneous AT from CD patients; and 40 (17%) ileocaecal mesenteric, 20 (9%) omental, and 20 (9%) subcutaneous AT from control patients (Figure 8.1). To explain further, for CD patient number 1 for example, a total number of 10 wrapping mesentery, 10 ileocaecal mesentery, 10 omental and 10

subcutaneous AT were embedded in matrigel as explained in the methods chapter 2 (section 2.6.1.).

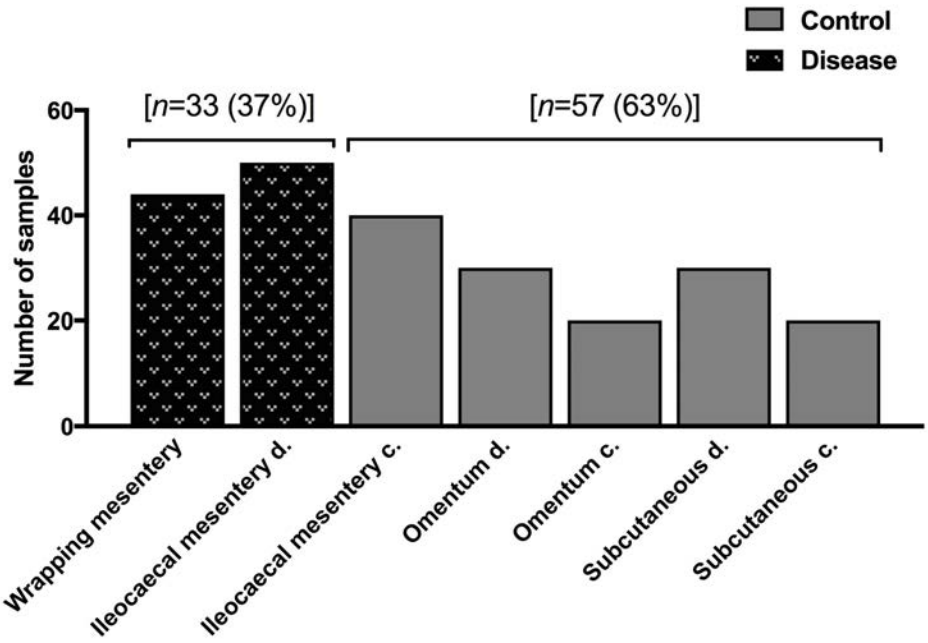


Figure 8.1. Adipose tissue types used in the analysis of matrigel functional assay. Test group including wrapping and mesenteric AT from CD patients, the remainder is considered the control group. Disease (d.). Control (c.).

8.3. Adipose tissues ratio of vascular sprouting versus no vascular sprouting

The ratios of AT producing vascular sprouting versus not producing vascular sprouting was measured after at day 11 of tissue incubation in the matrigel assay (chapter 2, section 2.6.1.). In brief, if there is no circumferential vascular sprouting, the AT was not included in the analysis of vascular sprouting quantification by ImageJ. But initially the test group (wrapping and mesenteric AT) and the control group were compared in the present or absent of circumferential vascular

sprouting. Out of 154, total samples cultured from CD tissue: 32/44 (73%), 30/50 (60%), 21/30 (70%), and 13/30 (43%) of wrapping mesenteric, ileocaecal mesenteric, omental and subcutaneous AT demonstrated vascular sprouting respectively. The difference in ratios were not statistically significant ($X^2_{(3)}$=7.5, p=0.06) (Figure 8.2, B). Furthermore, out of 80 total samples cultured from the control patients: 38/40 (95%), 18/20 (90%), and 15/20 (75%) of ileocaecal mesenteric, omental and subcutaneous AT demonstrated vascular sprouting respectively. The differences in ratios were not statistically significant ($X^2_{(2)}$=5.4, p=0.07) (Figure 8.2, A).

