# Mesenteric Fat in Crohn's Disease

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## DECLARATION

I, Jack Frederick Broadhurst 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.

# ABSTRACT

Crohn's Disease is a chronic inflammatory disorder of the bowel affecting approximately 1 in 800 people in the UK. The terminal ileum is most commonly affected and the mesentery becomes thickened, a phenomenon known as 'fat wrapping'. The cause is not understood.

Elemental feeding can induce remission in Crohn's disease and polyunsaturated fatty acid (PUFA) content may be the cause of a reduction in inflammation. Particular attention has focused on n-3 and n-6 PUFA content of elemental feeds.

The aim of this study was to further characterize mesenteric fat in Crohn's disease and to examine the effects of different PUFA on mesenteric inflammation in vitro.

Samples of adipose tissue were collected from patients undergoing intestinal resection for Crohn's disease and from controls. These were cultured in media and elemental (E028 and Emsogen) feeds containing different concentrations of n-3 and n-6 PUFA.

Significant findings were that mesenteric (MF) and omental (OM) adipose tissue released more inflammatory cytokines IL-6, leptin and MCP-1 when cultured in media rich in n-6 PUFA compared to media rich in n-3 PUFAs. OM mean IL-6 concentrations were 18.6(3.1-21.8)ng/mL in n-6 PUFA vs 3.07(0.62-19.10)ng/mL in n-3 PUFA (p=0.018), MF IL-6 concentrations were 3.77(0.76-9.52)ng/mL in n-6 PUFA vs 1.5(0.42-2.61)ng/mL in n-3 PUFA (p=0.03). OM Leptin concentrations were 0.42(0.08-0.90)ng/mL in n-6 PUFA vs 0.08(0.07-0.14)ng/mL in n-3 PUFA (p=0.006), MF Leptin concentrations were 0.27(0.13-2.62)ng/mL in n-6 PUFA vs 0.12(0.07-0.31)ng/mL in n-3 PUFA (p=0.033). OM MCP-1 concentrations were 18.80(4.39-31.5)ng/mL in n-6 PUFA vs 1.83(0.69-4.82)ng/mL in n-3 PUFA (p=0.002) and MF MCP-1 concentration were 4.59(2.20-13.72)ng/mL in n-6 PUFA vs 1.20(0.82-3.39)ng/mL in n-3 PUFA (p=0.006).

These findings show that n-6 PUFAs stimulate a greater inflammatory response from omental and mesenteric fat in vitro and may assist in formulating a more effective elemental feed for inducing remission in patients with active flares of Crohn's disease.

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# ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| ATG16L1 | Autophagy 16-like 1 |
| ATL | Aspirin triggered lipoxin |
| BMI | Body Mass Index |
| BSA | Bovine serum albumin |
| CARD15 | Caspase recruitment domain family member 15 |
| CD | Crohn's disease |
| COX | Cyclooxygenase |
| CSF | Colony stimulating factor |
| DHA | Docosahexanoic acid |
| DNA | Deoxyribonucleic acid |
| DNBS | Dinitro benzene sulphonic acid |
| DSS | Dextran sulphate sodium |
| E028 | Elemental 028 |
| EGM | Endothelial growth media |
| ELISA | Enzyme-linked immunosorbant assay |
| EPA | Eicosapentaenoic acid |
| FW | Fat wrapping |
| IBD | Inflammatory bowel disease |
| IGF | Insulin-like growth factor |
| IFN | Interferon |
| IL | Interleukin |
| IQR | Interquartile range |
| LCT | Long chain triglyceride |
| LOX | Lipoxygenase |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| LX | Lipoxin |
| MAP | Mycobacterium avium subspecies paratuberculosis |
| MCT | Medium chain triglyceride |
| MCP | Monocyte chemoattractant protein |
| MF | Mesenteric fat |
| MIF | Macrophage migration inhibitory factor |
| MIP | Macrophage inflammatory protein |
| NEFA | Non-esterified fatty acid |
| NF | Nuclear factor |
| NOD2 | Nucleotide-binding oligomerisation domain 2 |
| OM | Omental fat |
| PAH | Polycyclic aromatic hydrocarbons |
| PAI | Plasminogen activator inhibitor |
| PPAR | Peroxisome proliferator-activated receptor |
| PUFA | Polyunsaturated fatty acids |
| RBP | Retinol-binding protein |
| RNA | Ribonucleic acid |
| SC | Subcutaneous fat |
| SCT | Short chain triglyceride |
| SD | Standard deviation |
| SFA | Saturated fatty acid |
| TGF | Transforming growth factor |
| TLR | Toll-like receptor |
| TNBS | Trinitro benzene sulphonic acid |
| TNF | Tumour necrosis factor |
| UC | Ulcerative colitis |
| VEGF | Vascular endothelial growth factor |

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# Chapter 1

# INTRODUCTION

## 1.1 Crohn's Disease

Crohn's disease is a chronic inflammatory granulomatous disease that can affect any part of the alimentary canal, although most commonly the terminal ileum and colon (Abraham and Cho, 2009). It typically presents with abdominal pain, weight loss and bloody diarrhoea. Children often present with growth failure as the onset of the disease is often more insidious in the young. It frequently results in a significantly impaired quality of life and a regular requirement for medication and surgery along with an increased risk of complications such as colorectal cancer.

Crohn's disease has a patchy distribution and a characteristic histological phenotype that separates it from the other inflammatory bowel disease, ulcerative colitis. However there is considerable cross over between the two diseases. The condition 'regional ileitis' was first recognised as a clinical entity by the American Burrill B Crohn in 1932 (Crohn et al., 2000) whom gave it his name. But 'chronic interstitial enteritis' had previously been described by a Scotsman T Kennedy Dalziel (Dalziel, 1989) in 1913 and the Polish A Lésniowski in 1903 (Lichtarowicz and Mayberry, 1988). Over 80 years significant progress has been made in demystifying aspects of its molecular pathogenesis and advances in treatment, however the aetiological origins of Crohn's disease remain obscure. Crohn's disease is considered to result from the complex interactions between genetic, immune-related, environmental and possible infectious triggers that integrate to cause a T-cell mediated chronic inflammatory response of the intestine. This increasingly sophisticated model fails to explain the proximal events that set the process in motion. There are inexplicable curiosities in all aspects of Crohn's disease and it remains to this day a modern medical enigma.

## 1.2 Epidemiology of Crohn's Disease

### 1.2.1 Incidence and Prevalence

Crohn's disease is predominantly a disease of the developed world where the incidence is substantially higher than in less industrialised nations. It is also a disease that most commonly occurs in the early years but persists with considerable morbidity throughout life. The global incidence of Crohn's disease is currently reported as 6-15/100,000 and the prevalence 50-200/100,000 (Cosnes et al., 2011)

But the epidemiology and geographical distribution are varied with a higher incidence reported in Europe, UK (Mayberry et al., 1979) (Mayberry and Rhodes, 1984), and North America (Vind et al., 2006, Yapp et al., 2000, Rubin et al., 2000, Loftus et al., 2007, Bernstein et al., 2006) perhaps suggesting a common aetiological factor. In more recent times the disease has also emerged in China, Thailand, India and North Africa (Thia et al., 2008). The highest reported incidence is in the Canterbury district of New Zealand at 16.5/100000 (Gearry et al., 2006). There are also areas of previously low incidence of Crohn's Disease such as Japan that are now reporting an increase (Thia et al., 2008). Intriguingly there are also countries reporting an increase among parts of its population but not others. Hungary has a much higher and rising incidence among the general population than the Romany gypsies (Karlinger et al., 2000) and the Maori population of New Zealand have a lower incidence than Caucasians (Economou and Pappas, 2008). Whether these differences are genetic or down to altered exposure to a responsible aetiological agent remain to be elucidated.

### 1.2.2 Age, Gender, Race and Geographical distribution

Crohn's disease is most commonly diagnosed in late adolescence and early adulthood which suggests a hormonal influence, but it may occur at any age (Loftus, 2004). There is also a 20-30% higher female preponderance in high incidence populations (Molinie et al., 2004, Gearry et al., 2006) but this is not replicable globally or in areas with a lower prevalence (Devlin et al., 1980). Crohn's disease was initially thought to have a lower incidence in non-white populations however a systematic review showed similar figures in Hispanics, Asians and African Americans (Hou et al., 2009). A UK study showed increased incidences of inflammatory bowel disease in migrant populations greater than the local population (17/100,00 vs 7/100,000)(Probert et al., 1992) and interestingly, age at the time of migration affects inflammatory bowel disease risk. Migrant children in British Columbia under the age of 15 years had the highest risk. Among some religious populations such as Jews and Mormons, an increased incidence of Crohn's disease has been shown (Penny et al., 1985). But this also varies geographically suggesting a combination of environmental and genetic factors (Shapira and Tamir, 1992). A north south geographical gradient was also reported with higher incidences in northern Europe compared to the south (Shivananda et al., 1996), however this gradient has become blurred with the rising incidence of

Crohn's. It may be that more specific local environmental factors are relevant as shown in a French study. A relative risk of >1.5 was found in 96 of 273 cantons (small administrative regions) in northern France and so there are no clear explanations for regional variations in IBD risk (Declercq et al., 2010). Occasional 'clusters' of disease have also been reported with multiple diagnoses in one hamlet (Allan et al., 1986) or even within common residences (Van Kruiningen et al., 2007).

## 1.3 Environmental and genetic risk factors and theories of pathogenesis

Crohn's disease is described as a complex interplay between environmental and genetic factors.

### 1.3.1 Genetic Factors

The familial nature of inflammatory bowel diseases and particularly the evidence of aggregation in twins studies are suggestive of a genetic component. Familial aggregation was first reported in the 1930s and a family history of inflammatory bowel disease is still the strongest risk factor for developing it (Russell and Satsangi, 2004). Patients with Crohn's disease have a first degree relative with Crohn's disease in 2.2-16.2% of cases. In the Jewish population the estimated lifetime risk of developing inflammatory bowel disease as a first degree relative of a patient with Crohn's disease is 7.8% compared to 4.8-5.2% in non-Jews. (Russell and Satsangi, 2004)

The strongest evidence for genetic factors contributing to susceptibility in inflammatory bowel disease comes from twin concordance studies (Tysk et al., 1988, Thompson et al., 1996, Orholm et al., 2000). They show a pooled concordance of 37.3% concordance of for Crohn's disease in monozygotic twins compared to 7% in dizygotic twins. (Baumgart and Carding, 2007).

Genome wide association studies (Barrett et al., 2008) have discovered over 40 loci for genes associated with increased susceptibility. The strongest associations are between CARD15 (caspase recruitment domain family member 15) which encodes the NOD2 (nucleotide-binding oligomerization domain 2) pathogen recognition protein and other loci such as the IBD5 locus, the interleukin-23 receptor and the autophagy gene ATG16L1 (autophagy 16-like 1)(Jess et al., 2005). These link dysregulation of the host gastrointestinal immune system and the host

microbiome (Molodecky and Kaplan, 2010) (Halfvarson et al., 2006). However the lack of complete penetrance cannot account for the disease aetiology because the risk with even the most common alleles is very low and furthermore genetics alone cannot account for the rising incidence of the disease (Binder, 2004). This lack of penetrance and rising incidence must be accounted for by additional environmental factors that enable progression from genotype to phenotype. Further weight to the environmental influence on Crohn's disease can be drawn from a study by Joosens (Joossens et al., 2007) where an immigrant father and four of his eight children developed Crohn's disease. All known genetic variants were analysed but there was no association between known genetic susceptibility factors and disease incidence within the family. Indeed there must be 'missing heritability' because all of the genes so far associated with Crohn's disease account for less than 20% of the total heritable risk of developing the disease (Maher, 2008).

### 1.3.2 Environmental Factors

Several theories of environmental causes of Crohn's disease have been postulated since the first description of the disease but these have ultimately not yet proved conclusive. These can broadly be broken down into factors that modulate either the physical intestinal content to produce an aetiological agent, the microbiological content of the intestine to produce an agent or the immune response to the unknown agent or a possible combination.

### 1.3.3 Infections

The resemblance of the granulomatous ileitis seen in Crohn's disease with paramyxovirus-mediated vasculitis or Johne's disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in cattle and deer gave rise to speculation that Crohn's disease may be caused by an infection (Baumgart and Carding, 2007). Crohn's disease was linked to a measles outbreak in Sweden (Ekbom et al., 1994) but the findings were not supported by other studies (Robertson and Sandler, 2001, Hermon-Taylor et al., 1995, ter Meulen, 1998). MAP has been found to a varying degree within the tissues of patients with Crohn's disease (Pierce, 2009) but also in controls suggesting a lack of causation (Sartor, 2005). A randomised controlled trial treating Crohn's disease with anti-

mycobacterial drugs did not show any significant benefit (Selby et al., 2007). Similarly herpes virus (Sura et al., 2010, Wakefield et al., 1992) and mycoplasma (Roediger, 2004) have been examined but not found to be convincingly causative of Crohn's disease. However, Cadwell was able to demonstrate that a virus-plus-susceptibility gene interaction could modulate the pathological response to experimental colitis in animals (Cadwell et al., 2010).

### 1.3.4 Physical antigens

Multiple physical agents have been considered. A proportion of granulomata found within Crohn's disease tissue are found to contain crystals, usually oxalate. So crystals and toothpaste were at one point hypothesised to be the responsible agents (Roge et al., 1991, Williams, 1991, Sullivan, 1990). Dietary submicron exogenous microparticles have also been examined (Butler et al., 2007) but exclusion diets showed no difference in disease activity (Lomer et al., 2005).

### 1.3.5 Dysbiosis, innate immune deficiency, intestinal permeability and antibiotics

Another suggestion is that Crohn's is caused by an increase in intestinal permeability that leads to greater movement of antigens across the intestinal epithelium with consequent increased immune exposure. Numerous studies have shown increased intestinal permeability in Crohn's sufferers and similar abnormalities in permeability have been identified in their asymptomatic relatives suggesting a genetic predisposition. This is collectively termed 'the leaky gut hypothesis' (Ma, 1997, Silva, 2009, Pravda, 2011). Crohn's has also been attributed to a deficiency in the intestinal innate immune defence, which protects hosts against organisms that have breached the epithelial barrier. This theory is supported by the virtually indistinguishable intestinal disease phenotype seen in genetic syndromes of neutrophil and monocyte dysfunction such as glycogen storage disorder 1b and chronic granulomatous disease (Dieckgraefe et al., 2002). It hypothesises that failure of inflammatory mediator production results in insufficient recruitment of neutrophils. This leads to inadequate removal of bacteria but which can be partly compensated for by NOD 2. However if the bacterial influx is not cleared the bacteria are phagocytosed by macrophages and

this results in the granulomatous reaction characteristic of Crohn's (Marks and Segal, 2008).

Related to the theories surrounding mucosal barrier function and immunity is the idea that the composition of the intestinal microbiota is the factor that has changed in Western society and which causes Crohn's disease. This is termed dysbiosis and represents an imbalance between harmful and helpful luminal microbes (De Hertogh et al., 2012, Friswell et al., 2010, Kau et al., 2011). The loss of helminths from our intestinal flora (Weinstock et al., 2002), the use of childhood antibiotics (Card et al., 2004), the hygiene hypothesis (Bach, 2002) and the cold chain hypothesis (Hugot et al., 2003) along with multiple dietary sources have all been suggested to explain these changes. Commensal bacteria modulate gene expression in several important intestinal functions, including mucosal barrier fortification, angiogenesis, xenobiotic metabolism and postnatal maturation (Hooper and Gordon, 2001). This symbiotic relationship occurs within the first 3 years of life as the bowel is gradually colonised. This colonisation could be altered by many factors, including maternal influences such as breastfeeding (Klement et al., 2004) or bottle-feeding (Fanaro et al., 2003), route of delivery at birth and genetic factors, or environmental influences such as hygiene (Gearry et al., 2010) or antibiotic use (Card et al., 2004, Blaser, 2011, Hviid et al., 2011).

### 1.3.6 Curiosities

There are many other interesting associations with Crohn's disease that have been reported but have yet to be explained. Appendicectomy has been shown to be protective in ulcerative colitis (Radford-Smith et al., 2002) but predisposes to Crohn's disease (Cosnes et al., 2006, Andersson et al., 2003). Similarly and respectively, smoking causes the same effect (Cosnes, 2004). The proposed mechanism is immunomodulation of the intestinal immune system and altered tolerance to the microbiota (Baumgart and Carding, 2007). Small but nonetheless interesting case reports have also described the development of Crohn's disease after gastric bypass surgery (Ahn et al., 2005) and in the biliopancreatic limb after duodenal switch (Pretolesi et al., 2006). The temporary but complete remission of Crohn's after an unrelated febrile illness has also been reported (Hoption Cann and van Netten, 2011).

### 1.3.7 Diet

A seminal study by Rutgeerts (Rutgeerts et al., 1991) comparing defunctioned and non-defunctioned bowel anastomoses showed that anastomotic recurrence of Crohn's disease after ileocaecal resection was dependent on faecal stream. Some factor or agent within the small bowel content is requisite for the development of Crohn's disease (D'Haens et al., 1998). Given the historical infancy of the disease, the strong environmental and geographic factors, and the association with the developed world, it is likely that this intraluminal factor is something that humans have either begun to start ingesting in the past 100 years or Western society has increased the quantity of its ingestion.

Diet may be involved in the aetiology of Crohn's disease through a variety of proposed mechanisms, which include modification of inflammatory processes, alteration of gut microbiota and toxic actions of nutrients on the intestinal mucosa. Proteins, lipids, fibre, minerals and phytochemicals have the potential to modify these processes.

Studies to examine the relationship between inflammatory bowel disease and diet are notoriously difficult perform prospectively. This is due to the low incidence of the disease and the near impossibility of standardising the diet of a sufficiently large number of subjects for long enough to develop enough patients with Crohn's disease to draw any conclusions. As a result most of the study data on diet in Crohn's disease is retrospective and complicated by recall and selection bias negating any convincing conclusions.

A consistent finding of retrospective case-control studies was an increased probability of Crohn's disease with a higher sugar consumption (Jarnerot et al., 1983, Mayberry et al., 1980, Silkoff et al., 1980). No plausible pathological mechanism for how sugars might cause Crohn's has yet been elucidated. The possibilities might include an unknown pathological effect of sugar, altered bowel microbiota, or a residual confounder if there is an as yet unidentified aetiological agent that is associated with sugar intake (de Silva et al., 2011). Importantly the French E3N and IBD in Epic prospective cohort studies reported no association with carbohydrate intake (Jantchou et al., 2010) and total sugar intake respectively (Hart, 2012). Similarly diets low in carbohydrate have not proved useful in

controlling flares or maintaining remission (Ritchie et al., 1987, Lorenz-Meyer et al., 1996)

Animal protein has also been associated with increased risk of inflammatory bowel disease (Jantchou et al., 2010). In Japan, a country with a previously low incidence of Crohn's disease, cultural changes have involved adoption of a westernised diet, and increased animal protein has been implicated in the emergence of Crohn's disease in Japan (Shoda et al., 1996). Mechanisms by which animal protein might be responsible are the breakdown of haem into reactive oxygen species (Andersen et al., 2012) as sources of heterocyclic amines, polycyclic aromatic hydrocarbons (PAH) and N-nitroso compounds which are carcinogens caused by cooking and processing meat (Santarelli et al., 2008) or by the fatty acids contained within it.

A positive relationship between dietary fat intake and Crohn's disease has been demonstrated (Amre et al., 2007, D'Souza et al., 2008). Polyunsaturated fatty acids (PUFAs) may be the dietary nutrients responsible for the effects on inflammation in Crohn's. They are present in meats, butters and margarine, plant oils and fish. They can be separated predominantly into two groups: omega-6 (*n*-6) and omega-3 (*n*-3), where the number denotes the position of the first double carbon bond in relation to the terminal methyl carbon. This gives the fatty acids similar chemical properties and so they share similar biosynthetic pathways. *N*-6 PUFAs are predominantly found in meat and sunflower and maize oils, whereas *n*-3 PUFAs are found in fish and flaxseed oils. PUFAs may modify inflammatory responses by a number of mechanisms: influencing biochemical compositions of mucosal cell membranes, increasing cellular stress, altering the balance of lipid signalling molecules and affecting nuclear receptors (Calder, 2009) and these will be discussed in greater detail later in the chapter. The biphospholid membrane of cells contains *n*-3 and *n*-6 PUFAs including eicosapentaenoic acid (EPA) and arachidonic acid (AA) respectively.

In the Western diet there is a predominance of *n*-6 PUFAs and a recent Dutch study reported an *n*-6:*n*-3 intake ratio of 8:1 in gastroenterology patients (Pot et al., 2009) but it has been suggested that our Palaeolithic diet would have more resembled 2:1 (Kuipers et al., 2010). Interestingly, the prospective EPIC trial found a significant association with baseline intake of linoleic acid, an *n*-6 PUFA, and the risk of developing ulcerative colitis (Tjonneland et al., 2009). Similarly margarine,

rich in linoleic acid, has been implicated in retrospective case-control studies (Maconi et al., 2010). Another large study looking at long-term dietary fat intake and the risk of ulcerative colitis and Crohn's disease found no association between total fat, saturated fat, unsaturated fat, *n*-3 or *n*-6 PUFA intake and the risk of developing ulcerative colitis or Crohn's disease (Ananthakrishnan et al., 2013). However Costea found that children who consumed a higher *n*-6:*n*-3 dietary ratio were more susceptible to Crohn's disease if they were carriers of specific genetic variants of genes controlling unsaturated fatty acid metabolism (Costea et al., 2014).

Analyses of fatty acid composition of subcutaneous adipose tissue have shown that an increased level of AA at baseline was associated with a 4-fold increased risk of development of ulcerative colitis (de Silva et al., 2010). Furthermore a recent systematic review of the role of diet and development of inflammatory bowel disease found that high intakes of total fat was associated with the development of Crohn's disease (Hou et al., 2011).

## 1.4 The clinical features of Crohn's Disease

### 1.4.1 Gastrointestinal Features

Patients with Crohn's disease have intestinal and extra-intestinal manifestations. Crohn's disease presentation can be subtle with intestinal symptoms dependent on the location and the severity of involvement. Often the first symptom is abdominal pain but others are anorexia, nausea, diarrhoea and weight loss. Perianal fissures and fistulae are common.

### 1.4.2 Extraintestinal Features

There are multiple extraintestinal features that are usually related to disease activity. Low-grade fevers, weight loss, growth retardation and delay in sexual maturation in children are common. Chronic undernutrition from either decreased oral nutrient intake secondary to abdominal discomfort combined with a protein losing enteropathy is considered to be the predominant cause of growth delay. However corticosteroid use in the treatment of Crohn's disease can also contribute to growth delay. Some females may experience secondary amenorrhoea due to active disease or weight loss. Other features include arthritis, mucocutaneous

lesions, cutaneous lesions such as erythema nodosum and pyoderma gangrenosum, and ophthalmic complications including episcleritis, iritis and uveitis. Gallstones, primary sclerosing cholangitis, autoimmune hepatitis, nephrolithiasis and osteopenia are all closely associated with the disease.

## 1.5 Pathology of Crohn's Disease

Crohn's disease can affect any part of the gastrointestinal tract. Diseased segments of bowel are often separated by areas of unaffected bowel. The inflammation is transmural involving the entire thickness of the bowel wall and often extends through the serosa. The Montreal classification recognises a distinction between stricturing disease – constant luminal narrowing and penetrating disease – defined by the formation of intra-abdominal fistulae and inflammatory masses or abscesses (Satsangi et al., 2006).

| **Montreal classification for Crohn's disease** | |
|---|---|
| Age at diagnosis | A1 below 16 y<br>A2 between 17 and 40 y<br>A3 above 40 y |
| Location | L1 ileal<br>L2 colonic<br>L3 ileocolonic<br>L4 isolated upper disease* |
| Behaviour | B1 non-stricturing, non-penetrating<br>B2 stricturing<br>B3 penetrating<br><br>p perianal disease modifier† |

**Table 1.** The Montreal Classification of Crohn's disease

*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present.
†"p" is added to B1–B3 when concomitant perianal disease is present.

### 1.5.1 Macroscopic histological features

Small ulcerations occur over Peyer's patches called aphthoid ulcers, which extend into longitudinal ulcers called serpiginous ulcers. These are usually on the mesenteric border. The bowel wall is thickened with involvement of the

submucosa, muscularis propria, subserosa and mesenteric fat. The mesenteric fat can extend from the mesenteric attachment to surround the affected segment, which is called 'fat-wrapping'. Fat wrapping is defined as being present when greater than 50% of the circumference is covered and occurs in up to 75% of surgical specimens (Sheehan et al., 1992). The associated mesentery is thickened and retracted. This fat wrapping is almost pathognomonic of Crohn's disease and will be discussed further later.