To investigate differences in the ability of AT to produce vascular sprouting, the ratios of vascular sprouting of each tissue type was compared between the CD and control tissues. The ability of mesenteric AT to produce vascular sprouting was statistically significantly lower in CD mesentery (62/94, 67%) than in the control (38/40, 95%) ($X^2_{(1)}$=12.5, p<0.001) (Figure 8.2, E). Similarly, the ratio of vascular sprouting in the subcutaneous AT in CD was statistically significantly lower in CD (13/30, 43%) than in the control (15/20, 75%) ($X^2_{(1)}$=4.9, p<0.05) (Figure 8.2, C). There were similar ratios of vascular sprouting in the omental AT in CD (21/30, 70%) and in control (18/20, 90%) ($X^2_{(1)}$=2.8, p=0.09) (Figure 8.2, D).

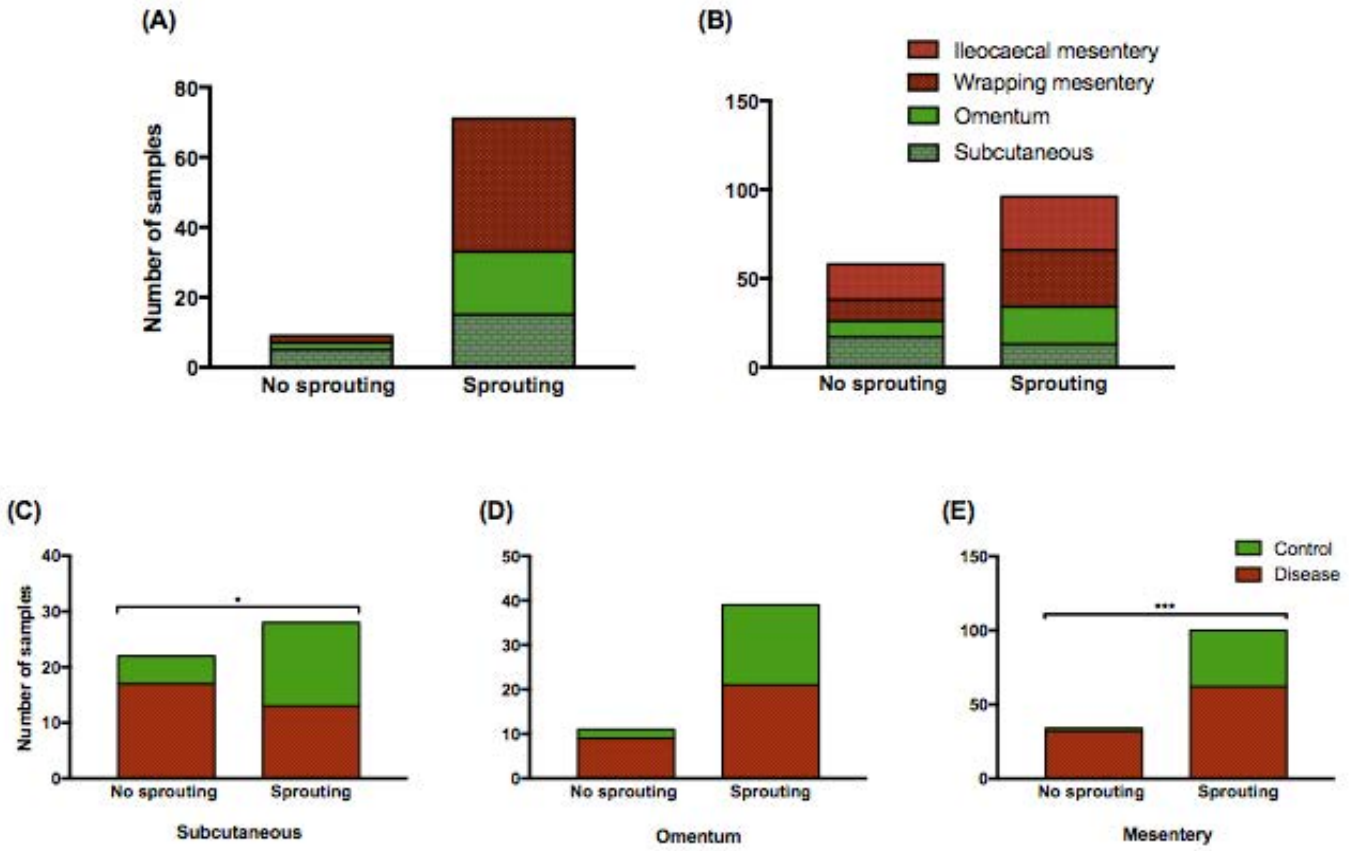


Figure 8.2. The ability of adipose tissues (AT) to grow vascular sprouting. Comparison between tissue types in the Crohn's disease (CD) (B) and control (A). The sprouting ratio comparison of CD and control mesenteric (E), subcutaneous (C) and omental (D) AT. Please note the test group is wrapping and ileocaecal mesentery of CD patients

8.4. Extent of vascular sprouting in adipose tissue

The extent of vascular sprouting was quantified using the imageJ software. Briefly, the growth surface area was divided on the AT surface area. This was performed every 6 hours until day 10 of incubation. Firstly, the results from the extent of growth on day 10 will be presented, following which the results of the speed of growth will be compared and presented in section 8.5.