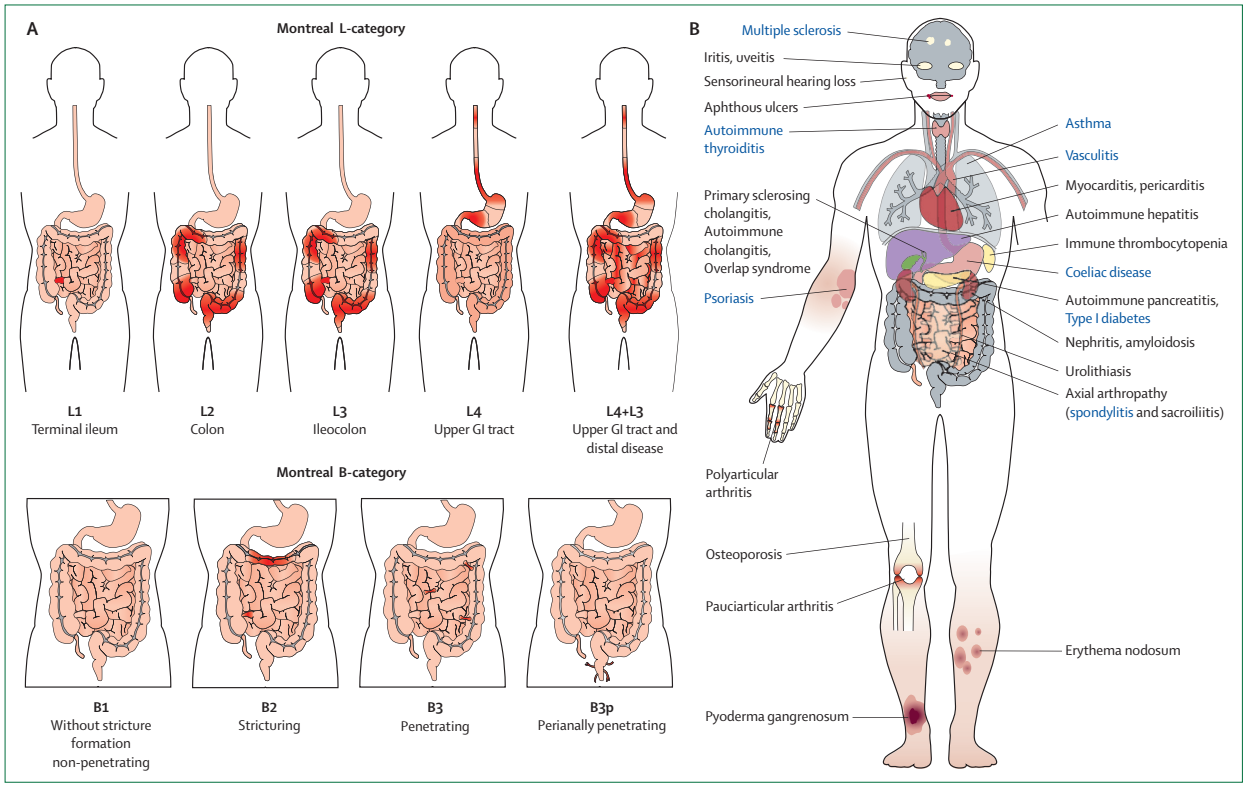



**Figure 1**. A diagram indicating the parts of the bowel affected by Crohn's disease, the extra-intestinal features and the Montreal classification. (Baumgart and Sandborn, 2012)

### 1.5.2 Microscopic histological features

Microscopically local chronic inflammation extends into the submucosa characterised by focal infiltration of neutrophils associated with lymphoid aggregates. Neutrophils and monocytes infiltrate the mucosal crypts leading to cryptitis and crypt abscesses. Aggregates of macrophages form non-caseating granulomata in up to 50% of cases, and there is associated local vasculitis and lymphangiectasia (Hendrickson et al., 2002, Van Kruiningen and Colombel, 2008).

## 1.6 Management of Crohn's Disease

The main goals of therapy of Crohn's disease are to induce clinical remission and then to maintain it. Unfortunately Crohn's disease tends to be relapsing and frequently requires surgery. Secondary aims of treatment are to heal the inflamed bowel, improve quality of life, prevent complications and to minimise the requirement for surgery.

### 1.6.1 Medical therapy

Since the aetiology of Crohn's disease remains elusive medical treatments target suppression and downregulation of the immune and inflammatory response. These include 5-aminosalicylates (mesalazine), immunomodulators (azathioprine, 6-mercaptopurine, methotrexate and ciclosporin), corticosteroids and targeted monoclonal antibodies to tumour necrosis factor-alpha (TNFα) (infliximab and adalimumab). The antibiotics metronidazole and ciprofloxacin are useful for controlling perianal sepsis. Unfortunately these medications have side effects including teratogenicity, growth retardation, osteopenia and increased risk of opportunistic infection (Toruner et al., 2008) and malignancy (Biancone et al., 2007).

### 1.6.2 Nutritional Therapy

Crohn's disease patients are frequently malnourished due to anorexia, malabsorption from inflamed segment, increased intestinal losses and the catabolic effect of chronic systemic inflammation. (Forbes et al., 2011). Parenteral and enteral nutritional therapies were sought to rectify the malnourishment. The observations that bowel rest coupled with total parenteral nutrition and that a defunctioning ileostomy proximal to the affected bowel segment result in significant clinical improvement, led to the concept of using nutrition as a primary therapy. Indeed this was first observed when a study looking at preoperative feeding showed an improvement in clinical disease status in the enteral support arm (Voitk et al., 1973).

Enteral feed is administered either orally or via nasogastric tube and is used in preference to the more invasive parenteral feed, which is associated with more complications. Parenteral feed is reserved for those in whom enteral feed has failed or in those with persistent vomiting, severe stenotic disease and short bowel syndrome (Forbes, 2006).

Exclusive enteral feeding has been shown to improve mucosal cytokine profiles and to reduce intestinal inflammation (Bannerjee et al., 2004, Breese et al., 1995). The degree of lipid and protein content has also been adjusted with demonstrable benefits (Gassull et al., 2002, Akobeng et al., 2000).

Multiple studies have been performed looking at the efficacy of enteral feeding and its comparison to corticosteroids in the induction of remission of Crohn's disease. Enteral feeding was found to be less effective than corticosteroids in early studies (Fernandez-Banares et al., 1995, Messori et al., 1996, Griffiths et al., 1995). A Cochrane systematic review published in 2007 also found that corticosteroids were more effective than enteral feeding at inducing remission (Zachos et al., 2007). However many of the studies analysed had methodological flaws with the potential confounding administration of other medications, but encouragingly a remission rate with enteral feed of over 50% can be expected and the best designed study finding a remission rate of 80% with enteral nutrition (Gonzalez-Huix et al., 1993). This success rate is considerably higher than most reported placebo effects looking at remission of Crohn's disease, (Zachos et al., 2007). However current international guidelines in the management of Crohn's disease do not recommend nutritional therapy as a first line treatment in adults (Dignass et al., 2010).

In children enteral feeding is already recommended as a first line treatment for Crohn's disease due to demonstrated efficacy (Day et al., 2006) and because of the obvious advantages over steroids. Foremost is the lack of side effects in comparison to the growth retardation and osteopaenia associated with steroids. Furthermore enteral feeding can result in mucosal healing which has been shown not to occur with corticosteroid use (Travis et al., 2006). One paediatric study of enteral feeding demonstrated remission rates at 8 weeks of 79% (Fell et al., 2000) and mucosal healing rates have been reported between 44% and 74% (Borrelli et al., 2006, Yamamoto et al., 2007). Elemental feed has also been shown to be helpful in the maintenance of remission of Crohn's disease and to accelerate withdrawal of steroid therapy (Akobeng and Thomas, 2007, Takagi et al., 2006).

#### *1.6.2.1 Types of enteral feed*

The mechanism by which enteral nutrition improves disease activity in Crohn's is unclear. Hypotheses put forward include upregulation of the intestinal immune

system, alteration of the commensal microbiome, reduction in gut permeability and a decrease in antigenic load presented to the gut mucosa (O'Morain et al., 1984, Cabre and Gassull, 2003).

Feeds can be broken down into two types, polymeric and elemental. Polymeric feeds contain whole protein that is thought to have high antigenicity, and elemental feeds, which are amino acid-based and are thought to be less antigenic. However many studies have been performed to test this and there is no convincing evidence that shows any difference in efficacy of remission between the two types (Gonzalez-Huix et al., 1993, Messori et al., 1996). One study did show polymeric preparations to be more effective in weight gain (Ludvigsson et al., 2004).

The effect of different lipid compositions of enteral feed was also analysed within the Cochrane Review (Zachos et al., 2007). No difference in efficacy was seen between high fat (>20g/1000kcal) and low fat (<20g/1000kcal) but a non-statistically significant trend was seen towards very low fat formulas (<3g/1000kcal). A trend was also seen towards very low long chain triglyceride (LCT) content (<5%). Conversely relapse rates were shown to be higher in patients with high versus low LCT content elemental feed (Bamba et al., 2003)

## 1.7 Mesenteric Fat, Lipids, and Crohn's Disease

### 1.7.1 Lipids and cellular physiology

A fatty acid is a carboxylic acid with a long aliphatic chain which is either unsaturated or saturated. Most naturally occurring fatty acids usually have an even number of carbon atoms in the chain between 4 to 28 and are usually derived from phospholipids or triglycerides. When not bound to other molecules they are called 'free' fatty acids. Fatty acids when metabolised yield large quantities of adenosine triphosphate and so are an important dietary source of energy. If there is a double bond between carbon atoms then a fatty acid is termed unsaturated and if there are no double bonds the fatty acid is termed saturated. Fatty acids are also subclassified in relation to the length of the chain.

- Short-chain fatty acids have chains with fewer than 6 carbon atoms
- Medium-chain fatty acids have chains between 6-12 carbons and can form medium chain triglycerides.

- Long-chain fatty acids contain chains between 13-21 carbons.
- Very long-chain fatty acids contain chains of 22 carbons or longer.

As mentioned above unsaturated fatty acids have one or more double bonds between carbon atoms. The two carbon atoms either side of the double bond can lie in either a *cis* or *trans* configuration. A *cis* configuration causes the two hydrogen atoms adjacent to the carbon double bond to extend out on the same side of the chain causing it to bend and restricting the conformational freedom of the fatty acid. The more *cis* double bonds the less flexibility in the chain and the greater the bend or curve in the molecule. The effect of *cis* bonds is that in a restricted environment such as part of a phospholipid in a lipid bilayer or triglyceride in a lipid droplet, they limit the ability of fatty acids to be closely aligned and can affect the melting temperature of the membrane or fat. A *trans* configuration means that the adjacent 2 hydrogen atoms lie on opposite sides of the chain and do not cause the chain to bend. Most *trans* fats do not occur naturally and are the result of human processing. The difference in geometry of fatty acids both saturated and unsaturated, plays an important role in biological processes and in construction of biological structures such as cell membranes.

There are several different systems of nomenclature used for fatty acids. They may have a common historical name, e.g. sapienic acid or a systematic name derived from the International Union of Pure and Applied Chemistry rules for the nomenclature of organic chemistry. Counting commences from the carboxylic acid end. Double bonds are labelled with *cis*-/*trans*- notation or *E*-/*Z*- notation, e.g. (9Z)-octadecenoic acid. In $\Delta^x$ nomenclature, the double bond is designated by $\Delta^x$, where the double bond is located on the *x*th carbon–carbon bond from the carboxylic acid end. A *cis*- or *trans*- prefix before each double-bond specifies the configuration of the molecule around the bond. For example linoleic acid is termed cis-$\Delta^9$, cis-$\Delta^{12}$ octadecadienoic acid. *N*–*x* (*n* minus *x*; also ω–*x* or omega-*x*) terminology both bestows names for specific compounds and organises them by their likely biosynthetic characteristics in animals. When counting from the terminal methyl carbon (designated as *n* or ω), towards the carbonyl carbon, the double bond is positioned at the $x^{th}$ carbon–carbon bond. For example, α-Linolenic

acid is classified as a *n*−3 or omega-3 fatty acid, and so it is expected to share biosynthetic properties with other fatty acids of this type. In mainstream nutritional literature the omega-*x* notation is popular but *n*−*x* notation is preferable in technical documents. Lipid numbers nomenclature takes the form *C*:*D*, where *C* is the number of carbon atoms in the fatty acid and *D* is the number of double bonds in the fatty acid. This notation can be confusing, as there are many different fatty acids that have the same numbers.

**Table 2 Common examples of unsaturated fats.**

| Common name | Chemical structure | $\Delta^x$ | *C*:*D* | *n*−*x* |
|---|---|---|---|---|
| Myristoleic acid | $CH_3(CH_2)_3CH=CH(CH_2)_7COOH$ | *cis*-$\Delta^9$ | 14:1 | *n*−5 |
| Palmitoleic acid | $CH_3(CH_2)_5CH=CH(CH_2)_7COOH$ | *cis*-$\Delta^9$ | 16:1 | *n*−7 |
| Sapienic acid | $CH_3(CH_2)_8CH=CH(CH_2)_4COOH$ | *cis*-$\Delta^6$ | 16:1 | *n*−10 |
| Oleic acid | $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ | *cis*-$\Delta^9$ | 18:1 | *n*−9 |
| Elaidic acid | $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ | *trans*-$\Delta^9$ | 18:1 | *n*−9 |
| Vaccenic acid | $CH_3(CH_2)_5CH=CH(CH_2)_9COOH$ | *trans*-$\Delta^{11}$ | 18:1 | *n*−7 |
| Linoleic acid | $CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$ | *cis*,*cis*-$\Delta^9$,$\Delta^{12}$ | 18:2 | *n*−6 |
| Linoelaidic acid | $CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$ | *trans*,*trans*-$\Delta^9$,$\Delta^{12}$ | 18:2 | *n*−6 |
| α-Linolenic acid | $CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$ | *cis*,*cis*,*cis*-$\Delta^9$,$\Delta^{12}$,$\Delta^{15}$ | 18:3 | *n*−3 |
| Arachidonic acid | $CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$ | *cis*,*cis*,*cis*,*cis*-$\Delta^5\Delta^8$,$\Delta^{11}$,$\Delta^{14}$ | 20:4 | *n*−6 |
| Eicosapentaenoic acid | $CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$ | *cis*,*cis*,*cis*,*cis*,*cis*-$\Delta^5$,$\Delta^8$,$\Delta^{11}$,$\Delta^{14}$,$\Delta^{17}$ | 20:5 | *n*−3 |
| Erucic acid | $CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$ | *cis*-$\Delta^{13}$ | 22:1 | *n*−9 |
| Docosahexaenoic acid | $CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$ | *cis*,*cis*,*cis*,*cis*,*cis*,*cis*-$\Delta^4$,$\Delta^7$,$\Delta^{10}$,$\Delta^{13}$,$\Delta^{16}$,$\Delta^{19}$ | 22:6 | *n*−3 |

**Table 3 Examples of common saturated fats**

| Common name | Chemical structure | *C*:*D* |
|---|---|---|
| Caprylic acid | $CH_3(CH_2)_6COOH$ | 8:0 |
| Capric acid | $CH_3(CH_2)_8COOH$ | 10:0 |
| Lauric acid | $CH_3(CH_2)_{10}COOH$ | 12:0 |
| Myristic acid | $CH_3(CH_2)_{12}COOH$ | 14:0 |
| Palmitic acid | $CH_3(CH_2)_{14}COOH$ | 16:0 |
| Stearic acid | $CH_3(CH_2)_{16}COOH$ | 18:0 |
| Arachidic acid | $CH_3(CH_2)_{18}COOH$ | 20:0 |
| Behenic acid | $CH_3(CH_2)_{20}COOH$ | 22:0 |
| Lignoceric acid | $CH_3(CH_2)_{22}COOH$ | 24:0 |
| Cerotic acid | $CH_3(CH_2)_{24}COOH$ | 26:0 |

Essential fatty acids are those required by the human body but which cannot be made in sufficient quantity from other substrates and so must be obtained from food. Fatty acids are required for oxidation for energy and to form phospholipids in cell membranes. Most fatty acids can enter the Krebs cycle and be oxidized for energy or stored as triacylglycerols. Specific fatty acids are precursors for signal molecules. Most non-esterified fatty acids are too strong acids to exist in a 'free' form and so are predominantly either bound to albumin or are within phospholipids and triacylglycerols. In well-fed humans most fatty acids are derived from diet rather than synthesised *de novo* so differ between individuals depending on diet, habitat and culture.

The term omega-3 (ω-3 or *n*-3) is the structural descriptor for a family of polyunsaturated fatty acids (PUFAs). As mentioned above *n*-3 indicates the position of the double bond that is closest to the methyl terminus of the acyl chain of the fatty acid. All *n*-3 PUFAs have the double bond on carbon number 3 where the methyl carbon is number one. The simplest *n*-3 fatty acid is a α-linolenic acid, which is synthesised from the *n*-6 fatty acid linoleic acid by desaturation, catalyzed by delta-15 desaturase. Humans and all animals do not possess this enzyme and so are unable to synthesise α-linolenic acid. Plants however do possess delta-15 desaturase and so are able to synthesize α-linolenic acid. Animals can only metabolise it by further desaturation and elongation, which ultimately yields

eicosapentaenoic acid (EPA) and then docosahexanoic acid (DHA). Of note is that the conversion of *n*-3 a-linoleic acid to EPA is in direct competition with the conversion of *n*-6 linoleic acid to arachidonic acid since the same enzymes are used. See Figure 2.

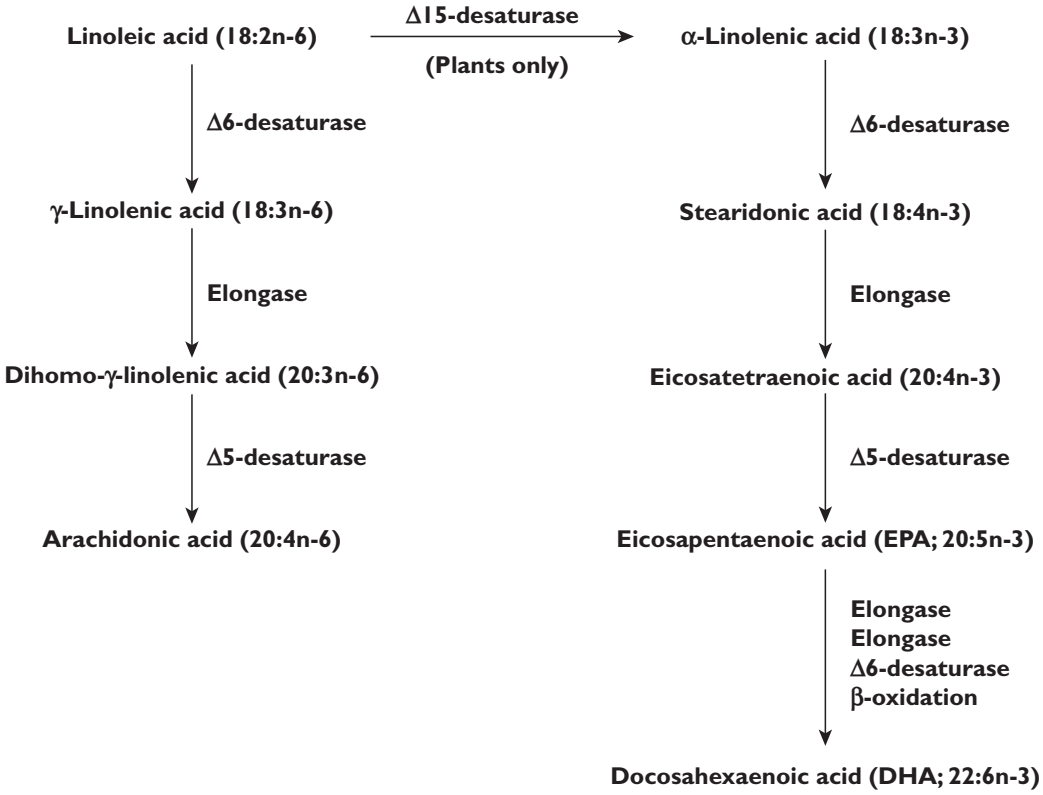



**Figure 2** The conversion of essential *n*-6 and *n*-3 PUFAs to their longer chain, more unsaturated derivatives (Calder, 2013)

The predominant *n*-6 PUFA found in the Western diet is linoleic acid and is typically consumed in 5 to 20-fold greater amounts than α-linolenic acid {British Nutrition Foundation. Briefing Paper: *N*-3 Fatty Acids and Health. London: British Nutrition Foundation, 1999}. The linoleic acid is converted within humans to arachidonic acid, which is the antecedent to many lipid-signalling molecules involved in inflammatory pathways called eicosanoids, including prostaglandins, thromboxanes and leukotrienes. Prostaglandins are synthesised though the cyclooxygenase-1 (COX-1) and COX-2 enzymes. Leukotrienes are produced by the

lipooxygenases (LOX). These enzymes metabolise *n*-3 PUFA, EPA, to prostacyclins, lipoxins and epoxy-eicosatrienoic acids that are less pro-inflammatory than those produced from *n*-6 PUFAs (Harris et al., 2009). The relative balance of *n*-3 to *n*-6 PUFAs will change the amounts of eicosenoids synthesised and alter the resulting degree of inflammation. *N*-3 PUFAs have many anti-inflammatory effects including decreased chemotaxis of leucocytes, decreased levels of adhesion molecules, reduced potent eicosanoid production, increased weak eicosanoid production, increased resolvin production, decreased inflammatory cytokine production and reduced T-cell proliferation. The mechanisms involved are listed in Table 4.

| Anti-inflammatory effect | Likely mechanism involved |
|---|---|
| Reduced leucocyte chemotaxis | Decreased production of some chemo-attractants (e.g. $LTB_4$); Down-regulated expression of receptors for chemo-atttactants |
| Reduced adhesion molecule expression and decreased leucocyte-endothelium interaction | Down-regulated expression of adhesion molecule genes (via NFκB, NR1C3 (i.e. PPAR-γ) etc.) |
| Decreased production of eicosanoids from arachidonic acid | Lowered membrane content of arachidonic acid; Inhibition of arachidonic acid metabolism |
| Decreased production of arachidonic acid containing endocannabinoids | Lowered membrane content of arachidonic acid |
| Increased production of 'weak' eicosanoids from EPA | Increased membrane content of EPA |
| Increased production of anti-inflammatory EPA and DHA containing endocannabinoids | Increased membrane content of EPA and DHA |
| Increased production of pro-resolution resolvins and protectins | Increased membrane content of EPA and DHA; Presence of aspirin |
| Decreased production of inflammatory cytokines | Down-regulated expression of inflammatory cytokine genes (via NFκB, NR1C3 (i.e. PPAR-γ) etc.) |
| Decreased T cell reactivity | Disruption of membrane rafts (via increased content of EPA and DHA in specific membrane regions) |

**Table 4.** A summary of the anti-inflammatory actions of *n*-3 PUFAs and the likely mechanisms (Calder, 2013)

It may be that through these mechanisms the fat content of the Western diet and elemental feed exert an effect in inflammatory bowel disease. Indeed in ulcerative colitis (UC) elevated levels of arachidonic acid and its metabolites prostaglandin $E_2$, leukotriene $B_4$ and thromboxane $B_2$ are found in the colonic mucosa of affected patients. (Boughton-Smith et al., 1983, Nishida et al., 1987). PUFAs may also affect intestinal inflammation via lipid signalling molecules that are involved in the control and resolution of inflammation (Gewirtz et al., 2002). These include lipoxins, $LXA_4$ and $LXB_4$ that are also formed from arachidonic acid through multiple routes and also aspirin-triggered lipoxin (ATL) (Serhan, 2005). Patients with ulcerative colitis have reduced $LXA_4$ synthesis (Gewirtz et al., 2002) and mice with blocked LXA synthesis develop IBD-like disease (Mangino et al., 2006). It could be that *n*-6 PUFAs are well tolerated by the majority of the population but that a subset may have a decreased ability to convert arachidonic acid to anti-inflammatory lipoxins and so the *n*-6 PUFAs are predominantly steered towards proinflammatory prostaglandin synthesis.

There are principally two mechanisms how *n*-6 metabolites might promote intestinal inflammation. They may regulate the mucosal barrier by affecting tight junctions with resultant impaired paracellular permeability or by dysregulation of the inflammatory response with consequent high levels of cytokines, eicosenoids and free radicals (Ferrer and Moreno, 2010). Furthermore, intestinal homeostasis and tolerance to commensal microbes is maintained by the nuclear factor κβ (NF-κβ) signalling and activation of peroxisome proliferator-activated receptor (PPAR), which results in down-regulation of cyclooxygenase-2 (COX-2) enzyme activity (Yang and Frucht, 2001) and the arachidonic acid cascade (Ferrer and Moreno, 2010). However the role of PPAR in IBD is not fully elucidated and work in this area is underway.

PUFAs may also contribute to Crohn's disease by directly adjusting the host microbiome. Approximately 2% of ingested PUFAs arrive undigested in the colon and can thus alter the survival of bacteria (Knapp and Melly, 1986). High-fat diets have been shown to change the bacterial flora in mouse models (Maslowski et al., 2009) and also to modify bile acid composition and bile acids, which may in turn change the composition of intestinal bacteria (Chen et al., 2010) or even exert their own inflammatory effect.

### 1.7.2 Adipose tissue

Interest in adipose tissue has soared after the revelation that it could act as an immune and endocrine organ rather than just be a depository for excess nutrients (White et al., 1992, Alberti et al., 2005, Schaffler et al., 2007, Pond, 2000, Mohamed-Ali et al., 1998). Different depots of adipose tissue in the body produce various sets of bioactive mediators related to specific metabolic and inflammatory pathways (Bertin et al., 2010). Indeed the accumulation of intra-abdominal fat is closely associated with the development of 'metabolic syndrome' (Alberti et al., 2005). Adipose tissue is a loose connective tissue composed mostly of adipocytes. In addition to adipocytes, adipose tissue also contains the stromal vascular fraction of cells including preadipocytes, fibroblasts, vascular endothelial cells and a variety of immune cells such as adipose tissue macrophages. Adipose tissue is derived from preadipocytes which in turn are derived from fibroblasts (Ali et al., 2013). Subcutaneous adipose tissue has minimal lymphoid tissue but greater angiogenic potential. Omental and mesenteric adipose tissue have a greater proportion of lymphoid tissue. Omentum contains milky spots of lymphoid cells and mesenteric fat contains the lymphatic drainage of the bowel with its concomitant lymph nodes. Omentum and mesenteric fat are often used interchangeably in the scientific literature termed 'visceral' fat. Although they both contain more lymphoid tissue and remain in lean patients when subcutaneous fat almost disappears, they are too different anatomically to be classified together. The interchelated lymphoid cells almost certainly release cytokines and exert secondary effects on the surrounding adipocytes in intact tissue. A recent study has shown that pro-inflammatory signalling in the adipocyte is necessary for adipose tissue remodelling and expansion (Asterholm et al., 2014).

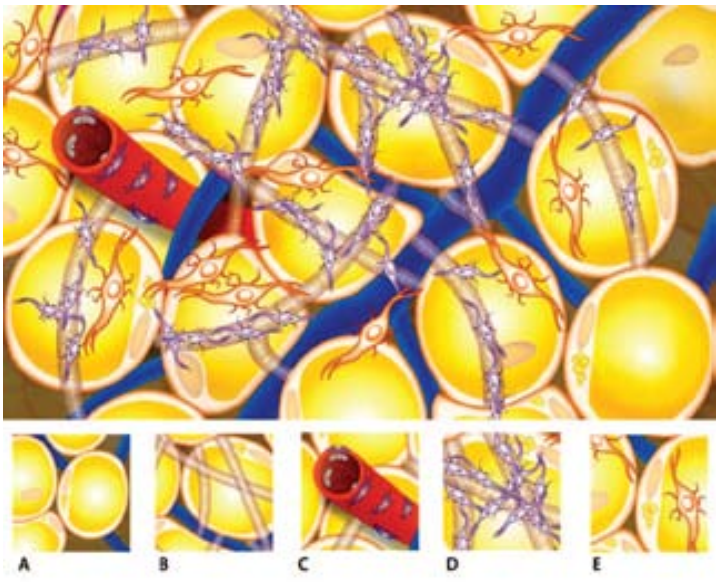



**Diagram 2. A.** Terminally differentiated mature adipocytes. **B.** Extracellular matrix (fibroblastic elements, most nucleated, undifferentiated cells adherent to ECM & Heterogeneous Perivascular Cells (Cell-To-Cell contacts). **C.** Pericytes-Endothelial Within Microvascular Elements (important in angiogenesis). **D.** Mesenchymal stem cells (Approximately 45% of nucleated cell counts in SVF). **E.** Pre-adipocyte (progenitor cells, near terminal differentiated form, adherent to adipocytes).
(Adapted From Alderman, D., Alexander, R.W.: *JOP* (2011) Vol. 3)

**Figure 3 The anatomy of adipose tissue and its cellular content**

### 1.7.3 Functions of Adipose tissue

#### *1.7.3.1 Storage, Thermal Insulation and Mechanical protection*

Adipose tissue is the predominant source of energy storage in mammals. Triacylglycerols are deposited in adipocytes to form fat depots. Adipocytes and adipose tissues are able to rapidly increase in size according to metabolic requirements. This ability for almost unlimited expansion makes it the most plastic tissue in the body.

#### *1.7.3.2 Adipogenesis*

Mature adipocytes form from pre-adipocytes, which are present within all adipose tissues and these can be recruited to differentiate to their mature form through out adult life (Ailhaud et al., 1992). This phenomenon is reversible with many reports describing mature adipocytes differentiating back to a preadipocyte state (Negrel et al., 1985, Ron et al., 1992). The number of fat cells reflects the balance of preadipocyte proliferation and apoptosis. Adipogenesis results from the transcripitional activation and gene expression within fibroblast-like cells destined to become preadipocytes. The preadipocytes must then receive mitogenic and adipogenic stimuli to continue to differentiate into the mature adipocyte by progressive attainment of the necessary morphological and biochemical characteristics (Ali et al., 2013).

#### *1.7.3.3 Lipolysis*

When carbohydrate availability for respiration is low during periods of fasting, adipocytes are able to respond to energy needs by hydrolysing triglycerides into glycerol and fatty acids. These then freely diffuse out of the cell and are circulated to wherever required. Stimulation of lipolysis is caused by many effectors including catecholamines, glucagon, growth hormone, cortisol, prostaglandins and cytokines such as TNFα, IL-1, IFNα, IFNβ and IFNγ.

#### *1.7.3.4 Immune and secretory function of adipose tissue*

Adipose tissue contains mature adipocytes and also many other cell types including preadipocytes, macrophages, endothelial cells, leukocytes and fibroblasts. These are able to express hormones and mediators collectively termed adipokines. (Trayhurn and Wood, 2004, Wozniak et al., 2009). These can be subdivided into hormone-like adipokines, chemokines and cytokines and others. See Table 5.

| | | |
|---|---|---|
| **Hormone-like adipokines** | Adiponectin | Omentin |
| | Visfatin | Vaspin |
| | Leptin | Adipsin |
| | Resistin | Chemerin |
| | Apelin | |
| **Cytokines and chemokines** | TNF | Monocyte chemoattractant protein-1 (MCP-1) |
| | IL-1 | Macrophage inflammatory protein-1 (MIP-1) |
| | IL-6 | Macrophage migration inhibitor factor (MIF) |
| | IL-8 | |
| | IL-10 | |
| **Others (hormones, growth factors, etc.)** | Macrophage colony-stimulating factor (M-CSF) | Granulocyte M-CSF (GM-CSF) |
| | Transforming growth factor (TGF) | Insulin-like growth factor (IGF-1) |
| | Serum amyloid A3 | C-reactive protein |
| | Complement system proteins | Plasminogen activator inhibitor-1 (PAI-1) |
| | Angiotensinogen | Retinol binding protein-4 (RBP-4) |

**Table 5.** Major mediators expressed by adipocytes (Bertin et al., 2010)

The recognition that proinflammatory mediators could be produced by adipose tissue led to it being considered an 'immunological organ' (Schaffler et al., 2007). Adipocytes, like macrophages and epithelial cells are able to produce pro-inflammatory (TNF, IL-6, IL-1β, IL-18) and anti-inflammatory (TGFβ, IL-10, IL-1RA) cytokines, chemokines (IL-8, MCP-1, MIP1α) and complement proteins from the innate immune system (Schaffler et al., 2007). They are also able to produce adipormones critical in the regulation of inflammation: leptin, adiponectin and resistin. Visfatin, omentin and vaspin are other adipormones that have been described but their function requires further investigation. Adipose tissue has also been shown to have phagocytic and anti-microbial properties (Cousin et al., 1999). Pre-adipocytes are able to convert into macrophages linking adipose tissue to

innate immunity (Charriere et al., 2003). In addition adipocytes can detect microorganisms through Toll-like receptors (TLR) and nucleotide-binding oligomerisation domain (NOD) proteins (Schaffler 2007}(Kopp et al., 2009). It was shown that specific TLR ligands could differentially upregulate Il-6 and MCP-1 release by adipocytes (Kopp et al., 2010).

#### *1.7.3.5 Depot specific differences in adipokine secretion*

Most of the research studies performed to date have compared subcutaneous fat to internal fat also referred to as visceral adipose tissue. Subcutaneous fat represents approximately 80% of total body fat mass. Typically omentum is used to analyse visceral adipose tissue as is can be easily resected without significant side effects to the patient. As a result less is known about how mesenteric adipose tissue compares to subcutaneous tissue. Similarly there may be significant variances between mesenteric and omental tissue both morphologically and in terms of adipokine secretion. Certainly the fact that increased visceral adiposity compared to subcutaneous adiposity predisposes to metabolic syndrome suggests that a functional difference exists between the depots (Hamdy et al., 2006, O'Connell et al., 2010). Leptin secretion and mRNA is increased in subcutaneous adipose tissue compared to visceral adipose tissue regardless of cell size or BMI (Lefebvre et al., Arner, 2001). IL-6, VEGF, PAI-1 and resistin secretion is greater from visceral adipose tissue (Arner, 2001).

## 1.8 Fat-wrapping

In Crohn's disease, one of the features previously mentioned is thickening of the mesenteric fat. The phenomenon of 'fat-wrapping' describes the macroscopic finding of over 50% of the bowel circumference to be covered by mesenteric fat (Sheehan et al., 1992) and loss of the bowel-mesentery angle. Surgeons used this observation, since Crohn's disease was first described, to help delineate the diseased segments requiring resection. 'Fat-wrapping' has also been called 'creeping fat' within the literature and defined less objectively as 'white adipose tissue hypertrophy extending from the mesenteric attachment and partially covering the intestinal circumference' (Desreumaux et al., 1999). However a strict definition seems unnecessary, as it is probably a gradual phenomenon and indeed occasionally fat can wrap the entire bowel circumference.

It was long assumed that this appearance only occurred in Crohn's disease but the pathognomonicity of fat-wrapping has recently been questioned (Golder, 2009). Fat wrapping has not been reported in any of the other granulomatous enteritides such as tuberculosis and yersiniosis (Sheehan et al., 1992) and is extremely rarely seen in any other condition. Fat wrapping is easier to detect in small bowel compared to large bowel due to the fat encroachment being restricted by taeniae coli but it is equally common (Sheehan et al., 1992).

Microscopically fat wrapping was found not to be associated with mucosal ulceration but only full thickness transmural inflammation (Sheehan et al., 1992) and as such has been shown to correlate poorly with the disease segment requiring surgical resection (Smedh et al., 1993). No correlation has yet been found between the prevalence of fat-wrapping and age, sex, weight, smoking, disease extent, disease chronicity or treatment. However the authors of the study speculate that since fat-wrapping is a sequel of mucosal ulceration (Kelly and Sutherland, 1988) that it is a pathological indicator of more chronic disease (Sheehan et al., 1992). Researchers then suggested that since fat wrapping is associated with transmural inflammation (assessed histologically by the depth of lymphoid aggregations) that the phenomenon occurs due to cytokine release from lymphoid tissue within the bowel wall (Borley et al., 2000).

Despite the knowledge of the existence of fat wrapping in Crohn's disease, very little attention has been devoted to studying it (Desreumaux et al., 1999) until the last decade when adipose tissue was discovered to be an endocrine organ. Histologically the mesenteric fat in Crohn's disease is markedly infiltrated with macrophages and T lymphocytes. In addition the thickened mesentery is due to hyperplasia of adipocytes, which have been reported to be 75% smaller than controls (Yamamoto et al., 2005, Peyrin-Biroulet et al., 2007). This is in contrast to increased visceral fat of obesity where adipocytes are enlarged and the tissue is hypertrophic. It remains unclear whether mesenteric thickening is a primary or secondary event in the development of Crohn's disease and there is a clear lack of understanding regarding the cause of its development.

## 1.9 Overexpression of mediators from Crohn's mesenteric tissue

Significant overexpression of cytokines and adipokines have been described from the mesenteric adipose tissue of patients with Crohn's disease including leptin, adiponectin, resistin, IL-6 and MCP-1 and this confirms that the fat is involved in the pathogenesis of Crohn's disease (Paul et al., 2006, Batra et al., 2009). Experimental models of colitis in mice have shown that during acute inflammation, the mesenteric adipose tissue is infiltrated with macrophages with raised expression of TNF, IL-6, MCP-1 (Karagiannides et al., 2006, Koon et al., 2009), however mesenteric thickening and fat wrapping were not seen, presumably due to the short duration of the inflammation.

The abnormal production of adipokines and cytokines from the mesenteric adipose tissue of Crohn's disease patients has been reported by many groups and those most relevant to this thesis will be described.

### 1.9.1 IL-6

IL-6 is a 21-18-kDa peptide released from a wide variety of sources including monocytes, macrophages, T cells, mast cells, fibroblasts, tumour cells, endothelial cells, epithelial cells, keratinocytes, skeletal muscle and adipose tissue (Van Snick, 1990, Pedersen et al., 2001, Mohamed-Ali et al., 1997). Lipopolysaccharide (LPS) induces IL-6 release from macrophages along with viral infections and other cytokines such as TNFα. (Nordan and Potter, 1986, Sehgal et al., 1988). IL-1β induces IL-6 secretion from adipocytes (Flower et al., 2003) and there is greater release from omental than subcutaneous deposits in obesity (Fried et al., 1998). Repression of IL-6 release has also been shown by glucocorticoids (Fried et al., 1998).

Il-6 is critical to the acute phase response that follows injury or infection. The pathway involves the local release of inflammatory mediators including IL-6, IL-1β, TNFα and interferons that then result in a systemic response involving fever, lymphocyte proliferation and the release of acute phase proteins from the liver. There is partial redundancy within this pathway and addition of any one of these cytokines will induce at least partially the response (Darlington et al., 1986). Although TNFα and IL-1β will cause most of the response only IL-6 can induce the entire pathway and stimulate release of all of the acute phase proteins. IL-6 is also required for B-cell differentiation, and T-cell activation and proliferation (Van

Snick, 1990). Multiple factors have been reported that upregulate IL-6 from adipocytes including β-adrenoceptor stimulation (Mohamed-Ali et al., 2001), IL-1 β (Flower et al., 2003), catecholamines and insulin (Vicennati et al., 2002), TNFα and exercise (Lyngso et al., 2002). It has also been suggested that adipocyte IL-6 release is not affected by food intake (Orban et al., 1999).

Il-6 is clearly a pro-inflammatory cytokine and is released by adipose tissue. In Crohn's disease it would be expected that IL-6 secretion from mesenteric adipose tissue involved in 'fat wrapping' would be elevated. However a 2006 study comparing mesenteric fat from diverticular, colon cancer and Crohn's bowel resections found a decreasing rate of IL-6 release respectively (Paul et al., 2006).

### 1.9.2 Leptin

Leptin is a 16-kDa non-glycosylated anorexia peptide, which modulates appetite, body weight and fat storage (Vaisse et al., 1996). It has a helical structure with close resemblance to Il-6 (La Cava and Matarese, 2004). Concentrations of circulating leptin increase proportionally with BMI (Frederich et al., 1995) and with age (Li et al., 1997). Leptin deficiency or dysfunction of the leptin receptor Ob-Rb is associated with morbid obesity in humans and mice. Leptin receptors are expressed in multiple tissues throughout the body including the brain and central nervous system (Campfield et al., 1996).

Congenital leptin deficiency leads to impaired T-cell proliferation and an increased mortality from childhood infections (Farooqi et al., 2002). Murine models of inflammation have shown that leptin deficiency provides a protective effect, including models of experimental colitis (Siegmund et al., 2002, Siegmund, 2004). Studies have also shown that leptin enhances lymphocyte activation, expression of adhesion molecules and cytokine production. Leptin increases the production of pro-inflammatory cytokines from macrophages and stimulates phagocytosis (Loffreda et al., 1998, Gainsford et al., 1996). It also promotes the T-helper cell Type 1 response and is involved in T-helper cell type 2 development (Batra et al., 2010). Leptin also stimulates lipolysis (Aprath-Husmann et al., 2001). In this way leptin can be considered to be pro-inflammatory with the capacity to modulate both adaptive and innate immunity (La Cava and Matarese, 2004, Batra et al., 2009). Leptin mRNA (Desreumaux et al., 1999) and protein overexpression (Paul et al., 2006) has been reported in the mesenteric fat of Crohn's disease patients.

### 1.9.3 MCP-1

MCP-1 is a 13-15 kDa chemokine known for its ability as a potent chemoattractant and activator of monocytes and macrophages. It is induced by multiple inflammatory mediators, including IL-1β, TNFα and IL-6. Multiple cell types have been shown to produce MCP-1 including mononuclear cells, mast cells, T cells, osteoblasts, fibroblasts, endothelial cells, bone marrow stromal cells, epithelial cells, microglia, astrocytes and smooth muscle cells. MCP-1 is also released by the stromavascular fraction of adipose tissue, pre-adipocytes and isolated mature adipocytes (Gerhardt et al., 2001, Xu et al., 2003). It can be considered pro-inflammatory and circulating levels are higher in patients with Crohn's disease (Herfarth et al., 2003). Studies into obesity have found greater levels of MCP-1 in visceral compared to subcutaneous adipose tissue (Bruun et al., 2005), however in Paul's study total MCP-1 secretion from whole mesenteric adipose tissue did not differ between Crohn's disease, colorectal cancer and diverticulitis (Paul et al., 2006).

### 1.9.4 Other known raised adipokines in Crohn's mesentery

Levels of the adiponectin mRNA and protein are increased in thickened Crohn's disease affected mesentery (Yamamoto et al., 2005) although the implications of this finding are not clear (Fantuzzi, 2008) given that there is no consensus on whether the protein is pro or anti-inflammatory. Circulating resistin levels are also raised in Crohn's disease in close relation to the degree of disease activity and resistin mRNA is also overexpressed in the Crohn's mesenteric fat. However, resistin is also raised in ulcerative colitis and diverticulitis so is not specific to fat wrapping (Paul et al., 2006).

### 1.9.5 Mesenteric bacterial translocation

It is also known from studies of Crohn's mesenteric fat that most total viable bacteria within the adipose tissue are located within adipocytes (Gay et al., 2005) and that adipocytes can respond to microbial antigens (Cousin et al., 1999) through their ability to express pattern recognition receptors such as Toll-like receptors and NOD proteins. Pre-adipocytes are also able to convert into macrophages (Charriere et al., 2003). A recent study demonstrated that bacterial translocation into adipocytes contributes to the inflammatory response in Crohn's disease thorough C-reactive protein production (Peyrin-Biroulet et al., 2011).

It has therefore been suggested that mesenteric thickening instead of being secondary to transmural inflammation and cytokines released by lymphoid aggregates may actually be due to bacterial translocation. There may be an alternative sequence to Crohn's pathogenesis. A different hypothesis has been proposed where an initial mucosal insult leads to bacterial translocation and mesenteric hyperplasia. Persistent bacterial infection within mesenteric lymph nodes leads to lymphangiectasia and lymphangitis with retrograde travel of inflammatory mediators resulting in mucosal ulceration at the mesenteric border (Behr, 2010). This model would be consistent with the pattern of discontinuous mucosal ulceration seen beneath the mesentery. However, a recent study found that adipokines modulate the macrophage compartment towards the M2 subtype and the authors contend that this suggests a protective role for the wrapping fat (Kredel et al., 2012).

### 1.9.6 Mesenteric lymph nodes

Indeed it may be that adipose tissue is fundamental to the immune function of lymph nodes within the mesentery with adipocytes supplying fatty acids to the lymphocytes (Pond, 2003). It has been shown that chronic inflammation of greater than six weeks duration can accelerate maturation of adipocytes around an experimentally stimulated lymph node, leading to hypertrophy of the adipose tissue (Mattacks et al., 2004). The effect was more marked in animals fed with a higher fat intake. A human study found higher levels of unsaturated fatty acids in perinodal adipose tissue compared with other depots but these differences were not replicated in Crohn's disease patients (Westcott et al., 2005). The same study also found depleted levels of PUFAs within the adipose and lymphoid tissue of patients with Crohn's disease and marked decrease in *n*-6 PUFAs within lymphoid tissue. The researchers suggested that this is due to a paracrine defect between perinodal adipose tissue and lymph nodes in patients with Crohn's disease (Westcott et al., 2005). There are significant site-specific differences in mesenteric and omental depots where the tissue contains greater amounts of lymphoid tissue, either in the form of lymph nodes or milky white spots in omentum or a greater stroma-vascular fraction and this may explain large variance seen later in the thesis. Although the medical literature has typically found *n*-3 fatty acids to be anti-inflammatory this finding may explain why some *n*-6 studies of elemental diet found clinical benefit (O'Morain et al., 1984, Gassull et al., 2002). One hypothesis to

explain this relationship between PUFAs, lymph nodes, perinodal adipocytes and mesenteric hyperplasia in Crohn's disease suggests that PUFAs from perinodal adipocytes activate the lymphoid cells of the adjoining node and so chronic stimulation leads to fat accumulation (Pond, 2001).

Given the paucity to date of research into mesenteric fat in Crohn's disease its role in the aetiology and pathogenesis of Crohn's disease can only be speculated upon. But there is clear evidence that it actively contributes to the intestinal and systemic inflammatory responses in Crohn's disease. It may be that fat-wrapping develops during sub-clinical periods of Crohn's disease in response to bacterial translocation. Perhaps it is an effort to contain the spread of pathogens and to prevent systemic infection through phagocytosis whilst not exerting a high inflammatory response. Adipocytes isolated from thickened Crohn's disease mesenteric fat and non-inflamed mesenteric fat do not respond to the same Toll-like receptor ligands and those adipocytes from the inflamed tissue are tolerant to lipopolysaccharide stimulation (Kopp et al., 2010). This might suggest that the inflammatory response in mesenteric adipose tissue can be shut down (Drouet et al., 2012). There is certainly more to be discovered in this regard.

## 1.10 Therapeutic lipids

The intimate relationship between adipose tissue physiology and lipid intake with Crohn's disease has been outlined above. But there is also plenty of interest in whether disease activity can be modulated using PUFAs therapeutically. This has mostly concentrated on *n*-3 PUFAs since their high intake was epidemiologically observed to be of benefit in Eskimo populations (Kromann and Green, 1980). *N*-3 PUFAs, particularly EPA and DHA, have since been shown to have many anti-inflammatory properties (Calder, 2006, Calder, 2009). By mechanisms mentioned previously *n*-3 PUFAs usually administered from fish oil result in incorporation of EPA and reduction of AA in membrane phospholipids and an according reduction in the production of pro-inflammatory eicosanoids and increased synthesis of anti-inflammatory equivalents. A reduction in resolvins created from DHA and EPA with anti-inflammatory properties has been reported (Weylandt et al., 2007). *N*-3 PUFAs also lead to a diminution of pro-inflammatory cytokine synthesis (TNF-α, Il-1β, Il-6 and Il-8) through either decreased nuclear transcription factors (NF-κβ) or PPAR-γ upregulation, lead to diminished leukocyte chemotaxis and reduce T-cell reactivity (Calder, 2006, Calder, 2009). It has also been shown that *n*-3 PUFAs are

incorporated to inflamed bowel mucosa (Hillier et al., 1991, McCall et al., 1989) and that they are protective in animal models of intestinal inflammation (Vilaseca et al., 1990, Nieto et al., 2002, Camuesco et al., 2005, Kono et al., 2010b). Some studies have reported low levels of *n*-3 PUFAs within patients with IBD (Siguel and Lerman, 1996, Kuroki et al., 1997) but this depletion was not found to be reproducible by other studies (Esteve-Comas et al., 1993, Fernandez-Banares et al., 1997, Figler et al., 2007).

### 1.10.1 *N*-3 PUFA use in Crohn's disease

A recent systematic review of *n*-3 PUFA therapeutic use in Crohn's disease was undertaken (Cabre et al., 2012). In active Crohn's disease two randomized controlled trials were considered eligible with *n*-3 PUFAs administered in enteral formulas either as supplements or total enteral nutrition (Nielsen et al., 2005, Grogan et al., 2012). Neither showed any significant difference toward *n*-3 enriched formulae improving disease activity or inducing remission. In quiescent Crohn's disease six studies were eligible (Belluzzi et al., 1996, Lorenz-Meyer et al., 1996){Belluzzi A 1997}(Romano et al., 2005, Feagan et al., 2008). *N*-3 PUFA supplements were used to maintain remission or to prevent postoperative recurrence of Crohn's disease. The paediatric study (Romano et al., 2005) showed a significant benefit with *n*-3 PUFAs but the relapse rate was inexplicably high and Belluzzi also showed a significant benefit that the other larger studies could not replicate (Lorenz-Meyer et al., 1996, Feagan et al., 2008). *N*-3 PUFAs were also shown to have no effect on postoperative recurrence rate. The overall conclusion of the review was that there was insufficient evidence to make a recommendation regarding the usefulness of *n*-3 PUFAs in Crohn's disease citing mainly methodological flaws in the study designs, in particular the lack of use of an adequately inert placebo.