The extent of vascular sprouting was significantly lower in CD wrapping mesenteric (mean=4.1, SD=3.8) in comparison to omental AT harvested from CD patients (mean=8.1, SD=5.4, $p<0.0001$) and AT harvested from control patients including: ileocaecal mesentery (mean=4.9, SD=3.2, $p<0.05$), and subcutaneous AT (mean=5.6, SD=3.4, $p<0.05$) (Figure 8.3). There was no statistical difference in the extent of vascular sprouting between the two tissue types in the "disease" group wrapping mesenteric AT and ileocaecal mesenteric AT (mean= 2.2, SD=1.7) and subcutaneous AT harvested from CD patients (mean=2.8, SD=2.4). The latter of which is also part of the control group (Figure 8.3). Comparison of the means of all types of AT to the combined extent of vascular sprouting in both wrapping and ileocaecal mesentery of CD patients (mean=3.2, SD=3) demonstrated a similar pattern as those seen when compared to wrapping mesentery only (Figure 8.4, A).

Vascular sprouting was statistically significantly (p<0.01) lower in the two tissue types that comprise the “disease” group CD wrapping and ileocaecal mesenteric AT (mean=3.2, SD=3) compared to all controls (mean=5.2, SD=4.1) (Figure 8.4, B). The level remained significantly (p<0.01) lower after excluding omental and subcutaneous AT of CD patients from controls (mean=4.9, SD=3.5) (Figure 8.4, C).