Although the general weight of scientific literature considers *n*-3 PUFAs to be anti-inflammatory in their effect in comparison to *n*-6 PUFAs, other studies have reported that *n*-6 PUFAs are beneficial. Gassull performed a double-blind randomized controlled trial which showed that a polymeric enteral feed supplemented with *n*-6 PUFAs resulted in an improved remission rate in comparison to the same feed with added monounsaturated fatty acids (Gassull et al., 2002).

## 1.11 Digestion and Absorption of fat from the intestine

If dietary fat is fundamental to the pathogenesis of Crohn's disease or if PUFAs are the therapeutic agent within elemental feed, it useful to consider the physiological processes behind the digestion and absorption of fat. Hydrolysis of triacylglycerols is initiated by lingual and gastric lipases, which break these into 1,2 – diacylglycerols and short chain fatty acids, but the dominant site of fat digestion is in the small intestine. Lipids are hydrophobic and poorly water-soluble but lipase is water-soluble and can therefore only work at the surface of fat globlules. Emulsification of fat by bile salts within the duodenum and the churning peristaltic movements of the small intestine allow pancreatic lipases to act upon a greater surface area of fat and thus improve digestion. The arrival of a fatty bolus of food in the duodenum stimulates the release of cholecystokinin, pancreozymin and secretin, which result in the secretion of bile and pancreatic enzymes. Monoglycerides and fatty acids associate with bile salts and phospholipids to form micelles. Short and medium-chain fatty acids are absorbed directly into the blood via intestine capillaries and travel through the portal vein to the liver. The micelles transport the poorly soluble monoglycerides and long-chain fatty acids to the surface of the enterocyte where they are absorbed. Because monoglycerides and fatty acids are nonpolar they are able to diffuse freely across the plasma membrane of the enterocyte. Within enterocytes the monoglycerides and fatty acids are re-synthesised into triacylglycerols, which are packaged with cholesterol and fat-soluble vitamins into chylomicrons, lipoproteins that transport lipids within the circulation. These are released at the basolateral surface of enterocytes and being too large to enter capillaries, chylomicrons enter lacteals, which are lymphatic capillaries within the villi. Chylomicrons enter the circulation via the lymphatic system at the thoracic duct avoiding first pass liver metabolism. This absorption of micelles and bile salts occurs predominantly in the terminal ileum, the site within the intestine most commonly affected by Crohn's disease.

## 1.12 Aims and Objectives

The aims of this study are to:

- improve knowledge and understanding of the changes occurring within mesenteric fat in Crohn's disease.
- analyse the direct effects of different PUFA concentrations within elemental formulae on adipose tissue with a view to their primary therapeutic use in adults.

The hypothesis is: fat content of elemental feeds has a direct effect on the inflammatory response of Crohn's mesenteric adipose tissue in vitro. This will be tested by collecting fresh adipose tissue and incubating it with elemental feeds of contrasting composition and analyzing the solutions for variance in inflammatory markers. The precise techniques will be explained in the following chapter.

# Chapter 2

# MATERIALS AND METHODS

## 2.1 Study design

There are certain inherent difficulties in studying Crohn's disease and these in part may explain why despite eighty years of effort the aetiology of Crohn's disease remains unclear. As yet no representative animal model of Crohn's disease exists. As the cause of Crohn's disease is unknown and it is a chronic disease, attempts to replicate it in an animal have been consistently flawed. Animal models of chemically induced colitis using dextran sulphate sodium (DSS), trinitro benzene sulfonic acid (TNBS) or dinitrobenzene sulfonic acid (DNBS) are well established (Wirtz et al., 2007) and are used predominantly for research into ulcerative colitis. A recent attempt to induce fat wrapping in a DNBS mouse model failed (Olivier et al., 2011). Non-epidemiological research into Crohn's disease therefore necessitates the use of human subjects or human tissue and this requires ethical approval.

Another difficulty in analysing Crohn's disease tissue is finding comparative tissue to use as a control. Patients without a significant health problem do not undergo bowel resection surgery. Therefore control patients must undergo removal of bowel for a separate disease. To be the ideal control the same segment would be removed but this control segment of bowel should be unaffected by any disease process.

In Crohn's disease, the terminal ileum is the most commonly affected segment and also the most commonly resected. The terminal ileum is also frequently affected by fat wrapping and by mesenteric thickening. Fortunately for this study, gastrointestinal surgical techniques that involve resection of the right colon require resection of the ileocaecal valve to enable an anastomosis of the two cut ends of bowel (See Figure 4). Resection of the ileocaecal valve necessitates the resection of at least 10cm of the distal terminal ileum, the same segment of bowel that is most commonly affected by Crohn's disease. There are therefore many gastrointestinal diseases affecting the colon alone that necessitate resection of normal terminal ileum and these can be used as controls.

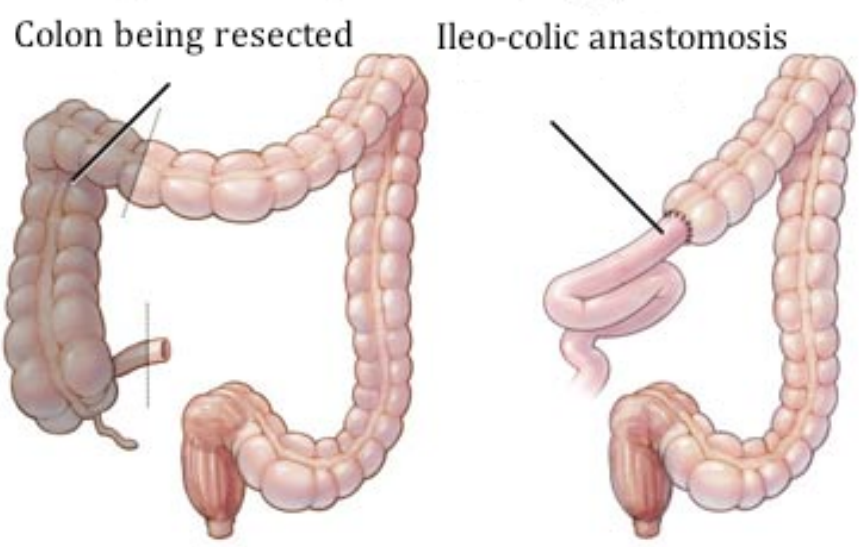



**Figure 4** A diagram demonstrating the bowel resected during a right hemicolectomy.

By definition patients undergoing colectomy are still not perfect controls because the patients are not healthy, however these are the closest conceivable controls that are ethically acceptable. The most common diseases in our hospital that require right hemicolectomy or total colectomy other than Crohn's disease, in respective order of frequency, are colorectal cancer and ulcerative colitis. Occasionally these same resections are performed for diseases of the appendix, functional disorders, trauma, diverticulosis and other rarities but these cases are not sufficiently frequent to be helpful in this study.

Colorectal cancer is common and many more right hemicolectomies are performed for cancer than for Crohn's disease. But for a number of scientific and ethical reasons, use of tissue from cancer patients is not appropriate for this study. Firstly patients undergoing resection for colorectal cancer in our centre are on average twice the age (78yrs) of those with Crohn's disease (36yrs) and ulcerative colitis (37yrs). It would be hard to accurately interpret differences between the adipose tissue depots of patients with Crohn's disease or colorectal cancer with such a wide age difference. Similarly cancers can cause systemic paraneoplastic syndromes through the autonomous release of humoral or immunologic factors

and this may discretely blur the interpretation of any findings. Secondly, the prognosis and further management of colorectal cancer after resection is dependent on the histologic findings. The analysis of cancerous involvement of mesenteric lymph nodes is paramount for management decisions regarding adjuvant chemotherapy and estimating prognosis. Tampering with or removing some of this tissue may therefore detrimentally affect the clinical outcome of the patient and so ethically it is not acceptable.

The remaining potential control group patients undergoing colectomy are those with ulcerative colitis, traumatic rupture, benign appendiceal disease, large right-sided polyps containing carcinoma-in-situ or functional bowel disorders. Relative to ulcerative colitis the other conditions are rare so it was decided that tissue from ulcerative colitics would be the primary controls. As previously mentioned there is considerable overlap between the two inflammatory bowel diseases however by definition ulcerative colitis is confined to the colon. The terminal ilea of ulcerative colitics ought not to be affected by the disease process. Rarely patients with ulcerative colitis can develop 'backwash ileitis'. The patient's disease is histologically proven to be ulcerative colitis affecting the colon, but the distal segment of ileum also shows non-specific mucosal inflammation. Fat-wrapping due to backwash ileitis has however never been described.

Ethical approval was obtained from the North West London Research Ethics Committee 1 to take human tissue for laboratory analysis and storage of tissue for future analysis, including organ culture, histology, mRNA and protein extraction. Three adipose depots were collected: subcutaneous fat (SC), omental fat (OM) and mesenteric fat (MF). The subcutaneous fat was taken from the site of surgical incision. The omentum was removed with the specimen. To attempt to standardise the mesenteric adipose tissue sample to improve comparability between cases, a 2x2cm sample was taken approximately 3cm from the mesenteric border and 3cm inferior to the ileocolic artery (See Figure 5).

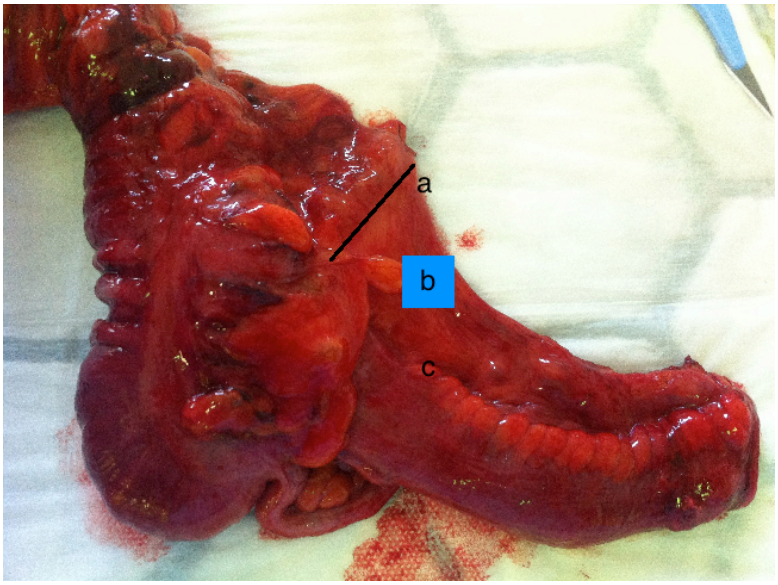



**Figure 5**. A photograph indicating the portion of mesentery removed for the study (b).

(a) and (c) represent the anatomical landmarks used to standardise the position, being respectively the ileocolic artery and the mesenteric border of the terminal ileum opposite the terminal ileal fat pad.

## 2.2 Study Population

### 2.2.1 Recruitment

Patients eligible for inclusion were approached after the decision to operate had been made. These patients were identified in outpatient clinic, the multidisciplinary team meeting or using the surgical department operations calendar. Patients were then either approached in clinic, by telephone or in acute cases on the day of surgery and they were then invited to enrol in the study. At this time the patient was given an information leaflet and signed a consent form. Data regarding duration of disease, treatment, social history and past medical history were all taken on a standardised collection sheet.

### 2.2.2 Medical history questionnaire

Pre-operatively a form was completed detailing the disease type, duration since diagnosis, indication for surgery, surgical procedure, other past medical history and drug history.

Age, gender, BMI, race, immigration status, and Montreal classification data was also collected.

### 2.2.3 Exclusion criteria

Patients with one or more of the following were excluded from the study:

- Previous terminal ileal resection
- Colorectal cancer
- Patients receiving intra-operative inotropic support
- Patients unwilling to provide informed consent.

### 2.2.4 Overall Population Characteristics

| | Crohn's | Ulcerative Colitis | Other Non-Crohn's |
|---|---|---|---|
| **N** | 19 | 15 | 3 |
| **Sex Male:Female** | 9:10 | 9:6 | 1:2 |
| **Median Age (range)** | 31 (16-68) | 35 (20-55) | 67 (19-68) |
| **BMI (mean)** | 22.8 | 24.7 | 25.5 |
| **Ethnicity (**Caucasian, Afro-Caribbean, African, Indian subcontinent**)** | 14:1:2:2 | 13:0:0:2 | 3:0:0:0 |
| **Immigration status (yes:no)** | 2:17 | 2:13 | 0:3 |
| **Smoker (yes:no:ex)** | 4:14:1 | 2:12:1 | 0:2:1 |

**Table 6**. The population characteristics of the patients recruited to the study

| Operation | Crohn's | UC | Other |
|---|---|---|---|
| **Ileocaecal resection** | 5 | | |
| **Right Hemicolectomy** | 7 | | 1 |
| **Subtotal Colectomy** | 3 | 3 | 2 |
| **Panproctocolectomy** | 1 | 12 | |
| **Small bowel resection/plasty** | 1 | | |
| **Ileocaecal and sigmoid resection** | 2 | | |
| **Total** | 19 | 15 | 3 |

**Table 7**. The operations underwent by the patients to enable retrieval of the terminal ileal mesenteric fat.

### 2.2.5 Specimen Collection

Patients had been deliberately fasted prior to anaesthesia for a minimum of 8 hours. Intraoperative fasting time prior to specimen collection varied depending on a multitude of factors including the operative time and difficulty of the surgery. Within the operating theatre, once the specimen of bowel was resected it was immediately handed from the surgeon to me. At this point it was laid out on a sterile trolley and photographed, see Figure 6. I then resected a 2x2 cm specimen of adipose tissue taken from the mesentery as close as possible to the point specified above. This was placed immediately into a sterile specimen collection pot containing 50mls CellGro® (see below). A sample of omental and subcutaneous adipose tissue was also taken at this point and placed into respective sterile pots containing 50mls of CellGro®. Occasionally there was insufficient subcutaneous fat in the malnourished patient to allow collection from this depot. All tissue samples were removed using sharp dissection whilst avoiding diathermy to prevent tissue damage. There was a wide variability in the time from ileocolic ligation to specimen collection due to the nature of the surgery which could not be standardised.

The specimens were then taken to the laboratory for analysis

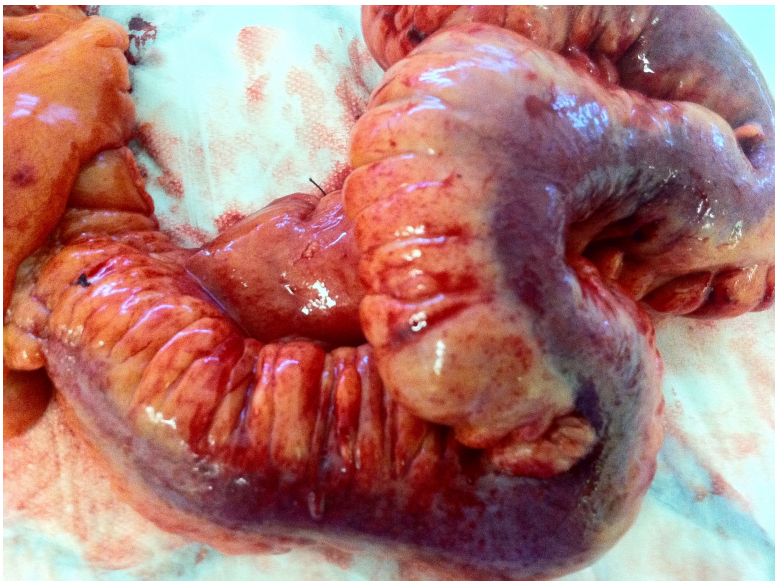


**Figure 6.** A photograph taken at collection immediately after resection. Fat-wrapping can be clearly seen.

## 2.3 Adipose tissue studies

### 2.3.1 Baseline Adipose tissue organ cultures

Subcutaneous, omental and mesenteric adipose tissue samples were dissected into 0.1g samples and then incubated in high-glucose serum-free medium (0.5mL), enriched with bovine serum albumin (BSA, 1 g/L), L-glutamine and phenol red (CellGro®, Mediatech Inc, Virginia, USA) with 1% penicillin/streptomycin for 24hrs at 37°C. At the end of the incubation period, the tissue samples were stored in foil packets at -80°C along with the culture supernatant. The supernatant was subsequently analysed to assess adipokine, cytokine and fatty acid secretion.

### 2.3.2 Viability of adipose tissue

Organ culture of adipose tissue has been validated and the tissue has been shown to be viable and respond to hormones for up to two weeks (Fried and Moustaid-Moussa, 2001). In the specific conditions of our laboratory adipose tissue was confirmed to be responsive to 1μM isoprenaline after 20 hours incubation in serum-free medium (Karastergiou and Mohamed-Ali, 2010).

### 2.3.3 Elemental Feed Adipose tissue cultures

The aim of these experiments was to see if elemental feeds with different content might exert different effects on adipose tissue ex vivo. As discussed in the introduction there is controversy surrounding the importance of *n*-3 and *n*-6 fatty acids in relation to the management of Crohn's disease and whether they provide inflammatory or anti-inflammatory effects.

## 2.4 Choice of Feeds

Elemental 028 Extra (Nutricia®) is the standard elemental feed used in our hospital for patients with acute flares of Crohn's disease and those with short bowel syndrome. We decided to use this feed as our standard due to its ready availability and to allow any results of our research to be translated clinically without delay. For comparison we used Emsogen (Nutricia®) elemental feed,

which was very similar in its standard nutritional content but varied in its fat composition. By keeping the different feeds as standardised as possible bar the fat content we aimed to attribute any differences we found experimentally to the fat content. Closer examination of the content is described below and the full nutritional content listed in Appendix 1.

## 2.5 Feeds

### 2.5.1 Elemental 028 Extra

Elemental 028 Extra is a liquid preparation which is used in children over 5 yrs old and adults as a sole source of nutrition or as a supplementary feed for the dietary management of Crohn's disease, short bowel syndrome, intractable malabsorption, enterocutaneous fistulae and radiation enteritis. It is provided ready mixed in liquid form and after careful dilution under sterile conditions with 2 parts CellGro® to 1 part Elemental 028 it was ready to use for adipose tissue culture.

### 2.5.2 Emsogen

Emsogen is designed for supplementary use in the management of severe malabsoprtion where long chain triglycerides are poorly tolerated and in cystic fibrosis. It requires supplementation with α-linolenic acid for use as a sole source of nutrition. It is a powder preparation. To make the 1:2 concentration of feed, Emsogen was first made up with distilled water to 20% solution under sterile conditions before dilution with 2 parts CellGro® to 1 part Emsogen.

| | Elemental 028 | Emsogen |
|---|---|---|
| **Nutrition Information** | **Per 100ml** | **Per 100ml** |
| Energy Kcal | 86 | 88 |
| Protein equivalent g | 2.5 | 2.5 |
| Total amino acids g | 3 | 3 |
| Carbohydrate g | 11 | 12 |
| Of which sugars g | 4.7 | 1.6 |
| Fat g | 3.5 | 3.3 |
| Of which saturates g | 1.3 | 2.6 |
| Monounsaturates g | 1.6 | 0.12 |
| Polyunsaturates g | 0.45 | 0.34 |
| % LCT | 65 | 17 |
| % MCT | 35 | 83 |
| Ratio n6:n3 fatty acids | 3.5:1 | 46.5:1 |
| % energy from linoleic acid | 3.6 | 3.4 |
| % energy from α-linolenic acid | 1 | 0.07 |
| Fibre g | Nil | Nil |
| **Fatty Acid Profile** | **g per 100g Fatty Acids** | **g per 100g Fatty Acids** |
| C6:0 | 0 | 1.4 |
| C8:0 | 20.1 | 59.2 |
| C10:0 | 14.9 | 20.4 |
| C12:0 | 0.1 | 1.9 |
| C14:0 | Trace | 0 |
| C16:0 | 2.6 | 2 |
| C18:0 | 1.6 | 0.27 |
| C18:1 (Oleic) *n*-9 | 46 | 3.9 |
| C18:2 (Linoleic) *n*-6 | 10.5 | 10.7 |
| C18:3 (α-linolenic acid) *n*-3 | 3 | 0.23 |
| C20:0 | 1.2 | 0 |
| C22:0 | 1.2 | 0 |

**Table 8.** The nutritional content of the two experimental elemental feeds.

Samples from each depot of adipose tissue were cultured in a 2:1 formulation of CellGro® and the specific elemental feed for 24 hours. The samples were then transferred to either 0.5 mLs of CellGro® or CellGro® with 1 μM isoprenaline for 4 hours. The specimens and supernatant were then stored at -80°C before subsequent analysis.

### 2.5.3 Adipokine assays

#### *2.5.3.1 ELISAs*

Enzyme-linked immunosorbant assays (ELISAs) were used to quantify adipokine and cytokines in adipose tissue cultures. IL-6, MCP-1 and leptin levels were

assessed with commercially available sandwich ELISAs (R&D Systems, Abingdon, UK). The microplates are pre-coated with a monoclonal antibody specific for the target cytokine. Standards were made up and with the diluted samples pipetted into the wells and incubated for the time indicated, so that any target cytokine was bound by the monoclonal antibody coating the well. After washing away the unbound substances an enzyme-linked polyclonal antibody specific to the target cytokine was added to the wells. Following a period of further incubation and the washing away of any unbound enzyme-antibody reagent, a substrate solution was added to the wells and colour develops according to the proportion of the cytokine bound in the initial step. The colour development is then stopped and the intensity measured with a microplate reader, as absorbance at 450nm with correction at 540nm. Intra- and inter-assay coefficients of variation are <5% and <10% respectively for all assays used.

The results were then divided by the sample dilutions to work out the true cytokine concentration.

The following dilutions were used for explant adipokine assays:

| **Adipokine** | **Dilution** |
|---|---|
| **IL-6** | 1:75 |
| **Leptin** | 1:10 |
| **MCP-1** | 1:100 |

**Table 9.** Adipokine ELISA dilutions

### 2.5.4 NEFA measurement

Non-esterified fatty acid (NEFA) in tissue culture supernatant was measured with an enzymatic colorimetric method (Wako HR Series NEFA-HR (2) kit, Wako Chemicals GmbH, Neus, Germany). The assay involves the acylation of coenzyme A by fatty acids, in the presence of acyl-CoA synthetase, followed by oxidation of acyl Co-A by acyl CoA oxidase and resultant production of hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase, allows oxidative condensation of 3-methyl-N-ethyl-N-(β-hydoxyethyl)-aniline with 4-aminoantipyrine and formation of a purple colour, the intensity of which is then measured spectrophotometrically.

Succinctly, 75 µL of tissue culture supernatant or NEFA standard (oleic acid at concentrations of 1.0 mmol/L, 0.5 mmol/L, 0.25 mmol/L, 0.125 mmol/L and 0.0625 mmol/L were added to a 96 well plate. They were incubated with reagent A (150 µL containing 0.31 mmol/L coenzyme A and 0.53 kU/L acyl-CoA-synthetase) for 15 minutes and with reagent B (75 µL containing 12 kU/L acyl-CoA-oxidase, 14 kU/L peroxidase, 2.4 mmol/L 3-methyl-N-ethyl-N (β-hydroxyethyl)-aniline) for 10 minutes before absorbance was measured at 550 nm.

## 2.6 Other Preliminary Methods for later analysis

### 2.6.1 Methods for later RNA extraction

#### *2.6.1.1 Collagenase Digest*

Approximately 4g of adipose tissue from each depot was weighed and minced under sterile conditions. The minced fat was then transferred into a 50ml universal tube to which was added 1ml of collagenase, 1.5 ml BSA and 7.5 ml CellGro®. The tubes were sealed with parafilm and incubated in a water bath at 37.0°C for 45 minutes. The resulting cellular suspension was filtered through a 250 micron sieve (Sigma-Aldrich, St. Louis, USA) and then transferred to a 15 ml flacon tube for centrifugation. The suspension was centrifuged for 5 minutes at 2000 rpm at 4°C. The adipocyte plug was separated and frozen fresh at -80°C and with 0.8ml of TRIzol® (Life Technologies, Carlsbad, USA), a monophasic solution of phenol and guanidine isothiocyanate designed to isolate separate fractions of RNA, DNA, and proteins from human cell and tissue samples. The supernatant was carefully poured off and the pellet washed 3 times in 1 ml of CellGro® and subsequently centrifuged again at 2000 rpm at 4°C. After the 3$^{rd}$ wash the pellet was re-suspended in 1 ml of CellGro® and then stored fresh at -80°C and with 0.8 ml of TRIzol.

#### *2.6.1.2 RNAlater®*

At the time of specimen collection in the operating theatre a subset of samples were immediately stored in RNAlater® (Life Technologies, Carlsbad, USA), an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. After returning to the laboratory the samples were then stored at -80°C.

### 2.6.2 Ex Vivo Angiogenesis Assay of Adipose Tissue

The role of angiogenesis in the expansion of adipose depots is not known. Recently Gealekman (Gealekman et al., 2011) was able to show that different depots of mouse adipose tissue has altered angiogenic capacity and in particular subcutaneous adipose tissue had the greatest in comparison to visceral adipose tissue. The thickened mesentery of Crohn's disease affected bowel is known to be very vascular. We attempted to replicate Gealekman's work to see if adipose tissue depots in Crohn's disease have different angiogenic capacity to controls. We also attempted to see if culture with different elemental feeds altered angiogenic capacity.

In a subset of patients 2 mg samples of the three adipose depots were embedded in an individual well of a 96 well plate containing 150 µL of Matrigel (BD Discovery Labware, Billerica, USA). Wells were filled with 150 µL of endothelial growth media (EGM-2 BulletKit, Lonza, Wokingham, UK) with 10% fetal bovine serum (FBS Gibco® Life Technologies, Carlsbad, USA). 100 µL was changed every second day for 14 days. Photographs were then taken at x20 magnification.

For the elemental feed experiment the EGM was mixed with each feed in a 2:1 ratio and substituted for the EGM in the protocol above.

## 2.7 Statistical Analyses

Data were analysed using IBM SPSS version 20.0 for Macintosh software (Statistical Package for the Social Sciences, IBM SPSS, New York, UK). Data in the text are expressed as mean (SD) or median (IQR) unless otherwise stated. Comparisons between depots were performed with Mann-Whitney for non-parametric data and correlations were determined by Spearman's Rho. Significance was defined as $p<0.05$

# Chapter 3

# CHARACTERISATION OF ADIPOSE TISSUE

## 3.1 Introduction

In this section are the results of the basal analysis of adipose tissue from subcutaneous, omental and mesenteric depots from the three disease cohorts, Crohn's disease, ulcerative colitis and non-Crohn's non-UC. NEFA concentration as a measure of lipolysis, IL-6, Leptin and MCP-1 concentrations were all measured after 24hrs incubation in CellGro® to determine if there were any basal differences between disease cohorts and adipose tissue depots. Furthermore to look at hormone responsiveness and to demonstrate viability, basal lipolysis and cytokine secretion profiles were performed at 4hrs and after 24hrs of organ culture. Organ culture with 1 microMolar Isoprenaline has previously been used to demonstrate increased lipolysis and thus tissue viability (Fried, S. K. & Moustaid-Moussa, N. 2001) and this solution was used in comparison with plain CellGro®. Additionally a single angiogenesis assay in matrigel was also performed and the results are presented.

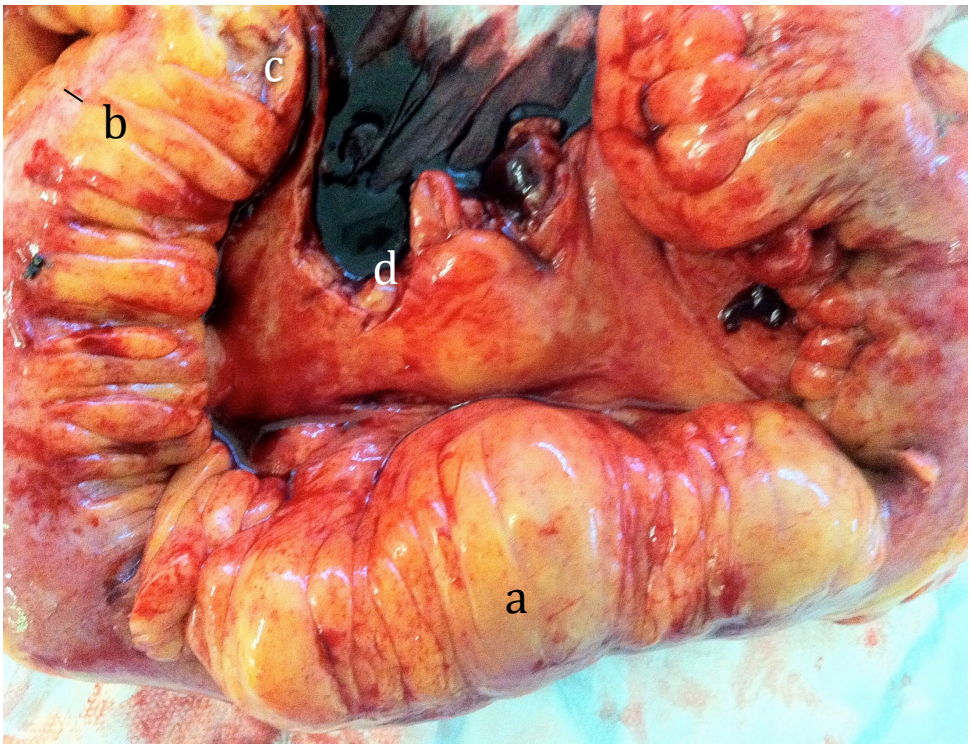



**Figure 7.** A photograph of an ileal resection specimen for Crohn's disease

The letter **a** demonstrates fat-wrapping; **b** demonstrates minimal serosa left 'unwrapped'; **c** shows the position of the piece removed (FW) for histologic comparison with standard piece (MF) removed in all cases shown at position **d**.

## 3.2 Basal Organ Culture

Adipose tissue samples from each depot from fasting patients and from both the Crohn's disease and ulcerative colitis cohorts were incubated in CellGro® with 1% penicillin/streptomycin for 24 hours in the conditions previously described, to look for any differences in basal production of cytokines and lipolysis. Measurement of non-esterified fatty acids (NEFA) was used as a measure of lipolysis and the cytokines IL-6, Leptin and MCP-1 were also measured.

### 3.2.1 NEFA

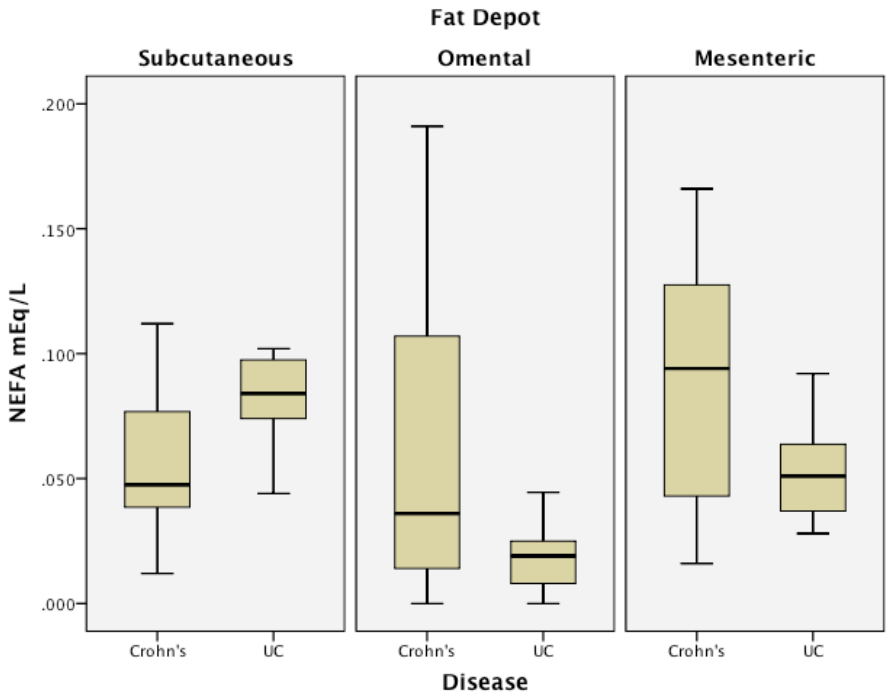



**Figure 8.** Net release of NEFA over 24h from 0.1g samples of whole tissue from mesentery, omentum and subcutaneous adipose tissue of CD and UC patients.

| | Crohn's | Ulcerative Colitis |
|---|---|---|
| **N** | 12 | 9 |
| **Sex (Male:Female)** | 5:7 | 7:2 |
| **BMI (kg/m²)** | 21.0 (19.6 – 24.0) | 23.1 (22.0 – 25.7) |
| **SC NEFA mEq/L** | 0.048 (0.033 – 0.092)<br>*† p=0.244* | 0.084 (0.059 – 0.100) |
| **OM NEFA mEq/L** | 0.036 (0.008 – 0.112)<br>*† p=0.270* | 0.019 (0.005 – 0.035) |
| **MF NEFA mEq/L** | 0.094 (0.039 – 0.128)<br>*† p=0.393* | 0.060 (0.037 – 0.079) |

**Table 10.** Net release of NEFA over 24h from 0.1g samples of whole tissue from mesentery, omentum and subcutaneous adipose tissue of CD and UC patients.

† Statistical difference between Crohn's and UC (Mann-Whitney)

| | SC | OM | MF |
|---|---|---|---|
| **Crohn's** | 0.048 (0.033 – 0.092)<br>*† p=0.587*<br>** p=0.176* | 0.036 (0.008 – 0.112)<br>*§ p=0.176* | 0.094 (0.039 – 0.128) |
| **UC** | 0.084 (0.059 – 0.100)<br>*† p=**0.002***<br>** p=0.171* | 0.019 (0.005 – 0.035)<br>*§ p=**0.007*** | 0.060 (0.037 – 0.079) |

**Table 11.** A table comparing net release of NEFA between subcutaneous, omental and mesenteric adipose depots.

† Statistical difference between SC and OM (Mann-Whitney)
* Statistical difference between SC and MF
§ Statistical difference between OM and MF

#### *3.2.1.1 Findings*

The only significant differences were between the amount of NEFA produced by subcutaneous and omental tissue and by omental and mesenteric tissue in ulcerative colitis. There were no significant differences between the two diseases. However although the mean values for Crohn's disease and ulcerative colitis are similar the ranges are consistently higher in the Crohn's disease depots (0.000 - 0.112 in CD OM vs 0.005 – 0.035 in UC OM). The reasons for this are unclear. It is possible that over 24 hours some lipolytic products may be re-esterified into triacylglycerols. Further more there may be depot or site-specific differences in the micro-anatomy of the tissue which might explain the large variances.

### 3.2.2 Basal IL-6, Leptin and MCP-1 after 24hrs incubation

#### *3.2.2.1 Graphs*

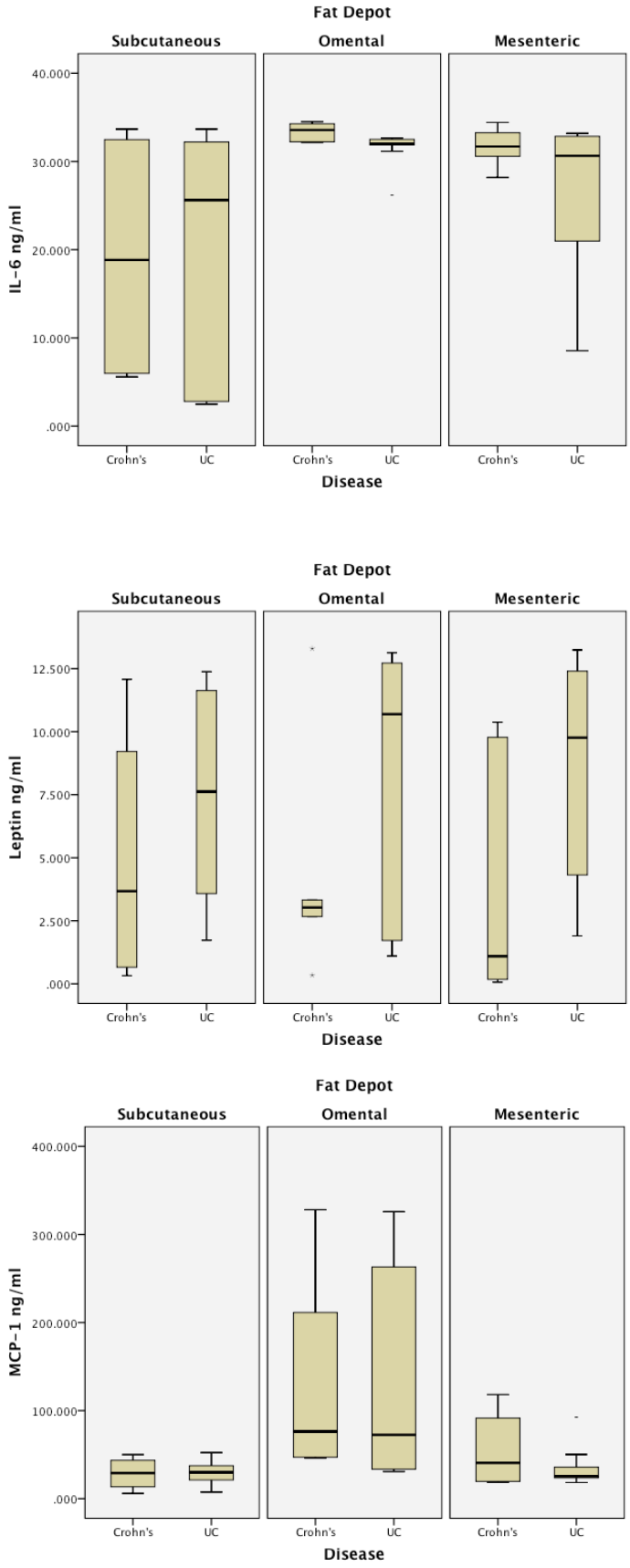



**Figure 9.** Graphs showing the different basal concentrations of IL-6, Leptin and MCP-1 between CD and UC after 24 h incubation in CellGro®.

### *3.2.2.2 Tables*

| | **Crohn's** | **Ulcerative Colitis** |
|---|---|---|
| **N** | 6 | 6 |
| **Sex (Male:Female)** | 3:3 | 2:4 |
| **BMI (kg/m²)** | 21.8 (18.3 – 29.7) | 23.5 (19.3 – 30.0) |
| ***IL-6*** | | |
| SC IL-6 ng/ml | 18.8 (5.8 – 33.1)<br>*† p=0.748* | 25.6 (2.7 – 32.6) |
| OM IL-6 ng/ ml | 33.6 (32.2 – 34.3)<br>*† p=**0.025*** | 31.9 (29.9 – 32.5) |
| MF IL-6 ng/ ml | 31.7 (30.0 – 33.5)<br>*† p=0.423* | 30.6 (17.9 – 32.9) |
| ***Leptin*** | | |
| SC Leptin ng/ ml | 3.67 (0.49 – 10.64)<br>*† p=0.394* | 7.62 (3.12 – 11.82) |
| OM Leptin ng/ ml | 3.03 (2.09– 5.82)<br>*† p=0.522* | 10.70 (1.56 – 12.82) |
| MF Leptin ng/ ml | 1.095 (0.15 – 9.93)<br>*† p=0.055* | 9.76 (3.71 – 12.61) |
| ***MCP-1*** | | |
| SC MCP-1 ng/ ml | 29.1 (9.8 – 46.8)<br>*† p=0.670* | 29.9 (17.8 – 41.2) |
| OM MCP-1 ng/ ml | 76.3 (46.7 – 240.6)<br>*† p=0.631* | 72.5 (32.8 – 278.8) |
| MF MCP-1 ng/ ml | 40.7 (19.4 – 98.2)<br>*† p=0.749* | 30.6 (22.4 – 60.7) |

**Table 12.** A table comparing the concentrations of IL-6, Leptin and MCP-1 released from subcutaneous, omental and mesenteric adipose depots between diseases.

*†* Statistical difference between Crohn's and UC (Mann-Whitney)

| | SC | OM | MF |
|---|---|---|---|
| ***IL-6*** | | | |
| **Crohn's** | 18.8 (5.8 – 33.1)<br>*† p=0.880*<br>** p=0.286* | 33.6 (32.2 – 34.3)<br>*§ p=0.078* | 31.7 (30.0 – 33.5) |
| **UC** | 25.6 (2.7 – 32.6)<br>*† p=0.262*<br>** p=0.522* | 31.9 (29.9 – 32.5)<br>*§ p=0.749* | 30.6 (17.9 – 32.9) |
| ***Leptin*** | | | |
| **Crohn's** | 3.67 (0.49 – 10.64)<br>*† p=0.831*<br>** p=0.522* | 3.03 (2.09– 5.82)<br>*§ p=0.337* | 1.095 (0.15 – 9.93) |
| **UC** | 7.62 (3.12 – 11.82)<br>*† p=0.749*<br>** p=0.423* | 10.70 (1.56 – 12.82)<br>*§ p=0.749* | 9.76 (3.71 – 12.61) |
| ***MCP-1*** | | | |
| **Crohn's** | 29.1 (9.8 – 46.8)<br>*† p=0.522*<br>** p=0.176* | 76.3 (46.7 – 240.6) §<br>*p=0.200* | 40.7 (19.4 – 98.2) |
| **UC** | 29.9 (17.8 – 41.2)<br>*† p=**0.037***<br>** p=0.749* | 72.5 (32.8 – 278.8)<br>*§ p=0.109* | 30.6 (22.4 – 60.7) |

**Table 13.** A table comparing the net release of IL-6, Leptin and MCP-1 between adipose depots.

*†* Statistical difference between SC and OM (Mann-Whitney)
* Statistical difference between SC and MF
*§* Statistical difference between OM and MF

#### *3.2.2.3 Findings*

The only significant differences were between the amount of omental IL-6 produced between Crohn's disease and ulcerative colitis and the amount of MCP-1 produced between subcutaneous and omental depots in ulcerative colitis. The numbers for these basal experiments are small with only 6 of each disease cohort so it is unsurprising that few reached statistical significance. There may be a trend appearing that in the ulcerative colitis cohort all of the depots seem to secrete greater amounts of leptin : 10.70 (1.56 – 12.82) vs 3.03 (2.09– 5.82) in omental fat and 9.76 (3.71 – 12.61) vs 1.095 (0.15 – 9.93) in mesenteric fat. This did not reach statistical significance but further analysis may validate this. Leptin levels may be affected by pre-operative fasting but there is no obvious flaw in the study design to suggest that colitics were fasted any more than Crohn's patients, if anything the opposite. Whole adipose tissue was studied so differences may be attributable to

other structures within the tissues such as lymphoid cells which may have contributed to the results as much as adipocytes. Subcutaneous tissue contains minimal lymphoid cells and so is probably the most representative of adipocyte function alone.

## 3.3 Catecholamine Sensitivity

This experiment aimed to prove that the adipose tissue samples were hormone sensitive ex vivo and still hormone sensitive after 24hrs incubation. Samples were cultured in either CellGro® for 4 hours or CellGro® and 1μM isoprenaline in CellGro®. At the endpoint the supernatants were transferred to sterile Eppendorfs and frozen at -80°C. For the 24 hour experiment samples were cultured in CellGro® for 24 hours and then transferred with to a well organ bath containing more CellGro® or 1μM isoprenaline in CellGro® for a further 4 hours. This technique is well described (Fried and Moustaid-Moussa, 2001) and previously successfully replicated in our laboratory (Karastergiou and Mohamed-Ali, 2010).

### 3.3.1 Differences in Lipolysis after 4hrs

| | **Crohn's** | | **Ulcerative Colitis** | |
|---|---|---|---|---|
| **N** | 7 | | 4 | |
| **Sex (Male:Female)** | 5:2 | | 2:2 | |
| **BMI (kg/m²)** | 22.2 (19.7 – 24.5) | | 23.1 (22.3 – 30.5) | |
| **4hr Medium** | **CellGro®** | **CellGro® + 1μM isoprenaline** | **CellGro®** | **CellGro® + 1μM isoprenaline** |
| **SC NEFA mEq/L** | 0.053 (0.047 – 0.065 )<br>*† p=0.056* | 0.073 (0.062 – 0.082) | 0.081 (0.059 – 0.120)<br>*† p=0.486* | 0.099 (0.067 – 0.136) |
| **OM NEFA mEq/L** | 0.052 (0.036 – 0.109)<br>*† p=0.805* | 0.053 (0.033 – 0.128) | 0.042 (0.024 – 0.058)<br>*† p=0.886* | 0.036 (0.023 – 0.051) |
| **MF NEFA mEq/L** | 0.077(0.070 – 0.103)<br>*† p=0.318* | 0.093 (0.079 – 0.115) | 0.071 (0.035 – 0.129)<br>*† p=0.686* | 0.070 (0.046 – 0.129) |

**Table 14.** A table comparing the differences in net NEFA release after culture of the three adipose depots for 4 hours in ether CellGro® or CellGro® + 1μM isoprenaline

*†* is the Statistical difference between the different 4h media

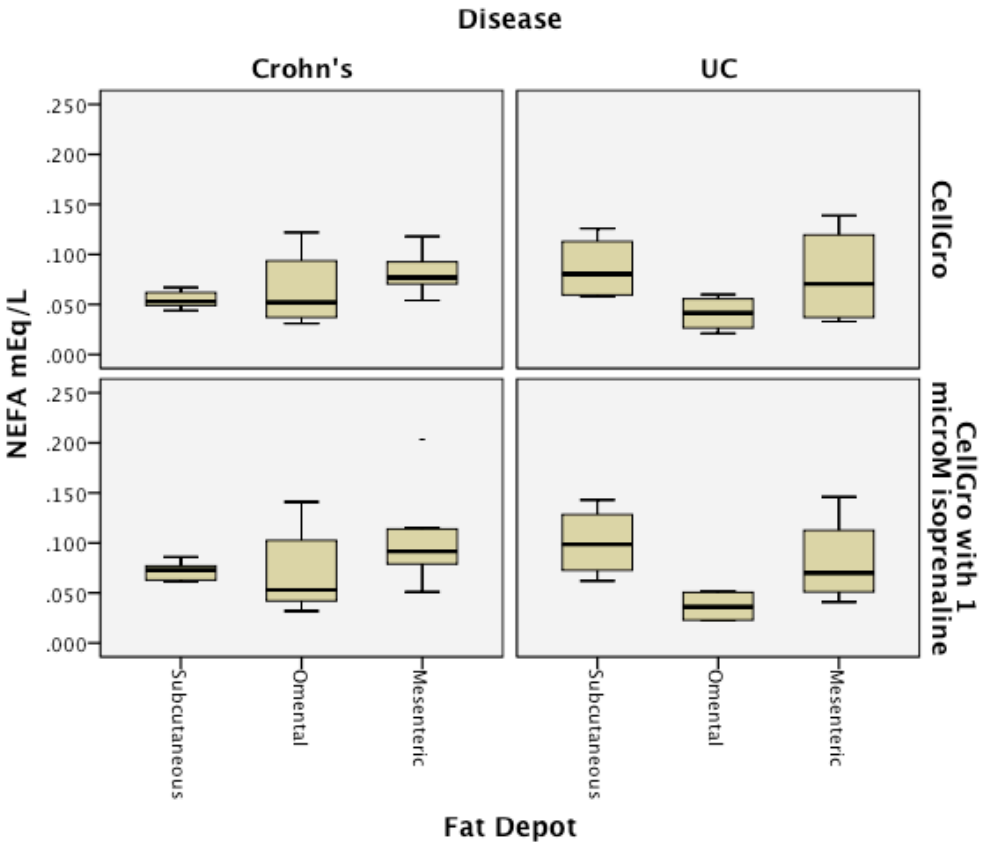


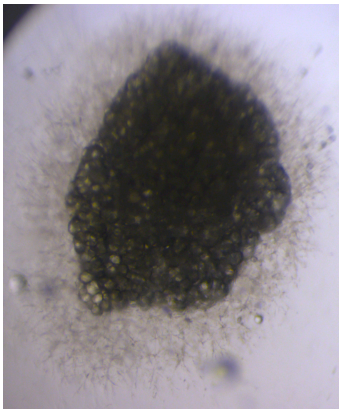

**Figure 10.** This graph shows the difference in net NEFA release after 4h culture in CellGro® or CellGro® + 1μM isoprenaline

### 3.3.2 Findings

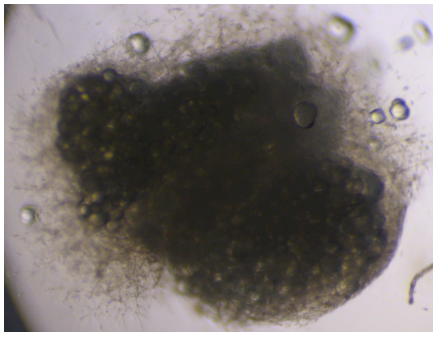
Only the 4hrly results are displayed above but the results from the 24hr experiment were the same. It was expected that both the 4 hourly and the 24 hourly experiments would demonstrate a marked difference in the degree of lipolysis seen after catecholamine stimulation in line with the literature and our laboratory's previous findings. Instead no statistical difference was seen in either experiment. Adipose tissue fresh from a living patient certainly ought to be responsive to isoprenaline if immediately cultured. We concluded that the isoprenaline solution was in some way flawed. This correlated with other findings within the lab. For financial reasons isoprenaline solution made by a previous fellow and frozen at -20C was initially used. Once this had been rejected and fresh solution made. We were able to confirm hormone-sensitivity and viability of adipose tissue in culture at 24 hours.