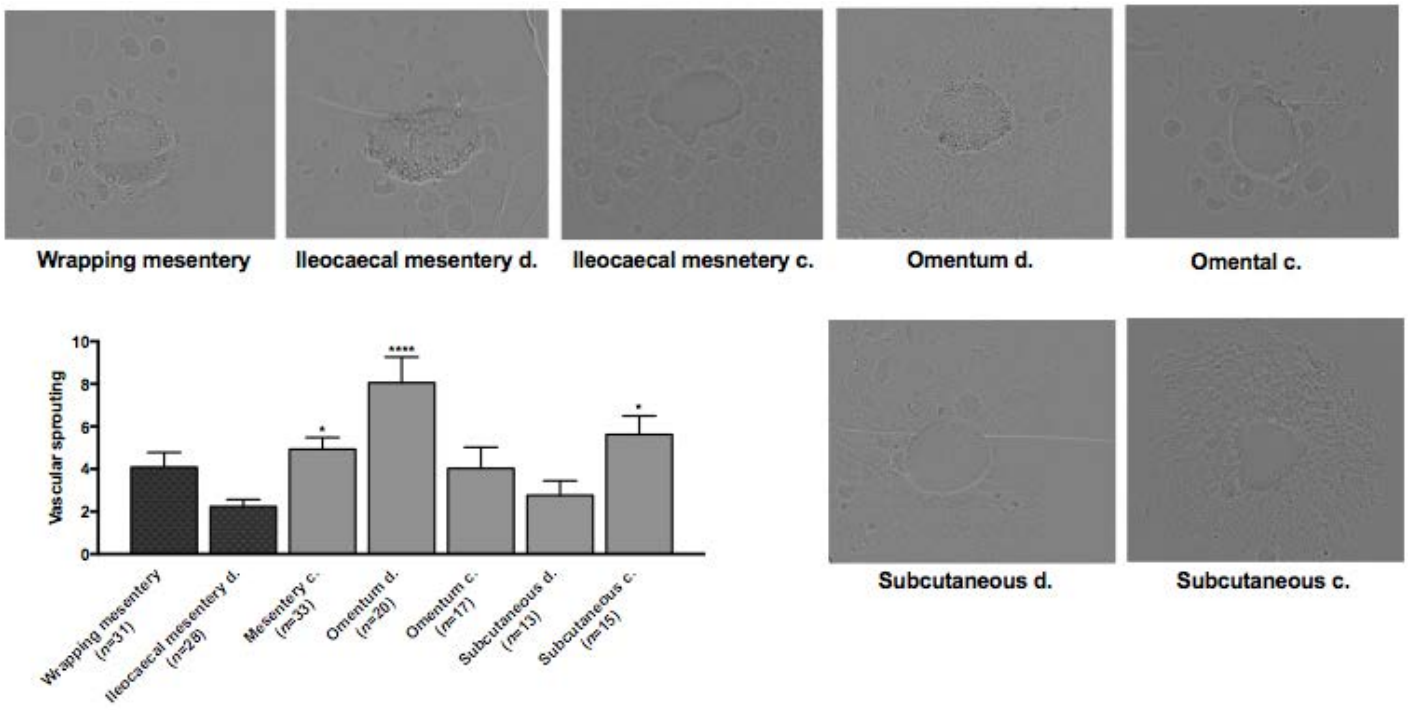


Figure 8.3. Vascular sprouting in adipose tissue (AT), comparing Crohn's disease (CD) (d.) wrapping mesentery to other AT, also from control (c) patients. The test group is the wrapping and ileocaecal mesentery from CD patients. *p<0.05. ****p<0.0001.