## 3.4 Matrigel Angiogenesis Assay

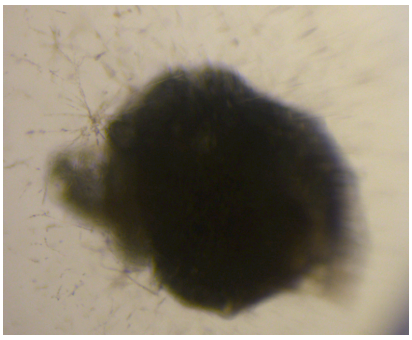
We aimed to replicate the angiogenesis assay of Gealekman as described in more detail earlier (Gealekman et al., 2011). From a surgeon's perspective thickened Crohn's mesentery appears very vascular. Similarly the histological analysis of Crohn's disease affected segments of bowel reveal local vascultis like changes (Hendrickson et al., 2002, Van Kruiningen and Colombel, 2008). If we were able to induce angiogenesis from adipose tissue samples in vivo then we might be able to show an effect of different elemental feeds on the degree of angiogenesis. Only one assay was performed with 2 samples from each depot of one patient with Crohn's disease (Age 42, BMI 24). After two weeks a photo of each sample was taken (see Fig 12)



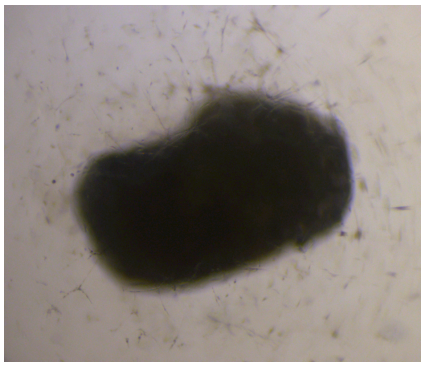
SC 1



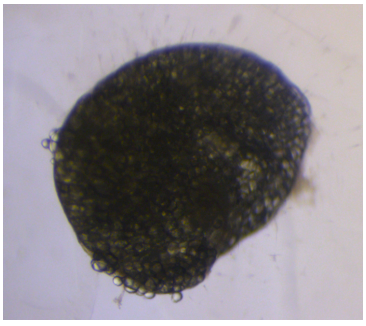
SC 2



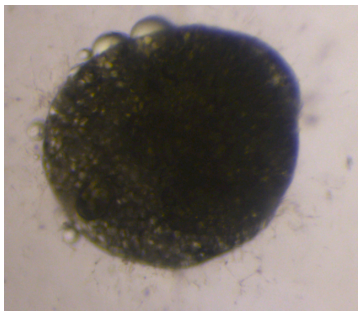
OM1



OM2



MF1



MF2

**Figure 11** The photos above depict the rate of angiogenesis from each depot after two weeks incubation in Matrigel with endothelial growth media (mag x10). SC – subcutaneous, OM – omental, MF – mesenteric. Not to scale.

### 3.4.1 Findings

Angiogenesis is clearly demonstrated in the pictures above validating the Gealeckman's technique. The subcutaneous adipose tissue samples appear to have had a much greater response than the visceral depots with the mesenteric fat showing the least response to the endothelial growth medium. Subcutaneous adipose tissue expands readily in obesity so could be expected to have angiogenic potential.

# Chapter 4

# ELEMENTAL FEED EXPERIMENTS

## 4.1 Introduction

The aim of these experiments was to see if elemental feeds with different content might exert variable effects on adipose tissue ex vivo. As discussed in the introduction there is controversy surrounding the importance of *n*-3 and *n*-6 fatty acids in relation to the management of Crohn's disease and whether they provide inflammatory or anti-inflammatory effects. The rationale behind the choice of feeds, Elemental 028 (*n*-6:*n*-3 ratio 3.5:1) and Emsogen (*n*-6:*n*-3 ratio 46.5:1) has been discussed earlier in the Methods chapter.

## 4.2 Choice of concentration

It was unknown whether adipose tissue would survive culture in elemental feed in vitro and it was also unknown at what concentration, if at all, Crohn's mesenteric fat might be exposed to the fat content of elemental feed. Given that the upper gastrointestinal tract produces up to 8 litres of fluid per day, most of which is reabsorbed along the small intestine, we deduced that the elemental feed would be diluted by the time it had reached the terminal ileum. An experiment was devised to determine the best concentration of elemental feed at which to culture the adipose tissue samples.

Subcutaneous, omental and mesenteric adipose tissue samples were collected from a patient with Crohn's disease. These samples were cultured in three concentrations of Elemental 028 Extra mixed with CellGro® containing 1% streptomycin/penicillin solution for 24 hours under standard incubator conditions as described in the Methods section. The three concentrations were 1:1, 1:2, 1:5 Feed:CellGro®. After 24 hours the adipose tissue samples were transferred to either CellGro® or CellGro® with 1 μM isoprenaline for 4 hours. The supernatants were then analysed for NEFA as a marker of lipolysis and thus viability and activity. The results are shown in Table 15 with percentage change (hormone-responsiveness) taken as a marker of the most effective concentration to use for further experimentation.

| | NEFA mEq/L | | % change |
|---|---|---|---|
| | CellGro® | CellGro® + 1 µM isoprenaline | |
| **1:1 concentration** | | | |
| **SC** | 0.358 | 0.462 | 29% |
| **OM** | 0.558 | 0.599 | 7% |
| **MF** | 0.310 | 0.306 | -1% |
| **1:2 concentration** | | | |
| **SC** | 0.196 | 0.275 | 40% |
| **OM** | 0.129 | 0.220 | 71% |
| **MF** | 0.148 | 0.190 | 28% |
| **1:5 concentration** | | | |
| **SC** | 0.102 | 0.146 | 43% |
| **OM** | 0.092 | 0.151 | 64% |
| **MF** | 0.123 | 0.157 | 28% |

**Table 15.** This table shows concentrations of NEFA after 24 h culture in the three concentrations of feed (1:1, 1:2, 1:5) and then 4 hours culture in either CellGro® or CellGro® with 1 µM isoprenaline.

### 4.2.1 Findings

The findings above showed the hormone-responsiveness of the adipose tissue after 24 hours of culture. Overall the 1:2 concentration of Elemental 028 Extra to CellGro® provided the greatest increase in NEFA (74%) and so it was decided that all adipose tissue cultures in the different elemental feeds in vitro would be performed at this concentration.

## 4.3 Feed experiments

### 4.3.1 NEFA

Initially the degree of lipolysis was measured by NEFA concentration in the supernatant of samples that were cultured in either of the two feeds for 24 hours and then for 4hrs in CellGro®.

#### *4.3.1.1 Difference in lipolysis between feeds and between diseases.*

| | Crohn's | | Ulcerative Colitis | |
|---|---|---|---|---|
| **Feed** | **E 028** | **Emsogen** | **E 028** | **Emsogen** |
| **N** | 9 | 4 | 3 | 3 |
| **Sex (M:F)** | 4:5 | 2:2 | 3:0 | 2:1 |
| **BMI (kg/m²)** | 22.2 (19.2 – 24.0) | 19.3 (20.3 - 20.9) | 23.7 (22.0 – 26.1) | 23.7 (22.0 - 26.1 ) |
| **SC NEFA mEq/L** | 0.17 (0.10-0.27) *† p=0.283* | 0.10(0.09-) | 0.15 (0.11 – 0.42) *† p=0.297* | 0.13 (0.03 – 0.16) |
| **OM NEFA mEq/L** | 0.19 (0.1 – 0.33) ***† p<0.001*** | 0.04 (0.03-0.08) | 0.21 (0.10– 0.27) ***† p=0.004*** | 0.05 (0.02 – 0.06) |
| **MF NEFA mEq/L** | 0.18 (0.12 – 0.32) ***† p=0.017*** | 0.09 (0.06-0.14) | 0.21 (0.10 – 0.33) ***† p=0.01*** | 0.05 (0.04 – 0.07) |

**Table 16.** Net release of NEFA is compared between feeds and between diseases.

*†* Statistical difference between the two feeds.

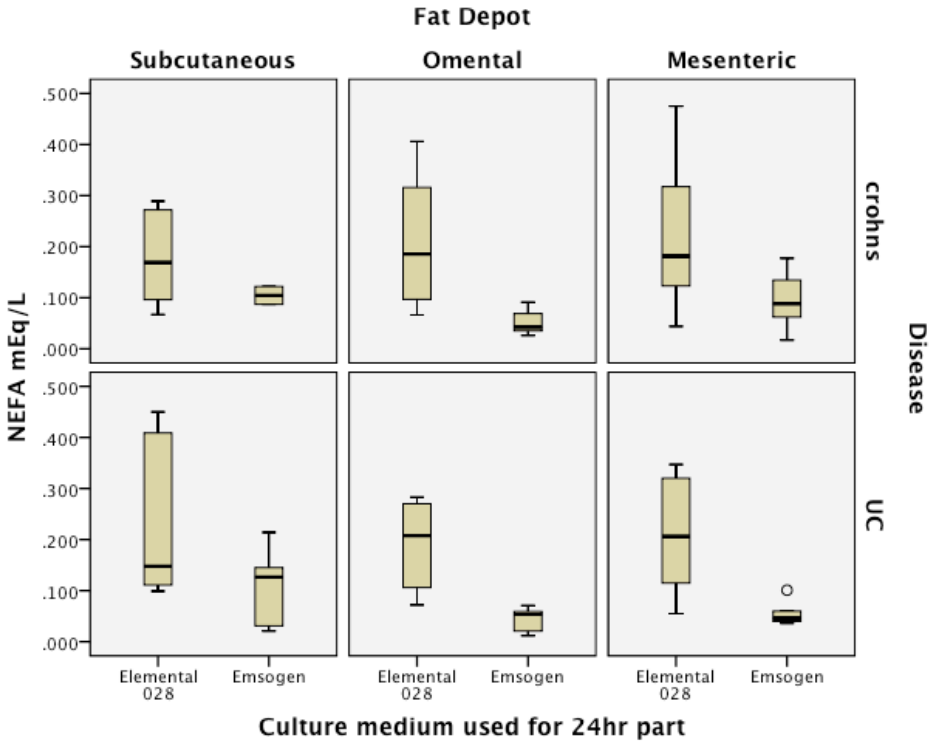



**Figure 12** This graph shows the difference in net NEFA release between feeds for the three adipose depots.

#### *4.3.1.2 Both cohorts combined*

The results when both diseases are combined together and the feeds compared directly are shown below in Table 17 and Figure 13.

| | **Both diseases combined** | |
|---|---|---|
| **Feed** | **E 028** | **Emsogen** |
| **N** | 12 | 7 |
| **Sex (M:F)** | 8:5 | 4:4 |
| **BMI (kg/m²)** | 23.7 (19.9 – 25.5) | 21.0 (20.2 -23.9) |
| **SC NEFA mEq/L** | 0.18(0.11- 0.27)<br>***† p=0.037*** | 0.12(0.07- 0.15) |
| **OM NEFA mEq/L** | 0.17 (0.10 – 0.27)<br>***† p<0.001*** | 0.05 (0.03-0.07) |
| **MF NEFA mEq/L** | 0.20 (0.12 – 0.32)<br>***† p<0.001*** | 0.08 (0.04-0.12) |

**Table 17.** Net NEFA release when both disease cohorts are combined compared between feeds.

*†* Statistical difference between the two feeds.

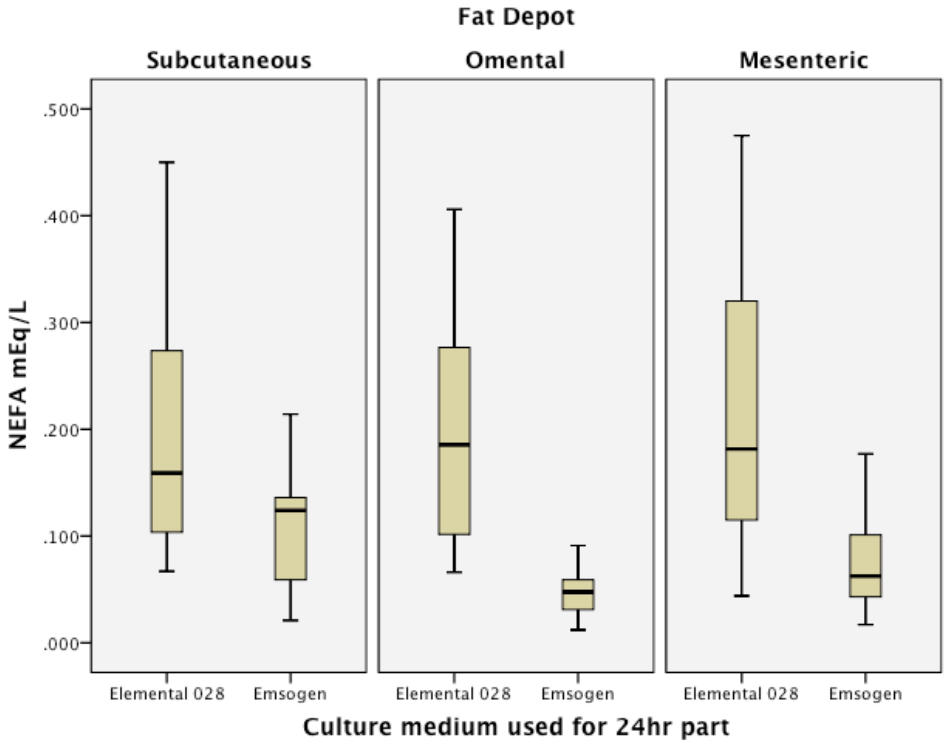



**Figure 13** The difference in NEFA release between the two feeds when both disease cohorts are combined.

#### *4.3.1.3 Findings*

For both the Crohn's disease cohort and the ulcerative colitis cohort there is a significant difference in NEFA concentration released from OM and MF adipose tissue depots into the two incubation solutions with a 2-3 fold increase in NEFA concentration in those samples that were cultured in Elemental 028 Extra. When both diseases are combined not only is the significance of the difference even more powerful but also there becomes a significant difference between the subcutaneous depots too (0.17 vs 0.05 mEq/L in OM and 0.2 vs 0.08 mEq/L in MF).

## 4.4 Cytokine Analyses

For the cytokine analysis six patients from each cohort had adipose tissue samples cultured in both feeds for 24 hours and then CellGro® for 4 hours. By ensuring that all samples from all patients underwent culture in both feeds absolute parity was ensured. The supernatants were then assayed for IL-6, Leptin and MCP-1 using ELISA.

### 4.4.1 IL-6, Leptin and MCP-1

The results of the ELISAs for all three cytokines are tabulated below.

| | Crohn's | | Ulcerative Colitis | |
|---|---|---|---|---|
| **N** | 6 | | 6 | |
| **Sex (M:F)** | 3:3 | | 2:4 | |
| **BMI ($kg/m^2$)** | 21.8 (18.3 – 29.7) | | 23.5 (19.3 – 30.0) | |
| **Feed** | **E 028** | **Emsogen** | **E 028** | **Emsogen** |
| ***IL-6*** | | | | |
| SC IL-6 ng/ ml | 0.16 (0.08 – 0.28) *† p=0.172* | 0.33 (0.18 – 0.95) | 0.61 (0.38 – 1.62) *† p=0.686* | 2.20 (0.22 – 7.26) |
| OM IL-6 ng/ ml | 2.81 (0.41 – 13.7) *† p=0.133* | 12.61(2.91 – 20.86) | 3.25 (0.73 – 20.89) ***† p=0.05*** | 21.38 (10.49 – 21.93) |
| MF IL-6 ng/ ml | 1.18 (0.19 – 2.21) *† p=0.204* | 3.65 (0.15 – 5.51) | 1.64 (0.79 – 5.46) ***† p=0.043*** | 6.56 (1.96 – 20.45) |
| ***Leptin*** | | | | |
| SC Leptin ng/ ml | 0.09 (0.07 – 0.44) *† p=0.400* | 0.13 (0.08 – 3.43) | 0.36 (0.09 – 0.82) *† p=0.386* | 0.56 (0.11 – 2.30) |
| OM Leptin ng/ ml | 0.07 (0.06 – 0.09) *† p=0.060* | 0.12 (0.07 – 0.48) | 0.11 (0.07 – 0.33) ***† p=0.028*** | 0.61 (0.38 – 1.86) |
| MF Leptin ng/ ml | 0.08 (0.06 - 0.14) *† p=0.056* | 0.14 (0.08 – 0.25) | 0.31 (0.10 – 1.57) *† p=0.119* | 1.38 (0.33 – 5.54) |
| ***MCP-1*** | | | | |
| SC MCP-1 ng/ ml | 0.69 (0.55 – 0.78) ***† p=0.024*** | 1.65 (0.87 – 9.97) | 2.4 (0.78 – 6.76) *† p=0.356* | 6.08 (2.67 – 13.56) |
| OM MCP-1 ng/ ml | 1.1 (0.64 – 4.16) ***† p=0.004*** | 10.34 (2.36 – 22.61) | 2.81 (0.89 – 39.37) *† p=0.119* | 28.71 (12.46 – 42.16) |
| MF MCP-1 ng/ ml | 0.89 (0.56 – 1.24) ***† p=0.018*** | 2.24 (0.76 – 4.80) | 3.21 (1.04 – 10.03) *† p=0.057* | 12.69 (4.55 – 20.32) |

**Table 18.** The net release of each cytokine is compared by feed and disease cohort

*†* Statistical difference between the two feeds.

#### *4.4.1.1 IL-6*

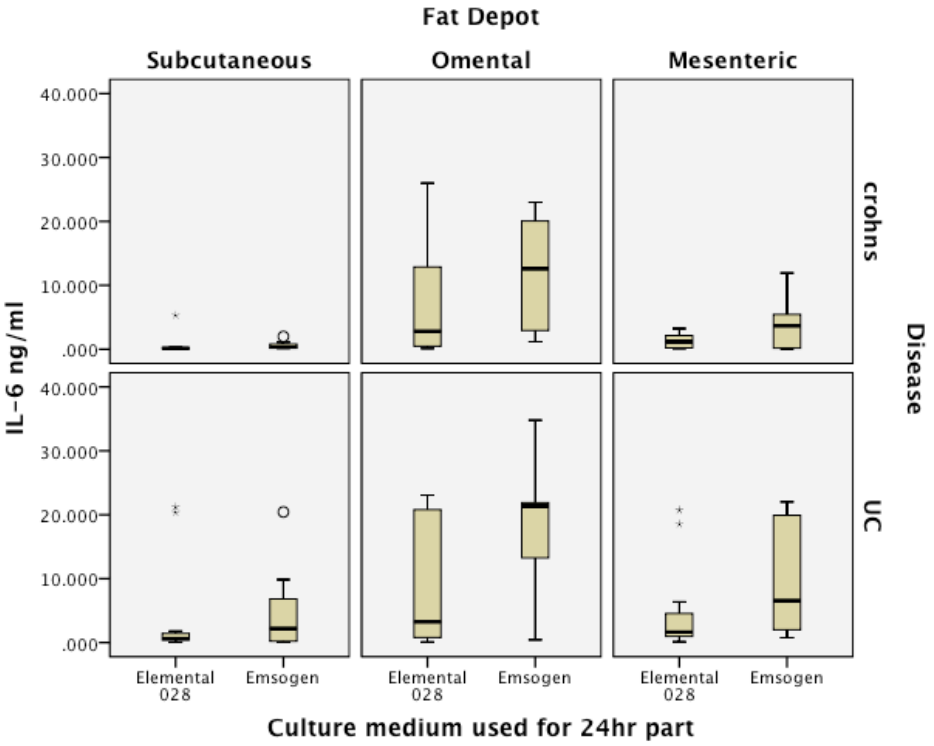



**Figure 14** A graph comparing net IL-6 release between feeds by adipose depot and disease

#### *4.4.1.2 Leptin*

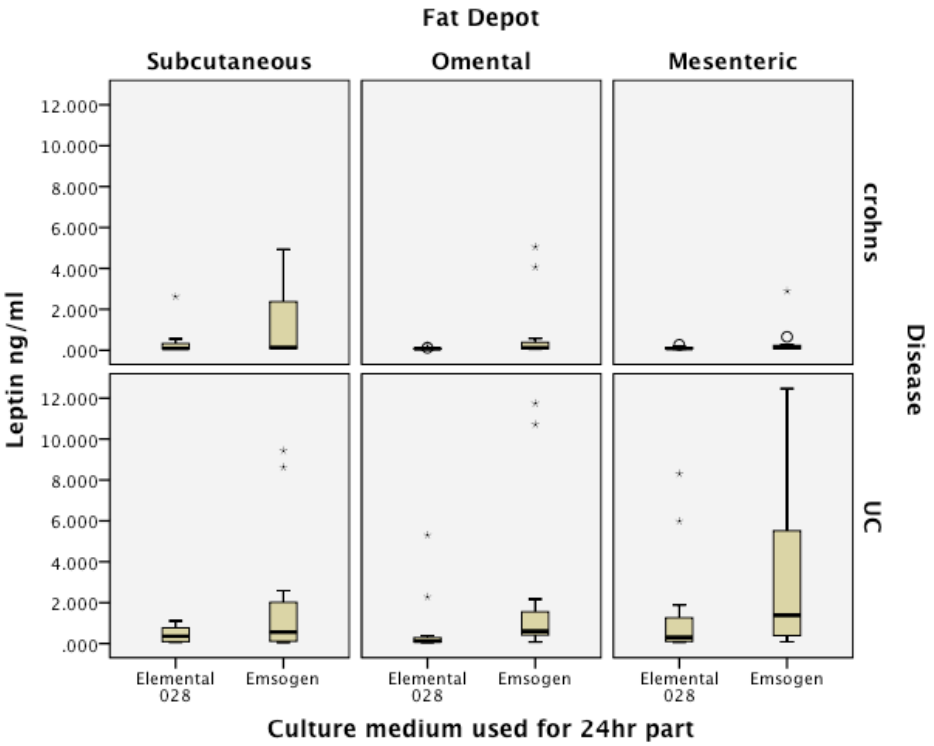



**Figure 15** A graph comparing net Leptin release between feeds by adipose depot and disease

#### *4.4.1.3 MCP-1*

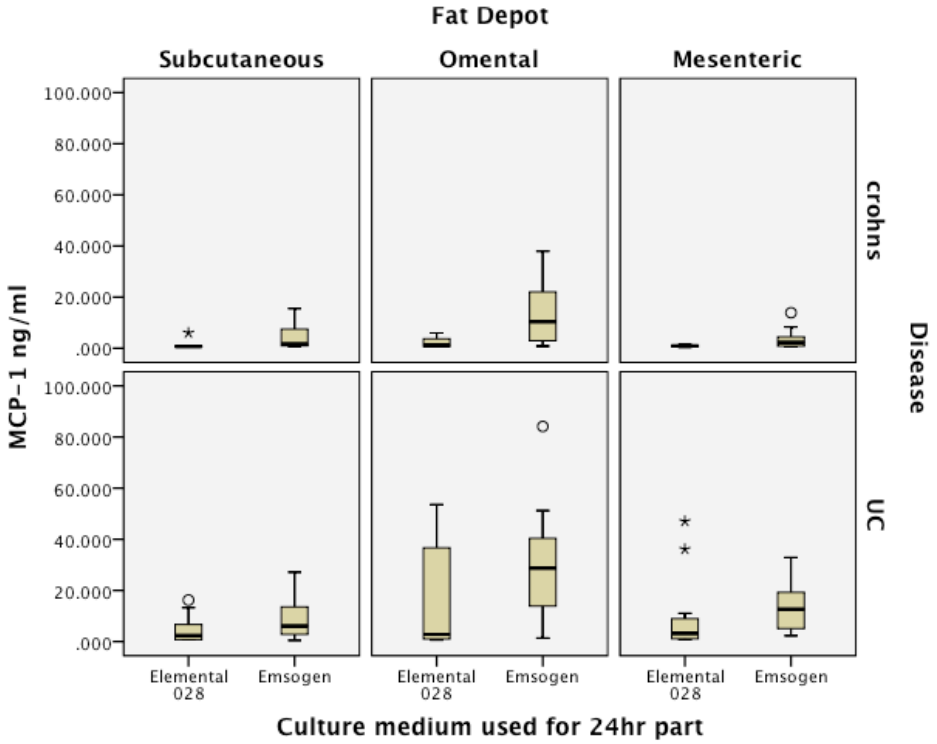



**Figure 16** A graph comparing net MCP-1 release between feeds by adipose depot and disease

### 4.4.2 Findings

There is a clear trend towards Il-6, Leptin and MCP-1 being raised in adipose tissue samples that were cultured in Emsogen in comparison to Elemental 028 Extra but this is statistically significant in only the six comparisons highlighted in Table 18.

## 4.5 Cytokine analysis with combined disease cohorts

To analyse solely the difference in effect between the two feeds, the results from the disease cohorts were combined and these are displayed in Table 19 and graphically in Figures 17, 18 & 19.

### 4.5.1 IL-6, Leptin and MCP-1 when both cohorts combined

| | **Both diseases combined** | |
|---|---|---|
| **N** | 12 | |
| **Sex (M:F)** | 5:7 | |
| **BMI (kg/m²)** | 23.1 (19.3 – 29.7) | |
| **Feed** | **E 028** | **Emsogen** |
| ***IL-6*** | | |
| SC IL-6 ng/ ml | 0.39 (0.11 – 0.99)<br>*† p=0.402* | 0.5 (0.2-2.65) |
| OM IL-6 ng/ ml | 3.07 (0.62 – 19.1)<br>***† p=0.018*** | 18.6 (3.1- 21.8) |
| MF IL-6 ng/ ml | 1.50 (0.42 – 2.61)<br>***† p=0.030*** | 3.77 (0.76 – 9.52) |
| ***Leptin*** | | |
| SC Leptin ng/ ml | 0.21 (0.08 – 0.64)<br>*† p=0.358* | 0.24 (0.09 – 2.30) |
| OM Leptin ng/ml | 0.08 (0.07 – 0.14)<br>***† p=0.006*** | 0.42 (0.08 – 0.90) |
| MF Leptin ng/ ml | 0.12 (0.07 – 0.31)<br>***† p=0.033*** | 0.27 (0.13 – 2.62) |
| ***MCP-1*** | | |
| SC MCP-1 ng/ ml | 0.81 (0.67 – 6.17)<br>*† p=0.070* | 3.38 (1.26 – 13.26) |
| OM MCP-1 ng/ ml | 1.83 (0.69 – 4.82)<br>***† p=0.002*** | 18.80 (4.39 – 31.5) |
| MF MCP-1 ng/ ml | 1.2 (0.82 – 3.39)<br>***† p=0.006*** | 4.59 (2.20 – 13.72) |

**Table 19.** The net release of each cytokine from the three adipose depots compared by feed and regardless of disease cohort.

*†* Statistical difference between the two feeds

### 4.5.2 IL-6

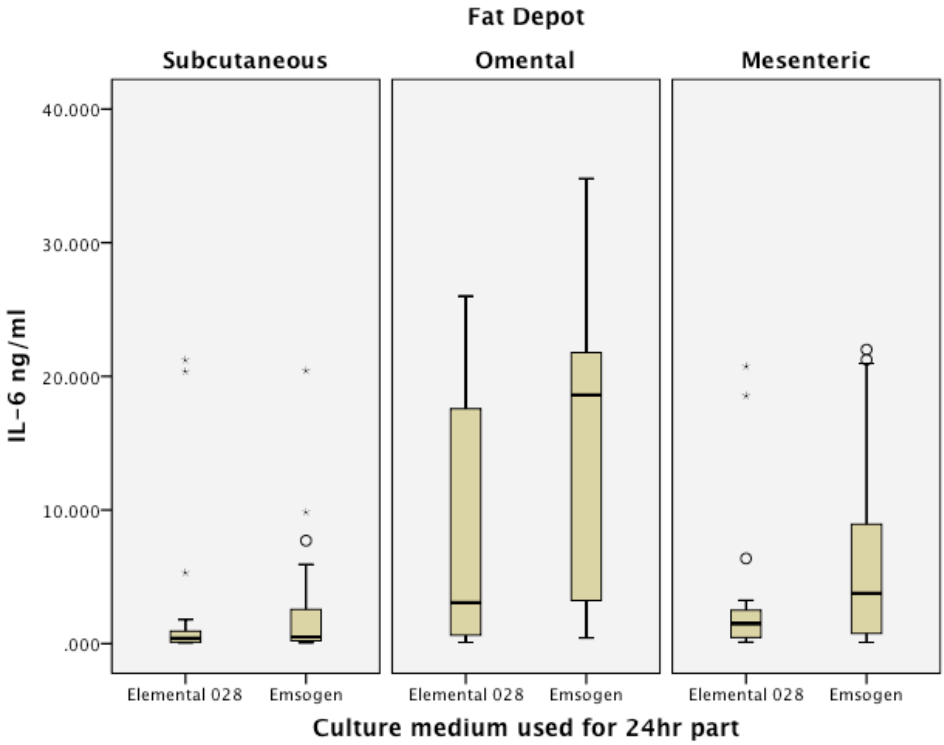



**Figure 17** A graph comparing net IL-6 release between the two feeds by adipose depot regardless of disease

### 4.5.3 Leptin

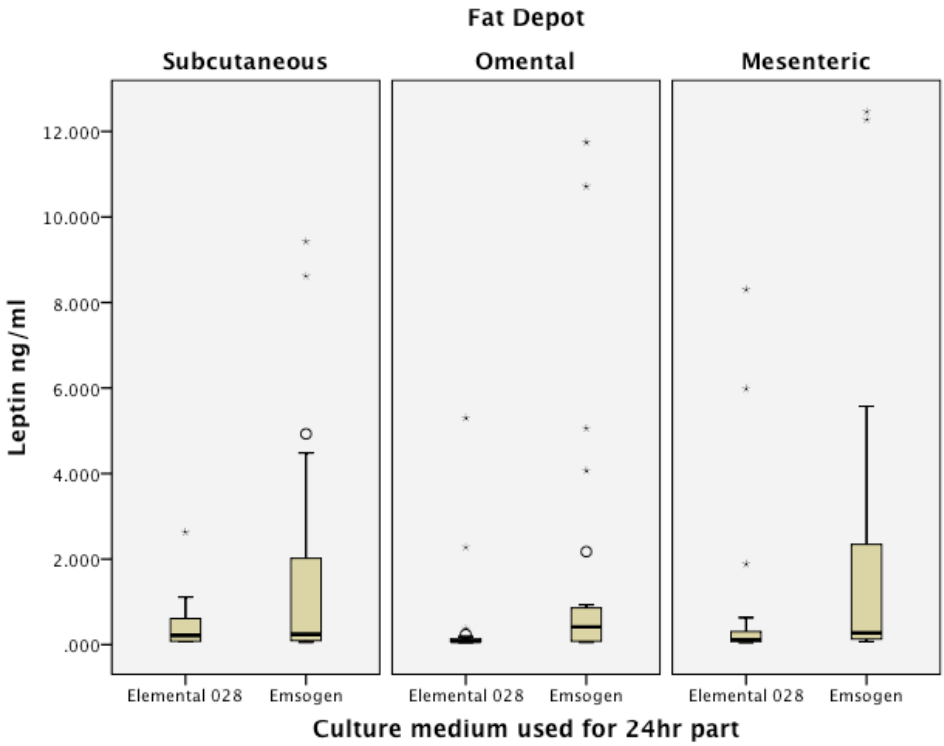



**Figure 18** A graph comparing net Leptin release between the two feeds by adipose depot regardless of disease

### 4.5.4 MCP-1

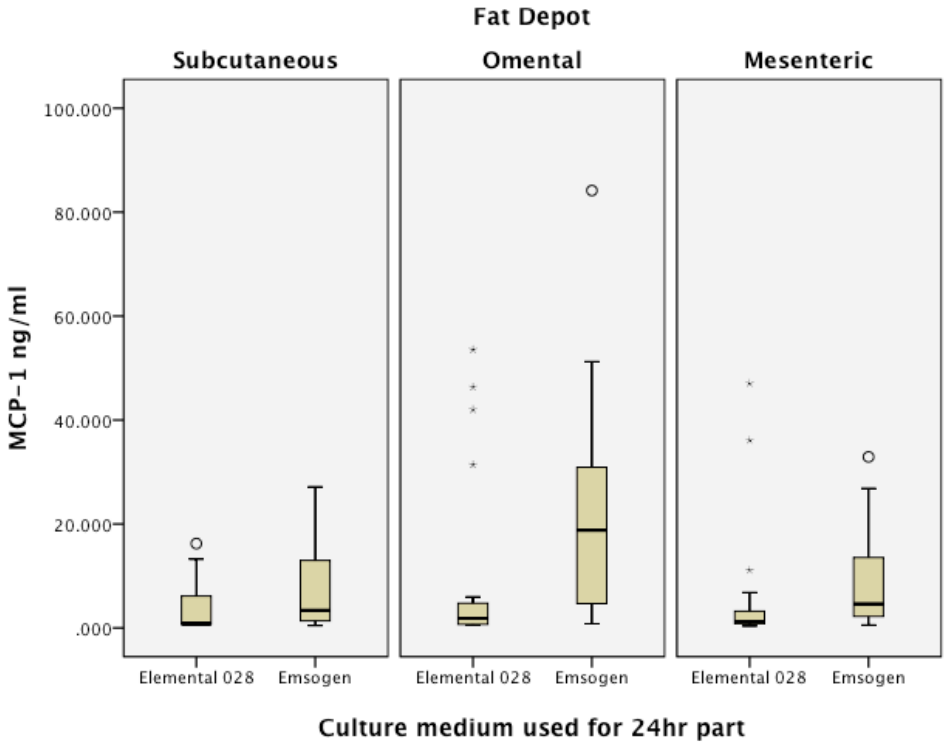



**Figure 19** A graph comparing net MCP-1 release between feeds by adipose depot regardless of disease

### 4.5.5 Findings

When both the disease cohorts are combined there is a highly significant difference between the concentrations of all IL-6 (p=0.018 and p=0.03), Leptin (p=0.006 and p=0.033) and MCP-1 (p=0.002 and p=0.006) between feeds for omental and mesenteric fat. The amount of all three cytokines is between 2 and 9 fold higher from tissues that were cultured in Emsogen than Elemental 028 Extra. Interestingly however, there was no significant difference between the subcutaneous depots in all three cytokine concentrations. This may be explained by the differences in the stromal vascular fraction and lymphoid tissue component of omental and mesenteric fat compared to subcutaneous tissue.

## 4.6 Overall data for IL-6

In an attempt to increase the power of the data all of the IL-6 assay results were combined regardless of disease cohort. This included the 12 patients whose tissues were cultured in both feeds and thus directly comparable and 12 cases that were individually cultured in only one of the two feeds (8 in Elemental 028 and 4 in Emsogen).

| | **Both diseases combined** | |
|---|---|---|
| **Feed** | **E 028** | **Emsogen** |
| **N** | 20 | 16 |
| **Sex (M:F)** | 9:11 | 7:9 |
| **BMI (kg/m²)** | 23.7 (19.6 – 29.6) | 22.0 (20.0 – 29.7) |
| **IL-6** | | |
| SC IL-6 ng/ml | 0.43 (0.18 – 1.78)<br>† $p$=0.159 | 0.99 (0.23 – 6.37) |
| OM IL-6 ng/ml | 2.05 (0.64 – 12.15)<br>† ***p*<0.001** | 19.07 (8.07 - 21.93) |
| MF IL-6 ng/ ml | 1.40 (0.23 – 2.88)<br>† ***p*=0.001** | 5.45 (1.96 – 18.69) |

**Table 20.** The net release of IL-6 from the three adipose depots compared by feed regardless of disease cohort and in all cases.

† Statistical difference between the two feeds

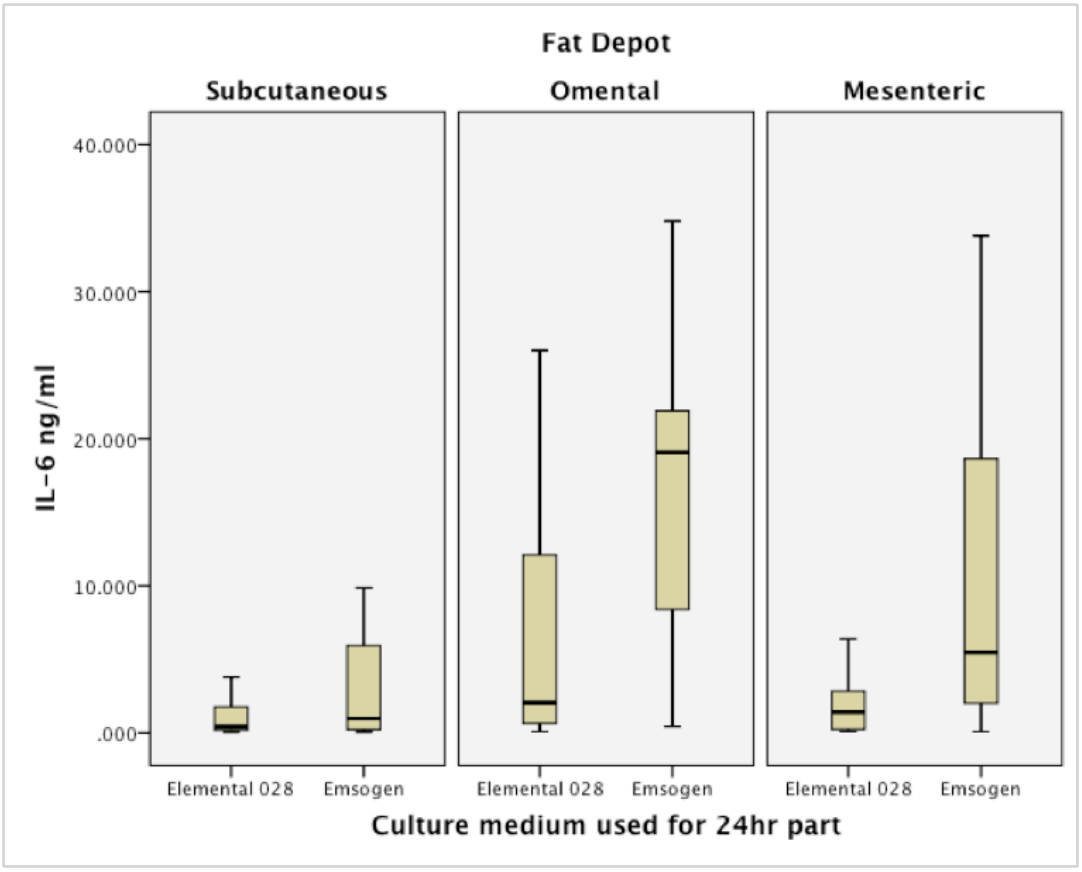



**Figure 20** The net release of IL-6 from the three adipose depots compared by feed regardless of disease cohort and in all cases.

### 4.6.1 Findings

When combined the difference in IL-6 release from omental and mesenteric adipose tissue between the two feeds becomes even more statistically significant with Emsogen resulting in far greater amounts of IL-6 release ($p<0.001$ and $p=0.001$ for OM and MF respectively). Again the effect is not replicated with subcutaneous fat.

## 4.7 Correlation between cytokines

Having found that IL-6, Leptin and MCP-1 concentrations were significantly raised after culture in Elemental 028 Extra compared to Emsogen, Spearman's rank correlation coefficient was used to measure dependence between the cytokines. It would be expected that the cytokines would correlate as they are all independent markers of inflammation and therefore should all rise with an inflammatory stimulus. Every cytokine analysis is included hence the high *n*-numbers. The results are tabulated below.

### 4.7.1 Overall correlation regardless of depot, disease or feed

| | | IL-6 ng/ml | NEFA mEq/L | Leptin ng/ml | MCP-1 ng/ml |
|---|---|---|---|---|---|
| **IL-6 ng/ml** | **Correlation Coefficient** | 1.000 | -.644 | .591 | .868 |
| | **Sig. (2-tailed)** | . | **.000** | **.000** | **.000** |
| | **N** | 381 | 188 | 239 | 239 |
| **NEFA mEq/L** | **Correlation Coefficient** | -.644 | 1.000 | -.672 | -.672 |
| | **Sig. (2-tailed)** | **.000** | . | **.000** | **.000** |
| | **N** | 188 | 361 | 50 | 50 |
| **Leptin ng/ml** | **Correlation Coefficient** | .591 | -.672 | 1.000 | .771 |
| | **Sig. (2-tailed)** | **.000** | **.000** | . | **.000** |
| | **N** | 239 | 50 | 239 | 239 |
| **MCP-1 ng/ml** | **Correlation Coefficient** | .868 | -.672 | .771 | 1.000 |
| | **Sig. (2-tailed)** | **.000** | **.000** | **.000** | . |
| | **N** | 239 | 50 | 239 | 239 |

**Table 21.** Correlation regardless of depot, disease or feed.

### 4.7.2 Correlation between cytokines released from cases cultured in both feeds

| | | IL-6 ng/ml | Leptin ng/ml | MCP-1 ng/ml |
|---|---|---|---|---|
| **IL-6 ng/ml** | **Correlation Coefficient** | 1.000 | .591 | .868 |
| | **Sig. (2-tailed)** | . | **.000** | **.000** |
| | **N** | 239 | 239 | 239 |
| **Leptin ng/ml** | **Correlation Coefficient** | .591 | 1.000 | .771 |
| | **Sig. (2-tailed)** | **.000** | . | **.000** |
| | **N** | 239 | 239 | 239 |
| **MCP-1 ng/ml** | **Correlation Coefficient** | .868 | .771 | 1.000 |
| | **Sig. (2-tailed)** | **.000** | **.000** | . |
| | **N** | 239 | 239 | 239 |

**Table 22.** Correlation between cytokines released from cases cultured in both feeds

### *4.7.2.1 Correlation of cytokines from subcutaneous fat only*

| | | IL-6 ng/ml | Leptin ng/ml | MCP-1 ng/ml |
|---|---|---|---|---|
| **IL-6 ng/ml** | **Correlation Coefficient** | 1.000 | .666 | .861 |
| | **Sig. (2-tailed)** | . | **.000** | **.000** |
| | **N** | 70 | 70 | 70 |
| **Leptin ng/ml** | **Correlation Coefficient** | .666 | 1.000 | .821 |
| | **Sig. (2-tailed)** | **.000** | . | **.000** |
| | **N** | 70 | 70 | 70 |
| **MCP-1 ng/ml** | **Correlation Coefficient** | .861 | .821 | 1.000 |
| | **Sig. (2-tailed)** | **.000** | **.000** | . |
| | **N** | 70 | 70 | 70 |

**Table 23.** Correlation of cytokines for subcutaneous fat only.

### *4.7.2.2 Correlation of cytokines from Omental fat only*

| | | IL-6 ng/ml | Leptin ng/ml | MCP-1 ng/ml |
|---|---|---|---|---|
| **IL-6 ng/ml** | **Correlation Coefficient** | 1.000 | .702 | .855 |
| | **Sig. (2-tailed)** | . | **.000** | **.000** |
| | **N** | 84 | 84 | 84 |
| **Leptin ng/ml** | **Correlation Coefficient** | .702 | 1.000 | .799 |
| | **Sig. (2-tailed)** | **.000** | . | **.000** |
| | **N** | 84 | 84 | 84 |
| **MCP-1 ng/ml** | **Correlation Coefficient** | .855 | .799 | 1.000 |
| | **Sig. (2-tailed)** | **.000** | **.000** | . |
| | **N** | 84 | 84 | 84 |

**Table 24.** Correlation of cytokines from omental fat only.

### *4.7.2.3 Correlation of cytokines from Mesenteric fat only*

| | | IL-6 ng/ml | Leptin ng/ml | MCP-1 ng/ml |
|---|---|---|---|---|
| **IL-6 ng/ml** | **Correlation Coefficient** | 1.000 | .676 | .879 |
| | **Sig. (2-tailed)** | . | **.000** | **.000** |
| | **N** | 84 | 84 | 84 |
| **Leptin ng/ml** | **Correlation Coefficient** | .676 | 1.000 | .823 |
| | **Sig. (2-tailed)** | **.000** | . | **.000** |
| | **N** | 84 | 84 | 84 |
| **MCP-1 ng/ml** | **Correlation Coefficient** | .879 | .823 | 1.000 |
| | **Sig. (2-tailed)** | **.000** | **.000** | . |
| | **N** | 84 | 84 | 84 |

**Table 25.** Correlation of cytokines from mesenteric fat only.

### 4.7.3 Correlation between IL-6 and NEFA

#### *4.7.3.1 Correlation between IL-6 and NEFA from Subcutaneous fat*

| | | IL-6 ng/ml | NEFA mEq/L |
|---|---|---|---|
| **IL-6 ng/ml** | **Correlation Coefficient** | 1.000 | -.449 |
| | **Sig. (2-tailed)** | . | **.001** |
| | **N** | 51 | 51 |
| **NEFA mEq/L** | **Correlation Coefficient** | -.449 | 1.000 |
| | **Sig. (2-tailed)** | **.001** | . |
| | **N** | 51 | 96 |

**Table 26.** Correlation between IL-6 and NEFA from subcutaneous fat

#### *4.7.3.2 Correlation between IL-6 and NEFA from Omental fat*

| | | IL-6 ng/ml | NEFA mEq/L |
|---|---|---|---|
| **IL-6 ng/ml** | **Correlation Coefficient** | 1.000 | -.645 |
| | **Sig. (2-tailed)** | . | **.000** |
| | **N** | 69 | 67 |
| **NEFA mEq/L** | **Correlation Coefficient** | -.645 | 1.000 |
| | **Sig. (2-tailed)** | **.000** | . |
| | **N** | 67 | 120 |

**Table 27.** Correlation between IL-6 and NEFA from omental fat.

#### *4.7.3.3 Correlation between IL-6 and NEFA from Mesenteric fat*

| | | IL-6 ng/ml | NEFA mEq/L |
|---|---|---|---|
| **IL-6 ng/ml** | **Correlation Coefficient** | 1.000 | -.709 |
| | **Sig. (2-tailed)** | . | **.000** |
| | **N** | 71 | 69 |
| **NEFA mEq/L** | **Correlation Coefficient** | -.709 | 1.000 |
| | **Sig. (2-tailed)** | **.000** | . |
| | **N** | 69 | 125 |

**Table 28.** Correlation between IL-6 an NEFA from mesenteric fat.

### 4.7.4 Findings

It is clear that the three cytokines significantly correlate and NEFA also significantly correlates with IL-6. This shows that they corroborate the findings of an increased inflammatory response with Emsogen despite being independents markers of inflammation.

# Chapter 5

# DISCUSSION

## 5.1 The Protocol

Crohn's disease is a chronic inflammatory disease of the intestine with an unknown aetiology. Thus far a combination of genetic, immunological and environmental factors have been shown to contribute to its pathogenesis but any more specific causative agent has yet to be elucidated. Management of the disease involves the use of toxic medications with unpleasant side effects and although surgery is used as a last resort over 75% of sufferers will require a resection within 10 years of diagnosis. The disease burden is high and the treatments are broad and damaging. Elemental enteral nutrition as a treatment of Crohn's disease is therefore an attractive alternative with no known deleterious side effects and an efficacy close to steroids in acute flares of the disease (Zachos et al., 2009, and Forbes et al., 2011). Enteral nutrition preparations have also been shown to maintain remission (Belluzi A, 1997). If the mechanism of action of elemental feed can be clarified perhaps its efficacy can be increased to supercede toxic medications and more insight into the aetiology of Crohn's disease can be gained. The fat content of enteral nutrition formulas appears to be significant (Gassull et al., 2002, Guerreiro et al., 2009, Forbes et al., 2011) and it was with this in mind that the protocol for this study was conceived. Furthermore, fat-wrapping and the thickening of mesenteric fat are pathognomonic to Crohn's disease and little is understood as to its specific aetiology. The objective of this study was to obtain samples of mesenteric fat from patients with Crohn's disease for comparison with controls and to compare the effect of two preparations of enteral nutrition, which varied predominantly in their fat content, on the release of inflammatory cytokines from whole adipose tissue.

Previous studies have used poorly matched controls for BMI and age with the use of elderly cancer patients or patients with acute inflammatory diseases such as diverticulitis (Paul et al., 2006). Similarly previous studies have compared mesenteric fat that was taken from varied positions on both the small and large bowel mesenteries and with relatively small numbers. This study was able to standardise the position of the mesenteric fat between groups and also similarly match the patients for age and BMI by using patients with ulcerative colitis as controls. Although we found significant differences in NEFA and cytokine production between the elemental feeds we did not find any difference between

the disease cohorts, although with greater numbers these may have become apparent.