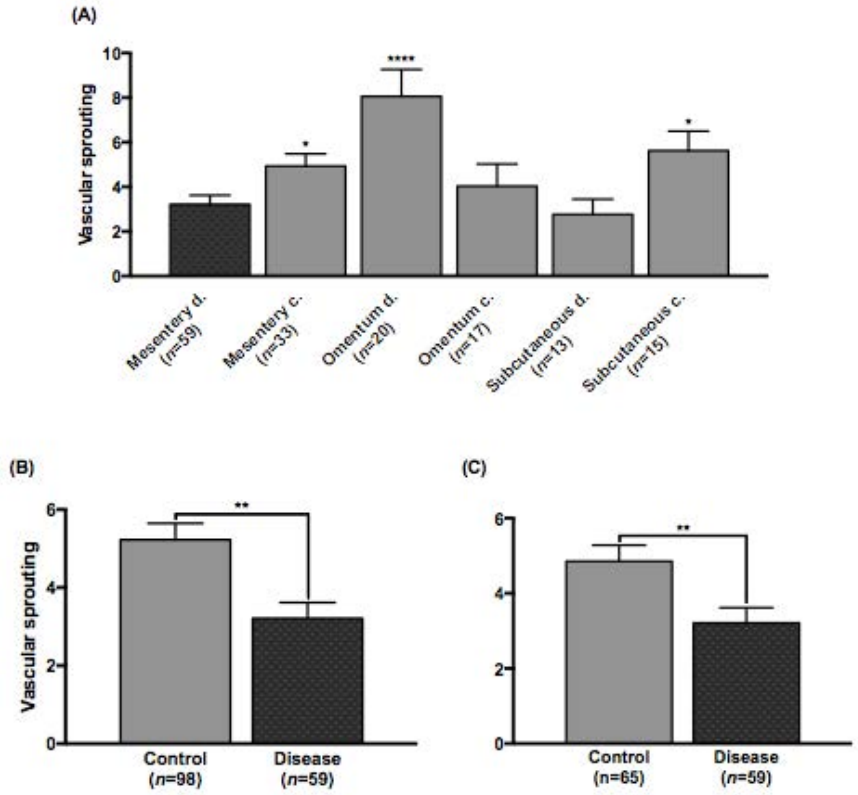


Figure 8.4. Combined Crohn's disease (CD) wrapping and ileocaecal mesenteric adipose tissue (AT) (test group) comparison to: all other tissue types individually (AT from non-CD patients and subcutaneous and omental AT form CD patients) (A); to combined AT "control group" (B); and to combined control AT excluding omental and subcutaneous AT from CD (C). CD (d.). non-CD Control (c.)

8.5. The speed of vascular sprouting

The speed of vascular sprouting was assessed by measuring the extent every 6 hours, starting at day 2 until day 10, on the same samples as described above and in chapter 2 (section 2.6.1). Inference statistics were generated using repeated measures ANOVA to demonstrate difference in the speed of sprouting at each point time.

The main column effect of difference in means of vascular sprouting at each time point of combined wrapping and mesenteric AT (test group) from CD patients were statistically significantly lower in comparison to ileocaecal mesenteric (mean diff.=-0.54, 95% CI [-0.7;-.04], p<0.0001) and subcutaneous AT from control patients (mean diff.=-1.2, 95% CI [-1.4;-0.9], p<0.0001). Similarly, the main column effect of difference in mean was also statistically significantly lower in comparison to omental AT (mean diff.=-2, 95% CI [-2.3;-1.9], p<0.0001) from CD patients (Figure 8.5). However, the main column effect of difference in mean was similar in comparison to subcutaneous AT (mean diff.=0.1, 95% CI [-0.13;0.3]) from CD patients and omental AT (mean diff.=-0.2, 95% CI [-0.4;0.01]) from control patients.

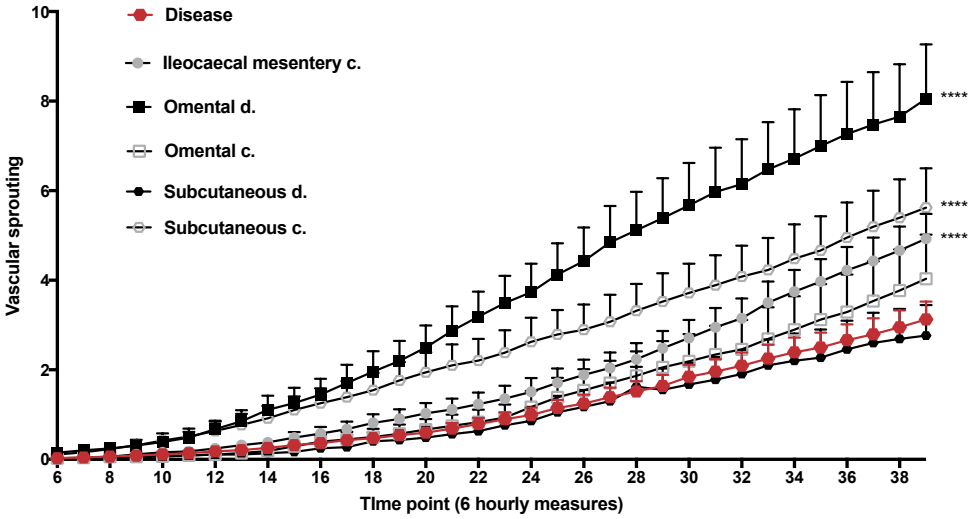


Figure 8.5. The speed of vascular sprouting comparing wrapping and mesenteric AT from Crohn's disease (CD) patients (test group, in red colour) to different types of control AT from CD (d.) and non-CD control (c.) patients. ****p<0.0001.

In addition, combined CD wrapping and mesenteric ATs' vascular sprouting at 6 hourly different time points compared to combined control tissue demonstrated statistically significantly lower means in the CD mesentery at every time point after the time point 20 which represents day 7 of tissue culture. Table 8.1 and figure 8.6, summarise the difference in means in each time point with their *p* values.

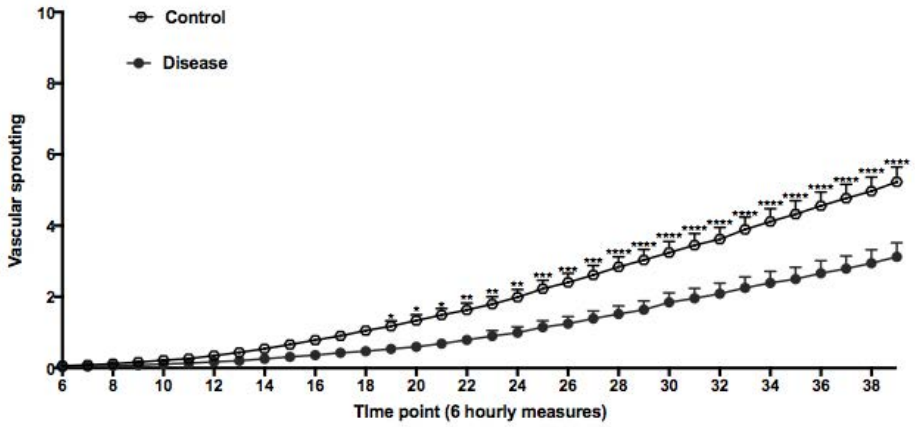


Figure 8.6. Comparison between combined Crohn's disease (CD) wrapping and ileocaecal mesenteric adipose tissue (AT) (test group) and combined AT from CD (subcutaneous and omental AT) and non-CD control patients. Different means and error bars are demonstrated at 6 hourly time points. *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

Table 8.1. Means of the extent of vascular sprouting at 6 hourly time point, comparing the test group (Crohn's disease wrapping and ileocaecal mesenteric adipose tissue(AT)) with the controls group (ileocaecal mesentery, subcutaneous and omental AT from non-CD patients and subcutaneous and omental AT from CD patients).

Time point	Control	Disease	Difference	*p* value
6	**0.06**	0.03	0.03	0.92
7	**0.09**	0.04	0.04	0.90
8	**0.12**	0.06	0.06	0.86
9	**0.17**	0.08	0.08	0.80
10	**0.22**	0.11	0.11	0.75
11	**0.27**	0.14	0.13	0.70
12	**0.35**	0.18	0.17	0.60
13	**0.44**	0.21	0.23	0.49
14	**0.54**	0.26	0.29	0.39
15	**0.66**	0.32	0.35	0.30
16	**0.78**	0.36	0.42	0.20
17	**0.90**	0.43	0.47	0.15
18	**1.05**	0.48	0.58	0.08
19	**1.18**	0.54	0.64	0.05
20	**1.33**	0.60	0.74	<0.05
21	**1.49**	0.69	0.80	<0.05
22	**1.63**	0.79	0.84	<0.05
23	**1.80**	0.90	0.90	<0.01
24	**1.99**	0.99	1.00	<0.01
25	**2.23**	1.15	1.08	<0.01
26	**2.41**	1.25	1.16	<0.001
27	**2.62**	1.39	1.23	<0.001
28	**2.84**	1.52	1.33	<0.001
29	**3.04**	1.64	1.40	<0.0001
30	**3.24**	1.85	1.40	<0.0001
31	**3.45**	1.96	1.49	<0.0001
32	**3.62**	2.09	1.53	<0.0001
33	**3.89**	2.25	1.64	<0.0001
34	**4.11**	2.39	1.72	<0.0001
35	**4.32**	2.50	1.82	<0.0001
36	**4.56**	2.66	1.90	<0.0001
37	**4.77**	2.79	1.98	<0.0001
38	**4.97**	2.95	2.02	<0.0001
39	**5.23**	3.12	2.11	<0.0001