## 5.2 Characterisation of Adipose tissue and Basal findings

This study aimed to determine if there was a difference in the in-vitro effects of elemental enteral feeds on adipose tissue by using lipolysis in the form of non-esterified fatty acid assays and inflammatory cytokine assays (Leptin, IL-6 and MCP-1) as differentiators. It was important to avoid carrying a bias through into the elemental feed studies by ensuring that there were no differences in the baseline levels between Crohn's disease and the control group (Ulcerative colitis). No significant differences in basal NEFA concentrations were found between the two cohorts (CD n=12 and UC n=9) see Table 10. A significant difference in NEFA concentration between the depots was seen in UC but not in CD with subcutaneous fat the highest 0.084 (0.059-0.100)mEq/l and omental fat the lowest 0.19(0.005-0.0350mEq/l, see Table 11. The converse was true of Crohn's disease although this was not statistically significant. The wide range of NEFA results may be explained by differences in fasting times pre-operatively of the patients and also variation in ischaemia time after ligation of the ileo-colic pedicle prior to placement into culture solution.

Similarly as seen in tables 12 and 13 little difference was found between IL-6, Leptin and MCP-1 concentrations after twenty-four hours of culture between cohorts and depots. The only significant differences found were between the amount of omental IL-6 produced between Crohn's disease and ulcerative colitis and the amount of MCP-1 produced between subcutaneous and omental depots in ulcerative colitis. The numbers (n=6 per cohort} were small making statistical significance hard to achieve.

Interestingly there did appear to be a trend (Figure 9 and Table 12 on page 69) with leptin concentrations higher in all depots in patients with UC compared to CD. Multiple studies predominantly examining obese patients have found that levels of leptin mRNA and release were higher in subcutaneous adipose tissue compared to omental adipose tissue by up to 2-3 fold (Arner, 2001, Montague et al., 1997, Van Harmelen et al., 1998, Russell et al., 1998). Our findings are not consistent with this but again the numbers are small. It has been shown that leptin mRNA is overexpressed in CD and UC compared to controls (Barbier et al., 2003) but no

difference in expression was seen between CD and UC. Increased leptin secretion after 24hrs of culture was seen in CD compared to colorectal cancer and diverticulitis controls (Paul et al., 2006) and circulating levels of leptin are also raised in CD (Karmiris et al., 2006, Valentini et al., 2009). Interestingly in these studies circulating levels of leptin were also raised in UC and not significantly different to CD. Our findings are consistent with leptin secretion being similar between UC and CD although there is a suspicion from the trend that with higher numbers leptin secretion may be significantly higher in UC compared to CD. Pre-operative fasting status nor ileocolic ischaemic time would not be sufficient to explanin this difference. The only study in the literature directly looking at IL-6 and MCP-1 secretion from CD mesenteric adipose tissue after twenty-four hours of culture compared this to colorectal cancer and diverticulitis patients. No significant difference was found between the concentrations of these cytokines between the CD and colorectal cancer control groups (Paul et al., 2006). We found no difference between these markers in CD and UC consistent with these findings see Figure 9. In Paul's study IL-6 and MCP-1 secretion were significantly increased in the diverticulitis group and the group concluded that these cytokines were related to a more general inflammatory response pattern not specific to CD. This suggests that they are good candidate cytokines for measuring the inflammatory response of adipose tissue to elemental formulas in culture.

## 5.3 The effects of elemental formulas on adipocyte organ culture

The aim of these experiments was to see if the differing fat contents of the elemental feeds would exert in vitro differences in the inflammatory response of adipose tissue in patients with Crohn's disease compared to controls. The elemental feeds are almost identical in all the macronutrient content but varied in their medium- and long-chain triglyceride content. E028 is rich in long-chain triglycerides (65%) whereas Emsogen has a high medium-chain triglyceride content (83%). The ratio of n6:n3 fatty acids is also significantly different with Emsogen much higher than E028 (46.5:1 vs 4:1). Other important differences are the mono-unsaturated fat content, which is twelve times higher in E028 and Emsogen contains twice as much saturated fatty acids.

The results of the NEFA lipolysis experiments showed that culture in E028 resulted in significantly greater amounts of NEFA production than in Emsogen, see Table 16, p79 when the diseases are separated. When Crohn's disease and

ulcerative colitis specimens are combined the results are even more conclusive, see Table 7, p80 and Figure 13, p81 with significantly greater NEFA release from the three adipose depots cultured in E028 compare to Emsogen, (SC- $p=0.037$ and both OM and MF $p<0.001$). The implications of this are that the adipose tissue specimens cultured in the lower n6:n3 ratio feed were healthier although this may be speculative. Much more persuasive are the results of the inflammatory cytokine assays. Concentrations of IL-6, leptin and MCP-1, all considered markers of inflammation in adipose tissue, were significantly higher in adipose tissue cultured in Emsogen compared to E028. Indeed when the disease vs control comparison was removed and the study concentrated purely on the effects of the two feeds on adipose tissue from any disease, production of IL-6 was 6x greater in omental fat, 2.5x greater in mesenteric fat, concentrations of leptin were 5x greater in omental fat and 2.5x greater in mesenteric fat and concentrations of MCP-1 were 9x greater in omental fat and 4x greater in mesenteric fat in specimens cultured in Emsogen See table 19, p85. All of these results were statistically very significant. Indeed the overall IL-6 data showed a 9 fold increase in IL-6 production in omental adipose tissue culture and 4 fold increase in mesenteric adipose tissue culture in Emsogen ($p<0.001$ for both). These data demonstrate an interesting effect. Foremost is the finding that Emsogen is clearly more pro-inflammatory than E028 when omental and mesenteric fat are cultured within it in-vitro. Furthermore, subcutaneous fat does not appear to undergo the same effect as the other two depots. None of the cytokines or NEFA assays of subcutaneous fat significantly differed between feeds. This study highlights the fundamental differences in cellular components between subcutaneous and visceral fat. Omental fat consistently produced higher concentrations of the inflammatory cytokines. This is most likely due to differences in the stromavascular fraction and lymphoid cells, lymph nodes versus milk spots, (Fried and Moustaid-Moussa, 2001) between the depots or possibly specialised perinodal adipocytes . Certainly the surgeon's moniker for omentum as being 'the policeman of the abdomen' appears justified. Many studies examining adipose tissue have compared visceral and subcutaneous depots where omental and mesenteric fat have been considered interchangeable and collectively called 'visceral'. These findings suggest this should not be the case.

The two feeds compared in this study are elemental. Multiple trials have assessed the efficacy of elemental feeds to induce remission and prevent relapse in Crohn's

disease but to date they have been shown to be almost as effective but not more so than steroids in the adult population (Forbes et al., 2011, Zachos et al., 2007). Proposed mechanisms for elemental feeds inducing remission include modulation of the enteric microbial environment, reduction of food antigens (Shah, 2007), and changes in intestinal permeability (Pravda, 2011) and gene expression (Hooper and Gordon, 2001). However, there may be a hitherto unexplored secondary effect on bowel function that modulates the disease process. An example might be possible changes in the regulation of the entero-hepatic circulation of bile salts that in turn beneficially alters the microbiome. A recent study was able to induce colitis in IL10 knockout mice using saturated milk-derived fat. The saturated fat promoted taurine conjugation of hepatic bile acids increasing the availability of organic sulphur for sulphite-reducing microbes and resulting in a proinflammatory $T_H1$ immune response and colitis (Devkota et al., 2012). This may give weight to the hypothesis that bile acid manipulation is relevant in the pathogenesis of inflammatory bowel disease or may be the mechanism by which elemental diet exerts its remitting effect.

In this study we have shown that the *n*-3 fatty acid rich elemental formula exerted a considerably increased anti-inflammatory effect on visceral fat depots in vitro and this may explain how the elemental feed induces remission in active Crohn's disease. The effects of *n*-3 and *n*-6 PUFAs on inflammation in the context of numerous diseases have been extensively researched. *N*-3 intake results in eicosapentaenoic acid incorporation and a decrease in arachidonic acid in membrane phospholipids leading to attenuated production of proinflammatory eicosanoids and increased production of their less pro-inflammatory or anti-inflammatory counterparts (Calder, 2006, Calder, 2009). Increased generation of EPA- and DHA-derived resolvins with anti-inflammatory actions have been described (Weylandt et al., 2007). Other anti-inflammatory actions of *n*-3 PUFAs include reduced synthesis of pro-inflammatory cytokines (such as TNF-a, IL-1b, IL-6 and IL-8) through either a decreased activation of some nuclear transcription factors (e.g. NF-κβ) or increase activation of PPAR-γ, reduced leukocyte chemotaxis and diminished T-cell reactivity (Calder, 2006, Calder, 2009). Conversely *n*-6 is traditionally considered to be an eicosanoid precursor and to exert a pro-inflammatory effect. It is correlated with Crohn's Disease Activity Index (CDAI) (Kuroki et al., 1997) and studies in animal models have shown that *n*-6

PUFA induce IL-1 expression and responsiveness to cytokines (James et al., 2000) while conversely *n*-3 PUFA inhibit these processes and reduce thromboxane $A_2$ and leukotriene $B_4$ production (Ioannidis et al., 2011). In this study design the two feeds were deliberately chosen for their distinct differences in *n*-6:*n*-3 ratio with Emsogen with a high ratio (46.5:1) compared to E028 with a low ratio (4:1). E028 with less *n*-6 PUFA resulted in significantly less inflammation.

Multiple other studies have been performed examining the effect of *n*-3 PUFA in active and quiescent Crohn's disease, mostly in the form of dietary supplementation with fish oil capsules (Belluzzi et al., 1996, Lorenz-Meyer et al., 1996, Romano et al., 2005, Feagan et al., 2008) with only a limited number of studies examining the effect of *n*-3 PUFA in elemental feeding (Nielsen et al., 2005, Grogan et al., 2012). A recent systematic review found that there was insufficient evidence to recommend *n*-3 PUFA as therapeutic agents in the treatment of Crohn's disease and ulcerative colitis (Cabre et al., 2012), however this was in part due to the numerical quality of the data and poor study designs. It also remains unknown whether elemental feeds, due to their almost pre-digested nature, are predominantly absorbed proximally in the small intestine upstream of the area of inflammation in Crohn's disease or if the fatty acids contained within the feeds reach the terminal ileum in sufficient concentrations to exert a direct immunomodulatory effect. If the latter does occur, our data show that *n*-3 PUFA appear to have an anti-inflammatory effect on visceral adipose tissue in vitro and this experiment may explain how elemental feeds containing *n*-3 PUFA exert a therapeutic effect in Crohn's disease.

Alternatively the differences in the performances of the two feeds may be explained by a separate variable component within the fat content. Medium chain triglycerides also seem to have immunomodulatory properties (Waitzberg et al., 1996, Wanten et al., 2000) and the Emsogen feed contained considerably greater quantities (83%) than E028. Animal model studies looking at trinitrobenzene sulfonic acid (TNBS)- induced ileitis in rats showed a decrease in mucosal inflammation in animals fed a mix of MCT and LCT diet compared to LCT diet alone (Tsujikawa et al., 1999). However a further study by the same group was only able to replicate the benefits in chemically-induced colitis and no difference in the degree of enteritis between LCT and MCT was found (Tsujikawa et al., 2001). Many other animal studies of chemically induced colitis have also shown beneficial

effects of MCT diets (Kono et al., 2010a, Mane et al., 2009). However the difficulties of translating the findings of a short term chemically induced inflammation in rats, to humans with inflammatory bowel disease of much longer and elusive aetiology are profound. There are also some clinical data suggesting that replacing part of dietary fat with MCT contributes to the primary therapeutic effect of enteral nutrition in Crohn's disease. One study of patients with active Crohn's disease assigned four separated elemental formulae with differing contents of LCT and MCT. Rates of remission were negatively correlated with LCT and inflammatory markers were decreased in patients receiving the lowest amounts of LCT leading the authors to suggest a pro-inflammatory effect of LCT and anti-inflammatory effect of MCT (Middleton et al., 1995). However other studies have not found any beneficial effect of MCT on disease activity (Khoshoo et al., 1996, Sakurai et al., 2002) and a clinical trial of low LCT versus high LCT whole protein diets in patients with active Crohn's disease also showed no difference in efficacy (Leiper et al., 2001). In our study MCT (greater in Emsogen) performed considerably less well than LCT (greater in E028) if indeed it exerted an effect at all.

A third variable within the fat contents of the feeds may have been the different composition of saturated and mono-unsaturated fatty acids. Both of these have been proposed as possible risk factors for inflammatory bowel disease (Hou et al., 2011, Sakamoto et al., 2005) but dietary formulas rich in MUFA seem to have neutral immunomodulatory effects both in vivo and in vitro (Buenestado et al., 2006, Granato et al., 2000). E028 contains twelve times more MUFA than Emsogen, and Emsogen contains two times more SFA than E028. A double blind randomised controlled trial that has generated much interest in the scientific literature found, contrary to expectation, that a diet containing high levels of MUFA in the form of oleic acid was no better than placebo at inducing remission and considerably less effective than a diet containing high levels of *n*-6 PUFA (Gassull et al., 2002). This conflicted with the group's previous findings where high MUFA levels feeds resulted in 80% remission in active Crohn's disease (Gonzalez-Huix et al., 1993) and the study by Middleton and colleagues using high MUFA level diets had remission rates of 50-90% concluded that remission rate was inversely proportional to levels of LCT (Middleton et al., 1995). In Gassull's study the levels of LCT were the same in both feeds, leading to their conclusion these were not the effector either. The explanation for these findings remains elusive. Cell culture

studies of lymphocytes have shown that the immunomodulatory effects of fat varies when the cells are cultured in isolated fatty acids or with mixtures of fatty acids (Karsten et al., 1994). Indeed both the unsaturated oleic acid (*n*-9) and linoleic acid (*n*-6) and the saturated stearic and palmitic acids affected cytokine production, with the saturated fats being the more potent effectors. The conclusion drawn was that the immunomodulatory effect is more dependent on the fatty acid profile rather than a single fatty acid. A study looking at the inflammatory response in mice in response to bacterial lipolysaccaride after five weeks of diets high in either coconut oil (MCT), olive oil (*n*-9), safflower oil (*n*-6) or fish oil (*n*-3) found reduced levels of IL-6 in the mice fed the *n*-3 and MCT diets, no difference in those fed *n*-9 diet and increased cytokine production in those fed the *n*-6 diet (Sadeghi et al., 1999). So it could be concluded that *n*-3 PUFA and MCT are anti-inflammatory and that *n*-6 PUFA and saturated fatty acids are pro-inflammatory. Adipose tissue culture in E028 resulted in considerably less inflammation than culture in Emsogen. Whether the reason for this is the lower *n*-6:*n*-3 PUFA ratio in E028 or the greater pro-inflammatory SFA content of Emsogen is not clear. However whichever of these potential effects predominates, both of them appear to exert greater effect than the purported anti-inflammatory effect of MCT, which was higher in Emsogen.

In regard to fat-wrapping and mesenteric thickening recent studies have raised significant new questions. The disease pathogenesis is still not entirely clear even in regard to the initial event. Is mucosal inflammation the initial step? Or is the mesenteric lymphatic system the critical player in the disease being primarily inflamed and causing retrograde inflammation in the bowel? A recent review (von der Weid et al., 2011) has highlighted the importance of the histological findings in Crohn's disease of extensive submucosal oedema, dilated lacteals and lymphocytic thrombi suggesting poor lymphatic drainage secondary to lymphatic obstruction or impaired contractile function of the mesenteric lymphatic vessels. They point out that limited attention has been paid to this area in the quest to understand Crohn's disease. In addition Shelley-Fraser has highlighted the connective tissue changes of fibrosis and muscularisation which are also characteristic of Crohn's and have received limited attention from, clinicians, diagnostic pathologists and researchers alike (Shelley-Fraser et al., 2012). In obesity visceral adipose tissue releases proinflammatory mediators and is infiltrated by macrophages and

modern theories define obesity as a state of chronic inflammation (Siegmund, 2012). It has also been shown that in patients with Crohn's disease mesenteric adipose tissue distant from the area of inflammation shows similar features to those seen in obesity (Zulian et al., 2011). Siegmund goes on to point out that Zulian et al have shown the CRP production triggered by local inflammation and bacterial translocation is the mechanism by which mesenteric fat contributes to maintaining the inflammatory response in Crohn's disease (Zulian et al., 2013). Their suggestion is that Crohn's disease is characterised by transmural inflammation followed by bacterial translocation. The mesenteric preadipocytes phagocytose the bacteria and stimulate a further local inflammatory response within the mesenteric fat, resulting in hyperplasia and smaller adipocytes (Peyrin-Biroulet et al., 2007) and a protective role for the tissue. Mesenteric adipose tissue in Crohn's disease has also been shown to have significantly fewer apoptotic cells than controls (Dias et al., 2014) and their inference is that this is to enable the maintenance of the inflammatory response. Recent studies in mice have shown that mesenteric adipocyte inflammation is necessary for tissue expansion and remodelling but also to maintain intestinal barrier function (Asterholm et al., 2014). This study has not shed any light on the causes of fat-wrapping or mesenteric thickening in Crohn's disease. It has successfully shown that an elemental feed with a lower ratio of *n*-6 to *n*-3 fatty acids appears to reduce inflammation of mesenteric adipose tissue in vitro. Given the recent findings discussed above what remains unclear is whether this would be a desirable effect in vivo. Indeed it may explain why *n*-6 fatty acids seemed to be protective in Gassull's study of 2002.

## 5.4 Limitations of the study

This study had limitations. Finding a control group to balance a cohort of patients undergoing bowel resection is difficult. In this study we used patients with ulcerative colitis due to their ready availability and similar age and body habitus. These are however two chronic inflammatory bowel diseases with considerable overlap and this may explain why no significant differences were seen between the two disease cohorts. Similarly it was also impractical in most cases to collect the Crohn's Disease Activity Index scores prior to surgery and to determine the patient's diet in the lead up to surgery. In particular it would have been most interesting to establish the lipid content to examine if this was associated with our

findings or shown to have an effect. Unfortunately for the study, none of the patient cohort were on elemental diet prior to surgery, which would have been interesting to explore. It was not possible to collect detailed dietary information from patients prior to their donation of adipose tissue. It would have been interesting to see how this correlated with the findings compared to the fatty acid composition of their adipose tissue. Pre-operative medication information was incomplete and was insufficient to measure any effect. The ischaemic time from ligation of the ileocolic artery to specimen collection was also not measured. All these factors may have exerted an effect and may explain the wide variance in the results.

Furthermore we had to make an assumption in interpreting this study that fatty acids contained within elemental feeds do indeed reach the terminal ileum to exert an immuno-modulatory effect. In what concentration this occurs if at all is unknown. Our initial dilution study may have no similarity to the true concentration of elemental feed in vivo. The adipose tissue in this study was cultured directly within elemental feed and CellGro®. This does not equate well to the physiological situation in vivo where elemental feed would be absorbed into the portal blood stream or lymphatic system and so whether the fat content of elemental feed even affects mesenteric fat is still not certain. A further limitation of the study was that the elemental feeds were not perfectly matched to identify the true effector differentiating the feeds. They were nearly identical except for the lipid content but it is not clear whether SFA concentration or the lower *n*-6:*n*-3 ratio caused the differences in the inflammatory response.

## 5.5 Clinical implications and future directions of research

The objective of this study was to evaluate the effects of different types of fat within elemental feed on the inflammatory response of human adipose tissue of patients with Crohn's disease in vivo. Many of the studies within the scientific literature are conflicting and this study clarifies that the fat content is not only important but exerts a significant physiological effect in vitro. This study also suggests that a feed that is high in *n*-3 PUFA and low in SFA might prove the most effective elemental feed for reducing mesenteric inflammation in patients with active flares of Crohn's disease.

Further research should involve work on the characterisation of the fat-wrapped adipose tissue collected during this study, in particular the histological analysis of the specimens, which is weak in the literature. Further work examining the absorption of elemental feed would be useful to elucidate its mechanism of action. Providing elemental feed to a patient without inflammatory bowel disease but with a distal defunctioning ileostomy to collect the effluent may assist this understanding. If elemental feed is shown to reach and be absorbed at the terminal ileum in concentrations similar to this study then a trial looking at rates of remission with a new feed high in *n*-3 PUFA and low in SFA could be undertaken.

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# APPENDICES

## Appendix 1 The Composition Of The Feeds

| | Elemental 028 | Emsogen |
|---|---|---|
| **Nutrition Information** | **Per 100ml** | **Per 100ml** |
| Energy Kcal | 86 | 88 |
| Protein equivalent g | 2.5 | 2.5 |
| Total amino acids g | 3 | 3 |
| Carbohydrate g | 11 | 12 |
| Of which sugars g | 4.7 | 1.6 |
| Fat g | 3.5 | 3.3 |
| Of which saturates g | 1.3 | 2.6 |
| Monounsaturates g | 1.6 | 0.12 |
| Polyunsaturates g | 0.45 | 0.34 |
| % LCT | 65 | 17 |
| % MCT | 35 | 83 |
| Ratio n6:n3 fatty acids | 3.5:1 | 46.5:1 |
| % energy from linoleic acid | 3.6 | 3.4 |
| % energy from α-linolenic acid | 1 | 0.07 |
| Fibre g | Nil | Nil |
| **Amino Acid Profile** | **g per 100ml** | **g per 100ml** |
| L-Alanine | 0.12 | 0.1 |
| L-Arginine | 0.26 | 0.23 |
| L-Aspartic Acid | 0.24 | 0.2 |
| L-Cystine | 0.01 | 0.01 |
| Glycine | 0.2 | 0.17 |
| L-Histidine | 0.15 | 0.13 |
| L-Isoleucine | 0.2 | 0.17 |
| L-Leucine | 0.34 | 0.30 |
| L-Lysine | 0.26 | 0.22 |
| L-Methionine | 0.15 | 0.1 |
| L-Phenylalanine | 0.25 | 0.24 |
| L-Proline | 0.24 | 0.20 |
| L-Serine | 0.15 | 0.12 |
| L-Threonine | 0.17 | 0.14 |
| L-Tryptophan | 0.07 | 0.06 |
| L-Tyrosine | 0.04 | 0.05 |
| L-Valine | 0.21 | 0.19 |
| L-Carnitine | 0.003 | 0.004 |
| Taurine | 0.006 | 0.006 |
| L-Glutamine | | 0.344 |
| **Fatty Acid Profile** | **g per 100g Fatty Acids** | **g per 100g Fatty Acids** |
| C6:0 | 0 | 1.4 |
| C8:0 | 20.1 | 59.2 |
| C10:0 | 14.9 | 20.4 |
| C12:0 | 0.1 | 1.9 |
| C14:0 | Trace | 0 |

| | | |
|---|---|---|
| C16:0 | 2.6 | 2 |
| C18:0 | 1.6 | 0.27 |
| C18:1 (Oleic) *n*-9 | 46 | 3.9 |
| C18:2 (Linoleic) *n*-6 | 10.5 | 10.7 |
| C18:3 (α-linoleic acid) *n*-3 | 3 | 0.23 |
| C20:0 | 1.2 | 0 |
| C22:0 | 1.2 | 0 |
| **Vitamins** | **Per 100ml** | **Per 100ml** |
| Vitamin A IU | 133 | 220 |
| Vitamin D IU | 19.2 | 20 |
| Vitamin E IU | 2.5 | 1.8 |
| Vitamin C mg | 5.7 | 5.7 |
| Vitamin K µg | 5 | 5 |
| Thiamin mg | 0.12 | 0.12 |
| Riboflavin mg | 0.12 | 0.12 |
| Niacin mg | 0.84 | 0.84 |
| Vitamin $B_6$ mg | 0.16 | 0.16 |
| Folic acid µg | 16.7 | 16.7 |
| Vitamin $B_{12}$ µg | 0.4 | 0.36 |
| Biotin µg | 3.6 | 3.6 |
| Pantothenic Acid mg | 0.4 | 0.4 |
| Choline mg | 18.3 | 18.3 |
| Inositol mg | 1.8 | 1.8 |
| **Minerals** | **Per 100ml** | **Per 100ml** |
| Sodium mg | 61 | 60 |
| Potassium mg | 93.2 | 93.2 |
| Chloride mg | 66.6 | 65.4 |
| Calcium mg | 45 | 49 |
| Phosphorus mg | 40 | 40 |
| Magnesium mg | 16.3 | 16.3 |
| **Trace Elements** | **Per 100ml** | **Per 100ml** |
| Iron mg | 0.84 | 0.84 |
| Copper mg | 0.08 | 0.08 |
| Zinc mg | 0.84 | 0.84 |
| Manganese mg | 0.12 | 0.12 |
| Iodine µg | 6.66 | 6.7 |
| Molybdenum µg | 6.66 | 6.7 |
| Selenium µg | 3 | 3 |
| Chromium µg | 3 | 3 |
| Osmolality | 695mOsm/L | 580mOsm/L |