8.6. Discussion

As in the case of any tissue that is changing mass, it is tenable to speculate that the expanding AT in CD, described as fat wrapping, would be accompanied by parallel changes in microcirculation (Crandall et al., 1997). However, if that expansion is pathological, it might be detrimental to the course of the disease and may be perpetuated by inadequate angiogenic mechanisms. Hence, balanced angiogenesis is required for the synthesis of an adequate capillary network, which is imperative to: ensure the delivery of oxygen and nutrients to the cells that make up the tissue; remove waste products; and allow the tissue to communicate with other body systems, such as the immune and hormonal systems (Cho et al., 2007, Christiaens and Lijnen, 2010). In order to assess the angiogenic mechanisms in CD mesentery, a multifaceted study was carried out including: tissue histology and immunohistochemistry, angiogenic genes and proteins expression and finally a functional assay. This experiment was designed to examine the angiogenic capacity of CD mesentery in comparison to other AT types from CD patients and controls. The multiple tissue type comparison was conducted keeping in mind that AT may differ in their angiogenic capacity. For example, the omentum was reported to exhibit more marked angiogenic capacity than the subcutaneous AT (Villaret et al., 2010). Based on the findings in the previous experiments, it appears that the angiogenic genes and proteins are down regulated in the mesenteric AT of the cohort of CD

patients included in this research project. Therefore, the hypothesis states that the angiogenic capacity of CD mesenteric adipose tissue is impaired in comparison to control.

The main findings in this set of experiments rejects the null hypothesis, and demonstrates that the mesenteric AT in CD patients has dysregulated angiogenesis. The test group was significantly less angiogenic than the control group, which included the mesenteric and subcutaneous AT of control patients and omental AT of CD patients. Furthermore, the speed of vascular sprouting of mesenteric AT from CD patients was significantly slower than the controls. In particular, the omental AT in CD patients demonstrated the most angiogenic capacity, and the fastest rate of vascular sprouting among all AT. The ratio of AT that manifest vascular sprouting versus no sprouting was similar between AT types in CD and controls. However, the mesenteric and subcutaneous AT in CD patients had significantly less ratio of vascular sprouting versus no vascular sprouting in comparison to the AT from control patients. Interestingly, these findings conform with the impaired angiogenic capacity observed in AT of patients suffering from obesity (Gealekman et al., 2011). Consequently, the expanding AT is sub-optimally vascularised. Indeed, Obesity has been described as a chronic inflammatory state due to the up regulation of proinflammatory adipokines and down regulation of anti-inflammatory adipokines expressed by the AT (Zabetian-Targhi et al., 2016). Similar analogy may be applied to the increased mesenteric AT mass in CD patients

with increase inflammation (chapter 3) and impaired angiogenic capacity. These features in addition to the dysregulated angiogenic gene expression may partly explain the chronic perpetuated imbalanced inflammatory (Borley et al., 2000) and angiogenic response in CD mesenteric adipose tissue.

Furthermore, the current evidence suggests that there is a strong association between obesity and metabolic syndrome (Carey, 1998). Particularly, patients with visceral obesity are more susceptible to metabolic syndrome (Pouliot et al., 1992, Bacha et al., 2003). In CD, patients with higher visceral to subcutaneous fat ratio are significantly more at risk of severe and complicated disease phenotype (Erhayiem et al., 2011). Although visceral fat in CD patients is mainly represented by mesenteric hypertrophy (Sheehan et al., 1992), it is plausible to speculate that the lack of angiogenic capacity of the mesentery AT in CD patients in this experiment with the presence of a macroscopically expanding mesentery reflects a mass that is not sufficiently vascularised, or marked by structurally and functionally impaired vasculature. Furthermore, in this experiment the angiogenic capacity of CD omentum was significantly higher than all other AT, a finding that agrees with the increase secretion of VEGF seen in the omentum in the tissue explant experiment (Chapter 6).

Another important finding is the angiogenic impairment seen in the subcutaneous AT of CD patients. Although, CD patients are generally

underweight (O'Keefe, 1996, Beattie et al., 2006), fat distribution appears to be favoured towards intra-abdominal rather than subcutaneous accumulation (Desreumaux et al., 1999). We already know that the intra-abdominal or white AT is linked to the development of metabolic disorders in obesity (Carey, 1998), as described above. However, in CD the increased intra-abdominal AT mass is independent of body mass index or metabolic abnormalities (Schäffler and Herfarth, 2005). Except for the omentum, the expression of angiogenic genes and proteins, specifically, IL6 and VEGF were down regulated in the mesenteric AT of CD patients included in this study (Chapters 4, 5 and 6). Also, the angiogenic gene profile was generally down regulated in the gene array experiment (chapter 5). Therefore, in this study, it has been confirmed on many levels including the matrigel functional assay, that angiogenesis is dysregulated or impaired in the mesenteric AT of CD patients. Although previous reports indicated an enhanced synthesis of inflammatory and angiogenic cytokines in CD mesentery, the contrary has been demonstrated in this cohort of CD patients. For example, leptin is a strong proangiogenic adipokine (Carino et al., 2008) that promotes angiogenesis in benign and malignant conditions (Barbier et al., 2003). The gene expression of leptin was down regulated by four fold in the mesentery of this cohort of CD. Leptin induces IL1β /IL1R singling pathway (Gonzalez and Leavis, 2003) that leads to the up regulation of VEGF (Coxon et al., 2002, Salven et al., 2002). IL1β and VEGF are also down regulated. The strength of positive correlation between

leptin, IL1β and VEGF was much stronger in the control tissue than in CD mesentery. The Spearman r for leptin on one hand versus IL1β and VEGF was 0.6 and 0.7 in the CD mesentery and 0.9 and 1 in the control mesentery respectively. The stronger correlation in the control AT marking a higher angiogenic capacity, where the weaker correlation in the CD mesentery marked a lower angiogenic capacity. Keeping in mind the complexity of the interplay between these factors, this suggests that a dysregulated gene expression in the correlation matrix described in chapter 5 may functionally be reflected as impaired angiogenic response.

There are several merits to this experiment including: 1) the use of a well-established assay for angiogenesis (Rojas-Rodriguez et al., 2014); 2) the comparison was made comprehensive, taking into account potential variation in the angiogenic capacity of different AT types; 3) random selection of tissue samples was attempted to ensure that cultured specimens are representative of the entire tissue; 4) the use of a time lapse microscopy allowed for analysis of dynamic changes, as opposed to the more commonly used endpoint analysis; and 5) the interpreter of the extent of vascular sprouting was blinded to the tissue type and the disease state, in order to avoid interpretation bias. Furthermore, the study of functional response confirms the findings of dysregulated and impaired angiogenic gene and protein expression seen in the previous experiments and histological specimens of CD mesentery, described in previous chapters, 4, 5 and

6. This was the first time to examine the angiogenic capacity of the mesenteric AT of CD patients and the findings may add further understanding of the angiogenic and inflammatory mechanisms in CD mesentery.

Despite the strengths described above there are unavoidable weaknesses including varieties of the cell types found in the cultured tissue. Although the ATs' angiogenic capacity may be dependant on the proangiogenic and antiangiogenic adipokines secreted by AT, these differ depending on the cell type. For example, adipocytes, preadipocytes, adipose tissue matrix, and stoma-vascular cell fraction secrete different levels of adipokines (Fain et al., 2004).

Further assessment of the angiogenic pathways, and mechanistic studies are required to understand the angiogenic response in the mesentery of CD and how this contributes to the pathogenesis. Assessments of the gene expression and functional assay at a cellular level would provide a more complete picture and would set the scene for identifying druggable targets, manipulating these in the model systems described, and taking forward for translation.

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